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Glycoscience and Microbial Adhesion

Volume Editors:
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Preface

In the post-genomic era, the chemistry and biology of carbohydrates have been recognized as among the most important research areas within the life sciences. It has been understood that glycosylation of primary gene products is regularly a basic fundamental of their biochemical features and that the glyco environment on eukaryotic cell surfaces, the so-called glycocalyx, is of utmost importance for prosperity and adversity of cell development and cell communication. As any molecular contact with a cell primarily concerns its nano-dimensioned sugar coat, glycobiological research has to be concerned with the mechanisms of such interactions as well as the consequences for cell biology. Wherever cells are in contact with the outside environment, as for example in the case of epithelial cells, their sugar decoration is utilized by microbes to adhere to and colonize the cell surface sometimes, eventually to form stable biofilms, which can be most detrimental to the host cell. This microbial adhesion process is mediated by a well orchestrated assembly of molecular interactions, among which carbohydrate-specific adhesion plays a decisive role.

This issue is dedicated to the role of carbohydrates in microbial adhesion processes and the research engendered within the glycosciences into this important biomedicinal matter. An interdisciplinary roundup of seven contributions has been combined to highlight glycoscience and to give an ample overview of the state-of-the-art of microbial adhesion research.

The field is introduced in the first chapter, which points out the complexity of the adhesion process involving multiple adhesins on a single microbe and their respective target receptors on host cells, and discusses the possibility of controlling bacterial infections via preventing the adhesion or invasion stages of microbial pathogenesis. The latter issue touches on a vision of an anti-adhesive therapy, which in our opinion receives some validation on the basis of the contributions collected herein.

The second chapter is a comprehensive summary of the polyvalent molecular architectures which have been conceived and synthesized in order to interfere in microbial adhesion processes on cell surfaces, an event where multivalent interactions most often are a prerequisite. Molecular constructs as described in this section can serve to investigate and manipulate fimbriae-mediated bacterial adhesion, as in the case of E. coli type 1 fimbriae-mediated bacterial adhesion, which is explored in all structure-biological details in the third chapter. In the following contributions in Chapters 4 and 5, more implications and reflections about bacterial adhesins and

bacterial carbohydrate recognition are conveyed, covering Gram-positive as well as Gram-negative bacteria, in Chapter 4 Streptococci and Staphylococci, and in Chapter 5, carbohydrate binding specificities of Helicobacter pylori.

In Chapter 6, "Bitter sweetness of complexity," the collected reflections on microbial adhesion are expanded by a perspective on a broader impact of glycosylation on cellular adhesion, motility and regulatory processes, paralleling the complexity of N-glycan structures on cell surfaces. It highlights particularly how structural details of *N*-glycans have been causally related to pathological scenarios, with a focus on β(1,6)-*N*-acetylglucosaminyltransferase.

In the final chapter, biofilm formation is reviewed, covering knowledge about structure and biosynthesis of polysaccharide intercellular adhesins (PIAs) which are central to biofilm formation. This comprehensive chapter explains all PIA-related principles of medical device-associated infections.

It is our hope, that this collection of expert articles, ranging from structural chemistry and structural biology to biochemistry and medicine, will be a stimulation and motivation for our colleagues in the life sciences. At the same time, we hope that these reflections on microbial adhesion will awake interest in and promote understanding of the complex processes associated with the glycocalyx and the multifaceted interactions between the host cell and its "guest," as well as the biological consequences resulting from this mutual interplay.

Spring 2009, Kiel and Dublin Thisbe Lindhorst and Stefan Oscarson

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Ins and Outs of Microbial Adhesion

Mumtaz Virji

Abstract Microbial adhesion is generally a complex process, involving multiple adhesins on a single microbe and their respective target receptors on host cells. In some situations, various adhesins of a microbe may co-operate in an apparently hierarchical and sequential manner whereby the first adhesive event triggers the target cell to express receptors for additional microbial adhesins. In other instances, adhesins may act in concert leading to high avidity interactions, often a prelude to cellular invasion and tissue penetration. Mechanisms used to target the host include both lectin-like interactions and protein–protein interactions; the latter are often highly specific for the host or a tissue within the host. This reflective chapter aims to offer a point of view on microbial adhesion by presenting some experiences and thoughts especially related to respiratory pathogens and explore if there can be any future hope of controlling bacterial infections via preventing adhesion or invasion stages of microbial pathogenesis.

Contents

1 Background

Adhesion to mucosal epithelial cells is generally regarded as an essential process in the life cycle of microbes such as *Neisseria meningitidis* (meningococci), typable *Haemophilus influenzae* and *Streptococcus pneumoniae*. These normally capsulate

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strains are noteworthy as common causative agents of bacterial meningitis, whereas non-typable *H. influenzae* and *Moraxella catarrhalis* that are devoid of the protective surface capsular polysaccharides tend to cause localized infections such as otitis media, sinusitis and pneumonia. However, all these species fairly frequently colonise their exclusive human niche, the upper respiratory tract, without infection. The duration of their asymptomatic carriage varies but may last for several months [5, 8, 34]. Thus, whilst in healthy individuals these bacteria remain confined to mucosal surfaces, they have the capacity to invade the epithelial barrier to disseminate further in susceptible individuals. As such, adhesion to mucosal epithelial cells can be regarded as an important first step in their pathogenic process. In addition, *ex viv*o investigations on biopsies from tonsils of subjects with no apparent disease have shown the presence of *N. meningitidis* within intact epithelial cells [37]; such a location generally results from high avidity adhesion to surface receptors. Intracellular location of nontypable *H. influenzae* has also been observed and has been linked to the persistence of the organisms despite antibiotic treatments in patients [43]. As an introduction, several aspects of cellular adhesion are described below which may lead to persistence in the host and/or disease. The aim is to present some common perceptible microbial strategies of host targeting and the diversity of mechanisms evident from studies predominantly conducted in vitro; their verification in vivo is often lacking as few good animal models are available for human specific bacteria. The paradigm of *N. meningitidis* is used frequently, but notably, similar mechanisms are also found in enteric and urogenital bacteria. Finally, the question of whether we can expect to be able to control microbial colonisation or infection by interfering with adhesion using glycans or other analogues of ligands/receptors is addressed.

2 Some Facets of Bacterial Adhesion

Jumping the first hurdle: The mucosal epithelial barrier consists of ciliated and non-ciliated cells with overlying mucus layers that are wafted around by the movement of cilia creating an unstable environment for adhesion. Attachment at such sites requires fast and effective adhesion strategies to overcome this 'mucociliary escalator'. In addition, at physiological *p*H, the net negative charge of the host cell surface creates a charge barrier against predominantly negative charge on most bacterial surfaces. As such, bacterial adhesion necessitates initial penetration of this barrier. For *N. meningitidis*, pili are considered important in charge penetration but their targeted sites on the host tissues are not random. In the nasopharynx, meningococcal pili bring about specific interactions with non-ciliated but not ciliated cells of the epithelium [38]. They are also regarded as primary determinants of bacterial specificity for human epithelial and endothelial cells. In addition, *N. meningitidis* integral outer-membrane adhesins, such as Opa and Opc proteins, are basic in nature and this property may enable better targeting of the host receptors. However,

both these adhesins are also known to bind to host glycans and proteins with specific interactions that are not entirely dependent on electrostatic charge [24, 45].

Redundancy in adhesion: Redundancy or possession of multiple adhesins/mechanisms of attachment is common for bacteria, enabling binding to multiple targets on a single cell. This creates high affinity interactions required for effective cellular invasion. Distinct adhesins often co-operate to increase cellular entry as exemplified by *N. meningitidis* pili-assisted increased invasion mediated by outer-membrane proteins (Figs. 1 and 3f) [10, 48]. Redundancy also becomes important for bacteria with a single host as one of the strategies for immune evasion common in such pathogens is phase variation (on–off expression of surface structures). This requires the presence of several distinct adhesion mechanisms raising obvious problems for devising effective anti-adhesion control measures against such pathogens

Diversity of receptor targeting mechanisms: It would seem self evident that microbes exhibiting host specificity and tissue tropism must possess specific host targeting mechanisms, although, host-specific iron-acquisition mechanisms may add to the niche specificity. However, specificity at a molecular level exists in a wider context also for microbes with more than one host. From numerous investigations on the mechanisms by which microbes target host molecules, it has become evident that mimicry of the natural ligands of host receptors is often adopted by microbes to engage with them.

Some protein binding mechanisms: Taking integrins as examples of microbial receptors, several diverse mechanisms can be recognised with distinct levels of mimicry playing a noticeable role. In some cases, true ligand mimicry is apparent as bacterial adhesins possess structural components closely resembling the natural ligand for engaging with the receptor at the ligand binding site. This is exemplified by the adhesins FHA (filamentous haemagglutinin) and pertactin of *Bordetella pertussis* (responsible for whooping cough). Both of these proteins contain the RGD sequence and can target RGD-binding integrins, an event that results in host cell signalling [20, 35]. Alternately, bacteria may bind to receptors via 'functional mimicry' of the ligand whereby a somewhat variant but nonetheless effective strategy leads to the stimulation of receptor-associated signalling pathways. For example, the protein of *Yersinia pseudotuberculosis* (an enteric pathogen with the ability to cause systemic disease) termed 'invasin' binds to several β1 family of integrins via key conserved residues required for ligand–integrin interactions [19]. In addition, invasin crystal structure has revealed this to be a case of enhanced functional mimicry as an optimized binding region on invasin imparts higher affinity interactions with the integrin compared with the natural ligand [11]. Interestingly, in the absence of invasin, *Yersinia* utilises another ligand, YadA which binds to the fibronectin receptor α5β1 indirectly by initially targeting fibronectin [18]. Such a mechanism of 'Pseudo ligand mimicry', in which natural receptor ligands are used as accessory bridging molecules, is also observed in the case of *N. meningitidis* Opc protein that binds to vitronectin and fibronectin, and via these to αvβ3 and α5β1 integrins (Fig. 2a) [42, 46]. Finally, 'receptor mimicry' involving expression of host-like receptors by microbes has been described for *Mycobacterium avium–intracellulare*

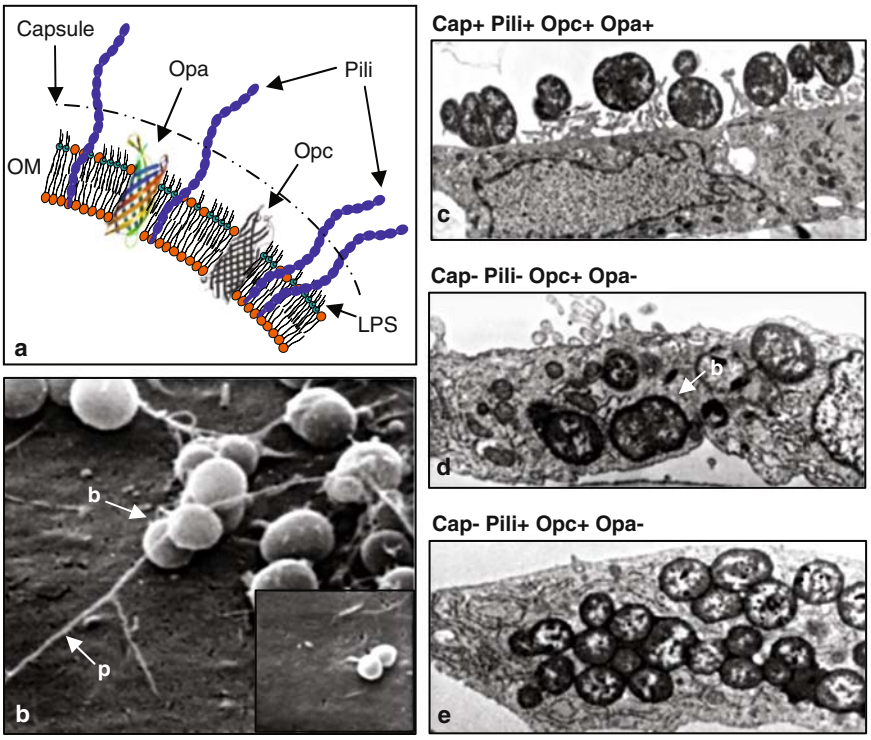

Fig. 1 Interplay between *Neisseria meningitidis* surface polysaccharides and adhesins in host cell targeting. **a** Some key common features of meningococcal disease and carriage strains that influence bacterial ability to adhere to and invade host tissues. OM: outer membrane. Meningococcal pili are long polymeric proteins which traverse the capsule and facilitate initial binding by overcoming the negative charge barriers at the cell–cell interface. They bind specifically to human cell receptors whose identity remains controversial to date. Opa and Opc are integral outer membrane proteins that have several surface exposed loops (four in Opa proteins and five in Opc) which participate in receptor binding. However, these adhesins are subcapsular and capsule can downmodulate their adhesion function in some settings. The ribbon diagrams are based on Opa-like *N. meningitidis* protein NspA (Neisseria surface protein A) [44] and the Opc structure as resolved by J. Derrick and others (adapted from [30]). (NspA diagram was kindly provided by Prof. Leo Brady, Department of Biochemistry, University of Bristol.) **b** The importance of pili (p) in mediating host cell interactions of capsulate bacteria (**B**) can be seen from the scanning electron micrographs (EM) of human endothelial cells infected with piliated meningococci. In contrast, very low binding of non-piliated bacteria is apparent in the inset of an equivalent field size at low magnification. In many cases, individual pili aggregate forming bundles that are often visible in EM (main picture, **b**) and can be seen making contact with the target cell surface. **c–e** *Evidence for interplay*. Transmission EM of the latter phenotype (capsulate, piliated and expressing the Opa and Opc adhesins) showing large numbers of adherent bacteria but none internalised (**c**). The cell entry inhibitory property of capsule is further apparent by comparison of **c** with **d** and **e** where capsule-deficient bacteria have been used to infect human endothelial cells. After a period of infection, a number of intracellular bacteria (**B**) were found in both cases. However, invasion potential of the phenotype in **e** (Opc+, Pili+) was much greater compared with **d** (Opc+, Pili−) [48]. (Note: Opa proteins were not expressed in the case of **d** and **e**.) As antibodies against Opc inhibit cellular invasion in both these cases, invasion is primarily driven by Opc.

