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Contrast Agents II

Optical, Ultrasound, X-Ray and Radiopharmaceutical Imaging

Volume Editor: Werner Krause

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Preface

The present volume on optical, ultrasound, X-ray and radiopharmaceutical contrast agents is the second in a series on diagnostics following a volume that was exclusively dedicated to magnetic resonance imaging. This volume covers compounds from four different imaging modalities starting with the most recent technology, optical imaging, followed by diagnostic ultrasound and the still dominant albeit more than one hundred year-old X-ray technique and concluding with radiopharmaceutical contrast agents.

Whereas contrast agents for optical imaging are not yet widely used – except for some dedicated techniques such as liver function tests – ultrasound is by now a well established procedure. However, since the machines are meanwhile so excellent – and nevertheless still improving –, contrast agent use is rather limited. X-ray imaging, on the other hand, is the mother of all imaging techniques and despite of being announced dead for repeatedly, is constantly being resurrected and each time gains in importance. The reason lies in the rapid further development of modalities such as computed tomography (CT) with ever improving sensitivity, reliability and speed.

Research in the field of contrast agents for optical imaging is vigorously growing due to the fact that non-ionizing radiation can be utilized and due to rapid progress in the development of optical instruments. The first chapter summarizes both the different techniques of optical imaging and the presently available and investigated classes of contrast agents such as carbocyanine dyes, tetrapyrroles, 5-aminolevulinic acid derivatives, and lanthanide chelates. Additionally, the medical applications of this technique are reviewed.

The second chapter approaches this field from a different angle, i.e. from the viewpoint of functional imaging. The main focus lies on functional imaging of the liver and the kidney. The reader is guided in this approach via a description of the technique followed by a review of the different markers that can be utilized. This article not only describes the chemistry behind the use of markers but, additionally, dedicates a significant portion to the biological understanding of how these compounds work. The scope of substances is far beyond optical imaging. It also includes other techniques utilizing for example X-ray and radiopharmaceutical contrast agents as biological markers.

The third chapter is dedicated to contrast agents for ultrasound imaging starting with the design, preparation and application of microbubbles. Additionally, the different presently available generations of contrast agents are

described, followed by the design of targeted agents and by applying the bubble principle to therapeutic drug and gene targeting.

The following three chapters describe different classes of X-ray contrast agents. The fourth chapter gives an overview on the chemistry of extracellular iodinated X-ray contrast agents starting with possible routes of synthesis. However, the main focus rests on analytical considerations with particular emphasis on the complex pattern of isomers. In particular dimeric compounds such as iodoxanol and iotrolan exhibit a plethora of different types of isomers such as enantiomers, diastereoisomers, *cis-trans* isomers, and rotamers. In this chapter, the correlation of HPLC peaks with individual isomers is described in detail.

The fifth chapter is dedicated to macromolecular iodinated contrast agents with low dispersity. They can be utilized as blood-pool agents. The synthesis, analysis and pharmacokinetics of this class of compounds is summarized and their diagnostic impact is evaluated.

The following chapter reviews tissue-specific X-ray contrast agents, in particular liver-specific substances. Although many different approaches including hepatocyte and Kupffer-cell targeting utilizing iodinated compounds have been followed, no small molecule could be obtained that is effective and safe. Two different approaches, however, do seem to be feasible and will be followed in the future. These are extracellular iodinated X-ray contrast agents encapsulated into liposomes and liver-specific lanthanide chelates.

The next two chapters are dedicated to radiopharmaceutical contrast agents starting with a detailed description of the chemistry of β^+ -emitting compounds based on fluorine-18. Particular emphasis is laid on the different radiolabeled precursors and their suitability for the rapid synthesis of compounds useful for positron emission tomography.

In the last chapter receptor-based diagnostic radiopharmaceuticals are reviewed including considerations on drug design, on receptors, and on receptor imaging with the objective of modifying the pharmacokinetics of these agents.

Presently, whereas optical imaging is looking for its place in the community of imaging modalities, ultrasound is highly established as a technique – with contrast agents still awaiting their final appraisal – and X-ray continues to be the prime imaging modality. On the other hand, radiopharmaceutical contrast agent have the potential to further establish molecular imaging with the ultimate goal of diagnosis on a molecular level.

Berlin, June 2002

Werner Krause

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Contrast Agents for Optical Imaging

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Optical imaging encompasses many different techniques which use light of the UV to the NIR spectral region to visualize tissue optical characteristics imparted through absorption and scattering of light, as well as emission of fluorescence. A variety of different probes for optical detection of diseases have been synthesized and characterized for their potential to engender disease-specific optical signals within the tissue. This review summarizes the essential literature in this research field, especially covering the chemistry of novel biocompatible probes from the cyanine dyes, tetrapyrroles, lanthanide chelates and some other entities. Different chemical, optical and pharmacological properties were exploited to provide extracellular dyes, target-specific conjugates, activatable probes or pro-drugs. In selected examples, the synthesis of such agents and their mode of action is described and accompanied with *in vivo* data demonstrating their diagnostic efficacy. Finally, a summary of medical applications is given with emphasis on the current and future role of optical contrast agents. Further progress in the design of designated dyes will be of great value for the establishment of novel optical diagnostic methods in routine clinical practice.

Keywords. Fluorescence imaging, Optical tomography, Contrast agents, Fluorescent dyes, Biomolecule conjugates

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Abbreviations

ALA	δ -aminolevulinic acid
CCD	charge-coupled device
CT	computed tomography
DOT	diffuse optical tomography
ELISA	enzyme linked immuno sorbent assay
ICG	indocyanine green
MPEG	methoxy(poly ethylene)glycol
MRI	magnetic resonance imaging
NIR	near-infrared
PD	photodetection
PDT	Photodynamic Therapy
PpIX	protoporphyrin IX
VIS	visible spectrum

1

Introduction

Optical imaging techniques for the assessment of tissue anatomy, physiology, metabolic and molecular function have emerged with tremendous vigour in the past few years. The attractiveness of this technology arises from the fact that fluorescent dyes can be detected at low concentrations and non-ionizing, harmless radiation can be applied repeatedly to the patient. Furthermore, the indisputable progress in the development of optical instrumentation in the last two decades (e.g. laser diodes, CCD cameras) has decisively contributed to the growing applicability of optical imaging techniques which have the advantage of being cheap, small in size and, therefore, easily at hand to solve clinical problems. The design of contrast agents for optical *in vivo* imaging of diseased tissues has likewise emerged and is reflected by an increasing number of publications in this area [1, 2]. Novel probes have been synthesized and characterized for their capability to monitor disease-specific anatomic, physiological and molecular parameters through their optical signals.

In the course of this review, the essential literature in this diagnostic field is discussed with a distinct focus on the chemistry of the optical contrast agents. The agents reviewed in this article were categorized by their structural family covering the cyanine dyes, tetrapyrrole-based compounds, fluorescent lanthanide chelates, fluorescein and oxazine dyes. For selected examples, the physicochemical, biochemical and pharmacological features contributing to the diagnostic efficacy of the compounds are illustrated. Alternatively, it might have been appro-

priate to group the compounds according to their pharmacological and biochemical properties, for instance whether or not a target-directed, molecular vehicle is employed, or the administered drug undergoes chemical conversion to report malignant conditions. These issues are rather considered as accompanying aspects throughout the article. In a subsequent chapter, the essential medical applications currently under experimental or clinical evaluation are discussed. Finally, some concluding remarks and future prospects are given.

2

Principles of Optical Imaging

2.1

Tissue Optical Properties

Under the term optical imaging a large variety of different disciplines is encompassed. In general, the method uses light within the ultraviolet (UV) and the near-infrared (NIR) spectral region to characterize the optical properties of tissue. Generally, the interaction of photons with tissue is based on the following three processes: absorption of light, scattering of light and emission of fluorescence. These parameters can be separately utilized to characterize tissue optical properties. A fundamental observation for optical diagnostic procedures relates to the fact that the penetration depth of light in living tissue strongly depends on the wavelength used [3], because the number of absorption and scattering events in tissue is a function of the wavelength. Normally, the extent of scattering in tissue decreases with increasing wavelength [4]. For wavelengths below 600 nm, absorption dominates scattering resulting in a small penetration depth of hundredths of micrometers up to a few millimetres, so that only superficial assessment of tissues in this spectral region is possible. Direct visual inspection or characterization using microscopic techniques is an established practice in medicine. Hence, the modality is capable of generating images of tissue structures with high spatial resolution, as does microscopy. A primary field of application is the examination of tissue surfaces using optical fibres incorporated into endoscopes or laparoscopes, as well as of ocular diseases through ophthalmoscopes and direct assessment of skin diseases or tissues during surgical procedures.

The absorption of light in tissue originates from oxy- and deoxyhaemoglobin, other porphyrins, melanin, and structures involved in cellular metabolism, such as NADH and flavins, as well as from several structural tissue components, such as collagen, elastin and lipo-pigments. Most of these compounds exhibit characteristic fluorescence spectra (tissue autofluorescence) throughout the visible (VIS) spectral region up to approximately 700 nm [5, 6]. Fluorescence from these intrinsic fluorescent markers can provide additional information on tissue structure and pathophysiological states [5, 7]. More specifically, the phenomenon of tissue autofluorescence has been thoroughly exploited to extract spectral patterns which indicate diseased tissue areas, e.g. in endoscopy of the gastrointestinal tract [6, 8–10], lymph node characterization [10] or cardiovascular diagnosis [11].

Imaging of larger tissues volumes requires light within the NIR spectral range (700–900 nm), because the absorption coefficient of tissue is relatively

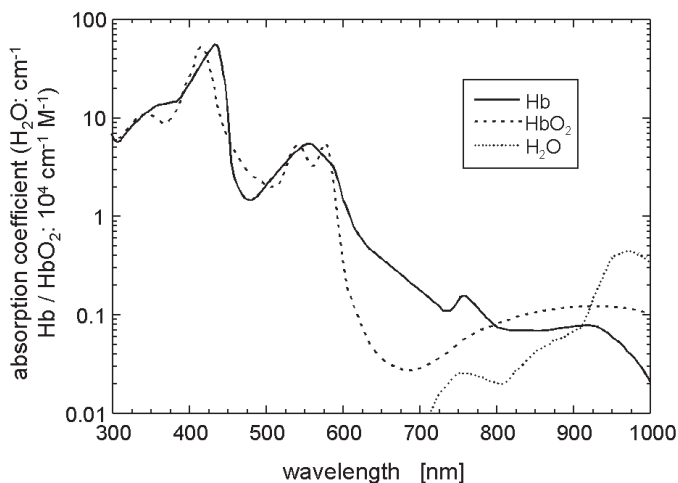


Fig. 1. Absorption coefficients of oxyhaemoglobin, deoxyhaemoglobin and water as a function of wavelength. The near-infrared window of tissue is defined as the spectral region between approximately 700–900 nm, where the absorption coefficients are at minimal levels

small resulting in penetration depths up to a few centimetres (Fig. 1) [12]. Thus, the identification of inhomogeneities exhibiting a difference in absorption or fluorescence compared to the bulk tissue is possible. However, due to scattering, photons do not follow straight paths when propagating through tissue and mathematical models of photon transport are needed to calculate the optical properties of tissue. This effect limits the spatial resolution of images obtained, so that morphological and structural tissue parameters are not accessible. Nevertheless, tissue absorption is mainly determined by oxyhaemoglobin, deoxyhaemoglobin and water, which exhibit a well defined minimum in absorption in the NIR spectral region (Fig. 1) and provide information that can be utilized to quantitatively calculate important physiological parameters, such as blood concentration (total haemoglobin) and oxygenation (ratio oxy/deoxy haemoglobin) [13]. These absorption data, together with tissue-dependent scattering properties, can be fitted by mathematical models to reconstruct the most probable photon propagation through tissue and generate a spatial map of tissue optical properties for a given illumination and detection geometry (see Chapter 2.2). This method has primarily been applied to detect breast tumours and image brain function, both of which have initiated a great deal of effort in the past few years (see Chapter 4). It is beyond the scope of this review to elucidate the underlying basics in more detail. For the specialized reader, references [4, 14–17] are recommended, in which the subjects of photon migration and diffuse light imaging are comprehensively described.

2.2

Function of Optical Contrast Agents

As in the established imaging modalities, such as X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and ultrasonic imaging (US), the

exogenously applied optical contrast agents principally provide the opportunity of engendering disease-specific signals within the tissue, thus enabling the display of physiological and molecular conditions which are characteristic of a certain disease state and progression. In principle, contrast enhancement is achieved when an administered agent changes the absorption, scattering or fluorescence properties of the diseased tissue to a different extent relative to the surrounding normal tissue. While it is difficult to influence scattering artificially, the absorption and fluorescence are easily affected by soluble dyes. Generally, the choice of the spectral range of absorption and fluorescence of the dye determines whether it is detectable on tissue surfaces (UV-VIS dyes) or from deeper located tissue areas (NIR dyes [18]). A prerequisite for sensitive detection of a contrast-enhancing dye is a high extinction coefficient at the desired absorption wavelength. If fluorescence photons are acquired, the compounds should exhibit large Stokes' shifts (spectral distance between absorption and fluorescence maximum) and high fluorescence quantum yields in physiological media.

The detection of fluorescent contrast agents is comparable to nuclear imaging methods, as in both modalities the photon sources (fluorescent dye or radionuclide) are distributed within the tissue. If fluorescence is recorded within the UV/VIS spectral region, both autofluorescence and the administered contrast agent will contribute to the observed signal, while in the NIR spectral region tissue autofluorescence is negligible due to the absence of endogenous NIR fluorophores (also see Chapter 2.1). In the latter case, the detected signal nearly exclusively reveals the distribution of the contrast agent. An important advantage of fluorescent over radiodiagnostic agents is that fluorescence can be excited continuously and is not limited like radioactive decay. A set of photophysical properties are accessible, ranging from changes in fluorescence quantum yield and fluorescence lifetime to alterations of spectral signatures. Moreover, for many dyes these parameters are influenced by local physiological or molecular conditions, such as pH, ions or oxygen and have therefore been utilized for their monitoring and quantification. Finally, light applied for fluorescence imaging is completely harmless.

2.3

Imaging Techniques

Two situations have to be taken into account: (i) imaging of superficial objects where reflected or scattered photons are directly measured, and (ii) diffuse imaging where photons are recorded after passing through relatively thick tissue and optical properties of the tissue are spatially reconstructed using mathematical models. Each of these applications requires different technical solutions and instrumental geometries. Images from superficial structures are generally obtained in reflection geometry by illumination with light of a desired wavelength and detection with suitable devices (e.g. CDD cameras). This process is similar to conventional photography. A possible way to image larger tissue volumes is to use transillumination geometry such that defined tissue areas are illuminated with light, and the transmitted, scattered light is detected in

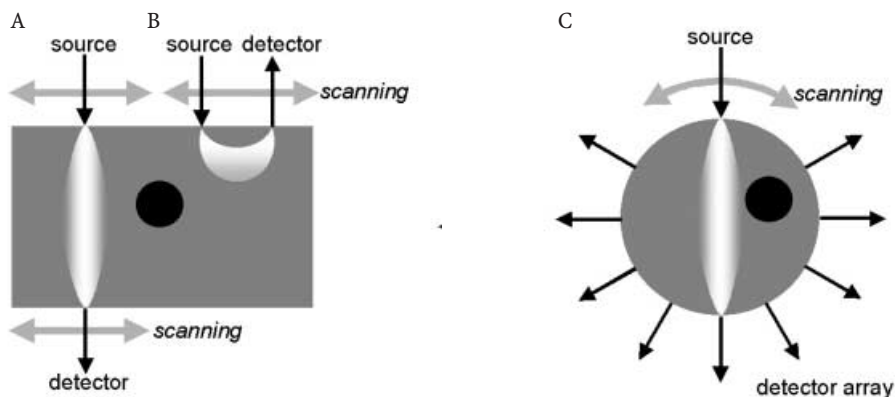


Fig. 2. Schematic illustration of imaging geometries. (A) transillumination of tissue by way of parallel scanning of source and detector leading to 2-dimensional projection images, (B) reflection imaging by detection of transmitted photons in close distance to the source and (C) optical tomographic imaging with a source and cylindrical detector array to detect multiple photon trajectories in different layers leading to a 3D data set. Light scattering results in a diffuse, banana or cigar – like shaped photon function which represents the most probable volumes where photons passed through when detected at a given position. Due to this effect a sharp demarcation of optical inhomogeneities in thick tissue volumes is not possible

180° projection geometry (in principal similar to X-ray mammography) [19–21]. Stepwise scanning of the desired area and image reconstruction from each single measurement of transmitted light intensity yields two-dimensional projection images. Alternatively, transmitted photons can be detected in reflectance geometry (0°) at close distance to the incident light beam [22]. Diffuse optical tomography (DOT) is based on the detection of photons at multiple positions and the mathematical reconstruction of three-dimensional optical images [4, 14–16]. Figure 2 shows a simplified sketch of possible imaging geometries. In principle, this technology permits the detection of transmitted, attenuated light at the incident wavelength as well as the sampling of red-shifted fluorescence of a contrast agent originating from within the tissue.

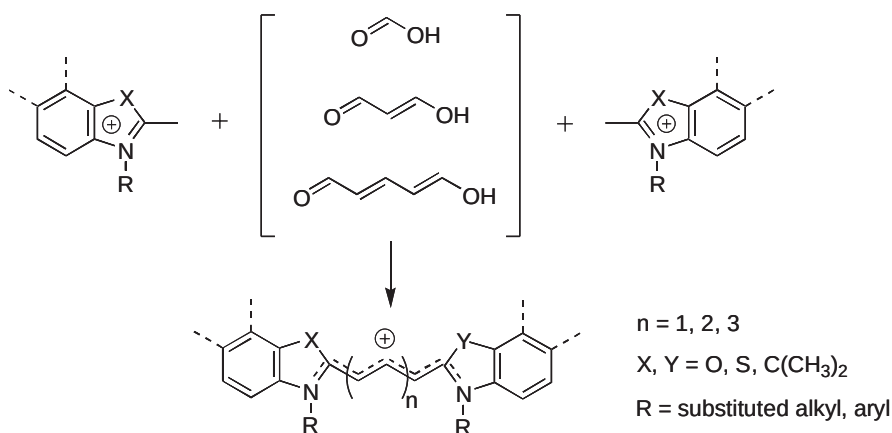
The information content of the detected light depends on the light source used for tissue illumination. Three different techniques are possible: (i) continuous wave (CW) light, (ii) intensity-modulated light of defined modulation frequency, and (iii) short (picosecond) laser pulses. While with technique (i) only the attenuation of intensity can be utilized to reconstruct optical images, technique (ii) additionally provides information on the phase shift of the modulated light relative to the initial light wave. Methods based on intensity-modulated light allow calculation of the absorption and scattering properties of the tissue separately from each other. Finally, pulsed light sources in technique (iii) and time-resolved detection with temporal resolution in the picosecond range permits a separation of early arriving photons from those, which underwent significantly more scattering events (late arriving photons). Here, the improvement of spatial resolution of images is possible by only utilizing the early photon fraction for image reconstruction [12, 16, 17].

3 Fluorescent Dyes as Contrast Agents

3.1 Cyanine Dyes

3.1.1 *Carbocyanine Dyes in Biomedicine and Analytics*

Cyanine dyes are structurally defined by a conjugated chain of an odd number of carbon atoms linked between two nitrogen centers forming a polymethine chromophore [23]. Among this class of compounds, carbocyanine dyes comprising benzoindolic or indolic entities at each side of the chromophore have been synthesized in a large variety of chemical derivatives. Basically, the carbocyanine structure is built up by reaction of two (identical or different) indolenines with a C1, C3 or C5 unit to yield a trimethine, pentamethine or heptamethine chromophore, respectively. Scheme 1 illustrates the underlying reaction principle.



Scheme 1. Principle of cyanine dye synthesis leading to trimethine ($n=1$), pentamethine ($n=2$) and heptamethine ($n=3$) chromophores. Structures comprising indolic subunits are usually named indocarbocyanine, indodicarbocyanine and indotricarbocyanine, respectively. Formic acid, malonic aldehyde, glutaric aldehyde are used in their protected dianilide or orthoester form. They can be applied as substituted derivatives to introduce residues into the polymethine unit. The indolic substructure might bear further residues or annelated aromatic rings

The length of the polymethine chain and the nature of the condensed exterior aromatic systems determines the absorption and fluorescence emission wavelength range, thus allowing the adjustment of the optical properties of the dye to a given detecting instrumentation. Carbocyanine dyes of visible to near-infrared absorption and fluorescence (approx. 450–900 nm) generally exhibit high molar extinction coefficients ($>150,000 \text{ M}^{-1} \text{ cm}^{-1}$) and good fluorescence quantum yields (up to 50 %). They were originally used in the photographic industry and

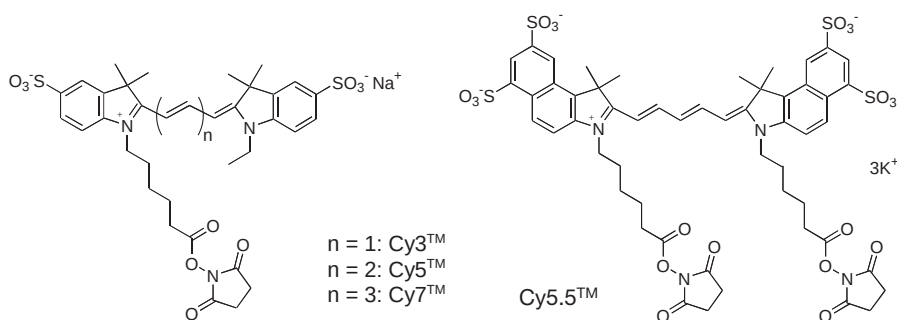


Fig. 3. Commercially available carbocyanine dyes with reactive groups for fluorescence labelling purposes. Cy3 (absorption maximum 550 nm, fluorescence maximum 570 nm), Cy5 (650 nm/670 nm), Cy7 (743 nm/767 nm) and Cy5.5 (675 nm/694 nm); Amersham Pharmacia Biotech, Freiburg, Germany

have also been designed as probes for a host of applications in analytical chemistry, biophysics, bioanalytics and biomedicine. These subjects are comprehensively reviewed e. g. in [24, 25]. In that respect, a primary synthetic goal has been to provide novel fluorescent markers for the application in immunoassays, screening assays or genetic analysis. Many of the structures developed for these purposes contain reactive groups e. g. N-hydroxysuccinimidyl esters [26–30], isothiocyanates [27, 31], iodoacetamido [32] or maleimido [33] functionalities. Figure 3 depicts a selection of chemical structures. The CyDyeTM-series (e. g. Cy3, Cy5, Cy5.5, Cy7) is commercially available (Amersham Pharmacia Biotech, Freiburg, Germany). Further chemical modification with non-biological residues affecting hydrophilicity and molecular weight or covalent reaction with target-specific biological vehicles, such as antibodies, proteins or peptides has extended the action spectrum of cyanine dyes towards *in vivo* probes for optical detection of diseases. For this purpose, particularly the NIR absorbing and fluorescing structures are currently of significance and are discussed in more detail in Chapters 3.1.2 and 3.1.3.

3.1.2

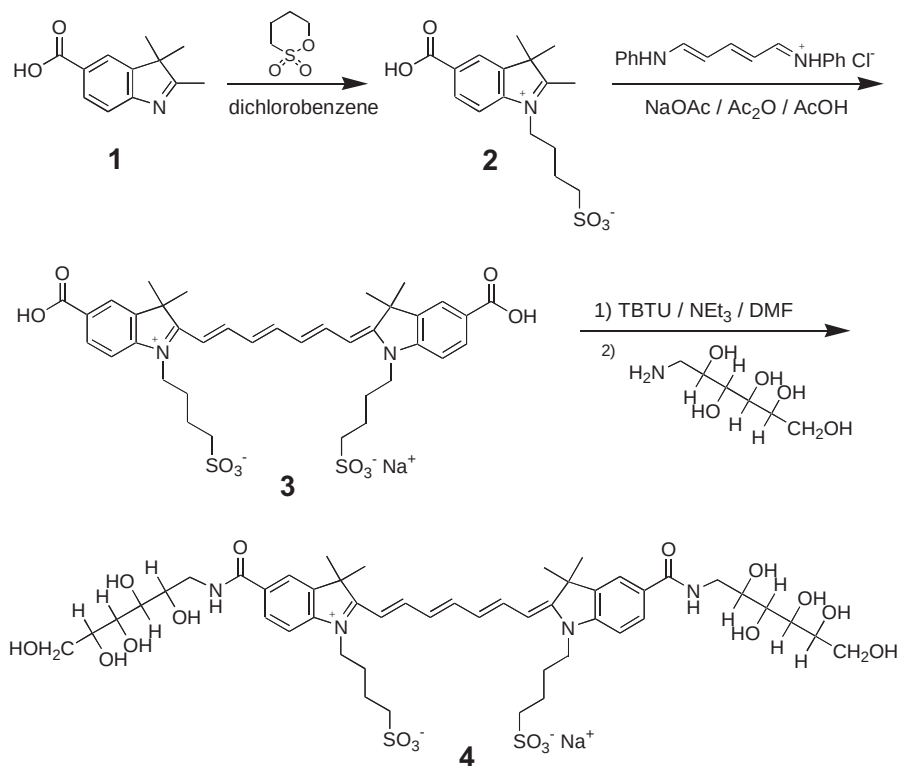
Non-Specific Carbocyanine Contrast Agents

A possible way to improve differentiation between normal and diseased tissues is the use of non-specific contrast agents which achieve contrast enhancement based on morphological and physiological properties of tumour tissue. Contrast agents for X-ray, CT and MRI are preferentially taken up by tumour tissue shortly after intravenous injection due to increased tumour vasculature, leaky endothelial structures and enlarged extracellular volumes [34], a phenomenon that is in principle exploitable for optical contrast agents.

A prominent representative example of a near-infrared absorbing cyanine dye is Indocyanine Green (ICG). This compound was first synthesized in the fifties [35] and was clinically approved as a diagnostic drug for the assessment of hepatic function and cardiac output, for which ICG exhibits favourable phar-

macokinetic properties [36, 37]. In the 1990s it was discovered to act as an imaging agent and is now frequently applied in fluorescence angiography to visualize vascular disorders in ophthalmology (see Chapter 4). More recently, ICG has been studied as a potential NIR contrast agent for the detection of tumours both in animals [38–40] and patients [41]. The rapid blood clearance of ICG providing only a short time window for contrast-enhanced investigations [40] and the rather poor fluorescence in physiological environment [40, 42] has initiated attempts to design structurally related agents with improved properties.

The synthesis of ICG-related indotricarbocyanine derivatives with carboxy, hydroxyalkyl and monosaccharide residues was the subject of our own work [40, 43]. The aim was to overcome some of the limitations of ICG by providing derivatives of substantially increased hydrophilicity and reduced plasma protein binding. It was assumed that such compounds might be able to leave the vascular space and extravasate into the extracellular compartment, with a certain degree of preference for tumour tissues. Starting from 4-hydrazinobenzoic acid (1), the glucamine-substituted indotricarbocyanine 4 was obtained in a 4-step synthesis through the dicarboxylic acid derivative (3) by amide bond formation with two D-glucamine molecules (Scheme 2) [40].



Scheme 2. Synthesis of the highly hydrophilic cyanine dye derivative bis-1,1'-(4-sulfobutyl)indotricarbocyanine-5,5'-dicarboxylic acid diglucamide (4) [40]

In animal studies, carbocyanine **4** showed improved efficacy as an optical contrast agent relative to ICG based on two features: (i) higher tumour concentration and tumor-to-normal tissue contrast shortly after injection [40] and (ii) a high fluorescence contrast at 24 h after injection [44, 45]. The absorption and fluorescence emission spectrum of carbocyanine **4** is typical for indotricarbocyanines and is illustrated in Fig. 4 together with a fluorescence image of a tumour-bearing rat showing elevated tumour fluorescence 24 h after administration.

Indotricarbocyanine dye derivatives with substantially increased molecular weight were designed by attaching monocarboxylated versions of the chromophore to either methoxy(poly ethylene)glycols (MPEGs) of different molecular

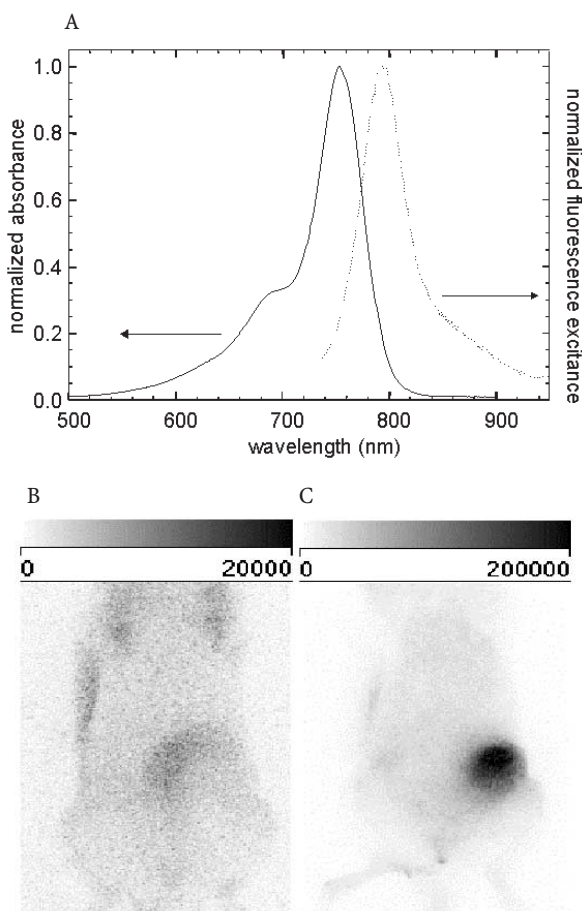


Fig. 4. (A) Normalized absorption (solid line) and fluorescence emission (dotted line) spectrum of diglucamide indotricarbocyanine **4** in bovine plasma (concentration $2 \mu\text{mol/L}^{-1}$); (B) fluorescence image of a tumor-bearing rat (MTLn-3 mammary carcinoma) in anterior view at 0 h and (C) 24 h after administration of **4** (dose: $2 \mu\text{mol/kg}^{-1}$ body weight), excitation wavelength 740 nm, detection bandpass 750–800 nm [45]. Copyright 2001 International Society for Optical Engineering (SPIE)

weight [46] or to biological macromolecules, such as transferrin or human serum albumin [47]. The aim of this approach has been to make use of the enhanced permeability of tumour vasculature (EPR-effect [48]) permitting macromolecules to extravasate preferentially into the tumour interstitium.

Although contrast enhancement with the agents covered in this chapter mainly relies upon a more distinct perfusion of tumours compared to normal tissue, as is achieved with ICG, and does not employ target-specific moieties, the value of contrast-enhanced tumour imaging was successfully demonstrated. These agents represent the classical format of a contrast agent.

3.1.3

Cyanine Dye Conjugates with Biomolecules

As discussed in Chapter 3.1.1, reactive cyanine dye derivatives are ideal fluorescent labels for tumour-specific carriers or vehicles, such as antibodies, proteins and peptides. In the literature, a variety of approaches can be found, where such dyes were employed in the design of fluorescent bioconjugates with enhanced selectivity for tumour-associated antigens.

The strategy of coupling cyanine dye labels to antibodies was first followed by Folli et al. and Ballou et al. [49–53]. The authors demonstrated target-specific uptake in experimental tumour models by planar imaging of superficial fluorescence patterns and fluorescence microscopy methods. However, as stressed critically by these and other researchers, antibodies have found only limited use as targeting vehicles for diagnostic agents in nuclear medicine and optical imaging [52]. The long circulation half-lives in the blood and their limited ability to penetrate into tumour tissue directly limits achievable signal-to-background ratios and does not allow separation of the signal of target-bound antibody from the background signal caused by the circulation of the antibody conjugate. Moreover, the allergenic and immunogenic potential of antibodies might hinder their use in purely diagnostic applications, especially for screening purposes.

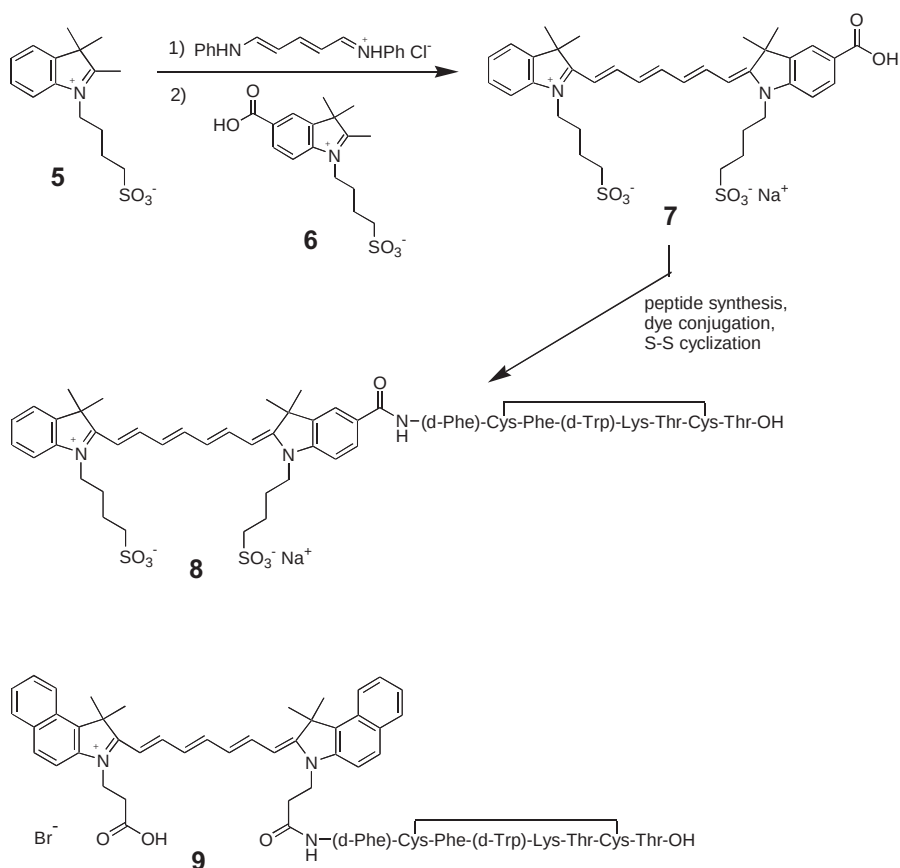
Generally, imaging contrast is improved when the circulating fraction of a systemically injected agent is cleared from the blood to the greatest possible extent while molecules localized in the target area maintain their concentration level. A possible way to approach this ideal situation involves the use of engineered antibody fragments of reduced molecular weight and preserved target affinity as target-specific vehicles for fluorescent dyes [54–56]. Neri and co-workers described antibody single chain fragments selected from phage display libraries [57] that were directed against an extracellular angiogenesis marker, a fibronectin isoform. This matrix protein is exclusively present in neoplastic blood vessels during angiogenesis. After labelling with fluorophores (Cy5 and Cy7, see Fig. 3) the antibody fragments were applied for *in vivo* fluorescence imaging of tumour angiogenesis in animal models [54, 55]. In addition, selective uptake in ocular neoangiogenesis in a rabbit model using a single chain fragment which was labelled with the photosensitizer Sn(IV)chorin e6 was demonstrated [58].

In typical labelling protocols, antibody or antibody fragment immunoconjugates with fluorophores are synthesized by incubating a buffered solution (pH

7.5–9) of the biomolecule ($0.5 - 10 \text{ mg/mL}^{-1}$) with molar excess of a reactive dye (typically 10–50 moles of dye per mole biomolecule) for a few minutes to several hours depending on dye reactivity and desired labelling ratio. Purification and separation from unbound dye is achieved by either dialysis, ultrafiltration or size exclusion chromatography. Inherently linked to the conjugation chemistry are suitable analytical methods, e.g. affinity chromatography or ELISA, to determine whether or not dye conjugation influences the binding affinity of the biomolecule. Moreover, the resulting fluorescent conjugates have to exhibit acceptable fluorescence properties that may depend on the dye used and on the dye-to-protein ratio in the final conjugate. In this respect it was demonstrated that labelling of an IgG antibody with Cy3 leads to fluorescence enhancement when comparing equal amounts of bound and free dye, while Cy5 and Cy7 cause severe fluorescence quenching particularly at labelling ratios above 2 [59].

A promising strategy for the development of target-specific contrast agents is the use of low molecular weight peptides as vehicles for diagnostic molecules. Many tumours are known to overexpress receptors for specific peptide ligands, e.g. somatostatin (SST) or vasoactive intestinal peptide (VIP) [60]. Pharmacologically optimized derivatives of these natural ligands have already been applied in radiolabelled form for the receptor scintigraphy of tumours. OctreoScan®, an ^{111}In -DTPA-conjugated SST analogue, is in daily routine clinical use as a radio-diagnostic agent for the detection of neuroendocrine tumours [61]. The literature reports some examples in which these principles were successfully adapted to optical receptor imaging by replacing the radiolabel by fluorescent cyanine dyes [62–66]. Achilefu et al. and Licha et al. described the synthesis and photophysical properties of cyanine dye conjugates with the SST analogue octreotate, a pharmacologically optimized octapeptide with high binding affinity to the SST receptor subtype 2 [62, 64]. In Scheme 3, the structures and synthetic access to cyanine dye-peptide conjugates are illustrated. The synthesis comprises the formation on non-symmetrically substituted cyanine fluorophores, in this example by reacting indolenine 6 and glutamic aldehyde dianilide in 1:1 molar ratio followed by the carboxyindolenine 7. In both of these publications it was shown that the conjugates are accessible by standard Fmoc solid-phase synthesis of the peptide, subsequent coupling of a indotricarbocyanine dye derivative with an activated carboxylic acid group to the N-terminal amino group, followed by cleavage of the conjugate from the resin and finally HPLC purification. Peptide cyclization through oxidative disulfide formation was either performed on resin (structure 9) [62] or after cleavage of the conjugate from the resin (structure 8) [64]. Photophysical characterization of the conjugates showed that the fluorescence properties of the conjugated dyes were less affected by the peptide leading to fluorescence quantum yields for indotricarbocyanine conjugates in the typical range of 10 % [64].

Fluorescence imaging experiments using SST-receptor positive animal tumour models revealed receptor-mediated uptake resulting in elevated fluorescence at the tumour site compared to normal tissue areas. In contrast to the *in vivo* imaging features of non-specific extracellular dyes or fluorescent single chain fragment conjugates, the highest contrast was achieved within just 1–2 h after intravenous injection and lasted up to 24 h [62, 65, 66]. This behavior is based on



Scheme 3. Synthesis of indotricarbocyanine-octreotate (8) [64] with the peptide linked at the 5-position of the benzolic system and chemical structure of the octreotate conjugate Cypate 9 [62, 66] with amide bond formation to the peptide through the propionic acid residue

receptor-mediated accumulation of the peptide conjugates in tumour cells, while non-bound molecules undergo rapid body clearance by the renal pathway [65].

Other compounds reported were conjugates with vasoactive intestinal peptide (VIP) and derivatives thereof [65, 67]. Based on VIP as the lead structure, it was shown that peptide conjugates with cyanine dyes could be prepared on solid cellulose supports to generate peptide libraries for drug screening and optimization [67].

R. Weissleder and co-workers have introduced an optical imaging approach, which is of fundamental difference to the approaches described above and utilizes the unique opportunity of modulating optical signals through intramolecular fluorescence quenching effects [68–72]. The probes described below represent “activatable” polymeric conjugates, which are not fluorescent upon intravenous injection, but are “switched on” when single fluorochrome fragments are liberated from the fluorescence-quenched polymeric

structure through cleavage by tumour-specific proteolytic enzymes. Thus, the agents are able to report information on protein function (here enzymatic activity).

In a first design approach, the contrast agent is composed of a poly(ethylene glycol) poly-(L-lysine) graft polymer, which is labelled with Cy5.5TM (see Fig. 3) at the ϵ -amino groups of free lysines. First, synthetic methoxy(polyethylene glycol) of 5,000 g mol⁻¹ molecular weight was modified with succinic anhydride, and the resulting carboxylic acid was activated with N-hydroxy(sulfo)succinimide. Conjugation with a 35,500 g mol⁻¹ poly(L-lysine) yielded a graft polymer of 450,000 g mol⁻¹ average molecular weight, which corresponds to a average composition of 92 MPEG molecules per poly(L-lysine). Finally, conjugation of Cy5.5TM with free lysines resulted in an average ratio of 11 dye molecules per polymer backbone (Fig. 5, structure 10) [68, 71]. In this bound state, the observed fluorescence intensity of the Cy5.5 fluorophore was 15-fold lower relative to the free dye. This drug exhibits increased fluorescence signals in the presence of the proteolytic enzymes cathepsin B and H by cleavage of the polylysine backbone and leads to a substantial signal increase both in cell culture and animal tumours [68, 70]. In Fig. 6 a fluorescence image of a tumour-bearing mouse demonstrates elevated tumour fluorescence 24 h after injection of the polymeric compound.

A further design stage for such activatable conjugates was achieved by incorporating a peptide sequence into the polymeric backbone. As illustrated in Fig. 5,

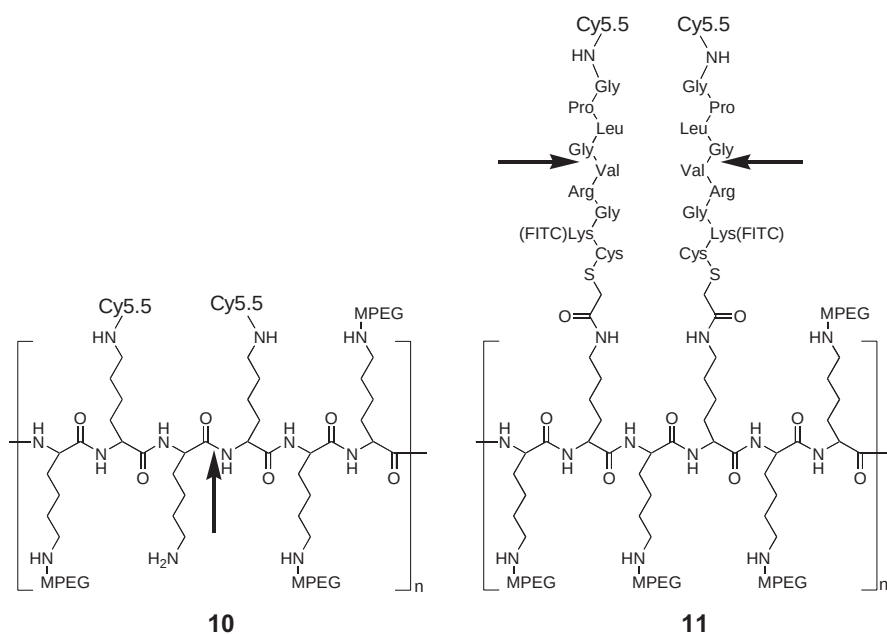


Fig. 5. Structures of Cy5.5-labelled poly(ethylene glycol) poly-(L-lysine) graft polymers 10 and 11. Arrows indicate the enzyme cleavage sites. Published with permission from Weissleder et al. [68, 69]

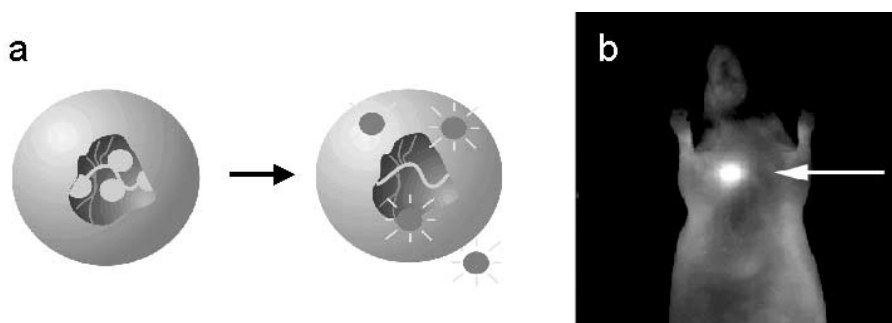


Fig. 6. (a) Schematic illustration of fluorescence activation process. Multiple fluorophores are attached to a polymeric carrier in close proximity to each other leading to quenching of fluorescence. Proteolytic cleavage of fluorophores enables the detection of fluorescence signals. (b) Fluorescence image of an LX1 tumour-bearing nude mouse at 24 h after injection of Cy5.5-labelled poly(ethylene glycol) poly-(L-lysine) graft polymer (dose: 10 μ mol dye/animal). Reprinted with permission from Weissleder et al. [68]. Copyright 1999 Nature Publishing Group

the graft polymer was modified with iodoacetamido groups, to which a peptide sequence was linked through selective reaction with the thiol group of a C-terminal cysteine [69]. Fluorophores are conjugated at the N-terminal amino group of the peptide and are again subjected to fluorescence quenching (structure 11). These peptide spacers can be easily modified to act as a substrate for tumour- or angiogenesis-specific enzymes with proteolytic activity, e.g. cathepsins [69] or matrix metalloproteinases (MMP's) [70, 72]. For compound 11, an increase in fluorescence intensity of up to 850 % in vitro in the presence of active MMP-2, a key proteinase involved in tumor angiogenesis, invasion and metastasis, was reported [72].

3.2

Tetrapyrrole-Based Dyes

3.2.1

Porphyrins, Chlorins, Phthalocyanines and Related Structures

Synthetic porphyrins, chlorins [73], benzochlorins [74], phthalocyanines [75] and expanded porphyrins [76] have been applied in a broad structural variety as photosensitizing agents for Photodynamic Therapy (PDT) of tumours [77, 78]. PDT involves the excitation of a systemically administered photosensitizer by light of an appropriate wavelength to induce the formation of cytotoxic singlet oxygen and radicals subsequently leading to apoptosis, cell death and tissue destruction [79]. While the therapeutic function of tetrapyrroles is not the main focus of this review, it is noteworthy to mention that these agents are potentially suited for diagnostic purposes, as many structures exhibit strong fluorescence in the VIS–NIR spectral region. The main rationale behind this type of application has been to utilize the fluorescence emission for real-time assessment of therapy progress and effectiveness during photodynamic treat-

ment. Data on biodistribution, pharmacokinetics and cellular localization can be directly obtained by measurement of the fluorescence of the administered drug. The term photodetection (PD) has been established in this context.

The first fluorescent tumour marker was a derivative of haematoporphyrin (HpD), which is not a uniform structure, but consists of various monomeric, dimeric and oligomeric porphyrins. During the 1960s and 1970s, this agent was thoroughly tested as both a photodynamic and diagnostic agent in patients, for instance for the fluorescence detection of esophageal, cervical or bladder carcinomas [80]. Other PDT compounds studied for their diagnostic capabilities were *meso*-tetra-*m*-hydroxyphenylchlorin (*m*-THPC) [81, 82], benzoporphyrin derivatives (BPD) [83], sulfonated phthalocyanines [84, 85], pheophorbides [86, 87] and derivatives of chlorin e6 [88–90]. Lutetium texapyrhin (Lu-TeX), an expanded porphyrin derivative with strong near-infrared absorption and fluorescence, is currently undergoing clinical trials as PDT agent for the treatment of several cancers and has additionally shown promise in the fluorescence angiography of ocular vasculature [91]. Fig. 7 illustrates the chemical structures of these compounds.

Compared to other classes of dyes, few attempts have been made to establish photosensitizing dyes as pure diagnostic agents, as in this context two major drawbacks of these compounds appeared: (i) the strong photodynamic activity and sometimes prolonged persistence of photosensitivity in several organs, particularly the skin, which is regarded as a potential safety concern, and (ii) the

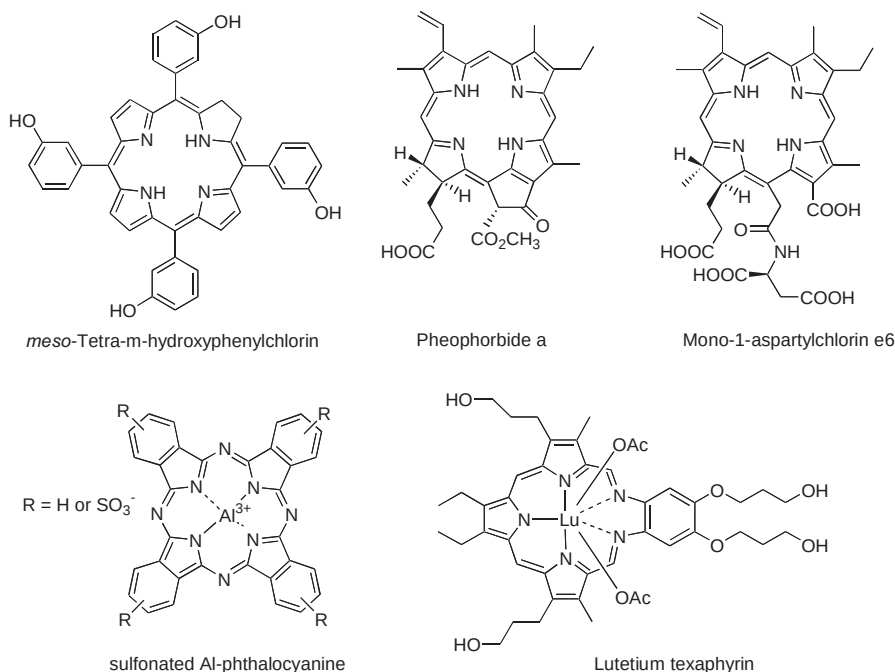


Fig. 7. Structures of photosensitizers studied as fluorescent agents for the detection of tumours

limited selectivity for diseased tissues with tumour/normal tissue concentration ratios usually not exceeding 3:1.

A few examples to render tetrapyrrolic compounds less phototoxic can be found in the literature. In one approach, carotenoid structures were employed for the synthesis of some carotenoporphyrin derivatives [92–94]. Figure 8 shows two structures by way of example. Due to similar photophysical properties of the two structural components, the excited triplet state of the porphyrin is quenched by the carotenoid moiety, thus inhibiting the formation of singlet oxygen, while its fluorescence capabilities are still preserved. Biodistribution studies revealed enhanced uptake into tumour tissue [39, 93, 95]. However, microscopy studies have shown that such compounds are associated with connective tissues in the tumors rather than with cancerous cells indicating low specificities for malignant transformation [96].

Another approach to minimize phototoxicity involved the synthesis of heterodimeric conjugates of two chlorine tetrapyrroles [97].

Similar to the approaches followed with cyanine dyes (Chapter 3.1.3), attempts to enhance the selectivity of photosensitizers by conjugating to target-specific vehicles have been described [98]. The approaches included conjugates with antibodies [99, 100], antibody fragments [58, 101], peptides [102], the serum proteins albumin and transferrin [103, 104] and estradiol [105].

3.2.2

δ-Aminolevulinic acid (Precursor of Protoporphyrin IX)

The mechanism of contrast enhancement using δ -aminolevulinic acid (ALA) [106] for PDT and PD differs fundamentally from other approaches where exogenous agents are applied. ALA is endogenously generated from glycine and succinyl CoA and represents an essential precursor in the heme biosynthetic pathway. Heme production takes place in almost all types of cells of the human organism and proceeds through different tetrapyrrolic intermediates. In the last step of the biosynthesis of heme, iron is incorporated into protoporphyrin IX (PpIX). This reaction is catalyzed by the enzyme ferrochelatase, which was suggested to have lower activity in many tumours [107]. Scheme 4 illustrates the essential intermediates in the heme biosynthesis. The administration of ALA as exogenous agent stimulates the intracellular synthesis of PpIX with a certain degree of (cell-type dependent) selectivity for tumours. This phenomenon engenders a temporarily increased intracellular PpIX concentration in tumour cells relative to healthy tissue. Upon irradiation with light, a cell-destructive, photodynamic effect is induced which has much higher selectivity for malignant cells and is accompanied by less side-effects than is usually achieved with exogenously applied photosensitizers. A comprehensive and detailed description of the principles of ALA applications in PDT, including biochemical, cell biological, pharmacological and clinical aspects, was published by Peng et al. [108].

While heme is not fluorescent, PpIX exhibits a fluorescence emission spectrum typical for porphyrins of this structural class (Fig. 9). Accordingly, PpIX fluorescence has been utilized to detect and visualize tumours and other tissue abnormalities in a large variety of clinical applications (see Chapter 4).

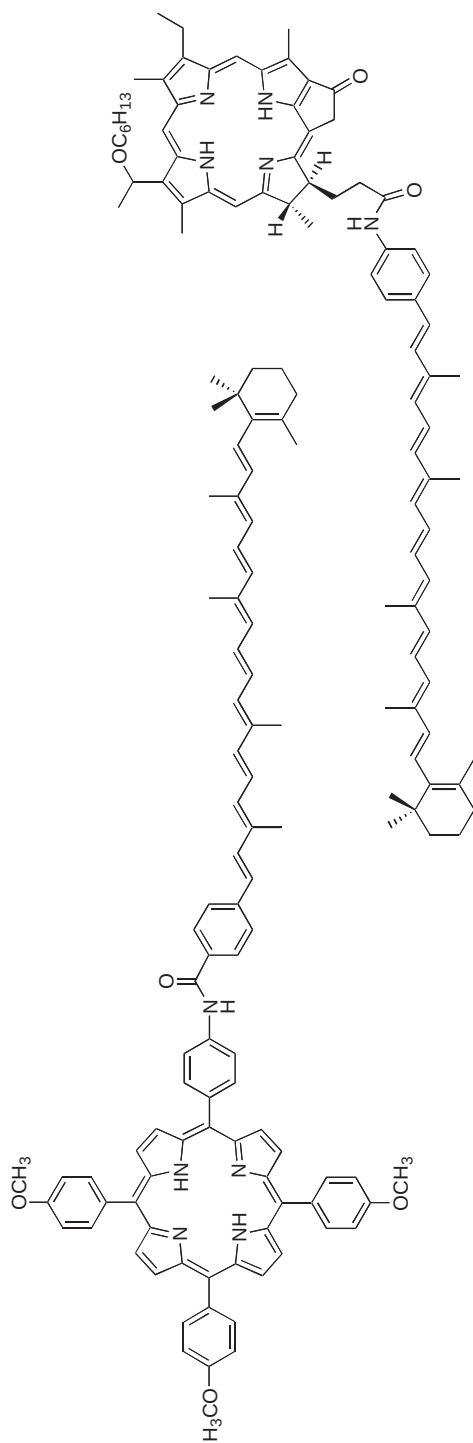
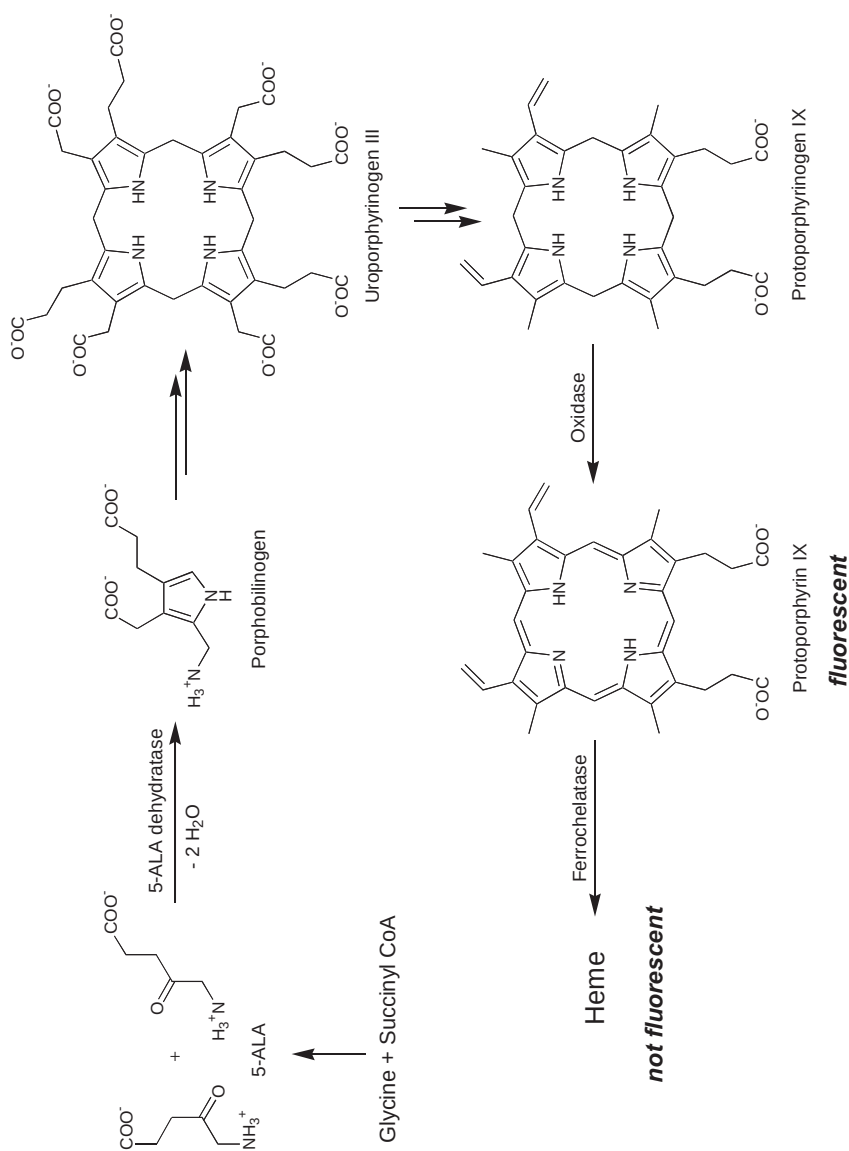


Fig. 8. Structures of carotenoporphyrins designed as fluorescent diagnostic agents with suppressed photosensitizing properties [39, 93, 95]



Scheme 4. The heme biosynthetic pathway. In vivo administration of δ -aminolevulinic acid induces accumulation of fluorescent protoporphyrin IX (PpIX) preferably in malignant tissues

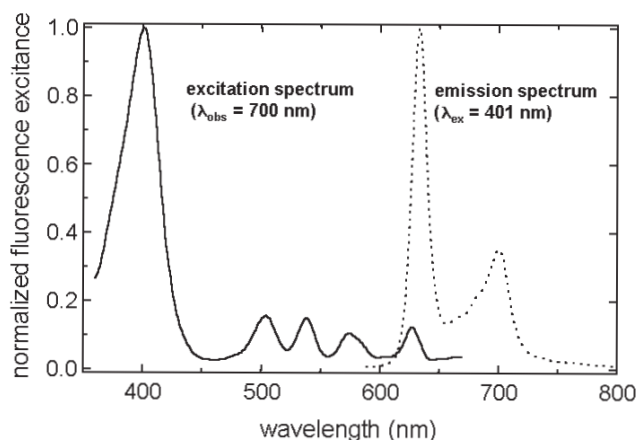


Fig. 9. Fluorescence excitation spectrum (solid line) and fluorescence emission spectrum (dotted line) of PpIX in methanol (kindly provided by B. Ebert, Physikalisch-Technische Bundesanstalt, Berlin)

The maximization of intracellular PpIX formation was achieved by optimization of treatment parameters (formulation, dose, way of application, etc.). A chemical approach directed towards improved ALA delivery to cells was followed by synthesizing ester derivatives of ALA, particularly alkyl esters of different alkyl chain length [109–111], ALA ethylene glycol esters and ALA-amino acids [112], which are converted into free ALA through ester cleavage by esterases. Many of these compounds penetrate more effectively into tissues and lead to enhanced amounts of accumulated PpIX [109–114]. In turn, approximately 30–150-fold lower ALA concentrations were required for comparable amounts of PpIX depending on the alkyl chain length used, with the highest benefit obtained with the n-hexyl ester [109, 113, 114].

3.3

Other Dyes

3.3.1

Fluorescent Lanthanide Chelates

Many of the lanthanides, e.g. La^{3+} , Eu^{3+} , Tb^{3+} , Nd^{3+} and Yb^{3+} , are known to form stable organometallic complexes with unique optical properties [115]. Particular attention has been directed towards Tb^{3+} and Eu^{3+} complexes which have been synthesized with large structural diversity [116–118] and applied in biotechnology and for the development of screening assays based on time-resolved detection techniques [25, 119–121]. When a UV–VIS absorbing aromatic structure is located close to the complexing moiety, the metal ion exhibits a characteristic emission spectrum upon light absorption through the aromatic system and subsequent intramolecular energy transfer of the absorbed energy to the metal ion. This ‘sensitized luminescence’ is characterized by very large Stokes’ shifts and long fluorescence lifetimes up to the millisecond range. The

emission spectrum is determined by the type of metal ion and typically consisted of several emission bands throughout the VIS–NIR spectral range. Terbium yields a bright green fluorescence (major peak at 550 nm) and europium fluoresces in red colour (major peak at 600 nm). Another focus for the chemical design of fluorescent rare earth complexes was driven by the fact that the optical properties of these compounds are sensitive to environmental conditions, such as pH, pO_2 , glucose, halide ions and alkali metals, permitting their use as chemical and biological sensors [122, 123].

The application of such compounds for the *in vivo* fluorescence detection of cancer has been proposed in the literature [124–127]. More specifically, a pycen-based macrocyclic complex with terbium, Tb-[N-2-pyridylmethyl)-N',N'',N'''-tris(methylenephosphonic acid butyl ester)-1,4,7,10-tetraazacyclododecane] or Tb-PCTMB (Fig. 10), has been studied as a fluorescent diagnostic agent for the detection of chemically induced colon tumours in rats [125, 126]. Bioconjugatable chelating derivatives of similar structure, consisting of a 1,4,7,10-tetraazacyclododecane system bearing a light-harvesting quinaldine structure, two phosphonic acids and a single carboxylic acid moiety for fluorescence labeling of target-specific biomolecules, have been described [128].

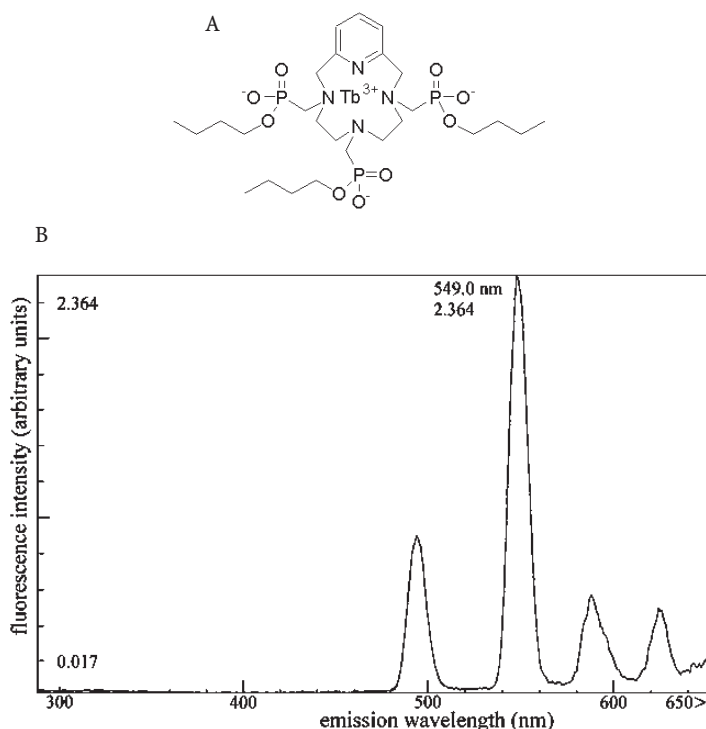


Fig. 10. (A) Structure and (B) fluorescence emission spectrum of Tb-[N-2-pyridylmethyl)-N',N'',N'''-tris(methylenephosphonic acid butyl ester)-1,4,7,10-tetraazacyclododecane] (Tb-PCTMB). Reprinted with permission from [126]. Copyright 1999 American Chemical Society

3.3.2

Miscellaneous Dyes

Probably one of the most frequently applied fluorescent dyes in bioanalytics, laboratory diagnostics and biomedical diagnostics has been fluorescein and its reactive derivatives, such as 5-aminofluorescein or fluorescein-isothiocyanate (FITC) and many other derivatized analogues [129]. In medical imaging, fluorescein is a routine drug for optical diagnosis in ophthalmology [130].

Other *in vivo* imaging applications include the preparation of fluorescent serum albumin conjugates with 5-aminofluorescein to yield diagnostic agents for the delineation of tumour margins during surgery [131]. In a different approach, the synthesis of fluorescent cobalamin derivatives employing FITC and other fluorescein-related fluorophors (Oregon Green, naphthofluorescein) was reported [132]. The use of these conjugates for the intraoperative visualization of cobalamin receptors was suggested. Finally mentioned is a phenoxazin-type dye, Ethyl Nile Blue A, which was used as a marker for the fluorescence-guided identification of pre-malignant lesions in animal models [133].

4

Medical Applications

Table 1 summarizes different modalities, detection principles and clinical indications which are currently at the level of experimental evaluation or in clinical use and employ exogenously administered optical contrast agents.

The only modality which is in daily and widespread routine clinical practice so far is the imaging of ocular diseases in ophthalmology. Fluorescein and ICG are established as fluorescent agents to enhance visualization of chorioretinal diseases, such as vascular disorders, retinopathies, neovascularization (age-related macular degeneration) or tumours [130, 134, 135].

The fluorescence-guided identification of human brain tumours and tumour margins during surgery was evaluated to facilitate the accuracy and safety of tumour resection and minimize the probability of tumour recurrence. For this purpose, ICG [136], fluorescein [137], 5-aminofluorescein albumin conjugate [131], and ALA [138] have been studied in selected patients or extended clinical trials.

In dermatology, the detection of PpIX fluorescence has shown promise for the visualization of skin cancers after topical application of ALA offering a convenient and simple method to monitor therapy progression and success [139–141].

Several types of superficial diseases in hollow organs have been monitored using fluorescence-guided endoscopy. Tetrapyrrole-based agents have been examined for this purpose [142] (see Chapter 3.2.1) but most promising results have been obtained with ALA. Clinical studies included the diagnosis of urinary bladder cancer [111, 143], bronchial cancer [144], various gastrointestinal diseases [8] and tumours in the oral cavity [145]. An essential aspect has been to improve the detection of dysplastic changes and carcinoma *in situ*, which escape the endoscopist's eye and require more reliable techniques. Beside ALA, other fluorescent agents of the kind presented here were suggested for these purposes [62, 65, 68, 125].

Table 1. Medical applications of optical imaging

Modality/Discipline	Principle	Signal	Clinical indication
Ophthalmology			Ocular diseases
Intraoperative diagnostics	Direct (microscopic) imaging	Reflected light, fluorescence	Complete resection of tumor margins
Dermatology			Skin tumors
Endoscopy			Tumours of the GI tract, lung, bladder, cervix, oral cavity
Cardiovascular imaging	Imaging via endoscopes or intravascular catheters	Reflected light, fluorescence	Atherosclerotic plaques
Optical mammography	Sequential and/or parallel detection of light at selected points; reconstruction of 2D, 3D images	Reflected or transmitted light,	Breast tumours
Brain imaging		fluorescence	Brain perfusion, stroke

Using flexible endoscopic catheters, intravascular fluorescence spectroscopy and imaging demonstrated that atherosclerotic plaques and other vascular abnormalities can be identified and characterized based either on endogenous fluorophores [11] or exogenous fluorescent dyes [146, 147].

Several approaches for the detection of breast cancer using NIR light and utilizing intrinsic tissue optical properties have been followed. Optical imaging of breast tissue dates back to 1929 [148] and was revived in the 1980s and 1990s when more powerful light sources and monitoring systems became available. Several clinical studies employing transillumination techniques based on modulated or pulsed light sources revealed the basic limitations of this modality. Suffering from low spatial resolution and the inability to differentiate between malignant and benign tissue, the clinical utility was limited and significantly inferior to X-ray mammography [12, 21, 149–152]. Diffuse optical tomography (DOT) using CW, modulated or pulsed light demonstrated advances in the quantification and 2D or 3D display of tissue absorption, scattering, vascularization and oxygenation. However, only limited numbers of patient data have been acquired and published so far [4, 15, 153–155]. The application of contrast agents to enhance the diagnostic value of optical breast cancer detection was proposed repeatedly [16, 17, 20, 39–41, 45]. The first contrast-enhanced imaging in a clinical setting was reported by Ntiachristos et al. who demonstrated uptake and localization of ICG in breast lesions using DOT [41].

Based on the same principles, DOT provides the opportunity to quantify cerebral blood flow and oxygenation in the brain [156]. Of particular clinical interest has been the application of ICG for neonates where imaging studies have

been performed in conjunction with the spectroscopic measurement of ICG clearance kinetics to minimize the risk of cerebral injury [157 – 159].

5 Conclusions

This review describes the current status of synthesis, experimental characterization, and clinical use of optical contrast agents for biomedical imaging. The current literature in this area obviously outlines the broad applicability these agents provide for the detection and characterization of diseases in living tissue. Various chemical, physical and biological principles were exploited for the development of novel imaging techniques. In that respect, a host of different chemical approaches were followed ranging from chemical derivatization of organic dyes and their functionalization with reactive groups, to solid phase conjugation and biolabelling techniques. Different photophysical parameters including the absorption coefficients, fluorescence quantum yields, fluorescence decay times and fluorescence quenching/recovery processes were featured to report information on morphology, physiology and molecular states.

The uniqueness of optical imaging, unlike any other imaging modality, derives from the fact that it is possible to combine conventional display of tissue volumes using direct-optical, transillumination or tomographic techniques, with the capability of gaining information on molecular properties and function due to the high instrumental sensitivity for optical signals. Considering the various principles of optical imaging methods but not disregarding the limitation of penetration depth and spatial resolution in thick tissues, a number of potential applications are envisioned to enter a stage where they are of significant value in clinical diagnostics. Indeed, optical diagnostic technologies are evolving throughout a broad spectrum of clinical disciplines including radiology, gynecology, gastroenterology, urology, surgery, ophthalmology and others. Recent progress in the development of sophisticated fluorescence-based bioanalytical assays, high-throughput screening methods and clinical *in vitro* diagnostics will create significant synergies. Finally, advances in imaging and laser technologies and the remarkable progress in biotechnology, from which new biological targets and designated biological vehicles will arise, will also have an important impact. Although a lot of effort is still necessary before optical contrast agents become widely established in clinical practice, it might be anticipated that optical imaging will play a vital role in clinical diagnostics and the development of novel therapeutic concepts in the future.

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Dynamic and Continuous Monitoring of Renal and Hepatic Functions with Exogenous Markers

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The liver and kidneys perform vital functions in the body and their numerous roles expose them to various forms of toxic materials and events. To prevent these organ failures, much effort has been devoted to the development of simple and reliable methods to monitor the functional status of the liver and the kidneys. All organ function measurements that use functional or clearance methods rely on the pharmacokinetics of endogenous biomarkers and various forms of exogenous radiopaque, radioactive, paramagnetic, photoactive, and organic molecules to monitor organ status. This review describes advances in the use of exogenous organ function-specific tracers for dynamic and continuous monitoring of renal and hepatic functions. Special emphasis is given to dynamic and continuous methods, which are illustrated with selected organ-specific markers. Thus, we reviewed the use of microsomal and cytosolic enzyme activities on lidocaine, caffeine, antipyrine, aminopyrine, coumarin, galactose and amino acids for dynamic measurement of hepatic function. The continuous measurement approach was illustrated by the clearance of indocyanine green. Similarly, the use of creatinine, iodinated aromatics, metal complexes, and organic dyes in dynamic and continuous renal function measurements is described. We conclude that the newer noninvasive continuous organ function monitoring with optical tracers, especially fluorescent markers, represents an important advance in patient care management.

Keywords. Renal function, Hepatic function, Near infrared fluorescent optical marker, Radioactive and paramagnetic metal complexes, Continuous and dynamic methods

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Abbreviations

CBT	Caffeine breath test
CT	Computed tomography
DMSA	Dimercaptosuccinic acid
DMX	Dimethylxanthine
DOTA	Cyclododecane-1,4,7,10-tetraazaacetic acid
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
FITC	Fluorescein isothiocyanide
GEC	Galactose elimination capacity

GFR	Glomerular filtration rate
HAMC	7-hydroxy-4-(aminomethyl)coumarin
ICG	Indocyanine green
MAMC	7-Methoxy-4-(aminomethyl)coumarin
MEGX	monoethylglycinexylidide
MMX	Monomethylxanthine
MRI	Magnetic resonance imaging
NIRS	Near infrared spectroscopy
OIH	<i>o</i> -Iodohippuric acid
PAH	<i>p</i> -Amino hippuric acid
PET	Positron emission tomography
SPECT	Single photon computed tomography
TMX	Trimethylxanthine

1

Introduction

This chapter reviews advances in the use of exogenous organ function-specific tracers for dynamic and continuous monitoring of renal and hepatic functions. The liver and kidneys are located in the abdominal cavity and perform vital functions in the body. Their numerous functions expose them to toxic materials that can result in chronic or acute diseases, which compromise normal organ function. Since organ failures are deleterious to health, much effort has been devoted to the development of simple and reliable methods to monitor the functional status of these organs. Static, dynamic (functional), and continuous (real-time) monitoring are the major approaches for evaluating them. The static method relies on intermittent or serial collection of biological fluids (for example, blood and urine) and subsequent remote analysis of the sample by various biological, chemical or physical techniques. The information obtained usually reflects end-point hepatocyte damage but may not detect fulminant, marginal, or early hepatic diseases [1]. A number of biochemical tests fall into this category, which remains the primary source of information for detecting and diagnosing many organ-specific diseases in clinical settings [2].

Dynamic methods¹, on the other hand, evaluate the physiologic activities of an organ over a short period and usually involve monitoring a marker's organ-specific activity in response to changes in physiologic functions. The marker and metabolites are conventionally quantified by remote or on-line analysis of discretely collected samples. While this approach is useful for measuring the functional capacity of the organ parenchyma, abrupt or moderate deviations from established baseline organ function may not sufficiently alter physiologic

¹ The terms *dynamic* and *continuous* are used to reflect methodological rather than physiological features. Thus, dynamic organ function monitoring uses discrete sampling or remote analysis to measure a marker's activity that reflects the functional capacity of the organ. This implies that continuous organ function monitoring procedures encompass dynamic processes but the events are measured on-line and in real-time.

activity within the test period, and hence may go unnoticed. Under such clinical conditions, continuous organ function monitoring is preferred.

Continuous monitoring of organ function displays in real-time the clearance profile or concentration of an organ function-specific marker in a pre-determined body compartment. This approach allows the early intervention by clinicians since instantaneous deviation from the established baseline reading may indicate the onset of an anomaly. However, it may not be useful for measuring dynamic functional reserve of the organ because subsequent activities after marker elimination from plasma may not be reflected in the clearance profile.

All organ function measurements that use functional or clearance methods rely on the pharmacokinetics of endogenous biomarkers and various forms of exogenous radiopaque, radioactive, paramagnetic, photoactive, and organic molecules to monitor organ status. Consequently, our primary focus is to review the exogenous markers and methods for organ function measurements.

2

Scope

Standard biochemical tests, methods not involving exogenous markers, and other static measurements are excluded, except in cases where a brief discussion is needed to place the subject in perspective. We recognize the enormous work on the diagnosis of pathologic tissues by various imaging modalities but this review is not about imaging studies. However, we have included pertinent information from imaging studies involving exogenous organ function markers that are relevant to our discussion. Some original and significant studies are indirectly cited when other works adequately described them. Omissions of important studies are not intentional and the interested reader is encouraged to consult the bibliographic section for further information.

3

Hepatic Function Monitoring

3.1

Basic Hepatic Anatomy and Physiologic Function

Strategically located in the upper part of the abdomen, the liver is the largest gland of the body. Its upper part lies close to the diaphragm and the lower segment rests on the abdominal viscera. The liver is divided into thousands of small functional units, the lobules, which are largely covered by peritoneum and the organ is held in place by intra-abdominal pressure. Microscopically, the liver cells (hepatocytes) are arranged in slabs and the cytoplasm of hepatic parenchyma contains unique particles that are amenable to staining and visualization by histochemical methods. The hepatocytes secrete bile that aids in fat digestion. Interwoven between the cells are highly porous capillaries that receive blood from the heart through the hepatic artery and from the intestine through the portal vein. This portal arrangement assures the passage of all ingested nutri-

ents and toxic products through the liver before entering the general circulation. The liver has many functions in the maintenance of life because of its size, strategic location, and huge hepatic parenchyma. These functions include blood storage and filtration, bile secretion, excretion of bilirubin and other xenobiotics, and many metabolic processes such as the conversion of glucose to glycogen and detoxification of drugs.

3.2

Hepatic Function Measurements and Disorders

The liver is the central organ that filters, stores, and detoxifies blood and its constituents. Thus, it is highly susceptible to a host of injuries because of its portal location and physiologic function. Blood distribution gradient exists within the liver and this heterogeneity results in differential exposure of various parts of the organ to injury. Hepatocytes closest to the portal vein and hepatic artery receive oxygen- and nutrient-rich blood supply, which makes them less susceptible to injury than those distal to blood supply [2].

Vulnerability of the liver to injury necessitates routine evaluation of hepatic function in patients and asymptomatic individuals to avert or control adverse clinical conditions. Thus, a plethora of methods has been developed for the diagnosis of liver diseases and dysfunctions. One such method uses physical palpation to determine alterations or changes in the orientation of the liver, which provides valuable information about the organ status but the quality of information is subjective and imprecise [3]. Another common method for the diagnosis of more serious hepatic injuries involves liver biopsies coupled with biochemical tests to determine the extent of liver injury and prognosis [4–7]. However, in acute and some chronic hepatic disorders, dynamic and continuous hepatic function monitoring would be advantageous.

3.3

Dynamic Hepatic Function Monitoring with Exogenous Organ-Specific Clearance Markers

Plasma clearance test is a good measure of hepatic blood flow but it does not necessarily evaluate the dynamic functional capacity of hepatic parenchyma [8]. Dynamic hepatic function tests generally rely on the role of the liver as the center for xenobiotic metabolism, and hence, reflect the functional hepatocyte mass or capacity. This is contrary to biochemical assays, which accurately quantifies end-point hepatocyte damage [1]. A good understanding of the biodistribution and metabolism of the marker is necessary to obtain reliable liver function tests. In general, the marker for dynamic hepatic function monitoring should possess the following properties [2];

- (1) Nontoxic to prevent further assault on the liver.
- (2) Effective in small doses in order to avoid significant side-effect.
- (3) Relatively small lipophilic molecule to improve hepatobiliary excretion and avoid complement activation.

- (4) Low hepatic extraction ratio and low protein binding to minimize reliance on hepatic blood flow.
- (5) Established hepatic metabolic route to define pharmacokinetics model.
- (6) Simple molecular design to avoid the production of complex metabolites that may interfere with detection and quantification techniques.

Although several markers for functional hepatic mass determination are currently used in clinical settings, most of them were originally used as drugs for various medical interventions. Nonetheless, these markers remain the mainstay of dynamic hepatic function monitoring, some of which are discussed below.

3.3.1

Markers for Dynamic Hepatic Function Monitoring by Microsomal Enzyme Activity

Cytochrome P450 enzymes reside in the endoplasmic reticulum in the centrilobular hepatocytes where metabolism of xenobiotics takes place. The role of these microsomal enzymes in the metabolism of lipophilic compounds forms the basis for monitoring functional hepatic mass by the rate of excretion of various compounds by the liver. Thus, any lipophilic molecule that is extracted by the liver and undergoes one of several reactions catalyzed by these enzymes can be used to assess hepatic function. Of these reactions, most of the markers discussed below favor the oxidative *N*-dealkylation metabolic pathway. This criterion requires the presence of *N*-alkyl group in the molecular structure of the probe. A major advantage of this biotransformation is the concomitant generation of *N*-dealkyl and CO₂ metabolites, which are amenable to biological fluid or breath analyses. Therefore, non-invasive determination of liver function can be performed by oral administration of drugs, followed by the detection of the gaseous metabolite. A major pitfall with the microsomal enzyme metabolism approach is the disposition of individuals to genetic variability of cytochrome P450 enzymes [9–11]. Nonetheless, when the potential sources of variability are adequately controlled, the microsomal enzyme marker approach is very reliable [12]. Some of the commonly used molecules are described below.

3.3.1.1

Lidocaine Clearance and Metabolism

Lidocaine [2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide] is a local anesthetic and antiarrhythmic drug whose plasma clearance and metabolism are widely used to measure hepatocyte metabolic capacity and hepatic blood flow, especially in patients with cirrhosis and chronic hepatitis [13–24]. This method is based on the efficient first pass elimination of lidocaine by the liver and subsequent biotransformation to monoethylglycinexylidide (MEGX) [25, 26]. In addition to MEGX, which is the primary metabolite of lidocaine (hence the name MEGX liver function test), the liver cytochrome P450 enzymes, including CYP3A3/4, convert lidocaine to a variety of other products through a series of oxidative *N*-dealkylation or hydroxylation reactions [27–40] (Fig. 1).

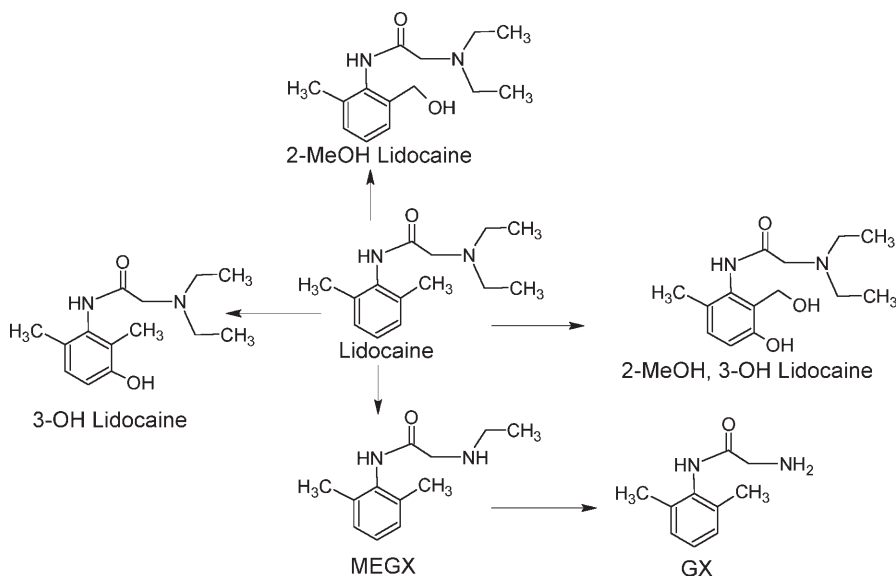


Fig. 1. Lidocaine and its metabolites: MEGX, monoethylglycinexylidide; GX, glycinexylidide

MEGX is readily detected by HPLC and fluorescence polarization immunoassay techniques [14, 21, 25, 40, 41]. The test is simple, normally requiring a one-time blood sampling, and informative because it depends on the capacity of the hepatic enzymes to metabolize lidocaine. While the analysis of lidocaine metabolites is rapid, this method has not been adapted for continuous hepatic function monitoring, which may be possible with the radiolabeled analogues such as ^{99m}Tc -Sn-lidocaine iminodiacetic acid [42].

The accuracy and precision of the MEGX test for mild or early hepatic disorders is still controversial because it is affected by many factors independent of disease processes, including genetic heterogeneity of P450 system, gender-specific variation, alteration of lidocaine plasma clearance by medication, and fluctuations in plasma clearance and hepatic metabolic rate of lidocaine [39, 41, 43–45]. The method is also bereft of standardized optimal MEGX sampling time point. Conflicting reports suggest 15, 30 or 60 minutes post injection of lidocaine, depending on the protocol used [16, 21, 43, 46, 47]. However, the MEGX test is generally reliable for monitoring and diagnosing advanced stage hepatic disorders.

3.3.1.2

Caffeine Clearance

Caffeine (3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) is a natural product from tea, coffee, and other plants, and is present in many beverages and food. It is primarily metabolized in the liver by cytochrome P450 enzymes to give *N*-demethylated xanthine and few other metabolites (Fig. 2) [2, 48–50]. About 17 caffeine metabolites have been identified in humans [51].

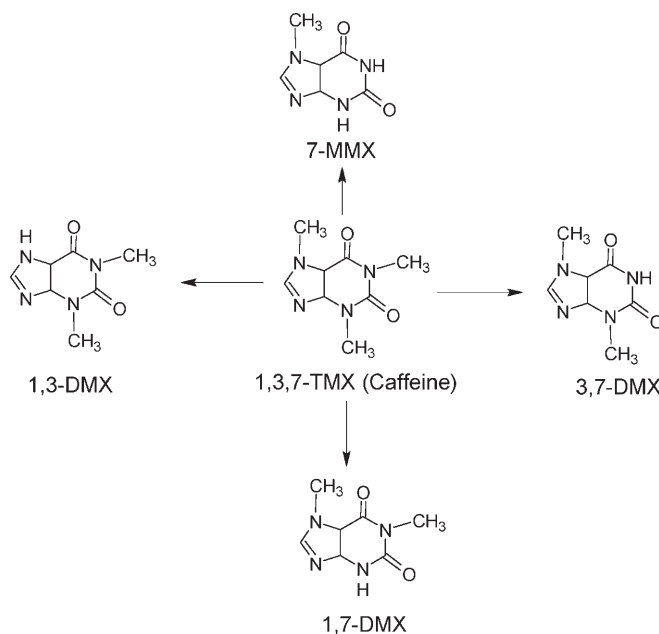


Fig. 2. Caffeine and some metabolites: TMX, trimethylxanthine; DMX, dimethylxanthine; MMX, monomethylxanthine

Due to its high oral availability, caffeine is normally dispensed orally but has also been administered intravenously to circumvent complication by oral delivery in critically ill patients [51]. Typically, the liver status is evaluated by analyzing caffeine and its metabolites in exhaled CO_2 (caffeine breath test, CBT) or in body fluids, including plasma, saliva, and urine [48–50, 52–56]. CBT is specific for cytochrome P4501A2 (CYP1A2) isoenzyme activity which removes the *N*-3 methyl group of caffeine to give the major caffeine metabolite, 1,7-dimethylxanthine (paraxanthine) and formaldehyde [2, 51]. The formaldehyde is further oxidized to CO_2 , which is used for CBT. To differentiate this metabolite from non-caffeine byproducts, the *N*-3 methyl group is conventionally labeled with ^{14}C or ^{13}C isotopes and the exhaled metabolite is quantified by differential mass spectroscopy ($^{13}\text{CO}_2$ to $^{12}\text{CO}_2$ ratio) or by a scintillation counter ($^{14}\text{CO}_2$) [52]. When body fluids are analyzed, it is not necessary to tag caffeine since the marker and a host of metabolites can be detected in plasma, urine, and saliva by highly sensitive HPLC and enzyme multiplied immunoassay techniques (EMIT) [57, 58]. If labeling is desired, there is little restriction on which carbon should be tagged.

Caffeine clearance can be completely non-invasive, simple, and reliable for monitoring the global functional hepatic capacity in advanced and chronic hepatic injuries. However, it may not be effective for detecting sudden hepatic malfunctions [59]. Analysis of caffeine metabolites also involves radioactive material or expensive equipment that is not conducive to critical or ambulatory care settings. Other factors that mitigate its usefulness include non-disease related variations resulting from differences in gender, age, drug interaction, and smoking

habits [12, 50]. As with all liver function agents that rely on cytochrome P450 isoenzyme metabolism, statistically significant differences may arise from genetic polymorphism of the enzymes rather than the diseased state of the liver. For example the caffeine clearance test does not detect certain types of diabetes [60]. However, a recent study identified and quantified sources of cytochrome P450 CYP1A2 variation and demonstrated that accounting for non-disease related factors diminishes the variation in the caffeine clearance test attributed to genetic polymorphism [12].

3.3.1.3

Antipyrine Metabolism

Antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazole-3-one) is an *N*-methylated analgesic drug which is primarily metabolized in the liver by microsomal cytochrome P450 enzymes [3, 61–68] (Fig. 3). The three main oxidative metabolites of antipyrine are 4-hydroxyantipyrine, 3-hydroxymethylantipyrine, and norantipyrine.

A combination of various properties of antipyrine gives it an advantage over other microsomal enzyme metabolizing drugs. These include high oral availability useful for non-invasive oral administration, lack of plasma protein binding which prevents environmental clearance variability, and fast distribution

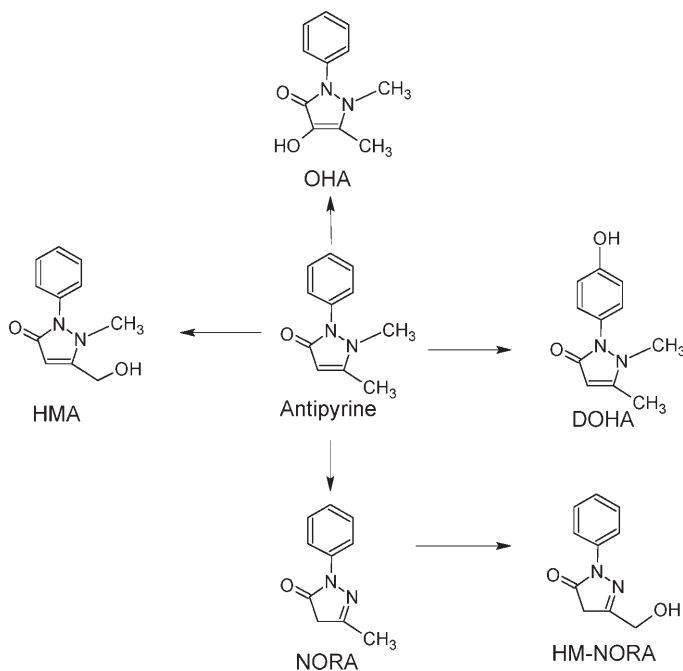


Fig. 3. Antipyrine and its metabolites: NORA, norantipyrine; HMA, 3-hydroxymethylantipyrine; OHA, 4-hydroxyantipyrine; DOHA, 4,4'-dihydroxyantipyrine; HM-NORA, 3-hydroxymethyl-norantipyrine [3, 72]

into body aqueous fluids, which allows rapid equilibration [69]. Its metabolites can be detected and quantified non-invasively as CO₂ by selectively labeling the *N*-2-methyl group with ¹³C or ¹⁴C isotopes, or in serum, urine and saliva [61, 64, 70, 71]. Tagging the *C*-3-methyl position is usually preferred for analysis of body fluids, if at all necessary [61, 72]. Methods used to quantify antipyrine and metabolites include HPLC [73–75], micellar electrokinetic capillary chromatography [70], and standard GC-MS. Analysis of ¹³C-enriched antipyrine in biological fluids by NMR is a direct, simple and sensitive technique [61, 72, 76] but the high cost of this instrument may prevent its routine use in clinical practice. Interestingly, the development of a high-throughput microplate reader assay for evaluating coumarin metabolite [77] and rapid diffusion immunoassay technique [78] may provide a widespread technology platform for the analysis and quantification of liver function markers.

Antipyrine clearance and quantification of metabolites represent a sensitive method for monitoring functional hepatocyte mass, especially in cirrhotic [66, 71, 79] and obstructive cholestatic [68] patients. However, the metabolism of antipyrine is not affected by certain hepatic disorders such as patients with massive liver neoplasm [80] or moderate to severe hepatitis C [64]. This attests to the difficulty in generalizing the reliability of information obtained from markers for evaluating functional hepatic reserve. Disorders that do not hamper the normal functioning of the metabolizing enzyme are not diagnosed by this method. This is in addition to individual variability reminiscent of other hepatic function tests that reflect microsomal enzyme metabolizing activities. One study also suggests the possibility of ethnic variability in the formation rate of certain antipyrine metabolites [63].

3.3.1.4

Aminopyrine Test

Aminopyrine (4-(dimethylamino)-1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazole-3-one) is an analgesic and antipyretic drug. In dynamic liver function evaluation, the aminopyrine breath test is commonly used to measure hepatic reserve in patients with acute and chronic liver diseases, and to monitor hepatic function during hepatectomy, organ transplant, and other medical interventions [71, 81–84]. A recent report suggests that the diagnostic capability of breath tests may be enhanced when multiple substrates are administered to patients [20]. Other studies have quantified aminopyrine metabolites in biological fluids and demonstrated the reliability of this approach [30, 85], especially when maintenance of endogenous CO₂ production or breath sample collection is difficult [86].

The structural feature of aminopyrine is similar to antipyrine except that aminopyrine has a substituent (dimethylamino group) at the *C*-4 position (Fig. 4). The exceptional efficacy of the aminopyrine breath test derives from the presence of multiple *N*-methyl groups that are readily removed by oxidative *N*-demethylation. Hence, further oxidation of several formaldehyde molecules produced by demethylation of aminopyrine gives large volumes of labeled CO₂ that is readily quantified. Some of the metabolites of aminopyrine are given in Fig. 4 [30, 85, 87–90].

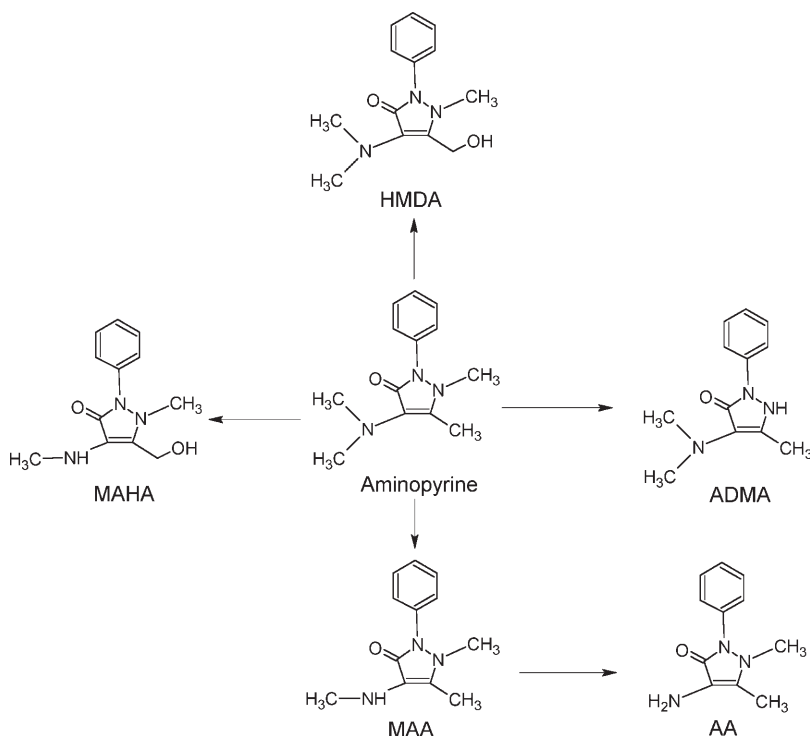


Fig. 4. Aminopyrine and metabolites: MAA, 4-monomethylaminoantipyrene; MAHA, 4-monomethylamino-3-hydroxymethylantipyrene; HMDA, 3-hydroxymethyl-4,4-dimethylaminoantipyrene; ADMA, 2-amino-4,4-dimethylaminoantipyrene; AA, 4-aminoantipyrene

Although aminopyrine breath test is non-invasive, precision of the data can be altered by many non-disease related factors such as gastric emptying and the patient's physical state [16]. In addition, most clinical tests currently in use monitor aminopyrine metabolite, $^{14}\text{CO}_2$, with the associated risk of radiation exposure to patients and personnel [16]. The use of ^{14}C may be supplanted by advances in the detection and quantification of ^{13}C -tagged molecules by gas isotope ratio mass spectrometry, infrared spectrometry, and other techniques [20, 81, 91–96]. Administration of repeated doses of the ^{13}C -labeled molecule is possible without the risk of increasing internal ionizing radiation in patients. With the recent observation that fasting is not necessary to obtain a reliable breath test, this technique can be extended to children and pregnant women who are unable to go without food for prolonged periods [97]. The exciting prospect of automatic and continuous measurement of gas-exchange, the development of precision gas analyzers, and advances in continuous flow isotope ratio mass spectrometry represent significant progress towards the development of a tandem dynamic-continuous hepatic function monitoring system [93, 98, 99]. Adaptation of these techniques to clinical settings, especially in critical and ambulatory care units, remains a challenge. Regardless of these developments, hepatic function breath

tests are indirect measurements that rely on a sequence of metabolic reactions that preclude absolute quantification of markers [100]. Aminopyrine is also a potent drug that is not ideal for routine diagnostic use. Studies have shown that it is a possible mutagen and has been implicated in some deleterious side effects [87]. However, its structural feature can be incorporated in the design of novel molecules for monitoring hepatic function by side-effects breath test.

3.3.2

Markers for Dynamic Hepatic Function Monitoring by Cytosolic Enzyme Activity

Determination of the level of cytosolic enzymes such as aspartate transaminase, alanine transaminase, and lactate dehydrogenase is part of standard biochemical liver function tests to measure hepatocellular necrosis [2, 101]. Cytosolic enzymes are not subject to genetic variations inherent in microsomal enzyme production. Liver cytosolic enzymes metabolize several molecules, of which galactose and amino acids are typical examples, used for hepatic function tests.

3.3.2.1

Galactose Clearance

The liver is an important site of carbohydrate and monosaccharide metabolism, a function that is used to measure the functional capacity of the liver. Consequently, the metabolism of several sugars such as fructose, sorbitol, and galactose has been explored as a possible indicator of functional hepatocyte reserve [11, 84, 102 – 105]. Of these, galactose clearance is widely used in clinical settings. Unlike fructose that is only partially metabolized in the liver, galactose is primarily metabolized in the liver, which accounts for more than 90% of its excretion from plasma [104]. In contrast to microsomal enzyme activity of the cytochrome P450 system that is affected by individual variability, galactose metabolism is controlled by a cytosolic enzyme, galactokinase, which is not influenced by genetic and environmental factors when appropriate conditions are used. At high plasma concentrations (500 mg galactose/kg body weight), the clearance of galactose follows zero-order kinetics between 20 and 60 minutes post administration [2, 107, 1046]. At this concentration, the rate-limiting phosphorylation of galactose by cytoplasmic galactokinase is saturated, hence the zero-order elimination appropriately reflects functional hepatocyte mass. At lower concentrations, the galactose clearance follows a first-order kinetics and measures effective hepatic blood flow. Advances in monitoring techniques have simplified the detection and quantification of galactose and its metabolites in expired CO₂ and body fluids. Time-consuming colorimetric and fluorometric galactose oxidase or dehydrogenase-coupled enzymatic reactions may be supplanted by the rapid and very sensitive HPLC method [107]. Also, the new single point measurement may replace the conventional method that determines galactose elimination capacity (GEC) by time-consuming serial collection and analysis of blood samples between 20 and 60 minutes post injection [104, 108 – 112]. In the new technique, a single blood sample at 30, 45 or 60 minutes post bolus injection of galactose has a good correlation with the cumbersome GEC method. One report, however, refutes the reliability of the single

point test in patients with advanced hepatic diseases [112]. A recent study on the single point concentration measurement in birds suggests that 80 min post administration gives the best correlation between single point and the GEC techniques [113]. Thus, the possibility of interspecies variation exist in galactose metabolic rate. The galactose breath test is also widely used in clinical settings [104, 105, 108]. The expired CO_2 from 1- ^{13}C or ^{14}C -labeled galactose metabolism is proportional to hepatocyte metabolic activity. Improvements in the detection and quantification of $^{13}\text{CO}_2$ by GC-FTIR analysis of the $^{13}\text{C}/^{12}\text{C}$ ratio may replace the radioactive ^{14}C approach in clinical settings [98]. Hopefully, these modifications will reduce the current disadvantages of the GEC test, which is time-consuming, technically cumbersome, invasive, and expensive [16].

3.3.2.2

Hydrophobic (Aromatic) Amino Acid Breath Test

The liver is also the principal metabolic center for hydrophobic amino acids, and hence changes in plasma concentrations or metabolism of these molecules is a good measure of the functional capacity of the liver. Two of the commonly used aromatic amino acids are phenylalanine and tyrosine, which are primarily metabolized by cytosolic enzymes in the liver [1, 114–117]. Hydroxylation of phenylalanine to tyrosine by phenylalanine hydroxylase is very efficient by the liver first pass effect. In normal functioning liver, conversion of tyrosine to 4-hydroxyphenylpyruvate by tyrosine transaminase and subsequent biotransformation to homogentisic acid by 4-hydroxyphenylpyruvic acid dioxygenase liberates CO_2 from the C-1 position of the parent amino acid (Fig. 5) [1, 118]. Thus, the C-1 position of phenylalanine or tyrosine is typically labeled with ^{13}C and the expired $^{13}\text{CO}_2$ is proportional to the metabolic activity of liver cytosolic enzymes, which corresponds to functional hepatic reserve. Oral or intravenous administration of the amino acids is possible [115]. This method is amenable to the continuous hepatic function measurement approach by monitoring changes in the spectral properties of tyrosine pre- and post-administration of the marker.

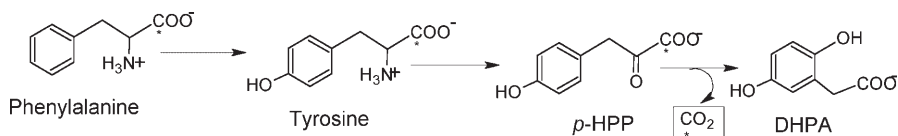


Fig. 5. Metabolism of labeled phenylalanine: *p*-HPP, *p*-hydroxyphenylalanine; DHPA, 2,5-dihydroxyphenylacetic acid (homogentisate)

3.3.3

Others

Generally, the liver is the center of drug metabolism, hence numerous drugs and methods to measure functional hepatic capacity are available. A host of these methods rely on the metabolic activity of cytochrome P450 enzymes, and some of the markers used include phenacetin, methacetin, trimethadione,

coumarin (catalyzed by CYP2A6), phenytoin (CYP2C6), chlorzoxazone (CYP2E1), and nifedipine (CYP3A3/4), erythromycin (CYP3A3/4), and S- mephenytoin (CYP2C19) [1, 10, 119–125]. A recent report suggests that simple ^{13}C -labeled methacetin breath test at 10 minutes post injection is useful for diagnosing hepatic dysfunction with exceptionally high sensitivity (98%) [114]. The dynamic hepatic function monitoring approach will enormously benefit from recent developments in high-throughput screening of biological fluids [126]. This method was successfully used to screen coumarin metabolites [77, 120, 127]. An important feature of the coumarin derivatives employed is the significant differences in the fluorescent properties of the liver marker 7-methoxy-4-(aminomethyl) coumarin (MAMC) and its metabolite, 7-hydroxy-4-(aminomethyl) coumarin (HAMC, Fig. 6) [127, 128]. Biotransformation of MAMC to HAMC results in emission (λ_{max}) shift from 396 to 470 nm. Thus, metabolism of coumarin can be monitored continuously and quantified by spectroscopic techniques.

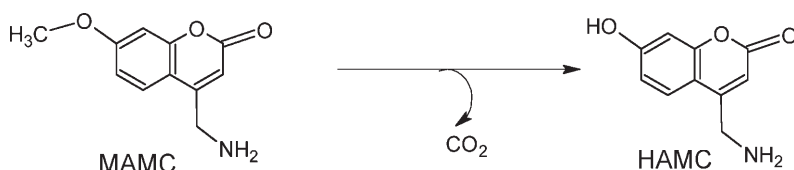


Fig. 6. Biotransformation of 7-methoxy-4-(aminomethyl) coumarin (MAMC, emission 396 nm) to 7-hydroxy-4-(aminomethyl) coumarin (HAMC, emission 470 nm) by cytochrome P450 enzyme CYP2D6

Newer and potentially better hepatic function index continues to captivate researchers. A recent report indicates that hippurate ratio is a good measure of functional hepatocyte capacity [13]. The procedure monitors the conjugation of glycine to *p*-aminobenzoic acid by liver enzymes and may provide unique information on functional hepatic reserve.

In nuclear medicine, the clearance of $^{99\text{m}}\text{Tc}$ -trimethylbromoimminodiacetic acid was reported to diagnose severely damaged livers but not mild injuries [129]. Another radioactive metal complex evaluated as a functional hepatic tracer, galactose $^{99\text{m}}\text{Tc}$ -diethylenetriaminepentaacetic acid conjugate of human serum albumin, is efficiently taken up by asialoglycoprotein receptor in the liver [130] and the regional effective hepatic blood flow and hepatic blood-pool volume can be determined by SPECT. However, the practical utility of these compounds for routine hepatic function evaluation is doubtful. SPECT, PET, MRI, and ultrasonography are currently used with or without contrast agents to diagnose hepatic disorders, but imaging studies are outside the scope of this review.

3.4

Continuous Hepatic Function Monitoring with Exogenous Organ-Specific Clearance Markers

This approach evaluates hepatic function by monitoring the clearance of liver-specific markers from a given biological compartment in real-time. Sudden

deviation of the clearance profile from an established pattern alerts the clinician to the onset of an abnormality that may require instant intervention. Continuous hepatic function monitoring is particularly useful in critical and ambulatory care settings where instant information about organ failure may be crucial for good patient care management.

In addition to hepatic blood flow and function, ICG plasma clearance is also a useful prognostic factor for selecting patients for hepatectomy [131]. A majority of model systems developed for continuous hepatic function monitoring rely on the clearance profile of indocyanine green (ICG), which is the primary focus of this section.

3.4.1

Indocyanine Green: A Liver-Specific Photodiagnostic Clearance Agent

Currently, an important measure of hepatic blood flow and biliary excretion relies on the elimination rate of ICG. This compound also plays an important role in evaluating various clinical conditions in ophthalmology [132], cardiac output, and circulating blood volume measurements [133–137]. Traditionally, hepatic function and blood flow are determined by discrete and invasive methods in which serial blood samples are collected and ICG concentrations are measured *ex vivo*. In acute hepatic disorders, organ transplant, and multiple organ failures, continuous monitoring of hepatic function would be advantageous. Consequently, several techniques to measure ICG concentration in real-time have been explored. Certain properties of ICG summarized below have endeared its widespread use in hepatology:

- (1) Lipophilic-hydrophilic character: ICG is an amphiphilic molecule consisting of a lipophilic polyaromatic polyene core and a water-solubilizing sulfonate group (Fig. 7).

As a surfactant, ICG exists predominantly in a monomeric form at low concentrations ($<5\ \mu\text{M}$) but rapidly aggregates at higher concentrations ($100\ \mu\text{M}$) [132]. Although administration of low ICG concentrations is preferred for *in vivo* applications in order to avoid aggregation, the rapid binding of ICG to plasma proteins prevents intramolecular association *in vivo* and may favor the use more concentrated ICG samples, if needed.

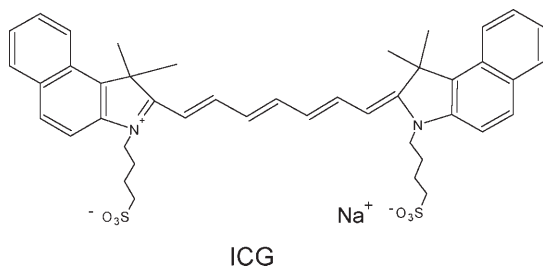


Fig. 7. Structure of indocyanine green (ICG)

- (2) Photophysical properties: ICG absorbs and fluoresces in the near infrared (NIR) region, the exact $\lambda_{\max}$ depending on the medium and extent of plasma protein binding. In aqueous solution, the monomeric form absorbs at 780 nm and the aggregates at 690 nm. This absorption maxima shifts to 805 nm in blood and emits at about 830 nm. The photophysical properties of ICG in the NIR are significant for its role in medical imaging and diagnosis [138–140]. Several endogenous chromophores absorb and emit radiation in the ultraviolet and visible regions. Such properties interfere with routine spectroscopic analysis of biological samples. To overcome this problem, interfering biomolecules must be removed before analysis or conjugation of dyes with desirable biological properties to the biomolecules become part of sample preparation protocol. The alternative method that uses photoactive molecules in the NIR takes advantage of the transparent window at 750–900 nm, where the absorption coefficients of endogenous chromophores are low. In addition to improved detection sensitivity, the radiation energy is much lower in the NIR than UV region, thus preserving sensitive tissue. Prolonged exposure of tissue surfaces such as the earlobe of fingertip to energetic radiation may damage healthy tissues. Coupled with the exceptionally high molar absorptivity ($250,000 \text{ cm}^{-1} \text{ M}^{-1}$) and NIR spectral characteristics, ICG is ideally suited for in vivo optical diagnostic applications.
- (3) Pharmacokinetics: When administered intravenously, ICG rapidly binds to plasma proteins and is exclusively cleared by the liver, and subsequently secreted into the bile [8]. This forms the basis of the use of ICG for monitoring hepatic blood flow and function. Two pharmacokinetics models, a mono-exponential decay, which describes the initial rapid clearance of ICG with a half-life of about 3 minutes (Eq. (1)) and a bi-exponential model, which incorporates the secondary phase clearance with a longer half-life (Eq. (2)), describe total clearance of ICG from plasma [132]. For real-time measurements by continuous organ function monitoring, the mono-exponential decay is preferred.

$$C = Ae^{-\alpha t} \quad (1)$$

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (2)$$

Where C is ICG plasma concentration, A and B are constants, α and β are ICG clearance rate constants, and t is time.

- (4) Metabolism: Various studies have shown that ICG is not metabolized but excreted largely intact in the bile [8, 132]. This is an important feature of markers for continuous function monitoring, because no deleterious byproducts are formed, which may ease regulatory approval.

The use of ICG to measure hepatic blood flow and function by spectrophotometric analysis of serial blood samples collected invasively was recognized more than 50 years ago [141], and the concept of non-invasive optical monitoring of physiologic function with ICG is not new [142–146]. However, advances in optical technology and the availability of miniature lasers for biomedical applications have resulted in the development of faster, simpler, and reliable optical methods for monitoring physiologic functions in real-time. While most of these methods rely on the absorption properties of ICG for continuous hepatic func-

tion and blood flow measurements, recent studies suggest that ICG NIR fluorescence spectroscopy is an alternative and highly sensitive method to measure hepatic blood flow [140, 147–150]. We will briefly review some recent studies in the absorption technique and discuss in more detail the fluorescence approach, since it represents an emerging novel approach.

3.4.2

Continuous Monitoring of Hepatic Function by Absorption Technique

ICG has a high molar absorptivity in the NIR region, which allows the detection of small quantities of the material in blood. Although the detection limit depends on instrument sensitivity, deviation from linearity has been suggested at a threshold of $3 \mu\text{g L}^{-1}$ [151]. The advantage of absorption mode is its ability to directly determine the absolute concentration of a marker in a well-defined compartment. Generally, any method that measures ICG concentration in blood as a function of time can be used to evaluate hepatic blood flow. Consequently, the application of NIR spectroscopy (NIRS) to the continuous monitoring of hepatic function has benefited from the enormous advances in the use of NIRS or pulse dye densitometry for determining cardiac output and blood volume [133, 134, 136, 137, 152, 153]. Typically, ICG is administered to a patient by bolus injection or continuous infusion, followed by continuous monitoring of the dye concentration or plasma clearance profile instead of by serial collection of blood samples at different times. Although the earlobe was the preferred site to monitor ICG plasma clearance in earlier years [145, 146, 153, 154], other vascular body parts, such as the fingertip and nose, are currently used [133, 134, 136, 137, 151, 152, 155–160]. A recent study also showed that non-invasive cerebral NIRS measurement of ICG elimination is equally as reliable as the conventional method [161]. A major problem with the earlobe system is that prolonged exposure to a light source may damage this delicate tissue by heat transfer to the earlobe, which may also increase blood flow that would affect data accuracy. In certain cases, the earpiece had to be held strongly in place, which decreased blood flow. These inconsistencies gave birth to the ICG fingertip monitor that is gaining widespread acceptance in biomedical research and medical practice [133, 136, 137, 156, 157, 162, 163]. To compensate for variations in blood volume at the fingertip, a correction factor was integrated into the ICG monitoring device to obtain reliable ICG measurement in real-time [163]. This method non-invasively measures blood ICG concentration and its disappearance is similar to the conventional discrete and invasive methods.

In a typical procedure, a finger probe equipped with light-emitting diodes and detectors transmit light simultaneously (at about 800 and 900 nm) to specified tissue volume, and the intensity of transmitted light is measured as a function of time. These two wavelengths are chosen to reflect the absorption of ICG at 800 nm (λ_{max}) and the low but comparable absorption of oxy- and deoxy-hemoglobin at 900 nm. Both hemoglobin and ICG have negligible absorption at 800 and 900 nm respectively. Thus, ICG concentration is measured at its λ_{max} (800 nm) to enhance sensitivity while using hemoglobin absorption at about 900 nm as an internal reference. The simple densitometric technique has evolved

into pulse dye densitometry [137], which uses the principle of pulse oximetry to measure ICG plasma concentration in the NIR region [164]. The device takes into account changes in light path induced by arterial pulsation when calculating the arterial blood ICG concentration.

3.4.3

Continuous Monitoring of Hepatic Function by Fluorescence Technique

Until recently, previous studies for continuous monitoring of hepatic function with ICG utilized the absorption mode. However, new studies demonstrate that the highly sensitive fluorescence technique can equally be used [148–150]. In addition to high sensitivity, in-depth analysis of the emission, excitation and polarization properties of fluorescence spectroscopy furnishes additional functional information about the dye molecule. In this system, the fluorescence profile emanating from the clearance of injected biocompatible dye is monitored with a small photodetector. Fig. 8 shows the in vivo fluorescence detection apparatus developed for continuous monitoring of organ functions [147, 148].

The procedure for this measurement is uncomplicated. A laser of appropriate wavelength for excitation of the agent under study is directed into one end of a fiber optic bundle. The other end is positioned a few millimeters from the ear. A second fiber optic bundle is also positioned near the ear to detect the emitted fluorescent light. The other end is directed into the optics and electronics for data collection. An interference filter (IF) in the collection optics train selects emitted fluorescent light of the appropriate wavelength for the test material. Once background reading of fluorescence is complete, a bolus injection of the photodiagnostic fluorescent agent is administered to the lateral tail vein of the rat and the rate of clearance from the vascular compartment is continuously monitored as a function of time.

This approach assumes that the blood clearance profile of the dye follows an open one-compartment pharmacokinetics model. The characteristics of such a

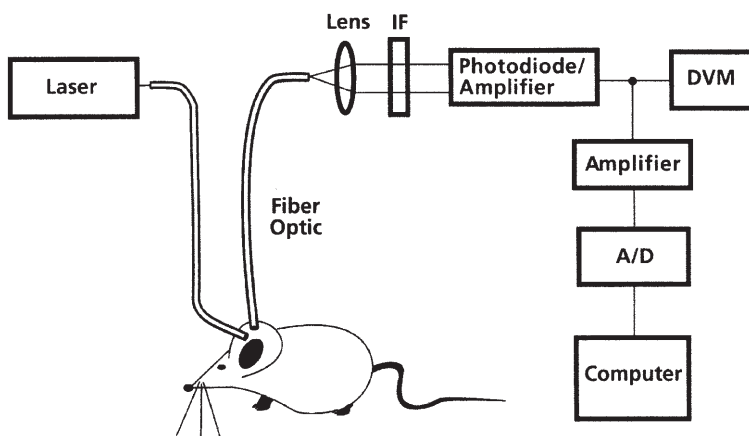


Fig. 8. Schematic of in vivo fluorescence detection apparatus

model require that there is no tissue absorption of the dye, the entire dye dose is in systemic circulation, it rapidly distributes between bloodstream and tissue, establishing rapid equilibrium (steady state), and that the dye elimination is dependent on excretion [165]. Experimentally, most of the photodiagnostic agents tested exhibit an apparent rapid distribution in blood and an exponential decay from plasma, which is characteristic of an open one-compartment pharmacokinetics model. The equation employed to fit the data is:

$$S = Ae^{-t/\tau} + B \quad (3)$$

where S is the fluorescent light intensity signal measured, and t is the time point of the measurement. The decay time τ , and constants A and B are deduced from the fitting procedure. Standard non-linear regression analysis software such as SigmaPlot® (Jandel Scientific Software, Rafael, CA) directly fits the data to Eq. (3). This methodology expands on a similar technique that employs optical densitometry (absorption) for the evaluation of cardiac output and liver function [144, 166].

In a typical procedure, a continuous wave laser (50 mW) excites ICG at 780 nm and the fluorescence signal is captured at 830 nm. Subsequent to a bolus injection in the tail vein of the rat, the time-dependent clearance of ICG from plasma can be monitored in real-time as shown in Fig. 9 for three rat data sets.

Three distinct regions are observed in the clearance curves. It begins with a region where data is gathered pre-bolus injection, and represents the baseline value for the subsequent experiments. The ICG plasma concentration rapidly peaks within a few seconds, followed by rapid exponential decay as the liver eliminates the dye from blood. Visually, the decay rates are similar for all three, and well within biological variability. After 15 minutes, approximately 90 % of the initial signal is lost. The ICG elimination from blood follows the single compartment pharmacokinetics model described by Eq. (3). After several experi-

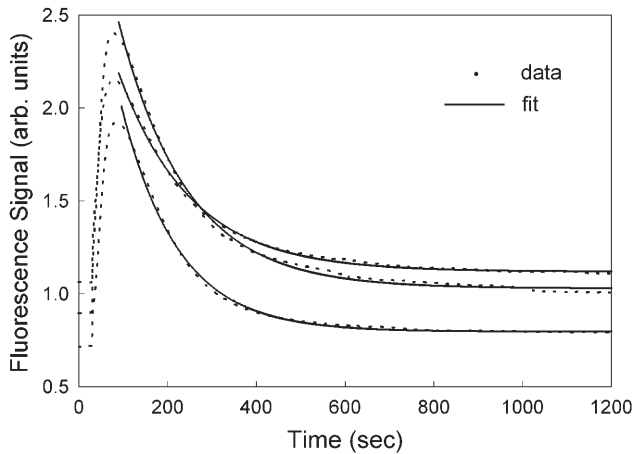


Fig. 9. In vivo fluorescence time-dependence for three rats with normal liver function after a bolus injection of ICG. The solid line is a single exponential fit to the measured data

ments in normal male Lewis rats, the average time constant for the clearance of ICG was $\tau = 145 \pm 16$ seconds (Table 1), which is in agreement with values reported in the literature [167].

To assess this methodology’s diagnostic capability in impaired livers, a partial hepatectomy was surgically performed on three rats, followed by measurement of the time dependence of fluorescence at the ear pre- and post-injection of ICG. Fig. 10 depicts the comparison of clearance rates between normal and impaired functioning livers. The time constants for the rats with normal and dysfunctional livers are shown in Table 1. The difference between the animals with normal and impaired livers is huge and quantifiable.

Invasive continuous hepatic function monitoring by the fluorescence procedure was also evaluated in rabbits [148]. In this study, a commercial catheter equipped with fiber optic technology for mixed venous oxygen saturation measurements (SVO_2) was modified to emit light at 780 nm and detect fluorescence at 840 nm. The catheter was placed into the right jugular vein and advanced

Table 1. Results of fits to Eq. (3) from rats with normal and dysfunctional livers

Normal ^a		Normal ^a		Abnormal ^b	
Rat #	τ (s)	Rat #	τ (s)	Rat #	τ (s)
1	153 ± 1	4	155 ± 1	7	1334 ± 1
2	122 ± 1	5	127 ± 1	8	1225 ± 4
3	163 ± 1	6	149 ± 1	9	1659 ± 2
Average (normal) τ (s): 145 ± 16				Average (abnormal) τ (s): 1406 ± 173	

^a Normal: Rats with normal functioning liver.
^b Abnormal: Rats with partial hepatectomy.

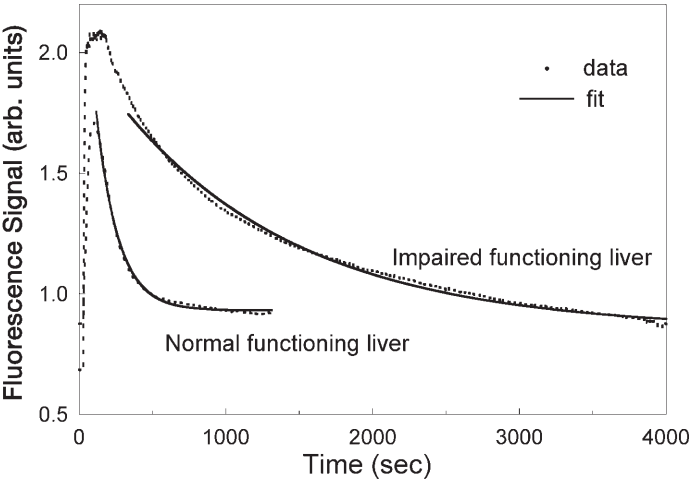


Fig. 10. Comparative in vivo fluorescence time-dependent clearance of ICG from plasma in rats with normal and impaired functioning livers

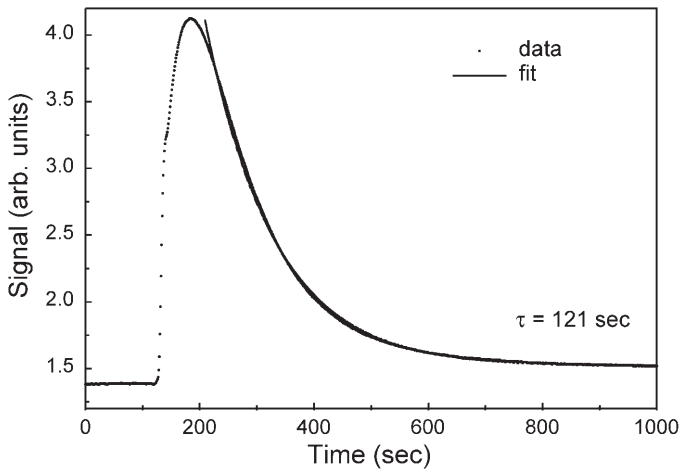


Fig. 11. Non-invasive in vivo fluorescence time-dependent clearance of ICG from plasma in a rabbit with normal functioning liver. The solid line is a single exponential fit to the measured data

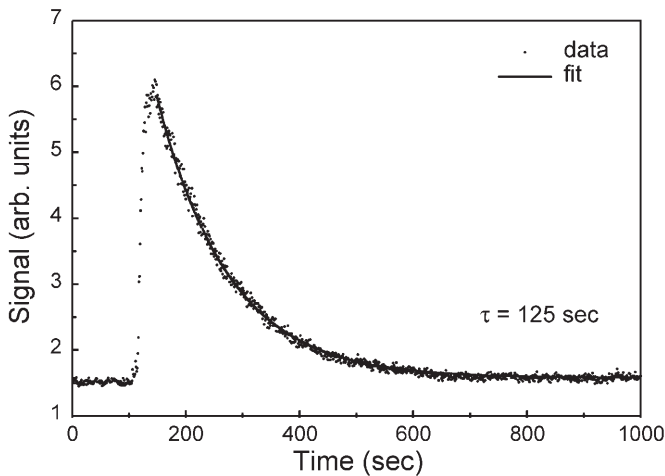


Fig. 12. Invasive in vivo fluorescence time-dependent clearance of ICG from plasma in a rabbit with normal functioning liver. The solid line is a single exponential fit to the measured data

towards the right atrium of the heart. This study demonstrates that ICG clearance profiles measured invasively and non-invasively are similar, as shown in Figs. 11 and 12.

Thus, in critically ill patients where catheters are conventionally inserted for diagnostic or therapeutic interventions, incorporation of the appropriate fiber optics to commercial catheters would enable the monitoring of hepatic function continuously and simultaneously with other medical interventions. Previous studies by the absorption method also found a close correlation between invasive and noninvasive ICG plasma elimination kinetics [157, 160].

4

Renal Function Monitoring

4.1

Basic Renal Anatomy and Physiologic Function

The kidneys are a pair of reddish brown colored and bean-shaped organs positioned on either side of the vertebral column in the abdominal cavity. The right kidney is usually 1 to 2 cm lower than the left kidney in order to accommodate the proximal part of the liver. They are protected by fibrous capsule and each kidney contains three distinct anatomical features consisting of the cortex, the medulla, and the excretory channels. Embedded in the kidneys are millions of functional units, the nephrons, each consisting of the Bowman's corpuscle, the proximal convoluted tubule, the loops of Henle, and the distal convoluted tubule. A network of capillaries, the glomeruli, nestles into the Bowman's capsule and plays an active role in glomerular filtration. Each kidney receives blood directly from the aorta through a large renal artery that rapidly branches into capillaries. This arrangement assures ultrafiltration under high hydrostatic pressure. The kidneys are vital organs that eliminate wastes from metabolism, adjust fluid and electrolyte balance, and participate in endocrine processes. These functions are accomplished through complex interrelated mechanisms involving the autoregulation of renal circulation, ultrafiltration of plasma at the glomerular membrane into Bowman's capsule, tubular reabsorption and secretion of solutes and water, and the transport of "stealth" filtrate and secretions to the bladder as urine. Thus, the kidneys assure homeostasis that is necessary for the maintenance of life. Detailed descriptions of these processes can be found in reviews and books on the kidneys [168–171].

4.2

Renal Function Disorders and Measurements

While most lipophilic and large molecules are primarily excreted by the hepatobiliary system, the kidney is the major excretory organ for many small organic and inorganic molecules, drugs and hydrophilic metabolites, maintenance of fluid balance, and bone metabolism. These functions expose the kidney to a number of clinical, physiological, and pathological conditions that may compromise renal function. Some renal disorders that necessitate clinical intervention are listed in Table 2.

Renal dysfunctions can be diagnosed by different methods, depending on the severity of the condition. Examination of urine, which is produced by the kidneys, provides an important indication of renal insufficiency. The urine output, color, odor, acidity, specific gravity, and constituents are important prognostic factors of kidney status. However, in critically ill patients and in acute renal failures induced by several diseases including multiple organ failures and diabetes, urine examination may be impractical and redundant. Such patients require reliable and simple methods to diagnose the onset of renal failure.

Table 2. Renal function disorders requiring medical intervention

Acute	Sub-acute	Chronic
Abnormal baseline or rapidly changing serum creatinine	Drug dosing based on renal function	Chronic glomerulonephritis
Acute cortical necrosis	Drug dosing of renally cleared agents	Chronic interstitial nephritis
Acute tubular necrosis (ATN)	Drug dosing of nephrotoxic agents	Diabetes mellitus
Contrast nephrotoxicity	Lupus nephritis flare	Lupus nephritis
Crush injury and myoglobinuria	Patients recovering from renal allografts	Gout
Hypertensive crisis	Patients recovering from hypotension/shock	Hypertension
Multi-organ failure syndrome	Patients recovering from dehydration	myeloma
Oliguria	Patients recovering from overhydration	Polycystic disease of the kidney
Obstetric emergencies such as abruptio placentae	Patients recovering from pre and post renal azotemia	
Obstructive uropathy	Patients recovering from surgery (vascular)	
Pre- and post-renal azotemia		
Renal allograft rejection		
shock/hypotension		

A plethora of methods has been developed to evaluate renal function by dynamic renography and remote analysis of the excretion of renal function markers. The underlying principle is that the kidneys excrete a majority of small hydrophilic molecules and their clearance, secretion, or fixation in the kidney is quantifiable. When a renal marker in plasma is filtered through the glomeruli, the accumulation of the filtrate in the Bowman's capsule. One or more of the following events may occur in the renal tubule once a marker is filtered or is in plasma [171]:

- (1) The marker remains in the plasma without binding to proteins, and the filtrate is neither reabsorbed nor secreted by the renal tubule. Clearance rate of the marker from plasma equals the glomerular filtration rate (GFR). The polysaccharide, inulin, typifies this class of markers.
- (2) In addition to its content in the glomerular filtrate, the marker is secreted by the renal tubule. Its clearance rate is used to measure renal blood flow but it overestimates the GFR. The aniline derivatives, *p*-amino hippurate (PAH) or radiolabeled *o*-iodohippuran (OIH), represent this group of renal markers. Note that PAH is partly filtered by the glomerular membrane, but molecules that are exclusively excreted by the renal tubules would be ideal for renal blood flow measurements.

- (3) The filtered substance is reabsorbed by the tubule. Some important molecules such as glucose and amino acids, and electrolytes such as sodium and chloride ions share this feature. Thus, their concentration in urine underestimates the GFR.
- (4) The filtered marker undergoes tubular reabsorption and secretion. The clearance of such markers depends on the relative rates of filtration, reabsorption, and secretion. Determination of renal function by these markers, typified by uric acid, is cumbersome.
- (5) The filtered and secreted marker may irreversibly bind to renal tubule, making their quantification in urine of little value. They are primarily used for static imaging of the kidneys and to obtain information about the functional renal mass. ^{99m}Tc -dimercaptosuccinic acid and ^{99m}Tc -glucoheptonate are good examples of such markers [172–175].

In the following section, we will focus on markers and methods for measuring the GFR because data for dynamic and continuous renal function measurements are available. In doing so, we acknowledge the importance of tubular secretion and fixation markers in the overall evaluation of renal status, and the reader is referred to some articles covering these subjects [174, 176–182].

4.3

Markers Excreted by Glomerular Filtration

Plasma constituents are exposed to the glomerular membrane and the rate at which a marker is filtered through this membrane is referred to as the glomerular filtration rate (GFR). Although glomerular filtration is only a part of the numerous functions of the kidneys, GFR is considered the hallmark of renal status [183–185]. It measures the permeability of the glomerular capillary wall, the surface area of the functional capillary filtration membrane, and the differences between the mean hydrostatic and oncotic pressures [186]. The GFR is expressed as the rate at which the marker in a given volume of plasma is cleared by the glomeruli per unit time (usually in mL min^{-1}). When exogenous markers are used, the compound is typically administered by constant infusion or bolus injection and the plasma or urine concentration is measured by serial or minimal (one or two) sample analysis. An ideal GFR marker should possess the following properties that must also be considered in designing new molecules [171, 184, 186]:

- (1) Molecular weight and molecular volume: The glomerular membrane is readily permeable to proteins and substances of less than 15 kDa and impermeable to compounds and complexes greater than 70 kDa. Molecules between these molecular weight ranges are partially permeable. However, very small compounds have the tendency to diffuse rapidly into tissues and may be readily reabsorbed and secreted by renal tubules. Hence, exogenous markers in the molecular weight range 400–12,000 Da are preferred for GFR measurements. The topology of the marker also plays an important role in the ease of its passage through the glomerular membrane. It is possible that large molecules will pass through the glomerular membrane by adopting a com-

pact molecular volume while relatively smaller compounds are excluded due to loose hydrodynamic molecular topology.

- (2) **Stability:** The marker for GFR measurement should be biologically inert, which implies the absence of binding to plasma proteins, reabsorption in the renal tubule, deleterious effect on renal function, and intact excretion of the filtrate in the urine without degradation. This biological inert criterion, albeit difficult to achieve synthetically, confers an enormous advantage for speedy regulatory approval, especially if small doses are used.
- (3) **Hydrophilicity:** The marker should be readily soluble in water. Incorporation of hydroxyl, amino, or charged ionic groups into the structural framework of the marker satisfies this requirement.
- (4) **Quantitative and qualitative analysis:** The marker should be amenable to simple quantitative plasma or urine analysis to obtain accurate measurements. Qualitative analysis should also be feasible, especially in clinical situations where instantaneous changes in clearance profile of the marker are measured.
- (5) **Excretion:** For direct monitoring, the marker must be eliminated exclusively by the kidneys. This criterion is particularly important in methods where marker clearance from plasma is monitored non-invasively.

These criteria limit the number of molecules that can be used to measure the global functioning of the kidneys by GFR. Blood urea nitrogen (BUN), one of original GFR markers, meets some of the criteria described above but variability of its concentration in blood may not be disease related and the glomerular filtrate is reabsorbed in the renal tubule. Inulin, which has a molecular weight of 5 kDa, is the gold standard for GFR determination in almost all circumstances. However, the administration and analysis of inulin is difficult and cumbersome for clinical applications. To simplify its analysis, many analogues of inulin have been prepared. These are primarily radiolabeled (HH ^3H , ^{14}C , and ^{51}Cr) and FITC-labeled inulin derivative [187–190]. However, simpler, sensitive, and non-radioactive methods to quantify inulin have emerged, and may revive interest in the routine use of inulin for GFR measurements [191–198]. A recent study identified potential sources of error that should be considered when selecting a method for inulin measurement [199].

4.3.1

Creatinine Clearance

Although the scope of this review limits us to methods related to exogenous markers, measurement of creatinine clearance or serum concentration is the bedrock of current clinical renal function management. Therefore, a brief discussion of its role is appropriate. Creatinine is a small cyclopentanamide endogenous molecule produced by the skeletal muscle and circulates freely in blood without binding to plasma proteins. It is readily filtered in the glomeruli, and in normal kidneys, its serum concentration and renal excretion rate is stable. Hence, increase in the serum creatinine concentration correlates with reduced glomerular filtration rate. Creatinine test has many advantages over

other markers. The marker is endogenous and therefore requires no regulatory approval, and does not elicit adverse immunologic reactions. The procedure can be completely noninvasive and the cost per test is low. Unfortunately, some of the reasons that make this test attractive also militate against it. Creatinine production depends on changes in muscle mass, age, and gender differences that are not related to renal pathology. The marker also undergoes tubular secretion such that the creatinine urine test consistently overestimates the GFR [184]. As a result of individual variations in creatinine concentration over time, a drop of 50 % in GFR may occur while the creatinine concentration remains within the “normal” range. Hence, this test is not sensitive to mild or moderate changes in GFR. In most clinical conditions, these fluctuations may not be life threatening and usually return to normal values without the need for urgent medical intervention. However, in critically ill patients, moderate changes in GFR may be important for timely diagnosis of renal insufficiency in a variety of renal diseases for which early treatment is essential. In such cases, a more reliable GFR marker is needed. Some studies suggest that other endogenous markers such as Cystatin C [183, 185, 200–213], and tryptophan glycoconjugate [214], may overcome some limitations of creatinine but none of these have gained wide acceptance in clinical practice.

4.3.2

Iodinated Aromatic Markers

Most contrast agents elicit nephrotoxicity because they are primarily excreted by the kidneys. However, when administered in small doses, they constitute a rich source of GFR markers. The two major classes of contrast agents that are finding clinical utility as GFR markers are iodinated aromatic compounds and metal complexes. Iodinated aromatics such as iohexol and iothalamate (Fig. 13) are commonly used as contrast agents for computed tomography (CT). They also have pharmacokinetics similar to inulin and hence are useful indicators of renal status [215]. The iodinated molecules used for GFR measurements consist of a triiodobenzene core and hydrophilic groups to enhance solubility in aqueous medium.

The presence of iodine in compounds facilitates their detection and quantification. Thus, the elimination of iodinated aromatics can be measured in blood

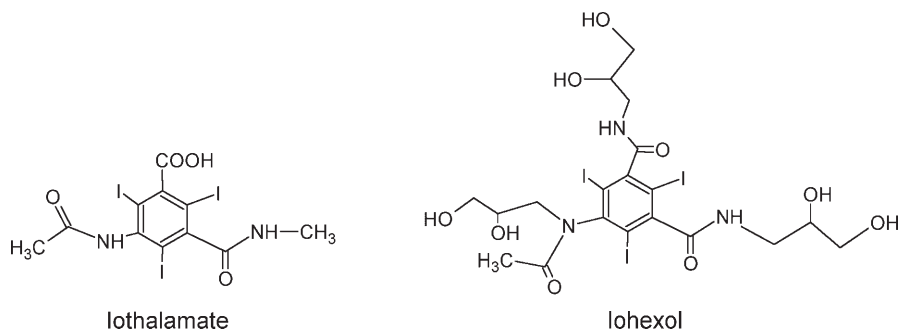


Fig. 13. Structures of two common triiodobenzene contrast agents used to measure GFR

or urine by colorimetric assay [216], HPLC [217, 218], capillary electrophoresis [219], inductively coupled plasma-atomic emission spectroscopy [220], and X-ray fluorescence [179, 218, 221–224]. These protocols require discrete sampling and remote analysis of biological fluids, which prevents the exposure of patients to ionizing radiation. Interestingly, GFR values can also be extracted from dynamic computed tomography (CT) scans non-invasively [225–232]. This approach combines functional information with high anatomical resolution. The functional capacity of each kidney can also be determined. The CT method is best used to obtain additional information when patients are undergoing the conventional contrast-enhanced CT procedure [230]. However, it is not suitable for routine GFR measurements due to exposure of patients to ionizing radiation, use of bulky and expensive equipment, administration of nephrotoxic iodinated contrast agents, and the high level of expertise required for instrument operation and data analysis. One study questions the validity of iohexol as a GFR marker, since it is reabsorbed in the renal tubule and further suggests that this contrast agent may alter normal mitochondrial oxidation mechanism [233].

Labeling of iodinated aromatics with radioactive ^{125}I or ^{123}I has proved to be a valuable approach to measure GFR in nuclear medicine. Prominent among these is sodium ^{125}I iothalamate, which is specifically marketed in the US for GFR measurement by the name Glofil®. Studies have shown that the clearance of this marker by the glomeruli is reproducible, simple, reliable and accurate, especially in children and those with advanced renal diseases [234]. This marker can also be administered by subcutaneous infusion to obtain GFR values without the need for urine collection [235]. Since very low doses (nanomolar scale) of radioactive aromatics are administered, monitoring of renal function may be achieved without disruption of normal physiologic functions. Concerns over radioactivity and associated handling costs may prevent the use of these compounds for routine GFR measurements.

4.3.3

Metal Complexes

Metal complexes continue to play a significant role in renal function monitoring in biomedical research and clinical medicine because they are excreted by the kidneys through the glomerular membrane. Of these, the two complexes routinely used for GFR measurements are radiolabeled diethylenetriaminepentaacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA). $^{99\text{m}}\text{Tc}$ -DTPA, an imaging agent, is exclusively cleared by the glomeruli and has become a staple in GFR measurements. The chemistry and pharmacokinetics of this compound and other $^{99\text{m}}\text{Tc}$ -based complexes have been adequately reviewed [182, 236]. This radioisotope has a half-life of about 6 h and emits a γ -ray of 142 keV on decay. The short half-life reduces the risk of cumulative exposure of patients to large doses of radiation and simplifies disposal protocol. Most hospital are equipped with scanners that capture radiation at about 142 KeV, thus the use of this agent does not necessitate additional equipment costs. However, it has not been possible to determine the exact structure of $^{99\text{m}}\text{Tc}$ -DTPA directly and

because of the short half-life, the administered product is usually formulated on demand. This prevents proper characterization of the injected dose, which becomes a major problem for GFR measurements since the protocol requires the use of small doses of materials. At low concentrations, the impact of impurities on GFR values becomes significant. EDTA is traditionally labeled with ^{51}Cr , which has a longer half-life (about 29 days) than $^{99\text{m}}\text{Tc}$. ^{51}Cr -EDTA is widely used today to measure GFR and validate newer methods. Although it accurately measures the GFR, it is not suitable for routine use due to the risk of unnecessary radiation exposure and costs associated with equipment, personnel training, and disposal of radioactive wastes. Structures of the three common ligands used to prepare GFR metal complexes are shown in Fig. 14 below.

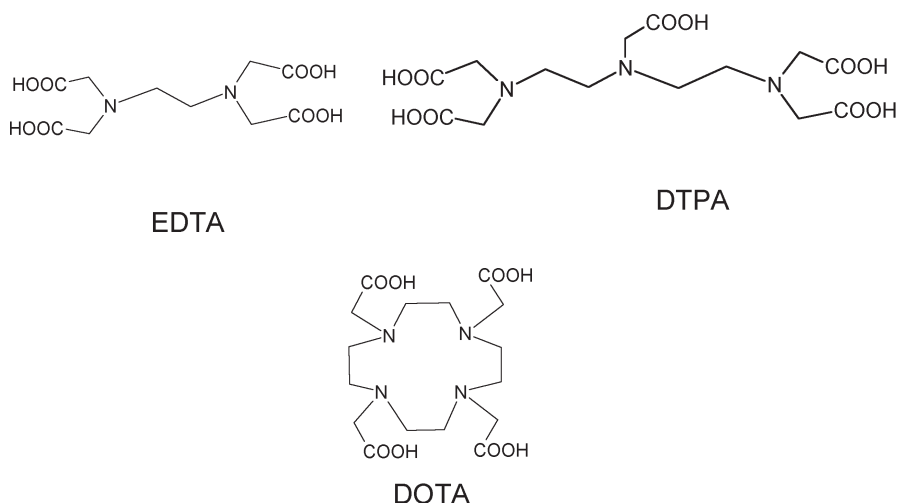


Fig. 14. Structures of some common chelators useful for GFR measurement: EDTA, ethylenediaminetetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; DOTA, cyclododecane-1,4,7,10-tetraazaacetic acid

The metal complex, Gd-DTPA and Gd-DOTA, are also cleared from plasma by the glomerular membrane and they have similar pharmacokinetics to the radiopharmaceutical analogues [237–247]. These paramagnetic complexes are conventionally used as contrast agents for magnetic resonance imaging (MRI). Their clearance profile in plasma and excretion in the kidneys can be monitored by discrete methods such as NMR analysis or the information can be extracted from dynamic kidney function images. As in nuclear medicine and CT, both functional and structural information can be obtained simultaneously. This approach has the advantage that it does not use ionizing radiation, but due to cost considerations, it may not be employed routinely in clinical settings, unless the patient is already undergoing MRI procedures.

The clearance of DTPA metal complexes by the glomeruli is primarily dependent of the ligand. Thus, other metal complexes may also be useful for GFR measurements. For example, colored metal complexes that are exclusively excreted

by the glomerular membrane may provide a simpler method to evaluate renal function by NIRS and optical imaging.

4.3.4

Organic Dyes

An important method for quantifying several renal function markers relies on the generation of colored species that are easily measured by spectrophotometric techniques. For example, inulin is first hydrolyzed and the fructose or sorbitol monomeric units are further derivatized to produce colored compounds. Similarly, colorimetric reaction of *p*-aminohippuric acid with dimethylaminocinnamaldehyde gives a deeply colored molecule whose intensity is proportional to PAH concentration [197]. Beyond these *in vitro* reactions, several dyes have been evaluated as possible renal function markers [248–252]. The challenge is to construct molecular designs that are biocompatible for *in vivo* renal function monitoring. Two approaches are possible. One approach is to use hydrophilic dyes and monitor their clearance by absorption or fluorescence techniques, and another is to conjugate dyes to biocompatible molecules that are renally excreted. Intuitively, dye-inulin bioconjugates would appear to be the best approach since inulin is an excellent GFR agent. Accordingly, some studies have demonstrated that this approach is reliable and simpler than radiolabeled inulin GFR measurements [187, 188, 253]. FITC-inulin is freely filtered by the glomeruli and is neither secreted nor reabsorbed in the renal tubules. By monitoring the fluorescence, minute quantities of the marker can be detected in blood and urine. Use of this marker to visualize the renal tubules by optical imaging or fluorescence microscopy is an added advantage. Recently, clearance of new FITC conjugates with polyaspartic acid was shown to be renal, and since a continuous method was used, these compounds will be discussed elsewhere in this review.

4.4

Continuous Renal Function Monitoring with Exogenous Organ-Specific Clearance Markers

Various methods developed for examining renal disorders rely on renography, renal blood flow, or the clearance of endogenous and exogenous renal markers. Imaging methods by CT, ultrasound, magnetic resonance, and scintillation furnish anatomical details of the kidneys to enable detection of abnormalities. Although recent studies have shown the capability of obtaining functional information with or without contrast agents, other less cumbersome methods are preferred when simple evaluation of the functional status of the kidneys is required, as in diabetic patients. In critical and ambulatory care settings where detection of moderate changes in renal function may alter the course of treatment, continuous renal function monitoring is essential. Continuous measurement methods have become indispensable for examining brain activity by electroencephalography, cardiac function by electrocardiography, and oxygen saturation by pulse oximetry. Instantaneous medical interventions

based on the outcome of these measurements have saved many lives, especially in critical and ambulatory care units. While renal and hepatic failures account for a significant number of deaths daily, no established methods exist for the continuous monitoring of these vital organs. Thus, in order to assess the status and to follow the progress of renal disease, there is a considerable interest in developing a simple, safe, accurate, repeatable, and continuous method for determining renal function, preferably by non-radioactive procedures. Three examples of recent developments in this direction illustrate this approach.

4.4.1

Continuous Renal Function Monitoring with Radiopharmaceuticals

Many radiopharmaceuticals are currently used to monitor renal function as an alternative to inulin and creatinine methods [178, 182, 254–257]. Initial studies began with the use of inulin derivatives of ^{51}Cr , ^{14}C , ^{125}I , and ^{131}I radioisotopes [189, 190, 258–260]. While these products eased the quantitative analysis of inulin clearance, they were unstable in vivo and sometimes altered the natural clearance of native inulin [260]. Advances in the development of radiopaque agents for X-ray radiography and stable radiopharmaceuticals for scintigraphy, coupled with the known contrast media renal excretion, facilitated the evaluation of these imaging agents for monitoring renal functions. Of these compounds, $^{99\text{m}}\text{Tc}$ -diethylenetriaminepentaacetic acid ($^{99\text{m}}\text{Tc}$ -DTPA) and ^{125}I -sodium iothalamate are widely used for renal function monitoring [182, 234, 261–264]. Dissatisfaction with the invasive and time-consuming discrete methods prompted several researchers to explore the possibility of external, non-invasive² monitoring of renal status in patients [265–277]. The radiopharmaceutical of choice is $^{99\text{m}}\text{Tc}$ -DTPA because of its proven safety in humans and the desirable properties of the $^{99\text{m}}\text{Tc}$ radioisotope [182].

The introduction of ambulatory renal monitor (ARM) to measure GFR in real-time represents a major advance in patient care management [266, 267, 270, 278, 279]. The equipment counts the radioactivity emanating from $^{99\text{m}}\text{Tc}$ -DTPA as a function of time and the clearance profile in blood is displayed on a monitor. Either a one or multiple-compartment pharmacokinetics mathematical model can be used to describe the biodistribution and clearance of the marker [280]. By defining intravascular and interstitial space as one compartment (the extracellular space), the clearance rate constant of $^{99\text{m}}\text{Tc}$ -DTPA reliably measured the GFR [281]. Typically, the ARM system is properly secured around the arm of the patient in a supine position prior to injection of the marker. Following the injection, the plasma concentration attains a maximum value before decaying exponentially in normal functioning kidneys. Calculating the slope of activity versus time plot gives the rate constant for the clearance of $^{99\text{m}}\text{Tc}$ -DTPA from the extracellular space, which correlates with GFR data obtained with ^{125}I -iothalamate by the conventional discrete method.

² The term *non-invasive* is misleading since intravenous injection of the radiopharmaceutical is required. Rather the term *minimally invasive* is preferred.

This approach is highly promising for monitoring the status of renal function in critical care setting. Particularly, proper management of contrast agent infusion can be achieved by using the ARM system to continuously monitor the renal status [267]. This method is simple, efficient, flexible, and has potential application when a nuclear medicine procedure is part of a diagnostic or treatment protocol. However, its acceptance for routine use in critical care unit is doubtful because of public perception of radioactivity, and the regulatory and health issues associated with radioactivity. Furthermore, inter-batch variation in the purity ^{99m}Tc -DTPA may affect the quality of information obtained from this marker. Also, the plasma protein binding of ^{99m}Tc -DTPA is associated with errors in GFR data. For measurements where moderate changes in renal function are critical, algorithms for data analysis should incorporate a correction factor for protein binding of the marker.

4.4.2

Continuous Renal Function Monitoring with Paramagnetic Agents

Clearance of DTPA and DOTA gadolinium derivatives is frequently used for dynamic monitoring of renal status [237, 241, 243–247, 282–289]. The clearance of gadodiamide, for example, is predominantly by glomerular filtration and has a high correlation with the clearance of ^{125}I -iothalamate [286]. This approach is very attractive because it combines high anatomical resolution with functional information. Tweedle et al. demonstrated the feasibility of continuous renal function monitoring with paramagnetic agents by using Gd-DTPA [282]. However, little progress has been made in this direction, probably due to adequate information obtainable from conventional MR renography. Miniaturization of magnets necessary for this approach may not be cost-effective based on current technology. Furthermore, since relatively large doses of the contrast agent are required (relative to radioisotopes), repeat doses may lead to deposition of toxic Gd metal in tissues. Hence, it is doubtful if this approach will be used for routine renal function monitoring. Nevertheless, recent advances in MR spectroscopy can overcome or minimize these problems.

4.4.3

Continuous Renal Function Monitoring with Photodiagnostic Agents

Various dyes are used directly or indirectly to evaluate renal function [248–252]. These markers are either used to study kidney morphology or estimate renal function by determining their clearance rate from the plasma or concentration in blood. For example, FITC-inulin conjugate has been examined as a renal agent but, as with previous markers, renal function was determined by remote analysis, which is not conducive in critical care situations. Most of these dyes absorb or fluoresce in the visible and near infrared regions of the electromagnetic spectrum, a range that falls with the purview of optical imaging and spectroscopy. Monitoring organ and physiologic functions by in vivo optical spectroscopy is accomplished by using a fiber optic equipped with a propagating light source to activate a chromophore in a compartment of interest and a

detector system to determine the clearance profile or marker concentration in the compartment. Some advantages of the optical method over conventional techniques include:

- (1) Use of non-ionizing low energy radiation that is relatively harmless to tissues.
- (2) Availability or design of biocompatible renal dye markers with unique biological, chemical, and photophysical properties.
- (3) Direct quantitative and qualitative analysis of the tracer by using highly sensitive absorbance or fluorescence techniques.
- (4) Administration of repeated doses is possible.
- (5) Simple ear or finger clip reduces a patient's anxiety and is less of a distraction.
- (6) Portable optical device that can readily integrate into established intensive care unit equipment.
- (7) Applicable to invasive (for example when the patient is already catheterized) or non-invasive techniques.

Previous studies have shown that fluorescein is not harmful to the kidneys at diagnostic doses but it is secreted and accumulates in the renal tubules [248, 290]. The bioconjugate, FITC-inulin, has been successfully used to measure GFR by discrete sampling approach [187, 253]. Recent studies, however, have demonstrated the feasibility of using new fluorescein bioconjugates for continuous renal function monitoring [147–150]. This technique assumes the agent is exclusively cleared from the plasma by the glomeruli such that the time-dependent curve of the emitted fluorescent light signal is indicative of the functioning of the kidneys. Some examples of the molecules studied are shown in Fig. 15.

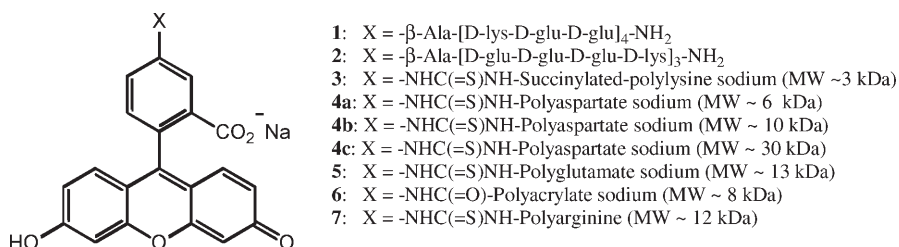


Fig. 15. Structures of fluorescein conjugates for evaluating renal function

These compounds are readily prepared by the reaction of fluorescein isothiocyanate with the polypeptide in a DMSO/water mixture and fluorescein-polyacrylate conjugate (**6**) is prepared by the reaction of fluoresceinamine with polyacrylic acid by using the mixed anhydride method [150]. In all cases, the absorption and emission maxima were similar at 490 and 525 nm, respectively, and reflect the photophysical properties of fluorescein itself.

A typical clearance profile and curve-fitting of these bioconjugates in a rat with normal renal function is shown in Fig. 16. The time constant is the relevant quantifiable measure for how fast the agent clears from the vascular system. Figs. 17 and 18 compare the plasma clearance profile of different fluorescein conjugates in normal and ligated rat kidneys. The clearance profile followed the

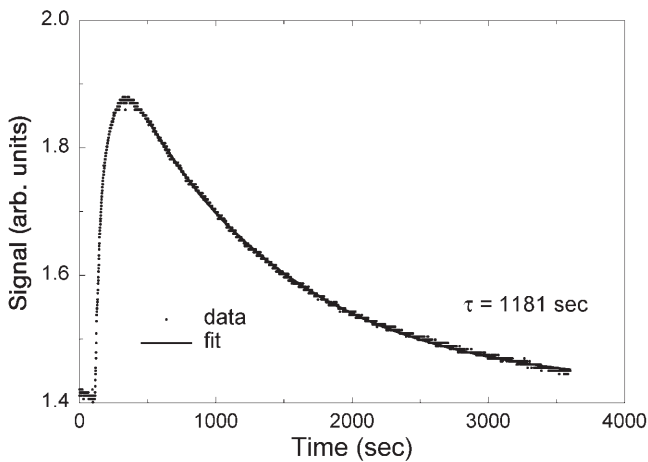


Fig. 16. Clearance curve of 4a in a normal rat with a fit to a single exponential

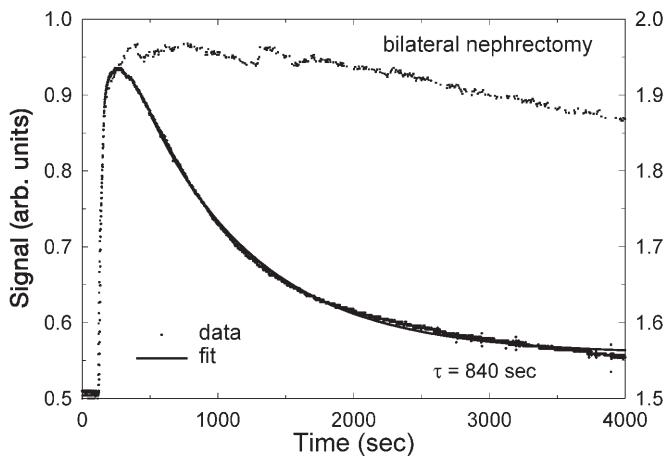


Fig. 17. Clearance curves of 4c in normal rat with a fit to a single exponential, and in a bilateral nephrectomized rat

single compartment pharmacokinetics model prior to bilateral nephrectomy, as predicted by Eq. (1). However, re-injection of the dye after nephrectomy led to a glaring deviation from normal clearance profile. Since the clearance organ was obstructed, the dye conjugate remained in blood during the experimental period.

While still preliminary, this study demonstrates the feasibility of evaluating the renal status in real-time by optical modality. This continuous renal function monitoring by the optical modality represents a new and minimally invasive method to detect kidney malfunctions. In addition to using relatively harmless radiation, the simplicity and portability of the equipment make this approach compatible for use in ambulatory and critical care. However, further studies

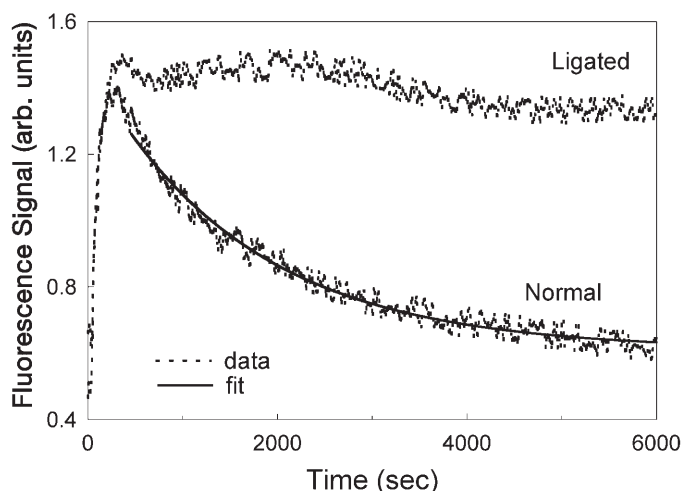


Fig. 18. The *in vivo* fluorescence time dependence after a bolus injection of fluorescein labeled poly-D-lysine in a single rat pre-kidney ligation (Normal) and post kidney ligation (Ligated). The solid line is a single exponential fit to the measured data

are required to elucidate the precise clearance (glomerular filtration or tubular secretion) pathway of the FITC bioconjugates. However, the slow clearance rate is consistent with a glomerular filtration pathway. Clearance of molecules from the plasma by the glomerulus is much slower than those removed by tubular secretion because the total surface area of the renal tubular capillaries is about 15 times greater than glomerular capillaries [182]. Thus, only a fraction of the marker (about 20% of renal blood flow) to the kidneys can interact with the glomerular membrane during each circulation cycle. Since depth is not an issue with this method (an ear or finger clip is proposed), a photodiagnostic agent that absorbs and fluoresces in the visible region will be equally effective as one that absorbs in the near infrared region.

5

Conclusions and Future Direction

Many markers are currently used for hepatic and renal physiologic function monitoring. Unfortunately, the majority of these markers was not designed for their current roles as organ-specific markers. Therefore, the search for optimal agents that have precise and predictable organ-related properties and that are amenable to routine, continuous, and noninvasive evaluation of physiologic functions continues. Such agents may benefit from integrating the structural framework of existing agents into the new molecules. Based on the ease of use and the rapid timescale of measurement, application of non-invasive and invasive *in vivo* fluorescence detection to monitor hepatic and renal functions may be especially useful for routine examinations and management of critically ill patients. However, further development of this methodology should include

improvements with respect to instrumentation (better detectors, better delivery and collection of light via fiber optics, optimizing laser power, etc) for increasing the sensitivity and signal-to-noise ratio over what is currently available. The dependence of the absolute fluorescent signal intensity on laser power and beam size, dye quantum efficiency, and in vivo capillary bed density needs further investigation.

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Ultrasound Contrast Agents: Development of the Field and Current Status

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Design, preparation and application of microbubbles as ultrasound contrast agents are discussed. Modern generation of ultrasound contrast materials can be prepared as aqueous dispersions of micron-size bubbles of water-insoluble gas (e.g., perfluorocarbon) coated with a thin shell made of protein, lipid or polymer. Such microbubbles can be injected intravenously for imaging blood flow and tissue and organ delineation and perfusion. Microbubbles can be outfitted with targeting ligands for selective contrasting of the areas of disease. Microbubbles can be selectively destroyed in the ultrasound field. So, if a drug or plasmid DNA is attached to a microbubble, selective release into the insonified areas of the body can be achieved for drug delivery or gene therapy. Several ultrasound contrast agents have received approval for clinical use so far. Their widespread application will allow rapid and inexpensive diagnostics of tissue perfusion abnormalities and location of tumor metastases.

Keywords. Microbubbles, Ultrasound contrast, Echo contrast imaging

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List of Abbreviations

- PEG poly(ethylene glycol)
DSPC distearoyl phosphatidylcholine
DPPG dipalmitoyl phosphatidylglycerol
MI mechanical index

**1
Introduction**

Ultrasound contrast agents were proposed nearly three decades ago – when it was accidentally discovered that air bubbles could be detected in the blood-stream after injections of agitated aqueous solutions [1, 2]. Since that time, slow but considerable progress was made, both in the ability to manufacture ultrasound contrast agents, and in the ability of ultrasound equipment to detect these agents at extremely high sensitivity, bordering on imaging of individual micron-size bubbles. There has been an explosive growth regarding publications on this subject, and it would not be possible to review all the existing literature (hundreds of publications) in a relatively short chapter. Therefore, we will only examine the general trends which will be illustrated by examples. Dozens of extensive reviews, including books and proceedings of numerous symposia, on various aspects and applications of ultrasound contrast agents are available (e.g., [3]). This is a multidisciplinary area of research, which interacts with a wide variety of specialties from acoustic physics to vascular physiology. The field is heavily commercialized. A substantial portion of research is done at companies and often published in the form of patents and brief conference abstracts. There are numerous trade names (which are of course the property of the owners of the respective trade marks).

We would like to show in this review that contrast echo imaging has several unique advantages that cannot be duplicated by the use of the other diagnostic imaging modalities, as well as some disadvantages, which limit the pace of the introduction of ultrasound contrast agents into widespread clinical practice. We will focus mostly on the type of ultrasound contrast agent known as “microbubbles”, which is an aqueous dispersion of micron-size particles that have a thin (often a monomolecular layer, ~ 2 nm-thick) membrane surrounding a hollow space filled with a gas. Due to their gas content, microbubbles possess exceptional compressibility and scatter ultrasound very efficiently. An ultrasound medical imaging system probe transmits an ultrasound pulse into the tissue. The probe then monitors the scattered echo signal, which is strongest in the case of microbubbles. Other types of ultrasound contrast agents, including liquid nanoemulsions and liposomes, will be mentioned briefly in this review. They require a much larger amount of material for successful detection by ultrasound.

2

Why Use Ultrasound Imaging Modality and Ultrasound Contrast Agents?

The general advantages of ultrasound are well known. They include the low cost of examinations and the relatively low cost of equipment (especially as computers are becoming faster and less expensive). Many thousands of ultrasound systems are in use.

Ultrasound equipment is the most portable of all the imaging modalities. It can be brought to the patient bedside and emergency room. Recently, ultrasound laptop/handheld portable imaging systems have become available and these can facilitate use in ambulances and field hospitals).

The ultrasound equipment is also readily available in private medical practice offices. It is certainly the imaging modality of choice for remote places and/or economically disadvantaged areas.

The examinations are performed in real time and can visualize motion (30 frames per second or even more); respectively, needle biopsies and tissue ablation procedures are often performed under the guidance of ultrasound imaging.

Ultrasound contrast agents offer the following general advantages: a very small intravenous injection dose is needed for imaging. New-generation contrast agents provide superb blood and liver contrast with as little as a fraction of a milliliter of the aqueous dispersion, which translates into sub-milligram quantities of the actual contrast material and microliters of gas.