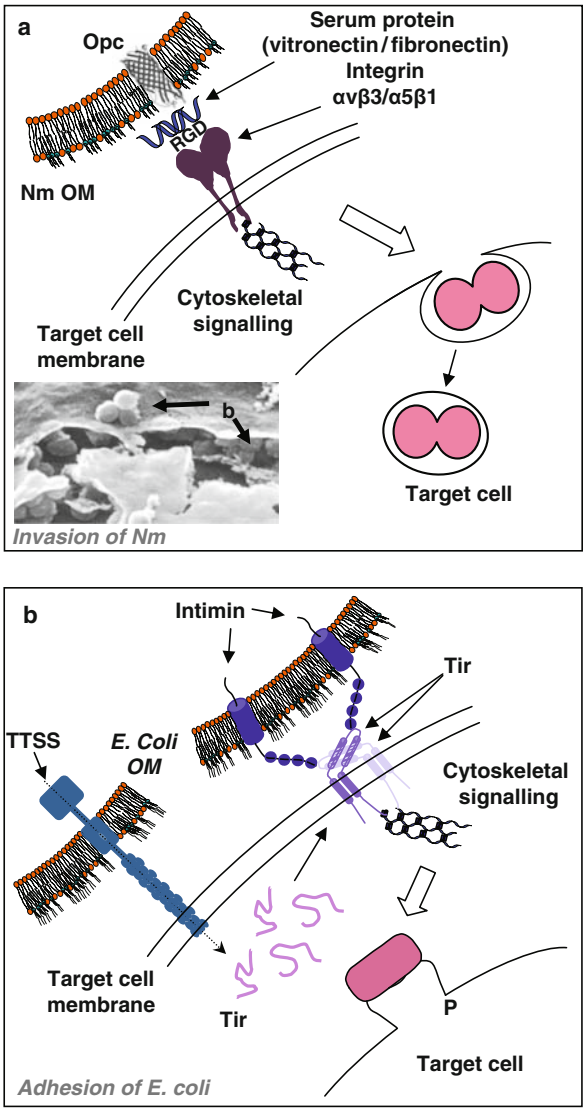

Fig. 2 Adhesion and invasion mechanisms of mucosal bacteria. **a** RGD-dependent targeting of human cellular integrins by *N. meningitidis*. Opc-expressing *N. meningitidis* (Nm) interacts with RGD-bearing serum proteins such as vitronectin and fibronectin present in the blood and subsequently via the formation of a trimolecular complex with integrin receptors on the apical surface of endothelial cells. This leads to cytoskeletal mobilisation and cellular invasion. *Inset*: Scanning electron micrograph of a fractured endothelial cell showing efficient invasion of human endothelial cells by capsule-deficient Opc-expressing bacteria (B) [46, 48]. **b** The Tir-Intimin system of *E. coli*. Enteropathogenic *E. coli* use their own protein Tir, which is injected into eukaryotic cells via the bacterial type three secretion system (TTSS). Tir is then inserted into the host plasma membrane and, in its dimeric form, binds to the intimin adhesin of the pathogens and leads to pedestal (P) formation on target cells at which bacteria become located [21, 23]. For detailed mechanisms see reviews [7, 9].

that can cause opportunistic infections in patients with AIDS. The bacteria contain β1 integrin subunit-like structures that enable them to bind to the integrin ligands including laminin, collagen and fibronectin, a process that is inhibited by anti-β1 integrin antibodies [31].

Other strategies of interest relate to mechanisms that lead to increased avidity of binding which can ultimately result in cellular invasion. Such a goal achieved via interactions involving multiple receptors on host cells have been illustrated in Figs. 1 and 3f for *N. meningitidis*. In other cases, distinct sites on a single receptor may be targeted. For example, some RGD-binding integrins have been shown to accommodate microbially coded RGD-bearing ligands and glycolipids at distinct sites. Such dual engagement appears to be required for cellular invasion [39]. Increased avidity may also be attained by upregulation of receptor expression on target cells. Numerous examples of such a strategy are available including *B. pertussis* upregulation of macrophage CR3 integrin via several mechanisms. Subsequent binding of the adhesin FHA to the integrin leads to macrophage entry that bypasses the phagocyte oxidative burst [35]. In other cases, target cell receptors upregulated via inflammatory cytokines are believed to form a potential basis of increased host susceptibility to infection (discussed below) or severity of infection. An example of the latter is the special pathology of acute cerebral malaria which appears to result from excessive adherence of infected erythrocytes to vascular endothelial CD36, ICAM-1, ELAM-1, and other receptors which are upregulated by circulating TNF-α during malarial infections [28].

Lectin-like interactions: As this subject has been addressed in the other chapters, only a few aspects relating to bacterial lectins are reiterated. In contrast to protein–protein interactions that can achieve a high degree of specificity and avidity, bacterial protein/host carbohydrate interactions generally allow greater promiscuity in receptor targeting via the common glycan substitutions of eukaryotic glycoproteins. In relation to the affinity of binding, some bacterial lectins can alter their conformational state in different environments to acquire high affinity for their targets. For example, it is proposed that high shear stress created by urine flows induces structural changes in the FimH lectin of uropathogenic *Escherichia coli* (UPEC) giving rise to a conformation with high affinity for its mannosyl receptor. This strategy may allow bacteria to attach to the colonising surface under high shear stress whilst allowing the bacterium to move by transient detachment under low shear stress [40].

Bacteria that generate their own receptors: Rather than using host molecules, enteropathogenic *E. coli* (EPEC) generate their own adhesion receptors. In a remarkable strategy, these pathogens utilize an injection system (the type three secretion system, TTSS) comprising a needle complex that inserts into the target cell membrane to deliver effector proteins such as Tir (translocated intimin receptor). Tir subsequently inserts into the host cell plasma membrane and acts as a receptor for the bacterial adhesin, intimin [21] (Figs. 2b).

The above examples were chosen to illustrate the diversity in microbial mode of niche colonisation. To stress the multifactorial and dynamic nature of colonisation and infection processes, the example of *N. meningitidis* is illustrated below.

3 Adhesion Mechanisms and Host Susceptibility: The Paradigm of *Neisseria*

The colonisation spectrum within respiratory neisserial species: commensalism to pathogenesis. Within the genus *Neisseria,* there are several species for which the only known host is man. Survival of the host must thus be a critical factor in influencing the evolution of their interaction mechanisms. Many neisserial species are harmless commensal organisms of the nasopharynx and are rarely associated with disease and include *N. lactamica* that shares numerous features with *N. meningitidis*. The latter, on the other hand, may colonise asymptomatically in up to 30% of a healthy population but has the capacity to become pathogenic and can cause fatal illness unless intervention measures are taken promptly. In this case, the disease is rare when compared to the occurrence of the bacterium in a healthy population and disease may be regarded as an accidental event, often arising as a result of immunedeficiency of the patient or other pre-disposing factors such as prior viral infections discussed below [4, 26].

Receptor modulation and transition from commensalism to pathogenesis. Susceptibility to infection by several respiratory colonisers including *N. meningitidis, H. influenzae* and *M. catarrhalis* increases markedly following influenza and/or respiratory syncytial virus (RSV) infections [1, 2, 4, 10, 12]. Further, epidemiological studies have shown spatial and temporal association between particular respiratory bacterial and viral infections. Since circulating inflammatory cytokines increase significantly following viral infections and since certain cellular receptors are upregulated in response to inflammatory cytokines, one potential mechanism may involve the upregulation of host receptors targeted by bacteria. Below, in vitro studies that examined this hypothesis have been briefly described.

Modulation of receptors by IFN-γ and consequences on bacterial adhesion and invasion was examined in a recent investigation. Besides epidemiological links between inflammation and infection, further impetus for this study was derived from the fact that the three mucosal opportunistic bacteria mentioned above all target CEACAMs (CEA-related cell adhesion molecules belonging to human carcinoembryonic antigen family (Fig. 3a), and that CEACAMs are expressed normally at low levels and are subject to upregulation by cytokines such as IFN-γ. In addition, studies on integrin upregulation have previously suggested that receptor density, by increasing the affinity of bacteria–host interactions, may encourage bacterial infiltration [19]. Indeed, transfected cell lines or IFN-γ treated epithelial cells expressing high levels of CEACAMs resulted in enhanced cellular invasion by *N. meningitidis* [10, 33]. Notably, whilst capsulate piliated bacterial interactions with cells expressing low levels of receptors do not lead to significant cellular invasion, a different scenario is observed in the setting of high surface density of CEACAMs. In this case, the meningococcal adhesins pili and Opa act synergistically in host cell binding which leads to greater invasion by capsulate bacteria (Fig. 3f). Since capsulate bacteria are serum resistant and meningococci can grow rapidly in blood, traversal of such a phenotype

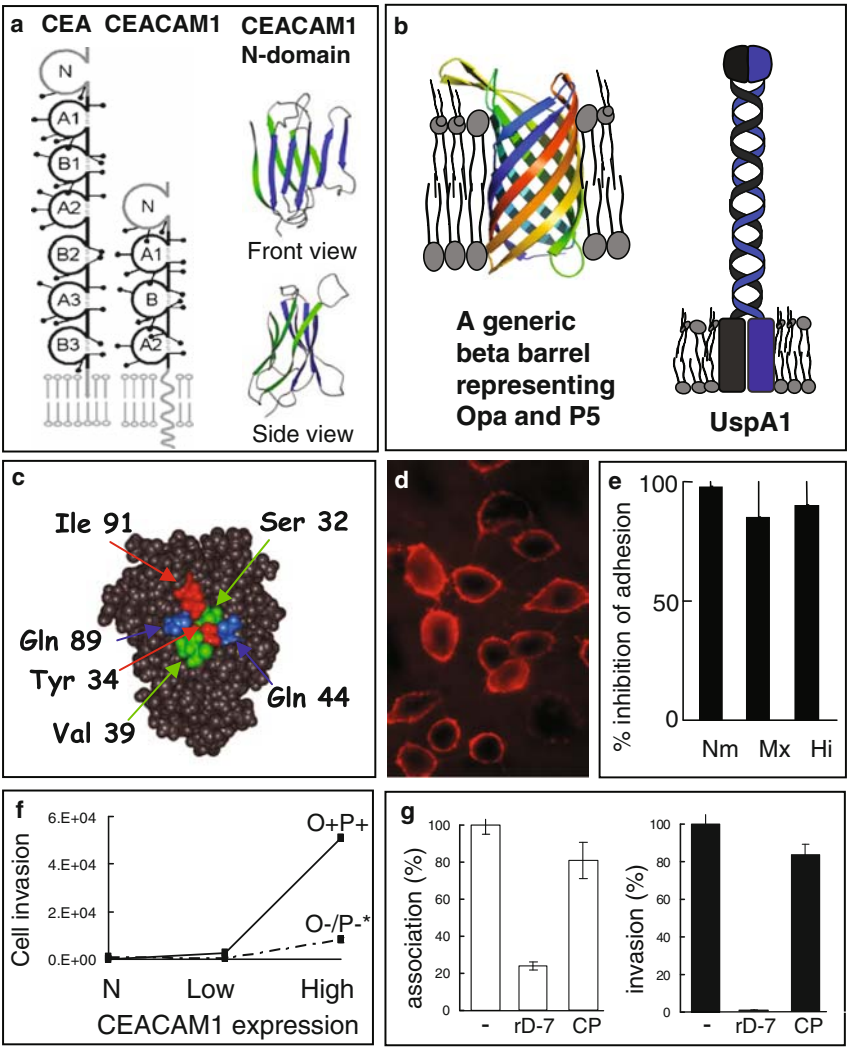

Fig. 3 CEACAM targeting of mucosal opportunistic bacteria. **a** Structure of the CEA-related cell adhesion molecules (CEACAMs). The molecular architectures of the GPI-anchored CEA and the transmembrane CEACAM1, two of the most widely expressed receptors, are presented. Glycans are shown as lollipop structures (http://cea.klinikum.uni-muenchen.de/) [17]. The family of molecules are characterised by their immunoglobulin variable (IgV)-like N-terminal domains and may contain none or several of Ig constant (Ig-C2)-like domains designated A1, B, etc. Ribbon diagrams of two views of the N-terminal domain (N-domain) of CEACAM1 (kindly provided by Dr. Andrea Hadfield, Department of Biochemistry, University of Bristol), required for the binding of several mucosal bacteria are shown. The immunoglobulin fold of the N-terminal domain consists of nine anti-parallel beta strands joined by loop regions arranged in two sheets (faces), ABED (*shown in green*) and C"C'CFG (CFG, for brevity; *shown in blue*). The ABED face is glycosylated whereas the CFG face is not; thus this protein face is fully exposed. **b** The molecular architectures of three of the bacterial ligands that bind to overlapping binding regions in the non-glycosylated CFG

across respiratory epithelium carries a high risk of widespread disease especially in a host with inadequate immunity against the pathogen (Fig. 4). In summary, adhesion appears not only to be essential for mucosal colonisation, but certain adhesin-receptor interactions could be key to microbial pathogenic potential.

Fig. 3 (continued) face of the receptor are shown [14, 16, 47]. The CEACAM-binding Opa proteins of *N. meningitidis* and P5 protein of *H. influenzae* are eight-stranded beta barrel molecules represented here by a generic structure based on that of NspA. However, at the amino acid level, the two proteins have little homology (11% similarity between P5 of *H. influenzae* strain Rd and OpaB of *N. meningitidis* strain C751) [16]. The genome of *N. meningitidis* encodes up to four different Opa proteins with variable surface-exposed loops. These variant Opa proteins nonetheless are able to bind to CEACAMs. The *M. catarrhalis* adhesin UspA1 that binds to CEACAMs is an extended 'lollipop'-like structure that exists as a trimer in the outer membrane. Notably, all three adhesins share little sequence similarities that could be assigned to their shared CEACAM-binding property. **c** A space filler model of the human CEACAM1 N-terminal domain showing the residues (in the CFG face) that are important in binding of the Opa proteins, with Ile-91 playing a central role. This residue is also central to the recognition of P5 and UspA1 [45]. **d–g** Complexities of ligand/receptor interplay and attempts at blocking invasion. A recombinant polypeptide (termed rD-7) based on UspA1, was produced that bound to CEACAM1 [15]. The efficient binding of rD-7 to surface expressed CEACAM1 is demonstrated by the red fluorescence of the indirectly labelled rD-7 in **d**. This immunofluorescence micrograph shows transfected HeLa cells expressing CEACAM1, which were incubated with rD-7 and the bound polypeptide was detected using rabbit antiserum against rD-7 followed by rhodamine conjugated anti-rabbit antibodies. The recombinant molecule rD-7 inhibits binding of multiple strains of the three species to HeLa-CEACAM1 cells (Nm: *N. meningitidis*, Mx: *M. catarrhalis* and Hi: *H. influenzae*) (**e**). In each case, mean % inhibition (and the range) of binding of ten strains in the presence of rD-7 compared to a control peptide is shown. The cellular invasion of capsulate *N. meningitidis* expressing either Opa or pili or both the adhesins was assessed using cell lines with distinct levels of surface CEACAM1 (**f**). For these experiments, untransfected HeLa cells were used as negative controls (N) in conjunction with HeLa transfectants expressing low or high receptor densities. Significant invasion of cells was only observed in cell lines expressing high levels of CEACAMs and both pili and Opa-expression was required for such invasion demonstrating the synergism between the adhesins in facilitating invasion by capsulate serum resistant phenotype. *Since the expression of Opa alone or pili alone in capsulate bacteria did not result in significant invasion, a single line is shown on the graph which represents their approximate invasion levels (O-/P-). The results illustrate one possible manner in which host cell susceptibility to invasion might increase during inflammation when circulating cytokines lead to receptor augmentation [10, 33]. The efficacy of rD-7 in blocking cellular adhesion or invasion was assessed in the setting of high receptor density. Capsulate (Opa+, pili+) meningococcal adhesion was reduced but not abrogated in the presence of the peptide, whereas invasion was completely abolished (**g**). Control peptide (CP) had no significant effect on cellular adhesion or invasion. The data indicate the key role of Opa-CEACAM interactions in driving invasion, and the potential of specific intervention measures in preventing this step

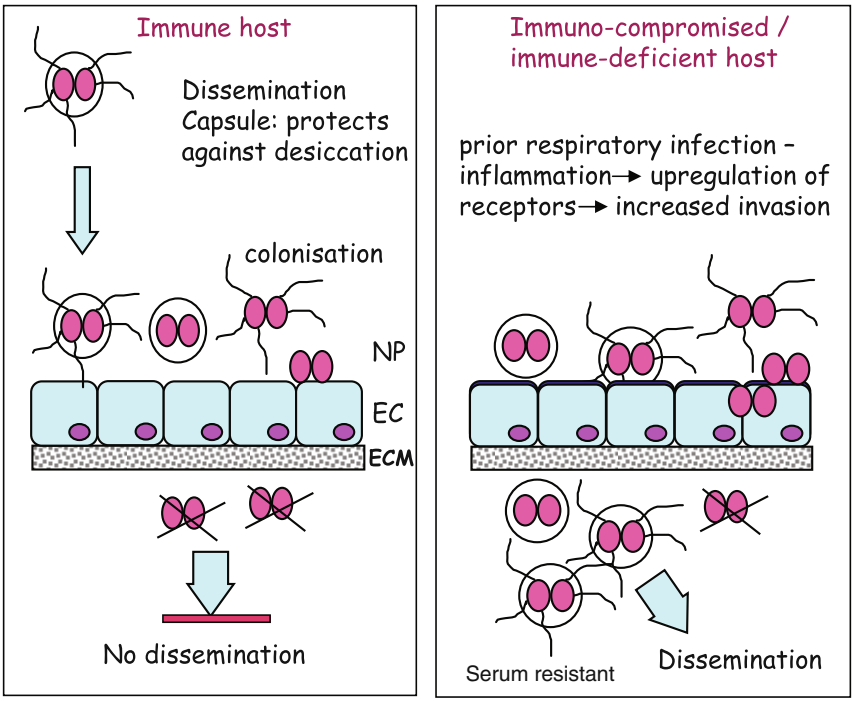

Fig. 4 Possible stages in the colonisation and pathogenic processes of *N. meningitidis.* A model based on epidemiological and in vitro experimental observations. Phase variation of surface structures is common in *N. meningitidis* and capsule-deficient phenotypes are found in the nasopharynx. Such a phenotype is invasive but is serum sensitive and does not survive in the blood. In the case illustrated on the right, where infiltration of capsule-expressing bacteria is possible, dissemination throughout the body may ensue unless the host has the ability to clear this phenotype efficiently from the blood (NP: Nasopharynx; EC: epithelial cell; ECM: extracellular matrix)

4 Potential Measures to Prevent Microbial Infection and Concluding Comments

The importance of invasion: Microbes require a foothold in a niche whilst avoiding elimination by the host. Within the array of mechanisms that have evolved to meet the latter requirement, epithelial cell invasion is regarded by many as a potential mechanism of hiding from professional phagocytes such as macrophages and neutrophils as well as other anti-microbials (antibodies, complement components, antimicrobial peptides, etc.). Whilst some microbes have evolved strategies to enter eukaryotic cells and multiply within, others may use this strategy merely to survive for periods and persist in the niche [6]. As noted above, meningococcal location within tonsillar epithelial cells has been reported. Bacteria then exit from these cells in either the apical direction to transmit to a new host or in the basolateral direction to enter the body. Armed with anti-phagocytic/anti-immune strategies such as

capsule, bacteria may survive in the blood and disseminate throughout the body (Fig. 4). Whilst this could be one scenario leading to disease, it is not a foregone conclusion that tissue invasion is required for infection. Indeed, mucosal damage, however small, may achieve the same effect by enabling bacteria to enter the body unhindered. However, the ability of a large number of mucosal pathogens to invade target cells or cross intact epithelial barriers does draw our attention to this potentially crucial step in the process of infection.

5 Natural and Artificial Anti-Adhesion/Anti-Invasion Measures

Receptor decoys in protein–glycan/protein–protein interactions. The intestinal pathogens *E. coli* and *Salmonella* use their mannose-binding lectins to target the abundant glycans of CEACAMs [22]. It has been suggested that in an evolutionary counter strategy, the GPI-anchored member, CEA (product of *ceacam5* gene), is shed daily in large amounts in the faeces, thus reducing pathogen load. CEA may thus be regarded as an arm of innate immunity that utilizes shed receptors as decoys [13]. In the case of the more recently recognised interactions of respiratory pathogens *N. meningitidis, H. influenzae* and *M. catarrhalis* with CEACAMs, it is becoming clear that the majority of the strains of these species bind preferentially to the transmembrane molecule CEACAM1, which may not be as readily shed. Interestingly, the pathogens utilise structurally divergent adhesins (Fig. 3b) to bind to a common protein site on the receptor (Fig. 3c) [3, 47, 49]. This focuses our attention on whether a common anti-adhesion strategy can be devised to control infections caused by them. Whether receptor decoys (perhaps in the form of small peptides) will prove effective in controlling bacterial infection in humans is at present unclear. However, since CEACAMs participate in several important physiological functions including homotypic interactions, any measures that may interfere with natural receptor functions need to be carefully evaluated. In a somewhat variant approach, infection preventing property of a CEACAM-binding recombinant peptide fashioned after a bacterial ligand, has been examined in vitro as outlined below.

The use of CEACAM-blocking peptides to inhibit invasion. Based on *M. catarrhalis* UspA1 adhesin that binds to CEACAMs, a recombinant peptide was generated with the capacity to bind specifically to CEACAMs (Fig. 3d) and block the binding of *N. meningitidis, H. influenzae and M. catarrhalis* to CEACAM expressing cell lines [15] (Fig. 3e). In vitro, the peptide significantly inhibits Opa-CEACAM-mediated cellular invasion of the Opa+, pili+ phenotype whilst not eliminating pilus-mediated adhesion which occurs via a distinct receptor. Importantly, this occurs in the post-inflammation models of infection in which CEACAM density of target cells is enhanced supporting high levels of cellular invasion [10, 33] (Fig. 3g). Thus, if the hierarchical importance of the receptors involved in mucosal barrier penetration can be identified, then it may be possible to inhibit selectively the route of cellular invasion without abrogating bacterial adhesion. This may have an added advantage in the case of the frequent mucosal

colonisers since the possibility remains that elimination of such bacteria could lead to colonisation by other unwanted agents and their presence on mucosa may serve to balance the local ecology and boost immunity. Whether such anti-invasive measures will be effective in vivo and whether they can be fashioned to prevent bacterial interactions without receptor interference and resultant side effects remains to be seen.

Vaccines. Perhaps the most effective strategy is to immunise with key adhesins/invasins to induce endogenous blocking antibodies. This approach, however, requires an extensive knowledge of the precise binding mechanisms of bacterial adhesins, which have proved extremely difficult in some cases. Adhesins with variant structures able to target a single receptor are not uncommon as exemplified by the Opa proteins of *N. meningitidis*. Such structural/antigenic variation is perhaps the greatest challenge to devising anti-adhesion vaccines based on bacterial ligands. Where the knowledge is available and the adhesin is largely invariant, successful use of adhesins has been made to prevent colonisation and disease in animal models, e.g., the FimH and the PapG adhesins of pathogenic *E. coli* [29, 32].

Anti-adhesion therapy for lectin-like interactions: the evidence for their efficacy. As is pertinent for this book, the final reflection should be on lectin adhesins of microbes. A number of enteric infections have been shown to be successfully prevented by administration of receptor mimics and have been reviewed recently [36]. One important example that illustrates the protective effects of glycans is the observation that infants fed on milk with high oligosaccharide levels are largely spared from high incidence of diarrhoea compared with those fed on low carbohydrate milk. This has been assigned to the presence of fucosylated oligosaccharides which are effective inhibitors of attachment of the enteric pathogen *Campylobacter jejuni* and of the stable toxin of enterotoxigenic *E. coli* [25]. Additionally, both carbohydrate mimics and antibodies to adhesins have been shown to be effective in a number of animal studies. However, human clinical trials using adhesion-inhibiting oligosaccharides have been disappointing for the respiratory pathogens *H. influenzae and S. pnemoniae*. Neither their incidence of colonisation nor otitis media could be prevented [41]. Similarly, for the enteric bacterium *Helicobacter pylori* (a causative agent of peptic ulcers and cancers), no reduction in colonisation was obtained [27], perhaps reflecting the multifactorial and dynamic nature of bacterial adhesion processes. It is entirely possible that high affinity blocking agents and cocktails of anti-adhesion drugs will finally succeed against some such pathogens! The only question is: Do we have the tools to study the complexities of in vivo dynamics at the host–microbe interface in order to devise effective anti-adhesion measures to combat problematic microbial diseases?

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Architectures of Multivalent Glycomimetics for Probing Carbohydrate–Lectin Interactions

Martina Lahmann

Abstract Well-defined multivalent glycoconjugates are valued tools in glycoscience and they are particularly valuable for the investigation of carbohydrate–lectin interactions. In addition to the relatively globularly shaped glycodendrimers many other designs have been realized. This chapter gives an overview on the common different architectures and their chemical synthesis by focussing on the achievements made since 2001.