The ultrasound contrast agents are manufactured from nontoxic natural or synthetic biodegradable materials (e.g., lipids or proteins), and a small amount of an inert low-solubility non-reactive gas (e.g., perfluorocarbon). These components have been shown to be harmless to the patient unlike the tens of milliliters of concentrated viscous solutions of the widely used X-ray contrast agents (which may sometimes result in nephrotoxicity).

Contrast-agent microbubble particles are similar in size and shape to red blood cells and in many instances behave rheologically similarly to the blood

cells [4] – hence making it possible to map organ and vascular lumen delineation, blood flow, tissue perfusion and tissue blood volume. This often helps in transforming non-diagnostic echo examinations into successful ones. Microbubbles can also be destroyed [5] or manipulated [6] by the ultrasound pulses, which may be helpful in determining the blood flow or the lymphatic drainage patterns within the tissue of interest.

The fact that only a small amount of contrast agent is needed for detection makes it possible to consider targeted imaging [7, 8], where the agent selectively binds to the receptors, e.g., on an inflamed endothelium, and marks the diseased tissue. Such agents have not been tested in the clinical trials, but their potentially high sensitivity might allow competition with some radiopharmaceuticals in the future. One particular example where ultrasound contrast targeting is already applied is the microbubble uptake by normal liver tissue [9]. Microbubbles are apparently phagocytosed by Kupffer cells in normal liver tissue, not in the tumor foci.

The interaction of ultrasound waves with microbubbles allows a drug (including genetic material for gene therapy [10]) to be released from the particles selectively at the desired insonified areas of the body, thus allowing targeted delivery of therapeutic agents. Currently, this approach is still far from clinical application.

3

What are the Problems with Ultrasound and Echo Contrast Agents?

The general disadvantages of ultrasound imaging are also well-known. Image artifacts may sometimes present a problem. In some patients and in some body habitus, the ultrasound propagation is attenuated by tissues, such as fat or bone. Ultrasound cannot be successfully applied for lung imaging since air in the lungs does not allow propagation of the sound waves.

Ultrasound imaging is operator-specific and requires experience; a skilled sonographer can obtain diagnostic quality images whereas the operator with insufficient training may obtain ambiguous results and this may become a legal liability issue in some countries. It is necessary to note here that the use of ultrasound contrast agents consistently helps to improve the image quality (the sonographer should be trained to use contrast agents).

The field of view for the ultrasound equipment is rather small, typically, not more than 20–30 cm and just a few centimeters at higher spatial resolution. Normally, a cross-sectional image in the plane of the imaging probe is obtained. Respectively, the operator must know where to place the transducer to locate the area of an expected lesion, as compared with a whole-body CT, the wide flat view of planar X-ray or MRI scans.

Ultrasound imaging is mostly two-dimensional. Real-time three-dimensional systems that give a field of view wider than a thin slice at a time are still quite rare and expensive. Quasi-3D systems based on manual lateral translation of the probe are gradually becoming available as software upgrades for existing equipment.

The image quality given by ultrasound is not as good as that provided by MRI or CT. Spatial resolution and the ability to image the anatomical details is inverse-

ly proportional to the frequency of the ultrasound used in a particular probe of the imaging system. High frequencies (e.g., 10–40 MHz) provide sub-millimeter spatial resolution and high image quality. The problem is that they only work for very short distances (skin, eyes etc). In order to image the organs located deeper inside the body (e.g., heart), frequencies of ~1.8–3.5 MHz are typically applied so that the signal can penetrate further. In this case, axial spatial resolution is a millimeter or worse.

Known or perceived disadvantages of ultrasound contrast agents are as follows. Microbubble agents with their size of several microns cannot escape from the bloodstream under normal conditions. Thus, the imaging cannot be performed in the areas outside the normal bloodstream. Essentially, microbubbles can only go where red blood cells do. Microbubble agents last only several minutes in the bloodstream. This makes their application less convenient for lengthy multi-plane examinations, and requires multiple boluses of contrast administration or a continuous infusion.

Particulate agents (especially gas-filled microbubble materials) are currently approached with caution by physicians, regulatory agencies and industry marketing, despite the fact that by 2001, perhaps up to ~100,000 patients (a rough estimate) will have received ultrasound contrast agents with very few (mostly minor) side effects manifested.

Because the design of the agent is new (micron-size particulates are seldom used in clinical practice as intravenous agents, especially in diagnostic imaging), a considerable investment and lengthy efforts are required to set up the manufacturing and achieve the agent approval by the regulatory agencies.

The limited range of the approved specific diagnostic indications for ultrasound contrast limits the likelihood of obtaining cost reimbursement from insurance providers for the contrast echo procedures (despite the obvious general diagnostic benefit). The reimbursement issue may obviously restrict the widespread use of contrast agents. More studies on the economics of contrast use will have to be performed to demonstrate to insurance providers the advantages of ultrasound contrast.

For all the above reasons, ultrasound contrast agents have not yet entered widespread clinical use.

4

Properties of an Ideal Ultrasound Contrast Agent

The ideal ultrasound contrast agent should possess the following set of characteristics. The agent should be absolutely safe and harmless for the patient. There should be no toxicity for the dose of contrast normally injected (therapeutic index, i.e., the ratio of the toxic dose of the agent to the dose used in normal procedures should preferably exceed 100:1). The agent should be completely biodegradable and biotolerable. The particles of the agent should not effect the tissue perfusion. The agent and the products of its decomposition should be degraded by the body and either safely excreted or degraded into the products of normal metabolic pathways. No long-term deposition of the agent or its components in the body can be allowed. At the same time, the agent should be stable

and robust in vivo and it should stay in the circulation as long as necessary to complete the diagnostic procedure. The agent should be easily detectable by ultrasound imaging systems and easily distinguished from the tissue background, providing bright contrast. The relation between the agent concentration and the ultrasound backscatter signal should be established.

The agent should demonstrate useful and clear diagnostic benefit. It should be proven that the agent does not merely improve the quality of the image and allow easier delineation of the anatomical structures, or diseased tissues, e.g., tumor metastases. It is now necessary to demonstrate in a statistically significant manner that the examination performed with the contrast agent is beneficial to making a clear diagnosis and helps to guide medical and surgical management of the patient. Use of echo contrast should also help to monitor the progress of therapeutic interventions and help to avoid other costly diagnostic procedures (e.g., MRI or nuclear medicine examinations).

The amount of contrast agent administered to the patient should be small, preferably in fractions of a milliliter. The administration procedure should be simple (a short intravenous injection is preferred). The agent should require minimal handling and preparation prior to injection. The contrast material should be stable during storage, preferably, with a shelf life of a couple of years. Freezer storage should be avoided; refrigerated long-term storage is acceptable; and room-temperature long-term storage is preferred. Contrast agents should be inexpensive and easy to manufacture. Large volumes of the agent should be made by short and flexible cycles with little involvement of the manufacturing personnel. Low-cost raw materials should be used. The manufacturing process should not require use of expensive equipment for long periods of time. Otherwise, high production costs would render the agent not affordable both for the industry and the customer.

Obviously, the criteria outlined above are rather stringent. However, we will show that it has become possible to satisfy most of them.

5

First Generation of Ultrasound Contrast Agents

It is easy to prepare microbubble-based contrast agents (several examples and approaches are listed in Table 1). The use of specialized or sophisticated equipment is not necessary. The apparatus needed consists of two syringes partially filled with an aqueous solution that also contain a small volume of air. The syringes are connected to each other via a standard 3-way stopcock [11]. As air and water are forced back and forth from one syringe to the other through the narrow stopcock valve orifice, a considerable shear force is generated, and air is dispersed in the aqueous phase, creating micro- and macrobubbles. Presence of solutes in the aqueous phase may further enhance microbubble formation. Plain isotonic saline, or solutions of renografin, dextrose, indocyanin green are normally applied [2]. These materials increase solution viscosity and/or act as surfactants. A viscous medium slows down the rate of microbubble movement to the top of the solution, where they will disappear (Fig. 1). Lower surface tension of the liquid-air interface results in more efficient dispersion of the gas in the

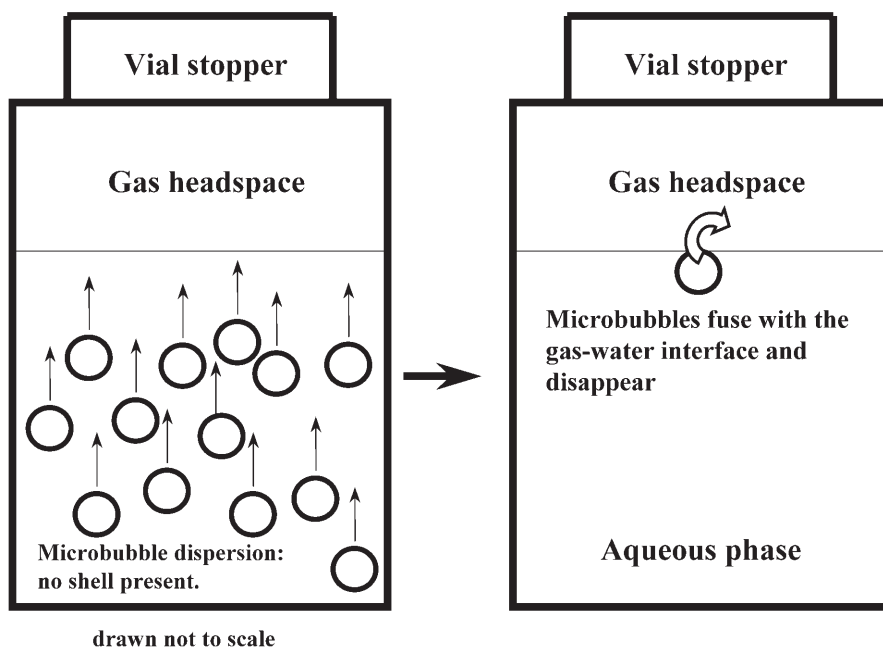


Fig. 1. Microbubbles that do not have a stabilizing shell float to the top of the solution and fuse with the gas-liquid interface

liquid. The molecules of surface-active compounds position themselves at the microbubble surface. In some instances, they can greatly stabilize the microbubble from destruction, essentially creating a shell. In addition to the two-syringe method (which may not be suitable for large-scale production), one may use other means of creating shear to disperse air in the liquid medium, e.g., sonication.

It is known that finely ground powders of sugar (e.g., galactose) or polysaccharides (dextran) can dissolve in water very rapidly. When water is added to this dry powder, a dispersion of microbubbles instantly forms as water penetrates the powder structure and dissolves the solid, leaving behind the pockets of air that turn into microbubbles as soon as they are surrounded by an aqueous phase from all sides. This is essentially the design of an early-generation contrast agent Echovist or SHU 454 (Schering), which received marketing approval in some European countries [12]. These bubbles have no protective shell; as they rise to the top of the aqueous solution, they fuse with other bubbles and with the air-water interface (Fig. 2). This means that the interval between the reconstitution of the dry powder and the time of loss of microbubbles in the preparation is quite short, even in the presence of a high viscosity sugar solution. In order to improve the microbubble stability *in vitro* and *in vivo*, a surfactant, such as palmitic acid, was added to Echovist formulation, turning it into the first-generation ultrasound contrast agent Levovist or SHU 508 (Schering) [13] (Fig. 3). Levovist is currently approved for use in some countries in Europe but not in US. A major advantage of Levovist over the agents of the previous generation (like

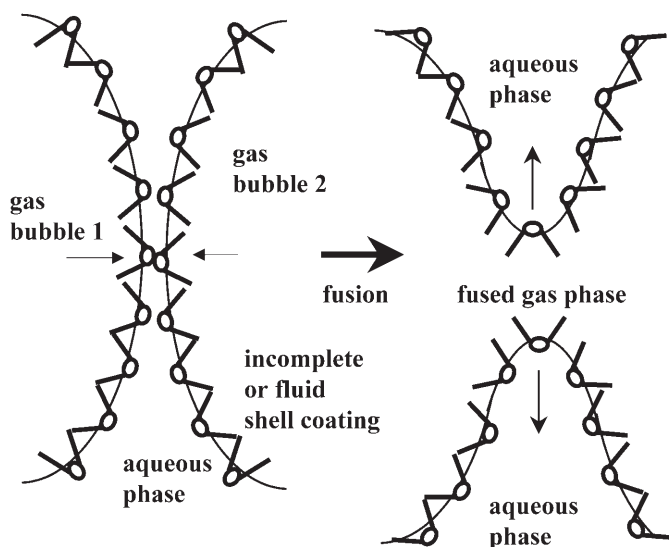


Fig. 2. Microbubbles that are stabilized only with a mobile surfactant layer will fuse on contact and disappear – long term storage in the aqueous phase is not feasible

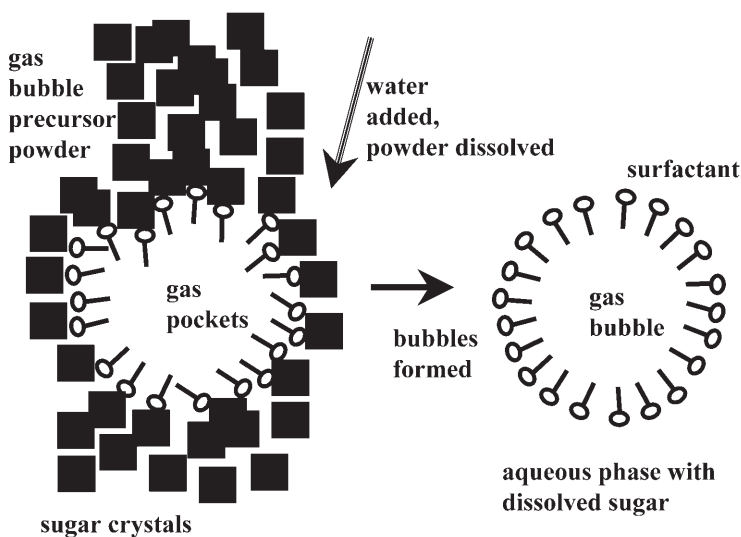


Fig. 3. A dry powder microbubble precursor agent: particles of water-soluble material (e.g., galactose) are coated with a surfactant (e.g., palmitic acid). Air or other gas is located in the spaces between particles. When water is added and galactose rapidly dissolved, gas-filled spaces between particles become individual microbubbles dispersed in the aqueous phase

Echovist or agitated saline) is its ability to pass through the lung capillary bed with the flow of blood. Earlier agents, due to their larger particle size and lack of a shell on the microbubble surface, were incapable of transpulmonary passage. Thus, they were incapable of traveling to the left side of the heart following intravenous injection, and had limited diagnostic applicability.

At about the same time the Echovist/Levovist strategy was developed, another approach was suggested – the use of protein shell to prepare microbubbles. It is well known that aqueous solutions of proteins (e.g., albumin) are capable of forming thick foams upon mixing, but these foams generally drain and disappear during storage. In order to create a stable shell coat for microbubbles, another step was necessary – denaturation of the protein [14]. A microbubble agent suitable for human use is based on human serum albumin, which is routinely administered in large quantities by intravenous infusions. The technique to form a protein shell requires heating of the protein solution to the temperature close to the globule melting point. Then, sonication is applied to disperse air in the albumin solution. The additional energy of ultrasound, combined with the ability of the gas-water interface to denature proteins, result in albumin denaturation and the formation of a thin membrane around individual air microbubbles. This membrane consists of denatured albumin. Such an agent, known as Albunex [15], was developed by Molecular Biosystems and received marketing approval in the US. The shell is quite stable; Albunex microbubbles can be stored for months with no detectable loss of particle concentration (Fig. 4).

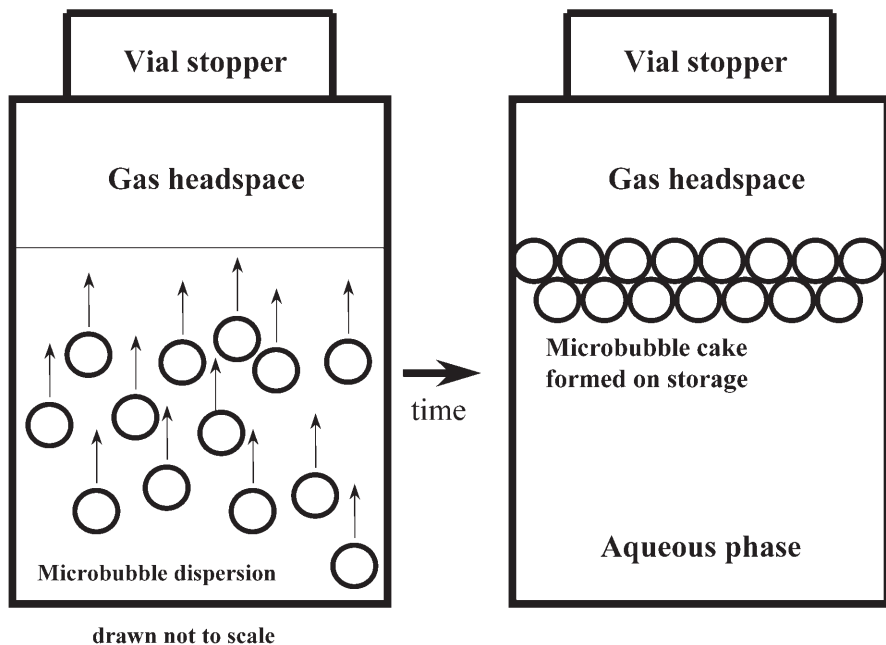


Fig. 4. Microbubbles coated with a protein or lipid shell form a stable cake on top of the aqueous phase. Microbubble fusion is inhibited. Long-term storage is feasible

Albunex was claimed to be the first ultrasound contrast agent capable of myocardial opacification after intravenous injection [16], but very large doses of the material had to be applied.

It is possible that the presence of oxygen in the air, combined with the action of high-intensity ultrasound can cause intermolecular crosslinking of albumin molecules in Albunex shell via formation of disulfide bonds [17]. Such cross-linking may further strengthen the shell. It is argued that active free radical species generated by sonication in the presence of oxygen may be responsible for this intermolecular disulfide bond formation. Substitution of air with other gases (nitrogen, helium, etc.) that did not contain oxygen resulted in a drastic loss of the yield of stable microbubbles. Use of myoglobin, which, unlike albumin, does not have free thiol groups, had the same effect. However perfluorocarbon-filled albumin-shelled microbubbles can be prepared with high yield. One would not expect medical-grade perfluorocarbon gas to contain oxygen. These bubbles presumably should not possess intermolecular crosslinking, but are very stable on storage (see below). Hence, intermolecular crosslinking may not be necessary for the microbubble stabilization.

Albumin-based air-filled microbubbles were reasonably stable on storage, but not very stable in the bloodstream. Air was rapidly lost and dissolved in the surrounding blood after administration to the patients. As a result, attempts to use this agent for perfusion studies in the clinical practice were not very successful, and it was limited mostly to opacification of the left ventricle and endocardial border delineation.

While the microbubbles with an albumin shell were already in development, other groups of researchers investigated the use of surfactants or lipid-stabilized microbubble shells. Pluronic-stabilized renografin-air bubbles were prepared, but their storage stability was unsatisfactory. It required sonication shear mixing immediately prior to administration into experimental animals [18]. Other materials fared considerably better. A combination of a hydrophobic Span and

Table 1. First-generation ultrasound contrast microbubble agents

Name/Shell composition/design	Gas content	Use/Approval Status	References
Mixed saline	air	Human use	[2]
Sonicated X-ray contrast	air	Human use	[2]
Sonicated Indocyanin Green	air	Human use	[2]
ST68 Sonicated Span/ Tween surfactant mixture	air	Animal studies	[19]
Filmix/lipid mixture shell	air	Animal studies	[21]
Albunex/denatured albumin shell/ dispersion in 5 % HSA aqueous solution	air	Approved in US, Europe	[15]
Echovist/no shell, dry carbohydrate matrix, requires reconstitution with water.	air	Approved in Europe	[12]
Levovist/palmitic acid, dry carbohydrate matrix requires reconstitution with water	air	Approved in Europe	[13]

hydrophilic Tween surfactants allowed preparation of a stable aqueous dispersion of microbubbles stabilized with the surfactant monolayer [19]. Stable cross-linked alginate polymer microbubbles were also prepared, but they were too big for intravenous use [20]. Lipid-stabilized bubbles were also suggested [21]. However, while these preparations may have provided shells sufficiently different from albumin shell (or Levovist) to ensure patent protection, their behavior in vivo was not sufficiently better.

6

Second Generation Contrast Agents: Composition and Design

During the 1990s, at least half a dozen major pharmaceutical and startup companies as well as groups of academic investigators were interested in improving on the limited success that had been achieved by the first-generation contrast agents (see Table 2 for some of the examples). An agent was needed with greatly increased circulation time (at least several minutes in the bloodstream), so that the question of tissue perfusion imaging could be tackled. Generally, two approaches, or a combination of both approaches, were applied. The first one was based on the use of low-solubility gases, mostly, fluorinated ones, like SF_6 , [22], C_3F_8 , [23, 24], C_4F_{10} , [25, 26] or the vapor of C_5F_{12} [27] or C_6F_{14} [28] in the balance of nitrogen gas. The second approach was based on the construction of a thick gas-impermeable protective microbubble shell [29].

7

Microbubble Gas Contents –

a Method to Improve Microbubble Stability in the Bloodstream

When low-solubility gases were incorporated inside the bubbles instead of air, destruction of the bubbles in the bloodstream was slowed down by several orders of magnitude. Typically, the increase in the size of a perfluorocarbon molecule by a single CF_2 group results in about an order of magnitude decrease in its solubility in water. While it is sometimes perceived that the diffusivity of high-molecular weight gas is also important for the issue of microbubble stability in the bloodstream, the actual diffusion mobility of smaller gas molecules (e.g., nitrogen, $M = 28$) is only several-fold higher than e.g., the diffusivity of C_4F_{10} ($M = 238$). Since the diffusion coefficient is inversely proportional to the molecule size, e.g., as cubic root of the molecular weight, that translates a tenfold increase in molecular weight into an approximately twofold decrease in diffusivity. However, the solubility in water is thousands of times less for the latter, and the stability of C_4F_{10} microbubbles in vivo in particular, is markedly improved (many minutes as compared to seconds). Similarly, the diffusion coefficient is only slightly higher for SF_6 than for C_4F_{10} , but the solubility of the latter is considerably lower, and so the stability of C_4F_{10} -containing microbubbles and their circulation time in the bloodstream is considerably improved. One of the examples of such direct comparison is Bracco's BR1/Sonovue microbubble agent, which contains SF_6 , [22] and the BR14 agent, which has the same shell but contains C_4F_{10} , and has a considerably longer life-span in the circulation [26].

In addition to the selection of gas for the microbubble core, a wide variety of approaches is available for the construction of the stable microbubble shell. The shells were initially copied directly from the first-generation microbubble design (e.g., Albunex to Optison). Later on, new shells were also developed which helped to improve microbubble circulation time and stability in the bloodstream.

8

General Design of the Ultrasound Agents and Microbubble Shell Design

The ideal microbubble shell would have to possess a set of often-conflicting properties, so, a compromise would have to be reached in many instances. If the bubbles are to mimic red blood cells *in vivo*, the shell should be flexible enough to allow microbubbles to pass through capillaries, otherwise, the bubbles might become lodged in the tissue and not be able to mark the blood flow. The shell should only slightly limit the vibration of microbubbles in the ultrasound field generated by echo imaging systems. If not, the microbubbles would not scatter ultrasound efficiently when driven by the pressure waves and would not provide a feasible contrast [30]. At the same time, a thick impermeable shell would be beneficial to extending the stability of microbubbles, reducing the rate of gas diffusion out of the microbubbles and increasing resistance to hydrostatic pressure [31]. Thicker shells might be used for the incorporation of drug substances for ultrasound-mediated drug delivery purposes [32]. Perhaps, a compromise should be reached, and, hopefully, the echogenicity would still be reasonably high while shell thickness would already improve the microbubble stability and also perhaps the load of the pharmaceutical agents. There were also indications that changes in the mechanical properties of the shell can influence the microbubble acoustic response, i.e., a change in the shell stiffness results in the change of the acoustic spectrum and resonance frequency of a microbubble. In particular, microbubble echogenic response at a certain frequency may be reduced.

It is possible that microbubble shell may be “shattered” during the interaction with an ultrasound pulse. Indeed, drastic variation of microbubble size, up to several-fold in less than a microsecond, has been reported [33], with linear speeds of the wall motion of microbubble approaching hundreds of meters per second in certain conditions. At these rates, it is easy to shatter the materials that would otherwise flow under slow deformation conditions. In some cases (e.g., lipid monolayer shells, which are held together solely by the hydrophobic interaction of the adjacent molecules), after such shattering the re-formation of the shell may be possible; in other cases – e.g., with a solid crosslinked polymer or a denatured protein shells – the detached “iceberg-like” pieces of the microbubble shell coat would probably not re-form and anneal, and the acoustic response of microbubbles to the subsequent ultrasound pulses would be different [34].

The materials used for the construction of the microbubble shell have to be fully biocompatible. They are either natural products (human albumin in the denatured albumin shell of Albunex and Optison), synthetic versions of natural

products (the lipids used for the construction of Sonovue shell) or combinations of synthetic biocompatible molecules. These may include biodegradable and biotolerable polymers (e.g., poly(lactic) acid derivatives in Acusphere's AI-700 [35] and cyanoacrylate polymer in Schering's Sonovist/SHU 563 A [36], or layered polymeric particles in biSphere [37]). Regardless of the shell composition, complete biocompatibility has to be proven, as well as lack of immune response to all the materials that constitute the microbubble shell.

Let us look in detail at some of the basic designs of the ultrasound contrast materials. There are generally three choices of design. First is a liquid emulsion of a low-boiling perfluorocarbon (e.g., dodecafluoropentane-based Echogen [38] or Sonogen/QW7437 [39] by Sonus Inc), which can generate gas microbubbles on demand *in vivo* or *in vitro* during the preinjection vacuum-activation procedure (phase-shift). The liquid nanoemulsion droplets of C_5F_{12} in the aqueous medium are stabilized by a fluorinated surfactant or a combination of surfactants (Echogen) or surfactants which carry a net negative charge (Sonogen). Essentially, this was a strategy to prepare microbubbles of the least soluble perfluorocarbon that is gaseous at 37°C (C_5F_{12}), without the use of a microbubble-protective shell. These agents have been tested in extensive clinical trials. Echogen was approved in 1998 by the European EMEA agency, but was not widely available. Approval was pending at the FDA in the US. The European application for Echogen was withdrawn in early 2001. There seems to be no further activity on these agents, which supports the preference of the shell-based stabilized microbubbles.

The second design of echo contrast materials is based on the dry powder material in the vial containing perfluorocarbon gas atmosphere. The powder in the vial is reconstituted with water prior to injection; at that time the shell-covered microbubble aqueous dispersion is formed. This is a direct continuation of the Echovist and Levovist first-generation agent approach, only the shell design is more advanced, and a low-solubility gas is used instead of air. Several agents are designed in a similar way, e.g., Bracco's Sonovue/BR1, recently approved in the EU, decafluorobutane-containing BR14 (in clinical trials), and Imagent (AFO145/AFO150) (Alliance Pharma, currently also known as Imavist by Schering) [40]. For Imavist/Imagent, an "approvable" letter was issued by the FDA. Another example is the decafluorobutane-based lipid-stabilized microbubble preparation Sonazoid/NC100100 [41]). This Nycomed-Amersham agent has undergone numerous clinical trials, and marketing approval is currently pending. All these agents are designed as a dry powder or cake of a water-soluble substance (e.g., polyoxyethylene) that rapidly dissolves after addition of water (the same as Levovist, Fig. 3). Such agents should probably be manufactured by high-shear mixing/sonication of gas into the aqueous lipid/surfactant dispersion with the formation of microbubbles. At the next step, the material is probably spray-dried or freeze-dried. As water removal is completed and vacuum released, vials are filled with a high-molecular weight gas. One may expect that AI-700 (Acusphere, Phase II clinical trials announced) [35] may have a similar design, where a spray-dried biodegradable polymer shell preparation of porous microparticles is placed in a vial with a perfluorocarbon atmosphere.

An intermediate approach between a second and third design types is used for the Definity agent, which was recently approved for marketing in the US (Dupont Pharma, earlier known as DMP115, also MRX-115 Aerosomes from ImaRx) [24]. It constitutes an aqueous dispersion of small (submicron) liposomes made of a phosphatidylcholine and PEG-phosphatidylethanolamine in a vial filled with an octafluoropropane atmosphere. Prior to injection into the patient, a sealed vial is placed in the arm of a mechanical shaker and rapidly mixed for a short time. The mixing process forms octafluoropropane microbubbles stabilized with the lipid shell [42]. Such a design allows a simplified production scheme. It is easy to manufacture liposomes reproducibly in large quantities (e.g., by a microfluidizer); they can be sterile-filtered and stored in bulk until packaging is performed. Finishing steps include filling the vials with aqueous liposome dispersion, filling in a heavier-than-air octafluoropropane gas and closing the vial stoppers. No expensive lyophilizer time is required for this product design. However, one must ensure that microbubbles, which are manufactured by the end user immediately prior to injection, should have a reproducible preparation yield and expected size distribution. To achieve that, mixing is performed in a controlled manner in an automated shaker device.

The third design suggests making microbubbles by the method of high-shear mixing, e.g., sonication, of perfluorocarbon gas (octafluoropropane as in Optison, or decafluorobutane, as in MP1950 or in PESDA) in the medium that would immediately form the shell on the surface of gas-to-liquid contact by self-assembly. This way, after preparation, the microbubble size distribution may be tested at the production site. Controlled storage conditions ensure that the microbubble size distribution, gas content and respectively the injected dose are within the specification limits. There is no need for additional preparation steps such as material reconstitution prior to injection. The material is stored as an aqueous dispersion, without lyophilization or freezing. It is counter-intuitive to expect bubbles stabilized with a thin film to stay intact in an aqueous phase for a year or more, but the bubbles are indeed stable. Optison (Mallinckrodt Inc/Nycomed Inc, or FS069, Molecular Biosystems) [23] as well as PESDA [25] microbubbles are prepared by denaturing human albumin at elevated temperatures during sonication, similarly to Albutex. A thin (about a dozen nm) crust of the material that is essentially similar to a boiled egg white is formed on the interface between the gas and albumin solution as this new contact surface is generated by high-shear sonic dispersion. MP1950 microbubbles (an experimental ultrasound contrast agent from Mallinckrodt Inc) are prepared similarly, by sonic dispersion of decafluorobutane gas in an aqueous micellar solution of DSPC phospholipid and PEG stearate, with other lipids if necessary [43]. A monolayer of phospholipid is assembled on the interface between the gas and aqueous phase. As the melting point of DSPC is $\sim 55^\circ\text{C}$, during the refrigerated storage the monolayer on the surface of the microbubble stays solid. Therefore, the ability of microbubbles to fuse during their collision is inhibited. To reduce the chance of microbubble fusion on storage even further, several mol percent PEG stearate is also implanted in the shell. It is positioned in such a way that the stearic acid tail is incorporated in the lipid monolayer. Stearic acid is attached to one terminus of a PEG chain. PEG polymer molecules protrude out

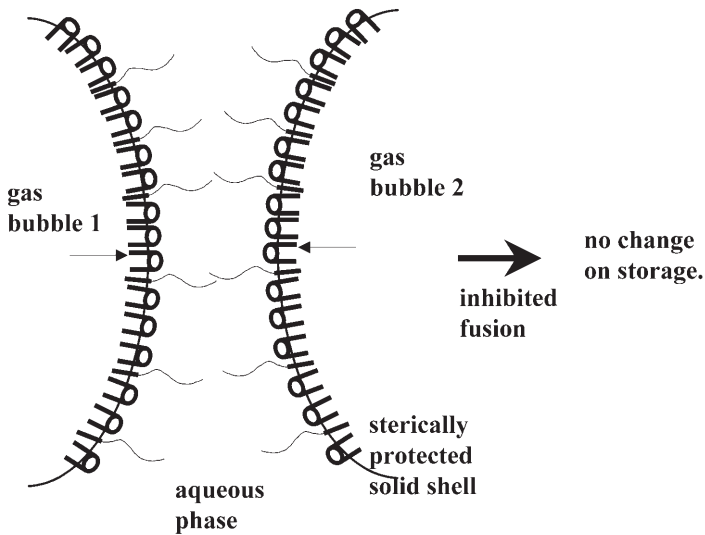


Fig. 5. Microbubbles coated with a shell of lipid/surfactant below the critical temperature, especially armored with an additional steric protection polymer brush layer, do not fuse. Long-term storage of pre-formed microbubbles in the aqueous phase is feasible

of the monolayer into the surrounding aqueous phase, creating a dense (e.g., ~10% by weight in a balance of water) PEG brush, ~5–10 nm thick. The exact brush characteristics depend on the length of the PEG chain and PEG surface density. The PEG brush on the surface of microbubbles serves as an additional repulsion barrier, which inhibits microbubble fusion so that long-term storage of microbubbles in the aqueous phase becomes possible (Fig. 5). In addition to or in lieu of the PEG brush, charged lipid molecules can also be incorporated in the shell, to use the electrostatic repulsion between the charged membranes of adjacent particles, as it is customary for the stabilization of colloid systems. Another benefit of a PEG brush on the microbubble shell surface may be an additional steric protection of contrast agent material, leading to longer circulation time and lower nonspecific binding *in vivo* (similar to Stealth liposomes [44] and Pluronic-coated polystyrene latexes [45]).

9

The Importance of the Microbubble Size Distribution

Size distribution plays a major role in the microbubble stability, behavior *in vivo*, and the microbubble acoustic response. The Rayleigh-Plesset equation which describes the microbubble response to pressure waves suggests that ultrasound scattering is proportional to the sixth power of the microbubble diameter [46]. It is not possible, however, to inject large bubbles (e.g., 0.1 or 1 mm in diameter) in the bloodstream, because they would be immediately lodged in the vasculature as emboli, severely limiting the blood flow. Fortunately, microbubbles with the size of several micrometers are still quite echogenic in the ultrasound

Table 2. Second- and next-generation ultrasound contrast microbubble agents

Name/Shell composition/design	Gas content	Use/Approval Status	Reference
Echogen/liquid microemulsion of C ₅ F ₁₂ in aqueous solution with Telomer surfactant (shell not present; pre-injection vacuum-activation used)	C ₅ F ₁₂	Approved in EU (withdrawn)	[38]
Sonogen/liquid C ₅ F ₁₂ microemulsion in aqueous solution with negative surfactant	C ₅ F ₁₂	Clinical trials completed	[39]
Definity/phospholipid + PEG-phospholipid liposome aqueous dispersion with gas headspace requires preactivation by high-speed mixing	C ₃ F ₈	Approved in US	[24]
Optison/denatured albumin shell dispersion of gas microbubbles in 1 % HSA	C ₃ F ₈	Approved in US and EU	[23]
Sonovue (BR1)/DSPC/DPPG shell/dry material with PEG; requires reconstitution	SF ₆	Approved in Europe	[22]
PESDA/denatured albumin shell dispersion of gas microbubbles in HSA/dextrose solution	C ₄ F ₁₀	Clinical Trials	[25]
BR14/DSPC, DPPG shell/dry powder with PEG; requires reconstitution	C ₄ F ₁₀	Clinical Trials	[26]
MP1950/DSPC + PEG stearate shell/dispersion of microbubbles in aqueous medium	C ₄ F ₁₀	Experimental Agent	[43]
Sonazoid (NC100100)/lipid shell/dry powder; requires reconstitution	C ₄ F ₁₀	New Drug Application	[41]
Sonovist/cyanoacrylate polymer shell/Imavist/Imagent/mixed phospholipid	air	Clinical Trials	[36]
	C ₆ F ₁₄	New Drug Application	[40]
dry powder; requires reconstitution	+ N ₂		
biSphere/layered microbubbles/adjustable shell thickness	fluorocarbon or air	Clinical Trials	[37]
AI700/poly-lactide-co-glycolide + PEG	fluorocarbon	Clinical trials	[35]
Quantison/thick shell made of denatured albumin by spray-drying	air	Clinical trials	[29]

frequency range of several MHz, which is most popular for diagnostic imaging. At this size, microbubbles can traverse through the microvasculature of the lungs with the flow of blood after intravenous injection and pass to the left ventricle and later to the heart muscle for imaging of myocardial perfusion. Smaller size microbubbles (~ 1 µm) are not very echogenic [47, 48]. The surface-to-volume ratio would be much higher in smaller bubbles, and they would lose encapsulated gas much more readily, both during the passage through the bloodstream and during the active events of bubble destruction in the ultra-

sound field (it takes much lower intensity of the ultrasound field to destroy smaller microbubbles). It would be most desirable to have a narrow size distribution microbubbles with the particle size small enough to allow free passage through the capillaries in all the tissues, but large enough to possess high echogenicity (perhaps $\sim 3-4\text{ }\mu\text{m}$ range). Extra-large ($\sim 10\text{ }\mu\text{m}$ mean size) Myomap microbubbles with a thick protein shell were proposed as a contrast agent that would lodge in the capillaries in the heart muscle to map myocardial blood flow [49]. However, blood flow through the capillaries that were blocked by the microbubbles would be greatly diminished. The suggested atrial route of administration of the agent also made it less feasible.

10

Gas Exchange from and to the Microbubbles

Deflation of microbubbles with the dissolution of gas in the surrounding medium was studied in detail by Epstein and Plesset [50] long before the microbubbles were suggested as ultrasound contrast agents. Obviously, if the medium is under saturated with the gas, there will be a flux of gas out of the bubble. The rate of gas loss from the bubble is dependent on the ability of the gas to pass through the shell (if any), dissolve in the surrounding medium and move away from the surface of the microbubble (either by diffusion or convection). For surfactant-coated bubbles in the aqueous medium, microbubble deflation takes place even if the medium is fully saturated with the gas. It is driven by surface tension, i.e., elevated Laplace pressure inside the bubble, which equals $2 \times (\text{surface tension})/(\text{radius of the bubble})$. Respectively, smaller bubbles would disappear progressively faster, and overall stability of the microbubbles in the aqueous phase should be limited. However, some of the ultrasound contrast microbubbles (e.g., Optison or MP1950) can be stored for many months as an aqueous dispersion without the loss of gas. We then have to assume that the shell of these microbubbles counteracts surface tension and brings internal bubble pressure down to ambient levels, so there is no driving force for the gas to leave the shell.

The rate of gas loss from a microbubble determines the effective contrast duration in the bloodstream. Scattering of ultrasound is proportional to the sixth power of microbubble size, so even a slight loss of gas from the bubbles may lead to a drastic reduction in contrast intensity. Monomolecular layers of lipid-based materials, as well as dozen-nm thick shells of denatured albumin, cannot serve as very efficient barriers to gas diffusion out of the bubbles. These shells also deform in response to external (e.g., arterial) pressure. Hence, these types of agents have to rely on the insoluble gas approach to ensure microbubble longevity in the bloodstream. Particles with thick shells, on the other hand, are well stabilized against gas loss. The extreme examples of such microspheres are the hollow glass or plastic beads with multi-micron shell thickness. These materials are available from the microbead manufacturing companies, but they are not biocompatible and therefore unsuitable for in vivo applications. Thick-shelled protein microbubbles prepared by spray-drying technology are quite stable against gas loss; however, they are not very echogenic [51]. Perhaps an

intermediate-thickness biodegradable shell (e.g., 50–200 nm) combined with an insoluble gas core would ensure optimal life-span *in vivo* and high ultrasound scattering efficacy.

The interaction of microbubbles with the milieu of the bloodstream does not necessarily constitute a one-way movement of gas out of the bubble. If the gas inside the bubble is poorly soluble in the aqueous medium, and another gas (e.g., air) is dissolved and saturates the surrounding medium, the process of rapid uptake of air into the bubble should take place for some of the larger microbubbles. Such microbubble growth was suggested in a series of modeling papers by Van Liew group [52, 53], and later expanded by Kabalnov et al [54, 55]. Only after the initial influx of air into the bubbles would the general deflation of microbubbles start. Once most of the gas inside the bubble is air, the deflation process might occur rapidly as it did for the unstable air-filled first generation microbubbles. Loss of perfluorocarbon from microbubbles *in vivo* does indeed occur, as confirmed by the fact that most of the perfluorocarbon from intravenously administered microbubbles is exhaled through the lungs within minutes after injection [56].

Interestingly, we have found that microbubble deflation in the ultrasound field is unusually slow for the microbubbles that contain perfluorocarbon gas when the surrounding medium is depleted of air and saturated with the same fluorocarbon gas [57]. So, perhaps in an air-saturated environment the ultrasound-mediated destruction of microbubbles may depend on the influx of air into the bubbles, not just on the perfluorocarbon loss.

11

Sensitivity of Microbubble Detection by Ultrasound

Success of a contrast agent in any imaging modality is determined by the amount of contrast material needed to achieve efficient contrast on the screen of a medical imaging system. Typically, injection of several milliliters (billions of individual microbubbles) of a first-generation contrast agent (e.g., Albunex) was required to achieve brief (seconds) contrast of blood in the ventricles of the heart. Obviously, bubbles were rapidly deflating, losing air into the bloodstream. Consequently, the bubbles lost most of their ability to scatter ultrasound before they had a chance to reach the imaging site. Another factor was the often-used higher-intensity transmitting power of the ultrasound systems, which contributed to the bubble destruction [58]. Higher transmitting power was a popular tool applied to improve the quality of the echo images. In these conditions, the contrast agent was instantly destroyed as soon as it reached the insonified site. Low transmitting power imaging as well as intermittent imaging schemes solved this problem. Advent of the second-generation agents (e.g., use of fluorine-based gases instead of the air core) improved the microbubble lifespan in the bloodstream from seconds to minutes. A dose of a fraction of a milliliter ($\sim 10^8$ microbubbles, fraction of a milligram of the shell material) is currently sufficient for an intravenous injection to achieve extended contrast in blood, in heart chambers or in the liver.

Another factor improving the sensitivity of microbubble detection is the development of specialized microbubble imaging modes by the ultrasound

equipment manufacturers. Initially, the Doppler function of the ultrasound equipment was applied for the selective bubble imaging (bubbles can emit ultrasound at the frequency different from the original driving frequency, especially during their destruction. This frequency change can be detected by Doppler ultrasound [9]). As microbubbles are capable of a non-linear response, they can generate second and third harmonic ultrasound backscatter signals, which can be detected with the ultrasound system probes and easily distinguished from the mostly-fundamental frequency tissue response. Some second-harmonic signal is still generated by normal tissue. So, this imaging mode does not prove to be very efficient for the selective imaging of microbubbles. A more advanced method for selective microbubble detection is based on pulse inversion, which improves signal-to-noise ratio by minimizing the signal from the surrounding tissues. Two pulses, one with a normal and one with an inverted phase signature, are transmitted consecutively by the transducer. Normal tissue responds to them in a similar (linear) manner. So, when the echo signals from that tissue are received by the transducer, they represent mirror images of each other and upon signal addition they cancel each other out with zero net result. Microbubbles respond nonlinearly (and differently) to these ultrasound pulses, and the product of the echo addition is not zero. Ultrasound equipment manufacturers use various names for such imaging modes (phase inversion, pulse inversion, contrast harmonic, etc). All of these approaches have greatly improved the sensitivity of microbubble detection, so that the first-generation air-based ultrasound contrast agent, Levovist, is making a strong comeback in its diagnostic imaging use, since it can now be detected in the tissues in small quantities, particularly by pulse inversion imaging [59]. In fact, pulse inversion contrast harmonic may allow detection of individual microbubbles (Fig. 6), which means that micron-size particles in the picogram weight range can be visualized by ultrasound. This result further strengthens the possibility of using ultrasound contrast in selective tissue targeted imaging (see below).

The response echo signal is not always proportional to the microbubble concentration. When it is low, the increase in the concentration of microbubbles (several thousand particles per milliliter) does result in the respective increase

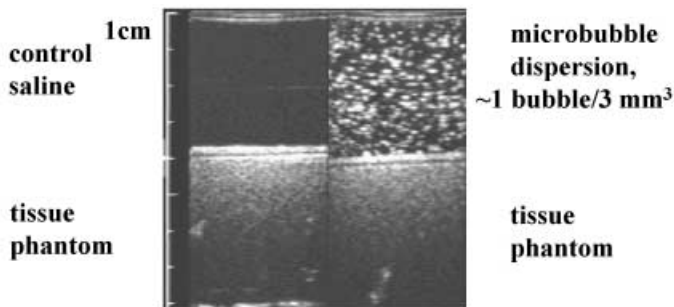
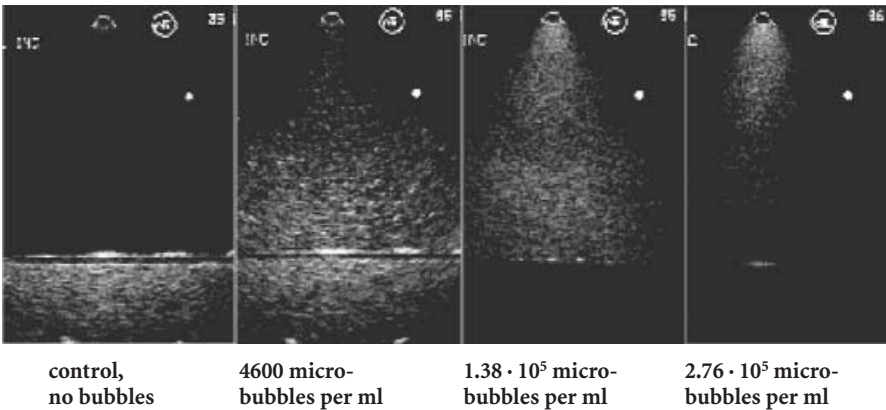


Fig. 6. Ultrasound imaging of a dilute microbubble dispersion in normal saline (*right*). Comparison with imaging of control normal saline (*left*). Imaging performed using a contrast pulse inversion scheme. Samples placed on top of an ultrasound tissue phantom (*bottom*)



Microbubbles in a saline bag placed above a tissue phantom.

Fig. 7. Concentration dependence of ultrasound backscatter signal by dispersions with increasing microbubble concentrations (from left to right). Imaging performed using a fundamental frequency scheme. Samples placed on top of an ultrasound tissue phantom (*bottom*)

of the echo signal (expressed as white speckles on the recorded image). However, a further concentration increase, in the range of hundreds of thousands of particles per milliliter, causes loss of the echo signal, first from the areas underlying the area of contrast, and then the contrast area itself (Fig. 7). The term used to describe this phenomenon is ultrasound attenuation. At high concentrations, microbubbles do not allow the passage of ultrasound through the area, mostly due to multiple scattering on the microbubble particles and the transfer of ultrasound energy to heat. Attenuation is dependent on the overall number of microbubbles in the beam, so it is stronger for thick slices of contrast-rich tissue, e.g., the right ventricular cavity of the heart after intravenous injection of contrast. This phenomenon often causes complete loss of echo signal at the bottom of the image immediately after contrast administration. One simple way to avoid attenuation is to avoid injecting a large dose of microbubbles as a rapid bolus, but instead to administer smaller doses of contrast at intervals. Continuous contrast infusion can also be applied.

Overall, the dose of microbubble contrast agents required for detection by ultrasound imaging is usually less than a microgram of material per gram of blood or tissue. This distinguishes these materials from X-ray and MRI contrast agents and makes them comparable to radiopharmaceutical tracers used in nuclear medicine. Microbubbles can be observed either when they are in the bloodstream, or when attached to solid support (see Fig. 8). At high surface microbubble concentration, attenuation is apparent, just as for microbubble dispersions (cf. Fig. 7). When microbubbles cover even a small percentage of a flat plastic dish, the backscatter signal is still quite strong. With surface coverage at 0.5%, microbubble weight was estimated at nanograms/cm² of the target surface [60]. This shows the potential of site-targeted microbubble contrast design.

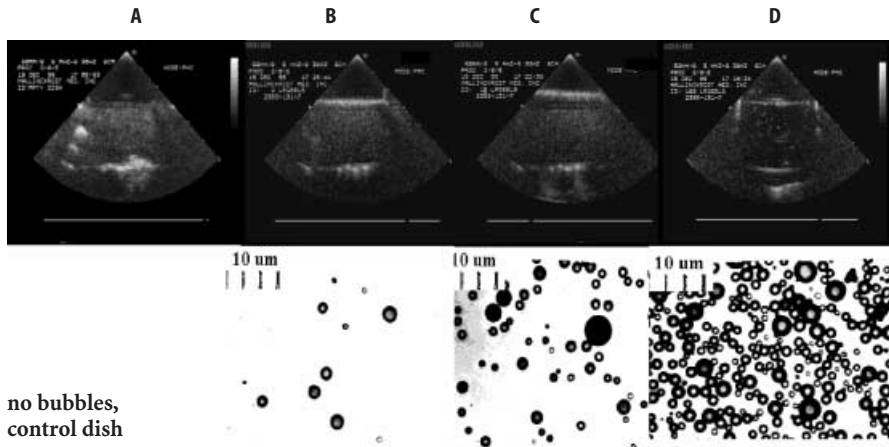


Fig. 8. Concentration dependence of ultrasound backscatter signal by plates coated with a layer of targeted microbubbles. Surface concentrations of microbubbles (as observed by bright-field optical microscopy, bottom) increase from left to right. Imaging performed using a fundamental frequency scheme. Samples placed on top of an ultrasound tissue phantom. Reprinted from *Advanced Drug Delivery Reviews* v. 37, A.L. Klibanov, Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging, p. 145. Copyright, 1999, with permission from Elsevier Science

12

Practical Applications of Microbubbles in Medical Imaging: General Principles

A general limitation to the usage of microbubble-based contrast agents is set by the restrictions on the particle size – several microns on average. Submicron microbubble particles are not very echogenic and rather unstable, so the space in the organism beside the areas where red blood cells travel is essentially off-limits if a regular intravenous administration of contrast is applied. Ultrasound contrast agents were discovered and initially developed in the cardiovascular imaging setting [1]. There are numerous applications in that area, some of them already approved by regulatory agencies (e.g., left-ventricle opacification and endocardial-border delineation for Optison [61]). However, because ultrasound equipment and the echo image quality are rapidly improving, contrast agents soon may not even be needed to aid obtaining anatomical information. The users would rather obtain functional information about tissues and organs. One example of such functional imaging is tissue perfusion. Ultrasound contrast agents were long ago proposed for imaging of myocardial perfusion defects, but years later this application has not yet reached widespread clinical practice. Many clinical trials have been performed, and successful procedural designs have been reported [62]. However, no regulatory approval has been received for this application yet. The reason for delay was most likely because several interdependent issues had to be clarified. These factors included the need for a long-circulating or infused contrast; the use of the improved phase inversion imaging



Fig. 9. Ultrasound images of the liver before (*left*) and 10 min after Sonazoid administration (delayed phase, where ultrasound contrast is accumulated in the normal liver tissue, *right*). Copyright, 2001, L. Needleman, Thomas Jefferson University, Philadelphia, PA. Reprinted with permission from [66]

modes to increase equipment sensitivity to microbubbles; and the fact that microbubbles can be destroyed by ultrasound (and it can be used to map influx of the contrast agent into the tissue). A complication specific to heart imaging is the constant three-dimensional movement of the heart and lungs, which makes frame-to-frame comparison difficult. Hopefully, further improvements in the ultrasound equipment will soon allow robust mapping of myocardial perfusion with existing contrast agents in widespread clinical practice.

Meanwhile, contrast echo imaging for the liver is rapidly becoming the imaging modality of choice for the detection of metastatic disease [63], hepatocellular carcinoma [64] and hemangioma [65]. There are ongoing clinical trials. This indication will hopefully find widespread use for gathering information about the condition of liver tissue. The wash-in and wash-out of contrast, as well as “late phase” imaging of microbubbles that were retained in the normal tissue, possibly via association with Kupffer cells, may allow tumor foci delineation and differential diagnosis of certain types of tumors (Fig. 9). The use of microbubbles that are selectively targeted towards normal liver tissue, may help [66, 67]. Even more important, contrast ultrasound may provide guidance in the therapeutic procedures in the ablation of metastases. Flow and contrast tissue accumulation data can be used to ensure that all the blood flow to the metastatic sites is blocked and all the malignant tissue is destroyed (e.g., by cryo- or radiofrequency ablation) [68]. Similar diagnostic information may be obtained in the case of contrast echo imaging of kidneys [69].

13

Practical Application of Microbubble Destruction by Ultrasound

It is now widely recognized that microbubbles can be destroyed by ultrasound irradiation [70]. This may be explained simply by the fact that when microbubbles are subjected to external pressure, they shrink and gas leaks out and dis-

solves in the surrounding aqueous medium. Destruction of microbubbles typically occurs at higher acoustic pressure values. In order to measure acoustic energy of the ultrasound wave, it has been proposed to compute Mechanical Index (MI) value = (peak negative pressure)/(frequency)^{1/2}. This number represents a relative semi-quantitative way of comparing the power settings of ultrasound for various frequencies, transducers and imaging systems. For modern ultrasound equipment, MI is normally displayed on the main screen of the imaging system, sometimes along with the Thermal Index. The latter is a measure of heat energy delivered to the tissue by ultrasound. Ultrasound system computer controls equipment settings so that the threshold safe value of the intensity of transmitted ultrasound is not exceeded (typically, MI < 1.9). Recently, the use of MI to predict microbubble destruction was strongly discouraged [71], but so far no replacement has been introduced, and MI still serves as a rough estimate of the transmitting power effects on ultrasound contrast.

Destruction of microbubbles is typically initiated as MI is increased to 0.2–0.3. The degree of destruction depends on pulse repetition (this is the number and rate of individual pulses in the sequence transmitted by the probes to obtain one picture frame) and the ultrasound frame rate. In modern equipment, the frame rate can be easily adjusted, from normal “real-time”, ~20–40 frames per second to very long intervals, up to 10 s or more between frames. Often, if tissue motion is involved (e.g., heart imaging) the ultrasound picture frame is taken in sync with some physiological parameter, e.g., at a particular phase of the EKG signal and breathing cycle. This allows frame-to-frame comparison of the tissue images. It is widely used to monitor tissue perfusion by contrast agents. One of these techniques involves varying the ultrasound frame rate, where first the ultrasound picture frames are separated by short intervals, and then gradually the interval is increased. Tissue content of contrast microbubbles in the areas of full perfusion is progressively replenished as the interval between the pulses is increased. The areas of poor perfusion demonstrate delayed filling with contrast and lower ultrasound signal plateau values. These perfusion defects can be easily detected on the ultrasound image as dark areas with low echogenic response. Such imaging strategy requires constant concentration of microbubbles in the bloodstream during the imaging period. This can be achieved by using a long-circulating contrast, i.e., microbubbles that stay in the bloodstream for a long time after a single intravenous injection (novel ultrasound agents may be necessary for this). Alternatively, existing contrast agents in the continuous slow infusion mode can be applied [72]. There are several schemes to perform such slow infusion: microbubbles can be diluted in an infusion saline bag, placed in a rocking syringe pump device, or delivered through a long thin tubing connected to an intravenous line.

14

Interaction of Microbubbles and Ultrasound: Physics

The progression of microbubble destruction in the ultrasound field strongly depends on the intensity and frequency of the ultrasound field. Generally, at low intensity (e.g., MI ~ 0.1, especially when imaging is performed in the intermit-

tent mode), no visible deflation of microbubbles can be detected. The most convenient method for observing microbubble destruction is optical microscopy: microbubbles are insonified either free-flowing in a thin capillary tubing [34] or attached to a solid support [73] and studied by microscopy. Typically, at $MI > 0.2$ most microbubbles demonstrate slow deflation; if insonation is intermittent, deflation of microbubbles coincides with the ultrasound pulses and ceases when ultrasound transmission is interrupted. When microbubbles are in close proximity to each other (e.g., a dense patch of microbubbles located several microns from each other on a solid support) the destruction slows down. This might occur due to the formation of a protective layer of liquid oversaturated with gas. The gas is then driven back to the bubble from the oversaturated surrounding medium. Alternatively, microbubbles that are located closely to each other are acoustically coupled and respond to sound waves differently.

At high mechanical index values ($MI \sim 1$ or more), microbubble destruction is much faster and may become violent. It has been shown with the use of a high-speed videocamera that, for a small period during the compression-rarefaction cycle, microbubbles subjected to high-intensity ultrasound may grow several-fold over the size of the original microbubbles and rapidly shrink to the size an order of magnitude less [33]. Prior to full collapse, the bubble may break into several pieces (Fig. 10). The rate of microbubble wall movement may reach hundreds of $m\ s^{-1}$. These events might cause damage to the endothelial lining of some capillaries, although the dose of contrast agent used in an animal experiment to demonstrate these events in the capillaries was hundreds of times more

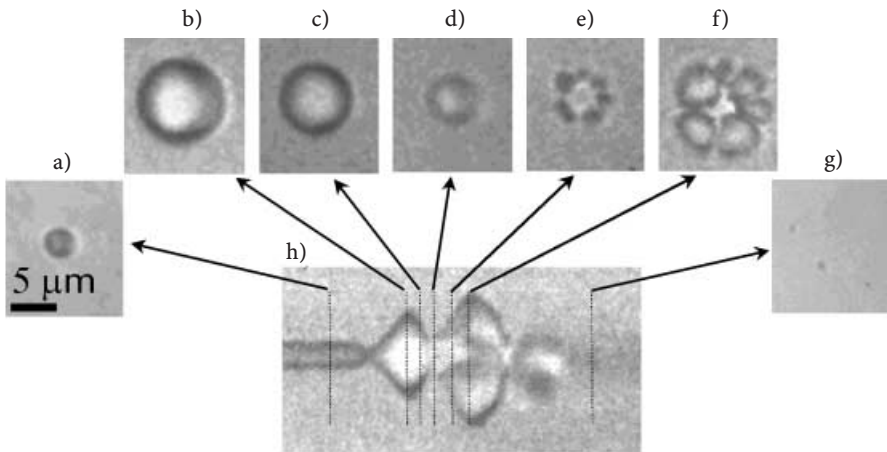


Fig. 10. Microbubble growth and collapse in the ultrasound field. Microscopy images taken with a high-speed camera during microbubble insonification. Optical frame images and streak image corresponding to the oscillation and fragmentation of a contrast agent microbubble, where fragmentation occurs during compression. The bubble has an initial diameter of 3 μm , shown in (a). The streak image in (h) shows the diameter of the bubble as a function of time, and dashed lines indicate the times at which the two-dimensional frame images in (a)–(g) were acquired relative to the streak image. Camera temporal resolution, 10 ns. Copyright, American Institute of Physics, 2000. Reprinted with permission from *Appl. Phys. Lett.* (2000), v. 77, p. 1057

than that normally used in humans. [74, 75]. At very high MI values, destruction of phagocytic cells that have taken up microbubbles may occur [76]. Therefore, it might be reasonable to moderate the transmitting power of ultrasound equipment when used in combination with microbubble-based contrast agents. This information confirms the validity of the statement on the front panel of an old ultrasound imaging system: "Use of minimum practical acoustical exposure is prudent".

15

Interaction of Microbubbles and Ultrasound: Targeted Drug Potentiation and Delivery Applications

In the world of drug delivery it was a long sought paradigm: to have a drug or a drug precursor injected in the bloodstream, but allow it to act only where and when it is required, turned on by an external "switch". The designs include temperature-sensitive liposomes that release the drug at the areas of local hyperthermia created by microwave irradiation. Alternatively, a photoreactive dye, like a porphyrin, would be photo-activated for tissue ablation via generation of reactive free radicals (e.g., for tumor therapy). However, these approaches have their limitations because both light and microwave radiation are difficult to deliver deep inside the body (recently, a needle-probe radiofrequency ablation has been used for tumor therapy in the liver). So, it is natural to suggest the use of the ultrasound waves that can penetrate deep inside the tissue and can be easily focused in a narrow beam. Ultrasound waves in combination with microbubble agents could be used for the ultrasound-mediated selective drug release and tissue delivery. Drugs can be co-administered with the microbubbles, to be activated by physical means in the presence of microbubbles. The first reported example of this approach is ultrasound-enhanced thrombolysis, where the efficacy of thrombolysis by a plasminogen activator enzyme was improved by ultrasound irradiation, particularly in the presence of microbubbles [77]. The action of ultrasound in this case is most likely purely mechanical, by breaking up the thrombus structure, assisting the influx of the thrombolytic enzymes into the area as well as removal of the degradation products. In the vicinity of a microbubble, microstreams of the surrounding aqueous medium were detected [78].

Another example of a delivery system based on microbubbles and ultrasound is the delivery of circulating microparticles (polymer latex beads) or fluorescent red blood cells outside of the capillaries into the surrounding tissues by the action of ultrasound on the co-injected Optison microbubbles [79]. Interestingly, polymer beads and red blood cells could be detected tens of micrometers away from the capillaries where the bubble destruction took place. This may imply that during rapid destruction of a microbubble in a very strong ultrasound field, adjacent microsphere beads in the bloodstream can be propelled deep into the surrounding tissues.

In a similar approach, entrance of plasmid DNA into the cells for transfection was improved by ultrasound [10, 80, 81]. The term "sonoporation" was suggested to describe this application in general. In order to bind plasmid DNA, microbubbles have to carry a net positive charge. Microbubble-DNA constructs

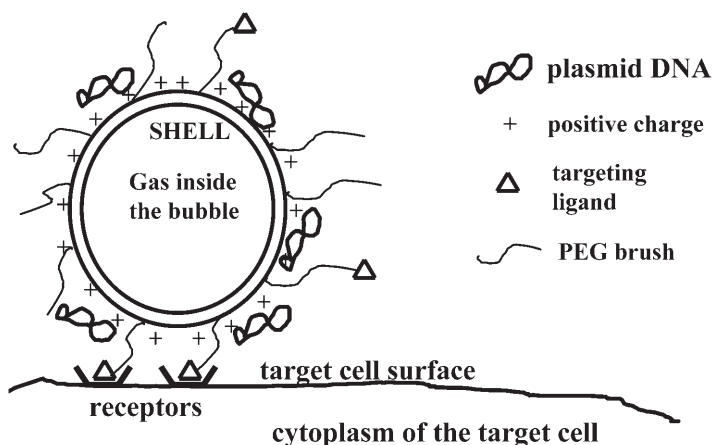


Fig. 11. Design of a plasmid DNA-microbubble complex for targeted DNA delivery

can be injected into the organism, either intravenously, intraarterially, or locally. Targeting ligands may be applied to ensure selective attachment of microbubble-DNA complexes to the cells of interest (Fig. 11). The complexes can then be activated by ultrasound, which would propel DNA into the surrounding cells or even deeper into the bulk of the tissue for transfection.

Not only plasmid DNA can be delivered via ultrasound-mediated microbubble destruction, but also smaller molecules such as antisense oligonucleotides [82], paclitaxel [32] and dexamethazone [83]. The drugs should be attached to the microbubble membrane either via a hydrophobic interaction, or connected to a microbubble shell by a covalent or noncovalent bond. During ultrasound irradiation, as the microbubble loses its gas core, microbubble shell fragments containing drug may be released from the bubble and go into the surrounding tissue, sometimes propelled by the action of ultrasound during microbubble collapse (see Table 3 for some examples of microbubble-based drug delivery agents).

In order to design a successful delivery system based on microbubble-ultrasound interaction, one has to apply the following strategy. The amount of the drug to be delivered should be small in order to accommodate it on the limited surface of microbubbles to be injected, perhaps under a milligram overall amount. Hence, potent drugs should be used. The drugs should possess low toxicity because one would expect that only a small percentage of the microbubbles administered to the patient would flow through the insonified target area, and that most of the drug-microbubble complex would eventually end up in the Kupffer cells in the liver after microbubble deflation [84], where the action of the drug may not be desirable, or would be metabolized into nontoxic products. If a drug is nontoxic and potent in itself, it would not even require selective targeted delivery. This further strengthens the suggestion of using plasmid DNA-microbubble complexes for ultrasound-mediated delivery, because plasmid DNA in the bloodstream or in Kupffer cells is rapidly degraded, but when delivered into the target cells by ultrasound, it may allow efficient expression

of the target proteins so that therapeutic effect could be achieved only in the insonified areas of the body.

16

Targeted Imaging of Microbubbles or Other Echo Contrast Agents

Tissue-specific imaging with ultrasound contrast agents was suggested long ago, but only recently has the development of the tissue-specific agents reached beyond the stage of concept patents and papers. The general idea is simple – an echo contrast agent outfitted with the targeting ligand administered to the patient is accumulated in the area of high target receptor density. Thus, ultrasound contrast enhancement of that area is achieved. The initial proposal was to prepare bicarbonate-filled targeted liposomes augmented with target-selected antibodies. Liposomes formed microbubbles upon pH-induced release of carbon dioxide gas, and targeted imaging could be performed [85]. Later, another liposome-based targeting agent was proposed – lyophilized liposomes with covalently attached antibodies. It was hypothesized that the oligolamellar structure of liposomes would make them echogenic. Successful targeting was demonstrated *in vitro* in model systems [86–88]. Unfortunately, some liposome-based agents possessed relatively low echogenicity and required the application of high frequency probes for imaging. As a further development of the liposome/nanoparticle scheme, a submicron emulsion of fluorocarbon liquid, stabilized with surfactants and having biotin-avidin-antibody sandwich or covalently attached antibody, was proposed as an MRI and ultrasound contrast agent [89, 90]. This emulsion agent also possesses low echogenicity in the bloodstream. However, it stays in circulation for a long time in order that targeted nanoparticles have sufficient time to accumulate at the target site (e.g., a fibrin clot). When nanoemulsion particles cover the target surface completely, some ultrasound contrast response is generated [90], but it is mostly demonstrated at higher frequencies. The use of a liquid fluorocarbon emulsion as a long-circulating contrast agent was first suggested years ago [91], but the amount of the material required for successful contrast was quite high. Overall, the use of gas-filled microbubble preparations for the design of targeted echo contrast remains a valid strategy. More recently, with the advent of pulse inversion imaging equipment and long-circulating stable microbubbles, this seems to be a most reasonable approach. Let us now focus on the design of targeted microbubble agents (see Table 3 for some examples).

Targeting ligands may be attached to the microbubble shell covalently or non-covalently, via a hydrophobic anchor inserted in the microbubble monolayer shell, or via an avidin-biotin sandwich, or a combination of both (Fig. 12). The advantage of an avidin-biotin coupling scheme is in its simplicity. Many targeting ligands, including antibodies, are already available in biotinylated form, or can be easily biotinylated. Therefore, this coupling scheme might serve as a first step for feasibility testing *in vitro* and *in vivo*. However, intravenous injection of avidin or streptavidin in the patients should be avoided. Likewise, humanized or fully human antibodies, preferably Fab or Fv fragments, or even shorter peptides or peptide mimetics, or oligosaccharides, should be used. Easily available

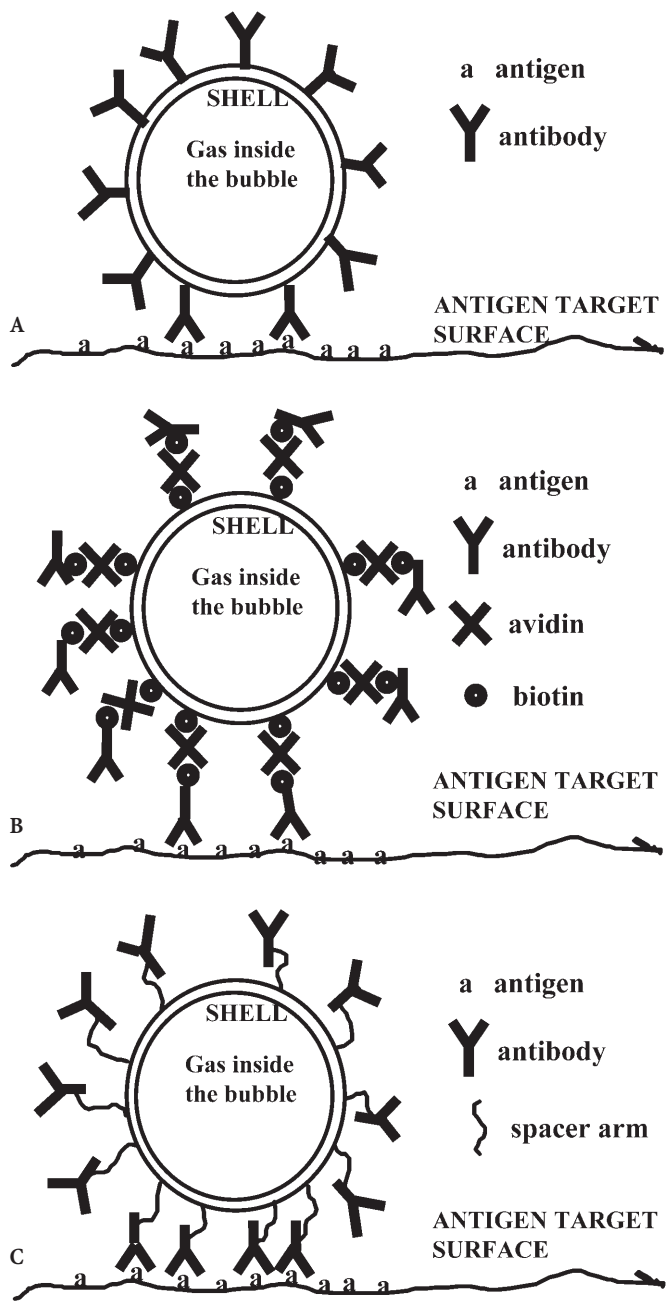


Fig. 12. Attachment of targeting ligands to the surface of microbubbles. A – direct, B – via avidin bridge, C – via a flexible spacer arm. Targeting of these microbubbles to a receptor-coated surface. Reprinted from *Advanced Drug Delivery Reviews* v. 37, A.L. Klivanov, Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging, p. 144. Copyright, 1999, with permission from Elsevier Science

mouse monoclonal antibody molecules (especially with their Fc fragments still attached) should be avoided. They may cause unnecessary immune responses in patients, i.e., development of human anti-mouse antibodies. Such a consequence is unacceptable for a diagnostic imaging agent.

Targeting ligands can be attached to the microbubble directly (Fig. 12 A) or via a flexible polymer spacer arm (e.g., polyoxyethylene, Fig. 12 C). Use of the spacer arm may allow for tighter binding in some situations. When the detachment force of the targeted microbubble removal from the receptor-coated surface was measured, microbubbles with spacer-ligand construct demonstrated superior retention as compared with the bubbles where the ligand was connected directly to the lipid anchor, without a spacer [92].

Recently, monoclonal antibodies were attached to gas-filled microbubbles using this spacer coupling technology. Testing *in vitro*, in a cell culture system, demonstrated selective accumulation of decafluorobutane-based lipid shell MP1950 microbubbles with covalently attached anti-ICAM-1 antibodies, onto the surface of activated endothelium [8]. Anti-P-selectin antibodies attached to such microbubbles via an avidin-biotin scheme showed selective accumulation in the areas of inflammation and ischemia/reperfusion injury in an animal model [93]. In the latter example, biotin was also connected to the microbubble surface via a flexible polymer spacer arm.

The ligand density on the surface of the microbubble is a function of the ligand size: larger ligands (like antibodies) may only allow attachment of hundreds of thousands of ligand molecules per microbubble (which is close to full surface coating). Smaller, peptide-based ligands may allow the attachment of a ten times higher amount of ligand molecules per bubble. The higher the surface density of the targeting ligand, the higher will be the chance of the microbubble retention on the target after their initial collision. One available example of a small ligand attached to microbubbles is an RGD-type peptide, connected to the surface of lipid-coated MRX-408 decafluorobutane microbubbles via a PEG spacer arm [94]. These peptide-targeted lipid microbubbles selectively attach to glycoprotein GPIIb/IIIa on the activated platelets, and can thus be targeted to thrombi. Targeted microbubbles on the surface of thrombi might be useful for targeted thrombus imaging; they may also aid in accelerating thrombolysis. It remains to be seen if targeted microbubbles attached to a thrombus will aid thrombolysis better than the microbubbles circulating in the bloodstream (see above).

There exists an even simpler alternative to the peptide-mediated targeting approach. Some lipids are used by the organism as markers for selective uptake of particles, cells or cell fragments into other cells. In particular, phosphatidylserine (PS) is normally used as a marker molecule for the uptake of aged red blood cells by Kupffer cells [95], or the scavenging action of neutrophils/macrophages on the fragments or apoptotic cells [96]. So, addition of PS to the shell of microbubbles results in their preferential rapid uptake to e.g., leukocytes in the area of inflammation. Leukocytes in the areas of inflammation adhere to the endothelium and can then bind particulate materials from the bloodstream, including lipid- and protein-based microbubbles [97], especially if these microbubbles contain PS [98]. This can be used to assess the areas of inflammation via

Table 3. Specialized/Third Generation microbubble contrast agents (all preclinical experiments)

Name/Shell composition/design	Application	Reference
MRX408/phospholipid + RGD peptide-PEG-lipid	Thrombus imaging and accelerated thrombolysis	[94]
MP1950/anti-ICAM-1 antibody attached via PEG spacer	Targeting activated endothelium	[8]
MP1950PS/ phosphatidylserine as part of the lipid shell	Targeting areas of inflammation by attachment to leukocytes	[98]
MP1950/anti-P-selectin antibody attached via PEG spacer and avidin-biotin bridge	Targeting activated endothelium	[93]
PESDA/Oligonucleotide attached by adsorption	Oligonucleotide delivery	[82]
MRX501/lipid shell + dexamethasone prodrug	Ultrasound-activated drug delivery	[83]
MRX552/lipid shell + soybean oil + paclitaxel	Ultrasound-activated drug delivery	[32]
ImaRx/positively charged lipid shell/plasmid DNA attached	Ultrasound-activated DNA delivery	[10]

preferential accumulation of ultrasound contrast. The number of microbubbles accumulated on leukocytes in the inflammation zones is sufficient for echo imaging. Microbubbles (with or without PS) can actually be completely endocytosed by leukocytes or macrophages. Endocytosis influences the acoustic response of a bubble: the whole cell effectively becomes a viscous extension of the microbubble shell [99]. It may also stabilize the bubble against gas loss, which may explain why air-containing Levovist bubbles can be retained in the liver for up to 30 minutes after intravenous injection of the contrast agent [9]. Of course, when a microbubble located inside a leukocyte is destroyed by a high-intensity ultrasound pulse, damage to the surrounding cell may occur. This might be used for the induction of leukocyte apoptosis and reduce inflammation [100].

Selective targeting of microbubble contrast agents to the specific receptors on the surface of endothelial lining may offer a way to visualize areas of active angiogenesis [101] and/or apoptosis. This type of imaging may be helpful in detecting actively growing tumors and monitoring the success of therapeutic interventions, making diagnostic imaging procedures an integral part of therapy schemes.

17 Future Directions of Contrast Ultrasound

Research in the field of ultrasound contrast materials will develop in two basic directions – the “simple” and the “complex”. The “simple” would mean wider clinical application of the store of available (or to-be-approved) contrast agents in clinical diagnostics. The success will depend not only on the quality of the new diagnostic procedures and results of the clinical trials with new ultrasound

equipment (especially for perfusion imaging). It will also depend on the ability of the pharmaceutical companies to support clinical trials and regulatory submissions. The degree of success of contrast ultrasound will also be dependent upon the progress achieved by the competing imaging modalities, such as MRI.

The “complex” approach is the discovery-level research in the areas of contrast agent targeting, molecular imaging and drug delivery (site-specific gene therapy in particular). Success of the “simple” approach may bring more attention to the “complex” research. Alternatively, discovery work may slowly develop on its own until a major breakthrough like a successful practical application in the areas of tumor therapy or gene therapy takes place.

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Chemistry of X-Ray Contrast Agents

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Iodinated contrast agents are widely used in X-ray diagnostic procedures such as angiography, urography and computed tomography. In this chapter, the synthesis and analytics of iodinated X-ray contrast agents are reviewed. General synthetic routes including patent applications and investigations on stability will be described for the different classes of available compounds. The analytical part covers physicochemical characteristics such as osmolality, viscosity, and partition coefficient as well as methods for the specific and unspecific determination of contrast agents in various matrices. Particular emphasis has been focused on the analysis of isomeric forms of the iodinated compounds.

Keywords. Iodinated X-ray contrast agents, Analysis, Synthesis, Isomerism

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1

History

Contrast agent research started as early as 1895, in the same year in which W.C. Röntgen discovered X-rays. Elements with high atomic numbers such as bromine, iodine, barium, bismuth and lead were soon discovered to be useful as contrast enhancers [1]. Strontium bromide and lithium and sodium iodide were studied as the first water-soluble contrast agents [2]. Later on, iodine became the leading contrast-giving atom for X-ray techniques. Today, all intravascular administrations require iodinated contrast agents. For visualization of the gastrointestinal tract, barium sulphate is used and, in rare cases, xenon gas is inhaled for the delineation of the pulmonary system.

The reasons that iodine took the leading role in contrast agent synthesis include its high absorption coefficient for radiation, its chemical versatility and relative inertness. Contrast agents are requested to be pharmacologically “invisible”, i.e. they should not exhibit any pharmacological activity in the organism. Their only role is to absorb X-rays. Accordingly, sodium iodide should be ideal from an absorption perspective – 85 % of the molecule is iodine – but not from a toxicity perspective. Free iodide ions affect above all the thyroids but also other organs and tissues. Organically bound, i.e. stable iodine, particularly if shielded by hydrophilic groups, is much better tolerated. Other factors contributing to contrast agent toxicity include the hydrophilicity of the molecule, and the osmolality and viscosity of the preparation. The historic development of X-ray contrast agents was a logical consequence of these considerations, moving from inorganic iodide, to organic mono-iodine compounds (Uroselectan A [3]), bis-iodine (Uroselectan B) and tris-iodine substances (diatrizoate [4]), from lipophilic to hydrophilic agents from ionic (diatrizoate) to non-ionic drugs (iopromide) and from monomers (iopromide) to dimers (iotrolan). A typical development sequence covering the history of contrast agent research is given in Fig. 1.

Hydrophilicity, osmolality and viscosity are directly related to the chemical structure of the contrast agents.

The pharmacokinetics of X-ray contrast agents used in angiography and urography are practically identical. These are characterized by extracellular distribution followed by renal excretion by glomerular filtration. The terminal half-life is approx. 1.5 h. An overview of the existing data on pharmacokinetics of X-ray contrast media in humans has been given by Bourin et al. [5].

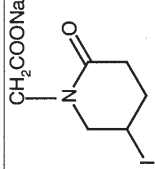
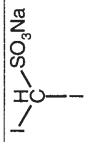
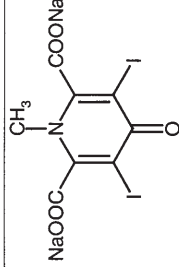
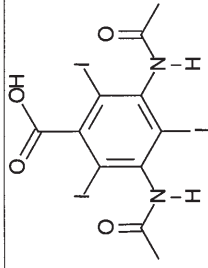
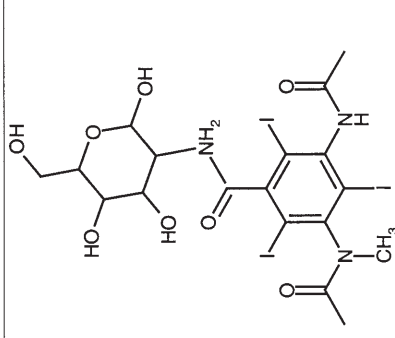
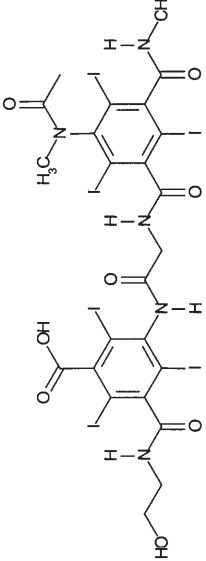
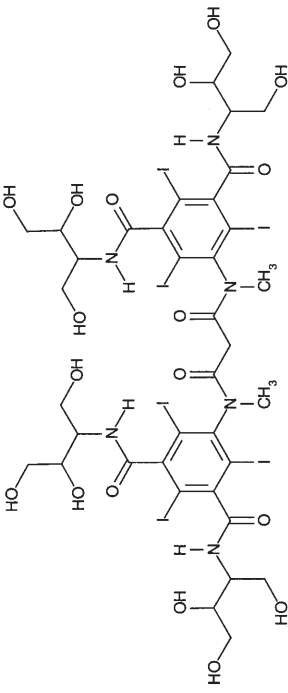
NaI						Metrizamide Nonionic monomer, 3 iodines
NaI	Uroselectan Ionic monomer, 1 iodine	Tenebryl Ionic monomer, 2 iodines	Uroselectan B Ionic monomer, 2 iodines	Diatrizoate Ionic monomer, 3 iodines		
						
	Ioxaglate Ionic dimer, 6 iodines				Iotrolan Nonionic dimer, 6 iodines	

Fig. 1. Historical development of iodinated X-ray contrast agents. The sequence of synthesis goes from top left to top right followed by bottom left to bottom right

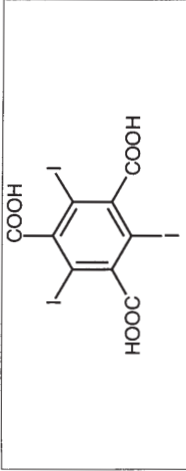
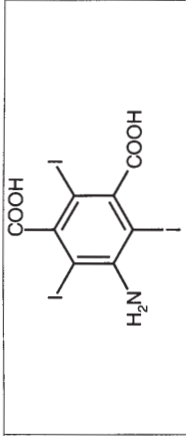
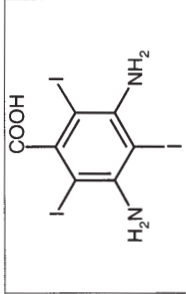
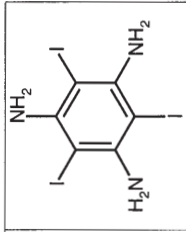
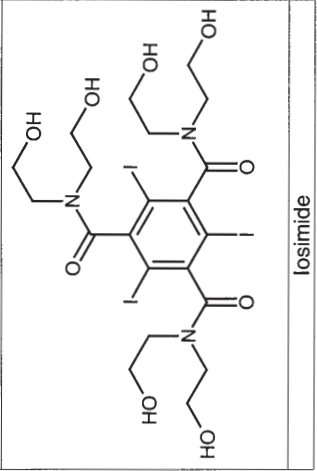
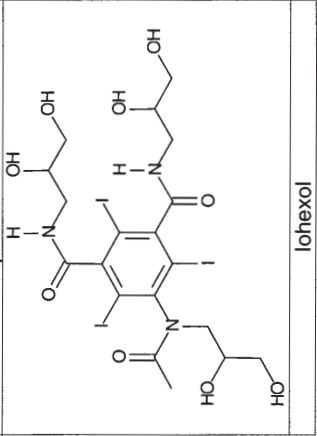
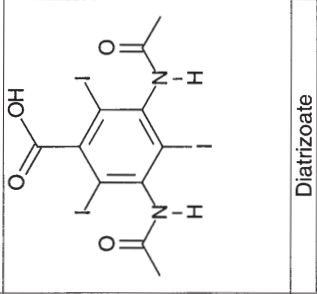
			
Trimesic acid	Isophthalic acid	Benzoic acid	Triaminobenzene
			No example available
Iosimide	Iohexol	Diatrizoate	-

Fig. 2. Theoretical types of X-ray contrast agents with triiodinated benzene rings

2

Basic Structures of Contrast Agents

All presently available X-ray contrast agents for intravascular injection are based upon the triiodobenzene ring substituted with two or three additional hydrophilic groups. In the case of biliary contrast agents (compounds that are taken up by the liver and excreted mainly by the biliary tract), two hydrophilic groups are introduced. For angiographic/urographic agents (compounds that stay within the extravascular distribution volume and that are excreted by the kidneys), three hydrophilic groups are introduced. In the following chapters, we will mainly concentrate on the most commonly used compounds, which comprise the latter class, contrast agents for angiography and urography.

In principle, four different types of triiodinated benzene rings are possible, which are illustrated in Fig. 2. They differ by the number of carboxyl groups directly attached to the benzene ring ranging from zero to three. For the first (three carboxyl groups) and last types (three other moieties such as amino groups), no examples have achieved approval by the authorities. The reason is synthetic difficulties which either made the synthesis impossible (amino or ether groups) or too expensive (carboxyl groups), although these two types would result in the most symmetrical contrast agents possible, particularly as non-ionic substances.

All presently available non-ionic monomers are illustrated in Fig. 3; all dimers including an ionic compound are listed in Fig. 4. The monomers are exclusively derived from aminoisophthalic acid. They only differ by their side-chains, which determine their physicochemical characteristics such as solubility, hydrophilicity, viscosity and osmolality. The aqueous solubility of X-ray contrast agents is generally extremely high being in the order of 1 g mL^{-1} . Most preparations of X-ray contrast agents are over-saturated solutions, which are, however, stable enough to allow handling and long-term storage without the danger of precipitation.

3

Synthesis of Contrast Agents

3.1

General Remarks

The most important step in the synthesis of iodinated contrast agents is the introduction of iodine into the molecule. Iodine is introduced either through substitution or by addition reactions. The iodination of double bonds, e.g. in the synthesis of Lipiodol (iodinated lipid formulation) is achieved either with I_2 , ClI or HI . Abrodil ($\text{ICH}_2\text{SO}_3\text{Na}$) – a formerly used contrast agent – can be obtained either from CH_2I_2 or from $\text{CHI}_3 + \text{Na}_2\text{SO}_3$. The benzene ring can be iodinated by a number of methods via the intermediate I^+ , which will react with positions of the benzene ring activated by neighbouring electronegative groups. If the electronegativity is not high enough, then the

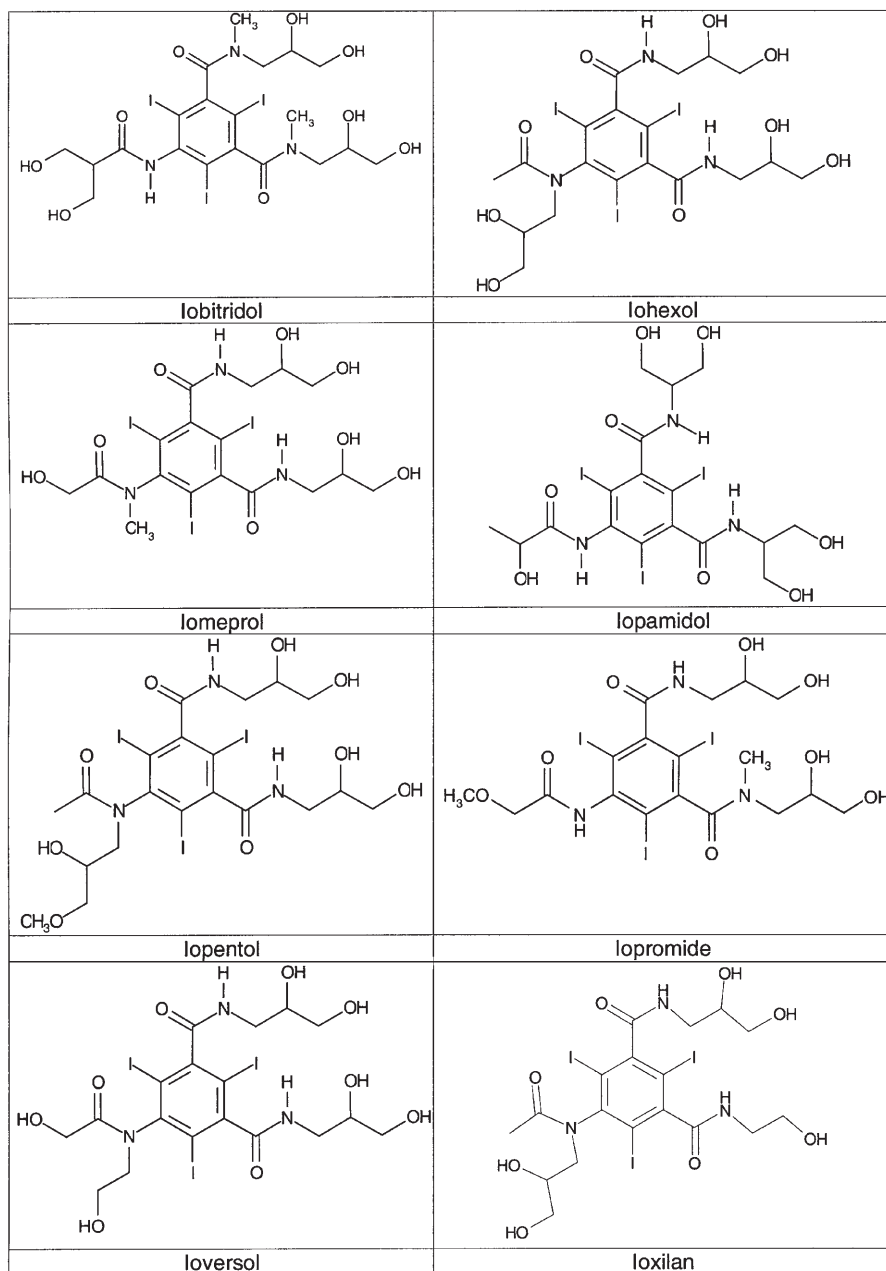


Fig. 3. Currently available non-ionic monomers

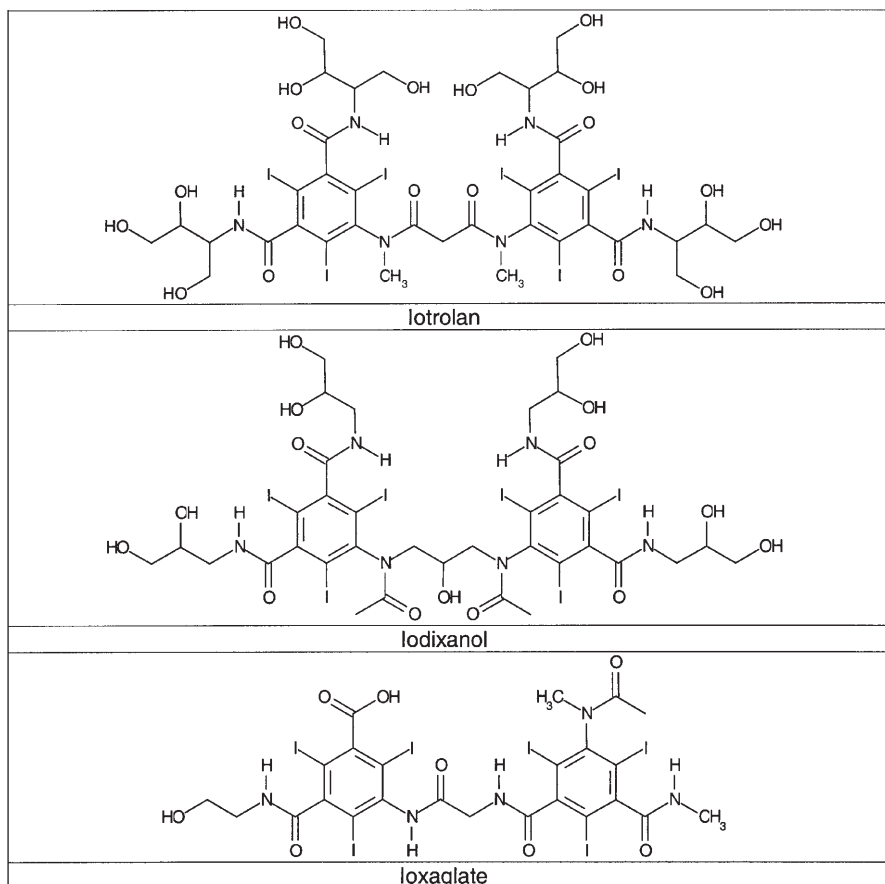


Fig. 4. Currently available dimers

number of iodine atoms introduced into the benzene ring is reduced, i.e. less than three. Normally, amine groups are used for activation. The active iodine ion (I^+) is generated in aqueous solution from CHI_3 . The resulting triiodinated compounds are usually less water soluble than their precursors and precipitate. Purification of the intermediates is easily performed. Following acylation of the amino groups, the synthesis of ionic contrast agents is complete. The acylation of the amino groups significantly increase their electronegativity and thereby stabilize the carbon-iodine bonds. As a typical reaction scheme for an ionic monomer, the synthesis pathway for diatrizoate is given in Fig. 5.

The synthesis of non-ionic contrast agents is much more complicated. Due to the higher aqueous solubility of the intermediates, purification processes have to be more sophisticated than for the ionic compounds. Moreover, for the carboxylic acid, salt purification procedures of ionic substances are no longer available for the non-ionic derivatives. Now, recrystallisation,

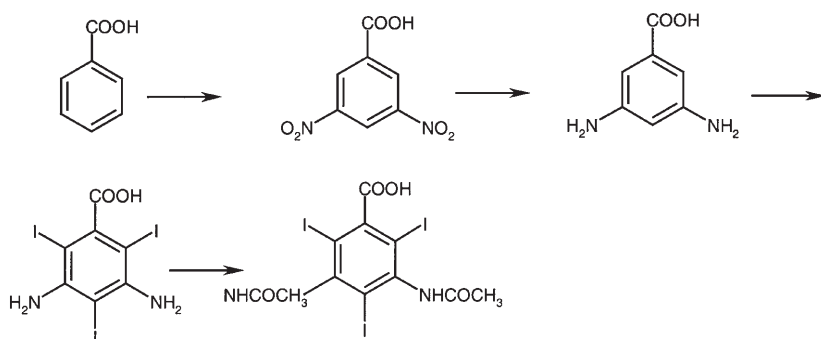


Fig. 5. Synthetic pathway to ionic contrast agents – reaction scheme for diatrizoate

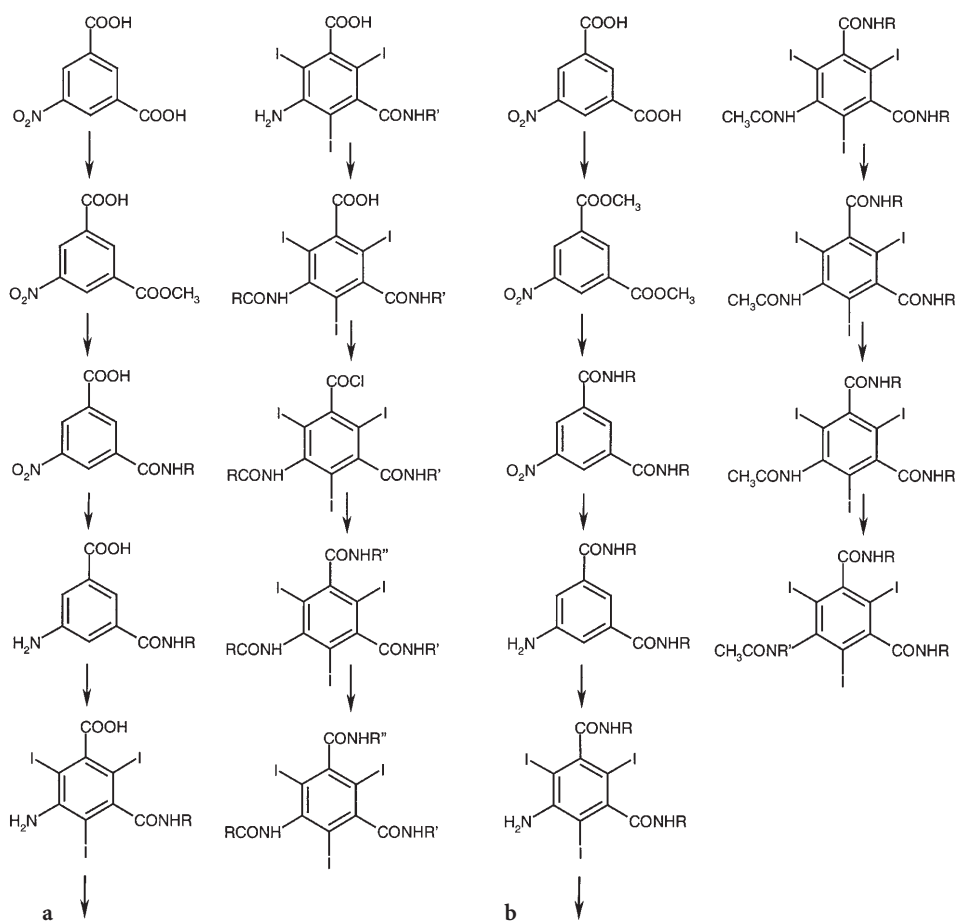


Fig. 6. Route of synthesis of nonionic contrast agents. **a** Synthesis of iothexol with a symmetrical structure. $R = R' = 2,3$ -propanediol. **b** Synthesis of iopromide with an unsymmetrical structure. $R_1 = \text{methyl}$. $R_2 = 2,3$ -propanediol. $R'_2 = \text{acylated } 2,3$ -propanediol

extraction and/or chromatography – much more expensive and yield-reducing procedures – have to be applied. Additionally, inorganic salts have to be eliminated in order to reduce the osmolality of the preparations. Three different synthetic routes are possible [6] leading to all currently available non-ionic X-ray contrast agents. Figures 6a and b illustrate the syntheses of one symmetrical, iohexol, and of one unsymmetrical contrast agent, iopromide, respectively.

The synthesis of ionic triiodinated contrast agents starts from either bisamino nitrophthalic acid or amino isophthalic acid. The nitro group is reduced to the amino moiety by hydrogenation. Iodine is introduced in aqueous solution with iodine monochloride. Due to the decrease in solubility the resulting triiodobenzene precipitates from the solution and can be recrystallised. Amino functions are then acylated.

Non-ionic compounds require significantly more synthesis steps, particularly for some unsymmetrical types. General features of the synthesis are identical to those used for ionic contrast agents, i.e. hydrogenation of nitro groups to give amino moieties, iodination with iodine monochloride, and acylation to protect/derivatise amino groups. In addition, amidation of carboxyl groups via carboxyl chlorides becomes necessary. Major efforts, however, have to be directed towards sophisticated purification procedures, since simple recrystallisation is often no longer effective. For some compounds, chromatographic and/or spray-drying techniques even have to be employed.

Priebe et al. [7] have described the synthesis and purification of iodixanol and its physical and toxicological properties, and analytical and spectroscopic data.

An even more challenging approach covers polymeric X-ray contrast agents derived either from linear or from branched backbones, e.g. dendrimers. An overview on this field of research has been published recently [8]. Additionally, the reader is referred to the chapter in this book by Idee et al. covering novel dendrimers with triiodobenzenes as building blocks.

3.2

Patents

Numerous patents have been filed for iodinated contrast agents starting from substances with only one iodine, e.g. 5-iodo-4-pyridone up to polymers with a large number of iodine atoms. Naturally, the largest portion of available patents covers the non-ionic triiodinated compounds, the class which dominates the present-day use of contrast agents. A selection of patents on non-ionic monomeric substances derived from triiodobenzene is summarized in Table 1 together with ionic triiodobenzene compounds. Additionally, dimers and other classes with iodine, such as tetraiodobenzene agents and, also, classes not derived from triiodobenzene are listed such as iodinated lipids or bromine-containing substances, e.g. perfluoroalkyl bromides or tribromobenzene derivatives. An overview on patents covering polymeric X-ray contrast agents has been published previously [8]. Table 1 not only covers patents on the synthesis of iodinated (halogenated) contrast agents, but also on other aspects such as analysis and degradation.

Table 1. Summary of patents on the synthesis, analysis and degradation of iodinated (halogenated) contrast agents. Major emphasis is put on agents used in angiography, urography and CT. I. Synthesis. II. Analysis. III. Removal of contrast agents. 1. Triiodinated ionic monomers. 2. Triiodinated non-ionic monomers. 3. Dimers. 4. Iodinated non-triiodobenzene derivatives. 5. Non-iodine compounds. 6. Others

Claims	Category	Patent No.	Inventor/Applicant	Reference
<i>Ionic triiodobenzene monomers</i>				
Acetylamino-2, 4, 6-triiodophenoxy-caproic acid from 3-acetylamino-phenol	I-1	GB979138	NN/Bracco	9
3-(<i>N</i> - β , γ -dihydroxypropyl-acetyl-amino)-5-acetylamino-methyl-2,4,6-triiodobenzoic acid	I-1	US3867431	Zutter/Bracco	10
3-(<i>N</i> -dihydroxypropyl-acetylamino)-5-acetylamino-methyl-2,4,6-triiodo-benzoic acid	I-1	GB1345665	NN/Bracco	11
Mixture of a nonionic X-ray contrast medium and an ionic X-ray contrast medium	I-1		NN/NN	12
Alpha ethyl- β -(2,4,6-triiodo-3-aminophenyl)-propionic acid	I-1	GB836884	Leo	13
Alkylammonium complexes of diatrizoic acid	I-1	US4735795	Ridgway/Squibb	14
5-[(3',5'-Bis(acetylamino)-2',4',6'-triiodophenyl)carbonyloxy]pentanoic acid and analogs	I-1	EP0603917	Bacon/Sterling Wintrop	15
<i>Nonionic triiodobenzene monomers</i>				
<i>N,N,N',N'</i> -tetra-(hydroxyethyl)-5-acetylamino-2,4,6-triiodoisophthaloyldiglycinamide	I-2	EP0129932	Korver/Dagra	16
5-Alkoxy-2,4,6-triiodo- or tribromo-isophthalic diamides	I-2	EP0185130	Fumagalli/Bracco	17
Iodinated or brominated, derivatives of amino-isophthalic acid	I-2	EP0118349	Dimo/Guerbet	18
Reverse osmosis as an alternative or substitute method for the purification of a crude diagnostic agent	I-2	US5221485	Johnson/Mallinckrodt	19
<i>N,N'</i> -bis(2,3-dihydroxypropyl)-5- <i>N</i> -(2-hydroxyethyl)glycolamido-2,4,6-triiodoisophthalamide	I-2	US4396598	Lin/Mallinckrodt	20
<i>N</i> -(<i>N</i> -alkyl-3,5-diacetamido-2,4,6-triiodobenzoyl)-glucosamine derivatives	I-2	GB1321591	NN/Nyegaard	21
1,4-Bis (acetylamino)- <i>N,N'</i> -bis[3,5-bis(2,3-dihydroxypropylaminocarbonyl)-2,4,6-triiodophenyl]2, 3-dihydroxybutane and analogues	I-2	EP0108638	Hansen/Nyegaard	22
Preparation of <i>m</i> -carboxamido- <i>o</i> -iodo- <i>N</i> -(β -hydroxyalkyl)aniline	I-2	US4396597	Kelly/Nyegaard	23

<i>N,N'</i> -bis(1,3-dihydroxy-2-propyl)-5-[<i>N</i> -(2-hydroxy-3-methoxypropyl) acetamido]-2,4,6-triiodoisophthalamide and analogues	I-2	EP0105752	Wille/Nyegaard	24
1,3-Bis(acetylamino)- <i>N,N'</i> -bis[3,5-bis(2,3-dihydroxypropylaminocarbonyl)-2,4,6-triiodophenyl]-2-hydroxypropane	I-2	US5349085	Hansen/Nyegaard	25
<i>N,N'</i> -bis(1,3-dihydroxy-2-propyl)-5-[<i>N</i> -(2-hydroxy-3-methoxypropyl)acetamido]-2,4,6-triiodoisophthalamide and analogues	I-2	US5204086	Wille/Nyegaard	26
Stereoisomer of a compound with at least one chiral centre, wherein said stereoisomer is in stereoisomeric excess	I-2	EP0992245	Frank/Goldham	27
(<i>N</i> -2-Hydroxyethylacetamido)-2,4,6-triiodo- <i>N,N'</i> -bis-(2,3-dihydroxypropyl) isophthalamide and analogues	I-2	US4250113	Holtermann/Nyegaard	28
Preparation of a contrast medium using acceptable buffer system the pH of which decreases with increasing temperature	I-2	GB2031405	NN/Nyegaard	29
Nanoparticulate diagnostic mixed carbamic anhydrides	I-2	US5472683	Illig/Kodak	30
3-Amido-triiodophenyl esters	I-2	US5668196	Robinson/Nanosystems	31
3,5-Bis alkanoyl amino-2,4,6-triiodobenzyl esters	I-2	US5603916	Singh/Nanosystems	32
Nanoparticulate diagnostic diatrizoxy ester	I-2	US5525328	Osifo/Nanosystems	33
Nanoparticulate diagnostic mixed carbonic anhydrides	I-2	US5643552	Illig/Nanosystems	34
3,5-Bis alkanoyl amino-2,4,6-triiodobenzyl esters	I-2	US5573750	Singh/Nanosystems	35
Nanoparticulate diagnostic mixed carboxylic anhydrides	I-2	US5573749	Illig/Nanosystems	36
Process of preparing nanoparticulate contrast agents using a premix of the contrast agent and a surface modifier, producing shear, impact, cavitation and attrition	I-2	US5543133	Swanson/Nanosystems	37
Nanoparticulate iodipamide derivatives	I-2	US5521218	Osifo/Nanosystems	38
Triiodoalkylphenyl esters	I-2	WO9706132	Bacon/Nanosystems	39
5-[3-(<i>N</i> -methylmethoxyacet amido)-5-methylcarbamoyl-2,4,6-triiodine-benzamidoacetamido]- <i>N</i> -(2-hydroxy)-2,4,6-triiodine-isophthalamide acid and analogues	I-2	US4264572	Klieger/Schering	40
5-Cyano-2,4,6-triiodoisophthalamide analogues	I-2	DE283 1496	Gries/Schering	41
3,3'-Thiodipropionic acid-bis-[3,5-bis-(2,3-dihydroxypropyl <i>N</i> -methylcarbamoyl)-2,4,6-triiodoanilide analogues	I-2	DE2926428	Muetzel/Schering	42

Table 1 (continued)

Claims	Category	Patent No.	Inventor/Applicant	Reference
5-Methoxyacetylamino-2,4,6-triiodoisophthalic acid [(2,3-dihydroxy- <i>N</i> -methylpropyl)-(2,3-dihydroxypropyl)]diamide	I-2	US4364921	Speck/Schering	43
5-Hydroxyacetamido-2,4,6-triido-isophthalic acid-(2,3-dihydroxy- <i>N</i> -methyl-propyl)-(2-hydroxy-ethyl)-diamide	I-2	US5183654	Speck/Schering	44
5-Amino-2,4,6-triiodoisophthalic acid bisamides	I-2	US4283381	Speck/Schering	45
<i>N</i> -hydroxyethylated 2,4,6-triidoaminoisophthalic acid bistrihydroxybutylamides	I-2	US4547347	Pfeiffer/Schering	46
Unsymmetrically substituted dicarboxylic-acid-bis-(2,4,6-triido-anilides)	I-2	US4395391	Pfeiffer/Schering	47
Esters of diatrizoic acid	I-2	US4567034	Charles/Squibb	48
Esters of diatrizoic acid	I-2	GB2157283	Charles/Squibb	49
Crystalline organic X-ray contrast agent having a surface modifier adsorbed on the surface thereof	I-2	EP0498482	Liverside/Sterling Wintrop	50
<i>Dimers</i>				
Recovery of iodine from iodo-aromatics	I-2/3	DE19702814	Schenk/Schering	51
Liquid phase acylation and subsequent <i>N</i> -alkylation process: addition of an alkylating agent to a solution containing the reaction products of said acylation	I-2/3	WO9808805	Ingvolstad/ Nyegaard	52
Vibrational (e.g. infrared, Raman or preferably near-infrared) spectroscopic monitoring of the reaction mixture	II-2/3	WO9823296	Bjoersvik/Nyegaard	53
Removing X-ray contrast agents from the waste water by recirculating the waste water several times and filtering it under pressure through semipermeable membranes	III-1/2/3	DE19542692 DE19542692	Haepcke/Inst Bioanalytik Umwelt Toxiko	54
3,3'-Thiodipropionic acid bis[3,5-bis(2,3-dihydroxypropyl)- <i>N</i> -methylcarbomyl)-2,4,6-triidoanilide] analogues	I-3	US4367216	Muetzel/Schering	55
Dicarboxylic acid bis-(3,5-dicarbomoyl-2,4,6-triidoanilides) wherein the amide radicals are different from one another	I-3	US5073362	Speck/Schering	56
Nanoparticulate diagnostic dimers	I-3	US5500204	Osifo/Kodak	57

<i>Iodinated compounds – non-triiodobenzene derivatives</i>				
Brominated, or iodinated natural or synthetic esters of C11 to C24 fatty acids	I-4	GB1081551	Guerbet	58
3,5-Iodopyridones	I-4	GB399463	Schering	59
Emulsions based on lipophilic iodized and/or bromated substances with emulsifiers and derivatives of cyclopenta-phenanthrene	I-4	US5445811	Norrind/Pharmacia	60
Polyiodinated phenyl fatty acid compounds	I-4	GB1400985	NN/Pharmacia	61
Emulsion of iodinated lipids	I-4	GB767788	Schering	62
6-Iodoethylated starch	I-4	DE3785849	NN	63
Iodized hydroxyethyl starch	I-4	US4744975	Sako/Nippon	64
Iodinated or brominated, derivatives of boron	I-4	Universal Pharma		
Heat-sterilisable O/W emulsion of iodised fatty acid esters	I-4	EP0391621	Kanda/Nippon	65
	I-4	US5545397	Universal Pharma	
	I-4	CN1074378	Meyer/Guerbet	66
	I-4	DE4111939	Foth/Fresenius	67
<i>Non-iodo compounds</i>				
Bromobenzene compounds	I-5	EP0073715	Dimo/Guerbet	68
Perfluorobromoalkyl ether	I-5	EP0120583	Clark/Sun Tech	69
Perfluoroalkylmonobromides	I-5	DE4116361	Felix/Hoechst	70
Perfluorobromoalkyl ether	I-5	US4640833	Clark/Adamantec	71
<i>Others</i>				
Polymeric contrast agents	I-6	WO0025829	Wolf/Nyegaard	72
Tetraiodinated or pentaiodinated benzene derivatives	I-6	US5693311	Meyer/Guerbet	73
Mixed bromo-iodo benzene compounds	I-6	EP0074307	Dimo/Guerbet	74
Phospholipid to which an X-ray contrast-giving moiety has been covalently linked	I-6	US5550263	Bengmark/ Karlsamns LipidTeknik	75
Emulsion for use as an X-ray contrast agent	I-6	EP0294534	Ajaxon/Kabivitrum	76

4

Stability of Contrast Agents

Iodinated contrast agents with iodine coupled to aromatic rings are very stable. However, under certain circumstances they are degraded, particularly by deiodination. Chellquist et al. investigated the degradation of the ionic monomer, diatrizoate [77]. The USP specification for free iodide is no more than 0.02 % (w/v). Nevertheless, the authors found several bottles of Hypaque, which contains diatrizoate, with iodide levels in the range 0.001–0.036 % (w/v). They reported that the degradation is triggered by preparation constituents such as glucose and copper ions. Solution stress studies containing diatrizoate and varying amounts of glucose and copper ions were conducted at three different pH values: 6, 7 and 8 in phosphate buffer at 60 °C. The decomposition of diatrizoate was monitored by HPLC. The greatest amount of decomposition occurred in those solutions prepared in pH 8 phosphate buffer and containing both copper ions and glucose. The authors suggested that glucose reacts with copper ions to form an activated species, which reacts to displace an iodide.

Gronewaller et al. studied the stability of iodinated contrast agents during angioplasty, i.e. during exposure to XeCl-Excimer laser light [78]. They irradiated 1 mL of iopromide, iomeprol, iopamidol or diatrizoate with a XeCl excimer laser (at 308 nm, pulse duration 120 ns, 50 Hz) using a 9 French multifibre catheter (12 sectors) and up to 20,000 pulses (106 J). Subsequently, they determined the concentration of the unchanged contrast agent by HPLC and free iodide. They found iodide liberation of up to 3.3 mgI mL⁻¹ and up to 19 % of the contrast agents changed their original molecular structure.

Priebe et al. [79] investigated the chemical stability of iodixanol under accelerating cleavage of the central bridge under ultraviolet irradiation by a Norrish Type-II reaction. Basic conditions (pH 14) combined with heat (60 °C) initiated a cyclisation reaction. On the other hand, less than 1 % iodixanol decomposed in solution heated to 140 °C for 2 days or under both basic conditions (pH 11, 20 °C, 5 days) and acidic conditions (pH 0.4, 80 °C, 5 days) or under an oxygen atmosphere (100 °C, 3 days).

Iodinated (aromatic) contrast agents are also very stable within the organism of animals or humans, i.e. they are not metabolised and are excreted unchanged. However, Jacobsen et al. [80] investigated the metabolic fate of iodixanol loaded into liposomes. Injected into monkeys, rats and dogs, the contrast agent was transported into hepatocytes, which it would not reach when administered without encapsulation. Subsequently, liver samples were analysed by HPLC and mass spectrometry. Two metabolites with UV spectra identical to those of the iodixanol *exo*- and *endo*-isomers and with a mass increase of 162 relative to iodixanol, were detected. Subsequently, the authors incubated iodixanol-liposomes or iodixanol in rat liver homogenates and found large amounts of iodixanol metabolites, whereas no metabolites were formed in other organ or tissue homogenates. Four metabolites could be identified. Two compounds were structurally related to the iodixanol *exo*-isomer, and two to the *endo*-isomer. Mass spectrometry techniques indicated that the metabolites were conjugates containing one or two glucose residues bound to iodixanol through *O*- α -glycoside-like linkages.

5

Analysis of Contrast Agents

The analysis of X-ray contrast agents has not been described in too much detail in the literature. Only scattered data for individual compounds can be found. In the following paragraphs, we will concentrate both on the determination of physicochemical characteristics, which allow for a classification of different contrast agents, e.g. into high and low-osmolar substances, and on the separation from by-products or biological material and on the determination of concentrations. Structural aspects of iodinated contrast agents have been described by Toennesen et al. [81].

5.1

Physicochemical Characteristics

The physicochemical characteristics of X-ray contrast agents strongly determine their pharmacological and toxicological behaviour and are therefore important features, particularly in guiding the search for improved substances.

5.1.1

Osmolality

The osmolality of a contrast agent solution is proportional to the number of independent particles in the solution and is strongly influenced by both the concentration of the contrast agent (or any other constituents) and the temperature of the solution. The osmotic pressure of a contrast agent preparation is given in milliosmol kg⁻¹ water (mosm kg⁻¹), in Megapascal (MPa) or in atmospheres (at). Conversion between the different units follows the equation 1 osm kg⁻¹ = 1000 mosm kg⁻¹ = 2.58 MPa = 25.5 at. In a multi-component system, the osmolality is defined as

$$\xi_m = \Phi \sum_i v_i m_i \quad (\text{a})$$

with ξ_m representing the osmolality (osm kg⁻¹), Φ the molal osmotic coefficient of the solution, v_i the number of dissociated species in solution and m_i the molal concentration (M kg⁻¹). The osmolality can easily be determined by vapour pressure osmometry using commercially available differential thermistor osmometers and defined temperatures. For calibration, the concentration dependence of the osmotic coefficient of saline is used. Details have been described, for example, by Miklautz et al. [82].

Currently available contrast agents can be classified into three different groups, high-osmolar compounds with osmolalities in the order of 1500 mosm kg⁻¹, low-osmolar agents with 600–700 mosm kg⁻¹ and isotonic substances with osmolalities similar to that of blood (300 mosm kg⁻¹). Data for individual contrast agents are summarized in Table 2.

High osmolality is directly related to certain types of side-effects such as pain at the injection site, cardiovascular effects (heart rate, blood pressure) and

Table 2. Physicochemical characteristics of iodinated contrast agents. Data have been collected from refs. 94 and 129

Agent	Molecular Weight	Butanol Partition Coefficient	Protein Binding (%)	Concentration (mgI mL ⁻¹)	Osmolality* (mosm kg ⁻¹)	Viscosity** (mPas)
Diatrizoate	810	0.044	8.8	306	1502	9.3
Ioxaglate	1406	0.086	14.0	320	584	16.2
Iobitridol	835	–	–	300	695	–
Iohexol	821	0.082	0.5	300	667	10.9
Iomeprol	777	0.105	0	300	521	4.5
Iopamidol	777	0.089	0	300	640	8.4
Iopentol	835	0.109	0	300	682	12.9
Iopromide	791	0.149	0.9	300	586	8.9
Ioversol	807	0.031	3.4	300	661	10.1
Ioxilan	805	–	–	300	585	9.4
Iodixanol	1550	0.028	4.2	320	290	20.7
Iotrolan	1626	0.005	1.8	300	291	17.4
				320	317	25.3

* at 37°C.

** at 20°C.

diuresis. The closer the osmolality is to that of blood, the lower is the overall side-effect ratio.

The measured osmolality of some contrast agents differ considerably from the theoretical value [83–85] due to the formation of aggregates in the solution by intermolecular bonding. Violon investigated the relative influence of different parameters on the experimental osmolality and established – based on seven compounds – a relationship allowing the calculation of the real osmolality of monomeric non-ionic contrast agents [86].

Sontum et al. [87] studied the aggregation behaviour of iohexol and iodixanol in aqueous solutions as a factor determining osmolality using light scattering and molecular diffusion. They found significant solvent–solute and solute–solute interactions and dynamic clusters containing at maximum two to three contrast agent molecules. However, no actual increase in the geometric size was found with increasing concentration. The authors therefore concluded that large colloidal-like structures or molecular aggregates were not present in the contrast agent solutions. These data are in contrast to those reported by Schneider who found higher forms of aggregates for some contrast agents, particularly for iopamidol and iodixanol [88]. It has been speculated that such aggregates could be the cause of the hypersensitivity reactions sometimes observed with iodinated X-ray contrast agents [89, 90].

5.1.2

Viscosity

The viscosity of a contrast agent preparation describes its ability to flow and is given in milliPascal × second (mPa s). Viscosity is strongly influenced by the con-

centration of the contrast agent and the temperature of the solution. For increasing concentration and decreasing temperature, viscosity increases. The determination of viscosities is performed with commercially available viscosimeters measuring the flow rate of the solutions in microcapillaries. The viscosities of contrast agent solutions with 300 mgI mL⁻¹ are in the range of 5–10 mPa s for monomeric compounds and > 15 mPa s for dimeric agents (see Table 2).

The viscosity of contrast agent solutions determines the maximal rate of injection. Since in some techniques, e.g. angiography or CT, extremely high injection rates are mandatory (up to 20 mL s⁻¹), the viscosity of the preparation might be a limiting factor. Side-effects related to high viscosity, on the other hand, have not been determined unequivocally.

5.1.3

Partition Coefficient

The partition coefficient in a system of aqueous buffer/organic solvent is a measure for the hydrophilicity or lipophilicity of the contrast agents. The organic solvent is either *n*-butanol (P_{bw}) or *n*-octanol (P_{ow}). The partition equilibrium between the aqueous and the organic phase is normally determined by a flask-shaking method. Typically, equal volumes of water-saturated *n*-butanol or *n*-octanol and aqueous diluted solutions of the contrast agent are equilibrated at 25 °C by rotation in centrifuge vials for approx. 25 h. The two phases are then separated by centrifugation and the concentrations of the contrast agent are measured. Since the concentration in the organic phase (C_o) is much lower than in the aqueous phase (C_w), the partition coefficient can be calculated according to

$$P_{ow} = C_o / (C_w - C_o) \quad (b)$$

Where C_o represents the concentration in the organic phase after partitioning and C_w the concentration in the aqueous phase before partitioning.

The partition coefficients of non-ionic monomeric contrast agents in the system water/*n*-butanol are in the range 0.03–0.15. Dimers are more hydrophilic, e.g. with a P value of 0.005 for iotrolan (Table 2).

Structural features such as electrical charge, hydroxyl groups in the side-chains and amino atoms of the amide groups reduce the lipophilicity (increase the hydrophilicity) whereas additional methyl groups sitting on amino moieties or free positions on the benzene ring increase the lipophilicity. Biliary contrast agents with a free position have $P_{ow} > 5$.

Some side-effects of angiographic contrast agents such as allergy-like events (nausea, vomiting) have been related to the lipophilicity (lack of hydrophilicity) of the contrast agents. However, clear-cut structure-activity relationships have not been established.

5.2

Determination of Concentrations

The concentration determination of triiodinated X-ray contrast agents has been described in the literature using a number of different methods. These

might be differentiated according to the target that is measured, e.g. aromatic rings, iodine or unchanged drug. Accordingly, the specificities of these methods differ significantly from low specificity for absorption methods to high specificity for any methods that additionally use separation steps such as HPLC. Examples of a spectrometric method are those described by Medzihradsky et al. [91] and by Schroder et al. [92]. The latter investigated the spectrophotometric determination of iodixanol in subcellular fractions of mammalian cells.

Iodine might be measured as a specific substitute for the contrast agent in those cases where it has been certified before that no change of the molecule – for example by biotransformation – has taken place. Since all angiographic contrast agents are excreted unchanged, the determination of iodine instead of the unchanged drug is justified. In other cases, e.g. in ecological studies when contrast agents have to be determined in waste water or soil samples, the contrast agent has to be measured by a method differentiating between unchanged drug and degradation products.

5.2.1

Iodine

Iodine concentrations can be determined by X-ray fluorescence spectrometry using a ^{241}Am source and measurement at suitable fluorescence wavelengths of iodine. Equilibration of the analyser is performed with either KIO_3 or a contrast agent solution such as iopromide. Typically, plasma or blood samples obtained in biotransformation or pharmacokinetic studies can be measured directly without dilution. Depending on the iodine content, urine samples are either processed undiluted or diluted with distilled water. The linear range of this method is $0.01\text{--}10\text{ mgI mL}^{-1}$. The precision (relative standard deviation for repeated measurements) has been reported as 1%, the accuracy (maximum deviation from nominal value) as $<5\%$. Details have been described by Pavel et al. [93] and Krause et al. [94]. Alternatively, inductively-coupled (ICP) mass spectrometry or ICP spectroscopy with iodine-specific wavelengths might be utilized [95–98]. Other methods include laser spectroscopy [99], direct potentiometry by a sensitive iodine electrode [100] and radiochemical methods [101] including neutron activation [102–104] or catalytic determination based on the Sandell-Kolthoff reaction [105, 106].

Despite their reliability, all these techniques are not drug specific; they are unable to distinguish the unchanged contrast agent and degradation or biotransformation products containing iodine that are potentially present in the sample.

5.2.2

Unchanged Drug

Whenever a higher degree of specificity is mandatory, other techniques have to be employed. A proton nuclear magnetic resonance (PMR) spectroscopic method has been described by Hanna [107] for the direct assay and identifica-

tion of iopamidol and iothalamate meglumine (ionic monomer) in commercial solutions and as a bulk material. Samples are prepared by diluting the contrast agent solution with D₂O or dissolving a powdered sample in D₂O. The internal standard was sodium acetate. Based on the resonance signals for the protons of the CH₃CO-group at 1.58 ppm (iopamidol) or 2.25 ppm (iothalamate), CH₃N group at 2.38 ppm (meglumine) and CH₃CO-group at 1.92 ppm (acetate) concentration can be determined with a mean $\pm$ SD recovery ($n = 10$) for iopamidol of $99.6 \pm 0.63\%$, for iothalamic acid of $99.7 \pm 0.66\%$ and for meglumine of $99.9 \pm 1.18\%$.

Jacobsen et al. described an HPLC/MS method for the determination and/or identification of iodixanol and its metabolites in biological samples from animals [108].

Lorusso et al. [109] described an HPLC method for assaying iomeprol in plasma and urine samples. Before chromatography, the biological fluids were treated with ion-exchange resins and iopamidol was added as internal standard. Detection was by UV spectrometry at 245 nm.

Arbuchi et al. reported on the determination of the non-ionic monomer, iopiperidol, in plasma and urine [110]. They used HPLC for the separation of iopiperidol and iopamidol from the endogenous components of the biological fluids, and their detection by UV absorption at 244 nm. Typically, they used LiChrospher RP-8 reversed-phase columns (25 cm $\times$ 4 mm I.D., particle size 5 μ m) and a LiChrospher RP-8 precolumn (2.5 cm $\times$ 4 mm I.D., particle size 5 μ m) to prevent contamination of the analytical column. Elution was performed isocratically with a 94:6 (v/v) mixture of 0.05 M KH₂PO₄ (pH 4.5) and acetonitrile at a flow-rate of 0.9 mL min⁻¹. The plasma samples were processed by adding an appropriate volume of the standard solution and perchloric acid (30 mL) for the precipitation of plasma proteins followed by centrifugation. The remaining solutions were injected directly into the HPLC system. Urine samples were handled likewise without the addition of perchloric acid. The mean absolute recovery was greater than 80 %. The precision and accuracy of the analytical methods were in the range 0.3–3.3 and –8.5 to 11 %, respectively. The detection limits of iopiperidol in plasma (0.1 mL) and urine (0.25 mL) were 0.2 and 0.4 mg mL⁻¹, respectively.

The same authors also described an equivalent HPLC method for the determination of the non-ionic dimer, iofratol, in plasma and urine [111]. Andresen studied the analysis of iopentol by HPLC [112]. Iodixanol might be analysed according to Jacobsen [113, 114].

The effect of varying the operating temperature from 6 to 90°C on the chromatographic performance of the *exo-exo* and *exo-endo* isomers of the X-ray contrast agent iodixanol in packed-capillary reversed-phase liquid chromatography was investigated by Molander et al. [115]. The authors showed increasing interconversion rates between the two isomeric conformers with increasing temperature. They found that temperature programming provided a significant decrease in the retention times in comparison with the isothermal separations while still preserving baseline separation of the isomers.

An example of a modified HPLC technique was reported by Jacobsen [116]. This author developed a fully automated HPLC method for the analysis of iodix-

anol in plasma samples using on-line dialysis for sample preparation. According to Jacobsen, optimal conditions were obtained with a static dialysis donor solution of 110 mL and 4 mL of recipient solution (dialysate) pulsed onto a trace enrichment column, giving maximum 55% dialysis efficiency in less than the chromatographic run time of 20 min. The method was sensitive down to 84 pmol mL⁻¹. Potentially, this technique might be adapted to the analysis of other contrast agents.

Jenkins et al. developed a capillary electrophoresis system for the measurement of iohexol as a marker of the glomerular filtration rate (GFR) with a run time of 5.25 min and a coefficient of variation (CV) of 4.3% at 80 mg L⁻¹ [121]. The GFR, calculated from the plasma clearance, had a reproducibility of 5.47%. A similar approach (liquid chromatography-mass spectrometry with positive electrospray ionization after enrichment by solid phase extraction) was applied by Putschew et al. for the determination of iodinated contrast agents in treatment plant effluents and surface waters [118].

The concentration of the X-ray contrast agents, diatrizoate, iopamidol, iopromide, and iomeprol, derived from radiological examinations in municipal sewage, sewage treatment plant effluents, rivers, and groundwater was investigated by Ternes and Hirsch using LC-electrospray tandem MS detection [119, 120].

Radioactively labelled contrast agents have been used to study biotransformation or biodegradation processes. Rode and Müller described the transformation of iodinated contrast agents by microorganisms such as *Trametes versicolor* [121].

5.3

Isomerism

5.3.1

General remarks

Iodinated contrast agents with polyhydroxylated carbon side-chains contain a number of asymmetric carbon atoms yielding numerous optical isomers which relate to each other as enantiomers or diastereoisomers. Sterically hindered non-asymmetric carbon or nitrogen atoms might result in additional asymmetry centres while the partial double bond character of the acyl-carbon-nitrogen bond of amide functions can lead to *cis/trans* isomerism. Such isomers are labelled rotamers when heating in solution is able to modify their ratio. Isomerism of iodixanol has been described by Priebe et al. [122], Fossheim et al. [123] and by Molander et al. [115].

5.3.2

Isomerism of Iotrolan

We have investigated the isomerism of Iotrolan by HPLC in order to correlate the pattern of HPLC peaks with rotamers, groups of diastereoisomers or with individual diastereoisomers [124].

5.3.2.1

The HPLC Pattern

At low resolution a typical HPLC chromatogram of an aqueous solution of iotrolan using a reversed phase column (ODS Hypersil) and an eluent system with water/methanol of 500:80 (v/v) contains three groups of peaks, denominated as G1, G2 and G3 (Fig. 7a). The ratio of G1:G2:G3 is approximately 68:4:28.

At higher resolution G1 and G2 split each into 5 baseline-separated peaks (G_{1-5} , G_{2-10}) at a ratio of approx. 1:4:6:4:1 while 7 still overlapping peaks are resolved in G3 (Fig. 7b and 7c) with a ratio of approx. 1:2:3:4:3:2:1. Two chromatographic systems are needed to achieve this resolution.

When G1, G2 and G3 are separated preparatively at low resolution HPLC, the fractions then concentrated to approximately the original concentration and finally heat-sterilized (120°C, 30 min) each fraction reassumes a ratio of G1, G2 and G3 as was present in the starting solution.

Repeating the above procedure with the fractions of each of the individual peaks as collected preparatively under high resolution HPLC from G1, G2 and G3 gave the following results:

- Each of the 17 individual peaks resulted in at least one new peak in the two other groups to which the starting peak did not belong.
- No rearrangement occurs within the individual groups G1, G2, G3.

These interchanges are summarized in Table 3 and shown schematically for each of the 5 peaks of G1 and G2 in Fig. 8. The results from peaks 11 to 17 of G3 are omitted in Fig. 8 for the sake of clarity. The lacking baseline-separation would

Table 3. Correlation table for the interchange of peaks upon sterilization. For example heating converts peak #1 into peaks #10 and #16

Results in peak #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1										x						x
2									x						x	
3								x				x	x	x		
4							x				x	x				
5						x				x						
6					x			?		x						
7			x								x	x				
8			x			?						x	x	x		
9		x												?	?	
10	?				x	x										?
11				x			x					x				
12			x	x			x	x			x			x		
13			x					x	x							
14		x	x					x	x			?			x	
15		x							x							
16	x									x				x		

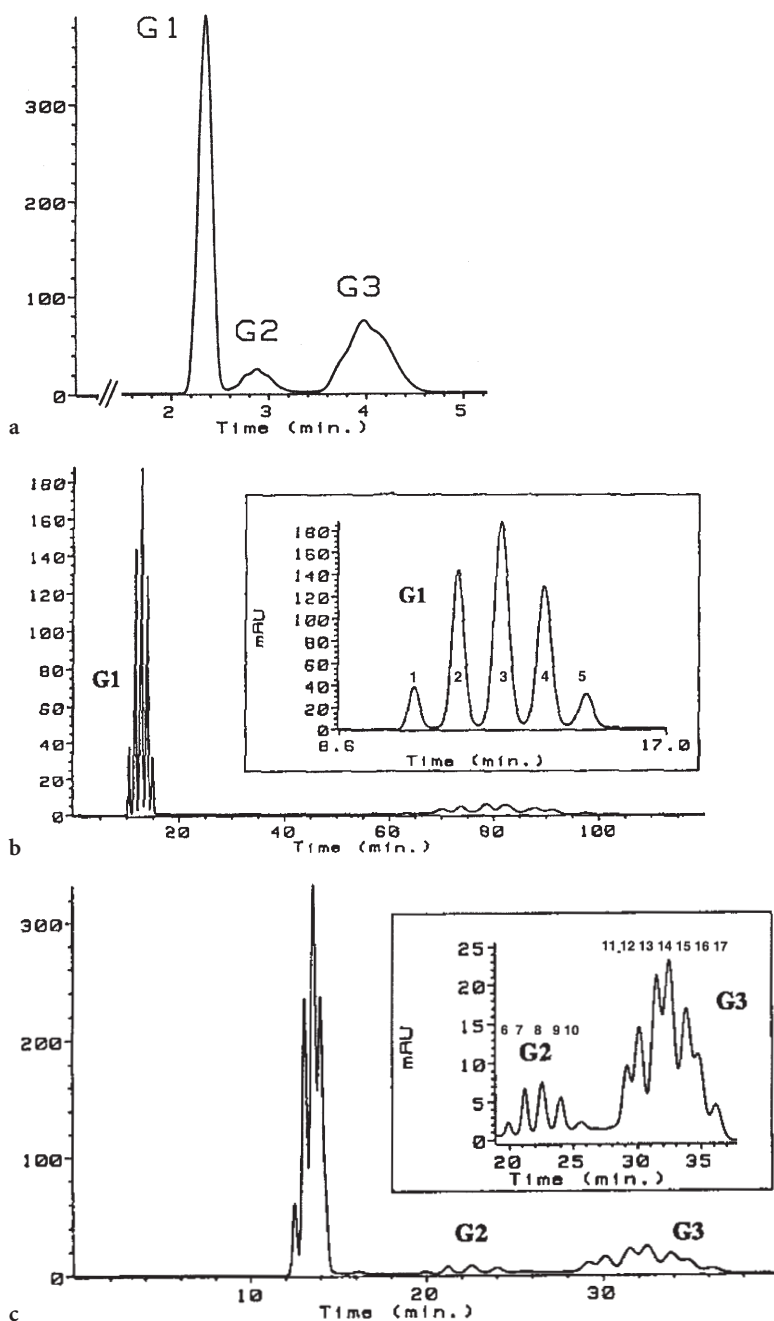


Fig. 7. Reversed-phase HPLC chromatogram of an aqueous solution of iotrolan. **a** Low resolution [ODS-Hypersil, 5 μ m, 250 $\times$ 4.6 mm water – methanol, 500:80 (v/v)]. **b** Low resolution (Superspher 100, 250 $\times$ 4 mm, water pH 3.4 (NH_4 -Formiate, 0.02 M), Na-heptane sulfonate (2 g L^{-1}), 1.5 mL min^{-1} , 40°C) Numbers 1–5 indicate the peak numbers. **c** High resolution (Superspher 100, 250 $\times$ 4 mm, water/methanol, 900:100, 0.4 mL min^{-1} , 40°C) Numbers 6–17 indicate the peak numbers

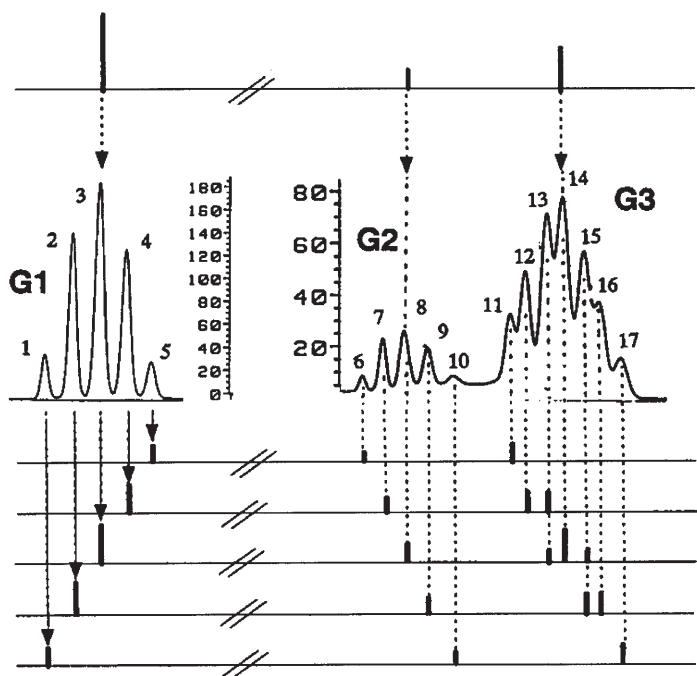


Fig. 8. Schematic representation of interchanges upon heat sterilization starting with the individual fractions containing peaks 1–10 as isolated by high resolution HPLC

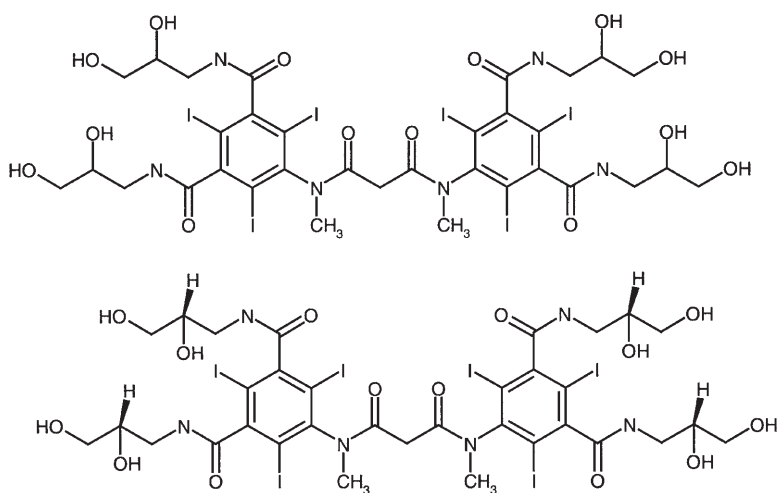
complicate the picture due to admixture of the not completely removable side-peaks. The numbering system selected for the peaks is found in Fig. 7b and c.

In Fig. 8 the vertical bars in the scheme on top of the diagram symbolize the respective isolated starting peak of the chromatogram below. The products of heat sterilizations are found on the horizontal lines below the chromatogram. The length of the bars indicates the relative peak areas obtained after sterilization.

For example, peak 1 of G1 produces peak 10 of G2 together with peak 17 of G3. Likewise peak 6 of G2 yields peak 5 in G1 and peak 11 in G3. Peaks 2 and 4 of G1 produce each just one peak in G2 but produce each two peaks in G3 with equal peak areas. Finally, peak 3 of G1 again gives just one peak in G2 but produces peaks 13, 14 and 15 in G3 with relative area ratios of 1:4:1.

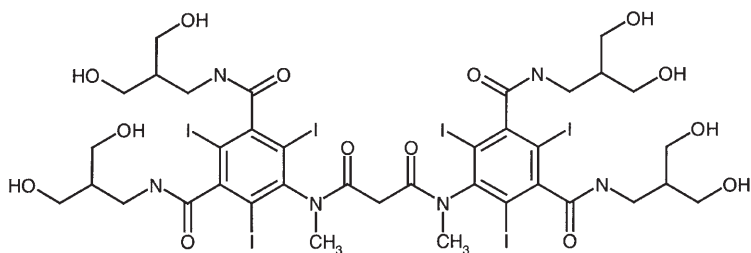
In summary, these experiments show clearly that heat sterilisation induces an equilibrium between the groups G1, G2 and G3, but that there is no equilibration between the individual component within each of the groups. For verification of these correlations, we utilized four model compounds with defined stereochemistry (Fig. 9) and performed HPLC analysis of aqueous solutions.

Whereas ZK37927 resulted in a single peak (Fig. 10), ZK33203, ZK34842, and ZK32349 showed a similar peak pattern as iotrolan, i.e. a main component with 60–70 % of the total peak areas followed by two more components with a larger peak area for the second one (Figs. 11–13).

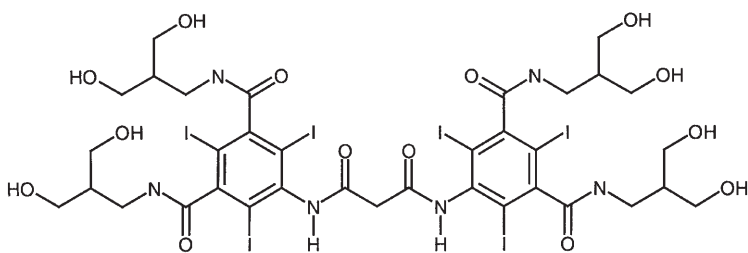


ZK 33203

ZK 34842



ZK 32347



ZK 37927

Fig. 9. Structures of model compounds with definitive stereochemistry



Fig. 10. Low-resolution HPLC chromatogram of ZK37927 (water/methanol, 850:150 (v/v))

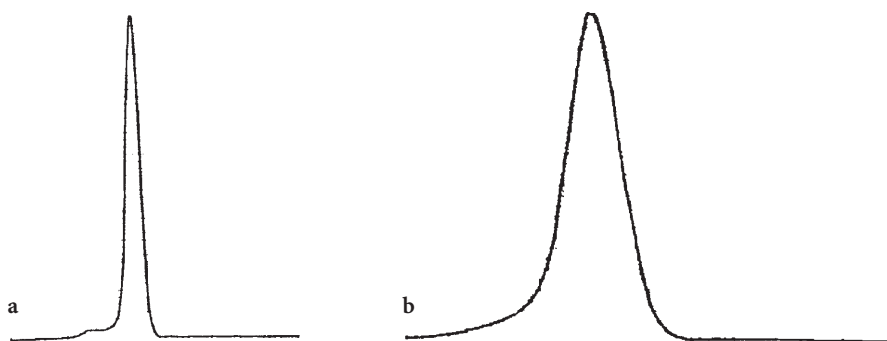


Fig. 11. Low-resolution HPLC chromatograms of ZK32347. **a** water/methanol, 650:80 (v/v). **b** water/methanol, 650:80 (v/v)+chloroform (8 g L^{-1})

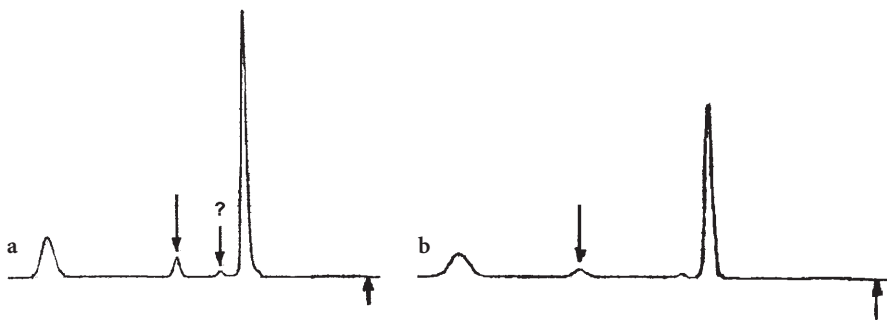


Fig. 12. Low-resolution HPLC chromatograms of ZK33203. **a** water/methanol, 650:80 (v/v). **b** water/methanol, 650:80 (v/v)+chloroform (8 g L^{-1})

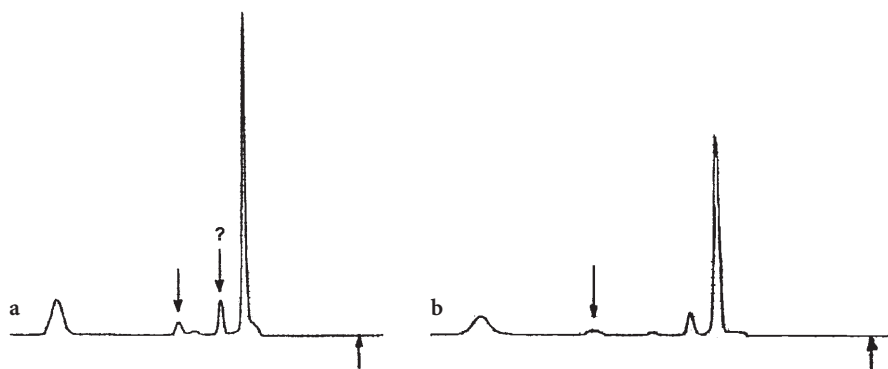


Fig. 13. Low-resolution HPLC chromatograms of ZK39482. **a** water/methanol, 650:80 (v/v). **b** water/methanol, 650:80 (v/v) + chloroform (8 g L⁻¹)

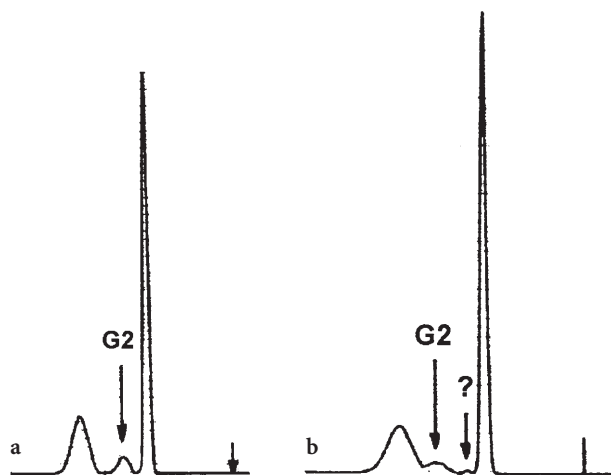


Fig. 14. Low-resolution HPLC chromatograms of iotrolan. **a** water/methanol, 850:150 (v/v). **b** water/methanol, 850:150 (v/v) + chloroform (8 g L⁻¹)

The addition of chloroform to the eluent system, water/methanol, moved one of the smaller peaks, indicated by an arrow in Figs. 11 – 13, towards longer retention times. The same behaviour can also be observed for G2 of iotrolan (Fig. 14). The addition of dichloroethane to the eluent is able to reverse the order of G2 and G3. Some minor additional peaks marked by a question mark in the chromatograms are most probably due to impurities without relevance to the present investigation.

Comparison of the relative peak areas of the model compounds with those of iotrolan shows a striking similarity (Table 4).

This similarity and the behaviour following the addition of chloroform might be interpreted that these peaks represent similar compounds/isomers. Accordingly, we will use the terms G1, G2 and G3 also for these groups of isomers. G1,

Table 4. Peak area ratios of the three main HPLC peaks (main component, component with 20–30 % of total area and the component that can be shifted by the addition of chloroform), normalized to 100 %, for four different dimers

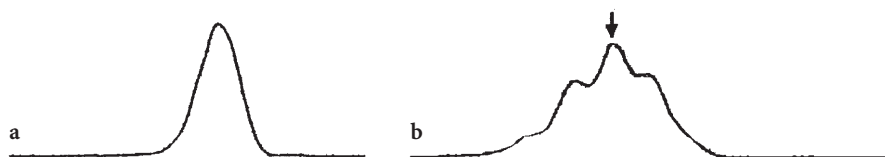
Compound	G1	G2	G3
	Main component	Peak influenced by chloroform	Second largest peak
ZK 34842	70.9	6.3	22.8
ZK 33203	70.3	6.2	23.5
ZK 32347	64.1	7.9	28.0
Iotrolan	68.7	3.9	27.4

G2 and G3 are in an equilibrium state, since thermal treatment (120 °C, 30 min) did not result in any modification of the relative peak ratios (chromatograms not shown).

Having shown at low HPLC resolution that both iotrolan and those model compounds with an *N*-methyl amide group in the malonic acid bridge can be split into the three groups, G1, G2 and G3 and that ZK37927 without this *N*-methyl moiety does not show this behaviour, we then tried to demonstrate the influence of the asymmetric centres in the side-chains on the fine structure of the HPLC chromatograms. To this end, we compared compounds with both (*R*) and (*S*) configuration in the side-chains (ZK33203), with exclusive (*R*) configuration (ZK34842) and without chirality in the side-chains (ZK32347).

Upon adding chloroform, splitting of group G1 due to diastereoisomers can be expected for compound ZK33203 only. Indeed, at very long retention times this effect can be observed. The resulting peak ratios resemble those described for G1 of iotrolan. Although the resolution was not high enough, a ratio of 1:4:6:4:1 might be assumed. For compounds ZK34842 and ZK32347, also at extremely long retention times, no indications for splitting were found (Fig. 15).

In the following paragraphs we will try to identify the isomers represented by the various HPLC peaks.

**Fig. 15.** High-resolution HPLC chromatograms of ZK33203. **a** water/methanol, 950:50 (v/v). **b** water/methanol, 950:50 (v/v)+chloroform (5 g L⁻¹)

5.3.2.2

Isomerism in the Bridge

For clarity, we will use the labels *a*–*f* and *a'*–*f'*, respectively, for individual bonds in the iotrolan molecule (Fig. 16).

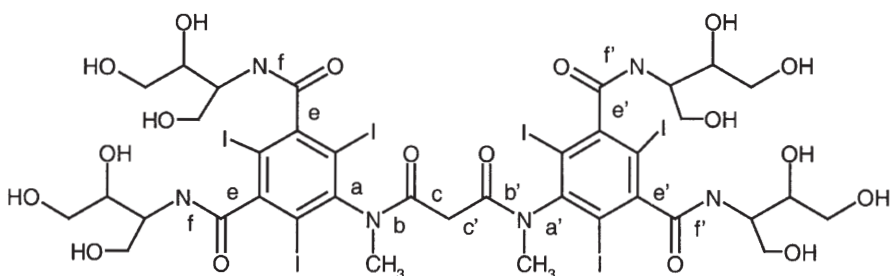


Fig. 16. Labeling of individual bonds in the iotrolan molecule

Steric restrictions preclude free rotation about the bonds *a* and *a'* as well as about the bonds *c* and *c'*. Bonds *b* and *b'* exhibit partial double bond characteristics due to the amide mesomerism. The energy barrier for rotation around these bonds is high enough (approx. 60 kJ mol⁻¹) to yield stable isomers at room temperature. Accordingly, an (*E*) and (*Z*) form can be assigned relative to each of the bonds *b* and *b'* resulting in (*E*)/(*E*), (*E*)/(*Z*) [(*Z*)/(*E*) in case of different substituents at the two aromatic rings] and (*Z*)/(*Z*) forms (Fig. 17). Assignment of these rotamers to the groups G1, G2 and G3 was possible by applying NMR spectroscopy to the respective fractions isolated by HPLC to give: G1 = (*E*)/(*E*), G2 = (*Z*)/(*Z*), G3 = (*E*)/(*Z*) (Fig. 18).

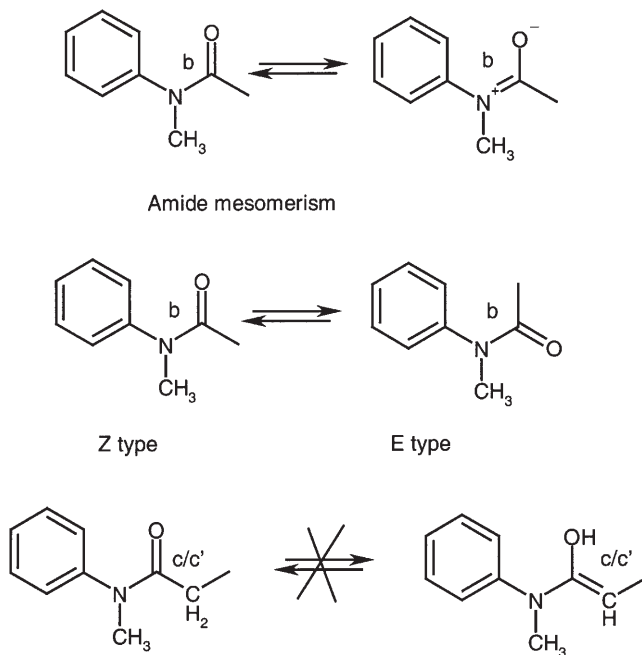


Fig. 17. Amide isomerism due to mesomeric forms resulting in (*E*) and (*Z*) isomers

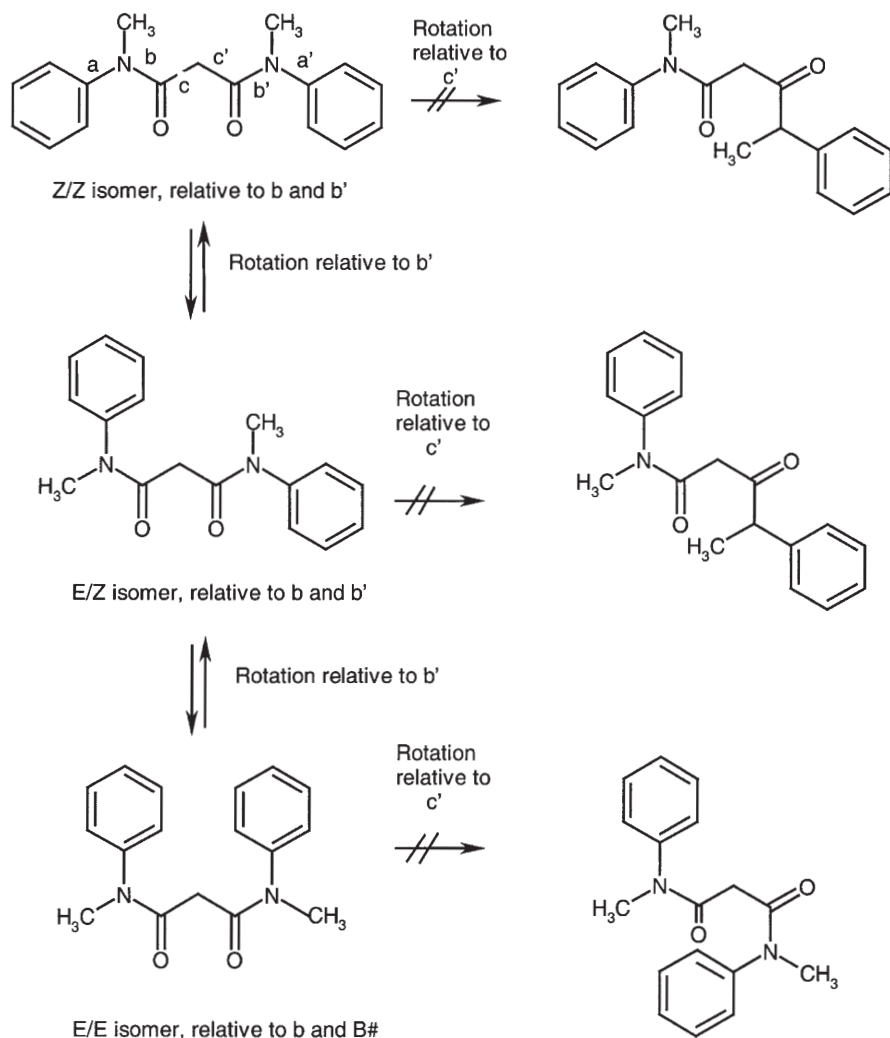


Fig. 18. Schematic representation of iotrolan rotamers. Rotation around bond c' is not possible

5.3.2.3

Isomerism in the Side-Chains

Having established the origin of the rotamers G1, G2 and G3, the splitting within each of these groups into further components due to the chiral centres in the amide side-chains was to be examined. These polyhydroxylated side-chains contain solely the *threo* (*RS*) form of 3-amino-butane-1,2,4-triol as a racemate (Fig. 19). Accordingly, from the possible 8 chiral centres only 4 have to be taken into consideration. These would lead – without considering other types of isomerism – to 16 isomers (8 pairs of isomers). Including (*E*)/(*Z*) isomerism, 48 isomers can be expected.

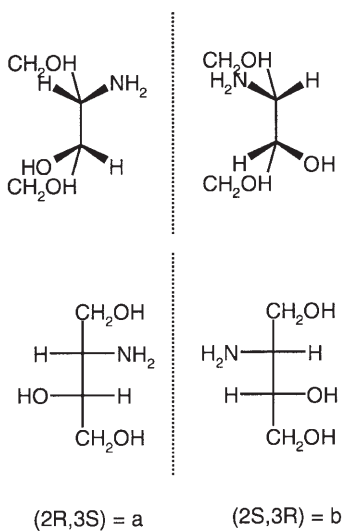


Fig. 19. Configurations of racemic 3-amino-butan-1,2,4-triol

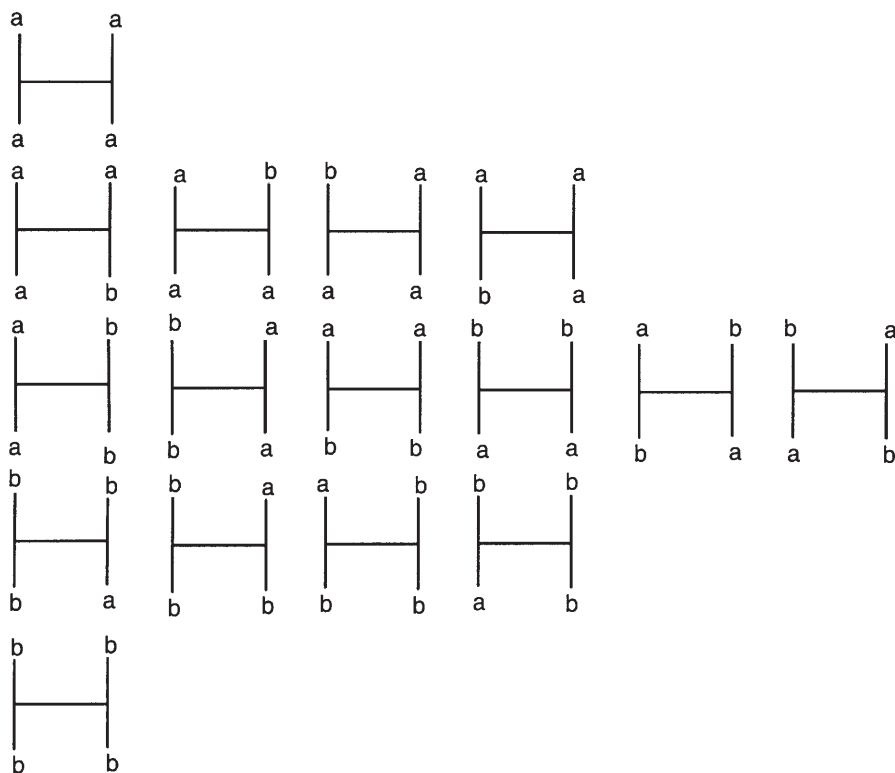


Fig. 20. Schematic representation of the 16 isomers of iotrolan

Assuming that the synthesis, i.e. the reaction of the tetra-acid chloride with racemic *threo*-aminotriol will result into a statistical distribution, the probability of formation of all isomers should be identical. These isomers are illustrated schematically in Fig. 20 where the configuration ($2R,3S$) of the 3-amino-butane-1,2,4-triol is abbreviated as “a” and ($2S,3R$) as “b”.

5.3.2.4

Atropisomerism of Iotrolan

Due to the steric hindrance of free rotation around bonds a and a' because of spatial requirements of the methyl groups on the amide-nitrogen and of the adjacent iodines on the aromatic rings, additional configurations are created. The *N*-methyl groups are thus arranged either above or below the plane of the aromatic ring while the acyl-C functions occupy the respective opposite sites. The Iotrolan molecule therefore might be considered as two tetrahedrons linked together by the malonic acid bridge. These tetrahedrons are stretched in one direction and the chiral axes coincide with the direction of stretching. The asymmetric centres are located within the tetrahedrons. They cannot, however, be located on a particular atom (Fig. 21). Accounting for the atropisomerism in the rotamers (E)/(E), (E)/(Z) [(Z)/(E)] and (Z)/(Z) results in structures shown schematically in Fig. 22.

A schematic representation is possible using a modified Fischer projection. For that purpose, the Iotrolan molecule is stretched to form a straight line connecting the two iodines in *para* positions to the nitrogens of malonic acid amide and arranging the aromatic rings in the paper plane. The tetrahedral wedges connecting methyl groups of the *N*-methyamide functions with the acyl-carbon of the malonic acid are thus perpendicular to the paper plane and are – by convention – no longer permitted to rotate. The other wedge is the line (R–R) which connects the two phthalic acid acyl groups bearing the side-chains through an amide bond. Abiding by these rules, the configuration of the two centres of atropisomerism is defined by the direction of the projection line which connects the two halves of the molecule as illustrated in Fig. 23. All rotameric isomers [(Z)/(Z), (E)/(Z) and (E)/(E)] are now identically represented by this symbolic abbreviation in form of projections.

Including the chiral centres of the side-chains into the modified Fischer projections, the illustration for an Iotrolan isomer having a ($2R,3S$)-configuration (designated as “a”) in all four side-chains gives the representation shown in Fig. 24. Since all possible side-chain combinations always have the *threo* configuration, the illustration can be simplified as given in Fig. 24, right side.