Keywords Carbohydrate–lectin interaction, Chemoselective ligation, Conjugation technique, Glycocluster, Glycoconjugate, Glycodendrimer, Multivalency

Contents

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Abbreviations

AFM	Atomic force microscopy
BOP	Benzotriazol-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate
BSA	Bovine serum albumin
Boc	*tert*-Butyloxycarbonyl
CB	Cucurbituril-based
CD	Cyclodextrin
ConA	Concanavalin A
CRM197	A diphtheria toxin mutant
DCC	Dicyclohexylcarbodiimide
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEPC	Diethyl phosphorocyanidate
DIPEA	Ethyldiisopropylamine
DMAP	4-Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOTA	1,4,7,10-Tetrakis (carboxymethyl)-1,4,7,10-tetraazacyclododecane
E. coli	*Escherichia coli*
EDCI	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ELLA	Enzyme-linked lectin assays
G	Generation
Gal	Galactose
Glc	Glucose
GlcNAc	*N*-Acetylglucosamine
GM1	Monosialotetrahexosylganglioside
GPC	Gel permeation chromatography
HBTU	*O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate
HIA	Hemagglutination inhibition assay
HIV-1	A human immunodeficiency virus
HOBt	Hydroxybenzotriazole
HSA	Human serum albumin
ITC	Isothermal titration calorimetry
Lac	Lactose (Galβ1-4Glc)
MALDI-Tof	Matrix-assisted laser desorption/ionization-time of flight
MALS	Multiangle light scattering
Man	Mannose
MD	Molecular dynamics
MS	Mass spectrometry

MRI	Magnetic resonance imaging
NK	Natural killer cells
NMM	*N*-Methylmorpholine
NMR	Nuclear magnetic resonance
PAMAM	Polyamido amino
PEG	Polyethylene glycol
PNA	Peanut agglutinin
PPI	Polypropylene imine
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RAFT	Regioselectively addressable functionalised template
SAM	Self-assembled monolayer
SPR	Surface plasmon resonance
T-antigen	Tumor antigen
TBTU	*O*-(Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
Tce	2,2,2-Trichloroethyl
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Tn-antigen	GalNAcα1Ser/Thr
UV–VIS spectroscopy	Ultraviolet–visible spectroscopy
WGA	Wheat germ agglutinin
XSAF	Extended X-ray absorption fine structure

1 Introduction

Geckos use a sophisticated assembly of submicroscopic foot hairs to walk along a ceiling upside down [1]. Such a "dendritic network" of hairs takes advantage of multiple adhesive forces, resulting in an efficient multivalency effect.

This observation has an analogy in the world of microbes, which need to adhere effectively to the surface of their host cells to escape the shear forces of body fluids. Bacteria utilize both protein–protein and carbohydrate–protein interactions for adhesion. Pathogenic *Escherichia coli* (*E. coli*) bacteria, for example, responsible for more than 80% of urinary tract infections [2], possess thin hair-like structures on their surfaces, called *pili* and the shorter *fimbriae.* Fimbriae specifically recognize carbohydrates. Type 1 fimbriae of *E. coli*, for example, can, via their FimH lectin domain [3], bind α-D-mannopyranosides. Lectins, e.g. [4–7] named after the Latin word "legere" to "pick out" or "choose" [8] are carbohydrate recognizing proteins found everywhere in nature, e.g. [9–13] and are intensively studied structures.

Like a gecko, fimbriated bacteria use multivalent contacts for adhesion. Moreover, "multivalency" is a general theme in biological systems and multivalent interactions are of importance for numerous biological processes, such as immune

regulation, cell-growth regulation or cell–cell recognition [14, 15]. Although an easy to understand principle at first sight, there are many different possibilities to construct (Fig. 1) multivalent interactions and to describe those theoretically, see e.g. [11, 16–27].

Detailed investigation of multivalent carbohydrate–protein interactions has gained increasing interest in glycosciences and thus a great deal of research has been devoted to the design and synthesis of chemical tools, such as multivalent glycomimetics of various architectures. With these devices in hand, a number of assays and biophysical methods, such as hemagglutination inhibition assay (HIA) [28] and enzyme-linked lectin assays (ELLA) [29], isothermal titration calorimetry (ITC) [30], or surface plasmon resonance (SPR) [31], to name but a few, can be utilized to probe carbohydrate–protein interactions.

As the affinity of monovalent carbohydrate ligands to their lectins is usually very weak (often even in the mM range) [32] and enhanced by multivalent interactions, multivalent carbohydrate ligands have become useful instruments during the last three decades [33]. It is known that the binding affinity of a ligand to the lectin is influenced by factors like size, spatial assembly and valency [34]. In order to prepare multivalent glycoconjugates, so-called neoglycoconjugates (e.g. Fig. 2) have been prepared. To find out more about the details of a specific binding event and

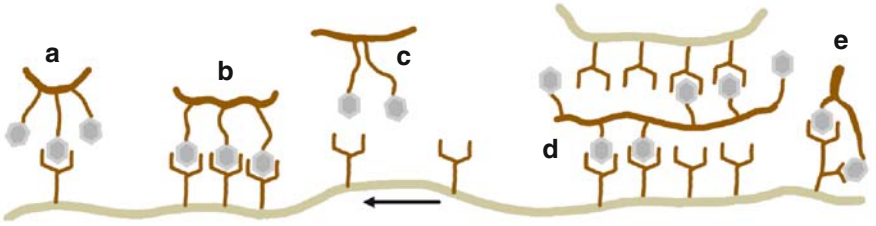


Fig. 1a–e Some possible modes of multivalent binding events. **a** Statistical multivalency. **b** Chelating. **c** Clustering. **d** Adhesion. **e** Sub-site binding

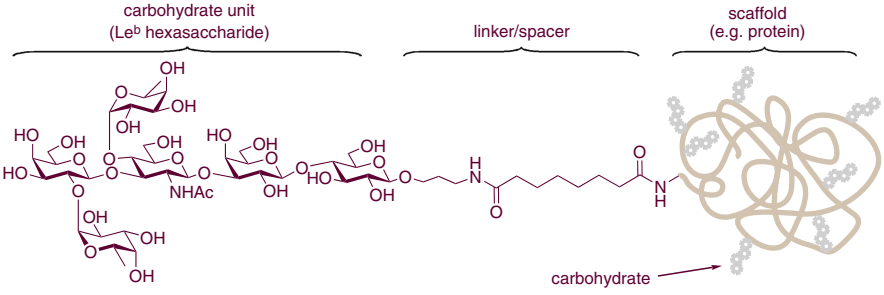


Fig. 2 A schematic representation of a (Le^b hexasaccharide) neoglycoconjugate [35]

mechanism and eventually to enhance the affinity of the binding, the presentation of the sugar ligand has to be optimized.

In neoglycoconjugates, a carbohydrate ligand is attached to a protein scaffold, often via a linker, in multiple copies. Proteins typically used are human serum albumin (HSA), bovine serum albumin (BSA) or a diphtheria toxin mutant (CRM_{197}) [36]. Also peptides and synthetic peptide scaffolds, such as polylysines, have been employed [37]. This approach leads to polydisperse material, with varying ligand loading, which can be determined by mass spectrometric analysis.

In addition, carbohydrate ligands can be multivalently arranged on defined synthetic scaffolds, leading to glycoconjugates with complete or almost complete homogeneity. Among those, glycodendrimers have been shown to serve as valuable tools for lectin binding studies and subsequent applications, e.g. [15, 16, 25, 38–48].

Since the first publication on the synthesis of glycodendrimers [49] in the mid-1990s followed by the suggestion to name these sugar covered macromolecules "glycodendrimers" [50], numerous excellent review articles have been published, e.g. [16, 23, 42, 51–60]. This chapter will focus on the synthetic achievements made with glycodendrimers and related macromolecules and their use in lectin research during the last few years, trying to cover the literature since 2001.

2 Multivalent Architectures

2.1 Dendrimers and Related Macromolecules: The Beginnings

Multivalent glycoconjugates turned out to be useful in many disciplines, i.e. material science, medicine, biosciences, and a broad range of possible applications has been identified. In 1978, Vögtle and co-workers [61] were interested in the construction of molecules containing large cavities to investigate host–guest interactions. They succeeded in making the required type of macromolecules by repeatedly performing a two-step reaction sequence comprising of Michael-type reaction of an amine and acrylonitrile followed by reduction, using a mono- or diamine as starting material. Each reaction circle turned the starting amino functions into a branching point, providing two new amino-terminate arms, thus doubling the number of terminal amino groups with each circle. The "iterativity" of the synthetic sequence prompted the authors to coin the term "cascade molecules". This approach was the birth of dendrimer chemistry.

Vögtle's early work on cascade molecules was followed by the synthesis of highly branched polyols in the laboratory of Newcome (Fig. 3) [62]. Since the first polyol structures reminded him of small trees, they were named "arboroles" (lat. *arbor* – tree). However, it has been the Greek-derived term "dendrimer" (δένδρον – tree, μέρος – particle), which has become the most popular term for highly branched monodisperse molecules with fractal character, as first used in a patent

from 1984, authored by Tomalia and co-workers [63]. They described radial molecules, which were intended for polymerization, leading to "starburst polymers" (Starburst™) with low polydispersity. In a subsequent paper [64], today's accepted nomenclature to describe the architecture of dendrimers has been introduced: The centre of a dendrimer contains the "core" (scaffold) to which layers of "dendrimer repeating units" are attached and the surface (shell) is covered with "terminal groups". The surface of a dendrimer can be changed by attaching additional molecules via the terminal groups (Fig. 4).

The (initiator) core is usually a small molecule exhibiting a few (at least two) reactive groups of the same kind. The main task of the dendrimer repeating unit is to multiply the number of reactive groups in every generation. Practically this can be achieved in different ways, but each time a layer of branching points is put on the growing dendrimer a new "generation" (*G*) of the dendrimer is produced. By this definition the core is generation zero (*G0*). Fractional generations can occur if two or more different molecules are used for each dendrimer repeating unit, e.g. methylester terminated intermediates (half generations, Fig. 5) are obtained when constructing *p*oly*am*ido *am*ino (PAMAM) dendrimers in a Michael condensation using diethylamine and methylacrylate.

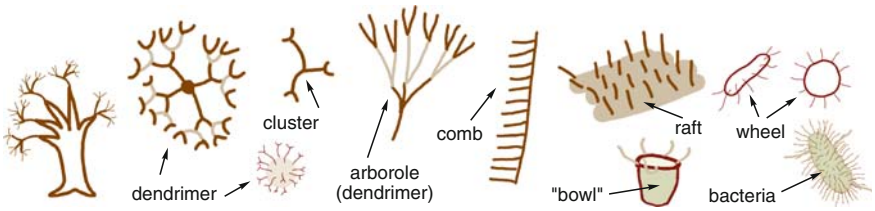


Fig. 3 Some archetypes of defined multivalent architectures compared to their natural counterparts

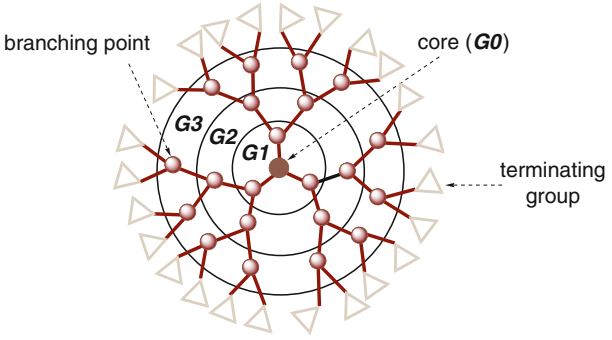


Fig. 4 A functionalized third generation dendrimer (*G3*)

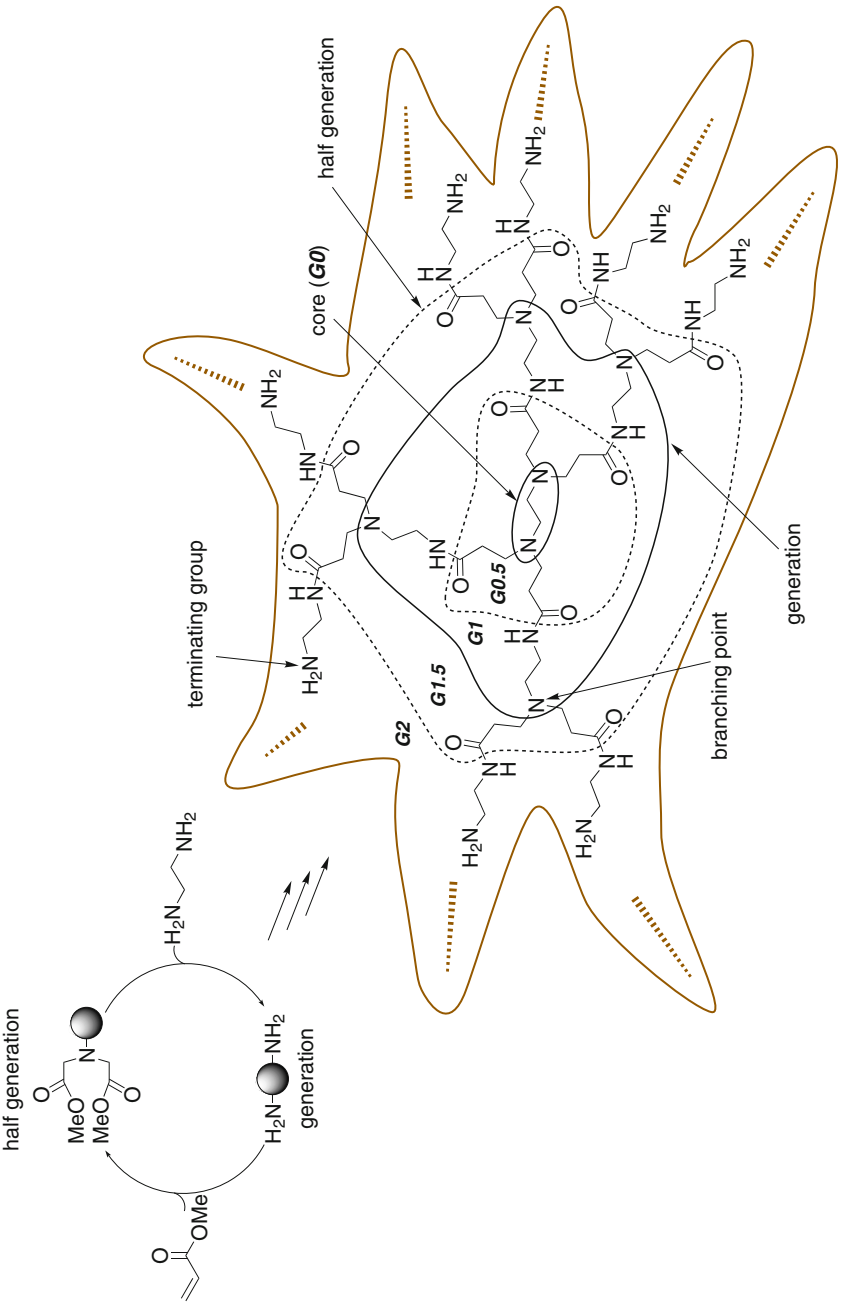


Fig. 5 PAMAM dendrimers like the depicted *G2* PAMAM dendrimer are synthesized in an iterative fashion as shown in the upper left corner

In general, a typical dendrimer has a well defined symmetric structure, consists of 2–32 generations (*G*2–*G*32) with molecular weights from 2 kD up to > 100 kD and diameters up to several nm. This is in the range of a medium sized protein. To gain an idea about the overall shape of a glycodendrimer MD (molecular dynamics) simulations have been used [39]. Although it has been suggested that larger dendrimers should be good mimics for proteins, there are some important differences: dendrimers are in general more loosely packed, while the density of functional groups on a dendrimer surface is much higher than in a protein of comparable size. For comparison, a *G*6-PAMAM has a diameter of approximately 6.5 nm, a molecular weight of about 29 kD and 128 primary amino groups accessible for modification [65], while human serum albumin has a similar diameter (ca. 6 nm, depending on the pH [66]), but has a molecular weight of 67 kD and about 30–35 ε-lysine amino groups typically available for conjugation.