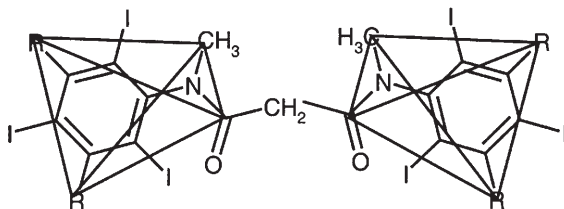


Fig. 21. Schematic representation of the chirality due to atropisomerism

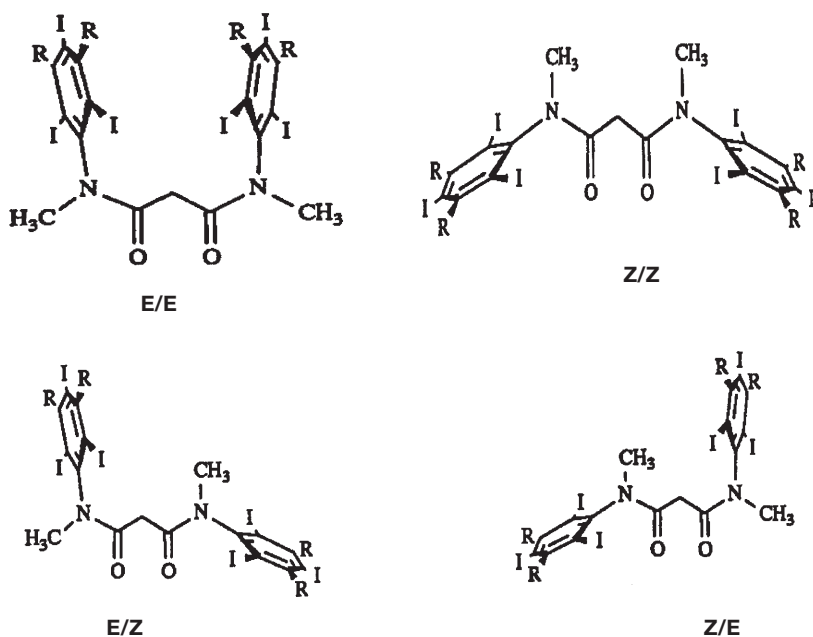


Fig. 22. Schematic representation of the steric arrangements accounting for rotamers and atropisomerism

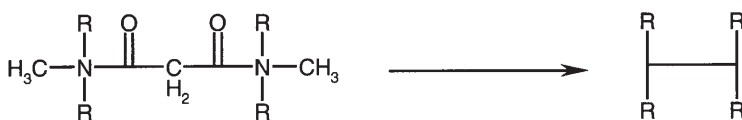


Fig. 23. Modified Fischer projection of iotrolan

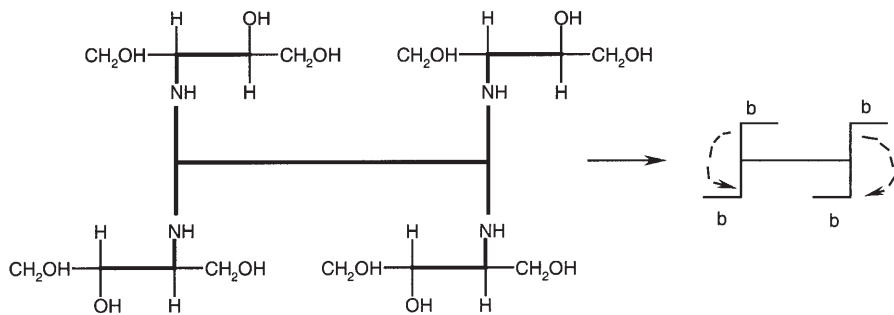


Fig. 24. Modified Fischer projection of the iotrolan molecule with a (2R,3S) configuration in all four side-chains

Having identified 6 centres of chirality, i.e. 4 due to the side-chains and 2 due to the atropisomerism, a total of 64 optical isomers are possible within each of the rotamers G1, G2, G3. However, this number is reduced to 16 configurations because of identities which can be visualized as follows: The Fischer projections of the iotrolan molecule are split at the bridge into two halves to give the

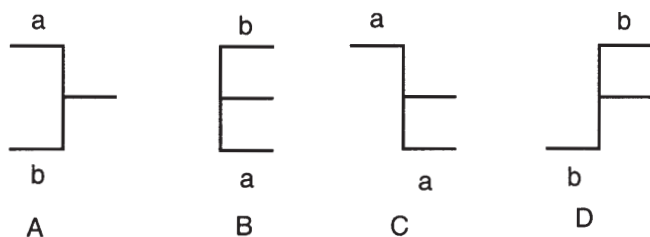


Fig. 25. The four combining possibilities in each half of the iotrolan molecule. The projection line in the middle of the molecule indicates the projection of the bridge

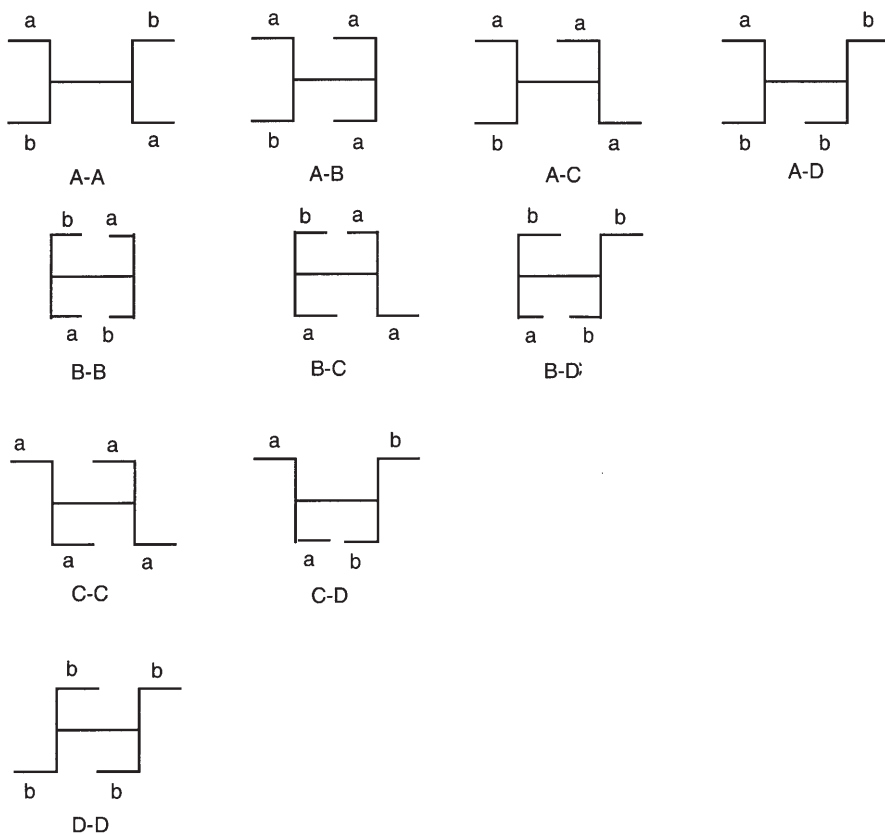


Fig. 26. The ten combining possibilities obtained by bonding the halves A-D from Fig. 25 with the malonamide bridge

moieties A, B, C and D bearing all combinations a and b of the side-chains as shown in Fig. 25. When the atropchirality is inverted by flipping the bridge projection over to the other side the identities become evident. By this, form A is converted into form B and vice versa while forms C and D remain identical with themselves as turning the projection in the plane does not affect the configurations. Rejoining the halves under all combinations of A–D results in the basic 10 structures shown in Fig. 26.

A new categorizing principle might be deduced from Fig. 26 which formally is the number of projections of the side-chain configurations, which are identical to the direction of the bridge projection. For example, for the combination A–A none of the side-chain projections correlates with the projection of the bridge, whereas for B–B all projections coincide. Formally, a *summarized chirality* is obtained, for which the chirality of the side-chains is overlapped by atrop-

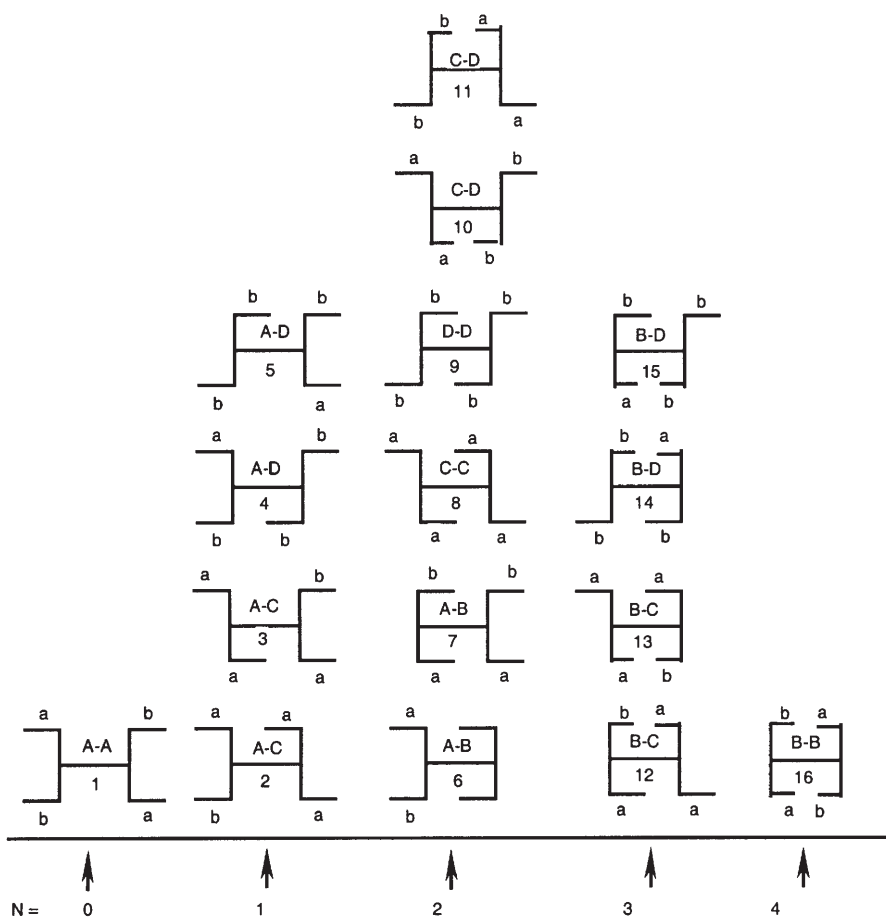


Fig. 27. Possible presence of the 16 isomers of iotrolan in the five groups of combinations from Fig. 26 sorted according to increasing numbers of agreement (N)

Table 5. Number of projections of the side-chains coinciding with the projections of the bridge

No. of corresponding projections	Combinations
0	A-A
1	A-C, A-D
2	A-B, C-C, C-D, D-D
3	B-C, B-D
4	B-B

Table 6. Presence of isomers sorted according to the coincidence of projections

No. of corresponding projections	Combinations	Molar amount	Remarks
0	A-A	1	<i>Meso</i> form
1	A-C	2	1 pair of enantiomers
	A-D	2	
2	A-B	2	<i>Meso</i> form
	C-C	1	1 pair of enantiomers
	D-D	1	<i>Meso</i> form
	C-D	2	
3	B-C	2	1 pair of enantiomers
	B-D	2	
4	B-B	1	<i>Meso</i> form

isomerism (Table 5). Accordingly, five groups of related combinations at a ratio of 1:2:4:2:1 are obtained. If we add the 16 isomers from Fig. 20 to this Table, the resulting molar ratio is 1:4:6:4:1 (Fig. 27, Table 6).

5.3.2.5

Structure and Chromatographic Behaviour

5.3.2.5.1

Separation into Groups G1, G2 and G3

As demonstrated earlier, splitting into groups G1, G2 and G3 – or the rotamers (E)/(E), (E)/(Z) [(Z)/(E)] and (Z)/(Z) – is only possible for those compounds that carry a methyl group at each of the two amide nitrogen atoms of the malonamide bridge, i.e. for model compounds, ZK34842, ZK33203, ZK32347, and for iotrolan. It can be expected that the relative orientation of the methyl and aromatic moieties towards the bridge – independent of the actual conformation of the molecule – will significantly influence the degree of exposition of hydrophilic and hydrophobic groups and thereby the observed retention behaviour.

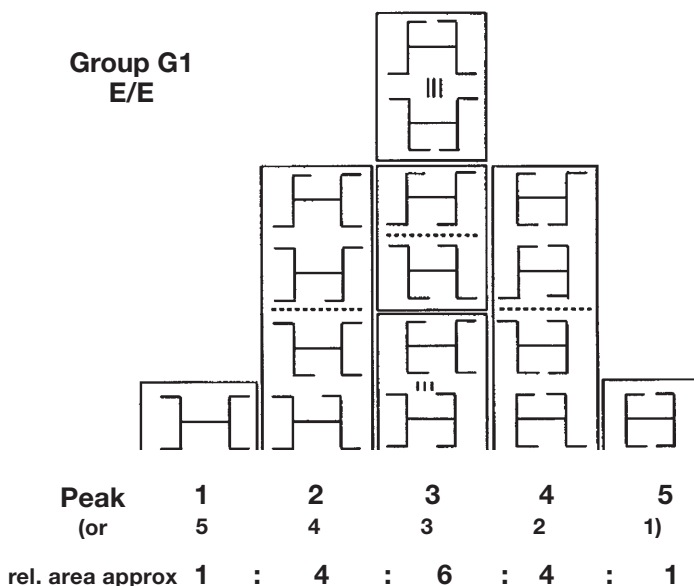


Fig. 28. Numbering for peaks of G1 (cf. Fig. 7 b) and isomer distribution in peaks 1–5. Dashed horizontal lines symbolize mirror planes (enantiomers); vertical triple lines indicate equivalent identities

5.3.2.5.2

Splitting of Groups G1 and G2

The G1 peak groups of iotrolan [(E)/(E)] and ZK33203, which both are synthesized from a tetraacid chloride and racemic amino-polyols, consist of five components. For iotrolan relative peak areas within G1 and G2 are approximately 1:4:6:4:1. For G1 of ZK33203, resolution was not high enough to determine the peak areas exactly. However, it might be assumed that they are similar to those observed for iotrolan. G2 of ZK33202 was not further investigated.

Again, applying the principle of a *summarized chirality* as governing the sequence of elution of the 5 peaks of G1 leads to the distribution of components shown in Fig. 28 extending the information from Fig. 27 by addition of mirror planes (dashed lines), indicating equivalent identities (vertical triple lines) and by enclosing *meso* forms and racemates in boxes to symbolize the relative molar ratios of the various forms. The compounds within one box relate to those in the other boxes as diastereoisomers.

Since we used a non-chiral HPLC system, resolution of enantiomers is not possible. Consequently the racemate (structures 8 and 9, Fig. 27) must appear in peak 3 as well as the two *meso* forms (structures 6/7 and 10/11) which each are present in double relative molar amounts. The two pairs of racemates (structures 2–5 and 12–15), each present with a relative molar abundance of 4, belong to peaks 3 and 4, since peaks 1 and 5 must, by necessity of a relative abundance of one for each form, occupy peak 1 and 5.

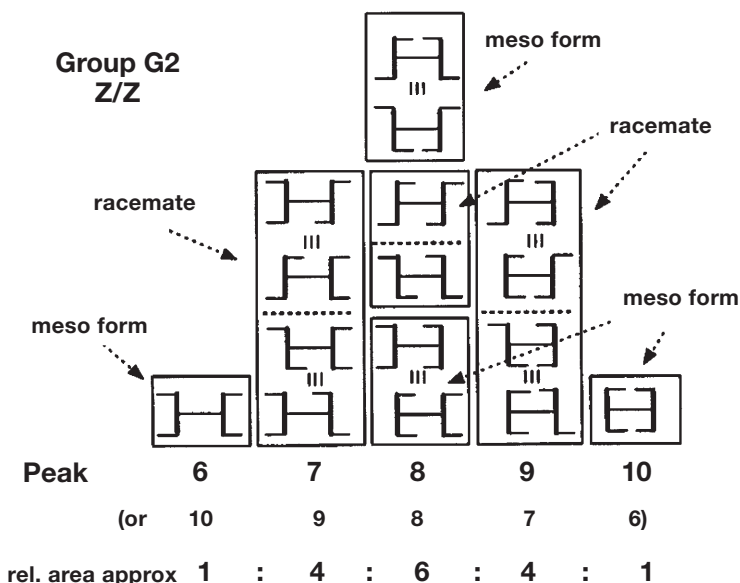


Fig. 29. Numbering for peaks of G2 (cf. Fig. 7c) and isomer distribution in peaks 6–10. Dashed horizontal lines symbolize mirror planes (enantiomers); vertical triple lines indicate equivalent identities

Proceeding from G1 with the (*E*)/(*E*) conformation to G2 with (*Z*)/(*Z*) implies a rotation about bonds *b* and *b'* as indicated by the bold lines in Fig. 29. As the centres of atropisomerism on each half of the molecule are not affected by this rotation, the projection of the side-chains must be adjusted such that the projections correspond to retention of the original configuration. The assignment of the peaks is made using the pattern interchange given earlier in Fig. 8.

5.3.2.5.3

Splitting of Group G3

Seven non-baseline separated peaks were found in G3 with relative ratios of the peak areas of 1:2:3:4:3:2:1 (Fig. 7c). As both, the (*E*)/(*Z*) and the (*Z*)/(*E*) conformations are to be considered because rotation is possible about either bond *b* or *b'*, the ratio is preferably represented as 2:4:6:8:6:4:2. The isomers produced by such rotation are summarised in Fig. 30. The double lines represent the rotated moiety of the iotrolan molecule starting from the respective (*E*)/(*E*) projection in the centre of the two columns while the solid bracket lines connect identical isomers.

Using again the pattern of interchanges (Fig. 8), assignment of peaks 11 and 17 to the respective *meso* forms derived from peaks 5 (G1) or 6 (G2) and respectively peaks 1 (G1) or 10 (G2) is trivial. Peak 12 results from peak 4 in G1 or peak 7 in G2. However, these starting peaks contribute in addition with 4 relative moles to peak 13 of G3 (Fig. 31). To achieve the relative area ratio of 6 in peak 13 of G3, one of the *meso* forms of peak 3 has to contribute the missing two relative

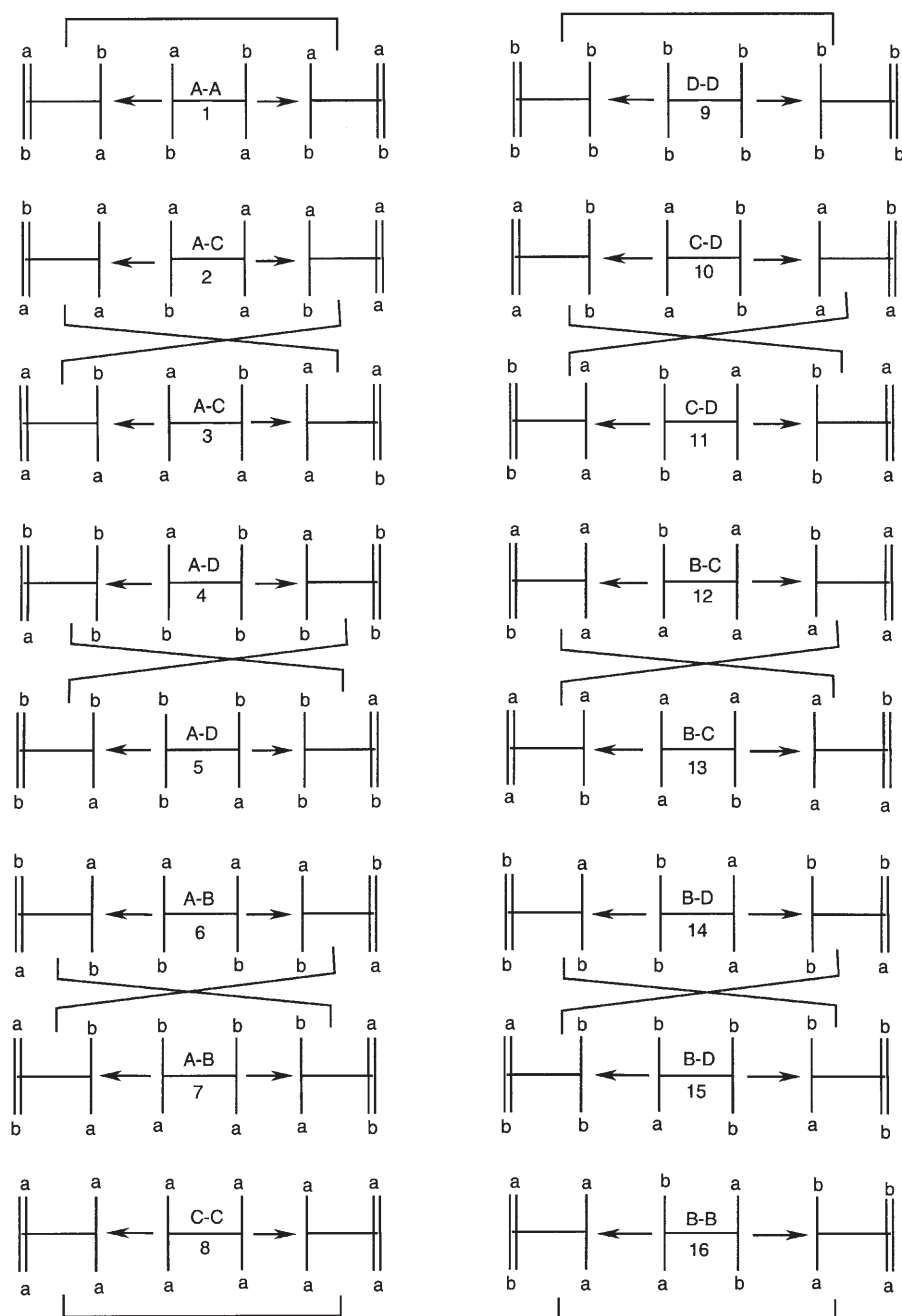


Fig. 30. Schematic representation of the formation of rotamers. The structures 1 – 16 (medium columns) are identical to those in Fig. 27. The arrows indicate rotation around bonds b and b' . The double line symbolizes the moiety of the molecule which was rotated. Solid bracket lines connect identical molecules

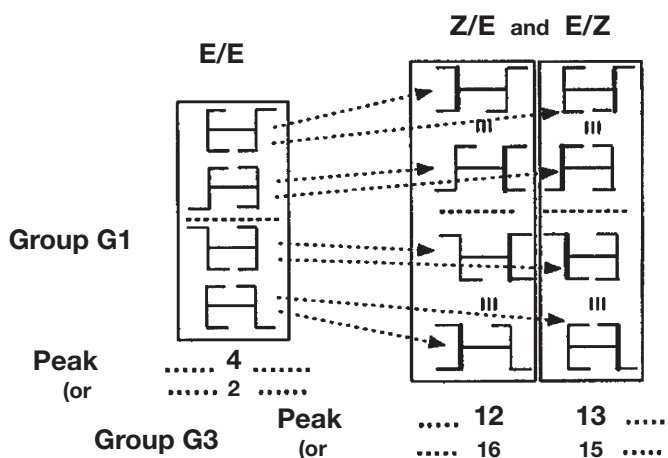


Fig. 31. Transformation of peak 4 (or 2) of G1 into peaks 12 and 13 (or 15 and 16) of G3 upon heat sterilization

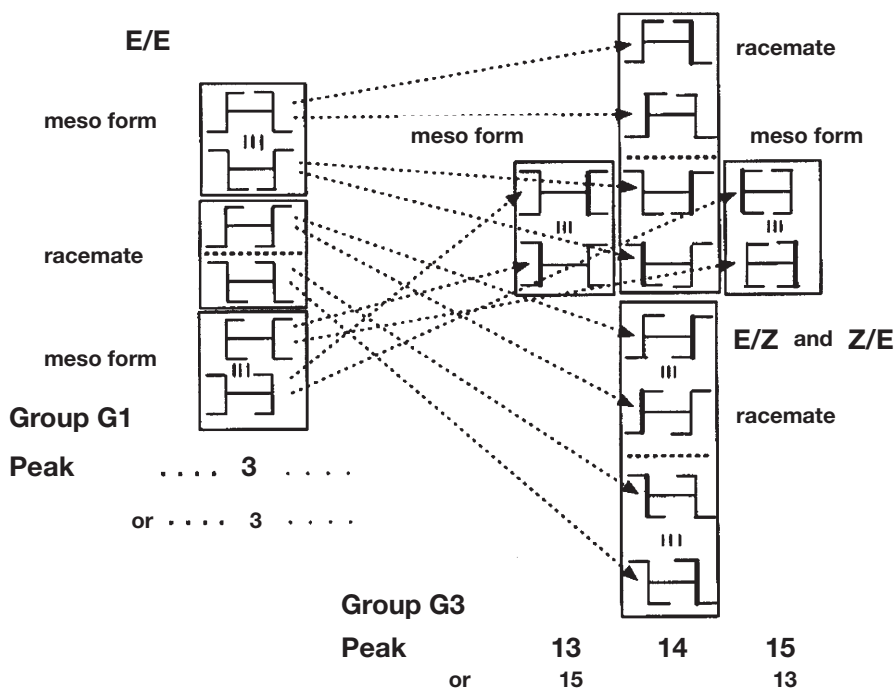


Fig. 32. Transformation of peak 3 of G1 into peaks 13, 14 and 15 of G3 upon heat sterilization

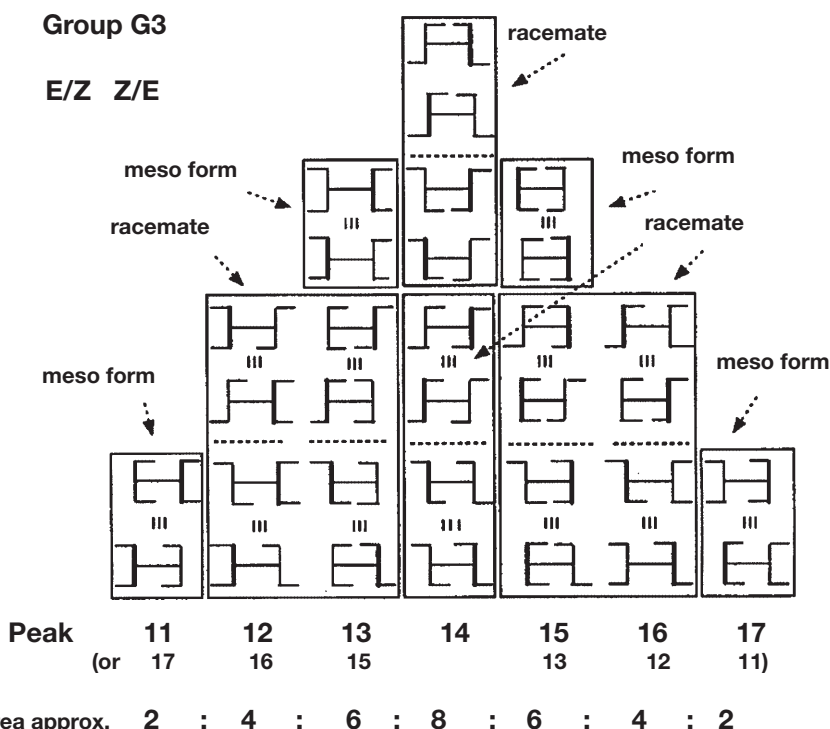


Fig. 33. Numbering for peaks of G3 (cf. Fig. 7c) and isomer distribution in peaks 11–17. Dashed horizontal lines symbolize mirror planes (enantiomers); vertical triple lines indicate equivalent identities

moles. The correlations regarding peaks 12, 13, 15 of G3 are outlined in Fig. 32. Filling the peaks 15–17 in an analogous manner gives the isomer distribution of G3 as shown in Fig. 33.

It should be pointed out at this point that the isomer distribution presented here fits the experimental observations, but that there still remains uncertainty regarding the sequence of elution of the various isomers except for those in the respective centre peaks of G1, G2 and G3. Due regard should be given to the fact that iotrolan represents a mixture of racemates and conformers which differ in their physicochemical properties as is demonstrated by their chromatographic separation. For the same reasons, the mode of preparation and purification may influence the composition of a given sample to some extent, but is not believed to bear significantly on the principles outlined herein.

6 Ecological Aspects

Iodinated X-ray contrast agents contribute in the sewage system to the burden of adsorbable organic halogens (AOX). In general, they are considered as not readily biodegradable.

Ternes and Hirsch [119] investigated the occurrence of iodinated X-ray contrast media derived from radiological examinations in German municipal sewage, sewage treatment plant (STP) effluents, rivers, and groundwater concentrating on four X-ray contrast agents, diatrizoate, iopamidol, iopromide, and iomeprol. They found a maximum concentration measured in STP effluents of 15 g L^{-1} for iopamidol. Median values in rivers and creeks were determined of up to 0.49 g L^{-1} for iopamidol and 0.23 g L^{-1} for diatrizoate. In ground water, these polar compounds were present at concentrations as high as 2.4 g L^{-1} for iopamidol.

To assess the potential environmental impact, studies on environmental fate and effects were conducted for a risk assessment. Steger-Hartmann et al. [125] calculated the predicted environmental concentration (PEC) in surface water and compared the resulting concentration of 2 g L^{-1} with the predicted no-effect concentration as derived from a battery of ecotoxicity tests. From these results, they obtained a ratio between PEC and the predicted no-effect concentration (PNEC) of 0.0002 and concluded that no environmental risk has to be expected in the aquatic environment.

Kalsch [126] used test systems with activated sludge, river water and river water plus sediment to study the degradation of ^{14}C -labelled diatrizoate and iopromide. He found that degradation of diatrizoate by and binding to aerobic-activated sludge was poor. Following a lag time of three weeks, deacetylation of diatrizoate started in systems with river water and sediment with a first-order rate constant of approx. 0.15 day^{-1} . He detected two metabolites that were stable until day 200 of aerobic incubation. Under anoxic conditions, further transformation of the aerobic metabolites was observed. For iopromide, Kalsch observed in activated sludge approx. 85 % degradation into two highly hydrophilic products. In water/sediment systems, one metabolite was formed with a delay of 20 days. The disappearance in river water was concentration-dependent with a lag time of 20 days. For both contrast agents, complete mineralisation to carbon dioxide was not observed.

The decomposition by the fungus, *Trametes versicolor*, of diatrizoate, iopamide and related triiodinated benzoates was investigated by Rode et al. [121]. All compounds were degraded, albeit not completely, under iodide release. Isolation and identification of metabolites suggested stepwise reductive de-iodinations as initial transformation steps.

7

Summary and Conclusions

Iodinated X-ray contrast agents have been in clinical use for more than seventy years and will continue to do so for the next decades. They are characterized by the use of extremely high doses, which lie in the upper gram range, and by extraordinarily good tolerability. Typically, in computed tomography, 100 mL of a solution containing 300 mgI mL^{-1} or 700 mg substance per mL are injected intravenously at injection rates of up to 5 mL s^{-1} , which gives a total dose of 70 g and a total injection time of 20 s. No other drug classes are used in such high doses. The chemical structures of the iodinated contrast agents have been refined in the last decades to such an extent that – consequently – their routes of synthesis

and methods of analysis have significantly increased in complexity. We have tried to summarize the present status of synthetic and analytical methods in use to prepare and characterize iodinated contrast agents.

Acknowledgement. Sincere thanks are expressed to our colleagues Dr P. Blaszkiewicz for many fruitful discussions on the issues of stereochemistry and for providing the model compounds, Dr G. Michl for assignment of the rotamers by NMR measurements and Mr K. Weigandt for all the HPLC work.

8

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Iodinated Contrast Media: from Non-Specific to Blood-Pool Agents

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In 1998, the commercial introduction of multislice computed tomography made X-ray functional imaging possible. Specific contrast agents that could remain within the intravascular compartment for a substantially longer time than conventional nonspecific agents may allow the quantification of local blood volume, tissue perfusion and permeability and thus the characterization of several types of lesions.

We describe a new series of monodisperse, macromolecular iodinated contrast agents. The synthesis, analysis and pharmacokinetics of these molecules are presented.

Their pharmacokinetic profiles are consistent with those of blood-pool agents, of either the rapid- or slow-clearance categories. Their diffusion through the endothelium was found to be low in vitro, thus confirming early confinement of these macromolecules, unlike nonspecific contrast agents. Preclinical safety data (including renal and immunological tolerability) are promising and should allow further evaluation of these compounds to be carried out.

Keywords. Blood Pool Agents, Monodisperse Iodinated Macromolecules, Computed Tomography, Pharmacokinetics, Safety Profile

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Abbreviations

BPA	blood-pool agent
C5/C0	the ratio between blood concentration measured at 5 minutes and the theoretical concentration, calculated by dividing the injected dose by the volume of the plasma compartment (evaluated at 25 and 30 mL/kg in the rat and the rabbit respectively)
CA	contrast agent
CH ₅₀	complement hemolytic activity 50 %
CT	computed tomography
DMAC	dimethylacetamide
ECG	electrocardiogram
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ESI-MS	electrospray ionization and mass spectrometry
FITC-BSA	fluorescein isothiocyanate-labeled bovine serum albumine
GFR	glomerular filtration rate
GPC	gel permeation chromatography
HOBT	1-hydroxybenzotriazole
HOCA	high osmolar contrast agent
HPLC	high-performance liquid chromatography
HU	Hounsfield Unit
IC ₅₀	inhibitory concentration 50 %
LD ₅₀	lethal dose 50 %
LDA	low-diffusion agent
MALLS	multi-angle laser light scattering
MIM	monodisperse iodinated macromolecules
MSCT	multislice computerized tomography
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
MW	molecular weight
NAG	N-acetyl- β -D-glucosaminidase (EC 3.2.1.30)
NS-CA	nonspecific contrast agent
PVP	poly(vinyl-pyrrolidone)
RBL-2H3	rat basophilic leukemia 2H3 mast cell line
RCBPA	rapid clearance blood-pool agent
RES	reticuloendothelial system
SCBPA	slow clearance blood-pool agent
SCE	size exclusion chromatography
T _{1/2α}	distribution half-life
T _{1/2β}	elimination half-life
V _D	volume of distribution

1

Introduction and Background

The first “modern” hydrophilic “nonspecific” iodinated CA was diatrizoic acid, which, formulated as a sodium and/or meglumine salt, is still used. This agent was first synthesized in 1953 [1]. Osmolality for this compound, at concentrations compatible with imaging (ie. 300–370 mgI mL⁻¹), is about 5–7 times that of plasma (1500–2100 mOsm kg⁻¹ H₂O). Other HOCA such as iothalamate, metrizoate and ioxithalamate, were synthesized and marketed over the next few years. In an attempt to reduce the osmolality of the injected solution and thus reduce pain and haemodynamic disturbances, metrizamide, a compound resulting from the condensation of metrizoic acid and glucosamine, was synthesized. It was the first nonionic CA.

The aggregation of metrizamide in solution further reduced its osmolality to about 485 mOsm kg⁻¹ at 300 mgI mL⁻¹ [1]. Unfortunately, this agent could not be sterilized by autoclaving and, consequently, had to be lyophilized and reconstituted extemporaneously. In the mid-1970s, stable nonionic compounds such as iopamidol or iohexol superseded metrizamide. Further improvement was reached by stabilization of the hydrophilic sphere against external disruption, as shown with iobitridol [2].

Another method for reducing osmolality of the injection was achieved by the synthesis of mono-carboxylic dimers, leading to the development of ioxaglic acid (600 mOsm kg⁻¹ at 320 mgI mL⁻¹). Because of specific properties on platelet functions and thrombin generation, this agent is widely used for interventional procedures [3].

In the 1990s, to achieve iso-osmolality with plasma, various teams synthesized nonionic dimers, which resulted in the marketing of iotrolan and iodixanol. However, these compounds suffer from high levels of viscosity (at 20°C and 320 mgI mL⁻¹: iodixanol = 25.4 mPa.s vs 15.7 mPa.s for the ionic dimer sodium/meglumine ioxaglate).

A series of so-called “compact dimers” has also been described [4]. These compounds were characterized by an even three-dimensionally distributed hydrophilicity due to the perpendicular ring conformation, and stabilization of hydrophilicity by hydroxylated amido groups characterized by high energy rotational barriers for (*E/Z*) isomers. Furthermore, their viscosity was low, allowing injection at 350 mgI mL⁻¹ (at this concentration and at 20°C, their viscosities were found to be between 19 and 26 mPa.s vs 44.5 mPa.s for iodixanol) [4].

The pharmacokinetic behaviour of all hydrophilic, NS-CA is well known. They all follow an open two-compartment model characterized by rapid distribution into the extracellular space volume and are excreted almost exclusively by glomerular filtration.

In humans, for all these compounds, V_D is about 0.15–0.27 L kg⁻¹ (consistent with the extracellular volume which, in humans, is about 0.25 L kg⁻¹), $T_{1/2\alpha}$ is 4–10 min, $T_{1/2\beta}$ is between 100 and 130 min and total clearance is 1–2 mL min⁻¹ kg⁻¹ [5]. These compounds are characterized by a rapid distribution: about 70 % of the injected dose is cleared from plasma (diffusion and excretion) within 2–5 minutes after injection [5]. Because of their high hydrophilicity, they do not enter the intracellular space to a significant extent.

Since there is direct proportionality between the iodine concentration within the tissue or vessel and X-ray attenuation, pharmacokinetic parameters can be calculated from time-density curves. In CT, 30 HU correspond to approximately 1 mg iodine g tissue⁻¹ [6]. This is a rather high local concentration of radio-opaque material, thus underlining the relatively low sensitivity of CT that counterbalances its high specificity.

Since 1998, X-ray imaging has experienced a major breakthrough with the introduction of MSCT scanners [7]. Since they can acquire four channels of helical data simultaneously, MSCT scanners allow substantially shorter acquisition times, retrospective creation of thinner or thicker sections from the same raw data, and improvement of three-dimensional rendering with reduction in helical artifacts [8]. MSCT allows rapid cardiac imaging during a single breath-hold. The first coronary artery angiographic images obtained with ECG-gated MSCT are encouraging [9].

Because of a) significant progress in spatial and temporal resolution provided by new MSCT scanners and b) the proportionality between the iodine concentration within the tissue and X-ray attenuation change at a fixed tube voltage, functional imaging appears possible using these new systems. Pathophysiological data strongly support functional imaging: tumoral angiogenesis is characterized by both an increase in new vessels (including capillary sprouts and non-endothelialized capillaries, as well as abnormal tri-dimensional organization) and an increase in permeability, such neo-vessels often being leaky, thus allowing large molecules such as plasma proteins to enter the stroma which helps formation of the matrix for new vessels [10]. Angiogenesis is a critical step in tumor growth, invasion and metastasis [11]. Increased microvascular permeability is also a major characteristic of inflammatory reactions [12]. Liver cirrhosis may also be an interesting indication in that the deposition of collagen fibers in Disse's space and the development of continuous basement membrane associated with this disease [13] may allow the microvascular permeability linked to NS-CA to be distinguished from that of BPA in the pathologic regions.

Functional CT may thus have five major goals:

- to differentiate malignancy from benignancy
- to detect occult malignancies (eg. hepatic metastases)
- to provide prognostic information
- to monitor the clinical results of therapeutic strategies
- to distinguish ischaemic from healthy tissue

Peak tissue enhancement is determined by blood volume but also depends upon cardiac output (which can vary from one patient to the other) and the volume of CA injected [10]. Furthermore, in the case of all presently available NS-CA, the intra- and extra-vascular components of enhancement cannot be evaluated separately [14]. It is also well known that the imaging window in CT angiography is very short and is classically compensated by the injection of high doses of NS-CA or the use of bolus-tracking software.

For functional CT procedures, the Patlak model or deconvolution-based methods are generally used for functional CT procedures in order to overcome this major limitation [10].

Because of their low molecular weight (<2000 Da), the standard NS-CA are extravasated to a massive extent on first pass in noncerebral areas. Thus, Canty et al. reported that first-pass extraction of a conventional nonionic CA averaged 33 % in normally perfused myocardial areas and 50 % in stenotic areas (where coronary blood flow was reduced by 50 %) [15]. These data may even have underestimated first-pass myocardial extraction of CA because of back diffusion of the molecule. In another model, approximately 80 % of the myocardial content of ^{125}I -iothalamate was found in the extravascular space 1 minute after intravenous injection in rats [16].

The validation of capillary permeability using CT techniques is difficult since they depend upon various molecular characteristics, among which volume is the most salient.

It thus appears desirable to design CA that could remain within the intravascular compartment (the so-called BPA) for a substantially longer time for several distinct reasons:

- *for anatomic imaging:*
- dose reduction by avoidance of re-injection and an increase in the temporal imaging window,
- better definition of small vessels (distality),
- detection of bleeding areas,
- *for functional imaging:*

quantification of

- blood volume,
- tissue perfusion
- permeability

Whatever the type of imaging considered (magnetic resonance imaging or X-rays), CA can be classified according to their pharmacokinetic behaviour into four categories (Fig. 1):

- *nonspecific CA* (Fig. 1 a) such as the above-mentioned and currently marketed molecules (iopamidol, iodixanol, ioxaglate, etc.). Their total body clearance is equal to the GFR.
- *low-diffusion agents* (Fig. 1 b). These molecules still diffuse through the normal endothelium but at a much lower rate than nonspecific CA because of a higher molecular volume. This volume, however, is not great enough to impede glomerular filtration. Consequently, their renal clearance is equal to the GFR.
- *rapid clearance BPA* (Fig. 1 c). These molecules are confined to the vascular compartment for a certain period and are rapidly excreted by the kidneys. Consequently, they are characterized by a restricted V_D and a clearance similar to the GFR.
- *slow clearance BPA* (Fig. 1 d). Whereas these agents are also confined to the vascular compartment, their molecular volume only allows a slow “sieving” through the glomerular fenestrae. Their renal clearance is consequently much lower than the normal GFR.

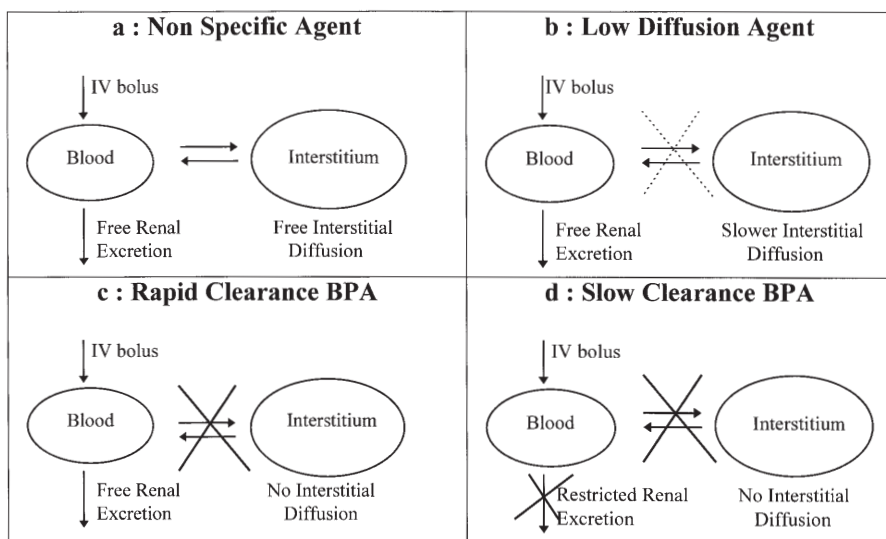


Fig. 1. Schematic compartmental pharmacokinetic representation of different classes of contrast agents: **a.** nonspecific agent (NSA; e.g. iobitridol); **b.** low-diffusion agent (LDA); **c.** rapid-clearance blood-pool agent (RCBPA; e.g. P743); **d.** slow-clearance blood-pool agent (SCBPA, e.g. P840). From Idée et al. (2001) *Invest Radiol* 26: 41, with permission (© Lippincott Williams & Wilkins)

To design BPA, various strategies have been followed by researchers: particulate systems (such as liposomes formulated with NS-CA [17–19], nanoparticles [20], micelle [21] or emulsion [22]) or macromolecules (either polymeric [23, 24] or monodisperse [25]). In the present article, we shall focus on monodisperse iodinated macromolecules, the most recent class of iodinated BPA published so far.

2

Chemical Synthesis and Characterization

Many factors affect the extracellular diffusion of CA. Briefly, the size, form, rigidity of the molecular structure and the charge seem to play essential roles in the process of diffusion through the endothelium. Dendrimers have many advantages. MIMs are iodinated dense structures with hydrophilic groups at the periphery (carboxylate or amido alcohol functional groups) making them water-soluble and biocompatible. They are monodisperse, globular, not readily deformed and rapidly achieve the steric hindrance required for lengthening vascular residence time.

These structures are constructed from iodinated repeat units of the type A-B₂ in which A can react with B (Fig. 2). The dichloride of triaminoacetylisophthalic acid was selected as the repeat unit and determines the multiplicity of two.

This triiodinated synthon has an amino functional group (A) that is potentially reactive with the chloride functional groups of the acid (B). The amino functional group is protected in the form of a phthalimido group.

The general structure of a MIM is shown on Fig. 3.

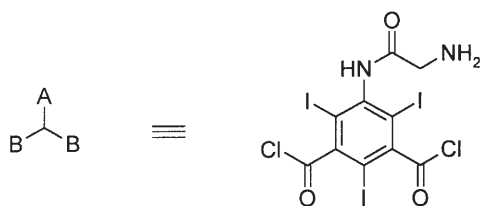


Fig. 2. Construction of MIM structures from iodinated repeat of the type A-B₂

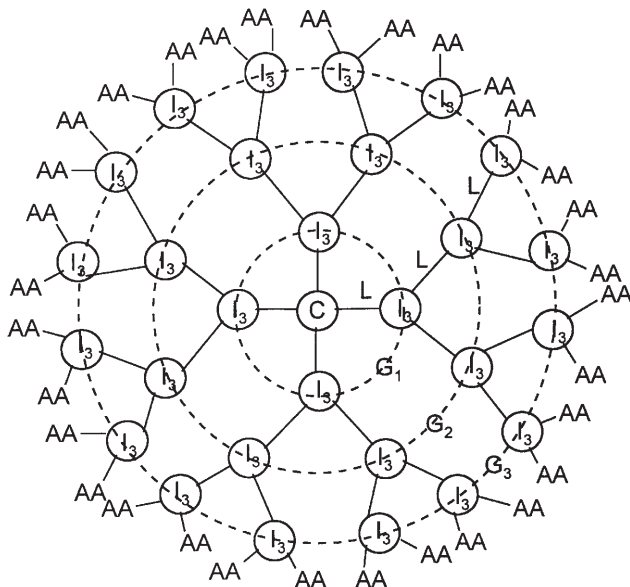


Fig. 3. Structure of an MIM with a tetracarboxylic core. C: polycarboxylic core of MIM G1: first layer of triiodinated rings. I₃: triiodinated aromatic ring G2: second layer of triiodinated rings. L: amido-acetyl bond G3: third layer of triiodinated rings. AA: amino alcohol

2.1

General Strategy for Synthesis

There are two defined methods of synthesis, divergent and convergent.

In the divergent method (Fig. 4), the molecule is assembled from the core to the periphery, while in the convergent method, the molecule is synthesized from the periphery to the core. In either method, the synthesis requires a stepwise process, attaching each layer to the previous one, purifying and then changing functional groups for the next stage of reaction.

For the divergent synthesis, the reaction sequence is amidification/chloridation. Once the polychloride of the acid is obtained, a partial or total amidification reaction with an amino alcohol yields a MIM, which is either ionic or non-ionic, respectively.

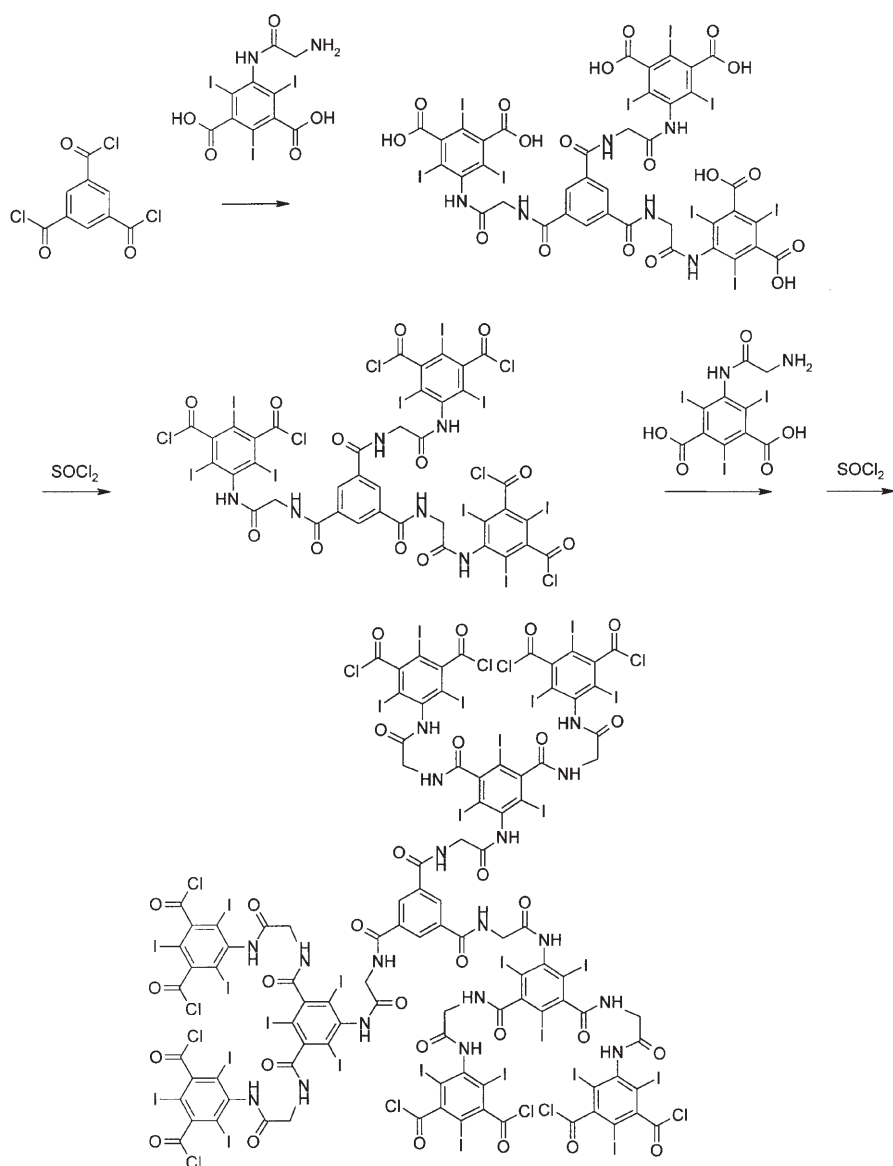


Fig. 4. Divergent synthesis strategy

This synthesis route is advantageous because hydrophilicity is imparted in the last step (as in a monomeric series) but it entails problems related to polydispersity and erratic purification of the intermediates.

The convergent synthesis was selected because it allows easier purification of the intermediates and better control of monodispersity because the number of reactive groups used to reach the next layer is limited.

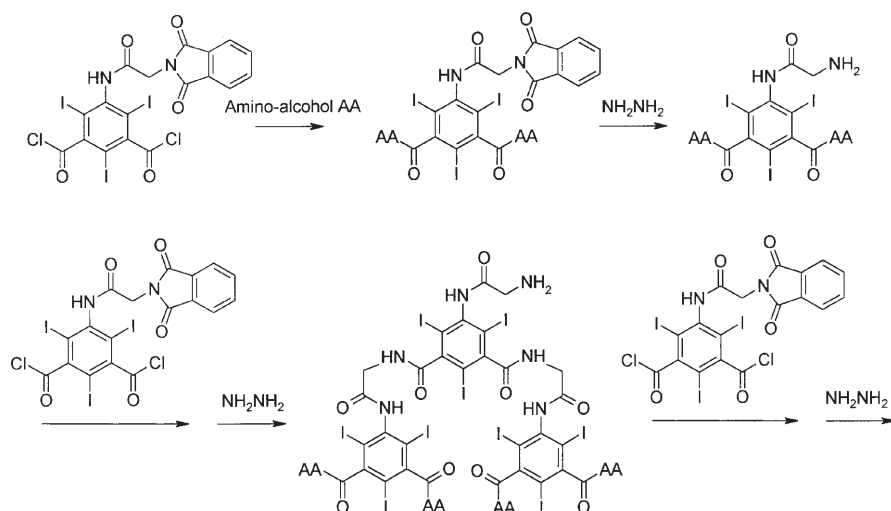


Fig. 5. Synthetic route of Gn substituent of MIMs

In the case of MIMs, the core substituents were synthesized from the dichloride of triiodophthalimidoaminoacetylphthalic acid (multiplicity = 2) after successive diamidification/deprotection sequences (Fig. 5). The substituents were then grafted onto the polycarboxylic central core by coupling.

Convergent MIM Gn synthesis strategy:



2.2

Choice of the Amino Alcohol

Amido alcohol functional groups increase the surface hydrophilicity and thereby reduce interactions with biological membranes or proteins. The amino alcohols chosen (HNR_1R_2 , where R_1 and R_2 each have at least one hydroxyl group) must provide the best compromise between hydrophilic coverage and viscosity while retaining high solubility.

These amino alcohols can be prepared by disubstitution of the benzylamine with a suitable halogenated or sulfonated derivative of the alcohol, followed by debenzilation (hydrogenolysis) using H_2 .

Amino alcohols studied, included those derived from glucose can be mentioned, i.e. HNR_1R_2 where $\text{R}_1 = \text{CH}_2(\text{CHOH})_4\text{CH}_2\text{OH}$ and $\text{R}_2 = \text{CH}_2(\text{CHOH})_4\text{CH}_2\text{OH}$ (disorbitylamine) (Fig. 6), or $\text{CH}_2\text{CH}_2\text{OH}$, $\text{CH}_2\text{CHOHCH}_2\text{OH}$.

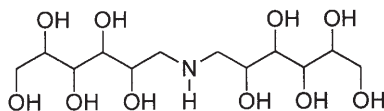


Fig. 6. Structure of disorbitylamine

2.3

Choice of the Core/Coupling Reaction

By adjusting the form, size and multiplicity of the core (Fig. 7), the pharmacokinetics (C5/C0 ratio, elimination in urine and bile) and toxicity can be modified.

Increasing the multiplicity of the core makes it possible to:

- obtain more compact molecules with a higher surface density and thus a better distribution of the hydrophilic covering,
- increase the conformational rigidity of the molecules and thereby lengthen the vascular residence time.

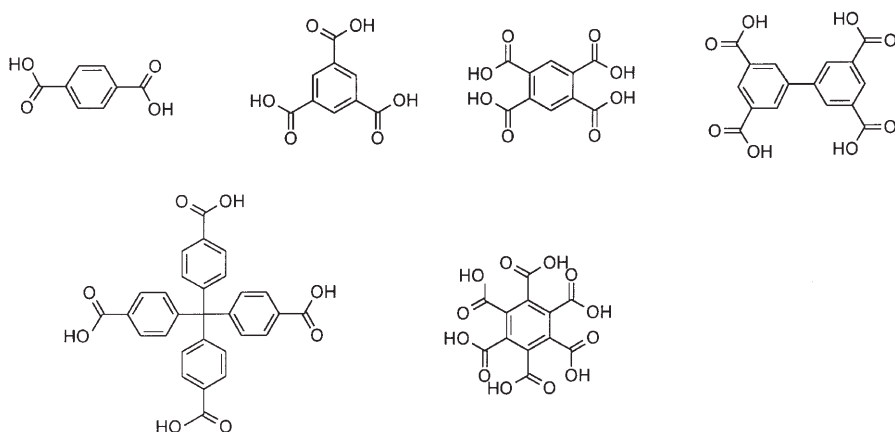


Fig. 7. Examples of polycarboxylic cores

Coupling the substituents to the polyacid core is a key step. The reaction must have a high yield to limit purification problems and show high selectivity between the amines and alcohols present to limit side reactions. The amidification reaction chosen is a coupling reaction used in peptide chemistry. The reaction is carried out at room temperature in the presence of a coupling reagent such as *N,N'*-dicyclohexylcarbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide or 1-ethoxycarbonyl-2-ethoxyl-1,2-dihydroquinoline, possibly in the presence of an activator such as hydroxybenzotriazole or *N*-hydroxysuccinimide (Fig. 8).

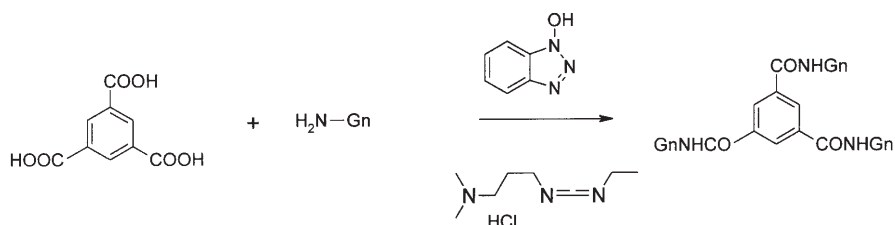


Fig. 8. Coupling a substituent with *n* layers to a core with a multiplicity of 3

2.4

Structure, Synthesis and Analysis of P743, P749 and P840

For details see references [26–28].

2.4.1

P749 and P743

P743 and P749 were MIMs obtained from the tetrakis core and substituents with two and three layers (G2 and G3), respectively. Use of the tetrakis core made it possible to obtain dense spherical products, with a homogenous hydrophilic covering at the periphery.

P743 (Fig. 9) was obtained from pure G2 substituents (with disorbitylamine as the amino alcohol) by dissolving them in DMAC under heat, and then carrying out the HOBT/EDCI-promoted coupling to the tetrakis core at room temperature. The crude reaction product was precipitated in ethanol. The powder was dissolved in water and the product was purified by ultrafiltration (cut-off at 5 kDa). Subsequent purification was performed by HPLC and SEC. The structure of the product was confirmed by ESI-MS (characteristic mass-to-charge ratio: 1433 and 1612). MW is 12,908.

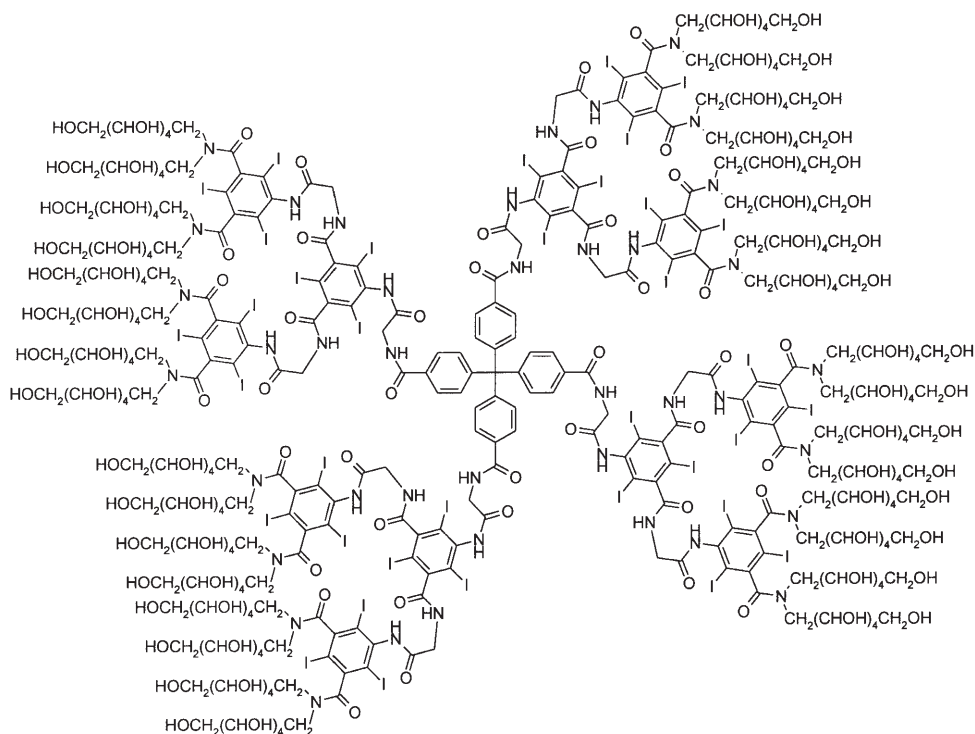


Fig. 9. Chemical structure of P743

2.4.2

P840

In contrast with the other MIMs, P840 was constructed by simple amidification of the G2 substituent on an iodinated core synthesized from the dichloride of triiodoaminoisophthalic acid (Fig. 10).

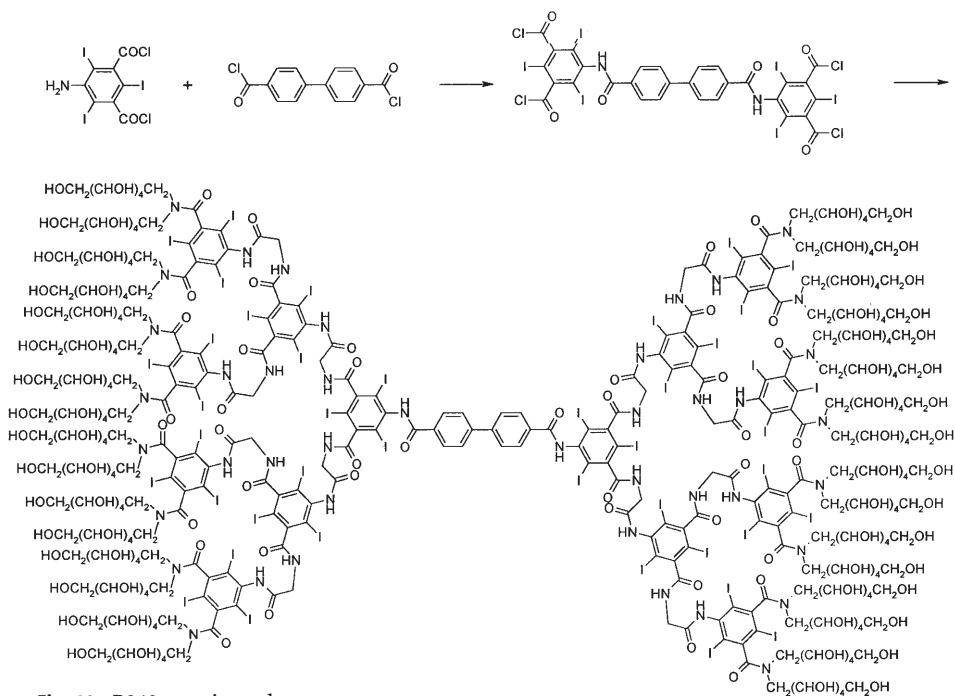


Fig. 10. P840 reaction scheme

2.4.3

MIM Analysis

MIM analysis was difficult to develop, as it required special HPLC and GPC conditions to determine the purity and mass spectroscopy to confirm the structures.

Combined synthesis/analysis operations were performed to characterize the MIMs and identify the impurities. The principal impurities were synthesized and isolated using HPLC. The intermediates and final products were purified on macroreticular resins.

The structures of the substituents with one, two and three layers, (denoted as G1, G2 and G3, respectively), as well as the MIM structures, were confirmed using ESI-MS. The molecular weights of the MIMs were also determined using laser light scattering (MALLS detector coupled with SEC).

Purity and monodispersity were established by GPC.

3
Main Physicochemical Characteristics

Osmolality of the test-solutions (initially hypo-osmotic because of the great number of iodine atoms per molecule) was adjusted with saline (Table 1). It is worth noting that viscosity of the test-solutions (150–200 mgI mL⁻¹) is compatible with intravenous injection.

Table 1. Physico-chemical properties of NS-CA and MIMs

	Iobitridol	Iodixanol	P743	P749	P840
Iodine Concentration (mgI mL ⁻¹)	300	320	152	183	187
Osmolality of the Test-Solution (mOsm kg ⁻¹)	695	293	367	444	318
Viscosity (37°C)	6.0	11.4	6.7	19.6	13.1

4
Pharmacokinetic Profiles

To further determine the blood pool behavior of the compounds, the C5/C0 ratio was calculated.

The pharmacokinetic profile of the RCBPA P743 was evaluated in rats and rabbits (Table 2) [25].

Table 2. Pharmacokinetic profile of P743 and the NS-CA iobitridol in rats and rabbits

	P749 (n = 3)	P743 (n = 3)	Iobitridol (n = 5)	P743 (n = 5)	P743 (n = 4)	Iobitridol (n = 6)
Dose (mgI kg ⁻¹)	300	300	300	150	300	300
Species	Rats	Rats	Rats	Rabbits	Rabbits	Rabbits
Class	SCBPA	RCBPA	NS-CA	RCBPA	RCBPA	NS-CA
T _{1/2β} (min)	31 ± 2	4 ± 0	19 ± 4	18 ± 5	22 ± 5	72 ± 6
V _D (mL kg ⁻¹)	65 ± 7	40 ± 1	195 ± 26	53 ± 7	47 ± 14	267 ± 18
Clearance (mL min ⁻¹ kg ⁻¹)	1.3 ± 0.1	7 ± 1	9 ± 1	3 ± 0	3 ± 0	3 ± 0
Mean Residence Time (min)	53 ± 3	6 ± 0	25 ± 8	19 ± 4	22 ± 3	90 ± 5
C5/C0 (%)	51 ± 3	21 ± 1	11 ± 1	49 ± 2	44 ± 1	16 ± 1

Elimination of P743 from the intravascular compartment is best described by a biphasic model in rabbits (Fig. 11).

The C5/C0 ratio was found to be higher for P743 than for the NS-CA iobitridol in both rabbits and rats (Table 2).

The clearance rate for P743 was found to be close to the creatinine clearance value in rabbits (about 3.5 mL min⁻¹ kg⁻¹) as well as in rats (6.2 ± 0.2 mL min⁻¹ kg⁻¹).

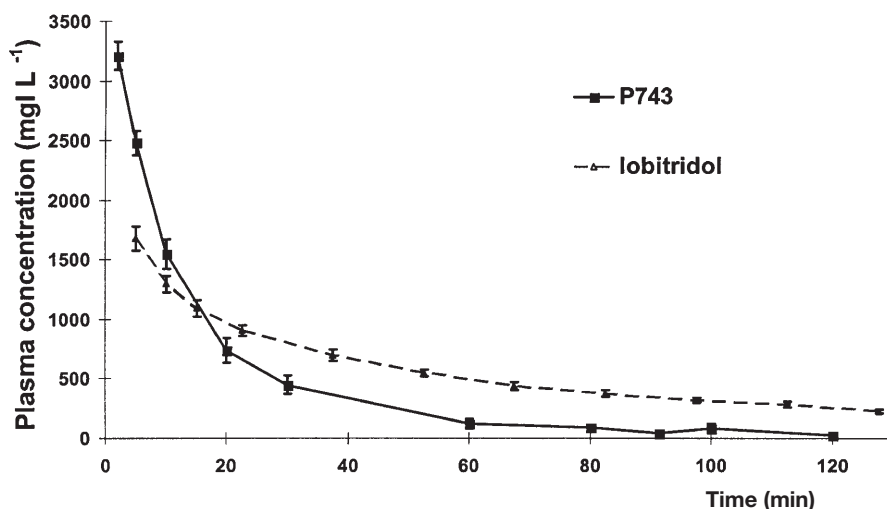


Fig. 11. Rabbit plasma pharmacokinetic curves for P743 and the non-specific agent iobitridol for a 120-minute period. From Idée et al. (2001) *Invest Radiol* 26: 41, with permission (© Lippincott Williams & Wilkins)

As shown in rabbits (Table 2), neither $T_{1/2\beta}$ nor clearance of the compound were dependent on the injected dose.

Taken together, these data are consistent with those of a BPA (low V_D , C_5/C_0 ratio greater than that of iobitridol) that is freely excreted by the kidney with a very short elimination half-life. Furthermore, no biliary excretion was observed up to 6 hours after intravenous injection of P743 at the dose of 300 mgI kg^{-1} in awake rats.

P749 also presented a V_D value close to the theoretical plasma volume in rats (i.e. 25 mL kg^{-1}) (Table 2). However, this compound presents interesting particularities that distinguish it from RCBPA and allow it to be categorized as a SCBPA: its $T_{1/2\beta}$ is longer than that of the iobitridol tested as a control and its clearance is far lower.

The pharmacokinetics of P840, another SCBPA, was investigated in rabbits, in comparison with the NS-CA iodixanol (Table 3).

The cumulative excretion of P840 at 48 hours post-injection was found to be $65 \pm 21\%$ of the injected dose in the urine and $4 \pm 1\%$ in the faeces. Thus,

Table 3. Pharmacokinetic profile of P840 and the NS-CA iodixanol in rabbits

	P840 (n = 5)	P840 (n = 5)	Iodixanol (n = 5)
Dose (mgI kg^{-1})	300	600	600
$T_{1/2\beta}$ (min)	102 ± 4	111 ± 7	54 ± 3
V_D (mL kg^{-1})	105 ± 7	105 ± 5	238 ± 26
Clearance ($\text{mL min}^{-1} \text{ kg}^{-1}$)	0.7 ± 0.2	0.7 ± 0.2	3.2 ± 0.4
C_5/C_0 (%)	71 ± 3	65 ± 3	19 ± 2

at this time-point, elimination for P840 was lower than that of a NS-CA (for comparison purposes, the cumulative excretion of iodixanol was 87% of the injected dose). However, completeness of the excretion was found following injection of P841, another SCBPA ($95.2 \pm 5.5\%$ of the dose excreted at 48 hours in rats), thus showing that a complete excretion can be achieved with such agents.

As shown by its C5/C0 ratio and V_D (Table 3), P840 also behaves like a BPA.

The V_D data suggest a restricted volume of distribution, but this value was higher than the strict intravascular plasma volume (30 mL kg^{-1} in the rabbit) and the value measured in the rat for P749.

P841 (clearance = $1.8 \text{ mL min}^{-1} \text{ kg}^{-1}$, $T_{1/2\beta} = 28.6 \text{ min}$, C5/C0 = 59%) showed a V_D value ($42.9 \pm 3.1 \text{ mL kg}^{-1}$) closer to the theoretical value in rats.

Such discrepancies between calculated and theoretical V_D can be explained by the fact that V_D (and clearance values) are calculated from data obtained after modelling of pharmacokinetic blood curves. This only gives a global overview of the distribution and excretion of test-compounds. This approach does not take into account the great heterogeneity of the endothelium structure that exists in the body (endothelia can be either continuous or fenestrated, they can incorporate large pores as in hepatic sinusoids which lack basement membrane or, conversely, they can be joined by so-called tight junctions and consequently tightly fused as in cerebral capillaries).

For example, diffusion of BPA is probably not restricted to the liver because of the specific structure of the endothelium in the sinusoids but, since the interstitial volume is low in this particular territory, it may be supposed that the contribution of the liver extravasation is negligible when compared with the whole capillary system.

5 Endothelial Permeability

Endothelial permeability can be assessed *in vitro*, i.e. in well-controlled experimental conditions allowing a rigorous comparison between CA and eliminating a number of parameters that may vary significantly following bolus injection in *in vivo* models.

Permeability of the RCBPA P743, SCBPA P749 and P840 and the NS-CA ioversol was evaluated using endothelial ECV 304 cells cultured until they reached confluence on Transwell-COL collagen-coated bicompartmental filters as previously described [25].

Table 4. In vitro endothelial permeability through an ECV 304 cell monolayer

	Ioversol 0.1 mmol L^{-1}	FITC-BSA 0.07 mmol L^{-1}	P743 0.1 mmol L^{-1}	P749 0.1 mmol L^{-1}	P840 0.1 mmol L^{-1}
Clearance (nL min^{-1})	771	69	228	137	180
Ratio to ioversol	–	0.09	0.30	0.18	0.23

Our data show that the clearance of the three MIMs tested was lower than that of the nonspecific and highly diffusible agent ioversol (Table 4). The model was validated by the use of FITC-BSA, which had a very low clearance from the upper part of the Transwell filters.

This model may be a very useful tool for structure-activity studies. As demonstrated for glomerular filtration [29], not only MW but also the size, shape or charge of the molecule most probably play a significant role here.

6
Safety Profile

6.1
Acute Toxicity

CA are classically injected as a bolus. Acute toxicity estimated by the lethal dose 50 % (LD₅₀) is a relevant parameter for comparing such molecules.

Various BPA were compared using their LD₅₀ (according to Reed and Muench's method [30]) following single intravenous injection (rate = 2 mL min⁻¹) in female mice of the Swiss strain.

Table 5. Acute toxicity of MIMs and the NS-CA iobitridol in mice

	Iobitridol	P743	P749	P840
LD ₅₀ Intravenous route (gI kg ⁻¹)	> 17.5	> 8.9	6.0	> 9.4
LD ₅₀ Intracerebral route (gI kg ⁻¹)	1.7	0.8	0.7	0.8

The maximum injected volume was 50 mL kg⁻¹ [25]. LD₅₀ was thus limited by the iodine concentration of the solution in the case of P743 and P840 and the NS-CA iobitridol. Thus, the intravenous acute systemic tolerance by the route intended for use in clinical practice was found to be satisfactory (Table 5).

6.2
Interaction between BPA and the Complement System

The question of complement activation in the physiopathology of NS-CA-induced anaphylactoid reactions remains open [5].

Among the several experimental models available to evaluate the involvement of the complement system [31], the measurement of hemoglobin released from antibody-coated sheep erythrocytes (CH₅₀ test) is frequently selected because of its simplicity. Briefly, the lower the CH₅₀, the higher the activation of the complement system. The CH₅₀ test has been extensively used to compare NS-CA [32].

When the units are mgI mL⁻¹ (i.e. in clinically-relevant conditions), it appears that all BPA tested do not interfere with the complement system in the tested concentration range (Table 6). As regards the effects of NS-CA on this model, data from the literature are rather conflicting [5, 32].

Table 6. Interaction of P743, P749 and P840 with the complement system (CH₅₀ test)

	Iohexol	P743	P749	P840
CH ₅₀ (mgI mL ⁻¹)	16	> 27	> 12.2	> 27
Ratio to CH ₅₀ for iohexol	–	> 1.7	> 0.8	> 1.7

6.3

Effects of P743 on Mast Cell Degranulation

Mast cells are large cells located immediately outside capillaries in many organs, including the lungs. As basophils, mast cells can release histamine or leukotriene B₄, a potent chemotactic agent.

The histamine- and leukotriene B₄-releasing potential of the RCBPA P743 has been investigated *in vitro* on the RBL-2H3 mast cell line [25]. RBL-2H3 cells (with permission of Dr RP Siraganian, National Institutes of Health, Bethesda, MD, USA) were replated on Eagle's minimum essential medium and incubated for 30 minutes at 37°C in 5% CO₂ with the test-solutions. Calcium ionophore A23187 was used as a positive control. The viability of mast cells was determined by means of a quantitative colorimetric assay that is based on the ability of viable cells to cleave the reagent MTT [33].

Table 7. Effects of P743 and iohexol on histamine and leukotriene B₄ release by isolated RBL-2H3 mast cells and cellular viability (MTT test)

	Histamine (nmol L ⁻¹)	Leukotriene B ₄ (pg mL ⁻¹)	MTT (% of control)
No Test Solution	38	0	–
Ionophore A23187 (1 mg L ⁻¹)	480	5881	101
P743 33 mgI mL ⁻¹	66	< 38	73
P743 65 mgI mL ⁻¹	56	< 38	87
Iohexol 30 mgI mL ⁻¹	49	< 7.5	106
Iohexol 75 mgI mL ⁻¹	69	17	89
Iohexol 150 mgI mL ⁻¹	101	41	76

Histamine release was found to be low with respect to P743. Furthermore, the pro-inflammatory potential of this compound was also found to be weak, as shown by the low amplitude of leukotriene B₄ release (Table 7).

6.4

Effect on Isolated Fibroblasts

The viability of mouse L-929 fibroblasts in the presence of P743 or P840 was tested by the MTT test.

The effects of the RCBPA P743 and the SCBPA P840 on *in vitro* fibroblast viability were similar to those of iohexol (Table 8).

Table 8. Inhibitory concentration 50 % of P743, P840 and iohexol on mouse L-929 fibroblasts (MTT test)

	Iohexol	P743	P840
IC ₅₀ (mgI mL ⁻¹)	51	46	47

6.5 Effects on Renal Function

NS-CA are classically regarded as being nephrotoxic in patients with preexisting chronic renal failure [34].

BPA were tested on a variety of experimental models in order to predict their renal tolerance in clinical conditions.

P743 was tested on a validated multiple-insult rat model [35] involving transient renal ischemia, dehydration, uninephrectomy and selective injection of a high dose (about 1.8 gI kg⁻¹) into the single remaining kidney and was found to have only minimal effects on GFR (evaluated by the endogenous creatinine clearance) and induced moderate enzymuria (NAG release into the final urine) or proteinuria. The effects observed were lower than those of the HOCA diatrizoate [25].

In another study, the effects of intravenously-injected P840 (600 mgI kg⁻¹) and iodixanol (900 mgI kg⁻¹) on GFR (evaluated by inulin clearance) were evaluated in anesthetized euvolemic pigs (n = 10).

Table 9. Effects of P840 and iodixanol on renal function in euvolemic pigs

	GFR (mL min ⁻¹ kg ⁻¹) at (min)				
	0	5	30	60	90
P840 (600 mgI kg ⁻¹)	1.14 ± 0.47	1.22 ± 0.47	1.09 ± 0.28	1.22 ± 0.38	0.87 ± 0.27
Iodixanol (900 mgI kg ⁻¹)	1.47 ± 0.12	1.41 ± 0.32	1.54 ± 0.30	1.08 ± 0.09	1.06 ± 0.19

Neither P840 nor iodixanol had major deleterious consequences on the GFR up to 90 min following injection (Table 9). The selected doses were different for P840 and iodixanol since the expected clinical dose of the SCBPA is lower than that of a classic NS-CA. Since acute renal failure is defined as a rapid and sustained decrease in renal function and since renal function is best evaluated by measurement of the GFR [36], such a parameter appears to be of particular clinical interest.

In the same study, neither the renal blood flow nor the renal vascular resistances were found to be affected by P840 (data not shown).

7 Discussion

Among the particulate systems synthesized as BPA, liposomal formulations present non-encapsulated CA levels of 40–60 % [17] which may potentially limit their value for perfusion (at first-pass) and permeability imaging. Furthermore, these agents are easily opsonized (at least for size > 200 nm) [6], i.e. their phagocytosis by neutrophils or macrophages is triggered by the association of antibodies and the C3 product of the complement cascade. This phenomenon is important in organs where the macrophage population (i.e. RES) is both large and active such as in the liver and the spleen. This specific behaviour has led these particulate agents to be tested as RES-markers in CT imaging [37, 38]. Unfortunately, liposomes incorporating NS-CA may be associated with tolerance problems, notably in humans [6, 39]. Several approaches have been selected to reduce the extent of opsonisation and thus to prolong the circulating time of liposomes, e.g. by coating them with PVP [40].

Unlike particulate systems, iodinated polymers are well tolerated [41] and are not trapped by liver or spleen RES [42]. However, their MW polydispersity (and thus unusual glomerular sieving) and conformational flexibility may explain their complex pharmacokinetic profile [41].

Macromolecular and monodisperse iodinated molecules such as those presented in the present article may be a promising alternative. Indeed, they allow either RCBPA or SCBPA pharmacokinetic profiles to be attained and their safety appears satisfactory.

The possibility of designing specific pharmacokinetic profile is a major advantage in that it allows the targeting of distinct imaging indications and provides a further tool for improving safety.

Preliminary imaging studies (CT scan vascular imaging) were carried out in rabbits with P743 [25]. While the first-pass profiles of this compound and that of the control NS-CA were similar, enhancement was two times higher after recirculation in the case of P743 (both CA being injected at the same dose, 300 mgI kg⁻¹ in the latter case) [25].

Functional imaging (and particularly that of permeability abnormalities) might be the better indication for BPA in CT. Distinction of benign versus malignant lesions and access to prognostic information are major challenges. The development of metastases may depend, in part, upon a subtle balance between angiogenesis inhibitors and activators generated from the primary tumour [43]. The great technological progress associated with CT, its high spatial resolution and the continuous improvement in the knowledge of the tumoural angiogenesis are strong arguments for the continuation of active research in this field.

8 Conclusion

We have described various types of monodisperse BPA. This innovative structural approach allows different pharmacokinetic profiles to be achieved and possibly to target several specific indications. Overall, their safety profile

appears to be satisfactory. Nevertheless, it seems that moving to the industrialization step will necessitate further development studies. Given the fast-growing advances in CT technology, future functional imaging, notably in oncology, may appear to be easier using these molecules than classic NS-CA.

9

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Liver-Specific X-Ray Contrast Agents

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Although the search for tissue-specific, in particular liver-specific, X-ray contrast agents has been the focus of extensive research efforts, no product is currently widely available. However, in recent years several approaches have reached the stage of clinical tests nourishing considerable hope that in the near future liver-specific agents will successfully be developed. The present chapter reviews the different classes of contrast agents, both the successful ones and the failures, and presents the status of achievements in this field of research and development. Included are hepatocyte-directed and Kupffer cell-targeted substances, iodine-containing and iodine-free compounds, water-soluble and particulate agents.

Keywords. Contrast agents, Triiodobenzenes, Liposomes, Metal chelates, Hepatocytes, Kupffer cells

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1

Introduction

Abdominal imaging with particular emphasis on the detection and characterization of liver lesions is one of the domains of computed tomography (CT), particularly since the advent of spiral CT. In CT, the normal liver parenchyma is characterized by a homogeneous density of 50–70 Hounsfield Units (HU), whereas for example metastases exhibit 10–20 HU lower densities. This difference in attenuation of normal and tumorous tissue is the basis for the detection of hepatic lesions. However, not all lesions show densities different from normal tissue so that a significant need exists for enhancing the density difference between normal and tumorous tissue. With an intravenous bolus injection dose of 100–150 mL of a currently available extracellular iodinated contrast agent with an iodine concentration of 250–400 mg mL⁻¹, for example with the monomeric compound iopromide or the dimer iodixanol, this can efficiently be achieved. However, due to the pharmacokinetics of these contrast agents, which are characterized by rapid distribution and extravasation into the extracellular space volume followed by fast renal excretion and also by a lack of membrane penetration, these drugs are not taken up by the liver parenchyma. Imaging of this organ, therefore, exclusively depends on the extracellular distribution mechanism, and the time window for imaging is limited to those parts of the distribution period in which the iodine concentration at the region of interest, i.e. the liver parenchyma, is high enough to allow for a significant difference in density of normal and hypo- or hyperperfused lesions. For the liver, this time window is in the range of 20–30 seconds for the arterial phase and similarly short for the portal venous phase which follows the arterial phase. Before and after these two particular periods, the image quality, i.e. the density difference between normal and tumour tissue, might not be sufficient for lesion detection.