There are two general strategies to synthesize dendrimers (Fig. 6). In the *divergent approach* dendrimer generations are sequentially added onto a core, thereby extending towards the dendrimer surface.

The preparation of a dendrimer according to the divergent approach is usually relatively easy, but requires a very high rate of transformation (>99 + %) in each step to avoid defects in the structure. Therefore, this strategy is mainly used for smaller dendrimers (<15 kD).

The second option is the *convergent approach,* as introduced in 1990 by Hawker and Frechét [67]. In this approach, dendritic wedges, so-called dendrons, are produced first and assembled on the core molecule in the last step, e.g. [68, 69]. A benefit of this route is that it is possible to mix wedges with different terminal groups with the opportunity to synthesize "designed" dendrimer surfaces and multifunctional dendrimers.

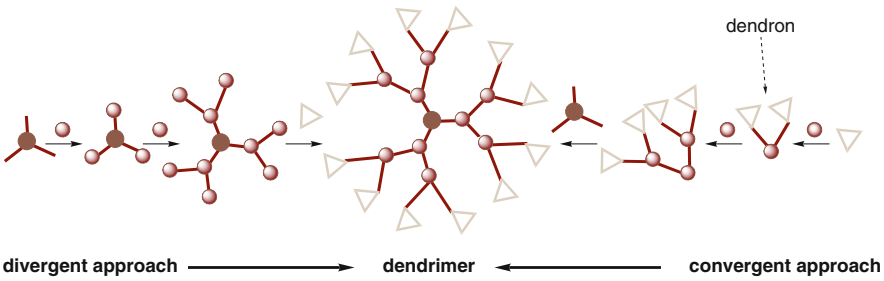


Fig. 6 Schematic representations of the divergent and the convergent approach for dendrimer synthesis

2.2 Dendrimers and Related Macromolecules Get Sugared

Some early "glycodendrimers" intended to make good inhibitors of influenza A virus hemagglutinin [49] were constructed following the divergent approach on a peptide core by solid phase methodology. Due to the lower symmetry of the core, the overall shape of these macromolecules was not as globular as claimed for Tomalia's Starburst® dendrimers [51].

The architecture of glycodendrimers is variable and includes, apart from the large group of carbohydrate-covered dendrimers, dendrimers which are constructed on a carbohydrate core or entirely constructed of carbohydrate building blocks. Still, one can think of many other topologies suitable as multivalent carbohydrate ligands (Fig. 3). Depending on the scaffold, more or less disk- or wheel-shaped structures with the sugar ligands attached at the rim can be expected when using aromatic scaffolds, e.g. [70], and this has been intended with the introduction of the cucurbituril scaffold [71]. Different types of bowl-like conjugates might be formed from cyclodextrins, calixarenes or carbohydrate cores. The use of sheet-like scaffolds (rafts) produces small multivalent patches with all ligands assembled in the same direction [72]. Very small multivalent ligands, carrying only a few carbohydrate epitopes (approximately two to four copies) comparable to a *G*1 dendrimer are sometimes called *glycoclusters*.

Quite often the naming of a multivalent carbohydrate ligand is deduced from the class of the core molecule, e.g. glycodendrimers constructed on calixarenes are called "glycocalixarenes". Wedge-like structures such as the glycodendrons can be selectively monofunctionalized and either used for tagging or linking on a core to get a typical glycodendrimer according to the convergent approach or for attachment to other types of scaffolds as polymers, sensor surfaces or proteins. Direct attachment of carbohydrate epitopes to suitable proteins, peptides, peptide analogues or polymers is also an option. A somewhat different approach to obtain relatively homogeneous multivalent presentations of carbohydrate ligands is to assembly them, covalently or non-covalently, on a gold surface, i.e. forming "glyco-SAMs" (SAM: self-assembled monolayer) [73–75] or quantum dots [76].

In glycobiology multivalent or multiantennary, respectively, neoglycoconjugates serve as important tools by mimicking the complexity of mammalian cell surfaces, since their epitopes are recognizable for lectins and selectines (mammalian cells, viruses, bacteria), e.g. [77]. Further, gaining better insight into the molecular interactions of carbohydrates may also allow researchers to interfere with disastrous consequences of malignant recognition processes causing cancer metastasis or viral adhesion, e.g. [78].

The value of glycodendrimers and other multivalent glycomimetics as well-defined multivalent glycoconjugates for studies in glycosciences was soon appreciated and both the range of applications and the number of publication on glycodendrimer synthesis started to grow. In recent years, the focus started to shift towards defined multivalent glycoconjugates with non-ball like topology (Fig. 3). The

advances in glycodendrimers have been thoroughly reviewed in many books, chapters and review articles, e.g. [16, 34, 40, 42, 52–56, 60, 79–81].

3 Designing Multivalent Glycomimetics

3.1 General Considerations

Various synthetic approaches to prepare multivalent carbohydrate ligands have been realized and compared to the synthesis of multiantennary oligosaccharides as found in native material of interest, and advantage is often taken by an overall more straightforward procedure for their assembly. In numerous examples synthetic efforts were reduced to the attachment of an important epitope, in many cases just a monosaccharide, to a suitable scaffold. Synthetic multivalent ligands can be designed with respect to the cores, the linker moieties, the surface epitopes and additional structural features accounting for the envisaged applications. This includes, apart from enhancing specificity and affinity, important aspects such as cytotoxicity (e.g. reducing the toxicity [82]) and biodegradability. The majority of glycodendrimers are prepared by standard solution phase chemistry, although solid phase chemistry was the approach used for the first reported glycodendrimers [49]. Even a standard DNA synthesizer has been successfully employed for the fast small-scale assembly of several multivalent glycodendrons with biological relevance using phosphoramidite chemistry [83], and recently the use of the Ellman's safety-catch variation of Kenner's sulfonamide linker [84] has been investigated to ensure high-yielding release of the glycodendrimers from the resin solid support under mild and chemoselective conditions (Fig. 7) [85].

3.2 The Diversity of Scaffolds and Cores

Almost any molecule exhibiting at least two copies of a reactive group can theoretically serve as core or scaffold for the synthesis of dendrimers and defined oligo- or polyvalent glycoconjugates. Since the "interior" of a multivalent glycoconjugate

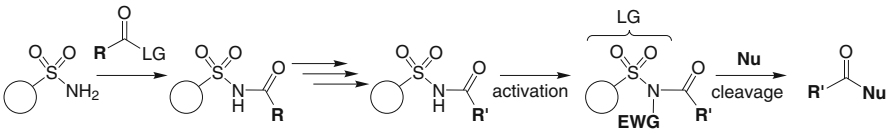


Fig. 7 Ellman's safety catch linker chemistry used for the synthesis of glycodendrons in a DNA synthesizer [85]

influences to some extent the overall properties of the target molecule, variation in the core and linker chemistry may lead to molecules with interesting and more specific biological properties [86, 87]. Some of the more common scaffolds used for the preparation of glycodendrimers and multivalent glycoconjugates have been collected here, leaving polymeric and protein scaffolds out.

3.2.1 PAMAM and PPI Scaffolds

Both the PAMAM (=Starburst™ [64]) and PPI (*p*oly*p*ropylene *i*mine dendrimers, Astramol™ dendrimers [88, 89]) scaffolds have been popular for glycodendrimer synthesis (Fig. 8). They contain tertiary amines at the generation-defining branching points and display primary amine groups at the periphery [90–98]. Dendrimers constructed on PAMAM scaffolds are so frequently used because these scaffolds are easy to handle, fairly bio-compatible and commercially available in various sizes. Also azido-functionalized PAMAM dendrimers have been used for the synthesis of triazole-ligated glycodendrimers [99]. Depending on whether non-protected or protected glycosides are used during the conjugation step, the crude products can be isolated either by precipitation or chromatographic methods, e.g. [93, 100, 101]. For instance, 64-meric cellobiosyl dendrimers were obtained (65%) by precipitation and further purified by gel permeation chromatography and HPLC [91]. Purification of glycodendrimers can be tricky due to the similarity of targets side-products, such as dendrimers with structural defect. This might affect the overall yields, e.g. after a long sequence of purification steps a 5% yield had to be accepted for a fucosylated trivalent non-PAMAM glycocluster [102]. Smaller glycoclusters, especially when protected sugar derivatives have been scaffolded, can be purified by normal phase silica gel chromatography [103].

To avoid structural defects, branching reagents are used in excess. Usually 1.25–2 equivalents per functional group are sufficient to obtain acceptable purity;

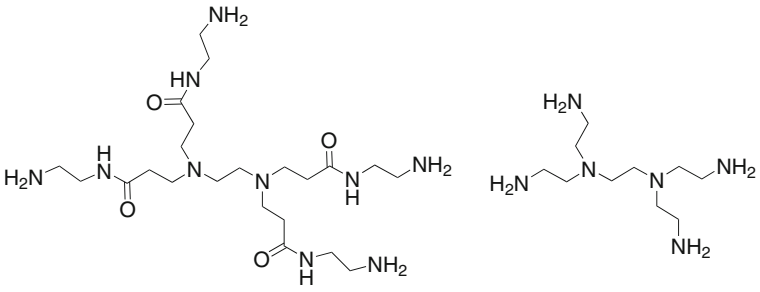


Fig. 8 First generation PAMAM (*left*) and PPI (*right*) scaffolds constructed on an ethylene diamine initiator core

however steric hindrance may cause problems. For example, only six GM1 (GM1: monosialotetrahexosylganglioside, structure Fig. 13) epitopes, isolated from bovine brain, could be attached to a second generation PAMAM scaffold despite a 2.5-fold excess of the precious material being employed [104]. The use of longer spacers can sometimes help to reduce the problem of steric hindrance [105]. With smaller carbohydrate epitopes multivalent scaffolding is more feasible, such as in the case of T-antigen (Gal1α-3GalNAc1α-R), which has been assembled onto a *G4*-PAMAM dendrimer in 79% yield, using only 1.1 equivalents of the glycoside building block per amino group [93].

In addition to PAMAM, PPI scaffolds have also been used [104], such as for the synthesis of a glycodendrimer with 64 galactosyl residues to target the liver Ashwell receptor [106]. PPI glycodendrimers bearing randomly sulfated galactosyl residues, mimicking the carbohydrate clustering found in lipid rafts, have been synthesized to compare the ability to inhibit HIV-1 infection of cultured indicator cells with dextran sulfate (Fig. 9). The latter is a known, potent, binding inhibitor of HIV-1. An average incorporation of 44 galactosyl residues (with an average of 2.4 sulfate groups per monosaccharide residue) on a 64-valent scaffold using standard peptide coupling conditions could be achieved and the testing results obtained indicated that these glycodendrimers inhibited the infection by HIV-1 isolates comparably well as the known dextran sulfate inhibitors. Thus these dendrimers may serve as the basis for the development of a novel class of HIV-1 binding antagonists [107–109].

3.2.2 Polyethylene Glycol Scaffolds

Polyethylene glycols (PEGs) are often used as linkers between the scaffold and the carbohydrate unit. Polyethylene glycols in combination with pentaerithritol as core structure have been used as soluble dendrimeric support material for the synthesis of the lacto-*N*-neotetraose tetrasaccharide with an average number of incorporated sugar moieties [110, 111]. Multivalent structures aimed at inhibiting binding to FimH were constructed on a similar core-spacer structural theme. A nitro-triol was used as an initiator core and extended with oligoethylenglycols of different length to allow the conjugated carbohydrate ligands to accommodate better to the FimH receptors of type 1 fimbriae. The carbohydrate ligands were attached to the scaffold

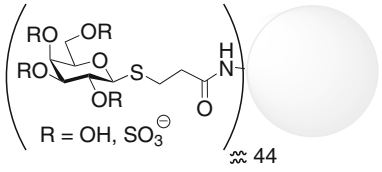


Fig. 9 About 44 sulfated galactosyl residues were attach to a PPI scaffold

as trivalent wedges which in turn had been linked via the 6-position of the methyl α-D-mannosides to a triacid [112]. Mannosylated glycodenderimers using glycerol and glycerol glycol polyether scaffolds were prepared on multigram scale with up to four ligands (63% in the coupling step, Fig. 10) [113].