In principle, with the presently available fast CT scanners and the availability of devices that are able to trigger the start of imaging as soon as the contrast agent bolus reaches the liver, this short time period does not pose too big a problem to the radiologist. However, in cases where a longer lasting delineation of lesions would be required, the extracellular agents meet their limits. Such cases are for example the CT-guided placing of biopsy needles in liver lesions or any tumour ablation procedures, from probe placing to real-time observation of the ablation process. With extracellular contrast agents, the injection of a new dose would be necessary. Since, however, these substances are not yet completely devoid of any side-effects, cumulative dosing is not recommendable.

Due to these particular kinetic limitations of the extracellular contrast agents, the search for liver-specific agents which should remain for a longer time in the liver in order to allow for a much larger imaging window has been extensively pursued for decades. In other imaging modalities, such as magnetic resonance imaging (MRI), tissue-specific contrast agents for the liver are already available. These comprise two different types of substance which can be characterized

according to their liver uptake mechanisms. Hepatocyte-specific compounds such as the metal chelates, mangafodipir (TeslaScan®; Nycomed, Oslo, Norway) or Gd-EOB-DTPA (Eovist®; Schering AG, Berlin, Germany) [1–3] are representatives of one group, and particle-derived compounds which are taken up by the Kupffer cells of the liver such as superparamagnetic oxide particles represent the second one [4]. One example of this latter type is ferucarbotran (Resovist®, Schering AG, Berlin, Germany).

These different approaches in the search for MRI liver contrast agents have also been tried for X-ray imaging. In the following chapters, the literature will be reviewed for compounds with potential as X-ray liver contrast agents.

2

RES-Specific Liver Contrast Agents

The use of the reticulo-endothelial system (RES) was the first approach to liver contrast agents. As an adjunct, spleen imaging would also be possible with a contrast agent that is taken up by the RES. The Kupffer cells of the liver, which represent 10 % of all hepatic cells, constitute the major portion (80–90 %) of all fixed macrophages and they are extremely effective in the phagocytosis of all types of particles. The downside of the use of this mechanism, however, is the concomitant release of toxic mediators that might and – as a matter of fact – often has made this approach non-feasible. Adverse events provoked by the mediators are changes in blood pressure (most often hypotension) and fever.

2.1

History of Particulate Contrast Agents

Contrast agents that are taken up by the RES usually have to be particles in order to be recognized by macrophages. The first such particle was thorium dioxide (Thorotrast), an extremely effective and – on a short-term basis – very well tolerated contrast agent [5]. Thorotrast was injected as a 25 % colloidal suspension and was in use from 1929 until the late 1950s. The agent is avidly taken up by the RES and is thus found in the liver, spleen, lymph nodes and bone marrow. Unfortunately, it is retained for many years after administration. Thorotrast is no longer used because thorium is an alpha emitter and thorium dioxide has an (estimated) biological half-life of 400 years. These characteristics make Thorotrast carcinogenic and it can cause angiosarcomas of the spleen and liver, leukemia and aplastic anemia [6].

The principle of using particulate contrast agents, however, survived the decision to no longer use thorium dioxide. The objective of research efforts focused on finding contrast agents with better tolerability. The efficacy of thorium dioxide was never questioned. Olsson investigated the use of colloidal iodine [7], Fischer that of stannic oxide [8], and Teplick used barium sulphate [9]. In subsequent years, three different approaches were followed: oily contrast emulsions that are taken up by macrophages, the synthesis of pro-drugs and encapsulation of iodinated contrast agents into liposomes.

2.2

Iodinated Lipids

Iodinated lipids have found widespread interest for many decades. The types and chemical classes varied and one of these agents (Lipiodol) is still in clinical practice for therapeutic purposes when mixed with additional cytotoxic drugs.

2.2.1

Iodized Oil

The first extensively tested RES-specific contrast agent on the basis of iodinated lipids was EOE-13 with its precursors, AG 52–315 and AG 60–99. EOE-13 is an aqueous emulsion of the iodinated ester of poppy seed oil and is able to selectively enhance the normal liver and spleen parenchyma [10–12]. However, severe adverse events such as headache, fever and chills prevented further use of these contrast agents [13–15], although their extent could be decreased by hydrocortisone given prior to contrast injection.

A later preparation of iodized oil was Lipiodol (Laboratoires Guerbet, France) or Ethiodol (Savage Laboratories, Melville, NJ, USA), which is a mixture of iodinated fatty acid esters of poppy seed oil with the main components monoiodostearate and diiodostearate. Lipiodol is a clean, bright, pale yellow fluid presented in 10 mL ampoules. It has been widely used in lymphangiography and hysterosalpingography. When injected into the hepatic artery of patients with hepatocellular carcinoma (HCC), iodized oil selectively accumulated in the tumour [16]. This observation led to relatively wide-spread use of iodized oil as a diagnostic agent for the detection of HCC, particularly in East Asia. Additionally, it was used as a carrier to transport therapeutic drugs into the HCC lesions [17]. Both approaches, the use of iodized oil for diagnostic and for therapeutic purposes, however, have encountered and continue to encounter significant opposition [18]. One of the main reasons was that its mechanism of action, including the mechanism of transport, is still not fully understood and – particularly for diagnostic purposes – its side-effect profile is not acceptable. Iodized oil has an extremely long residence time (several weeks or even months) in the liver. It is washed from the arteries into the veins and remains there until finally cleared following uptake into Kupffer cells [19]. A lot of effort has been invested in the optimization of the use of iodized oil, such as changing the dosage, modifying the oily preparation and even using different routes and modes of administration. However, despite all these efforts, in the Western hemisphere, iodized oil never gained the same importance as in East Asia.

2.3

Triiodobenzene Derivatives

2.3.1

Pro-Drug Approach

The underlying principle of the pro-drug approach consists in chemically modifying water-soluble contrast agents such that the new compounds exhibit

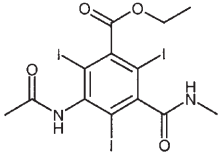
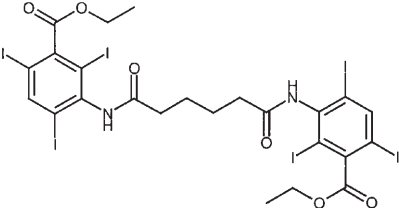
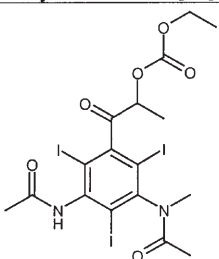
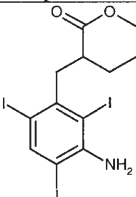
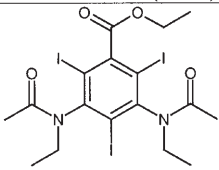
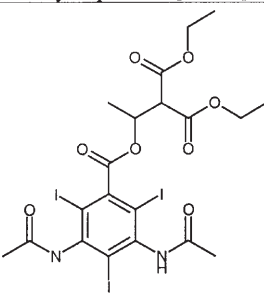
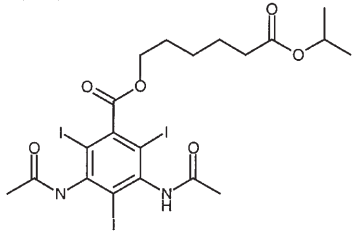
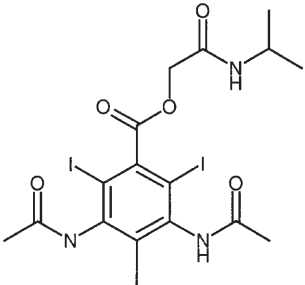
	
Ethyl iothalamate [20]	Ethyl iodipamide (IDE) [21]
	
Metrizic acid ester (IEEC) [25]	Ethyl iopanoate [31, 32]
	
Ethyl diatrizoate [26, 27]	Diatrizoic acid ester #1 [26, 27]
	
Diatrizoic acid ester #2 [26, 27]	Diatrizoic acid ester #3 [26, 27]

Fig. 1. Triiodobenzene derivatives with low water solubility evaluated as RES-specific X-ray contrast agents. Figures in brackets indicate the corresponding reference

decreased water solubility and are – as any particles – taken up by macrophages. Subsequently, these substances are degraded to either provide the original water-soluble contrast agents or other highly water-soluble breakdown products that are excreted by the kidneys. Examples for this pro-drug approach are esters of water-soluble compounds (Fig. 1) such as iothalamate ethyl ester and iodipamide ethyl ester, IDE [20, 21].

Following intravenous injection of IDE into rats, the contrast agent is actively accumulated in the liver and spleen for more than 2 hours and is then slowly cleared from the organism in approximately two days [22]. Lauteala et al. showed that the particles were phagocytized by polymorphonuclear neutrophils (PMNs) of rat lungs but did not cause any emboli or any damage due to aggregate formation in the capillaries [23]. In dogs, an intravenous dose of 75 mgI kg^{-1} IDE caused an increase of 40–60 HU in liver attenuation, which persisted from 5 minutes to ten hours post infusion. No enhancement of tissues other than liver and spleen was observed. IDE was completely eliminated from the liver within seven days [24].

Leander et al. [25] evaluated a pro-drug of metrizoic acid, IEEC (Fig. 1), as a potential contrast agent for CT scanning of the liver in tumour-bearing rabbits in comparison to the triiodinated extracellular agent, iohexol, at doses of 100 mgI kg^{-1} (IEEC) and 570 mgI kg^{-1} (iohexol), respectively. In the images obtained with the particulate contrast agent, the attenuation in the normal liver parenchyma and the contrast between normal liver and lesion was significantly higher compared with the images obtained with iohexol.

Liversidge et al. [26, 27] prepared more than 50 compounds with low water solubility on the pro-drug principle starting from extracellular triiodinated X-ray contrast agents such as diatrizoic acid, iothalamic acid, urokonic acid or metrizoic acid. Some of these compounds (cf. Fig. 1) were subsequently tested by Gazelle et al. [28–30]. The formulations were aseptically prepared adding a non-ionic polymer for colloidal stabilization, milled to uniform size and surfactants and stabilizers were also added. Particle size was less than 400 nm, generally below 200 nm. The iodine concentration was 89 mg mL^{-1} . The viscosity was low at 1.5–2.2 mPa s. CT experiments in rabbits at doses of 1.5 or 3 mL kg^{-1} showed a high variability of biodistribution depending on the type of formulation. The preparations were either rapidly taken up by the liver and spleen with high CT density in these organs or circulated for a long time in the blood achieving low attenuations of the liver and spleen. Maximum enhancements of these compartments were in the range of 260–280 HU (blood, liver) and 900 HU for the spleen.

The CT behaviour of three alkyl esters or amides of diatrizoic acid (Fig. 1) in rabbits after i.v. injection of 3 mL kg^{-1} (89 mgI mL^{-1}) was reported by Gazelle in 1994 [28]. The particles had sizes of 200–300 nm. The ester compound with the short alkyl chain circulated for a long time in the blood, and the ester with the longer chain was rapidly taken up by the liver achieving CT densities in this organ of more than 225 HU. The amide showed a blood-pool behaviour similar to that of the short-chain ester. The ethyl ester of diatrizoic acid (EEDA), formulated as a nanoparticulate contrast agent with a diameter of 200 nm, was positively tested in a rabbit model with liver abscesses [30]. EEDA proved to be superior to iohexol in detecting these abscesses.

Ethyl esters of diatrizoate and iopanoic acid were synthesized by Yang et al. [31, 32] and encapsulated into poly(D,L-lactide) by dissolving the components in methylene chloride followed by emulsification with 1 % polyvinyl alcohol. After heating at 50°C for 4 h, the organic solvent was evaporated and the remaining suspension was filtered through a 5- μm filter. The particle size obtained by this process was 1 μm , the iodine concentration was 50 mg mL⁻¹. Biodistribution studies in rats showed significant uptake into liver and spleen. In CT studies in rabbits, a prolonged enhancement of these organs was observed. Instead of the abovementioned ethyl esters of triiodobenzene derivatives, alternatively, ethiodol could be encapsulated into polyvinyl alcohol resulting in a similarly efficient liver contrast agent. Studies in humans with these agents have not been reported.

Ethyl diatrizoate-based nanoparticles were also studied by Rubin et al. [33], who applied a special grinding procedure and the concomitant use of surfactants to achieve particle sizes of 200 – 400 nm. By varying the type of surfactant, the characteristics of the nanoparticles could be modified. Imaging studies in rabbits showed that the selected surfactant had a significant effect on the efficacy, i.e. on the distribution behaviour, which ranged from a long-circulating blood-pool type to an agent that was rapidly taken up by the liver.

The cyclic carbonate of the triiodinated extracellular contrast agent, ioxilan (Fig. 2), was studied by Li [34, 35]. The compound was synthesized by adding carbonyldiimidazole to ioxilan using catalytic amounts of sodium methoxide. The particle preparation was produced by a solvent extraction/evaporation method in acetone/methylene chloride and final filtration through a 5- μm filter. The average diameter of the particles was 1.1 μm and the iodine content was 45 %. Aggregation of the particles was not observed in aqueous solution or in rat plasma. Degradation of the particles was possible under basic conditions. In plasma, enzymatic degradation took effect. The acute toxicity in mice as measured by LD₅₀ was approx. 1 gI kg⁻¹, whereas for ioxilan itself, the LD₅₀ is in the range of 15 gI kg⁻¹. Following intravenous injection in rats at doses of 100, 200 and 270 mgI kg⁻¹, maximal liver enhancements of 23, 38 and 110 HU were observed. The attenuation enhancement persisted for one hour and started to decrease two hours after injection.

The biodistribution of particles with different sizes of 290 nm (“nanoparticles”) and 1.7 μm (“microparticles”) was investigated in rats by Li et al. [36] following i.v. injection at doses of 50 mgI kg⁻¹. Both types of particles were taken

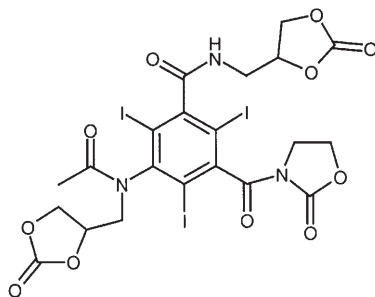


Fig. 2. Structure of ioxilan carbonate [34, 35]

up in the liver and spleen. In the blood, there was no significant difference between the biodistribution of the two particle types, whereas the smaller ones achieved a significantly higher liver concentration than the larger ones. The spleen concentration, on the other hand, was significantly lower.

2.3.2

Polymeric Contrast Agents

Torchilin et al. synthesized an iodine-containing amphiphilic block-copolymer consisting of iodine-substituted poly-L-lysine which is able to form micelles in aqueous solution [37]. The two components of the block-copolymer were methoxy-poly(ethylene glycol) propionic acid (MPEG-PA) with a molecular weight of 12 kDa and poly[ϵ , *N*-(2,3,5-triiodobenzoyl)]-L-lysine. The particle size of the micelles was approx. 80 nm, and the iodine concentration was 20 mg mL⁻¹. Biodistribution studies in rats showed significant and prolonged enhancement of the aorta, the liver and spleen.

2.2.3

Liposomes with Iodinated Contrast Agents

Liposomes, as carriers for diagnostic or therapeutic drugs, have been the focus of extensive studies over the past decades. Liposomes are vesicular particles that are composed of a membrane formed out of lipids with a polar head group and one or preferentially two long non-polar side-chains (Figs. 3, 4). Typical lipids



Fig. 3. Bilayer of a liposomal membrane composed of hydrophilic headgroups – represented as circles – with two long alkyl chains

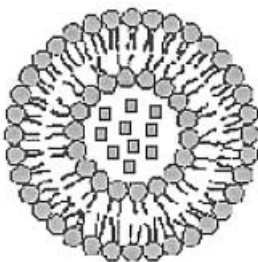


Fig. 4. Schematic representation of a unilamellar liposome with encapsulated contrast agent (squares)

are soy phosphatidylcholine (PC), distearoyl phosphatidylcholine (DSPC), or dipalmitoyl phosphatidylcholine (DPPC). For stabilization, cholesterol might be added. Negative charge can be introduced into the liposome membrane by adding stearic acid or negatively charged glycerides such as dimyristoyl phosphatidylglycerol (DMPG) or dipalmytoyl phosphatidylglycerol (DPPG). There are four different types of liposomes available (Fig. 5). These are small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MLV) and multivesicular vesicles (MVV). SUVs and LUVs consist of one single membrane. They are only differentiated by diameter with SUVs covering the range 20–50 nm. MLVs have an onion-like structure with more than one membrane. MVVs are liposomes within a liposome, i.e. one large vesicle has swallowed several smaller ones.

Liposomes are useful as carrier systems for therapeutic or diagnostic drugs. Depending on the chemical structure of the loaded compound, it can either be incorporated into the membrane layer as is observed for lipophilic drugs or stored in the aqueous compartment at the centre of the vesicle (hydrophilic substances).

Liposome formulation is affected by a number of factors including the type of lipids used (charged vs. uncharged, surface-modified vs. unmodified), the chemical nature of the active agent, and the intended size. Several methods of preparation have been described in the literature such as thin film hydration, sonication, extrusion, ethanol injection, detergent dialysis or reverse phase evaporation. Out of these methods, extrusion through two or more polycarbonate filters of defined pore size under pressure might be best suited. This procedure produces well-defined liposomes with uniform particle size distribution. The vesicle properties of the liposomes can further be improved by subjecting the liposomes to freeze-thaw cycles before extrusion.

Important criteria for the evaluation of liposomal quality are drug-to-lipid ratio, encapsulation efficiency, size, and lamellarity. The higher the drug-to-lipid ratio, the higher is the payload of the carrier and the lower the systemic burden

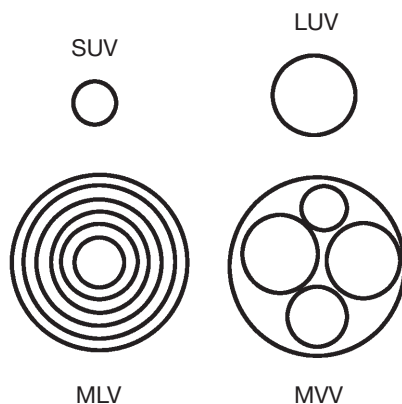


Fig. 5. Types of liposomes: SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; MVV, multivesicular vesicle

by the lipids. Accordingly, highest payloads would be expected for LUVs. However, as is easily anticipated, this type of structure is less stable than SUVs, MLVs or MVVs with their downside of lower payloads.

The incorporation of iodinated contrast agents, particularly triiodinated substances used for angiography and urography, has been studied extensively. Compounds like iopamidol or iopromide pose a number of problems that have to be overcome. These are their extremely high hydrophilicity, their need for very high concentrations to be effective and, as a consequence, the high osmotic pressure of their solutions. The last of these problems can be overcome by not eliminating unencapsulated contrast agent from the preparation so that the osmotic pressure inside and outside the liposome is of similar magnitude. The downside of this approach is the presence of free drug with its different pharmacokinetics compared to the liposomally entrapped substance.

Practically all available iodinated extracellular X-ray contrast agents have been encapsulated into liposomes using different lipids and methods of preparation. Table 1 gives a short and intentionally incomplete overview of some of the approaches. The first liposomal contrast agent preparation that was tested in humans contained diatrizoate [48]. The injected dose was up to 0.5 mL kg⁻¹. The preparation was effective even in plain radiography where lesions down to 0.8–1.0 cm could be detected in patients. However, adverse events such as fever and hyperthermia, which occurred in 30 % of the patients, limited further use. We have incorporated iopromide into MLVs that were prepared from phosphatidyl choline (PC), cholesterol and stearic acid at a molar ratio of 4:5:1 using the ethanol-evaporation technique [44]. The liposomes can be stored freeze-dried and they are reconstituted before use by

Table 1. Selected liposome preparations with encapsulated iodinated contrast agents

Type	Contrast Agent	Size (μm)	Encapsulation (%)	Iodine/Lipid Ratio	Reference
SUV	Metrizamide	0.1		0.1	38
SUV	Diatrizoate		8.5	0.7	39
	Iohexol	0.6	6.2	0.5	
	Iotrolan		6.7	0.5	
LUV	Diatrizoate			1	40
LUV	Diatrizoate	0.2–0.5		1	41
MLV	Iosefamate				42
MLV	Metrizamide	0.5		0.1	43
MLV	Diatrizoate	3	28		44
	Iopamidol	4	38		
MLV	Iopromide	0.5	30–40	1	44
MLV	Diatrizoate				45
	Iotrolan				
Various	Various				46
Various	Various				47

adding a 135 mM mannitol solution. After resuspension, their mean size was 0.5 μm and the osmolality was 600 mosm kg^{-1} . This preparation was well tolerated in animals with an LD_{50} in rats of approx. 3 gI kg^{-1} and the imaging efficacy was good in rats, rabbits, dogs and monkeys [49–59]. The tolerability was, however, not sufficient for human use. In a phase I trial, adverse events were observed, which resulted in discontinuation of the study. Later, these adverse events could be attenuated by modifying (reducing) the size and type of the liposomes and the type of buffer that was used for the preparation of the lipid suspension. A further reduction was observed by pre-medication with acetylsalicylic acid [60].

Leander et al. described ready-to-use liposomes that were effective and well tolerated in humans [61]. The iodinated contrast agent was the non-ionic dimer, iodixanol. The particle size of the liposomes was 350 nm with an iodine concentration of 70–80 mg mL^{-1} encapsulated. They injected doses of 30, 70 or 100 mgI kg^{-1} intravenously and found maximal enhancement values of 45 HU in the liver and 240 HU in the spleen. The uptake into these organs was not linearly correlated with the injected dose. No serious or unexpected adverse reactions were reported. Dose-dependent changes in leukocyte counts and body temperature were seen.

A series of iomeprol-containing liposomes were evaluated in animals by Petersein et al. [62] and in healthy volunteers by Spinazzi et al. [63, 64]. BR2 and BR21 are liposomes made of phosphatidyl choline (PC), dipalmitoyl phosphatidic acid (DPPA) and cholesterol at a molar ratio of 2:1 (PC + DPPA/cholesterol) with an iodine content of 260 mg mL^{-1} (BR2) and 320 mg mL^{-1} (BR21), respectively, and a size of 0.4 μm . BR2 contains 40 mg lipid mL^{-1} and BR21 20 mg mL^{-1} . In rabbits, BR2 tended to provide a higher and more persistent CT enhancement than BR21.

After intravenous injection of BR21 at doses of 0.5–2.5 mL kg^{-1} into healthy volunteers, no serious side-effects occurred. All other adverse events were minor and transient. Vital signs, ECG and clinical laboratory parameters were not affected. The authors conclude that BR21 appears to be safe and well tolerated in healthy volunteers.

2.4

Bromo Compounds

Perfluorooctylbromide emulsions (PFOB) have been described as a bromine-based particulate contrast agent that is taken up selectively by the liver and spleen after i.v. injection [65]. It might also be used for ultrasound and magnetic resonance imaging [66]. Behan et al. [67] investigated the safety and dose response of PFOB in cancer patients with hepatic metastases. They injected doses of 0.5–3.0 mL kg^{-1} of the emulsion and found a persistent increase in the density of blood, liver, and spleen with maximal enhancements of 55, 39, and 317 HU, whereas lesions enhanced only minimally (7 HU or less). At higher doses, peak enhancement of the liver and spleen was delayed to 24 h. However, adverse effects occurred in 14 of 18 patients including lower back pain in six, delayed fever in eight, and malaise in three.

3

Hepatocyte-Specific Liver Contrast Agents

Hepatocytes are the dominant cell type in the liver constituting approx. 70 % of all liver cells. They are responsible for the uptake of substances from the blood and for the excretion by the biliary system. Specific requirements have to be met by compounds to be able to enter the hepatocytes. These are definite ranges of molecular weight, lipophilicity and polarity or charge. Additionally, receptor-mediated transport systems of the hepatocytes pose further requirements on the structure of the substance.

Three different approaches have been evaluated in the search for hepatocyte-specific CT contrast agents. These are, first, the modification of biliary contrast agents which are taken up by the hepatocytes of the liver and which are excreted into the bile. The disadvantage of this class of compounds is that the steady-state concentration in the liver is very low and not sufficient for imaging. In CT, a target concentration of iodine of 1 mg per g of tissue results in a difference in density that is at the limit of detection (30 HU). Concentrations in the liver of conventional biliary contrast agents are far from this threshold. Significant efforts therefore have been directed to the synthesis of new biliary contrast agents that achieve higher steady-state levels in the liver. The second approach is the modification of parenteral nutritional preparations by the introduction of iodine into some of the carrier molecules. And the third route of research is to use metal chelates, which had been synthesized as contrast agents for MRI also for CT purposes with the lanthanide ion replacing the iodine of presently available CT contrast agents. All three approaches will be covered in this review.

3.1

Iodine-Containing Compounds

In this section the first two approaches, modification of biliary contrast agents and iodination of nutritional lipids will be described.

3.1.1

Iodobenzene Derivatives

3.1.1.1

Diiodobenzene Derivatives

Tetraiodophenolphthalein probably constitutes one of the oldest contrast agents tested for biliary imaging (Fig. 6). As early as 1909 it was discovered that this drug is excreted by the liver [68] and in 1923 it was found that it was concentrated in the biliary bladder [69]. Other compounds of this class include erythrosin and Rose Bengal. However, except for Rose Bengal labelled with ^{131}I iodine and used for liver function testing, these compounds never gained major importance in clinical use. A further diiodo derivative is choloselectan (Fig. 6).

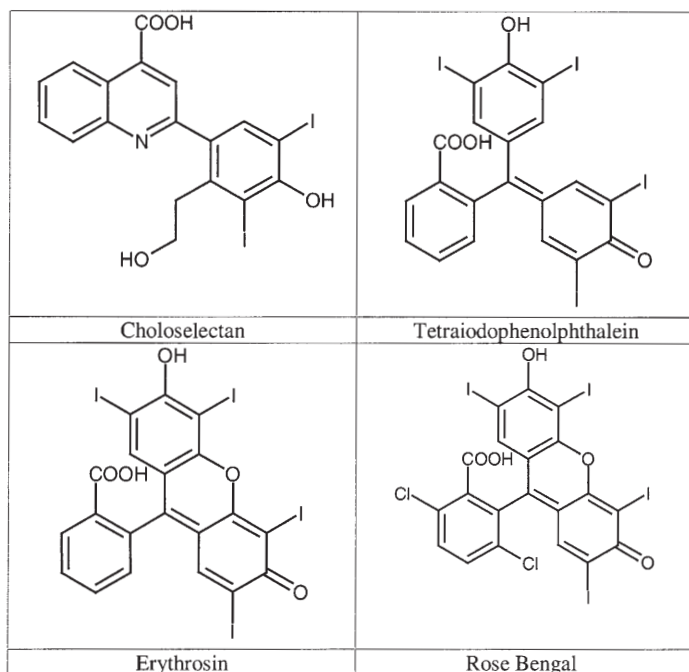


Fig. 6. Structures of diiodobenzene derivatives as described in [59]

3.1.1.2

Triiodobenzene Derivatives

Hepatocyte-specific liver contrast agents derived from the highly substituted triiodobenzene moieties used for angiography and urography, such as iohexol or iodixanol, are produced by reducing the extraordinarily high hydrophilicity of these extracellular compounds to such an extent that they are now able to pass membranes. This can be achieved by different measures, e.g. by omitting one of the hydrophilic substituents on the benzene ring and/or by reducing the hydrophilicity of the substituents by eliminating hydroxyl groups. Another prerequisite is the introduction of one or two negative charges. Following these modifications, the pharmacokinetics of the triiodinated compounds is now changed from extracellular distribution to uptake by the hepatocytes followed by biliary excretion or – after re-secretion into the blood – followed by renal elimination.

Figure 7 gives a comparison of typical examples of monomeric and dimeric extracellular and hepatocyte-specific triiodobenzene-derived contrast agents.

Iopromide, a currently used contrast agent for urography, angiography and computed tomography is characterized by a total of four hydroxyl groups. Other extracellular agents contain up to six hydroxyl groups, for example iohexol, ioversol or iobitridol. They are all non-ionic. Their partition coefficient in the system *n*-butanol/water is in the range of 0.149 (iopromide) to 0.082 (iohexol).

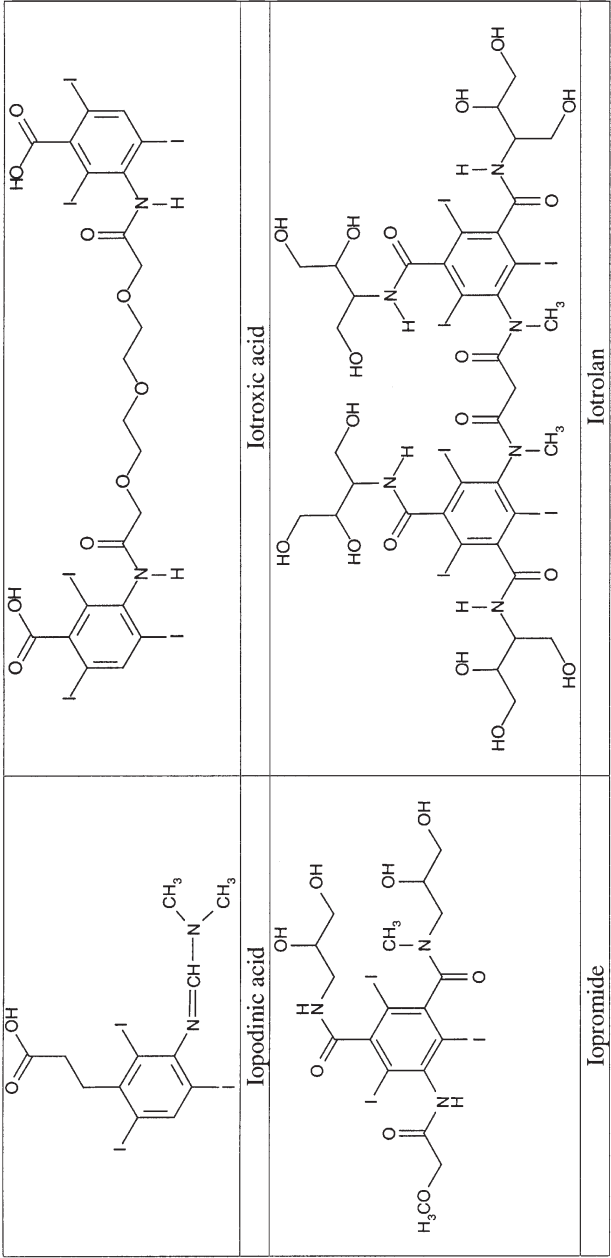


Fig. 7. Comparison of the two different types of extracellular and biliary contrast agents. One monomeric and one dimeric example are illustrated from each type. Iopromide and iotrolan (extracellular type) are extremely hydrophilic whereas iopodinic acid and iotroxic acid (biliary type) are both ionic and very lipophilic

Iopodinic acid, a monomeric biliary contrast agent, does not contain hydroxyl groups any more. Moreover, position 4 of the benzene ring is unsubstituted and the substituent in position 2 is highly lipophilic.

Iotrolan, a dimeric urographic contrast agents has 12 hydroxyl groups and its partition coefficient in the system *n*-butanol/water is 0.005. Iotroxic acid, a dimeric biliary contrast agent, is without any hydroxyl groups and, again, positions 4 and 4' of the triiodobenzene rings are unsubstituted.

Figures 8 and 9 summarize other structural types of monomeric and dimeric hepatospecific X-ray contrast agents. All monomeric compounds are characterized by a free 4-position, by one negative charge in the form of a carboxyl group and by substituents lacking hydrophilic moieties such as hydroxyl groups. Two of the dimeric substances also have free 4-positions, one, iosefamate, has a

Phenobutiodil	Iophenoxic acid	Iopanoic acid
Cinamioidil	Iobenzamic acid	Iocetamic acid
Tyropanoate	Bunamiodyl	Ipodate

Fig. 8. Monomeric biliary contrast agents

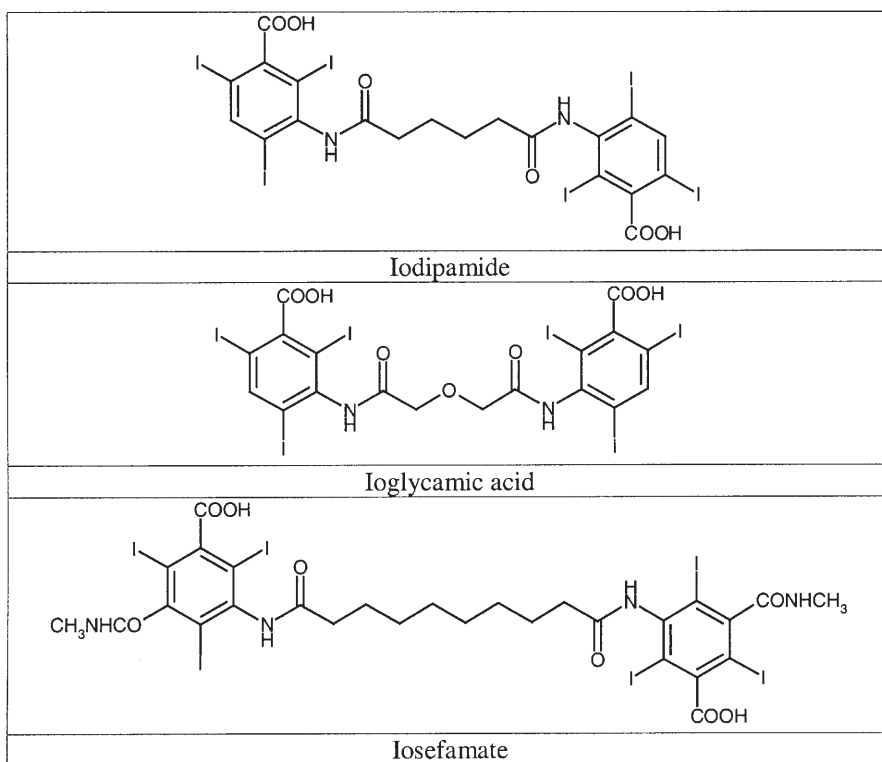


Fig. 9. Dimeric biliary contrast agents

methylamide group in position 4. All dimeric contrast agents have two negative charges, i.e. two carboxyl groups, one on each ring. Due to the low hydrophilicity of these substances, the iodine concentrations of the preparations are much lower than those of the angiographic agents. For the latter the upper limit is 400 mgI mL^{-1} which is equivalent to approx. 900 mg of substance per mL. The concentrations of biliary contrast agents are typically 50 mgI mL^{-1} (iopodinic acid). The corresponding viscosity at 20°C is 1.3 mPa s and the osmolality is 290 mosm kg^{-1} . The lower hydrophilicity leads to increased protein binding of these compounds. For example, iodipamide is bound to plasma albumin at 85%, whereas the protein binding of urographic contrast agents such as iohexol is negligible.

Some of these triiodinated compounds are orally active, i.e. they are absorbed from the gastrointestinal tract after oral administration and imaging of the biliary system is possible following this route of administration. Examples are iopanoic acid, iophenoxic acid and sodium ipodate. A prerequisite for oral absorption is a balance of relatively hydrophilic and lipophilic moieties in the molecule. Numerous investigations have been performed to establish structure-activity relationships for this class of compounds, e.g. by Archer and Hoppe [70, 71]. Sodium salts are better absorbed than the free acids [72].

The mechanism of uptake into the hepatocytes and of the excretion into the bile is active transport. In principle, excretion into bile is possible both as the unchanged drug and in the form of metabolites, which is more often observed. Conjugation with glucuronic acid or sulfates of biliary acids is quite common and depends on the structural differences of the contrast agents. Examples include the glucuronidation of iodoaliphonic acid [73, 74] both in the ether and the ester forms, which is observed for one third of the dose, whereas two thirds are excreted in the unchanged form. Iopanoic acid is exclusively excreted as an ester glucuronide [71]. Non-conjugated (unchanged) drug can be re-absorbed after biliary excretion. This might lead to a long retention of the contrast agent in the body due to continued excretion and re-absorption.

The triiodobenzene structure of biliary contrast agents has for many decades been the lead structure for numerous efforts to synthesize liver contrast agents. However, up to now it was not possible to come up with a substance that is useful as an X-ray contrast agent for liver imaging. The main reasons are that – even for computed tomography, which is the most sensitive X-ray imaging technique presently available – very high concentrations of 1 mgI mL⁻¹ or gram of tissue are necessary in order to be able to draw meaningful diagnostic conclusions. Unfortunately, the pharmacokinetics of liver uptake and excretion into the bile are such that the concentration in the liver always remains far below 1 mgI g⁻¹ of liver tissue. The reasons might be that either the uptake is saturated early or that the elimination from the liver is much faster than the uptake so that steady state concentrations in the liver never reach the necessary high values.

Another problem is the significant inter-species difference observed for these pharmacokinetic steps. Whereas in some species, e.g. in the dog, concentrations above 1 mg g⁻¹ tissue could easily be achieved, this was not the case in others. Unfortunately, humans belong to the group with lowest liver uptake and steady-state concentration, respectively [75].

Recent efforts to synthesize triiodinated benzene derivatives that are taken up by the liver have been published by Ranganathan [76]. This group synthesized lipophilic and/or amphiphilic derivatives of the angiography contrast agent, diatrizoate (Fig. 10).

The partition coefficients of these compounds ranged from 25 (bis-methyl diatrizoate) to 10¹⁰ (derivative of #2 with a C18 chain length of the carboxylic acid). Tissue distribution studies in mice showed good liver uptake of these substances, particularly for those with intermediate chain lengths. No human data have been reported so far.

3.1.1.3

Tetraiodobenzene Derivatives

The mono-morpholide of tetraiodophthalic acid has been tested as biliary contrast agent (Fig. 11). It is easily synthesized by tetraiodination of phthalic acid using concentrated sulphuric acid and iodine. Although its diagnostic characteristics were quite satisfactory, the compound did not find clinical use due to toxicity [77].

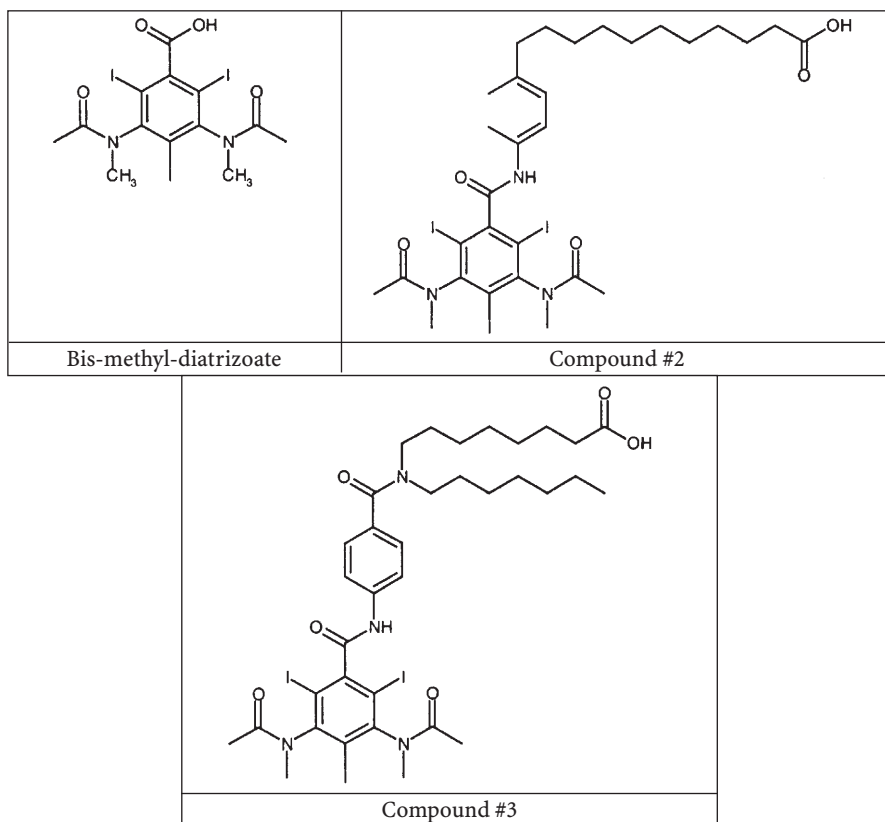


Fig. 10. Lipophilic compounds evaluated as hepatocyte-specific liver contrast agents [67]

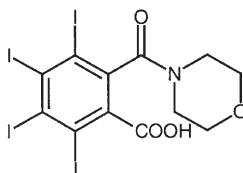


Fig. 11. Structure of tetraiodophthalic acid monomorpholide [68]

3.1.2

Iodinated Lipids

3.1.2.1

Iodinated Triglycerides

Iodinated triglycerides (ITG) were most extensively investigated by the group of Weichert et al. who synthesized a plethora of different compounds and formulated them in many different ways including different sizes. Only some of these

contrast agents will be described in more detail in this summary. Recently, the group published data on the physicochemical properties, the biodistribution and the imaging behaviour of an ITG [78, 79]. The earliest reports by this group date back to the beginning of the eighties.

The rationale for this type of contrast agent is to use the endogenous metabolic pathway of lipid metabolism in the liver for the transport of iodinated substances. Chylomicron remnants are naturally occurring lipoproteins in the blood that are responsible for the transport of lipids into the liver. Three different mechanisms for this transport are discussed: direct uptake by the low-density lipoprotein receptor; transport to the low-density lipoprotein receptor-related protein (LRP) mediated by heparan sulfate proteoglycan (HSPG) or direct HSPG-LRP uptake and direct HSPG uptake. One of the prerequisites for particles to be transported by these mechanisms is a mean diameter of less than 100–300 nm.

Weichert et al. synthesized polyiodinated triglycerides and incorporated them into the lipophilic core of a synthetic lipid emulsion (LE) that is similar to chylomicron remnants. In addition to imaging of the anatomy of the liver, the function of this organ – as determined by the transport efficiency of the ITG-LE – was an objective for this class of contrast agents. One of the last and most promising ITGs was 2-oleoylglycerol-1,3-bis[7-(3-amino-2,4,6-triiodophenyl)] heptanoate (DHOG, [80]). The structures of DHOG and a palmitoyl derivative with shorter linkers are given in Fig. 12. Formulations with iodine concentrations of 25–50 mg mL⁻¹ were prepared by dissolving the lipid components in organic solvents followed by evaporation under vacuum. After adding aqueous glycerol, the emulsion was micronized using high-pressure shear mixing. The resulting particle size of the ITG-LE was 100–200 nm. Following sterile-filtration, the preparation could be stored at room temperature.

A study on the biodistribution of ITG in rats showed a dose-dependent behaviour. At doses of 12.5 and 25 mgI kg⁻¹, large portions of the dose could be recovered in the liver and spleen, whereas at 105 mgI kg⁻¹, uptake into the liver was prolonged following an extended period of retention in the blood volume. The maximum CT liver enhancement, however, remained proportional to the injected dose. When the mean particle size was increased to 1.5 µm, the selectivity for liver uptake was lost.

Longino et al. [81] demonstrated that with a formulation composed of 2.4% dioleoyl PC with a cholesterol:DOPC molar ratio of 0.4, which was emulsified at 14,700 psi, 35°C for 10 min, a reproducible production of these ITG-LE could be established. The use of cholesterol was mandatory in generating a stable emulsion near the target size.

When the surface of the chylomicron remnants of ITG-LE was modified by pegylation, liver uptake was inhibited and the resulting contrast agent circulated for a prolonged time in the blood pool [82]. Pegylated and non-pegylated ITG-LE could also be used in combination in tumour-bearing rabbits [79] by injecting the two components only minutes apart. CT images obtained after the two ITG-LE injections were superior to those after iohexol.

In addition to DHOG, the basic structures of other ITG-derived contrast agents were also described by Weichert et al. [80]. All these compounds were

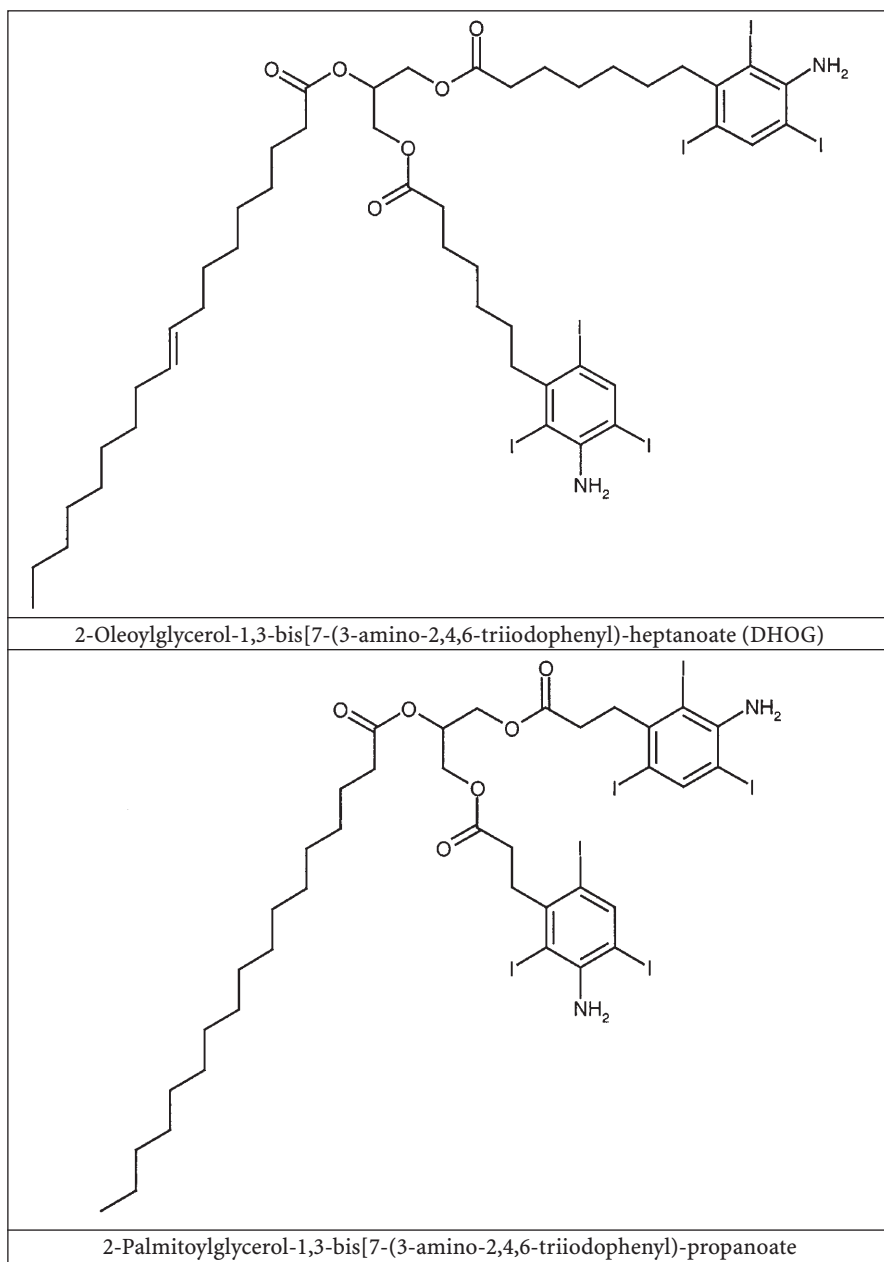


Fig. 12. Structures of iodinated triglycerides with two triiodobenzene groups [71]

glyceryl 2-oleoyl 1,3-bis[ω -(3-amino-2,4,6-triiodophenyl)]alkanoates. They showed rapid uptake into the liver with a long persistence in this organ. Interestingly, substances with longer alkyl chains were eliminated more rapidly than those with shorter chains.

Weichert also synthesized sterol esters of triiodinated biliary contrast agents [83], such as cholesteryl, pregnenolone and dehydroepandrosterone iopanoate (Fig. 13). Only the first compound accumulated in the liver. The reason probably was its resistance to hydrolysis.

Further structures evaluated by Weichert et al. for their ability to localize in the liver included derivatives of 1,3-dipalmitoylglycerol 2-[ω -(3-amino-2,4,6-triiodophenyl)]alkanoates [84]. Due to significant differences of in vivo hydrolytic stability, only some of the compounds – the stable derivatives – accumulated in the liver.

In addition to mono- and bis-triiododerivatives, trisubstituted lipids were also synthesized by Weichert et al. [85]. The compound with the highest accumulation in the liver out of this series was glyceryl 1,2,3-tris[3-(3-amino-2,4,6-triiodophenyl)]propionate (Fig. 13).

So far, none of these iodinated lipids has reached phase I clinical trials.

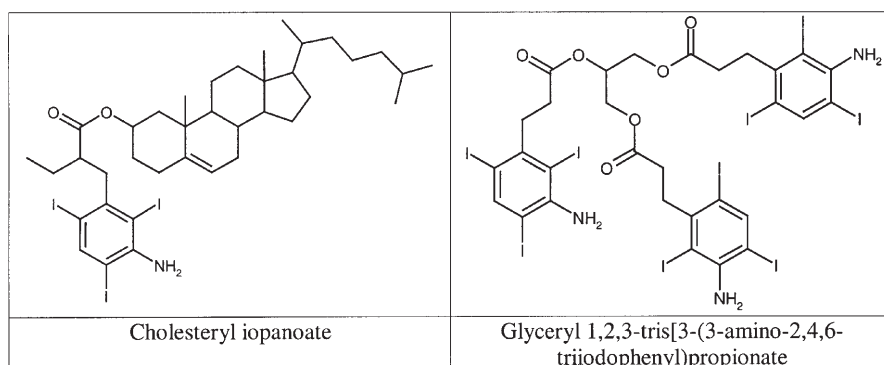


Fig. 13. Chemical structures of cholesteryl iopanoate and glyceryl 1,2,3-tris[3-(3-amino-2,4,6-triiodophenyl)]propionate [71]

3.1.2.2

Iodinated Fatty Acids

The basis for contrast agents derived from nutritional products was Intralipid, which had been prepared from phospholipids obtained from soy bean oil and egg yolk [86]. Intralipid was chemically modified by iodination into Intraiodol, which contained 50 mgI mL⁻¹. The particles formed in the emulsion were mostly taken up by the hepatocytes and to a lesser extent by the RES [87, 88]. Intraiodol was better tolerated than EOE-13 or its precursor, AG 60.99. The addition of cholesterol to Intraiodol resulted in NRI 757 with an iodine concentration of 42 mg mL⁻¹. Further modifications by replacing the iodinated triglycerides of Intraiodol by ethiodized poppy seed oil and reducing the cholesterol content to

8% resulted in FP 736–03 [89–91]. Replacing the ethiodized poppy seed oil by ethiodized linoleic acid gave FP 736–04, which is a 10% oil-in-water emulsion with an iodine content of 62 mg mL⁻¹. The concentration of cholesterol is 7%. Phenylalanine serves as a further stabilizer. The mean droplet size is 250 nm. This contrast agent was extensively studied by the group of Magnusson et al. who reported on the use of this agent in experimental animal models and humans [92, 93].

In healthy male volunteers a dose-escalating study with iodine doses of 1–2 mL kg⁻¹ was performed. Liver enhancement, as measured by CT, reached values of 15–30 HU in a dose-dependent manner. The most common side-effects were headache, rhinitis, sore throat and bitter taste, which occurred at late time points (6 hours) after administration.

3.2

Non-Iodinated Hepatocyte-Specific Liver Contrast Agents

Metal chelates have long been tested as contrast agents for CT in indications like urography or angiography. However, Gd-DTPA (Magnevist®, Schering AG, Berlin, Germany) with only one gadolinium ion, a concentration of only 0.5 M of gadolinium, which is equivalent to 76 mg Gd per mL and with a pharmacokinetic profile identical to that of extracellular iodinated compounds with three or even six iodine atoms which have a concentration of 3 M of iodine (up to 400 mgI mL⁻¹) was not able to replace the iodinated agents due to insufficient contrast [94–97]. Only in patients who do not tolerate iodinated compounds at all, did these gadolinium chelates gain certain importance.

It was therefore highly surprising that chemically modified DTPA (Fig. 14), for example ethoxy benzyl-DTPA (EOB-DTPA) chelating a lanthanide ion should be useful as a CT liver contrast agent [98]. The synthesis of this class of compounds and its use in magnetic resonance imaging (MRI) has been described by Schuhmann-Giampieri [99]. Likewise, MRI imaging characteristics in rats and nonlinear pharmacokinetic modelling have been reported by this group. Data in humans including safety, pharmacokinetics and preliminary imaging results have been published by Hamm et al. [100].

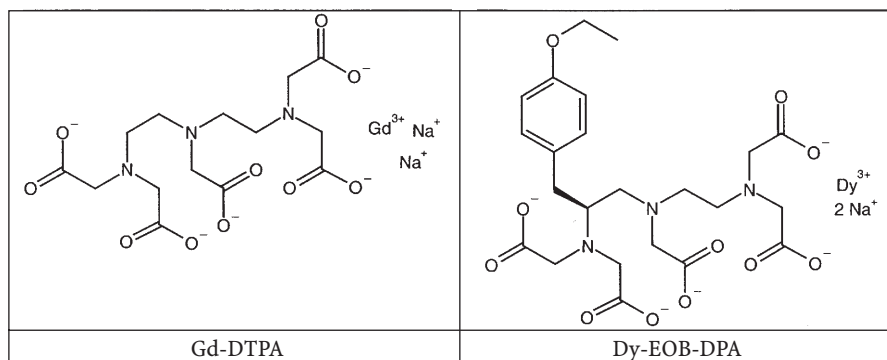


Fig. 14. Structures of Gd-DTPA and Dy-EOB-DTPA [88]

A number of different metal ions have been chelated with the EOB-DTPA ligand. The resulting preparations were 0.25 M in metal ions, which is extremely low compared to iodinated extracellular X-ray contrast agents, which are approx. 3 M in iodine. Out of these complexes, those containing the lanthanides, Pr, Gd, Dy, Yb, and Lu showed the highest stabilities, whereas La and Ce complexes and also Pb chelates were much less stable as determined by binding to bone meal suspensions. The partition coefficients in the system *n*-butanol/water of the chelates were approx. 0.01 for most of the complexes, exceptions being Pr-EOB-DTPA with 0.04 and Pb-EOB-DTPA with 0.001. The osmolalities at 37°C were in the range from 718 (Ce) to 914 mosm kg⁻¹ (Pr). Protein binding was between 6 and 8%. Biliary excretion in anaesthetized rats following injection of 0.5 mmol kg⁻¹ was in the range 49–61%. The concentration in the liver of rats 15 min after a dose of 0.5 mmol kg⁻¹ was approx. 2 µmol per g liver which is equivalent to approx. 300 µg Gd per g for the gadolinium chelate. Liver imaging in rats using CT showed an increase in density of 10 HU, 30 min after an intravenous dose of 0.5 mmol kg⁻¹. In rabbits, an increase of 15–30 HU was observed during an observation period of two hours [98].

Both Gd-EOB-DTPA and Dy-EOB-DTPA were also tested in humans [101, 102]. For Dy-EOB-DTPA, imaging was performed in two patients. In patient #1, 27 metastases were found by plain CT (without injection of a contrast agent). The number of metastases increased to 40 after the injection of the extracellular agent, iopromide (300 mgI mL⁻¹), at a dose of 120 mL and to 41 after injection of 0.25 mmol kg⁻¹ Dy-EOB-DTPA. In patient #2, one metastasis was detected by plain CT and following iopromide injection. After Dy-EOB-DTPA, the number of metastases found, increased to three. However, the most important result of this study was the long time window during which the liver lesions could be detected. For Dy-EOB-DTPA, the time window was 90 minutes (the whole observation period), whereas for iopromide, metastases could only be observed in a period of 20–40 seconds. This class of compounds might therefore be useful for all procedures that require longer time periods, such as for example the exact placement of biopsy needles or the placement of tumour ablation probes. Additionally, the process of tumour ablation might be followed on-line following placement of the probe with one single injection of the contrast agent. For any type of interventional procedures in the liver, EOB-DTPA-type chelates of lanthanide metal ions might therefore be useful.

4

Summary and Conclusions

Although computed tomography is developing rapidly and new imaging techniques continue to improve resolution, sensitivity and speed of the procedure, there remains a strong demand for liver-specific CT contrast agents. At present, no such agent is available for widespread clinical use. However, in recent years significant progress has been made to come closer to this goal. Four different approaches have reached the stage of clinical testing. These are two hepatocyte-specific contrast agents and two RES-specific compounds. Representatives of the first group are iodinated lipids (FP-736-04) and metal chelates (Gd and

Dy-EOB-DTPA). The second group is represented by liposomes with different extracellular iodinated contrast agents that are encapsulated and by PFOB. Efficacy does not seem to be an issue for these agents. The future will show whether their safety will be sufficient for further development.

5

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Chemistry of β^+ -Emitting Compounds Based on Fluorine-18

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Positron emission tomography (PET) is a non-invasive imaging technology which provides a unique window on the physiology and function of living organisms. It uses short-lived positron emitting isotopes to trace labelled compounds in vivo. Among those radioisotopes, fluorine-18 is a nuclide of choice: it has a longer half-life (110 min) and a positron energy (0.635 MeV) lower than the other commonly used positron emitter, carbone-11 ($t_{1/2}$: 20 min, 0.96 MeV). This makes fluorine-18 very attractive in terms of synthesis time, biological studies and resolutions of PET scans. Fluorine-18 is generally produced in a cyclotron via the $^{20}\text{Ne}(d,\alpha)^{18}\text{F}$ or $^{18}\text{O}(p,n)^{18}\text{F}$ nuclear reactions. The availability of either labelled molecular fluorine [^{18}F]F₂ or labelled fluoride [^{18}F]F[−] allows flexibility in the development of synthetic routes to organic compounds. However, [^{18}F]fluoride which is available in high specific activity is preferred for most tracer applications. The aim of this review is to emphasise the scopes and limitations of the chemistry with fluorine-18. The synthetic methodology of the different ^{18}F -labelled precursors, their uses in rapid reactions and the potential applications of the new ^{18}F -radiopharmaceuticals are reviewed. Apart from basic publications, most references cited have been published in the past 10 years.

Keywords. [^{18}F] Fluorine, [^{18}F] Fluoride, Positron emitter, Synthesis, Radioligand

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1

Introduction

The understanding of biological processes in living species is based, to a large extent, on the study of the biodistribution, metabolism and elimination of chemical entities. Positron Emission Tomography (PET), a medical imaging technique, has become a powerful technique since it is non-invasive and uses tracers labelled with radionuclides which form stable covalent bonds to carbon and thus have identical properties to the parent drug [1–3]. A positron emission tomograph allows an accurate quantification of the distribution of radioactivity in the body and offers possibilities to study physiology, molecular biology, energy metabolism, drug-receptor or drug-enzyme interactions and the fates of radiotracers in living tissues [4]. In addition, modifications of the biodistribution by physiological stimuli or pathological processes can also be measured. PET is accomplished by coincidence detection of the two energetic, body-penetrating photons (511 keV, 180° apart) which are emitted upon positron annihilation. It has been demonstrated that PET information is complementary to that obtained with conventional X-ray, single photon emission computed tomography (SPECT), and magnetic resonance imaging [5, 6].

Compounds labelled with positron emitting radionuclides have been developed as radiotracers to study human brain and cardiovascular functions. The PET data are strongly dependent on the radiotracers that are injected (cf., e.g. [7]). Factors such as the biodistribution of the molecule of interest, its selectivity as well as its blood brain permeability and its type of binding in the tissue are important considerations to be made before selecting the tracer for PET studies. The choice of the appropriate radionuclide, the position of labelling, the stereochemistry of the molecule and the mode of administration are also key factors. All these aspects are not being discussed here. This chapter reviews the different aspects of ^{18}F -chemistry, developed over the past 5–10 years (see [8–13] for precedent reviews). It summarizes the synthetic methodologies, which have been explored to overcome some of the limitations inherent in the use of short-lived isotopes. No comments will be made on the biological behaviour of the new synthesized radiotracers.

The article has been organized around the different labelled precursors available directly from a cyclotron ($[^{18}\text{F}]\text{F}_2$ and $[^{18}\text{F}]\text{fluoride}$) and the main ^{18}F -reagents easily prepared from them and developed for the synthesis of radio-pharmaceuticals. A short introduction will present first some characteristics of the chemistry with short-lived isotopes.

2

General Introduction to the Chemistry of β^+ -Emitting Compounds

For comparison, all the radiochemical yields (RCY) of labelled compounds are expressed in percentages from the cyclotron-produced labelled precursor. They are decay corrected from the end of bombardment (EOB) except when stated.

2.1

Physical Properties of Currently Used Positron Emitters

Positron emitters present physicochemical properties well adapted to the radiolabelling of markers of perfusion, metabolism or receptor density. In Table 1, the main physical characteristics of fluorine-18 are compared to those of the most common radionuclides used in medical imaging (Positron Emission Tomography "PET", Single Photon Emission Computed Tomography "SPECT") or in pharmacological studies. Due to the short half-lives of the radioisotopes used, it is possible to synthesize radiopharmaceuticals (no-carrier-added "n.c.a" synthesis) that are not intentionally contaminated with the corresponding non-radioactive substrate. Their high specific radioactivity allows the injection of tracer amounts unable to perturb a biological system or to saturate a receptor.

Table 1. Comparative physical properties of the most common used radionuclides

Radionuclide	Half-life	Decay ^a	Maximum specific activity ^b (GBq mol ⁻¹)	mg GBq ⁻¹	mg Ci ⁻¹
^{11}C	20.4 min	β^+ (99 %)	3.4×10^{11}	3.5×10^{-8}	1.2×10^{-6}
^{13}N	9.96 min	β^+ (99 %)	6.9×10^{11}	2.0×10^{-8}	6.9×10^{-7}
^{15}O	2.07 min	β^+ (99.9 %)	3.4×10^{12}	4.7×10^{-9}	1.6×10^{-7}
^{18}F	109.7 min	β^+ (97 %)	6.3×10^{10}	3.0×10^{-7}	1.0×10^{-5}
^3H	12.3 years	β^- (100 %)	1.1×10^6	2.7×10^{-3}	0.1
^{14}C	5730 years	β^- (100 %)	2.3×10^3	6.0	224
^{125}I	60 days	γ (EC)	8×10^7	1.5×10^{-3}	5.8×10^{-2}
$^{99\text{m}}\text{Tc}$	6 h	γ (IT)	1.9×10^{10}	5.2×10^{-6}	1.9×10^{-4}

^a EC: electron capture; IT: Isomeric Transition.

^b Defined as the number of decay N per second and per mole.

2.2

Characteristics of the Reactions with Short-Lived Isotopes

The chemical reactions involving positron emitters have to be specially designed to take into account the short half life of the radionuclide, the limited number of labelled precursors and the sub-micromolar amounts of these precursors. Moreover, the reactions must be possible without any addition of the stable isotope (especially when ligands of receptors are synthesized). Several practical considerations that influence the design of positron-emitter radiotracers with a high specific radioactivity and their experimental handling have been reviewed [4, 8, 14 – 19].

The synthetic route should aim at incorporating the label as late as possible in the sequence. This requires the development of rapid syntheses (generally not more than 3 h for ^{18}F compounds) including HPLC purification and formulation of the radiopharmaceutical for intravenous injection. The large amounts of reagents compared to those of the labelled substrate [20] usually lead to rapid reactions. However, unexpected labelled compounds can also arise from side reactions of reagents in excess or from reactive impurities present in the reaction medium [21].

Each step of the synthesis usually needs optimisation of reaction conditions (time, temperature, solvents, concentrations). Different techniques of reaction activation can also be used. Microwave heating has been shown to give faster, cleaner and more selective reactions [22, 23] than conventional heating. Ultrasound, although promising [24], has not known the same development. Finally, catalysed reactions involving palladium complexes have been developed in carbone-11 chemistry [25] over the last few years. They have not been widely studied in fluorine-18 chemistry.

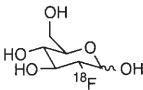
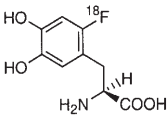
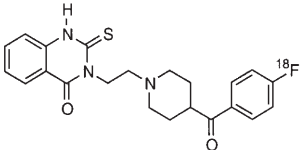
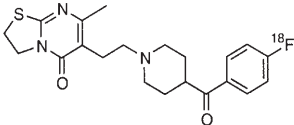
Radiation protection and automation of synthetic procedures have to be carefully considered when planning synthesis of ^{18}F -radiotracers. They require the simplification of all the manipulations or transfers and this point will not be discussed here.

2.3

Main Biological Studies Using ^{18}F Tracers

The main radiopharmaceuticals labelled with fluorine-18, routinely prepared ($[2\text{-}^{18}\text{F}]$ fluorodeoxyglucose $[^{18}\text{F}]\text{FDG}$ [26 – 28], $[^{18}\text{F}]\text{fluoro-L-DOPA}$ [29], $[^{18}\text{F}]\text{altanserine}$ [30, 31], $[^{18}\text{F}]\text{setoperone}$ [32]) are presented with their uses in Table 2. For comparison, the most common tracers labelled with carbon-11 (methionine [33], palmitic acid [34], flumazenil (RO 15.1788) [35], PK 11195 [36], raclopride [37], deprenyl [38], Way-100635 [39], McN-5652Z [40], CGP 12177 [41]) are shown in Table 3. By far, $[^{18}\text{F}]\text{FDG}$ is the most widely studied, particularly in oncology for the diagnosis of tumours, detection of sub-clinical diseases, assessment of therapy responses, and detection of recurrence. ^{18}F -Steroids [42], ^{18}F -proteins or peptides, or ^{18}F -labelled tissue specific agents have also been synthesized for the detection and monitoring of various malignancies [43].

Table 2. Main ^{18}F -radiotracers currently used

Radiotracer	Structure	Use
$[^{18}\text{F}]\text{FDG}$		Glucose metabolism (oncology, cardiology, brain): the first radiopharmaceutical for PET studies
$[^{18}\text{F}]\text{Fluoro-L-DOPA}$		Presynaptic dopaminergic metabolism (Parkinson disease and other movement disorders, schizophrenia, neurodegenerescence)
$[^{18}\text{F}]\text{Altanserin}$		Serotoninerig receptors ($5\text{HT}_{2\text{A}}$)
$[^{18}\text{F}]\text{Setoperone}$		Serotoninerig receptors (5HT_2)

Figures 1 and 2 compare the uses (oncology vs receptors) of tracers labelled with carbon-11 and with fluorine-18 [44].

2.4

General Introduction to Fluorine-18 Chemistry

The biological properties of a compound are determined by a number of parameters (for example its solubility, lipophilicity, degree of ionisation, stereochemistry and electronic factors). When replacing fluorine-19 with a radioactive isotope of fluorine, the radiotracer has essentially the same properties as the unlabelled compound (the small isotope effect is usually negligible [45]). However, many molecules that are of interest for biological applications do not originally contain fluorine. Introduction of a fluorine atom will thus change the molecule's physical properties and consequently its biological behaviour [46]. For a tracer application, the labelling strategy should be directed toward the position(s) that will have as little effect as possible on the characteristics of the parent molecule.

There are several radioactive isotopes of the naturally occurring fluorine-19: two β^+ emitting (positron): ^{17}F ($t_{1/2}$: 64.5 s) and ^{18}F ($t_{1/2}$: 109.7 min) and six β^- emitting: ^{20}F ($t_{1/2}$: 11 s), ^{21}F ($t_{1/2}$: 4.4 s), ^{22}F ($t_{1/2}$: 4.1 s), ^{23}F ($t_{1/2}$: 2.2 s), ^{24}F ($t_{1/2}$: 0.34 s), ^{25}F ($t_{1/2}$: 59 ms) [47]. For radiolabelling procedures, ^{18}F is the most practical to work with thanks to its relatively long half-life. ^{18}F is considered an excel-

Table 3. Main ^{11}C -radiotracers currently in use

Radiotracer	Structure	Use
$[^{11}\text{C}]$ Methionine		Protein synthesis, metabolic abnormalities
$[^{11}\text{C}]$ Palmitic acid		Myocardial metabolism of fatty acids
$[^{11}\text{C}]$ Flumazenil (RO 15.1788)		Benzodiazepine receptors
$[^{11}\text{C}]$ PK 11195		Benzodiazepine receptors
$[^{11}\text{C}]$ Raclopride		Dopamine receptors (D_2)
$[^{11}\text{C}]$ Deprenyl		Monoamine Oxidase-B inhibitors
$[^{11}\text{C}]$ Way-100635		Serotonin receptors (5HT_{1A})
$[^{11}\text{C}]$ McN-5652Z		Serotonin transporters
$[^{11}\text{C}]$ CGP 12177		β -adrenergic receptors

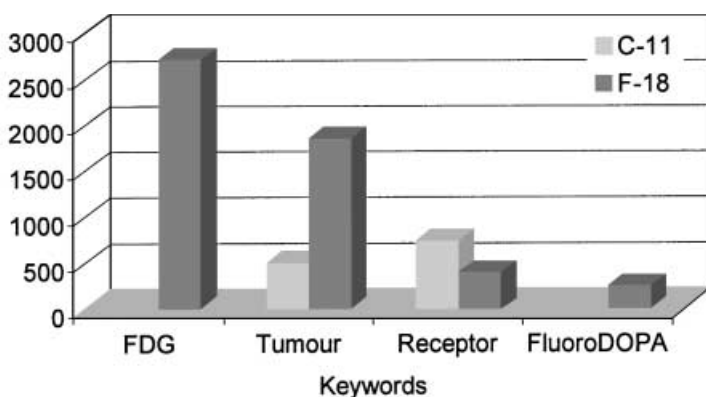


Fig. 1. PET Studies using positron emitters (number of hits per keyword)

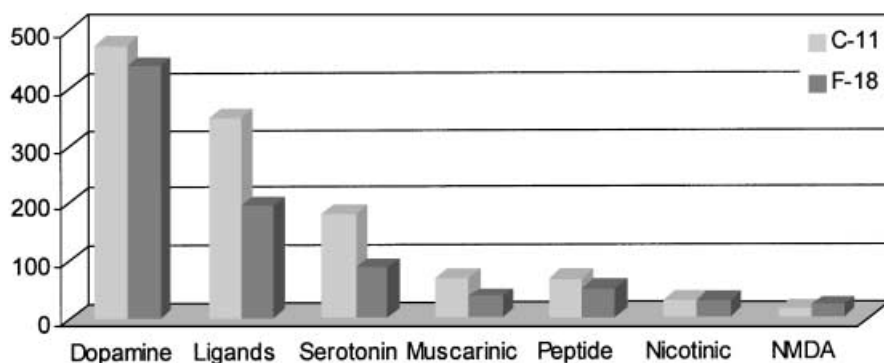


Fig. 2. PET Studies of receptors (number of hits per keyword)

lent positron-emitting radionuclide because of its nuclear (cf. Table 1) and chemical properties.

Generally ^{18}F -labelled analogues are designed by replacement of an hydrogen [8, 10] but fluorine is isostere to oxygen (van de Waals radii: 147, 120 and 152 pm respectively for fluorine, hydrogen and oxygen [48], bond lengths: 133, 110 and 143 pm respectively for C-F, C-H and C-O [46–49]. The electronegativity of fluorine (4.0) is close to that of oxygen (3.5) in its ability to function as a hydrogen bond acceptor [50].

2.4.1

Methods for the Production of Fluorine-18

Fluorine-18 is readily available from cyclotrons by using a wide variety of nuclear reactions. The most common methods to produce ^{18}F are given in Table 4. They will not be discussed here as more elaborated details can be

Table 4. Currently used methods of ^{18}F production

Nuclear reaction	Target material	Beam energy (MeV)	Product	Specific radioactivity
$^{20}\text{Ne}(\text{d}, \alpha)^{18}\text{F}$	0.1 % F_2/Ne	18 or 23	$[^{18}\text{F}]\text{F}_2$	30 – 370 MBq μmol^{-1}
$^{20}\text{Ne}(\text{d}, \alpha)^{18}\text{F}$	15 % H_2/Ne	14	$[^{18}\text{F}]\text{HF}$	0.1 – 1 TBq μmol^{-1}
$^{18}\text{O}(\text{p}, \text{n})^{18}\text{F}$	H_2^{18}O	15	$[^{18}\text{F}]\text{F}^-$	0.01 – 7 TBq μmol^{-1}

obtained from different reviews [10, 18, 51, 52]. Depending on the method used, either electrophilic ($[^{18}\text{F}]\text{F}_2$) or nucleophilic ($[^{18}\text{F}]\text{HF}$, $[^{18}\text{F}]\text{F}^-$) species can be obtained. The same nuclear reaction $^{20}\text{Ne}(\text{d}, \alpha)^{18}\text{F}$ is sometimes used to produce $[^{18}\text{F}]\text{HF}$ [53]. However, the use of the Ne/H_2 target remains difficult.

$[^{18}\text{F}]\text{Fluoride}$ is usually prepared from the $^{18}\text{O}(\text{p}, \text{n})^{18}\text{F}$ reaction on ^{18}O -enriched water [52]. This route is highly attractive because it is a high-yielding reaction, the radioisotope is produced with a high specific radioactivity and all the radioactivity is potentially useful for labelling in contrast to $[^{18}\text{F}]\text{F}_2$ for which only 50 % of the total radioactivity is used.

In order to lower the cost of the nuclear reaction, enriched water is recovered after trapping the ^{18}F -fluoride ions on an ion exchange resin [54, 55]. For this reason and also because of scarcity of enriched water, the exploration of different target strategies is still searched. In examples, a frozen $[^{18}\text{O}]\text{carbon dioxide}$ gas target [56] and irradiation of 2-fluoroaniline by 30–40 MeV photons $[^{19}\text{F}(\gamma, \text{n})^{18}\text{F}]$ [57] was shown to be efficient. However, the specific radioactivity still remains very low.

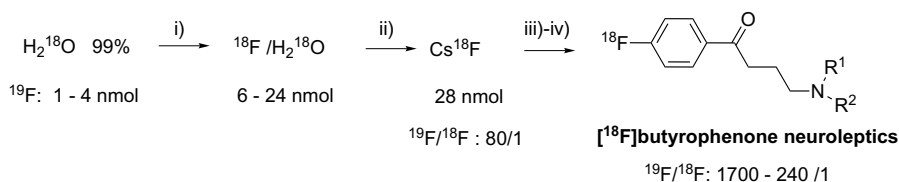
The $^{16}\text{O}(^3\text{He}, \text{p})^{18}\text{F}$ reaction is also occasionally used for $[^{18}\text{F}]\text{fluoride}$ production. The yield is low but it uses natural instead of enriched water as the target material. However the cost of helium-3 is also significant.

2.4.2

Specific Radioactivity

When the tracer of interest is toxic or able to saturate easily in vivo the receptor sites, it is necessary to design a synthetic procedure allowing one to obtain the radioligand with the highest specific activity possible. In fluorine-18 chemistry, the specific radioactivity depends first on the production of fluorine-18. $[^{18}\text{F}]\text{F}_2$ is always produced with a low specific radioactivity due to the need of adding the stable isotope in the target. The specific radioactivity has to be measured by titration of the iodine formed when the target content is trapped in a potassium iodide solution [58]. It can also be deduced from the amount of $[^{18}\text{F}]\text{fluorobenzene}$ obtained by reaction of ^{18}F -molecular fluorine with trimethylphenyltin [53].

Irradiation of enriched water (>95 %) with protons (16 MeV) is the most common method used to produce no-carrier added $[^{18}\text{F}]\text{fluoride}$. Fluorine being less ubiquitous than carbon, ^{18}F -radiotracers can be obtained with very high specific radioactivities (50–500 GBq μmol^{-1}). The theoretically possible



Scheme 1. i) irradiation; ii) Cs_2CO_3 ; iii) *p*-nitrobenzonitrile, DMSO, 140 °C, 10 min; iv) cyclopropyllithium, Et_2O ; v) HCl , MeOH , 110 °C, 7 min; vi) $\text{R}^1\text{R}^2\text{NH}$, KI , 100 °C, DMF-THF

value ($6.3 \times 10^4 \text{ GBq } \mu\text{mol}^{-1}$) however is never reached due to contamination with the stable isotope originating from the radionuclide production, the solvents, chemicals and other sources (impurities not removed by the purification procedures). Usually the amount of carrier is less than 1 μmol . High specific radioactivities are obtained by using 98 % or higher [^{18}O] enriched water. An example of the decrease of the specific radioactivity along the synthesis of [^{18}F]butyrophenone neuroleptics has been described [20] (Scheme 1). Before irradiation, target water contains 1–4 nmol per 0.5 ml of ^{19}F and after irradiation and addition of cesium carbonate, 28 nmol per 0.5 ml. This value represents a ratio $^{19}\text{F}/^{18}\text{F}$ of 81/1 before starting the chemical synthesis. At the final stage of the preparation of the butyrophenone neuroleptics, this ratio can reach 1700–240/1.

2.4.3

Positive Features of Labelling with ^{18}F

Fluorine is not always present in biological molecules and ^{18}F -radiotracers are often analogues of the corresponding hydrogenated compound. The introduction of a fluorine-18 atom in a biomolecule presents several advantages both for chemistry and biology. Compared to carbon-11 ($t_{1/2}$: 20 min), the longer half-life potentially allows time for complicated, multi-step radiolabelled procedures. Appropriate ^{18}F -labelled tracers provides tools for following processes over a longer time than 1 h with the PET camera. Moreover, (see Sect. 2.4.2) the tracers can be obtained in high specific radioactivities.

The introduction of ^{18}F in a tracer molecule can be used to block metabolism as, for example, with [^{18}F]FDG, or to detect metabolic processes. Introduction of a fluorine also changes the lipophilicity of the compound. Correlations between lipophilicity ($\log P$) and the ability of a labelled compound to cross the blood brain barrier has been measured by PET (as an example see [59]).

Fluorine-18 decays largely by positron emission (β^+ : 97 %, electron capture: 3 %) and the positron is of relatively low energy (max: 0.635 MeV). As a consequence, it has the shortest linear range of the four isotopes (^{11}C , ^{13}N , ^{15}O , ^{18}F) and images of the distribution of a tracer labelled with fluorine-18 have the highest resolution (Table 5).

Finally, of the radionuclides commonly used in PET, only ^{18}F , with its half-life of approximately 2 h, can be incorporated into agents and shipped to centres that are not too far from the production site.

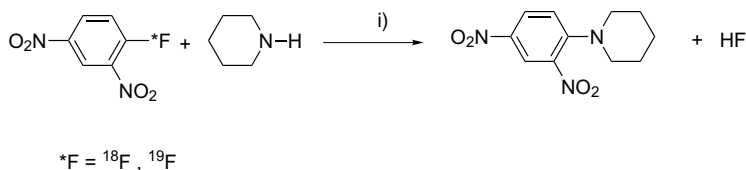
Table 5. Comparison of maximum energy and linear range of the commonly used positron emitters

Radionuclide	Maximum energy (MeV)	Maximum linear range in H ₂ O (mm)
¹¹ C	0.96	4.12
¹³ N	1.19	5.39
¹⁵ O	1.72	8.20
¹⁸ F	0.635	2.39

2.4.4

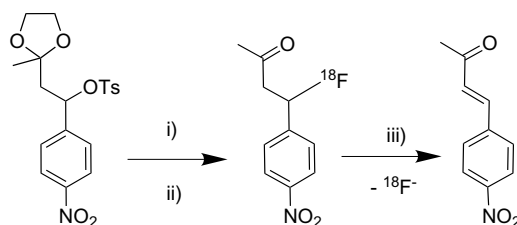
Isotope Effects

Natural fluorine consists entirely of the isotope ¹⁹F and fluorine isotope effects (F KIE) are expected to be small. They were measured for the first time in 1993 [45] in the SNAr reaction of ¹⁸F/¹⁹F fluorodinitrobenzenes with piperidine (Scheme 2). Their value (1.0262 ± 0.0007) [45, 60] suggests a substantial C-F bond breaking in the rate-limiting step of the reaction.

**Scheme 2.** i) THF, 22 °C

F-KIE have also been determined in order to probe the influence of steric effects on the mechanism of the reaction of 2,4-dinitrofluorobenzene with 2- and 4-methylaniline respectively [61]. The ¹⁸F/¹⁹F KIE using isotopically labelled and unlabelled substrates were determined to be 1.0005 ± 0.0030 for 4-methylaniline and 1.0119 ± 0.0037 for 2-methylaniline in DMSO at -30°C . The large F KIE for 2-methylaniline, compared to that observed with its regioisomer 4-methylaniline, suggests that the rate-limiting step is the leaving group departure for this sterically more hindered nucleophile, whereas the insignificant F KIE, for the less sterically hindered 4-methylaniline, indicates rate-limiting addition of the nucleophile.

Fluorine-18 was also used as a tool for the study of the base promoted HF elimination from 4-fluoro-4-(4'-nitrophenyl)butan-2-one [62] (Scheme 3). The F KIEs values were respectively 1.0037, 1.0047 and 1.0013 with formate, acetate or imidazole used as the base. The size of the fluorine KIEs corresponds to 5–15% of the estimated maximum for complete C-F bond breakage and is consistent with a postulated E1cB-E2 like or E1cB_{ip} mechanism.