3.2.3 Ester-Based Dendrimers

The majority of globular shaped glycodendrimers are constructed on PAMAM or related scaffolds, and thus robust groups as amides or tertiary amines are predominant in these cores. Rare exceptions are the Boltorn™ glycodendrimers which apply ester linkages (Fig. 11). This cheap scaffold is mainly used in its polymeric form as additive for plastics and coatings. The first example published in 2003 reported the synthesis of a 16-valent (60% yield) and 32-valent (68% yield) mannosylated dendrimer. The carboxylic groups were introduced to the dendritic support using succinic anhydride in the presence of DMAP in pyridine and covered with unprotected aminoethyl mannoside (1.2 equivalents per reactive site) under standard peptide coupling conditions. The products showed a high solubility in physiological media and low toxicity. Binding studies to the mannose-binding lectin *Lens culinaris* demonstrated the existence of a clear interaction between the mannose derivative systems and the *Lens* lectin [115]. Similar structures were used to investigate the interaction between DC-SIGN, a C-type lectin receptor of dendritic cells recognizing high mannose structures and involved in the initial

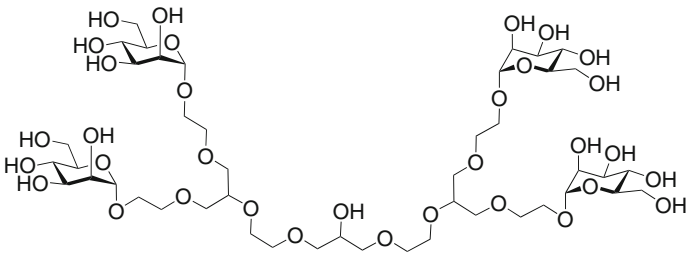


Fig. 10 A tetravalent polyether based glycodendron presented in [113]

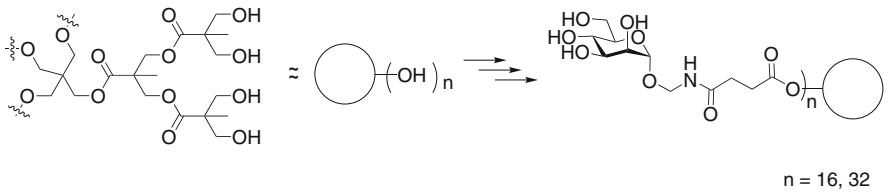


Fig. 11 A fragment of a "perfect" Boltorn™ polymer and a schematic representation of the prepared dendrimers [114]

steps of numerous infectious diseases. The 32-valent mannosylated Boltorn™ dendrimer was able to block specifically the transreceptor function of DC-SIGN in the *Ebola* virus model [116]. Binding constants in the submicromolar range were obtained, assuming that the multivalent conjugate successfully mimicked the natural DC-SIGN organisation as microdomain in the dendritic cells plasma [117]. Recently, biocompatible single walled carbon nanotubes have been designed using mannosylated Boltorn™ dendrons as coating. Triazole ligation was employed for dendron fixation to the nanomaterial and evaluation of the coated nanotubes showed reduced cytotoxicity [118].

3.2.4 Aromatic Cores

Common core structures are aromatic systems (Fig. 12), e.g. derivatives of trisubstituted benzene [70]. These can in turn be extended either by functionalized aromatic systems or by other functionalized linkers, e.g. carbohydrate-based spacers [119] or additional monosaccharides, e.g. furanoside derivatives [120]. A similar architecture has been employed for anthracene capped chiral dendrimers [112, 121].

A 3,5-di-(2-aminoethoxy)-benzoic acid core and branching unit was used to attach up to eight copies using long PEG-based linkers (>20 carbon atoms per chain) of a GM1 mimic for inhibition of cholera toxin binding. The protected clusters were produced in 65, 40 and 14% yield for the di-, tetra and octavalent structures respectively. While the monovalent GM1 mimic showed an expected inhibition in the mM range, the multivalent scaffolds led to increased binding. The best inhibition was obtained with the octavalent analogue, although the highest relative potency per sugar epitope was achieved with the tetravalent conjugate [105] (Fig. 13). The multivalent GM1 mimics were isolated as inseparable mixtures containing up to 33% cyclic imides formed in the linker-unit during deprotection. Possible effects on conformational changes in the GM1 mimics were mentioned, while an accurate IC_{50} concentration could not be determined since the limit of the assay was reached. A similar study has been carried out with analogous lactosyl-

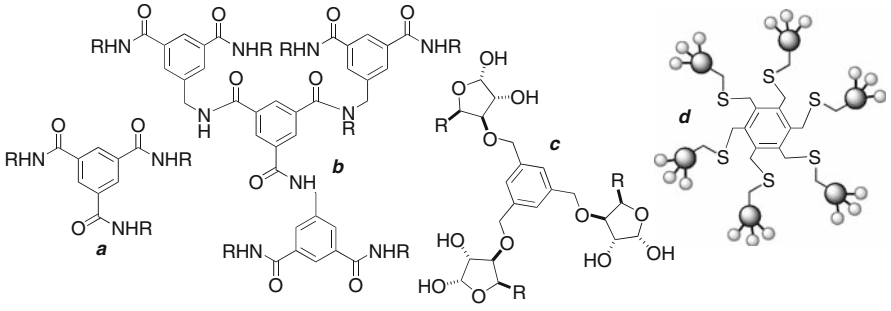


Fig. 12a–d Some reported aromatic core units. **a**,**b** [70]. **c** [120, 121]. **d** [122]

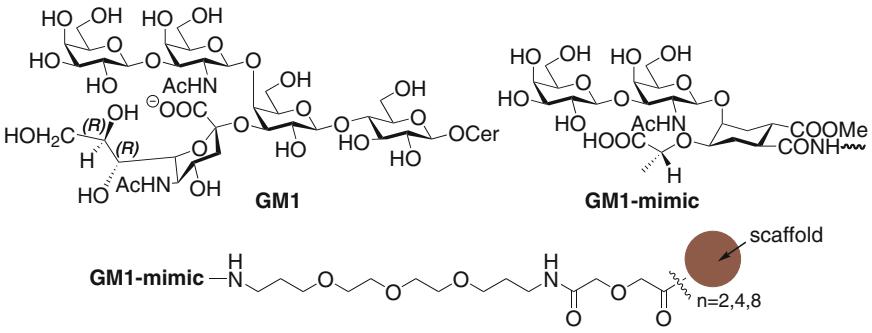


Fig. 13 The GM1 structure, its mimic and a schematic representation of the multivalent products reported in [105]

decorated dendrimers [123]. The same core structure was also used to study the adhesion of other bacterial lectins [124]. Recently polyphenylenes have been introduced as scaffolds for glycodendrimers [125]. An interesting architecture has been presented by Roy et al. using oligothiols as initiator core. Trivalent mannosylated clusters, prepared using triazole chemistry, were attached to these cores by almost quantitative S_N2 reactions [122].

3.2.5 Calixarenes, Cyclodextrins and Cucurbituril Cores

Larger scaffolds like calixarenes [58, 126, 127], and cyclodextrins have also been proven useful for the construction of glycodendrimers. Tetravalent glycosylated calixarene clusters have been prepared via isothiocyanate ligation. The obtained thiourea groups did serve not only as conjugation linkers but also as binding units for anionic substrates as shown by NMR spectroscopy and ESI MS experiments (Fig. 14). Turbidimetric analysis indicated specific interactions with Concanavalin A (ConA) and peanut lectin (PNA). Thus, the use as site specific molecular delivery systems was suggested [128]. Octa- and tetravalent calixarene-based glycoconjugates exhibiting *N*-acetyl-D-glucosamine residues have been prepared (50–95% yield, linked via a β-thioureido group) and tested as binding inhibitors in agglutination assays using the wheat germ (*Triticum vulgaris*) agglutinin (WGA) and erythrocytes. The inhibitory ability had been found to be dependent on the presence of the spacer and on the shape and rigidity of the calixarene skeleton [126].

In similarity to calixarenes, cyclodextrins (CDs) can host smaller guest molecules in their cavity. Thus, glycodendrimers built on these cores could possibly function as site-specific molecular carriers. An interesting idea in this sense has been realized by Djedaïni-Pilard, Fernandéz and collaborators to allow both the tuning of lectin binding and the prevention of undesired receptor-blockage with empty carrier molecules, a common problem in designing site specific drug delivery systems. The rim of βCDs was decorated with one bi-functional wedge, comprising

a mannosyl core cluster targeted for ConA binding and a tyrosin unit, the latter small enough to be included in the βCD cavity. The self-inclusion of the small tyrosinyl unit resulted in an unfavorable ligand presentation, yet adding a cavity-competing molecule released the tyrosinyl residue and switched the mannosyl core cluster into a perfect-fit position. Further addition of a cavity-competing scavenger could tune the binding event (Fig. 15) [129].

Several βCDs glycodendrimers decorated with different sugar epitopes, e.g. galactose, lactose and lactosamine, using direct linking and spacer arms of different length were synthesized (approx. 5.5 equivalents sugar thiol per 6-iodo βCD group, 7 days, 73–91% yield) to evaluate both the inclusion complexation behavior and the affinity to ConA [130, 131] (Fig. 16, left). Heptasubstitution of the scaffold led to a slight increase of the binding constant values, but doubling the number of ligands did not gain further affinity to ConA. The host capacity is influenced on both steric effects and the properties of the guest molecule [130]. The idea has been extended

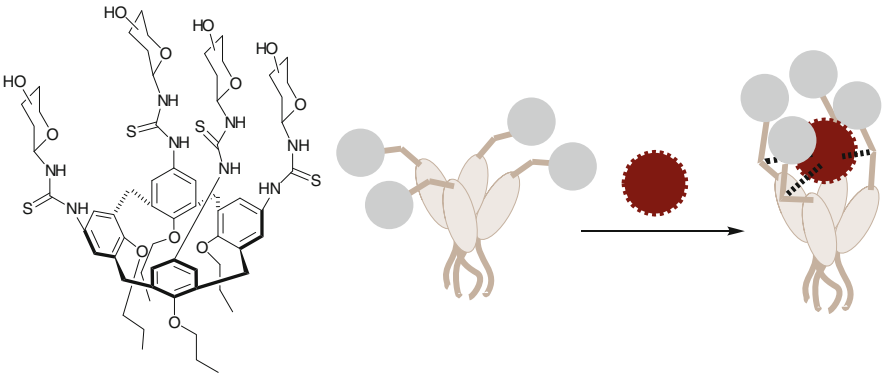


Fig. 14 A glycodendrimer constructed on a calixarene scaffold (*left*) and the proposed binding mode (*right*) presented in [128]

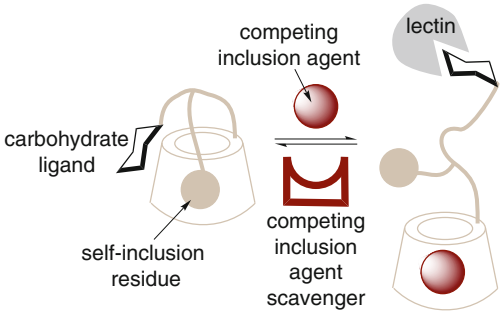


Fig. 15 A schematic representation of the reported switching of carbohydrate –lectin binding through allosteric supramolecular interactions (adapted from [129])

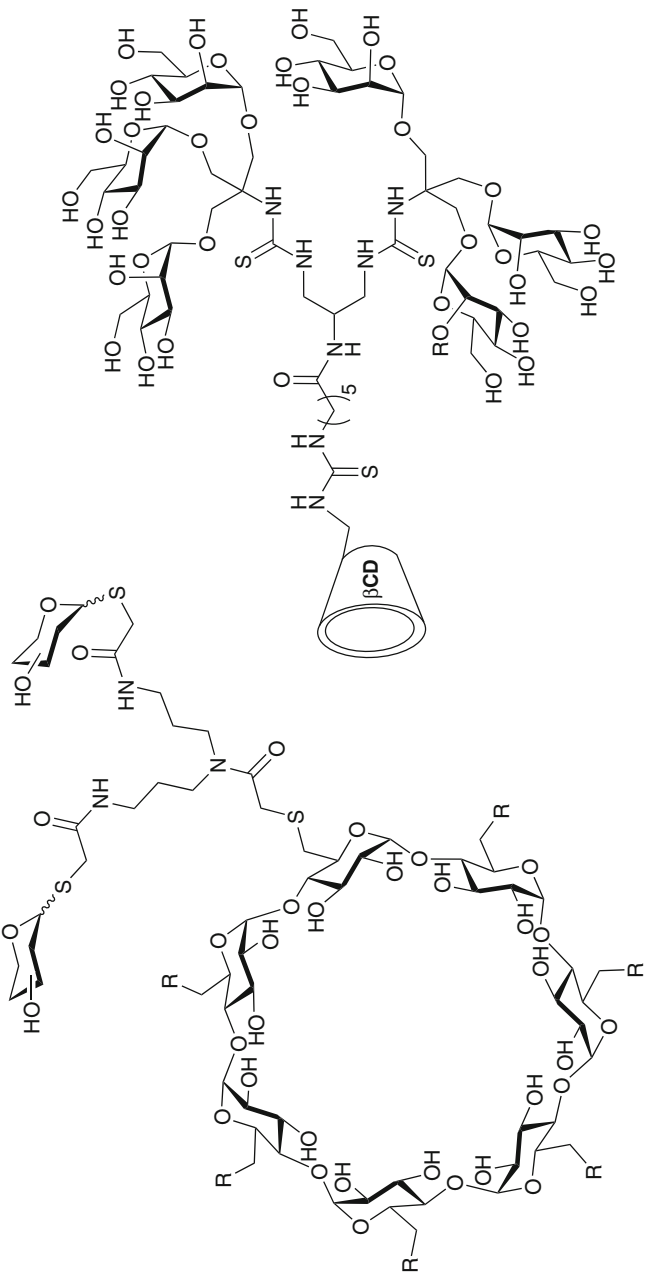


Fig. 16 A βCD glycoconjugate (*left*) [130, 131] and a higher branched version (*right*) reported in [85, 133]

into a chemo-enzymatic approach producing a heptavalent sialyl Le^x cluster. The 6-iodo βCD derivative was coupled with *N*-acetylglucosamine (GlcNAc) as its sodium thiolate and the attachment of all additional sugar residues was aided by suitable enzymes [132].

Mannosylated βCD clusters differing in the ligand valency and geometry were prepared and assessed for their binding efficiency towards ConA and a mammalian mannose/fucose specific cell surface receptor from macrophages. This series of dendrimers was synthesized to explore the structural prerequisites to allow inclusion complex formation of the anticancer drug docetaxel (Taxotere®) as the target guest. Three major building blocks were prepared (branched mannosylated wedges, branching units, 6′-amino-6′-deoxy βCD) and assembled using different spacer elements in a divergent manner (Fig. 16, right) [133]. A similar arborole like structure was obtained by synthesizing the multivalent wedge on a solid phase resin. The conjugation of the single copy hexavalent wedge was achieved by Ellman's safety catch linker chemistry using the monoamino βCD template as nucleophile in 68% yield as estimated from initial resin loading [85].

Other cavities mainly targeted as drug-delivery vehicles, have been exploited. Cucurbituril-based (CB) carbohydrate clusters (carbohydrate wheels) were synthesized by photoreaction of allyloxylated CB-6 with acetylthioglycosides. Twelve glucosyl, galactosyl and mannosyl residues (4 equivalents per reactive site, 2 days in MeOH) were introduced in 77, 76, and 83% yield, respectively, and finally deacetylated [71] (Fig. 17). The mannosylated CB cluster's affinity to ConA was examined by turbidimetric analysis. A 1,100-fold excess amount of methylmannoside had to be added to obtain a transparent solution as consequence of complete disruption of the cross-linking interaction.

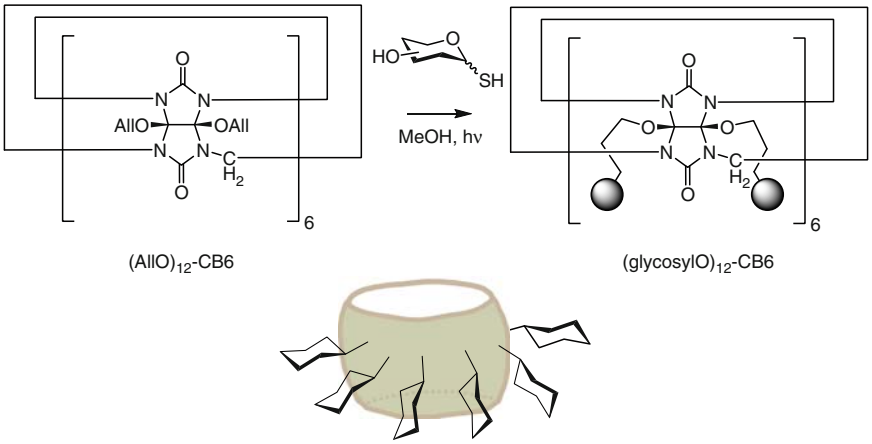


Fig. 17 Synthesis of CB glycoclusters and a schematic representation of principal structure (adapted from [71])

3.2.6 Carbohydrate-Centered Dendrimers

Monosaccharides and oligosaccharides can serve as core structures to lead to carbohydrate-centered dendrimers. Various examples can be found in the recent literature [134–139]. “Octopus glycosides” [140] has been used as quite descriptive name for this type of dendrimers for which a perallylated carbohydrate is usually an early synthetic intermediate. Besides monosaccharides, di- and trisaccharide cores have also been employed for this approach together with non-branching linkers, such as in the synthesis of a raffinose-based cluster mannoside [135]. Fine-tuned reaction conditions enabled to attach up to 11 mannosyl residues using glycosylation chemistry (Fig. 18) [141]. Moreover, the concept could be extended to even more privileged structures, employing an orthogonal linker at the anomeric position of the carbohydrate core, allowing the synthesis of octopus glycosides with a fluorescent probe or a biotin label, i.e. [134].

3.2.7 Inositol Cores

Inositols, although more robust than carbohydrates, are less readily available and only rarely used as cores for glycocluster synthesis. Recently, *scyllo*-inositol has been utilized as scaffold for the synthesis of a multivalent glycoconjugate according to a bi-directional synthetic route (Fig. 19). This allowed the number of terminal sugars to be varied, as well as attachment of the glycoclusters to a carrier (e.g. for the construction of microarrays [142]).

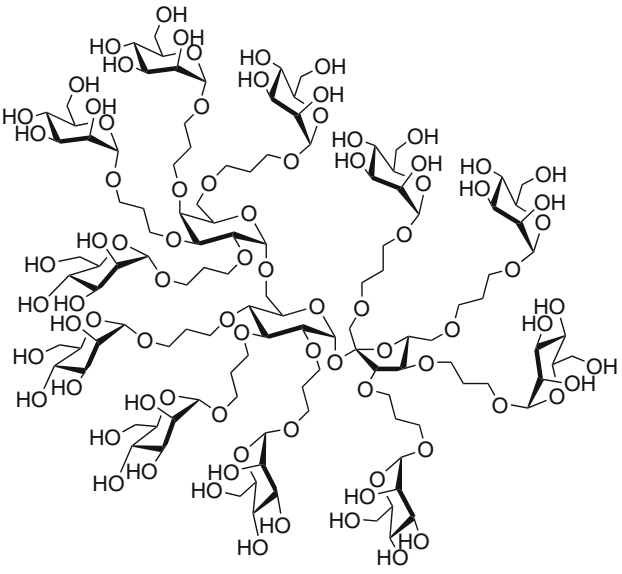


Fig. 18 The mannosylated raffinose octopus glycoside presented in [137]

3.2.8 Carbosilane-Based Glycomimetics

After some first examples around year 2000 [143–145], carbosilane-based glycoconjugates have become popular and their advances have been reviewed recently [57]. The claimed advantages are their neutral nature and biological inertness [146]. Three (64%), four (55%) and six (44%) copies of lacto *N*-neotetraose have been attached via substitution chemistry to the carbosilane scaffolds depicted in Fig. 20 [147]. Presented in the same paper are the binding constants of lactotriaose (GlcNAcβ1-3Galβ1-4Glc) decorated carbosilane clusters to the WGA which showed a 2,500-fold enhanced affinity of the hexavalent glycodendrimer to the lectin compared to free lactotriaose. Up to eight sialic acid residues have been clustered on carbosilane scaffolds and their biological response against *influenza* virus sialidases was investigated showing that some of the dendrimers have inhibitory potency for the sialidase [148].

Carbosilane dendrimers with three (86%), four (72%) and six (75%) galabioses (Galα1-4Gal) were prepared to evaluate their use as artificial inhibitors against Shiga toxins produced by *E. coli* O157:H7 [149]. Other carbosilane dendrimers carrying multiple copies of the globotriaosyl (Galα1-4Galβ1-4Glcβ1-) ceramide trisaccharide on the surface were synthesized and evaluated towards their biological activity against the Shiga toxins [146]. Recently, the synthesis of a highly photoluminescent globotriaosyl-functionalized silole glycocluster has been reported. Six peracetyl-protected carbohydrate portions were introduced by substitution of the bromo-terminated core with thiolates. The deprotected cluster represents the first reported hydrophilic silole derivative [150].

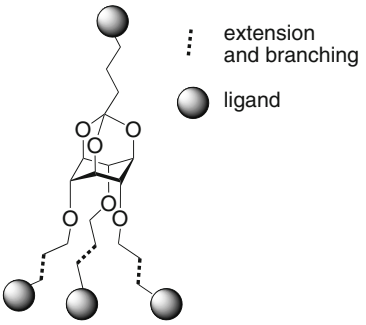


Fig. 19 An inositol based glycoconjugate [142]

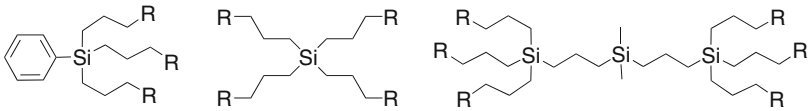


Fig. 20 Different carbosilane cores used, e.g. in [147]

3.2.9 Comb-Like Scaffolds

Highly symmetric structures with an overall globular shape do not always provide the most advantageous presentation of multiple ligands. Also a linear design can attain a high degree of symmetry as has been shown with the synthesis of comb-like glycodendrimers using chemoselective ligation to introduce the Tn-antigen (GalNAcα1Ser/Thr) carbohydrate portions [151]. A short amino acid sequence containing alternating lysine residues in the backbone, determining the branching points, was prepared by solid phase synthesis (Fig. 21). The set of the glycodendrimers obtained were tested as inhibitors with different plant and mammalian lectins. One of the dendritic compounds inhibited unexpectedly natural killing (NK) cells in both rats and humans. Spatial arrangement, as well as the number of ligands and the conjugation of the Tn antigen with immunoadjuvant compounds, has been assumed to be key factors in controlling an efficient immune response against these comb-like glycodendrimers. One drawback when employing linear scaffolds is their flexibility and possibility to form superstructures, for example by coiling up. Thus the scaffold has to be rather rigid, as in the example depicted in Fig. 21 [151], where an accumulation of amide bonds furnishes an overall stiffness of the molecule.

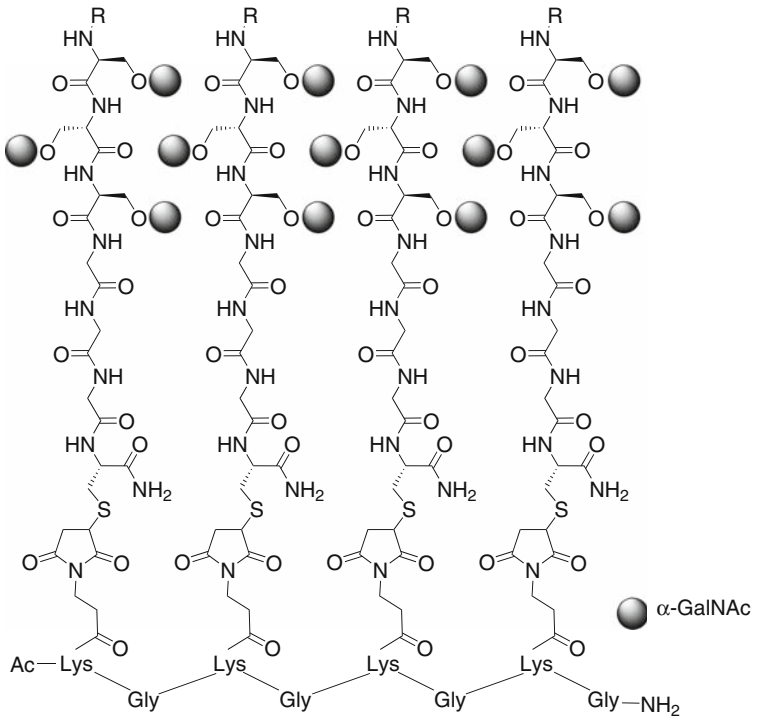


Fig. 21 An example of a comb-like scaffold [151]

3.2.10 Raft-Scaffolds

Control of the spatial arrangement of the carbohydrate ligands to achieve optimal binding features is a typical challenge in designing multivalent glycoconjugates. The use of RAFT (*r*egioselectively *a*ddressable *f*unctionalized *t*emplate) scaffolds is another approach to tackle this problem (Fig. 22) [152]. Smaller peptide rings are assumed to reassemble the support, which is needed for the synthesis of defined multivalent patches. As in most peptide-based glycoconjugates, lysine residues are used for the attachment of sugar ligands. Dendritic wedges constructed on orthogonally protected cyclopeptidic scaffolds using aminooxylated carbohydrates ($Lac\beta1ONH_2$, $GalNAc\alpha1ONH_2$ and $Man\alpha1ONH_2$) have been synthesized as carbohydrate-based recognition elements, to allow the eventual preparation of defined RAFTs with mixed carbohydrate content [72].