Scheme 3. i) $[^{18}\text{F}]\text{KF}/\text{K}_{222}$, MeCN; ii) *p*-TsOH, acetone- H_2O ; iii) formate or acetate or imidazole

3

Electrophilic Radiofluorinations

Methods of $[^{18}\text{F}]\text{F}_2$ production used today require carrier fluorine (0.1 % in Ne) (see Sect. 2.4.1). As a consequence, $[^{18}\text{F}]\text{F}_2$ or milder reagents synthesized from it cannot be used for the synthesis of radiopharmaceuticals with high specific radioactivity (see [8, 10, 63]).

3.1

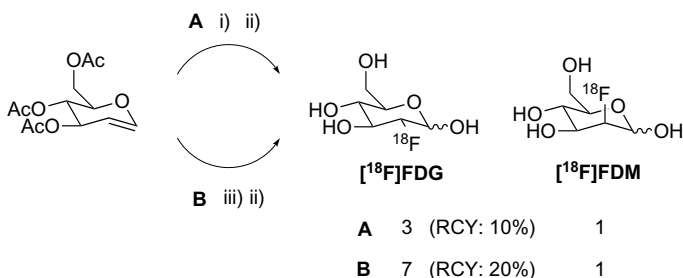
Electrophilic Radiofluorinations via $[^{18}\text{F}]\text{F}_2$ or $[^{18}\text{F}]\text{AcOF}$

The simplest reagent is ^{18}F -molecular fluorine and it provides a facile mean of introducing ^{18}F into electron-rich compounds (alkenes or aromatic rings). The procedures are usually fairly simple since the labelling is performed in one of the last reaction steps. However, electrophilic fluorination of aromatic compounds using F_2 is generally a poor and non-selective method. The oxidizing strength of fluorine may lead to exothermic radical chain reactions with the formation of side products and tars. However, it was found that the reactivity of F_2 could be moderated and controlled at low temperatures using F_2 diluted with an inert gas (0.1 – 0.5 % in neon) [58] or in a strong acid medium [64]. These methods, easily adapted to radiofluorinations since the carrier F_2 in the target is similarly diluted (0.1 – 2 % in Ne), was used as early as 1977 to synthesize $[^{18}\text{F}]\text{FDG}$ from 2,4,6-tri-*O*-acetyl- D -glucal [26, 65]. To decrease the reactivity of electrophilic fluorine, different fluorinating agents have been synthesized from $[^{18}\text{F}]\text{F}_2$ ($[^{18}\text{F}]\text{CH}_3\text{COOF}$ “AcOF”, $[^{18}\text{F}]\text{R}^1\text{R}^2\text{NF}$, $[^{18}\text{F}]\text{XeF}_2$). $[^{18}\text{F}]\text{Acetyl hypofluorite}$ can be prepared in about 50 % yield by reaction of $[^{18}\text{F}]\text{fluorine gas}$ in a column filled with AcOK/AcOH [66] (Scheme 4).



Scheme 4. i) AcOH/AcOK

Its reactivity [67] compared to $[^{18}\text{F}]\text{F}_2$ towards triacetylglucal is shown in Scheme 5. In polyfluorinated reagents, only one fluorine atom can be substituted for an ^{18}F isotope and thus the radiochemical yield is limited (i.e.: 50 % when using F_2).



Scheme 5. i) [^{18}F] F_2 , 0.1 – 2 % Ne, CFCl_3 ; ii) HCl ; iii) [^{18}F] AcOF

3.1.1

^{18}F -Fluorine Addition

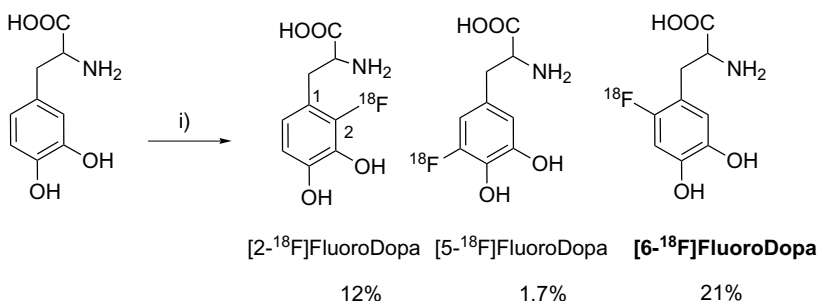
[^{18}F] F_2 addition on the double bond of triacetoxylglucose [26], followed by deprotection of the acetate functions (Scheme 5), was the first method used to produce [$2\text{-}^{18}\text{F}$]fluorodeoxyglucose. About 10% of [^{18}F]fluorodeoxymannose (FDM) were also formed in the reaction (Scheme 5).

3.1.2

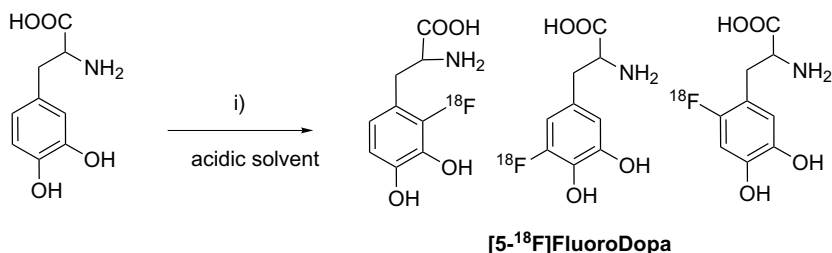
Hydrogen Substitutions

The substitution of a hydrogen for a fluorine-18 has been widely used. Direct radiofluorination of L-DOPA [68] (3,4-dihydroxyphenyl-L-alanine) yields the three possible stereoisomers [$2\text{-}^{18}\text{F}$], [$5\text{-}^{18}\text{F}$] and [$6\text{-}^{18}\text{F}$]fluoro-L-DOPA in 12, 1.7 and 21 % yields respectively (Scheme 6). A complex HPLC purification leads to the preferred [$6\text{-}^{18}\text{F}$]fluoro-L-DOPA in 3 % radiochemical yield.

Recently, it has been shown that the reactivity and selectivity of [^{18}F] F_2 in the electrophilic fluorination of L-DOPA can be modulated by using different acidic solvents (Scheme 7). These conditions have been exploited to produce clinically useful quantities of the regioisomer [$5\text{-}^{18}\text{F}$]fluoro-L-DOPA [69].



Scheme 6. i) [$^{18}\text{F}_2$], -65°C , HF



solvent	RCY %	2- ¹⁸ F	5- ¹⁸ F	6- ¹⁸ F
HF/BF ₃	40	35	5	60
HF	30	35	5	60
TFA	9	90	10	0
50% TFA in AcOH	5.3	56	44	0
10% TFA, 2% Ac ₂ O in AcOH	7.6	57	43	0
HOAc	0	0	0	0

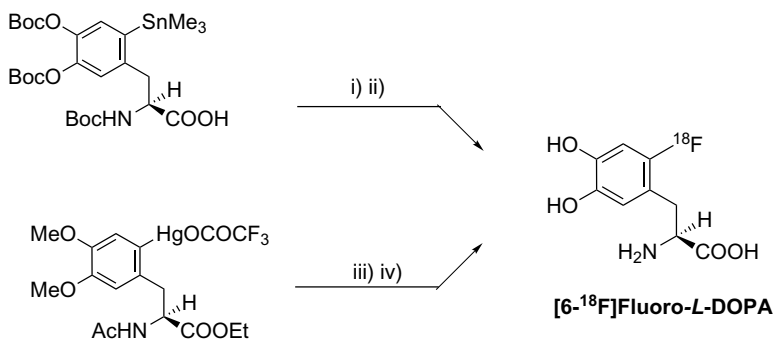
Scheme 7. i) [¹⁸F]F₂

The direct electrophilic radiofluorination of aromatic rings of unprotected L-phenylalanine, L-tyrosine have also been studied. Trifluoroacetic acid is the solvent of choice but the high reactivity of electrophilic fluorinating agents yields a mixture of regioisomers [70]. Uracil, using [¹⁸F]F₂ in acetic acid, was used to prepare [5-¹⁸F]fluorouracil [71], a drug of choice for the visualization of a variety of tumours. The direct radiofluorination of purines with [¹⁸F]F₂ is the only synthetic approach available to date for the preparation of [8-¹⁸F]fluoroganciclovir, [8-¹⁸F]fluoropencyclovir and [8-¹⁸F]fluoroacyclovir. Useful quantities are routinely produced for monitoring HSV1-tk gene expression in living animals [72].

3.1.3

¹⁸F-Fluorodemetalations

The selective electrophilic aromatic substitution carried out by displacement of a metallic substituent (Hg, Sn) (¹⁸F-fluorodemetalation) using [¹⁸F]F₂ or [¹⁸F]AcOF remains a method of choice to introduce a fluorine atom on a specific position. In the early preparations of [6-¹⁸F]fluoro-L-DOPA, the reaction of a 6-substituted mercuric derivative with [¹⁸F]acetyl hypofluorite yielded the expected compound in 11 % yield [73, 74]. Reaction of a mercuric precursor, free or on a modified polystyrene support “P-CH₂-COOHg(DOPA precursor)” allows the preparation of [¹⁸F]fluoro-L-DOPA in an overall yield up to 23 %. The polymer supports are easily prepared, require no special treatment for storage and are convenient to use in automated production [75].



Scheme 8. i) [^{18}F] F_2 , CFCl_3 , RT, 10 min; ii) HBr 48 %, 130 °C, 10 min, RCY: 26 – 33 %, 45 – 50 min total synthesis time; iii) [^{18}F] AcOF , CHCl_3 ; iv) HI , 150 °C, 25 min, RCY: 12 %, 50 min

^{18}F -Fluorodestannylation is now the preferred demetallations for the electrophilic route to [^{18}F]fluoro-L-DOPA (Scheme 8). The radiochemical yields obtained using [^{18}F] F_2 [76, 77] are superior to those using [^{18}F] AcOF [78]. This approach has been recently used for the preparations of [^{18}F]fluoro-*m*-tyramines [79], [2- ^{18}F]oxoquazepam, an agonist of benzodiazepine receptors [80], of [7- ^{18}F]fluorotacrine (9-amino[7- ^{18}F]fluoro-1,2,3,4-acridine), a potential PET agent for mapping acetylcholinesterase [81] and of 2-[^{18}F]fluorobenzidine, precursor of benzodyes, potential radioligand to image amyloid deposits [82] (Table 6).

3.2

Radiofluorinations via N- ^{18}F Compounds

Although a very powerful reagent for electrophilic fluorinations, the *N*-fluoro compounds [83] (fluoro-pyridones, fluoro-*N*-sulfonamides) have not been widely used in fluorine-18 chemistry probably because they are prepared from ^{18}F -fluorine.

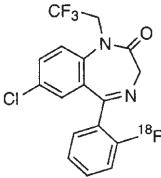
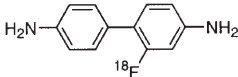
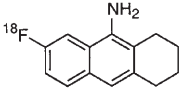
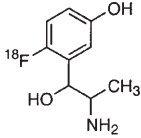
^{18}F -Fluorination of carbanions (R-Li , R-MgX), first carried out with [^{18}F]perchloryl fluoride, [84] can reach 45 % when using *N*-[^{18}F]fluoro-*N*-sulfonamides [85] (Scheme 9). 1-[^{18}F]Fluoro-2 pyridone [86], an easy to use selective fluorinating agent [87], is prepared (48 %, 35 min) by reacting [^{18}F] F_2 with 2-(trimethylsilyl)pyridinium triflate. This reagent was shown to be a useful labelling fluorine transfer reagent in unusual fluorine/metal exchange reactions (Scheme 10).

3.3

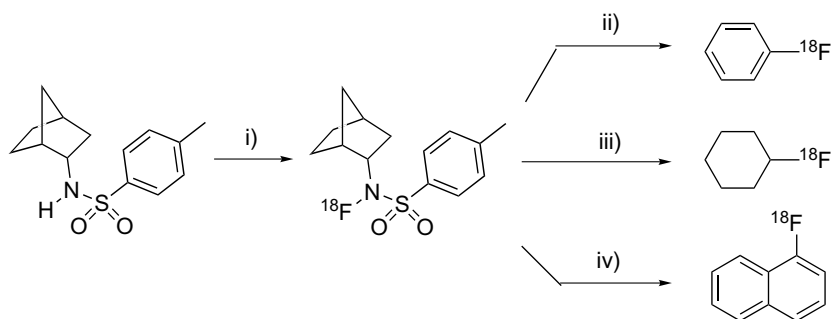
Radiofluorinations via [^{18}F] XeF_2

Xenon difluoride labelled with positron-emitting ^{18}F has been prepared by reaction of cyclotron produced [^{18}F] F_2 with xenon [88]. This low-yielding method requires high pressure. [^{18}F] XeF_2 was also obtained by treating sulfuryl chloride fluoride solutions of XeF_2 in fluorinated ethylene propylene vessels with Bronst-

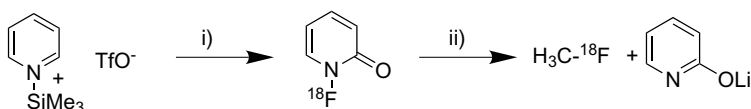
Table 6. Examples of fluorine-18 demetallations

Radiopharma- ceutical	Fluorinating agent	Exchange	Synthesis time (min) ^a	Radio- chemical Yield (%) ^b
 [2- ¹⁸ F]oxoquazepam	[¹⁸ F]AcOF, MeCN	SnMe ₃	^c	25-25
 [¹⁸ F]fluorobenzidine	[¹⁸ F]F ₂ , HCCl ₃ , AcOH	SnMe ₃	20	22
 [¹⁸ F]fluorotacrine	[¹⁸ F]F ₂ , HCCl ₃ , AcOH	SnMe ₃	20	41
 [¹⁸ F]fluorometaraminol	[¹⁸ F]AcOF, MeCN	HgOAc	60	60

^a From EOB, ^b decay corrected, ^c not given.

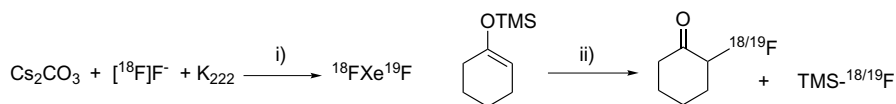


Scheme 9. i) [¹⁸F]F₂, Freon, –78 °C, 45 %; ii) Ph-M, –78 °C, Et₂O, M=MgBr, RCY: 40 %, M=Li, RCY: 61 %; iii) cyclohexyl-MgBr, –78 °C, Et₂O, RCY: 29 %; iv) 1-naphthyl-MgBr, –78 °C, Et₂O, RCY: 53 %



Scheme 10. i) [¹⁸F]F₂, then AcOEt and silica gel, RCY: 48 %; ii) H₃C-Li, -80 °C, 10 min, quantitative yield

ed or Lewis acids ([¹⁸F]HF, [¹⁸F]SiF₄ or [¹⁸F]AsF₅) that had themselves been prepared from [¹⁸F]fluoride. The method is not for widespread use. Recently [89], it has been shown that Cs⁺-Kryptofix-222 complex is a convenient alternative to Pyrex glass as a catalyst for the exchange ¹⁸F for ¹⁹F on XeF₂ in chlorinated solvents under mild conditions (50 min, CH₂Cl₂, RT radiochemical yield: 37–84 %). The reaction is inhibited in acetonitrile. The reactivity of [¹⁸F]XeF₂ with 1-[(trimethylsilyl)oxy]cyclohexene was studied under different conditions (Scheme 11). The mechanism of the reaction is strongly influenced by the solvent, reaction vessel and catalyst. Pyrex or Cs⁺-Kryptofix-222 complex acts as a Lewis catalyst leading to reactions via an ionic mechanism. In the absence of these catalysts, or in MeCN solutions, a weak Lewis base, XeF₂ remains unionized and reacts by single electronic transfer.

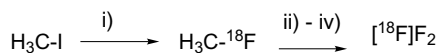


Scheme 11. i) XeF₂, CH₂Cl₂, RT, 50 min, 60 %; ii) ¹⁸FXe¹⁹F, CH₃CN, 16 min, 33 %

3.4

¹⁸F-Fluorine Gas from ¹⁸F-Fluoride

The carrier-added electrophilic [¹⁸F]F₂ obtained as described previously is limited to applications in which a high specific radioactivity is not required. In order to improve the specific radioactivity of radiopharmaceuticals prepared via a direct fluorination, [¹⁸F]F₂ can now be prepared in high specific radioactivity from [¹⁸F]fluoride and in 30–60 % yields. In the procedure, ¹⁸F-labelled fluoromethane is synthesized from aqueous [¹⁸F]F⁻ and CH₃I (Scheme 12). It is then mixed with carrier F₂ in an inert neon matrix and the constituents are atomized in an electric discharge (20–35 kV, 400 μA, 10 s). A rearrangement and ¹⁸F for ¹⁹F exchange take place [89]. The specific radioactivity of [¹⁸F]F₂ is regulated by the amount of carrier fluorine used in the exchange reaction. This method has shown its efficiency in the routine preparation of [6-¹⁸F]fluoro-L-DOPA [90], of [¹⁸F]CFT (specific radioactivity at the end of synthesis: 10–19 GBq μmol⁻¹) [91] and of some other ligands [92].



Scheme 12. i) [¹⁸F]KF/K₂₂₂, CH₃CN; ii) GC separation; iii) F₂/Ne 150 nmol – 1.5 μmol; iv) electric discharge

3.5

^{18}F -Fluorination in an Electrochemical Cell

An electrochemical cell [93, 94] was used to obtain an efficient anodic deposition of no carrier added ^{18}F -fluoride solubilized in the target water. The radioisotope is electrochemically adsorbed on the anode (glassy carbon electrode) and can be easily dried. An opposite electrical field releases the radionuclide directly into a solution of a phase transfer catalyst in dipolar aprotic solvents. The nucleophilic fluorination can be performed simultaneously if the electrochemically and thermally induced desorption of radioactivity is done in the presence of the precursor. However, the yields remain poor (3% in the electrochemical n.c.a ^{18}F fluorination of anisole).

4

Nucleophilic Radiofluorinations

Nucleophilic substitutions with ^{18}F fluoride have been largely developed both in aromatic ($\text{S}_\text{N}\text{Ar}$) and aliphatic (generally $\text{S}_\text{N}2$) series. Nucleophilic additions remain rare. ^{18}F -Nucleophilic radiofluorinations usually do not require any carrier and thus enable the synthesis of products with high specific radioactivity. The S_N can be performed either directly on a suitable and generally complex precursor of the target molecule or indirectly via a small labelled precursor. Both approaches present drawbacks: the first one generally leads to poor yields and the second requires multistep synthesis and more sophisticated automation processes.

This section will present successively the different ^{18}F -labelled reagents, the usual conditions of radiofluorinations and then a critical view of the strategies of synthesis of ^{18}F -precursors, their derivatives and finally their uses for the preparation of ^{18}F -radiopharmaceuticals.

4.1

Reagents and Conditions

^{18}F Fluoride anion as a fluorinating reagent does not offer all the synthetic possibilities of "electrophilic" fluorine. Indeed, ^{18}F fluoride anion, produced by irradiation of enriched water (cf. Sect. 2.4.1) in the nuclear reaction $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$, is provided in aqueous solution. Its highly solvated form is a poor nucleophile [50] but a strong base [95]. The need for a reactive naked fluoride requires its combination to large cations (tetraalkylammonium) [96], cesium, rubidium [97] or the presence of crown ether [98] or aminopolyether [99, 100] with potassium. Although soluble in a large variety of solvents, tetrabutylammonium and tetramethylammonium fluorides have been less used probably because they are more difficult to obtain anhydrous [101] and they are less stable at high temperatures [102].

Typically, from the experimental point of view, the radiofluorinating agent ^{18}F MF ($\text{M}=\text{K}, \text{Cs}, \text{Rb}$) is prepared as followed: the aqueous ^{18}F fluoride, obtained from the target, is passed through an anion exchange resin to recover

[^{18}O]water. [^{18}F]Fluoride is then eluted with an aqueous solution of carbonate (K, Cs, Rb). The salt (in the presence of K_{222} with K_2CO_3) is then dried by successive additions of dry acetonitrile followed by azeotropic distillations. For sensitive substrates or products, substitution of part of the carbonate by the less basic and non nucleophilic oxalate was shown to be efficient [105].

Finally, impurities (water, certain metal ions, competitive anions) [103] can render [^{18}F]fluoride non-reactive. The metal ions are supposed to complex the carbonate ion and thus reduce the pH which in turn reduces the efficiency of the reaction. Methods to purify the [^{18}F]fluoride anion have been developed (see, e.g. [104]).

The radiofluorinations are generally carried out in dipolar aprotic solvents. Dimethylsulfoxide (DMSO) gives rapid reactions. However, it is an oxidizing agent and its high boiling point (189°C) can make difficult its complete removal. When possible, acetonitrile is a good alternative. Sulfolane [106], dimethylacetamide [105] have been shown superior in some cases.

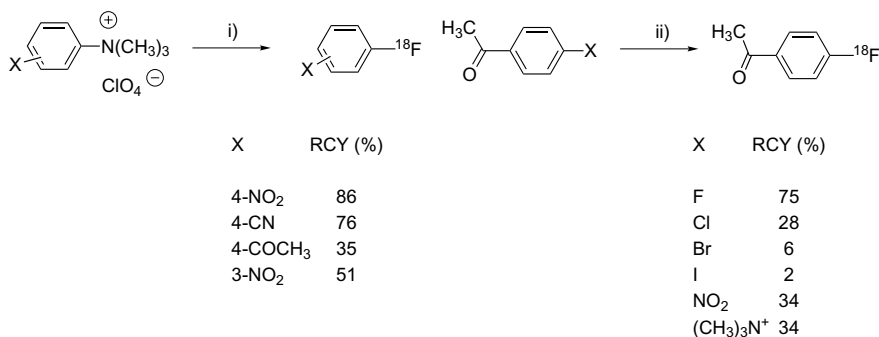
Nucleophilic radiofluorinations can be simplified through the use of polymeric quaternary ammonium hydroxide for both trapping and nucleophilic activation of [^{18}F]fluoride from target water. A synthesized 4-aminopyridinium resin but not the commercially available Dowex and Amberlite resins has been efficient to prepare [4- ^{18}F]fluorocyanobenzene and [^{18}F]fluoroalkylketones [107]. This methodology is now used in the General Electric FDG MicroLab system for the routine production of [^{18}F]fluorodeoxyglucose. It begins to be applied to the preparation of other ^{18}F -radiotracers [108, 109].

4.2

Nucleophilic Aromatic Substitutions

The most successful approach for preparing high specific radioactivity ^{18}F -substituted aromatic compounds is the nucleophilic aromatic substitution reaction [10, 97]. This reaction is only possible if activated (electron deficient) aromatics are used. Aromatics which don't bear a strong electron withdrawing substituent EWG (nitro, cyano, acyl group on the appropriate positions) and a good leaving group LG (NO_2 , Cl, F, $^+\text{NMe}_3$) give no or poor fluorination yields.

Exchange reactions of substituted nitrobenzenes have been studied in detail (Scheme 13). They have shown that the EWG activating effect in the ^{18}F for $^+\text{NMe}_3$ displacement in DMSO at 140°C increases in the order: $\text{EWG} = 3\text{-NO}_2 < 4\text{-CH}_3\text{CO} < 4\text{-CN} < 4\text{-NO}_2$ and that the NMe_3^+ cationic group is a better leaving group than the best neutral ones including NO_2 and F itself. The trimethylammonium group [110, 111] presents some other advantages: compounds bearing this function are stable, easy to handle and generally afford high radiochemical yields upon substitution. Moreover, they are normally insoluble in most organic media whereas the neutral arylfluorides are readily soluble in organic solvents and thus radiotracers of high specific radioactivity can be obtained due to the relatively easy separation of the labelled products from the starting material. ^{18}F -Fluorodemethylations are the major limitations to the use of this leaving group. These reactions have been exploited to prepare [^{18}F]fluoromethane from a non-volatile precursor [112].



Scheme 13. i) [¹⁸F]CsF, DMSO, 140 °C, 20 min; ii) [¹⁸F]F⁻/(COOK)₂/K₂CO₃/K₂₂₂, DMSO, 160 °C, 20 min

Aromatic fluorine for halogen (F-X) exchange reactions (DMSO, 160 °C, 20 min) in an [¹⁸F]fluoride-cryptand-oxalate system using 4'-halo-acetophenones (F, Cl, Br and I) has also been studied. The relative efficacy of the exchange is the following one: F-F > F-Cl > F-Br > F-I, the radiochemical yield for the exchange F-F being similar to that of the commonly employed NO₂ or ⁺NMe₃ displacements [113].

Microwave technology has been successfully applied to improve both yields and time in ¹⁸F-chemistry [22, 23, 114]. However, differences in the reactivity can be observed. In example, in the radiofluorination of 4-fluoro-4'-nitrobenzophenone, the precursor is so activated that all available [¹⁸F]fluoride reacts within 15 s to replace either the nitro or the fluoride leaving group [22].

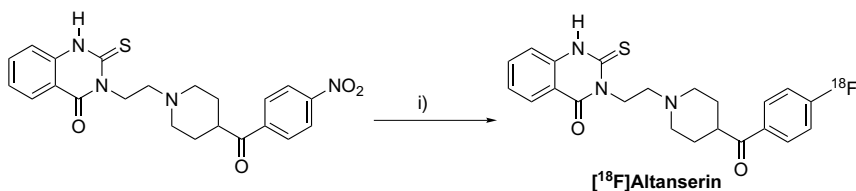
4.2.1

One-Step Synthesis of Complex Structures

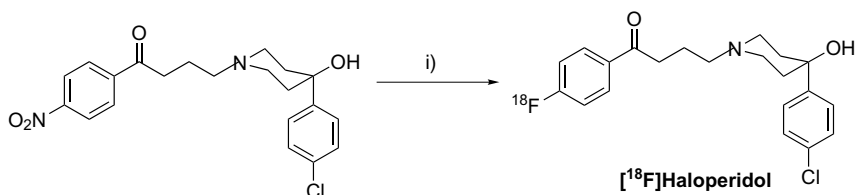
4.2.1.1

Direct Nucleophilic Exchange

The direct exchange of a leaving group for a fluorine-18 is often the preferred method for routine preparation of radiopharmaceuticals. Scheme 14 presents the one-step synthesis of [¹⁸F]altanserin [30, 115], one of the most used ligand for mapping 5-HT₂ receptors.



Scheme 14. i) [¹⁸F]KF/K₂₂₂, microwave, 5 min, HPLC, RCY: 10 % EOS, 110 min total reaction time



Scheme 15. i) $[^{18}\text{F}]\text{F}^-/(\text{COOK})_2/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO, DMF or DMA, 160°C , 30 min, RCY: 21 %, 90 min total reaction time

Another example is given in Scheme 15 with the radiosynthesis of the butyrophenone neuroleptic $[^{18}\text{F}]\text{haloperidol}$. The direct nucleophilic ^{18}F -fluorination has required several optimisation studies to reach a 21 % radiochemical yield [105].

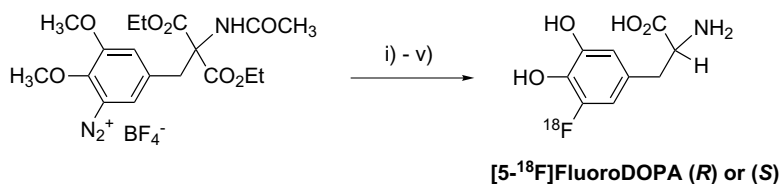
This one-step radiofluorination being not always possible (see for instance the preparation of ^{18}F -sulfonylureas [116]), small ^{18}F -labelled molecules have been synthesized and their rapid transformations or reactions with different types of substrates studied.

4.2.1.2

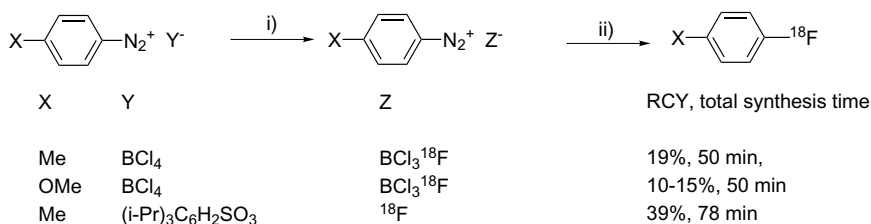
Balz Schiemann and Related Reactions

The Balz Schiemann decomposition is the most common method used for the introduction of a fluorine atom on an aromatic ring not bearing electron withdrawing substituents. It has been studied in fluorine-18 chemistry as early as 1967 [117]. The main drawback of this reaction is that only one fluorine atom from the tetrafluoroborate is transferred and thus the maximum radiochemical yield is 25 % and in practice as little as 2–15 % are observed [118]. However, using $[^{18}\text{F}]\text{HF}$, this labelling method has been successfully applied to the preparation of $[^{18}\text{F}]\text{fluorophenylalanine}$, $[3\text{-}^{18}\text{F}]\text{fluorotyrosine}$ [115] and $[5\text{-}^{18}\text{F}]\text{fluoro-L-DOPA}$ [119] (Scheme 16). A modified Schiemann reaction ($[^{18}\text{F}]\text{KF}$, HBF_4) has allowed the synthesis of ^{18}F -fluconazole, an antifungal agent [120] (RCY: 1–2 % uncorrected, 220 min).

In order to use the potentiality of the Balz Schiemann reaction and to increase the specific radioactivity, the tetrafluoroborate anion was substituted for the tetrachloroborate. The fluorination of *p*-toluidyl diazonium tetrachloroborate,



Scheme 16. i) $[^{18}\text{F}]\text{HF}$, D_2^{18}O , 20 min, 60°C ; ii) vacuum distillation; iii) $120\text{--}125^\circ\text{C}$, xylene 20 min; iv) HBr 48 %, H_2O , 20 min; v) chiral HPLC RCY: 24 % for each enantiomer, 100 min total synthesis time



Scheme 17. i) X=Me, Y=BCl₄⁻: [¹⁸F]F⁻/*n*-Bu₄NOH, *o*-xylene; X=OMe, Y=BCl₄⁻: [¹⁸F]F⁻, K₂CO₃; X=Me, Y=2,4,6-(i-Pr)₃C₆H₂SO₃⁻: [¹⁸F]F⁻/Na₂CO₃/15-Crown-5; ii) 110 °C, 30 min, *p*-chlorotoluene or *o*-xylene

as a model compound, has been carried out using either [¹⁸F]tetrabutylammonium fluoride or [¹⁸F]KF (without K₂₂₂, this latter giving a stable complex with the diazonium cation). On pyrolysis, the [¹⁸F]fluorotrichloroborate gives the expected ¹⁸F-fluoroarene in 18% radiochemical yield (Scheme 17) [121]. This methodology has been applied to the synthesis of [4-¹⁸F]fluorophenol [122]. The development of this strategy has been limited by the difficulties encountered to prepare the tetrachloroborate in a pure and dry form and by the formation of chloroarenes during the thermal decomposition. To improve the accessibility to ¹⁸F-fluoroarenes, the pyrolysis of different 4-toluidyldiazonium salts in the presence of [¹⁸F]fluoride has been studied (Scheme 17). The 2,4,6-tri-isopropylbenzenesulfonate was shown to be the most efficient, [4-¹⁸F]fluorotoluene being obtained in 39% yield (78 min total synthesis time).

Triazene decomposition, which can be a good alternative to the decomposition of tetrafluoroborate, has also been studied in details for the preparation of ¹⁸F-fluoroaromatics [123–125]. Although a wide variety of experimental conditions have been tried, the radiochemical yields remained low (2% for [¹⁸F]haloperidol). Better yields are obtained by ¹⁸F for ¹⁹F nucleophilic aromatic substitutions. Decomposition of aryl diazo sulfides to fluoroaromatics is not yet adapted to the preparation of ¹⁸F-fluoroaromatics [126].

4.2.2

[¹⁸F]Nitrobenzenes and Applications

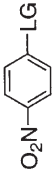
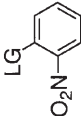
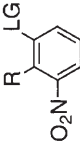
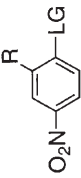
4.2.2.1

Synthesis of [¹⁸F]Fluoronitrobenzenes

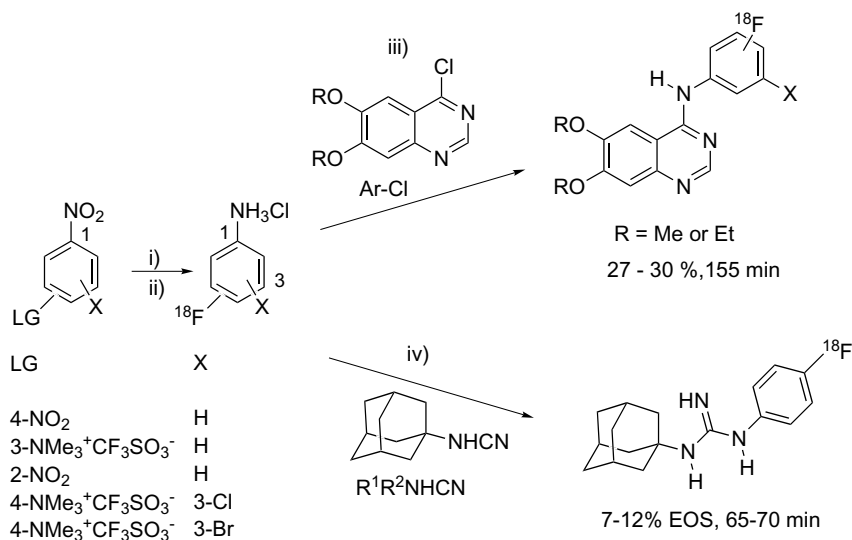
The preparation of [2-¹⁸F] or [4-¹⁸F]fluoronitrobenzenes has been widely studied [111, 127–132] due to the potential applications of these ¹⁸F-precursors. High yields of [4-¹⁸F]fluoronitrobenzene (or its 2-regioisomer) are usually obtained when the leaving group is in ortho or para orientation to the nitro group.

Recently it has been shown that [3-¹⁸F]fluoronitrobenzene can be obtained in yields up to 77% when the radiofluorination is carried out in DMSO at 150 °C for 20 min. No reaction occurs in acetonitrile. Lower yields (respectively 17 and 23%) are obtained in the radiofluorination of 2,6-dinitrotoluene and 2,4-dinitro-

Table 7. Preparation of [¹⁸F]fluoronitrobenzenes

Substrates	LG	R	Reactions conditions ^a	RCY ^b %	Notes
	Cl		[¹⁸ F]RbF, 165°C, 15 min	35 – 50	^c
	NO ₂		[¹⁸ F]RbF, 150°C, 20 min	87	^d
	NO ₂		[¹⁸ F]KF/K ₂₂₂ , 160°C, 3 min	70 – 85	^e
	NMe ₃ ClO ₄		[¹⁸ F]CsF, 120°C, 20 min	91	^f
	NMe ₃ OTf		[¹⁸ F]KF/K ₂₂₂ , 120°C, 10 min	87	^g
	NO ₂		[¹⁸ F]RbF, 150°C, 20 min	58 – 90	^d
	NO ₂		[¹⁸ F]KF/K ₂₂₂ , 130°C, 10 min	75 – 90	^h
	NO ₂	H	[¹⁸ F]KF/K ₂₂₂ , 150°C, 20 min	50 – 77	ⁱ
	NO ₂	H	[¹⁸ F]F/KHCO ₃ /K ₂₂₂ , 160°C, collidine	84	^j
	NMe ₃ OTf	Me	[¹⁸ F]KF/K ₂₂₂ , 130°C, 10 min	38	^h
	NMe ₃ OTf	Cl	[¹⁸ F]CsF, 130°C, 5 min	33 – 80	^h
	NMe ₃ OTf	Br	[¹⁸ F]CsF, 130°C, 5 min	75 – 80	^h

^a All the reactions were carried out in DMSO. ^b Radiochemical yields at EOB, corrected for decay. ^c [128]. ^d [127]. ^e [129]. ^f [110]. ^g [111]. ^h [132]. ⁱ [130]. ^j Instantaneous reaction carried out in MeCN/H₂O 9/1 [131].



Scheme 19. i) $^{18}\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$ or Cs_2CO_3 , DMSO, 130°C , 5–10 min; ii) NaBH_4 , Pd/C, MeOH, RT then HCl; iii) Ar-Cl, DMF, 85°C , 10–15 min; iv) LG=4- NO_2 , X=2- CH_3 ; $\text{R}^1\text{R}^2\text{NHCN}$, HCl, CH_3CN , 120°C , 12 min

zene, $[4\text{-}^{18}\text{F}]\text{fluorohydrazinobenzene}$ [133], $[4\text{-}^{18}\text{F}]\text{fluorobenzenesulfonylchloride}$ [134] or $[4\text{-}^{18}\text{F}]\text{fluorophenol}$ [122]. $[4\text{-}^{18}\text{F}]\text{fluoroanilines}$ were also the key precursors in the synthesis of ^{18}F -fluorophenylpiperazines including $[^{18}\text{F}]\text{RP 62203}$ [129, 135, 136] a highly selective antagonist of 5-HT₂ receptors [137] (Scheme 18) or in the preparation of ^{18}F -labelled σ receptor ligands [138] and ^{18}F -fluoroanilinoquinazolines [132] to study the epidermal growth factor receptor involved in human neoplastic lesions (Scheme 19). ^{18}F -Labelled benzenediazonium is also a possible route for the radiolabelling, under mild reaction conditions (pH 8, RT, 5 min), of tyrosine and tyrosine containing peptides H-LysTyrSer-OH [139].

4.2.3

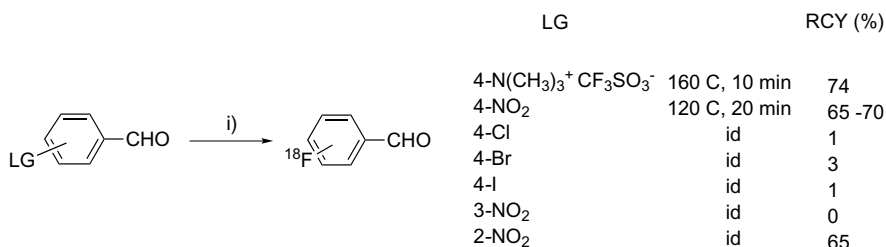
$[^{18}\text{F}]\text{Fluorobenzaldehydes}$. Applications

4.2.3.1

Synthesis of $[^{18}\text{F}]\text{Fluorobenzaldehydes}$

$[^{18}\text{F}]\text{Fluoroarylaldehydes}$ are easily obtained in moderate to high yields from the corresponding nitro [140, 141] or trimethylammonium triflate [110, 111] (Scheme 20) using $^{18}\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$ in DMSO, at 120°C for 10 min. Yields up to 70–74% can be reached.

The radiochemical yields of radiofluorinations can be strongly different for closed structures [141]. Some examples are given in Scheme 21. By measuring the ^{13}C NMR chemical shifts of the aryl carbons, it was shown that the yields can be correlated with the electron density at the reaction centre.

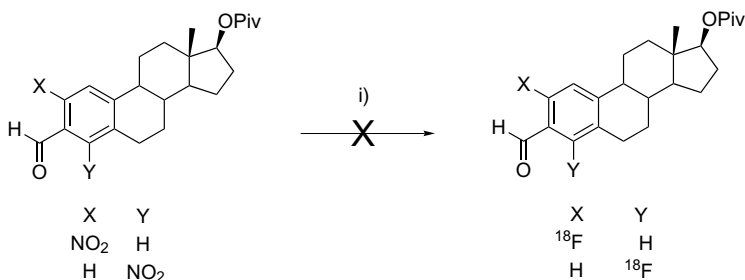


Scheme 20. i) [¹⁸F]F⁻/K₂CO₃/K₂₂₂, DMSO

R ¹ , R ²	RCY (EOB) %	¹³ C NMR
		δ (ppm) C ₆
H, H	78	149.58
O-CH ₂ -O	51	146.08
O-C(CH ₃) ₂ -O	42	145.76
OCH ₃ , OCH ₃	23	143.80
O-CH ₂ Ph, O-CH ₂ Ph	24	143.65
H, OCH ₃	5	142.24
O-(CH ₂) ₂ -O	0	123.70

Scheme 21. ¹⁸F-Fluorination of 2-nitrobenzaldehydes: correlation between the ¹³C-chemical shifts and the radiochemical yields

In more complex structures, ¹⁸F-fluorodenitration ([¹⁸F]nBu₄NF or [¹⁸F]KF/K₂₂₂, 150 °C, DMSO) are not always successful [142] and a combination of all the electronic effects of the different substituents (alkyl substituents at the meta and para positions of the estrogen systems in the example given in Scheme 22) have to be taken into account.

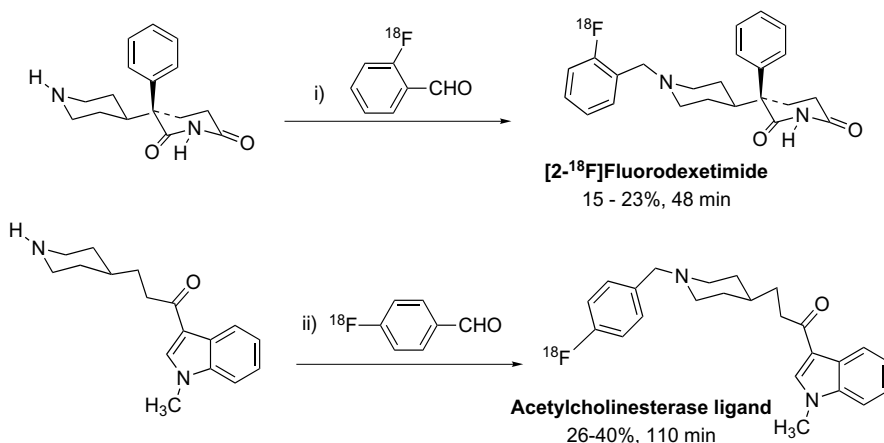


Scheme 22. i) [¹⁸F]F⁻/n-Bu₄NOH or [¹⁸F]F⁻/K₂CO₃/K₂₂₂, DMSO, 150 °C

4.2.3.2

Reductive Aminations of [^{18}F]Fluorobenzaldehydes

Both [2- ^{18}F] and [4- ^{18}F]fluorobenzaldehydes are key intermediates in the synthesis of *N*-[^{18}F]fluorobenzylamines via reductive aminations. This methodology has been successfully applied to the preparation of a potential acetylcholinesterase inhibitor [143] and of fluoro analogues of dextetimide, potent ligand of muscarinic cholinergic receptor [144] (Scheme 23).

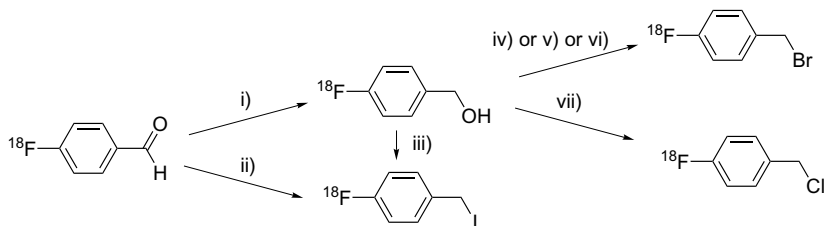


Scheme 23. i) NaBH_3CN , CH_3COOH , 120°C , 10 min; ii) NaBH_3CN , CH_3COOH , 135°C , 15 min

4.2.3.3

Reductions of [^{18}F]Fluorobenzaldehydes and Further Applications

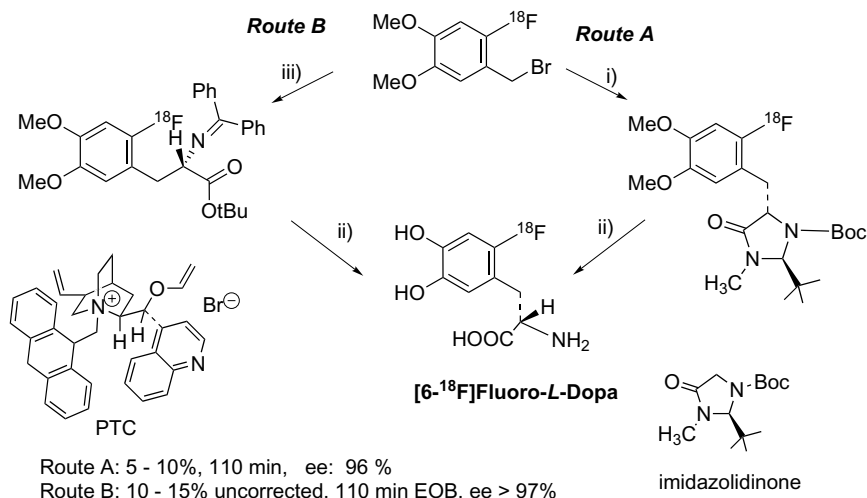
Different methods (summarized in Scheme 24) have been used to transform ^{18}F -fluorobenzaldehydes into ^{18}F -fluorobenzylhalides. Most of them are based on reduction into the corresponding ^{18}F -fluorobenzylalcohol (LiAlH_4 [145], NaBH_4 [140b, 146], NaBH_3CN [147], SiH_2I_2 [148, 149]) followed by halogenation (HI [150, 151], HBr [147], P_2I_4 , Ph_3PBr_2 [152], SOBr_2 [145, 146, 153], SOCl_2 [154] with time-consuming operations (condensation, evaporation, extraction). In order to simplify the synthetic procedures especially for automation, the reduction of



Scheme 24. i) LiAlH_4 or NaBH_4 or NaBH_3CN ; ii) SiH_2I_2 ; iii) HI or P_2I_4 ; iv) HBr ; v) SOBr_2 ; vi) Ph_3PBr_2 ; vii) SOCl_2

[^{18}F]fluorobenzaldehyde has been recently carried out in nearly quantitative yields onto a C18 cartridge by flowing an aqueous solution of NaBH_4 on the C18 cartridge containing the ^{18}F -aldehyde [152]. [$4\text{-}^{18}\text{F}$]Fluorobenzyl alcohol is eluted from the column and collected in a vessel containing solid P_2I_4 or Ph_3PBr_2 where it is transformed efficiently into the corresponding [$4\text{-}^{18}\text{F}$]fluorobenzyl-halide (radiochemical yield: $>90\%$ in 10 min for the iodide and 50–60% within 30 min for the bromide).

An important application of these precursors is the asymmetric synthesis of aminoacids, the key step being an enantioselective benzylation using a chiral auxiliary (route A, Scheme 25) [155] or a chiral phase transfer catalyst (PTC) (route B, Scheme 25). This latter approach avoiding the use of dry reagents is particularly adapted to automated synthesis and enables the production of more than 7.4 GBq (200 mCi) of [$6\text{-}^{18}\text{F}$]fluoro-L-DOPA from 55.5 GBq (1.5 Ci) of starting [^{18}F]fluoride [157].



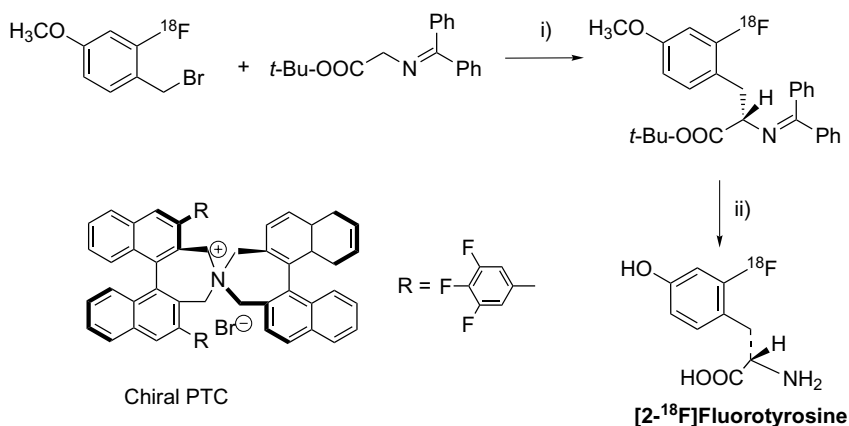
Scheme 25. i) imidazolidinone lithium enolate, THF, -78°C , 5 min; ii) HI 47%, 200°C , 20 min then HPLC; iii) Schiff base, PTC, CsOH , H_2O , toluene, 0°C , 10 min

A similar strategy, using a recently described PTC [158], was used to transform [$2\text{-}^{18}\text{F}$]fluoro-4-methoxy-benzylbromide into [$2\text{-}^{18}\text{F}$]fluoro-L-tyrosine (ee $>97\%$, 25% decay corrected, 100 min) (Scheme 26) [158b].

4.2.3.4

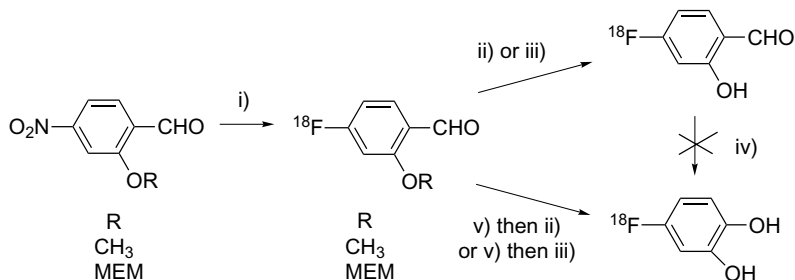
Oxidations of [^{18}F]Fluorobenzaldehydes to [^{18}F]Fluorophenols

The preparation of fluorinated catechols and phenols and particularly fluorinated derivatives of the catecholamines and aminoacids has led to the search for methods which do not induce racemization of chiral precursors. Benzaldehydes and alkoxybenzaldehydes are considered as latent phenols and catechols respec-



Scheme 26. i) Chiral PTC, toluene, CsOH or 50 % KOH, 10 min, 0°C; ii) HI, 200°C, 20 min then HPLC, RCY: 25 %, 100 min, ee > 97 %

tively after oxidation and deprotection. A careful choice of phenol protecting groups and reaction conditions has allowed the preparation of ^{18}F -fluoromethoxyphenols in 18–28 % in less than 2 h synthesis time (Scheme 27) [159]. This methodology has been successfully applied to the synthesis of [4- ^{18}F]fluorophenol [160]. However, Baeyer Villiger oxidation of [4- ^{18}F]fluoroarylketones are preferred for complex structures.

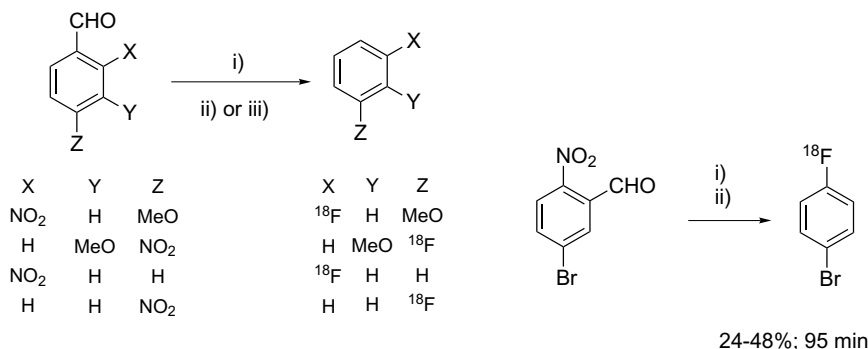


Scheme 27. i) [^{18}F]F $^-$ /K $_2$ CO $_3$ /K $_{222}$, DMSO, 120°C, 25 min, 50–60 % (R=Me), 30–40 % (R=MEM); ii) R=CH $_3$: BBr $_3$, CH $_2$ Cl $_2$, 0°C, 25 min, 50–60%; iii) R=CH $_3$ OCH $_2$ (MEM): F $_3$ CCO $_2$ H, 110–120°C, 20 min, 60–70%; iv) H $_2$ O $_2$, MeOH, KOH, 100°C, 10–30 min; v) H $_2$ O $_2$, MeOH, KHSO $_4$, 110–120°C, 25 min, R=Me: 70–80%; R=MEM: 30 %

4.2.3.5

Decarbonylation of ^{18}F -Aromatic Aldehydes and Applications

Due to its strong activating effect in nucleophilic aromatic substitutions and to the possibility of its removal by decarbonylation, the aldehyde function has been used for the preparation of [^{18}F]fluoroarenes not bearing electron-withdrawing substituents. Decarbonylations, possible in the presence of Pd/C [161], are more efficient in terms of time (15 min vs 1 h) and yields (80 %) when using Wilkin-



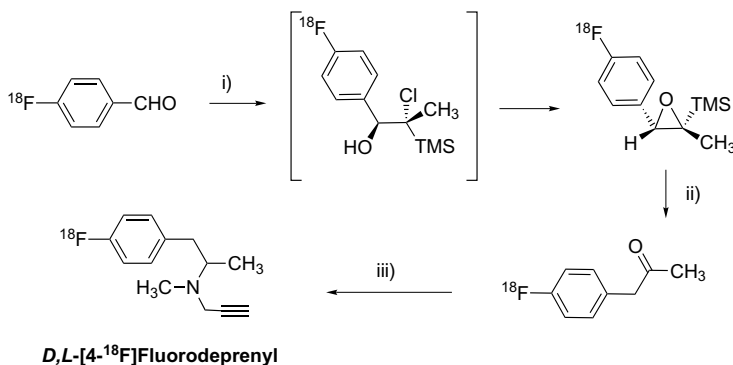
Scheme 28. i) [¹⁸F]⁻F⁻/K₂CO₃/K₂₂₂, DMSO, 145 °C, 20 min; ii) Rh(PPh₃)₃Cl, 1,4-dioxane, 150 °C, 15 min, 85 %; iii) 10 % Pd/C, 1,2,3,4-tetramethylbenzene, 250 – 255 °C, 50 – 60 min, 60 – 70 %

son catalyst, RhCl(PPh₃)₃ [162]. The reaction is fairly general and it has been applied to the synthesis of [4-¹⁸F]fluorobromobenzene (Scheme 28) [163, 164].

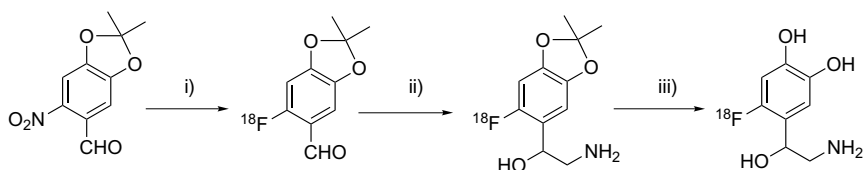
4.2.3.6

Nucleophilic Addition to [¹⁸F]Fluorobenzaldehydes

In order to synthesize [4-¹⁸F]deprenyl, MAO inhibitor, a strategy based on the conversion of an ¹⁸F-labelled aldehyde to its homologous ¹⁸F-methyl ketone [165] was developed. The target compound does not possess an electron-withdrawing group on the aromatic ring and thus makes impossible the direct introduction of an ¹⁸F via a nucleophilic aromatic substitution. The key step of the synthesis involves the instantaneous reaction of the ¹⁸F-aldehyde with the anion of (1-chloroethyl)trimethylsilane at –75 °C followed by spontaneous cyclization into the ¹⁸F-epoxide while warming to room temperature. After acidic rearrangement of the corresponding epoxide, reductive alkylation with NaBH₃CN leads to *D,L*-[4-¹⁸F]fluorodeprenyl in 11 % in a total synthesis time of 90 min (Scheme 29).



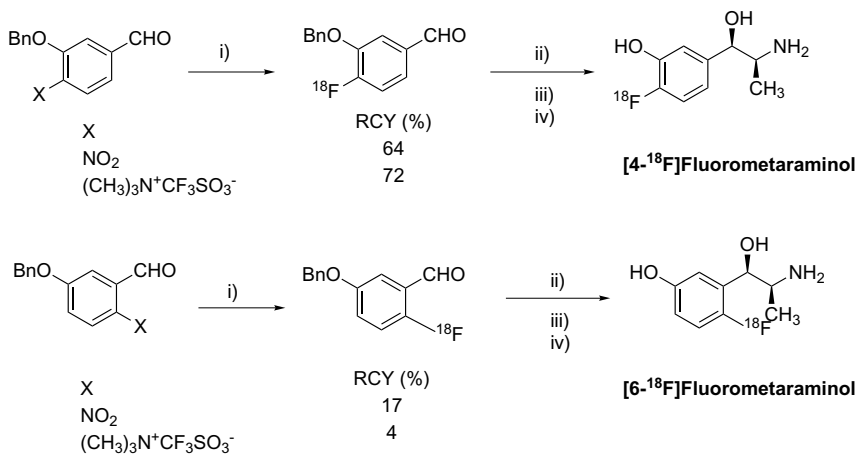
Scheme 29. i) (CH₃)₃Si-C(Li)(Cl)CH₃, –75 °C, 5 min; ii) 18 N H₂SO₄, reflux, 5 min, 50 %; iii) *N*-methyl-propynylamine, CH₃OH, NaBH₃CN, molecular sieves, 65 °C, 15 min, 35 %. RCY: 11 %, 90 min total synthesis time

**[6- ^{18}F]Fluoronorepinephrine**

Scheme 30. i) KF/K_{222} , DMSO, 120°C , 40–45 %, 18 min; ii) Me_3SiCN , ZnI_2 10 min RT then LiAlH_4 , 50°C , 10 min; iii) HCOOH 100°C , 5 min then semi preparative HPLC then chiral HPLC; RCY: 6 % for each enantiomer, 128 min total synthesis time

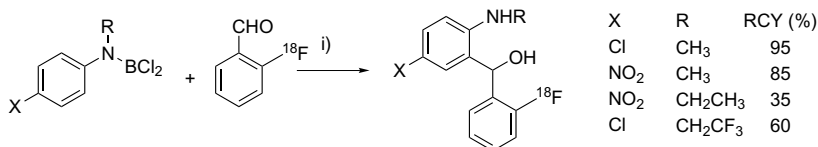
^{18}F -Fluorobenzaldehydes have also been used as starting materials for the preparation of enantiomers of $[\text{6-}^{18}\text{F}]$ fluoronorepinephrine, a myocardial marker [166]. The key step is the formation of a protected ^{18}F -cyanhydrine which is reduced into an amino alcohol. Deprotection, purification and resolution on a chiral column provide both enantiomers (Scheme 30).

A similar approach using a nitro derivative as precursor of the amino alcohol was developed to synthesize $[\text{4-}^{18}\text{F}]$ - and $[\text{6-}^{18}\text{F}]$ fluorometaraminols. Here again, the radiochemical yields of the radiofluorination appear strongly dependent on the position and the nature of the leaving group (Scheme 31)[167].



Scheme 31. i) $^{18}\text{F}\text{-F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO, 130°C , 15 min (NO_2 precursor), 130°C , 2 min (ammonium precursor); ii) EtNO_2 , NaOH , MeOH , 0°C , 5 min, 60–70 %; iii) Pd/C , HCOONH_4 , MeOH , 150°C , 50–60 %; iv) C18 HPLC (diastereomeric separation), chiral HPLC (enantiomeric resolution)

^{18}F -Labelled 2-amino-2'-fluorobenzhydrol, intermediates in the synthesis of amino benzodiazepinones, has been synthesized in an elegant one-step procedure [168] based on the specific ortho coupling of an anilinodichloroborane with an aldehyde [169] (Scheme 32).

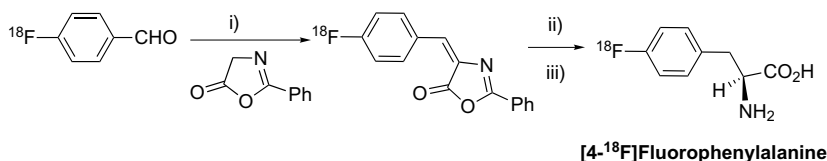


Scheme 32. i) NEt₃, dichloroethane-hexane, 20–60 °C, 5–20 min

4.2.3.7

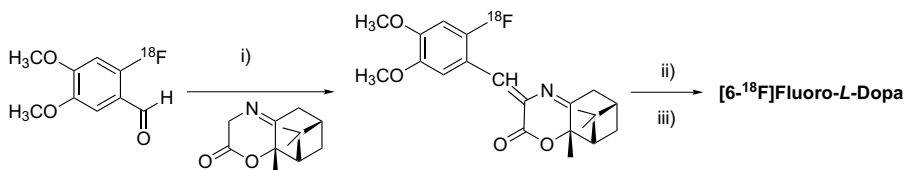
Condensation Reactions of [¹⁸F]Fluorobenzaldehydes

In order to decrease the number of chemical steps in the synthesis of L-[4-¹⁸F]fluorophenylalanine, a potential marker for probing protein synthesis [140a], [4-¹⁸F]fluorobenzaldehyde has been directly condensed with an imino-lactone. Hydrolysis then chiral HPLC yield the *S* amino acid in 5 % radiochemical yield (Scheme 33).



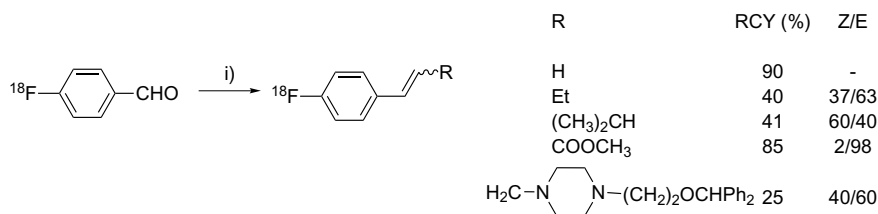
Scheme 33. i) 1,4-Diazabicyclo[2.2.2]octane, ethanol, 140 °C, 10 min 80–85 %; ii) AcOH, HI, red P, 220 °C, 10 min, phosphate buffer pH 4; iii) chiral HPLC RCY: 5 %, 120 min total synthesis time

Direct condensation of 4, 5-dimethoxy-[2-¹⁸F]fluorobenzaldehyde with an asymmetric chiral inductor [170] followed by L-selectride reduction of the olefinic double bond and hydrolysis leads to [6-¹⁸F]fluoro-L-DOPA in 3 % radiochemical yield and an ee higher than 90 % (total synthesis time: 125 min) (Scheme 34). This method avoids the preparation of ¹⁸F-fluorobenzylhalides.



Scheme 34. i) NaH, THF, 20 min; ii) *tert*-BuOH, THF-ether, L-selectride, –78 °C, 10 min; iii) H₂NOH.HCl, AcOH, EtOH, H₂O then HI, 220 °C, 15 min

Transformation of [4-¹⁸F]fluorobenzaldehydes into [4-¹⁸F]fluorophenyl-alkenes using the Wittig reaction has been relatively unexplored. Examples are shown in Scheme 35. It requires the in situ generation of the ylid [171] by reaction of the phosphonium bromide with propylene oxide [172]. These conditions, successfully used in carbon-11 chemistry [173], have however the drawback of leading to a mixture of *Z* and *E* stereoisomers.



Scheme 35. i) Ph₃P⁺CH₂R, Br⁻, propylene oxide, 1,2-dichlorobenzene, 160 °C, 10 min

4.2.4

[¹⁸F] Fluoroarylketones and Applications

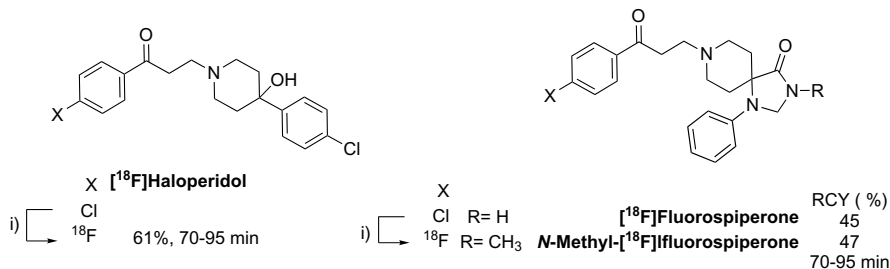
4.2.4.1

Synthesis of [¹⁸F]Fluoroarylketones

[4-¹⁸F]Fluoroarylketones are easily prepared in the direct ¹⁸F to NO₂ exchange. However, the carbonyl being less efficient than a nitro or a cyano group in promoting the aromatic nucleophilic substitution, yields are generally lower than those obtained in the preparation of [¹⁸F]fluoronitrobenzene or [¹⁸F]fluorobenzonitrile [97, 128].

A systematic study of parameters critical to the reproducible production in high yield of ¹⁸F-fluoroarylketones [174] has shown that the trimethylammonium triflate displacement gives the highest yields. The reaction is particularly sensitive to the amount of the base (K₂CO₃/K₂₂₂) used to trap [¹⁸F]fluoride. Under microwave activation (700 W) the optimal radiofluorination time is 2.5 min. Attempts to use the optimised conditions to prepare the ortho isomer failed, the major reaction being the competitive nucleophilic aliphatic substitution leading to [¹⁸F]fluoromethane (see [112]).

Using the chloro-precursor and less basic radiofluorination conditions, high yields of ¹⁸F-neuroleptics have been obtained (Scheme 36) [113].



Scheme 36. i) [¹⁸F]F⁻/(KOOC)₂/K₂CO₃/K₂₂₂, DMSO, 160 °C, 20 min

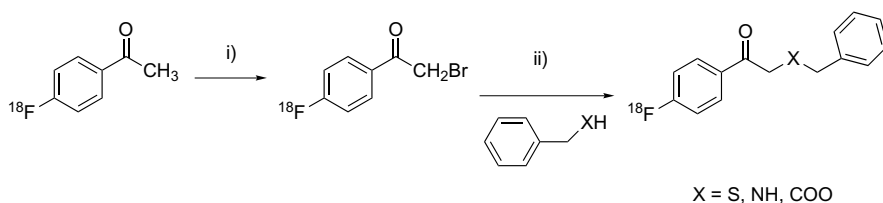
4.2.4.2

[4-¹⁸F]Fluoroacetophenone. Ketalization and Peptide Labelling

The ketalization of [4-¹⁸F]fluoroacetophenone required a careful adjustment of reaction conditions [175]. It has been carried out in order to prepare fluorine-18 labelled progestin ketals, potential imaging agents for breast tumours.

Transformation of [4-¹⁸F]fluoroacetophenone into [4-¹⁸F]fluorophenacyl bromide has been studied [174, 176]. The solid phase method [177] appears the method of choice (Scheme 37).

This precursor is a promising reagent for the radiolabelling of proteins and peptides which contain free thiols or carboxylic acids [178].

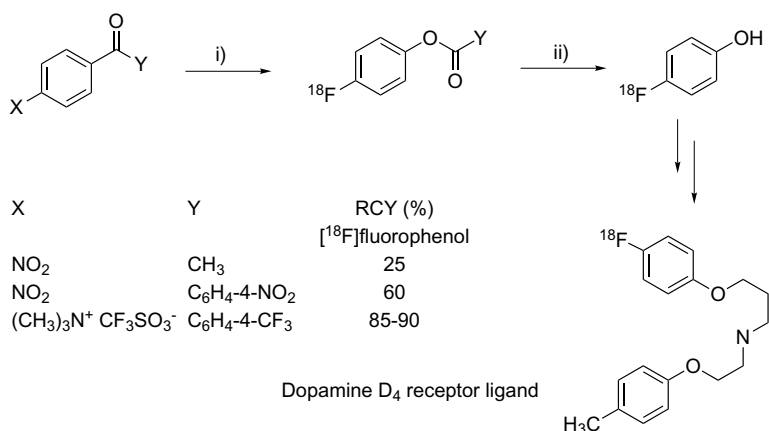


Scheme 37. i) A-26, Br₃⁻, THF, 60 °C, 10 min 61 %; ii) PhCH₂XH, CH₂Cl₂

4.2.4.3

Baeyer Villiger Oxidations of [¹⁸F]Fluoroarylketones

Oxidation of ¹⁸F-labelled phenone derivatives using *m*-chloroperbenzoic acid in trifluoroacetic acid yields [4-¹⁸F]fluorophenol in 25–40% decay corrected [160]. This yield was improved (up to 60%) by using H₂O₂ under acidic conditions and benzophenones bearing an electron deficient aryl group (Scheme 38) [179]. The methodology was applied to the synthesis of a highly selective dopamine D₄ antagonist (D₄/D₃ > 100).

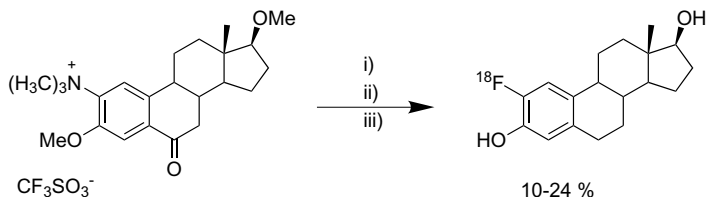


Scheme 38. i) H₂O₂, H₂SO₄, AcOH; ii) NaOH then HCl

4.2.4.4

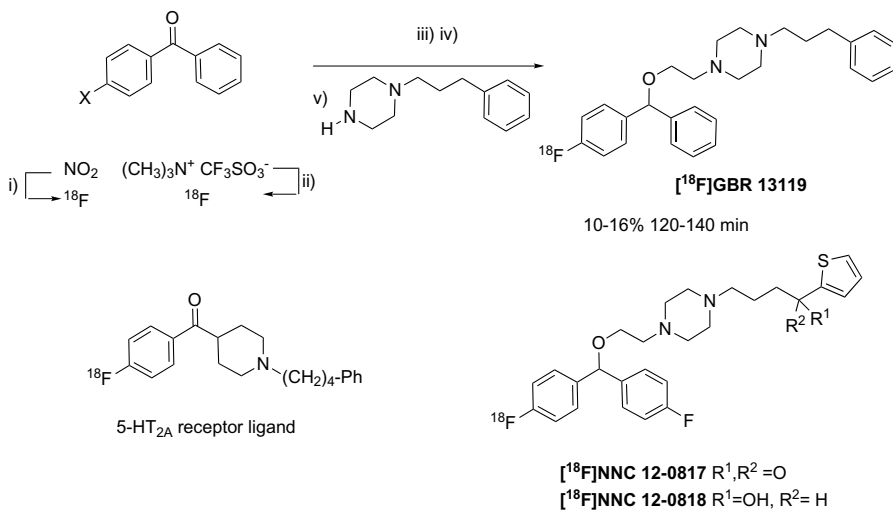
Reduction of [^{18}F]Fluoroarylketones

Like aldehyde, the ketone function provides sufficient activation on an aromatic ring for the nucleophilic substitution. Reduction of the carbonyl group after incorporation of the ^{18}F -fluoride ion yields ^{18}F -alkyl aromatics [177]. This methodology using ^{18}F for trimethylammonium exchange, then reduction of the carbonyl compound, have proved to be useful for the synthesis of a [^{18}F]fluorophenol at high specific activity [141] (Scheme 39).



Scheme 39. i) [^{18}F]nBu₄NF, DMSO, 150 °C, 45 min, 20–50 %; ii) AlCl₃, LiAlH₄, RT, 5 min, 95 %; iii) EtSH, AlBr₃, 30 min, 50 %

[^{18}F]Fluorobenzophenones are also precursors, after reduction into the corresponding alcohol, of different complex structures such as a 5-HT_{2A} receptor ligand [180] and several dopamine transporter ligands: GBR 13119 [181], GBR 12909 [182] and analogues [183], NNC 12-0817 and 12-0818 [184] (Scheme 40).



Scheme 40. i) [^{18}F]F⁻, *n*-Bu₄NOH, DMSO, 165 °C, 25 min, 52 %; ii) [^{18}F]F⁻, K₂CO₃, K₂₂₂, DMSO, 155 °C, 20 min, 60–95 %; iii) LiAlH₄, 0–5 °C, 1 min, 55–85 %; iv) SOCl₂, 100 °C, 20 min; v) *N*-substituted piperazine, mesitylene, 155 °C, 25 min, 25–60 %

4.2.5

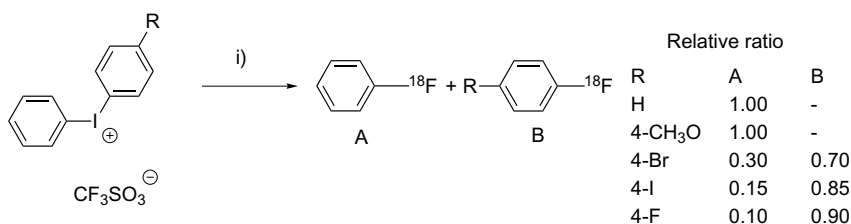
[¹⁸F]Fluorohalobenzenes and Applications

4.2.5.1

Synthesis of [4-¹⁸F]Fluorohalobenzenes

The synthetic potentialities of palladium mediated cross-coupling reactions (Heck, Suzuki, Stille, Buchwald-Hartwig) have led to the search for a practical synthesis of [4-¹⁸F]fluoroiodo and [4-¹⁸F]fluorobromobenzenes. [¹⁸F]Fluoroiodobenzene can be obtained in poor yields from the nucleophilic exchange ¹⁸F/trimethylammonium triflate. More efficient is the reaction of [¹⁸F]fluoride with a suitable diaryliodonium salt (Scheme 41) [185]. However, this methodology seems more difficult to use with complex structures (see, e.g. [142, 186]). In these exchanges on para substituted diaryliodonium salts, the nucleophile always prefers attacking the less electron rich aryl group.

[4-¹⁸F]Fluorobromobenzene has also been prepared in a two-step process (radiofluorination, decarbonylation) from bromonitrobenzaldehyde [162, 163] (see Scheme 28).

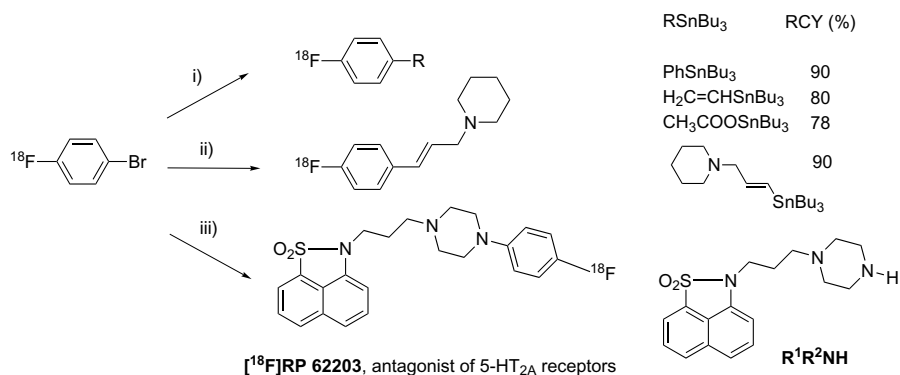


Scheme 41. i) [¹⁸F]KF/K₂₂₂, under N₂ (20 psi), 85 °C, 40 min

4.2.5.2

Cross-Coupling Reactions of [4-¹⁸F]Fluorohalobenzenes

Very efficient cross coupling reactions of tin derivatives with [4-¹⁸F]fluorobromo and [4-¹⁸F]fluoroiodobenzenes have been carried out [162, 163] (Scheme 42). Reaction of the appropriate piperazine with [4-¹⁸F]fluorobromobenzene under Hartwig-Buchwald conditions [187] yields [¹⁸F]RP 62203 in high specific activity and biologically usable amounts. This method appears superior to the one described previously starting from [¹⁸F]fluoronitrobenzene (Scheme 18).

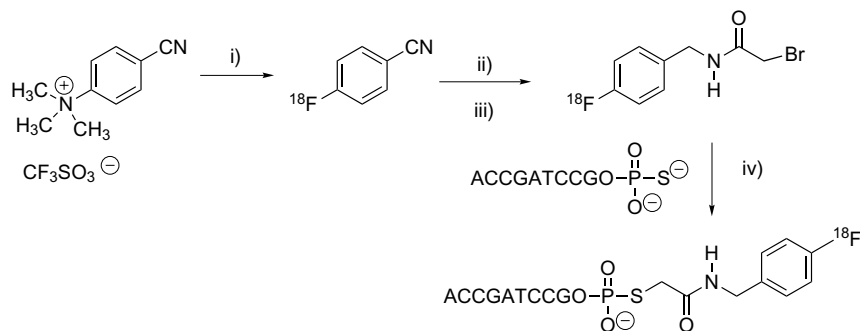


Scheme 42. i) R-SnBu₃, Pd₂dba₃, AsPh₃, DMF-dioxane, 115–120 °C, 5–30 min; ii) R-SnBu₃, BnClPd(PPh₃)₂, CuI, DMF-dioxane, 120 °C, 10 min; iii) R¹R²NH, Pd₂dba₃, P(*o*-tolyl)₃, *tert*-BuONa, toluene, 110 °C, 15 min, 60 % from [¹⁸F]fluorobromobenzene. [¹⁸F]RP 62203 RCY: 10–12 % from [¹⁸F]F[−], 150 min total synthesis time

4.2.6

[4-¹⁸F]Fluorocyanobenzene and Oligonucleotide Labelling

[4-¹⁸F]Fluorobenzonitrile can be obtained in high yields by nucleophilic exchange of a trimethylammonium group [20]. It has been transformed into *N*-[4-¹⁸F]fluorobenzyl- α -bromoacetamide in order to label oligonucleotides [188]. The main advantage of this labelled precursor is the stability of the Csp²-F bond. Solvolysis of the C-F bond is often noticed when radiolabellings are carried out with ¹⁸F-labelled fluoroalkyl compounds. The labelling strategy is simple, reliable, independent of backbone modifications and of the sequence of the nucleotide bases. It should be of general applicability provided that the oligonucleotides bear a phosphorothioate group at their 3' end (Scheme 43).



Scheme 43. i) [¹⁸F]KF/K₂₂₂, DMSO, 120 °C, 20 min, 60–85 %; ii) LiAlH₄, THF, 120 °C, 2 min, quant.; iii) BrCOCH₂Br, H₂O-CH₂Cl₂, 25 °C, 2 min, 65–90 %; iv) Phosphate buffer, pH 8, MeOH-H₂O, 80 °C, 15 min, 70–90 %

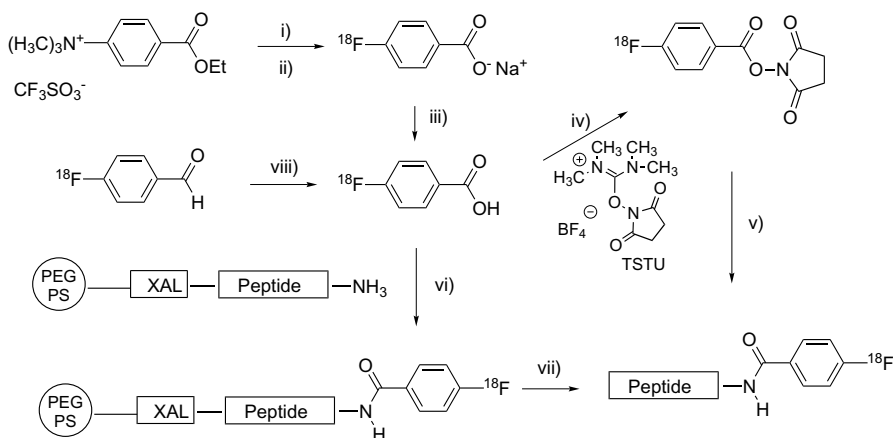
4.2.7

[¹⁸F]Fluorobenzoic Acid Derivatives. ¹⁸F-Labelled Peptides

[4-¹⁸F]Fluorobenzoic acid has been prepared by oxidation (Jones reagent) of the corresponding ¹⁸F-aldehyde (80 °C, CH₃CN, 10 min, RCY>95%) [189]. Short-lived isotopes such as fluorine-18 become more and more interesting in radioimmunodetection and the control of radioimmunotherapy. ¹⁸F-Labeling of proteins and peptides can only be achieved via prosthetic groups. *N*-Succinimidyl-[4-¹⁸F]fluorobenzoate appears as one of the most promising [190]. It reacts almost exclusively with the lysine residues of the proteins through formation of stable amide bonds. The original synthetic procedure starts with the nucleophilic aromatic ¹⁸F-fluorination of 4-trimethylammonium benzaldehyde triflate followed by oxidation with potassium permanganate [191]. A more recent approach avoids this laborious, difficult-to-automate oxidation and the liquid phase extraction by using a 4-trimethylammonium triflate benzoate as the precursor. This method also takes advantage of *O*-(*N*-succinimidyl) *N,N,N',N'*-tetramethyluronium tetrafluoroborate (TSTU) for esterification, at low temperature, of fluorobenzoic acid. A similar approach to the preparation of a radio-ligand for imaging angiotensin converting enzyme has been described [192].

The solution phase synthesis of peptides often results in mixtures of labelled, unlabelled compounds along with other impurities and requires extensive purification. A route using a solid phase approach [polyethylene glycol-polystyrene PEG-PS derivatised with a xanthen-2-oxoalate (XAL) linker] was recently developed [193] (Scheme 44).

This method presents several advantages: it is fast (20 min total synthesis time), efficient, clean and readily automated. A high specific radioactivity (20–25 GBq μmol⁻¹ starting from 10–50 MBq of [¹⁸F]fluoride) is obtained for mod-



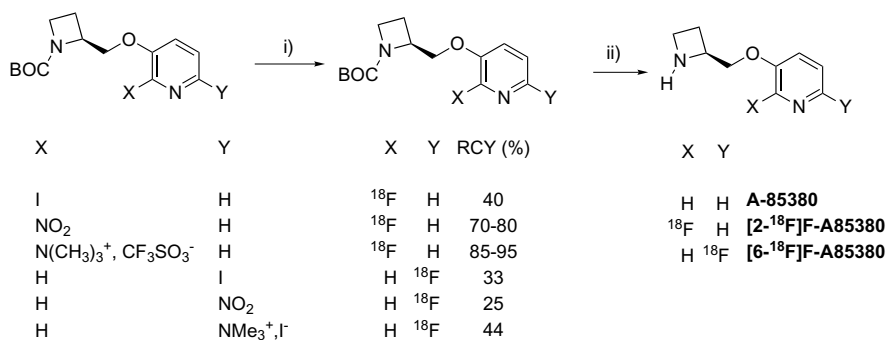
Scheme 44. i) [¹⁸F]F⁻/(KOOC)₂/K₂CO₃/K₂₂₂, DMSO, 90 °C, 10 min, 85%; ii) 1 mol/l NaOH, 90 °C, 8 min; iii) 1 mol/l HCl (CH₃)₄NOH; iv) TSTU, CH₃CN, 2 min, 90 °C then AcOH; v) Peptide: NEt₃, 5 min, RT. Protein: borate buffer, pH 8.5, 10–15 min; vi) coupling agent, 3 min; vii) trifluoroacetic acid, 7 min, 70–80% EOS from [¹⁸F] fluorobenzoic acid; viii) KMnO₄

el peptides (containing most naturally occurring amino acids except cysteine and tryptophan prone to oxidation).

4.3

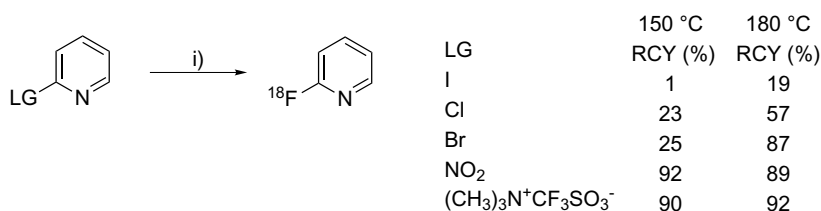
^{18}F -Nucleophilic Heteroaromatic Substitution

Compared to homoaromatic and aliphatic substitutions, nucleophilic substitutions with fluoride ion (^{19}F or ^{18}F) of heteroaromatic derivatives have not been widely studied. $[6\text{-}^{18}\text{F}]$ Fluoronicotinic acid diethylamide has been prepared in a reasonable yield (40%) from the corresponding 6-chloronicotinic acid. However, a high temperature ($>200^\circ\text{C}$) is required [194]. $[2\text{-}]$ and $[6\text{-}^{18}\text{F}]$ Fluoronicotine have been obtained in 30–40% radiochemical yield from the corresponding 2- and 6-bromopyridine derivative respectively and $[^{18}\text{F}]$ cesium fluoride [195] in DMSO at 210°C for 30 min. With the need for efficient radiolabelling of potent nicotinic acetyl choline receptors ligands F-A-85380 {fluoro-3-[2(*S*)-azetidylmethoxy]pyridine} and norchlorofluoroepibatidine {exo-2-(6-fluoro-3-pyridyl)-7-azabicyclo [2.2.1] heptane}, substituted pyridines bearing different leaving groups have been synthesized and evaluated in nucleophilic ^{18}F -radiofluorinations. The trimethylammonium group, either as a triflate [196] or an iodide [197] is slightly superior to iodo [198] or nitro leaving groups [199, 200] both in 2- and 6-positions (Scheme 45). The difference observed in the radiochemical yields (25 vs 80%) of $[6\text{-}^{18}\text{F}]$ A-83580 [197] and $[2\text{-}^{18}\text{F}]$ A-83580 [196] suggests that the 6-position in the pyridine ring is less reactive than the 2-position towards nucleophilic aromatic substitution.

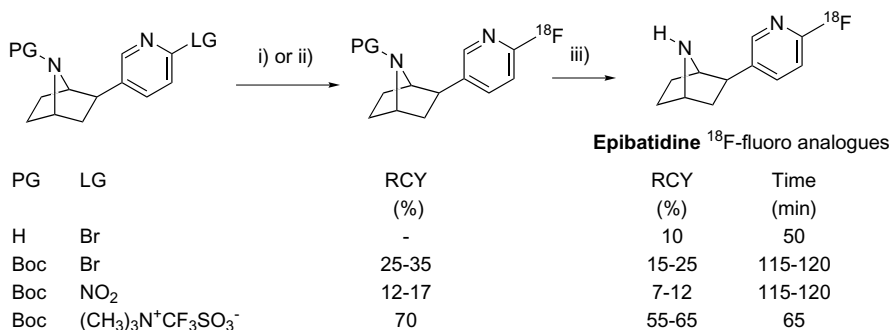


Scheme 45. i) $[^{18}\text{F}]\text{F}/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO; 190°C , 15 min ($\text{X}=\text{I}$, $\text{Y}=\text{H}$), 30 min ($\text{X}=\text{H}$, $\text{Y}=\text{I}$), 150°C , 20 min ($\text{X}=\text{NO}_2$, $\text{Y}=\text{H}$), 2 min [$\text{X}=(\text{CH}_3)_3\text{N}^+\text{CF}_3\text{SO}_3^-$, $\text{Y}=\text{H}$], 5 min [$\text{X}=\text{H}$, $\text{Y}=(\text{CH}_3)_3\text{N}^+\text{I}^-$], 15 min [$\text{X}=\text{H}$, $\text{Y}=\text{NO}_2$]; ii) $\text{CF}_3\text{COOH}-\text{CH}_2\text{Cl}_2$, 2 min

Systematic studies of the radiofluorination of 2-substituted [201] and 3,6-disubstituted pyridines [202–204] were described (Scheme 46). The trimethylammonium leaving group with the triflate counterion appears the precursor of choice in the preparation of ^{18}F -fluoroanalogues of epibatidine (Scheme 47).

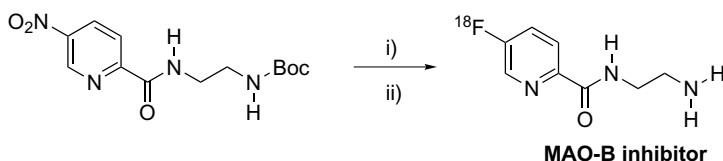


Scheme 46. i) $[^{18}\text{F}]\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO



Scheme 47. i) $[^{18}\text{F}]\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO, PG=H: 190 °C, 15 min; PG=Boc: LG=Br 180 °C, 10 min; LG=NO₂ 150 °C, 10 min; ii) PG=Boc, LG=(CH₃)₃N⁺CF₃SO₃⁻ $[^{18}\text{F}]\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO, 120 °C, 10 min; iii) CF₃CO₂H, 120 °C, 10 min

Introduction of a fluorine-18 in β -position to the pyridine nitrogen has been carried out in 37 % radiochemical yield by nucleophilic exchange ^{18}F for NO₂ in the synthesis of a potential MAO-B imaging tracer (Scheme 48) [205].



Scheme 48. $[^{18}\text{F}]\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO, 135 °C, 20 min; ii) 20 % HCl, 10 min, 135 °C. RCY: 10 %, 120 min total reaction time

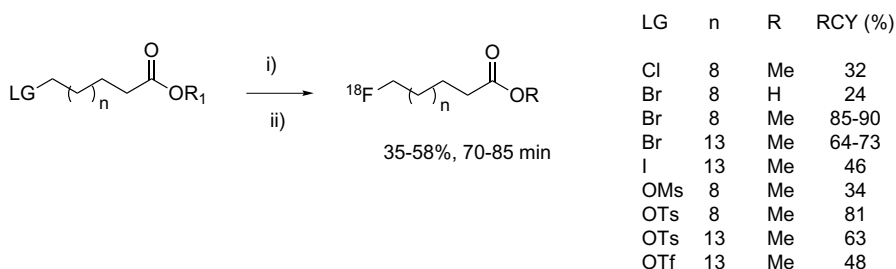
4.4

Nucleophilic Aliphatic Substitution

4.4.1

Direct $\text{S}_{\text{N}}2$ Reaction on Complex Structures

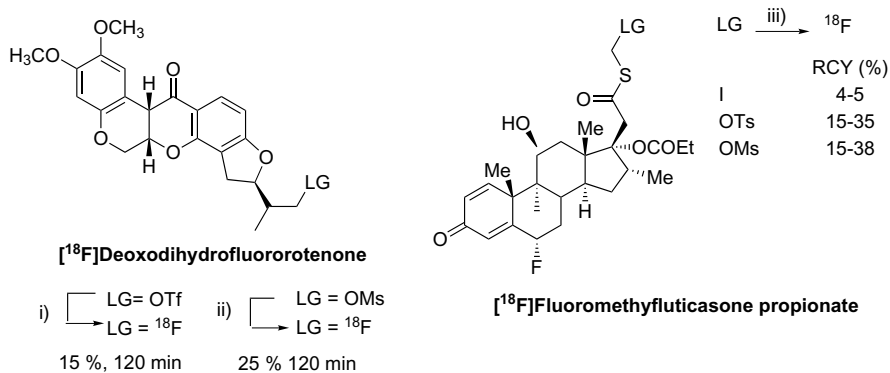
Direct nucleophilic $\text{S}_{\text{N}}2$ exchanges ^{18}F /leaving group (mesylate, tosylate, nosylate, triflate, iodide or bromide) in a complex structure using $[^{18}\text{F}]\text{fluoride}$ anion are more frequent in aliphatic than in aromatic series. The choice of the leaving group is strongly dependent on the substrate to label [206] and it is often neces-



Scheme 49. i) [^{18}F]KF, CH_3CN , 110°C , 10 min; ii) $\text{R}=\text{Me}$: 0.5 N KOH-MeOH reflux, 15 min

sary to test several of these groups to optimise the radiochemical yield. For instance, the tosylate and bromide were shown to be the most reactive for the preparation of [ω - ^{18}F]fluorofatty acids or analogues (Scheme 49) [207, 208].

Scheme 50 presents some recent examples of direct nucleophilic exchanges $^{18}\text{F}/\text{TsO}$ compared to $^{18}\text{F}/\text{MsO}$ or $^{18}\text{F}/\text{TfO}$ in the synthesis of radiotracers directly usable for PET studies [209, 210] and chosen for their structural diversity.



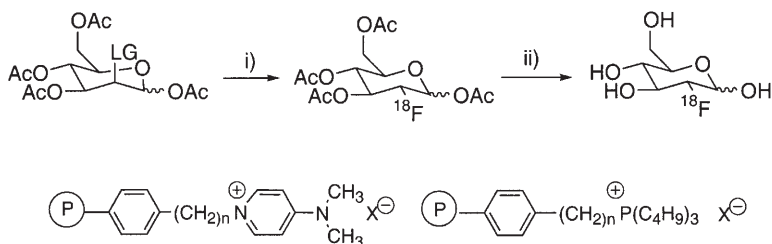
Scheme 50. i) [^{18}F]n-Bu $_4\text{NF}$, CH_3CN , 10 min, 65°C ; ii) [^{18}F]KF, CH_3CN , 12 min, 115°C ; iii) [^{18}F]KF/K $_{222}$, 125°C , 30 min

This strategy has potential disadvantages. Many substrates contain more than one nucleophilic atom (e.g., hydroxyl, phenol, amino) and introduction of a good leaving group only at the desired position is complicated by the need for protecting groups. Also, the stability of alkyl sulfonate esters is variable and side reactions during the ^{18}F -labelling step may be problematic. For these reasons, most of the syntheses are carried out on protected substrates.

4.4.2

$\text{S}_{\text{N}}2$ Reaction on Protected Complex Structures

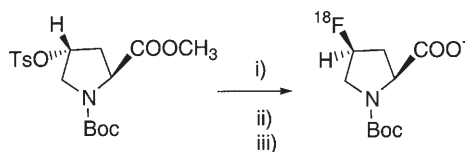
The most useful and important positron emitting radiopharmaceuticals, 2-deoxy-2- ^{18}F fluoro-D-deoxyglucose [^{18}F]FDG, is prepared in a two-step



Scheme 51. i) $[^{18}\text{F}]\text{F}^-/\text{resin}$, 100°C , CH_3CN 0.7 ml min^{-1} ; ii) 1 mol/l aq HCl , 110°C , 10 min then Biorad AG11A8

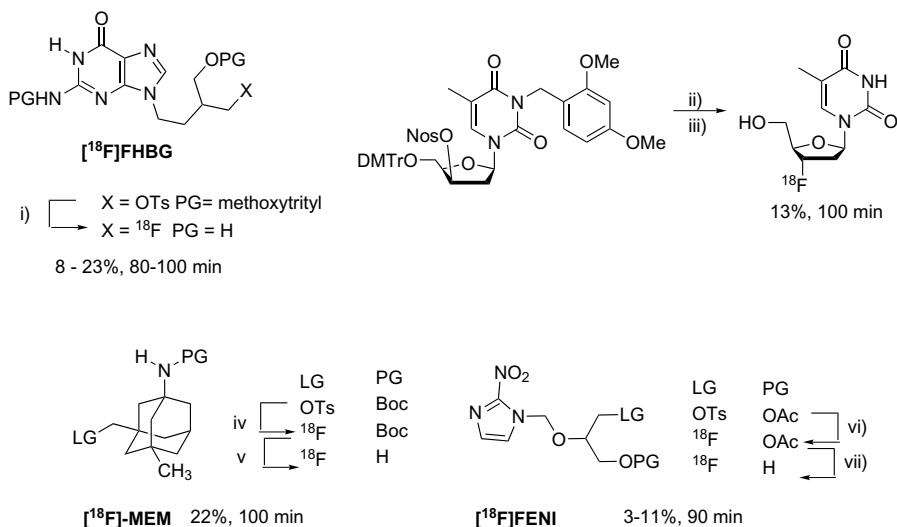
process. The nucleophilic reaction of $[^{18}\text{F}]\text{fluoride}$ with tetra-*O*-acetyl-2-triflate- β -mannose [100, 211] is followed by deprotection of the ester functions (Scheme 51). Many improvements of the original synthesis have been published. One of them is the use of quaternary pyridinium or phosphonium salts immobilized on divinylbenzene cross-linked polystyrene gel to trap the $[^{18}\text{F}]\text{fluoride}$ anion and to carry out the ^{18}F nucleophilic exchange [212, 213]. Radiochemical yields of over 70% can be reached. This method allows an easy recovery of $[^{18}\text{O}]\text{water}$, avoids contamination by toxic Kryptofix (K_{222}) or tetrabutylammonium bicarbonate and makes the automation easier. A cation-exchange resin column under flow rate control [214] can also be used to shorten the hydrolysis reaction time. Using this kind of synthesizer, time required for FDG synthesis was reduced to about 25 min (from about 45 min) and the yield was increased to about 80% (from about 30–70%).

This solid phase radiofluorination was also shown efficient for the stereospecific and semi-automated preparation of *cis*- and *trans*- $[4\text{-}^{18}\text{F}]\text{fluoro-L-proline}$, markers of the collagen synthesis [109] (Scheme 52).



Scheme 52. i) $[^{18}\text{F}]\text{F}^-/\text{quaternary 4-aminopyridinium resin}$, $85\text{--}90^\circ\text{C}$, 10 min ; ii) 0.1 mol/l aq. HCl , 120°C , 20 min ; iii) a) 1 mol/l aq. NaOH , RT , 15 min ; b) $0.2\text{ mol/l aq NaH}_2\text{PO}_4$. RCY: 16%, 80 min total reaction time

Scheme 53 gathers a few other recent examples of structurally different ^{18}F -radioligands prepared in two rapid chemical steps: a guanine derivative $[^{18}\text{F}]\text{FHBG}$ [215, 216] to image viral infection and gene therapy, a memantine derivative $[^{18}\text{F}]\text{-MEM}$, ligand of the *N*-methyl-D-aspartate receptor complex [217], $3'\text{-deoxy-[3-}^{18}\text{F}]\text{fluorothymidine}$ for visualizing cellular proliferation in vivo [218] and $[^{18}\text{F}]\text{FENI}$, a potential agent for imaging hypoxic tissues [219]. The radiofluorination conditions (cf. Scheme 53) are very similar.

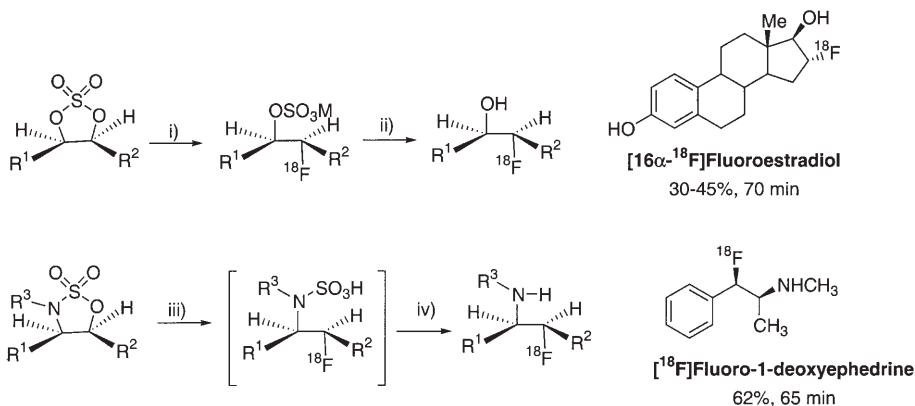


Scheme 53. i) [^{18}F]KF, MeCN, 115 °C, 20 min; ii) [^{18}F]F $^-$ /K $_2$ CO $_3$ /K $_{222}$, MeCN, 100 °C, 10 min; iii) ceric ammonium nitrate, MeCN, EtOH, H $_2$ O, 100 °C, 3 min; iv) [^{18}F]F $^-$ /K $_2$ CO $_3$ /K $_{222}$, DMSO, 125 °C, 20 min; v) 20% HCl, 5 min, 100 °C; vi) [^{18}F]F $^-$ /K $_2$ CO $_3$ /K $_{222}$, DMF, 110 °C, 5 min; vii) 0.05 mol/l NaOH, EtOH/H $_2$ O (1/1) 1 min, 40 °C

4.4.3

Ring Openings by [^{18}F]Fluoride

Reaction of [^{18}F]fluoride anion on cyclic precursors (epoxides [220], cyclic sulfonates [221], cyclic sulfamidates [222, 223]) is an efficient strategy for the stereospecific synthesis of β - ^{18}F -fluoroalcohols or β - ^{18}F -fluoroamines. Typical examples are shown in Scheme 54.

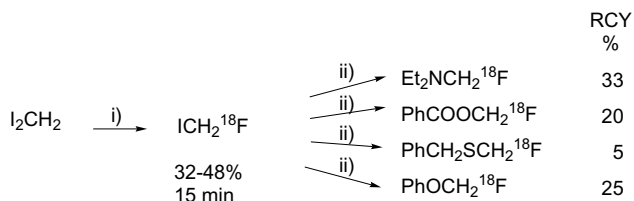


Scheme 54. i) [^{18}F]F $^-$ /K $_2$ CO $_3$ /K $_{222}$, CH $_3$ CN, 100 °C, 10 min; ii) 0.1 N HCl, 140 °C, 40 min; iii) [^{18}F]F $^-$ /K $_2$ CO $_3$ /K $_{222}$, 90 °C, CH $_3$ CN, 20 min; iv) Et $_2$ O-H $_2$ SO $_4$, RT, 25 min

4.4.4

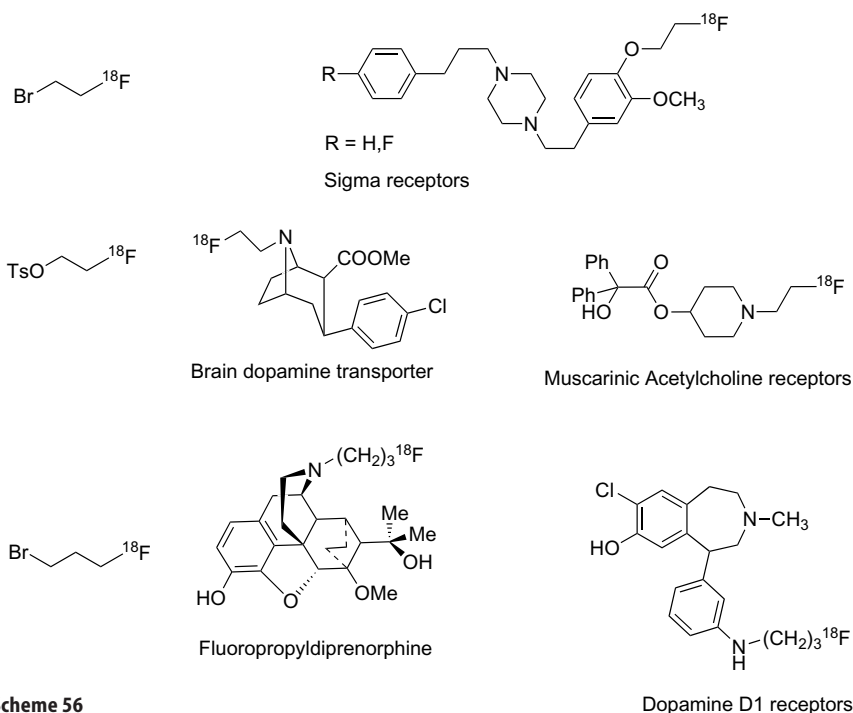
¹⁸F-Fluoroalkyl Precursors: Synthesis, Derivatives and Applications

[¹⁸F]Fluoroiodomethane, a valuable synthetic precursor for the introduction of a fluoromethyl group into radiopharmaceuticals, has been prepared from diiodomethane (Scheme 55) [224]. Its reactivity toward amine, acid, thiol or phenolate functions has also been studied.

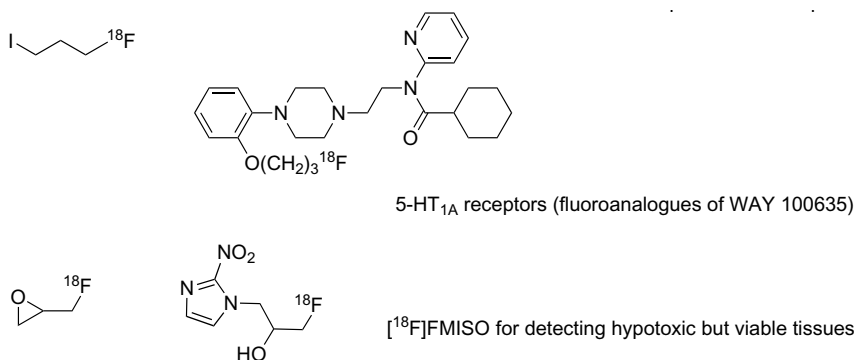


Scheme 55. i) [¹⁸F]F⁻/K₂CO₃/K₂₂₂, CH₃CN, 25 °C; ii) Et₂NH or PhCOOH or PhCH₂SH or PhONa, 80 °C, 10 min

Some of the main [¹⁸F]alkylating agents ([¹⁸F]bromofluoromethane [225], [¹⁸F]bromofluoroethane [226, 227], [¹⁸F]tosyloxyfluoroethane [228, 229], [¹⁸F]bromofluoropropane [230, 231], [¹⁸F]fluoroiodopropane [232], [¹⁸F]tosyloxyfluoropropane [233], [¹⁸F]epifluorhydrin [234, 235]) are presented with examples of their uses in scheme 56.



Scheme 56

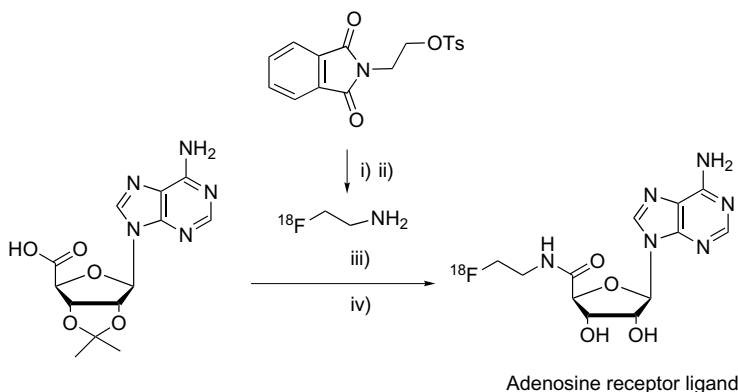


Scheme 56 (continued)

4.4.5

$[2\text{-}^{18}\text{F}]\text{Fluoroethylamine}$

$[2\text{-}^{18}\text{F}]\text{Fluoroethylamine}$ has been synthesized in a two-step sequence from a suitable phthalimide [236] and used to prepare an adenosine receptor ligand [237] (Scheme 57). This route is more efficient than the direct substitution of a tosyl group (24 % vs 1 %).

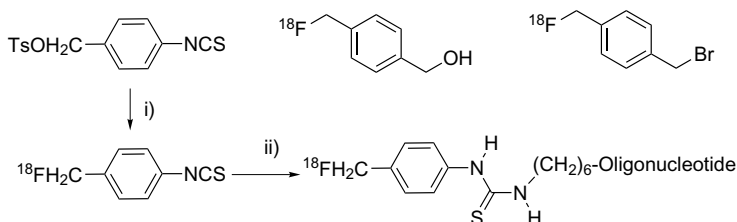


Scheme 57. i) $[\text{F}^{18}]\text{KF}/\text{K}_{222}$, CH_3CN , 78 °C; ii) H_2NNH_2 , H_2O , RCY: 27 %, 30 min total synthesis time; iii) pyridine, 60 °C, 40 min, RCY: 80 – 94 %; iv) 90 % formic acid, RCY: 30 %

4.4.6

^{18}F -Fluoromethylarene Derivatives

$[4\text{-}^{18}\text{F}]\text{Fluoromethylbenzylalcohol}$, $[4\text{-}^{18}\text{F}]\text{fluoromethylbenzylbromide}$, $[4\text{-}^{18}\text{F}]\text{-fluoromethylbenzene isocyanate}$ (Scheme 58), obtained from the corresponding bromo or sulfonyl [238, 239] derivatives, have been used mainly for the labelling of oligonucleotides [240] or peptides [241].



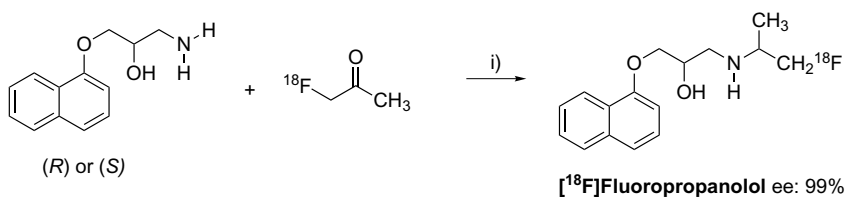
Scheme 58. i) [^{18}F]KF/K₂₂₂, CH₃CN, 110–120 °C, 15–20 min, RCY: 13–15 %, 40 min total synthesis time; ii) H₂N-(CH₂)₆-oligonucleotide, buffer, RCY: 8 %

4.4.7

^{18}F -Alpha Fluoro-Substituted Carbonyl Compounds

[^{18}F]Fluoroacetate, a rodenticide, has been prepared in 24 % yield (non-decay-corrected, in 70–90 min synthesis time) [242] from *O*-mesylglycolate using [^{18}F]tetrabutylammonium fluoride in the presence of tetrabutylammonium hydrogencarbonate.

The nucleophilic exchange [^{18}F]F[−]/TsO[−] is preferred for the synthesis of [^{18}F]fluoroacetone. This precursor, obtained in 45–60 % and 15 min, has been used for the labelling of β -adrenergic ligands [243, 244] (Scheme 59).

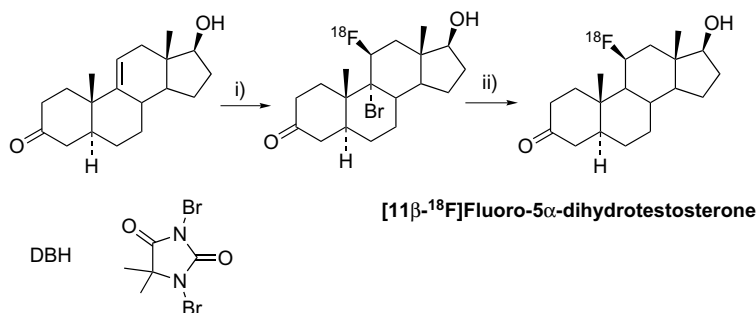


Scheme 59. i) NaBH₃CN, CF₃COOH, CH₃CN, 100 °C, 30 min, 20–25 %

4.5

^{18}F -Bromofluorinations

Nucleophilic displacement using [^{18}F]fluoride works well in aliphatic systems where reactive halides or sulfonates esters can undergo substitution at unhindered sites. In order to introduce a ^{18}F fluorine atom in a secondary or tertiary position, a two steps strategy was developed. It involves a ^{18}F -bromofluorination of alkenes, followed by reductive debromination (*n*-Bu₃SnH, AIBN). [^{18}F]BrF is usually generated in situ from [^{18}F]potassium fluoride and 1,3-dibromo-5,5-dimethylhydantoin (DBH) in sulfuric acid. This methodology was successfully applied to label steroids at the 11 β and 6 α positions [245] (Scheme 60) and to prepare [^{18}F]fluorocyclohexanes [246].



Scheme 60. i) [^{18}F]F $^-$, CH_2Cl_2 , DBH, H_2SO_4 , 40°C, 15 min; ii) $n\text{-Bu}_3\text{SnH}$, C_6H_6 , AIBN, 85°C, 20 min, RCY: 2.4–3.2%, 180 min total synthesis time

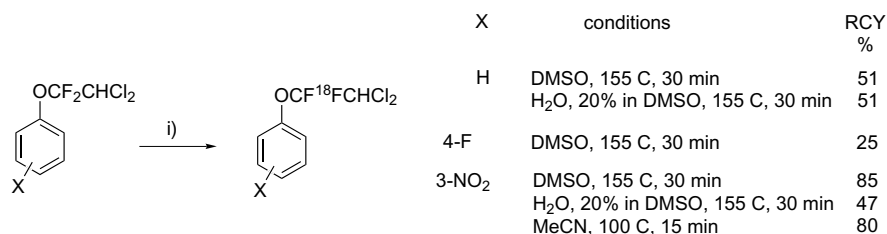
4.6

Labelling of Polyfluorocompounds

4.6.1

Labelling of a Trifluoromethyl Group

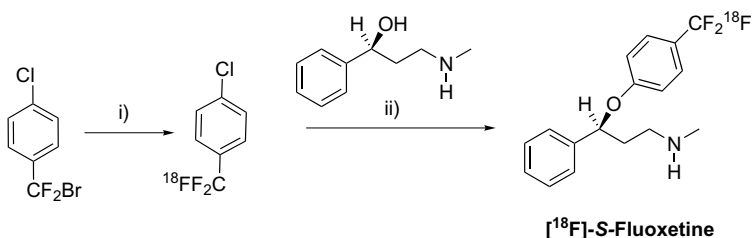
The trifluoromethyl group is found in a wide variety of pharmaceuticals. Besides its effect on the electron density, its high lipophilicity improves transport characteristics *in vivo* and facilitates lower dosage rates. ^{18}F for ^{19}F isotopic exchange reaction has been used to synthesize [1- ^{18}F]1,1-difluoro-2,2-dichloroethyl-arylethers. The substitution proceeds in good to excellent yields (>85%) and is strongly dependent on the reaction conditions. Substitution occurs even at room temperature in DMSO or in acetonitrile containing up to 20% of water (Scheme 61) [247].



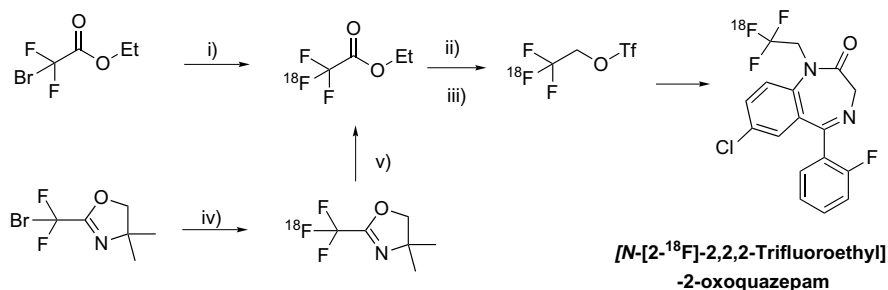
Scheme 61. i) [^{18}F]KF/ K_{222}

The radiosynthesis of [^{18}F]fluoxetine, a selective serotonin uptake inhibitor (Scheme 62), is one of the rare examples in which a Br atom has been substituted for an ^{18}F isotope [248]. It suffers from a low specific radioactivity due to decomposition of starting material.

[2- ^{18}F]Trifluoroethyltriflate, a precursor developed for *N*-alkylation of a 1,4-benzodiazepine-2-one, was synthesized in a three-step procedure from ethyl bromodifluoroacetate, the key reaction being again the nucleophilic exchange $^{18}\text{F}/\text{Br}$ to give ethyl [2- ^{18}F] trifluoroacetate (Scheme 63) [249]. Protection of the



Scheme 62. i) $^{18}\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, tetramethylenesulfone, 120°C , 4 min. RCY: 25–30 %, 60 min total synthesis time; ii) NaH, dimethylacetamide, 120°C , 45 min. RCY: 9–10 %, 150 min total synthesis time



Scheme 63. i) $^{18}\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DMSO, 125°C , 20 min; ii) AlH_3 , 40°C , 2 min, THF; iii) $(\text{CF}_3\text{SO}_2)_2\text{O}$, 2,6-lutidine; iv) $^{18}\text{F}^-/\text{K}_2\text{CO}_3/\text{K}_{222}$, DME, microwave, 50 W, 30 s, 50 % v) H_2SO_4 , EtOH

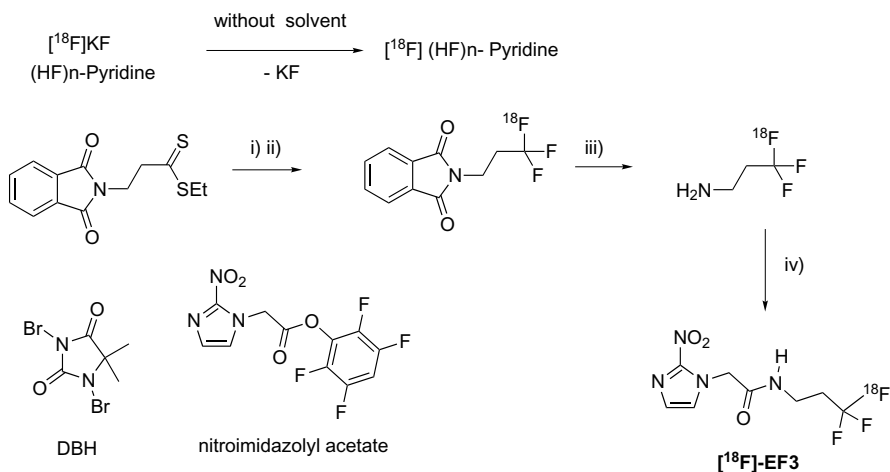
carboxylic functionality in an oxazoline ring appeared necessary to reach high specific activities [250].

Recently [251], the preparation of $[3\text{-}^{18}\text{F}]$ trifluoropropylamine was achieved in three steps from *N*-phthalimido-3-aminopropane dithioate by oxidative- ^{18}F -fluorodesulfurization, then hydrazinolysis of the resulting *N*-phthalimido- $[^{18}\text{F}]$ trifluoropropylamine with an overall chemical yield of 40 % and within 60 min from $[^{18}\text{F}]\text{HF}$ (Scheme 64). This new labelled precursor was used to synthesize the marker of hypoxia $[^{18}\text{F}]\text{-EF-3}$.

4.6.2

Labelling of Perfluorohydrocarbons

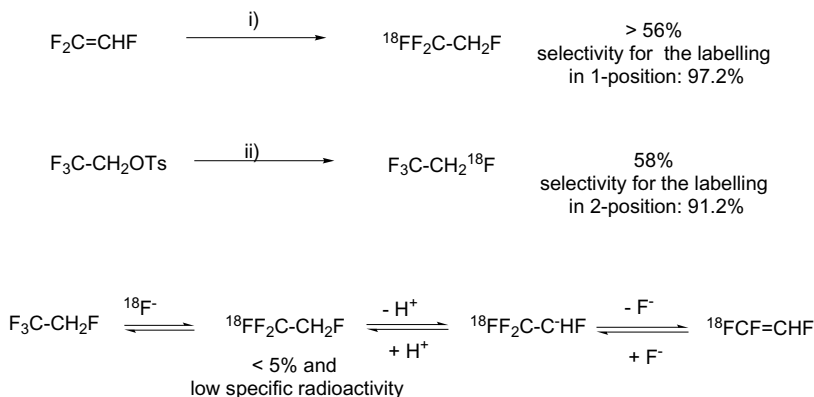
1,1,1,2-Tetrafluoroethane (HFA 134a) is a substitute for chlorofluorocarbons (CFCs) and is also being considered as a substitute drug propellant. In order to understand the fate of this compound after inhalation, HFA 134a was regioselectively labelled with fluorine-18. $[1\text{-}^{18}\text{F}]$ 1,1,1,2-Tetrafluoroethane was prepared by nucleophilic addition of no-carrier added $[^{18}\text{F}]$ fluoride to trifluoroethylene in acetonitrile at 95°C for 25 min and $[2\text{-}^{18}\text{F}]$ 1,1,1,2-tetrafluoroethane by nucleophilic displacement $^{18}\text{F}/\text{OTs}$ [252–254]. The position of fluorine-18 in labelled 1,1,1,2-tetrafluoroethanes (HFA 134a) was determined using an isotope separator. A very high selectivity was observed for the labelling in the 1-posi-



Scheme 64. i) [^{18}F]-(HF) $_n$ -Pyridine, DBH, 6 equiv F^- , CH_2Cl_2 , 10 min; ii) (HF) $_n$ -Pyridine, DBH, 48 equiv F^- , CH_2Cl_2 , 10 min; iii) N_2H_4 , H_2O , 75°C , 10 min. RCY: 40%, 60 min total synthesis time; iv) nitroimidazolyl acetate, $0-20^\circ\text{C}$, 30 min. RCY: 5% overall yield, 90 min total synthesis time

tion. However, the selectivity measured for labelling HFA 134a in the 2-position (the CH_2F group) was lower than expected. The authors suggest a possible direct exchange at the 1-position (the CF_3 group) during the labelling reaction. This study warns against any assumption of site specificity for ^{18}F -labelling based on a simple formal representation of the labelling reaction.

The specific radioactivity of the prepared [$1\text{-}^{18}\text{F}$]1,1,1,2-tetrafluoroethane is two to three orders of magnitude lower than expected for the specific radioactivity of the cyclotron-produced n.c.a [^{18}F]fluoride. This implies that free fluoride is generated in the reaction (Scheme 65).



Scheme 65. i) [^{18}F]F $^-$ /K $_2\text{CO}_3$ /K $_{222}$, CH_3CN , *tert*-BuOH, 160°C , 9 min, 20–35% (EOS); ii) [^{18}F]F $^-$ /K $_2\text{CO}_3$ /K $_{222}$, CH_3CN , 115°C , 25 min, 50%

When the addition is carried out in acetonitrile- d_3 , mono and di-deuterated [$1-^{18}\text{F}$]1,1,1,2-tetrafluoroethanes are obtained, suggesting that the solvent acts as a source of protons. This reaction was the first demonstration of an efficient addition of [^{18}F]fluoride to a fluoro-olefin: it achieves the equivalent of the addition of hydrogen [^{18}F]fluoride.

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Conclusion

In spite of its difficult chemistry, fluorine-18 remains the most attractive nuclide (half life, positron energy) for PET studies. This review has summarized the different synthetic approaches to fluorine-18 labelled radiopharmaceuticals. ^{18}F -Electrophilic fluorination remains of limited use in the preparation of radio-tracers for mapping receptor sites. It could become a method of choice if electrophilic sources of fluorine-18 become available with a high specific radioactivity. Promising results have been made in this field for the last five years. ^{18}F -Nucleophilic substitution is the most used reaction in aromatic, heteroaromatic (pyridine) and aliphatic series. No new ^{18}F -labelled fluorinating reagents have appeared over the past ten years and the efforts have been mainly concentrated on the development of small ^{18}F -labelled precursors or on the improvement of radiolabelling methods. Some efficient reactions have permitted the synthesis of ^{18}F -fluoroarenes not bearing electron-withdrawing groups. However their applications to more complex structures is still a challenge. Finally, ^{18}F -fluorine has appeared as a tool in reaction mechanism studies thanks to its isotope effect.

There are now more than 200 PET centres worldwide and, compared to single photon emission computed tomography (SPECT) or magnetic resonance spectroscopy (MRS), PET is the most sensitive technique and gives quantitative data. While there are currently a wide variety of site-specific ^{11}C - and ^{18}F -radio-tracers available for PET studies of receptors, transmitter reuptake sites and enzymes, the search for novel therapeutic approaches will continuously generate new targets for which there is no existing radiotracers. The development of new synthetic methods which proceed rapidly, reliably and in high yield is an important issue. These advances in rapid organic chemistry would contribute to PET development by improving the quantitative nature of the experiments, by giving tools for drug research or for imaging specific sites. As PET is an expensive technology, a challenge is now to synthesize radiopharmaceuticals which can be used, with the same compound, after a suitable radiolabelling, either in PET (for quantification and characterization) or in SPECT (for routine clinical studies).

6 References

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Fundamentals of Receptor-Based Diagnostic Metalloradiopharmaceuticals

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In the last decade, a large number of radiolabeled small biomolecules have been studied for their potential as radiopharmaceuticals for diagnosis and radiotherapy of various diseases. This review will focus on some fundamental aspects of receptor-based diagnostic radiopharmaceuticals, including radiopharmaceutical design, receptors and receptor imaging, choice of biomolecule, and modification of pharmacokinetics.

Keywords. Radiolabeled biomolecules, Receptor ligands, Radiopharmaceuticals

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1

Introduction

Radiopharmaceuticals are drugs containing a radionuclide, and are used routinely in nuclear medicine departments for the diagnosis or therapy of various diseases. They are mostly small organic or inorganic compounds with definite composition. They can also be macromolecules such as monoclonal antibodies and antibody fragments that are not stoichiometrically labeled with a radionuclide. Diagnostic radiopharmaceuticals contain gamma-emitting isotopes for scintigraphic imaging or positron-emitting isotopes for positron emission tomography (PET). In general, diagnostic radiopharmaceuticals are used in low concentrations, in the range of 10^{-6} to 10^{-8} mol/l, and are not intended to have any pharmacological effect. The aim of the diagnostic application is the detailed description of the morphologic structure of organs and above all the testing of their physiological function through the accumulation of radiotracer. In addition, diagnostic radiopharmaceuticals provide a non-invasive method of assessing disease states and monitoring the effect of treatment.

Radiopharmaceuticals can be divided into two primary classes: those whose biodistribution is determined exclusively by their chemical and physical properties and those whose ultimate distribution is determined by their receptor binding or other biological interactions. The latter class is often called receptor-based target-specific radiopharmaceuticals. The introduction of ^{111}In -DTPA-Octreotide for the diagnosis of somatostatin receptor positive tumors has spurred the search for new target-specific radiopharmaceuticals. A large number of radiolabeled small biomolecules have been studied for their potential use in diagnosis and radiotherapy of various diseases. Many excellent review papers have recently been published covering a broad range of topics [1 – 29]. This review will focus on some fundamental aspects of receptor-based diagnostic radiopharmaceuticals, including radiopharmaceutical design, receptors and receptor imaging, choice of biomolecule, and modification of pharmacokinetics.

2

Strategies for Radiopharmaceutical Design

There are several strategies for the design of receptor-based target-specific metalloradiopharmaceuticals (Fig. 1) [11]. The integrated approach involves the replacement of part of a known high affinity receptor ligand with the requisite “unnatural” metal chelate in such a way that there are minimal changes in size, conformation, and receptor binding affinity. The metal chelate is a vital part of the receptor binding motif. Unfortunately, this approach often results in a more synthetically challenging target molecule or the loss of receptor binding affinity [16]. Apparently, the replacement of the C-C or C-heteroatom bonds with M-N or M-O or M-S coordination bonds has significant impact on the size and conformation of the targeting molecule, which are critical for receptor binding. The introduction of the metallic core can also change the lipophilicity of the receptor ligand.

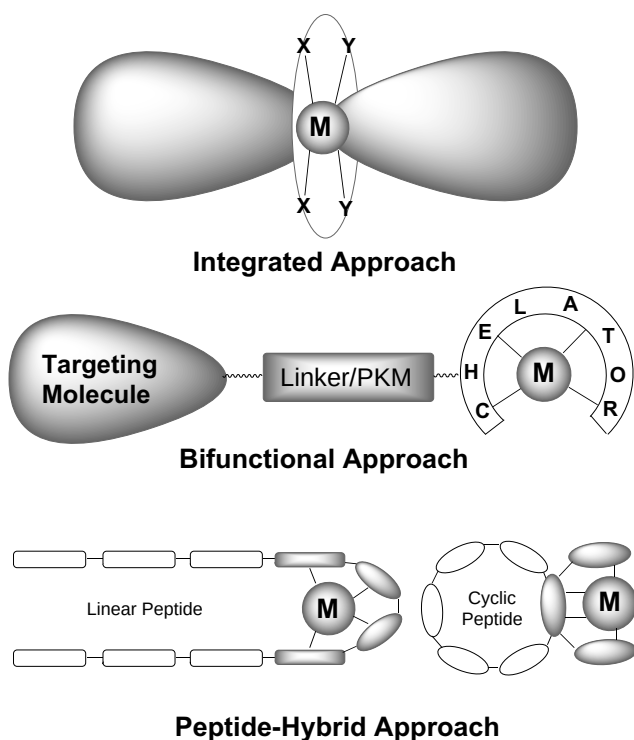


Fig. 1. Schematic representation of three approaches in radiopharmaceutical design

The bifunctional approach uses a high affinity receptor ligand as the targeting biomolecule, a bifunctional chelator (BFC) for conjugation of the biomolecule and chelation of the radionuclide, and a linker as the pharmacokinetic modifier (PKM) [11]. The targeting biomolecules can be macromolecules, such as antibodies or antibody fragments, small peptides, or non-peptide receptor ligands. The choice of the BFC is determined by the nature and oxidation state of the metal ion. It should be noted that the biomolecule is only one part of the radiopharmaceutical. The PKM and metal chelate are equally important. If the radiolabeled receptor ligand does not have a good pharmacokinetic profile, it is still difficult to develop into a product for routine clinical use even if the targeting biomolecule has high receptor binding affinity and specificity.

In the bifunctional approach, the metal chelate is often far enough from the receptor-binding motif to minimize possible interference with receptor binding. This is the more popular approach for the development of receptor-based target-specific radiopharmaceuticals, in part, due to the likelihood of retaining the receptor binding affinity with a careful selection of the BFC for the radiolabeling. Most of the receptor-based target-specific diagnostic radiopharmaceuticals (Fig. 2) approved or under clinical investigation use the bifunctional approach.

In the peptide-hybrid approach, the radionuclide (^{99m}Tc or ^{188}Re) is chelated by a tripeptide sequence (such as Gly-Gly-Gly, Cys-Gly-Gly, or Cys-Gly-Cys)

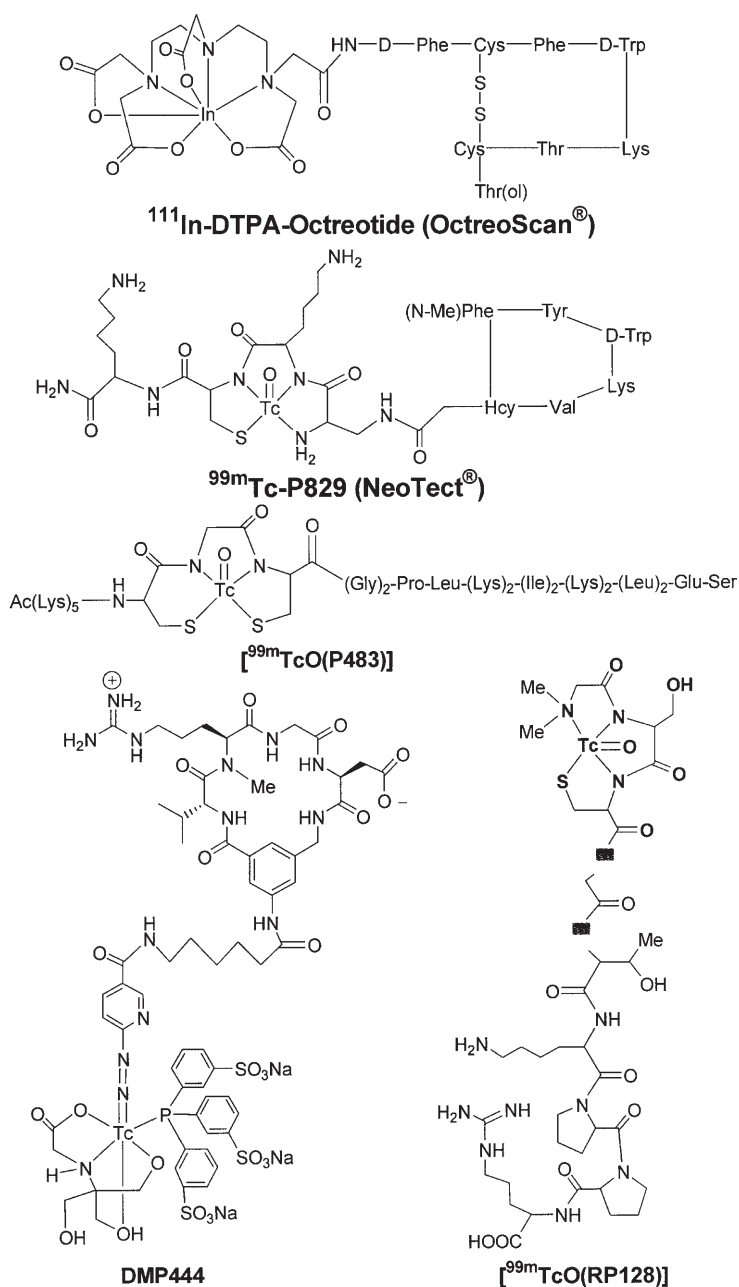


Fig.2. Selected receptor-based radiopharmaceuticals using the bifunctional approach

containing an N_4 , N_3S , or N_2S_2 donor set. The tripeptide sequence can be part of either a long linear polypeptide or a cyclic peptide backbone. The radionuclide can also be incorporated as part of a macrocyclic peptide framework. The unchelated linear peptide has a relatively low binding affinity for the intended receptor and becomes a high affinity receptor ligand once it bonds to the metal ion. The chelation of the radionuclide results in a constrained and metal-containing macrocyclic peptide with increased receptor binding affinity. This approach has been used to prepare ^{99m}Tc - and ^{188}Re -labeled α -melanocyte-stimulating hormone peptide analogues [29, 30].

3 Receptors and Receptor Imaging

Receptors are defined as entities that can recognize a receptor ligand with high affinity and selectivity. The receptors are proteins embedded in the cell membrane which separate the extracellular environment from the intracellular compartment. A receptor is often characterized by its biological properties, including high ligand affinity, specificity, saturability, and distribution in relation to the physiologic response [31, 32]. Receptors are different from enzymes even though they are all proteins. The most important property of an enzyme is its catalytic activity, which is influenced by the nature of substrate, temperature, and pH. Receptors have no catalytic activity, but rather produce a physiologic change directly. In addition, enzymes are often found in much higher concentration and to have higher capacity than receptor systems.

Receptor imaging relies on the localization of a radiolabeled compound that binds to receptors with high affinity and specificity. Many important biological functions are controlled by receptors. It is also believed that changes in receptor concentration are related to certain disease states. All of these provide the biological foundation for receptor imaging, a non-invasive way of assessing biochemical changes in disease states and monitoring the effect of treatment of various diseases. The high specificity of receptor binding results in selective uptake and distribution of the radiolabeled receptor ligand at the tissues that are known to contain substantial concentration of the target receptor. It is this high receptor binding affinity and specificity that makes receptor imaging advantageous over traditional scintigraphic imaging using simple ^{99m}Tc complex radiopharmaceuticals and over other diagnostic modalities such as MRI.

Receptor binding is the first step in both pharmacological and hormonal signaling, and is often followed by a cascade of biological effects after binding to receptor. Some receptor ligands bind to the receptor site, compete with the “native ligand”, and cause a cascade of biochemical effects. They are usually called “agonists”. Some receptor ligands bind to the receptor site with comparable binding affinity, and cause no physiologic and biochemical reactions. They are considered as antagonists. For a receptor ligand to be useful as a targeting molecule it should have very high receptor binding affinity with IC_{50} value in the nanomolar range. Low receptor binding affinity will result in low radioactivity uptake and short residence time at the receptor site.

The location of the receptor determines the design of the radiopharmaceutical, the selection of the BFC, and the degree of tolerance of the receptor ligand to chemical modification such as the use of different PKM groups. If the receptors are intracellular, the radiolabeled receptor ligand has to cross the cell membrane to reach the receptor site. In this case, the receptor ligand is usually small and has little tolerance for chemical modifications [16]. The metal chelate is most likely neutral unless a specific intracellular transport mechanism is accessible. If the receptor is extracellular, the radiolabeled receptor ligand will not have to cross the cell membrane to interact with the receptor. In this situation, there is usually a higher degree of tolerance towards chemical modifications [17]. The metal chelate can be neutral or charged depending on the pharmacokinetic requirements. Most of new radiopharmaceuticals approved or under clinical investigation are targeted to extracellular receptors [11].

The choice of a receptor system depends on the targeted disease and the relationship between the disease and the expression of receptors. The receptor concentration has to be sufficiently high. There is no value in designing a radiolabeled receptor ligand for a clinically important disease if the concentration of the receptor is not sufficient for external imaging. The receptor must be able to recognize the receptor ligand with high affinity and specificity. Otherwise, the new radiopharmaceutical will not be able to provide the necessary target uptake and target-to-background ratio, and it will be difficult to differentiate various diseases or disease states with high sensitivity and specificity.

The clinical application of a new radiopharmaceutical for receptor imaging will depend ultimately on its sensitivity, the counts recorded for a given amount of activity in a specific foci [22]. There are many factors that could influence the sensitivity of the radiopharmaceutical. In general, the higher receptor concentration results in a greater percentage of the injected dose of the radiopharmaceutical in the diseased tissue, and gives better sensitivity [33]. Receptor heterogeneity results in a lower number of binding sites for the radiolabeled receptor ligand and leads to decreased accumulation of radioactivity at the diseased tissue and a loss of sensitivity for detection [33]. Internalization of receptors often results in higher target uptake and longer duration of the radioactivity. It is very important to remember that only a small portion of the radiopharmaceutical is delivered to the receptor site. The higher the amount of radiopharmaceutical delivered to the receptor site, the better the sensitivity of the radiopharmaceutical will be [11].

4

Targeting Biomolecules

The targeting biomolecules can be macromolecules such as antibodies and antibody fragments, which are known for their high binding affinity and specificity for their antigens. They can also be small biomolecules, including peptides, peptidomimetics, and non-peptide heterocycles. They can be native biomolecules such as hormones and growth factors, which play a significant role in the cell signal transduction, cell proliferation, and cell growth. They can also be synthetic small biomolecules that mimic the receptor-binding motif of a native macromolecule such as antibody.

The selection of a specific class of biomolecules for receptor-based imaging is largely dependent on the targeted disease, the receptor population at the diseased tissue, receptor-binding affinity, internalization of the receptor, and the size and lipophilicity of the targeting biomolecule. In general, the selected receptor ligand should be an antagonist and have high receptor binding affinity with IC_{50} values preferably in the nanomolar or sub-nanomolar range. Internalization of the receptor is beneficial for the accumulation of radiolabeled receptor ligand at the diseased tissue. Small receptor ligands often give much better mobility and accessibility to the receptor sites and exhibit faster blood clearance than macromolecules.

4.1

Antibodies and Antibody Fragments

In the last two decades, monoclonal antibodies and their fragments have been studied for their potential applications in both diagnostic and therapeutic nuclear medicine. Radiolabeled monoclonal antibodies are often referred as “magic bullets”. Although considerable progress has been made in this area [34–38], clinical studies with radiolabeled antibodies and antibody fragments have often demonstrated limited accumulation in the target, and relatively slow blood clearance due to their high molecular weight, resulting in only modest target-to-background ratios. The lack of effectiveness of radiolabeled antibodies is attributed in part to the inaccessibility of macromolecules to the tumor cells in solid masses and in part to the heterogeneous distribution of tumor associated antigens on the tumor surface [15]. There is little doubt that antibodies and antibody fragments have high receptor binding affinity and high specificity. However, if the radiolabeled antibodies cannot be delivered to the target efficiently and clear from the non-target organs fast enough, they are not good targeting molecules for receptor imaging or radiotherapy.

4.2

Growth Factors

There are a number of polypeptides called “growth factors” which are directly and specifically involved in stimulating cell division [39]. These growth factors are secreted by cells and interact with specific membrane-bound glycoprotein receptors. Growth factors and their receptors are important determinants of normal and pathological growth reactions. Through signal transduction pathways, receptor binding of peptide molecules triggers the production of intracellular downstream effector molecules, which ultimately cause cell proliferation. Very often the receptor ligand binding can activate one or more plasma membrane-associated transducing systems which generate intracellular signals. Upon binding the receptor, the specificity and magnitude of the response is believed to be mainly due to the type and abundance of the receptor rather than the availability of the growth factor.

Growth factor receptors located on the cell surface are often water-soluble because the peptide growth factors cannot diffuse across the plasma membrane

[25, 39]. Growth factors exert their effect by binding to their receptors, generating tyrosine kinase activity. A cell may alter its receptivity to a peptide growth factor by “up-regulation” or “down-regulation” of the number of surface receptors, or alter the affinity of the receptor for the growth factor, or receptor occupancy may result in a quantitative change in the intracellular signal [39]. When tumor cells undergo proliferation and invasion, certain growth factor receptors are highly up-regulated compared to the normal cells. Some of them are continuously internalized and recycled back to the cell surface. This forms the biological foundation for diagnostic imaging using radiolabeled peptide growth factors.

It should be noted that normal cells also require growth factors for differentiation and proliferation. Radiolabeled receptor ligands can also bind to normal tissues via these receptors, resulting in high background activity and radiation burden to normal organs. This would appear to contraindicate the use of growth factor receptors as molecular targets. However, malignant cells often show over-expression of growth factors since oncogenes encode either growth factor or their receptors [26, 40]. Oncogenic transformation can be characterized by gene amplification, which can also lead to over expression of growth factor receptors on malignant cells, or alterations in gene structure, resulting in mutant gene products characterized by re-arrangements, point mutation, and deletions. Both the receptor over-expression and mutated receptor generation offer opportunities for the enhanced tumor uptake [26, 40].

4.3

Peptide Growth Hormones

Metabolic processes, cell growth and differentiation are regulated by hormones, which are produced by specific glands [20, 41]. Hormones can be divided into four main groups: peptides, steroids, iodothyronine, and neurotransmitters. The regulation of metabolic processes, cell growth and differentiation is achieved by an interaction of hormones with specific cellular receptors located on the cell surface, cytoplasm or in the nucleus. The interaction between the hormone and its receptor must elicit a signal that is transmitted to the regulatory steps of a metabolic pathway in order to result in specific metabolic effect. Each receptor represents an element that serves two functions: (1) recognition and binding of a specific hormone and (2) transformation of the receptor binding into a signal that modifies cellular metabolism and/or growth [25, 41].

The native ligands for these receptors are often polypeptides that have regulatory functions in several normal organs, mainly in brain, pituitary, gut, endocrine system. These peptide receptor ligands have been named accordingly, i.e., neuropeptides, gastrointestinal peptides, regulatory peptides, or hormones. It is possible to prepare synthetic molecules, similar to native hormones that bind to a hormone receptor. These synthetic biomolecules are often termed “analogs”, which can express an action similar to that of the native hormone (agents) or determine effects opposite to those induced by the hormone (antagonist). These synthetic analogs can be very useful for the diagnosis of certain endocrine tumors.

Table 1. Peptide receptors expressed by human primary tumors or tumor cell lines (adapted from references [41–43])

Receptors	Tumor type
LHRH receptors	Breast tumor, prostate tumors
Bombesin/GRP	SCLC, colonic carcinomas, glioblastomas
Gastrin receptors	Gastrin carcinomas, colonic carcinomas
VIP receptors	Colonic carcinomas, gastrin carcinomas, pancreatic carcinomas, lymphomas
CCK receptors	SCLC, pancreatic cancers, neuroblastomas, meningiomas, medullary thyroid carcinomas
Tachykinin receptors	Colonic carcinomas, astrocytomas, lymphomas
Opioid receptors	SCLC, neuroblastomas, breast tumors, thymomas
Somatostatin receptors	Neuroendocrine tumors, lymphomas, renal cell carcinoma, breast tumors (50%), tumors of nervous systems

A variety of peptide receptors have been discovered in a large number of human tumor lines and in human primary tumors. These include receptors for luteinizing-hormone releasing hormone (LHRH), bombesin or gastrin release peptide (GRP), vasoactive intestinal peptides (VIP), cholecystokinin (CCK), substance P (SP), opioids, and somatostatin. Table 1 shows some selected peptide receptors expressed by human primary tumors [20, 39–43]. While most of the receptors listed have been identified on tumor cell lines, somatostatin receptors were characterized in both primary human tumors and their metastases. Table 2 lists selected examples of radiolabeled peptide growth hormones as diagnostic radiopharmaceuticals. The radiolabeled regulatory peptides include somatostatin analogs [44–53], bombesin analogs [54–57], CCK-B peptides [58–60], and vasoactive intestinal peptide (VIP) [61–64] for tumor imaging.

Somatostatin is a regulatory hormone that plays an inhibitory role in the normal regulation of several organs, including the central nervous system, the hypothalamus and pituitary, the gastrointestinal (GI) system, and the exocrine and pancreas. These actions are mediated through high affinity membrane receptors that occur in various densities throughout these tissues. Somatostatin

Table 2. Selected examples of radiolabeled receptor ligands as radiopharmaceuticals

Receptor ligand	Receptor	Targeted disease	Reference
Somatostatin analog	Somatostatin	Tumor (neuroendocrine)	44–53
VIP peptides	VIP	Tumor	61–64
Bombesin Analogs	Bombesin/GRP ^a	Tumor	58–60
α -MSH ^b	α -Melanocyte	Tumor (breast, prostate)	29, 30
CCK-B peptides	Cholecystokinin	Tumor	58–60

^a Gastrin release peptide.^b Melanocyte stimulating hormone.

receptors are over-expressed in a variety of human tumors. The neuroendocrine tumors show the highest incidence of somatostatin receptor over-expression.

Octreotide is a somatostatin analog that, like the native hormone, inhibits growth hormone excretion, but it has a longer half-life for the prolonged inhibitory effect in target organs expressing somatostatin receptors. Using radiolabeled somatostatin analogs, tumors positive with somatostatin receptors can be visualized using planar scintigraphy, SPECT or PET. ^{111}In -DTPA-octreotide has become one of the most commonly used radiopharmaceuticals for tumor imaging. A $^{99\text{m}}\text{Tc}$ -labeled somatostatin analog, $^{99\text{m}}\text{Tc}$ -P829 (NeoTect) [48, 51–53], has recently been approved by FDA as a new radiopharmaceutical for imaging tumors.

It should be noted that some normal organs, including the pituitary and the exocrine and endocrine pancreas, also express somatostatin receptors. That may explain the fact that most radiolabeled somatostatin analogs all show high accumulation in the pituitary and the pancreas. For diagnostic radiopharmaceuticals, the amount of injected radioactivity is relatively low so that the accumulation of radioactivity may not cause significant irreversible damage to these normal organs.

4.4

Peptides as Molecular Recognition Units for Receptor Binding

Most antibodies interact with antigens via one or more small peptide sequences. The interactions include ionic, hydrogen bonding, and Van der Waal forces. It is now possible to synthesize small peptides that have the same amino-acid sequences as the second or hypervariable regions of the heavy chain of the antibody. Small peptides are necessary elements in many fundamental biological processes and in many cases the affinities of small peptides for their receptors are significantly greater than that of antibodies [11, 25]. They can also tolerate harsher chemical conditions for modification or radiolabeling. Small peptides are easy to synthesize and modify, less likely to be immunogenic, and can have rapid blood clearance. The faster blood clearance results in adequate T/B ratios earlier so that it is practical to use $^{99\text{m}}\text{Tc}$, which is the preferred radionuclide for diagnostic nuclear medicine. All these factors make small peptides excellent candidates for the development of target-specific radiopharmaceuticals.

There are also disadvantages in using small peptides as targeting biomolecules. The first obstacle is the rapid proteolysis in plasma by endogenous peptidases and proteases. The peptides must be engineered to inhibit their enzymatic metabolism in order to increase their biological half-life in plasma. There are several ways to block the enzymatic destruction of peptides, including the use of D-amino acids, substitution of the peptide bonds, replacement of the disulfide bond with a thioether linkage, and insertion of unusual amino acids. Another challenge in using small peptides as targeting biomolecules is the loss of binding affinity when conjugated to the metal chelate. In many cases, the peptide molecule contains only 4–6 amino acid residues. The attachment of the metal chelate has a significant contribution to the overall size and molecular weight of the radiopharmaceutical, and may cause conformational changes in the peptide receptor binding sequence. Whenever possible, the attachment of the metal

Table 3. Selected examples of radiolabeled peptides as diagnostic radiopharmaceuticals

Receptor ligand	Receptor	Targeted disease	References
Cyclic RGD peptides	GPIIb/IIIa/platelets	Thrombosis	94–106
Fibrin α -chain peptides	Fibrin	Thrombosis	114
CSVTCR	TSP ^a /platelets	Thrombosis	115
RGD-peptidomimetic	GPIIb/IIIa/platelets	Thrombosis	109–113
Tuftsins antagonists	Tuftsins	Infection	80–83
fMLF	Neutrophil/WBC ^b	Infection	71–79
Antimicrobial peptides	Neutrophil	Infection	84–86
RGD-containing peptides	Vitronectin ($\alpha_v\beta_3$)	Tumor	89–93
YIGSR	Laminin	Tumor	87, 88

^a Thrombospondin.

^b White blood cell.

chelate should be kept apart from the receptor binding sequence of the peptide, and the size of the metal chelate should be minimized by the choice of an appropriate BFC.

Very few linear peptides bind to their target with high affinity (0.1–10 nmol/l) and high specificity [65, 66]. Linear peptides are often degraded rapidly in serum by protease. The combination of low receptor binding affinity, lack of specificity, and rapid degradation makes linear peptides non-optimal targeting molecules for the development of receptor-based diagnostic radiopharmaceuticals [18]. It has been shown that cyclization of RGD-containing peptides via various linkers, including S-S disulfide, thioether-S, and rigid aromatic rings or other heterocycles, leads to increased receptor binding affinity and selectivity [18, 67–70]. However, there is little evidence to show that any particular mode of cyclization will result in high affinity receptor binding. One thing that is clear is that the cyclic peptides with the conformation at the receptor-binding motif similar to that of the natural receptor ligand are likely to have higher receptor binding affinity and better selectivity.

Table 3 shows some selected peptide motifs which have been used for the development of receptor-based diagnostic radiopharmaceuticals. These include ^{99m}Tc-labeled chemotactic peptides [71–79], tuftsins receptor antagonists [80–83], and antimicrobial peptide [84–86] for imaging focal sites of infection, laminin receptor-binding YIGSR [87, 88], vitronectin receptor antagonists [89–93] for imaging tumor, cyclic GPIIb/IIIa receptor antagonists [94–113], fibrin α -chain peptide [114] and thrombospondin receptor analog [115] for imaging thrombi.

4.5

Peptide Receptor Ligands from Phage Display Peptide Library

There has been substantial progress in the construction of phage-displayed peptide libraries and in screening methods with the libraries to isolate peptide receptor ligands [66, 116–119]. Phage display peptide libraries offer opportunities to characterize peptide binding specificity of important proteins, such as

Table 4. Selected examples of short peptide motifs derived from phage display peptide libraries (adapted from [116])

Peptide sequence	Proteins receptor
RGD, NGR, CRRETAWAC	$\alpha_5\beta_1$ Integrin
RGD, KGD	$\alpha_{IIb}\beta_3$ (IIb/IIIa) Integrin
RGD, RGD-4C	$\alpha_v\beta_3/\beta_5$ Integrins
VSWFSHRYSPFAVS	$\alpha_6\beta_1$ Integrin
GYEDGYAGPILYN	$\alpha_M\beta_2$ (IIb/IIIa) Integrin
XXXX*XXX, Y* ^{E/M} NW	SH2 domain
RPLPP, APPLPPR	SH3 domains

Single letter abbreviations of amino acids: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe, G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; X = variable; Y = Tyr; Y* = phospho-Tyr.

antibodies involved in inflammatory reactions [119, 120] or integrins that mediate cellular adhesion [121]. Identification of a class of potential peptide lead compounds often leads to the development of novel peptidomimetic drugs with better pharmacokinetics and bioavailability for therapeutic pharmaceuticals. For radiopharmaceuticals, however, both peptides and peptidomimetics are equally important as long as they have the required receptor binding affinity and selectivity.

Table 4 lists some examples of proteins that bind short peptide motifs derived from phage display peptide libraries [66, 116 – 121]. Of particular interest is the use of phage display peptide libraries for screening peptides that bind to integrins. Each of the integrins ($\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$) has a slightly different peptide binding specificity and favors a distinct cyclic RGD-containing peptide. For example, peptides selected for binding to $\alpha_5\beta_1$ contain CRGDGWC while those for $\alpha_v\beta_5$ have the CDCRGDCFC (RGD-4C) peptide sequence. Sometimes, unexpected novel peptide receptor ligands are discovered using the phage display. For example, the peptide CRRETAWAC was found to be very specific for $\alpha_5\beta_1$ with no binding to other members of the integrin family.

4.6

Non-Peptide Receptor Ligands

In addition to numerous peptide receptor ligands, many non-peptide receptor ligands have also been studied and used as targeting biomolecules for diagnostic metalloradiopharmaceuticals. Table 5 lists some selected examples of radio-labeled non-peptides as diagnostic radiopharmaceuticals. These include leukotriene B₄ (LTB₄) receptor antagonists for imaging focal sites of infection [122 – 124], neurotransmitter receptor antagonists and dopamine transporter brain imaging [125 – 129], folate receptor antagonists [130, 131], vitronectin receptor antagonists [132], and tyrosine kinase inhibitors for tumor imaging [133].

Table 5. Selected non-peptide receptor ligands for diagnostic radiopharmaceuticals

Receptor ligand	Receptor	Targeted disease	Reference
LB ₄ Antagonists	LTB ₄ /WBC ^a	Infection/inflammation	122 – 124
Folate analogues	Folate receptor	Tumor	130, 131
VnR ^b antagonists	vitronectin receptor	Tumor	132
TKI ^c	TKI receptor	Tumor	133
^{99m} Tc-TRODAT	Dopamine transporter	Parkinson's disease	125 – 128
^{99m} Tc-Technepine	Dopamine transporter	Parkinson's disease	129

^a White blood cell.^b Vitronectin receptor.^c Tyrosine kinase inhibitors.

5 Pharmacokinetics and Pharmacokinetic Modifiers

For radiopharmaceuticals, the term “pharmacokinetics” refers to the distribution and elimination of the radionuclide following administration of the radiopharmaceutical. Figure 3 shows possible excretion routes and bio-interactions of a radiopharmaceutical in the biological system. Renal excretion is always preferred since the radiopharmaceutical can be cleared rapidly from blood without significant metabolic degradation. Lipophilic molecules tend to be excreted via hepatobiliary system and are often metabolized by enzymes in the liver. This will create a significant problem due to slow clearance and radiation burden to liver and GI tracts. The biological interactions include receptor binding, protein binding, and chemical reactions of the radiopharmaceutical with metal ions or native chelators. While receptor binding is a necessary step for the radiolabeled recep-

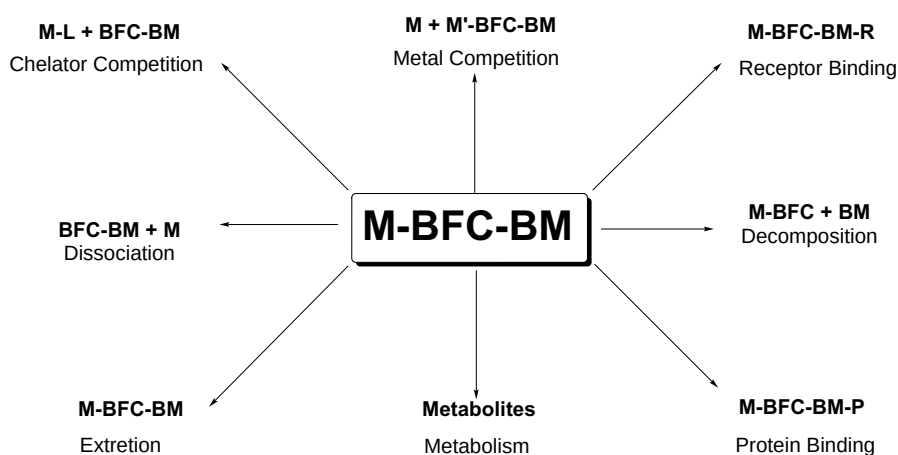


Fig. 3. Distribution and elimination of a radiopharmaceutical following administration (M-BFC-BM: M=radionuclide; M'=metal ion in the blood stream; BFC=bifunctional chelator; BM = biomolecule; L = competing chelator)

tor ligand, protein bonding is often detrimental, and has dramatic impact on the blood clearance, renal excretion [134], and the target-to-background ratio. The competition between native chelators, such as amino acids, and metal chelate may cause early release of the radionuclide and decrease the target uptake.

The main pharmacokinetic consideration is that the radiolabeled receptor ligand has a high target uptake with diagnostically useful target-to-background ratio in a short period of time. To achieve this goal, the radiopharmaceutical should have a fast blood clearance via renal excretion. The time for the radiolabeled receptor ligand to reach the target should be short. Otherwise, it will take a long time to get diagnostically useful images. The receptor-binding rate should be fast and the dissociation rate slow. In this way, the accumulation of radioactivity at targeted tissues can be maximized. The new radiopharmaceutical must have a rapid renal clearance to avoid accumulation of radiotracer in the GI tract, which may obscure the visualization of abdominal targets.

The pharmacokinetics of a radiopharmaceutical can be systematically altered by chemical modification of the targeting biomolecule or metal chelate, and the use of a pharmacokinetic modifying linker. The chemical modification of a biomolecule can be achieved by introducing various hydrophilic or lipophilic groups onto the side-chains of the targeting biomolecule. Sometimes a peptide sequence, such as polyaspartic acid, can be used to improve the hydrophilicity. The chemical modification of the metal chelate can be achieved using BFCs with different charge and hydrophilicity.

The choice of the linker depends upon the pharmacokinetic requirements for the radiopharmaceutical. Figure 4 shows several types of linkers (cationic, anionic, or neutral). The linker can be a simple hydrocarbon chain to increase lipophilicity, a peptide sequence (polyglycine, polyserine, or polyaspartic acid) to improve the hydrophilicity and renal clearance, or a poly(ethyleneglycol) linker to slow extraction by the hepatocytes. It has been reported that linker groups have significant effect on biodistribution of ^{99m}Tc -labeled biomolecules [95, 96]. Metabolizable linkers have been used for ^{111}In -labeled somatostatin analogs [135]. A tetrapeptide linker Gly-Gly-Gly-L-(p-NO₂)-Phe-CONH₂ that is cleaved between Gly and Phe residues has been used to modify pharmacokinetics of ^{90}Y -labeled antibodies [136, 137].

6

Receptor-Based Radiopharmaceuticals and Molecular Nuclear Medicine

In the last decade, research in the radiopharmaceutical industry has focused on receptor-based metalloradiopharmaceuticals for *in vivo* characterization of diseased tissues and monitoring biological changes in diseased tissues at the molecular level. There are a number of reasons for the direction change. These include competition from other imaging modalities for anatomic definition, the success in measuring biochemical pathways using PET, and improvements in both PET and SPECT (single photon emission computed tomography). Imaging modalities, such as X-ray computed tomography (CT), ultrasound (US), and nuclear magnetic resonance imaging (MRI), are better suited for anatomic analysis of diseased tissues. However, it is very difficult to use these modalities

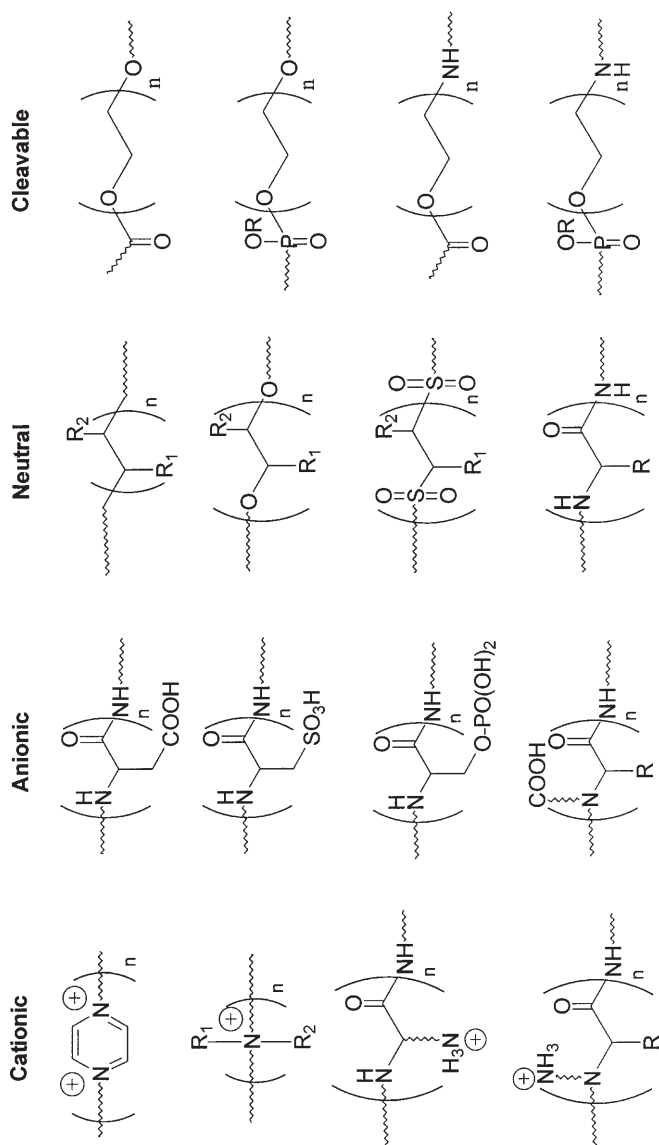


Fig. 4. Linkers for modification of pharmacokinetics of radiopharmaceuticals

to monitor biochemical changes in the diseased tissues mainly due to the fact that MRI and CT often require much higher concentration of the contrast agent to achieve a reasonable target-to-background ratio.

Development of a receptor-based target-specific radiopharmaceutical requires a good understanding of fundamentals in biology, chemistry, and nuclear medicine. As many advances in science have led to the characterization of cellular structure and function, the evolving face of molecular nuclear medicine has mandated that we all to some extent become a molecular biologist in order to understand the fundamental biochemistry of receptor imaging. On the other hand, studies of various diseases by molecular imaging will lead to more detailed characterization of cellular structure and function, and to further advances in molecular biology.

Molecular nuclear medicine is not just limited to diagnostic applications of radiopharmaceuticals. It also involves the use of receptor-based radiopharmaceuticals for the treatment of diseases such as cancer. Therapeutic radiopharmaceuticals are often labeled with high-energy β -particle emitting radionuclides and offer opportunities for selective in vivo cell destruction. Early experience with ^{90}Y -labeled somatostatin analogs has been extremely encouraging for the treatment of somatostatin receptor positive neuroendocrine tumors [2–4]. One can always hope that these new receptor-based therapeutic radiopharmaceuticals can find wide use for the treatment of cancer even though they may not be the elusive “magic bullet”.

7

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