3.2.11 Pentaerythritol and Other Small Cores for Glycoclusters

After the synthesis of high-valent glycodendrimers had been a central goal in the field (the lactosylated 128-mer has been one of the largest (PAMAM) dendrimers published [43]), glycoclusters with typical valencies between three and six have emerged to be valuable tools for the investigation of carbohydrate recognition domains. Some examples are presented in Fig. 23a–d.Three copies of a branched trisaccharide have been attached to a triacid core (Fig. 23a) in 56%. The structure was intended to mimic a possible antitumor β-glucan protein isolated from barmy mycelium of *Grifola frondosa* (Maitake) with a molecular weight of 95 K [153]. The triacid scaffold can easily be synthesized from acrylic acid *tert*-butyl ester and nitromethane. Reduction with Raney-Ni converts the nitro group into an amine if required [154]. Pentaerythritol (Fig. 23b) is generally useful for the preparation of tetravalent cluster [155, 156]. Mannosylated clusters based on pentaerythritol were

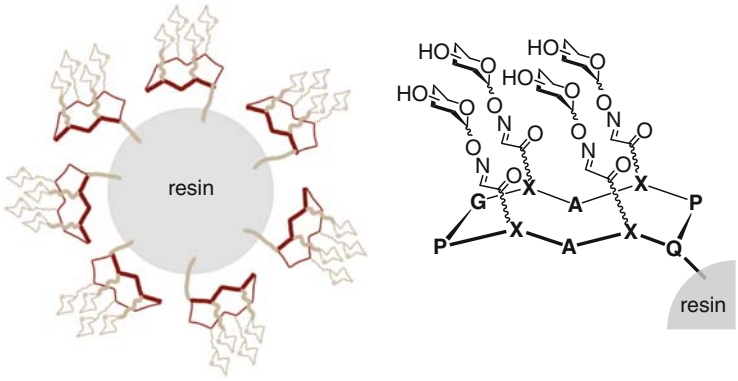


Fig. 22 A cyclic peptide forms the RAFT scaffold [72]

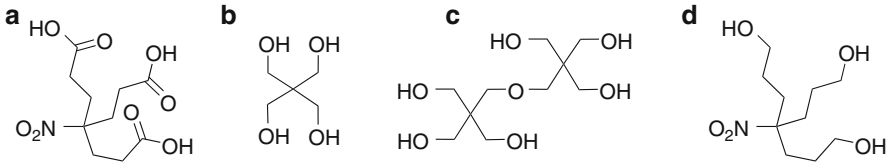


Fig. 23 Some typical core structures employed for the synthesis of glycoclusters

tested for their capacity to block binding of *E. coli* to yeast mannan in vitro. Compared to methyl α-D-mannoside a more than 200-fold enhanced inhibitory potency was reported for the mannosylated cluster [157]. A small library of mannosylated pentaerythritol and bis-pentaerythritol (Fig. 23c) clusters with different linker has been synthesized and the reported data showed a potential as therapeutic candidates for treatment of urothelial infections caused by *E. coli* [158].

3.2.12 Exotic Cores

This paragraph presents a collection of unusual core structures used for the synthesis of distinct monodisperse glycoconjugates, where most examples were not initially targeted to investigate carbohydrate–protein interactions. Thus, a porphyrin core has been functionalized with four perbenzoylated and twelve peracetylated βGlcNAc residues in 39 and 16% yield, respectively [159]. Up to six glycosyl groups have been attached to a ferrocene sandwich and the effect on the inclusion of the ferrocene moiety in the cavity of βCD have been studied. It has been claimed that conformational changes occurring in these structures would open the way to tertiary structure associated effects as known from proteins [160]. Recently a [60]fullerene has been used as support for glycodendron synthesis. Two mannosylated dendrons presenting six monosaccharides were connected through two adjacent imino bridges to the all-carbon framework [161]. Photoisomerisable glycodendrimers have been constructed on an azo-benzene core which was terminated with up to four mannose residues and up to eight lactose units (Fig. 24) [162]. The conjugates were used for binding studies. ConA was the target lectin for the mannosylated conjugates and the lactose covered compounds were used with PNA. ITC studies with the isomer-enriched glycoclusters revealed a general better binding for the *cis*-isomer than the *trans*-form [162].

3.3 Linkers Merge Sugars and Scaffold?

Numerous different spacer units have been used to link carbohydrate moieties to scaffolds in order to produce multivalent glycoconjugates. In classical dendrimers, the linker is often short and treated more or less as a connecting unit only, although

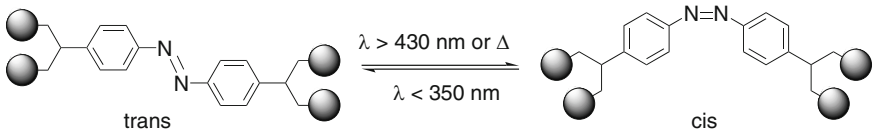


Fig. 24 A photo-switchable glycocluster [162]

the nature of the linker will have an impact on the accessibility of the sugar epitope both for a reagent during synthesis and for a tested receptor. Often, the choice of the linker is governed by the chemistry used and not by the desirable biological properties of the targeted product. It has been recognized that the spacer arms are not passive elements and influence the binding ability according to their chemical nature [87, 131]. However, it has been difficult to choose systematically a linker in order to guarantee proper ligand presentation according to exact geometrical criteria [112, 163]. For spherical dendrimers as obtained from PAMAM scaffolds, the initiator core can give a good indication of the overall shape of the glycodendrimer, and the spacing between the linker arms. MD simulations as well as XSAFs studies provide a valuable tool for this type of investigation [39, 164].

Hydrophobicity of linkers has also been shown to be of importance and sometimes more lipophilic linkers turn out to be the better choice. Inhibition of the adhesion of F1C-fimbriated recombinant *E. coli* and the *P. aeruginosa* strains PAO and PAK was studied with multivalent conjugates terminated with up to four βGalNAcβ1-4Gal disaccharides. Multivalent effects up to a factor of 10 were observed and different binding modes discussed. Clearly the lipophilic linkers were beneficial for the binding, but continuing research is required to understand fully the findings [124]. Three mannosylated clusters differing in the length of the branching spacer were synthesized and tested for their affinity to the type-1 fimbrial lectin. None of the new clusters was a better inhibitor than the reference cluster with a zero-length branching spacer. Interestingly, the most flexible cluster showed an almost doubled affinity to the lectin [165]. Depending on the purpose of the glycoconjugate, aromatic systems and squaric esters in the linker units should be used with awareness, but rigid linkers can be very sensible to save entropic loss. However, if non-fitting linkers are used this will be detrimental for the activity of the conjugate.

3.4 "Additional Gadgets": If Multivalency is Not Enough

Numerous homogenous glycoconjugates have been synthesized and used to study carbohydrate–protein interactions. Sometimes it is convenient to have a reporter system integrated into the conjugate. Depending on the function, this additive should or should not interfere with the receptor site. Glycoconjugates have been equipped with almost everything which seems to be useful, e.g. a biotin label,

fluorescent [166], or photoactive labels [167] and many more. As an example, PPI-based glycodendrimers carrying mannose or lactose epitopes were labelled with radioactive technetium ^{99}Tc [168] to study the biodistribution pattern of these constructs in mice to explore the potential of these systems as drug carriers. DOTA-like [DOTA = 1,4,7,10-tetrakis (carboxymethyl)-1,4,7,10-tetraazacyclododecane] chelators are well known to form Ln(III) chelates of high thermodynamic and kinetic stability. Glycodendrimers constructed on PAMAM scaffolds were equipped with a DOTA monoamide-linked dendron. This type of glycodendrimers might have a future as candidates for medical imaging agents (MRI) and gamma scintigraphy [169]. An octavalent *N*-acetyl-glucosamine functionalized PAMAM with an in vitro high affinity for the recombinant lymphocyte receptor NKR-P1A was equipped with a fluorescent marker to trace the fate of the glycodendrimer in a mouse model [170].

4 Assembling Multivalent Glycomimetics

4.1 How to Choose a Chemical Method?

The chemistry used to produce defined multivalent glycoconjugates is rich and varied. Nevertheless, suitable reactions for the synthesis of rather large and highly symmetric structures have to meet a number of requirements. The most important prerequisite for such reactions is a high, close to quantitative yield in order to make purification easier and to diminish defect structures. In addition, mild reaction conditions are required as many sugar derivatives such as fucosides, sialic acid derivatives or deoxy sugars, i.e. [102], are highly sensitive and easily destroyed. Since purification of the target structures can involve enough problems in itself, the employed reagents should yield no problematic by-products. Furthermore, high chemoselectivity is appreciated to minimize detouring protecting group chemistry. To reduce the number of steps as well as to allow the usage of saccharide structures isolated from natural sources, it is advantageous if the synthesis can be carried out in water or buffer systems.

4.2 Chemoselective Ligation or Click Reaction?

Many classical reactions have been used to link a carbohydrate epitope to multivalent scaffolds. During recent years “chemoselective ligation” [171, 172] and “click” chemistry have become popular. The term “chemoselective ligation” is used for reactions which allow selective bond-formation of two complementary functional groups in the presence of other, non-complementary functional groups under particularly “bioorthogonal” conditions [173]. Amide bond formation is possibly the

most common conjugation reaction, but not a perfect example of a chemoselective ligation, since the reaction is not completely chemoselective because ester formation is sometimes observed. Among the examples for chemoselective ligation reactions, so-called "click" reactions [174] form a sub-group, lacking the claim of a "biological environment" for the reaction conditions [175].

4.3 Synthetic Methods to Assemble Multivalent Glycomimetics

Section 4.3.1 will give an overview of some common synthetic methods used for the preparation of defined multivalent glycomimetics.

4.3.1 Oxime Ligation

This type of chemoselective ligation has been widely used for the preparation of glycoconjugates during the last few years, e.g. [72, 175–177], since alkoxime formation is a robust, high yielding and highly chemoselective reaction. This functional group is formed under slightly acidic conditions by reversible condensation of an aldehyde and an alkoxylamine (Fig. 25). A major advantage of this ligation technique is that it requires neither a coupling reagent nor chemical manipulations except mixing of the two components [178].

In the case of a carbohydrate-aldehyde source, an isomeric mixture of products is to be expected, consisting mainly of the *E*-isomer and some of the *Z*-form of the open-form sugar alkoxime and only minor amounts of the two anomeric pyranosyl oximes (Fig. 26) [179].

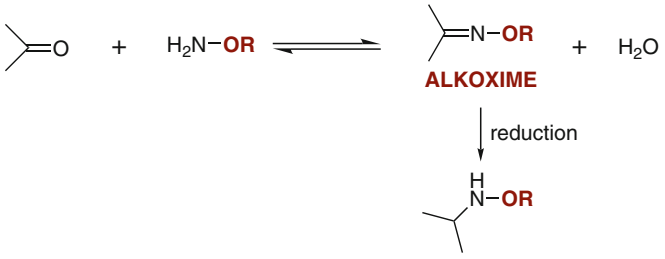


Fig. 25 The general formation of an alkoxime

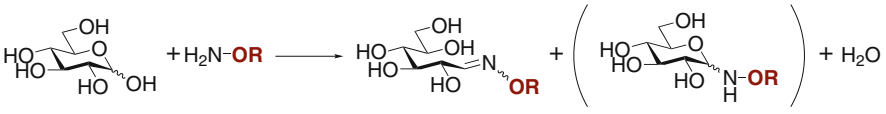


Fig. 26 An isomeric mixture is expected when preparing sugar alkoximes from a carbohydrate–aldehyde source

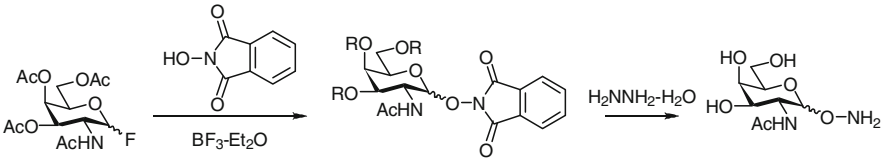
The formation of mixtures and open chain products can be circumvented by reversing the functionality. In this case the carbohydrate delivers the oxyamino group which is then reacted with a carbonyl source to furnish the oxime linkage. A relatively convenient method to introduce the oxyamino function into a sugar moiety is via glycosylation using *N*-hydroxyphthalimide, e.g. employing the respective sugar fluoride with the aglycone in the presence of BF_3-etherate, and subsequent removal of the phthalimido group (Scheme 1 [72]).

PAMAM scaffolds have been decorated with carbohydrates via oxime ligation [177]. RAFT scaffolds have been functionalized with D-mannose, *N*-acetylgalactosamine and lactose via the reversed oxime ligation, i.e. the sugar molecules were aminoxylated (cf. Scheme 1) and then reacted in aqueous acetic acid with the RAFT scaffold provided on solid support and presenting four glyoxaldehyde functions. Excess sugar was recovered and RP-HPLC analysis of a reaction sample, cleaved from the resin, showed only the expected product [72].

4.3.2 Triazole Ligation

Another chemoselective ligation reaction is the [2 + 3] cycloaddition between an azide and an alkyne. This reaction has been discovered by Huisgen and was lately named "click-reaction" by Sharpless and Meldal [180, 181]. Whereas the Huisgen 1,3-dipolar cycloaddition leads to two isomeric triazole products at high temperature, "click chemistry" is performed under the catalysis of Cu(I), thus changing the reaction mechanism from a concerted to a step-wise route and resulting in the formation of the 1,4-substituted triazole as the only product, usually isolated in high yields [174, 182–186].

Various conditions have been evaluated for the Cu(I)-mediated triazole synthesis [186]. This reaction allowed the fast and efficient preparation of glycodendrimers with up to 27 carbohydrate residues (fucose, mannose, and lactose residues) in reproducible high yields (up to 92%). The resulting PEGylated glycodendrimers were completely characterized by NMR and MALDI-Tof and have demonstrated an increased capacity to aggregate lectins with higher generations. This novel glycoconjugate architecture benefiting from the advantageous properties of PEG was envisioned, i.a. (inter alia) as a potential tool for the study of carbohydrate receptor interactions (Fig. 27) [187, 188]. Microwave-assisted preparation of mono - and



Scheme 1 Introduction of the oxyamino group into the anomeric position of an *N*-acetyl galactosamine [72]

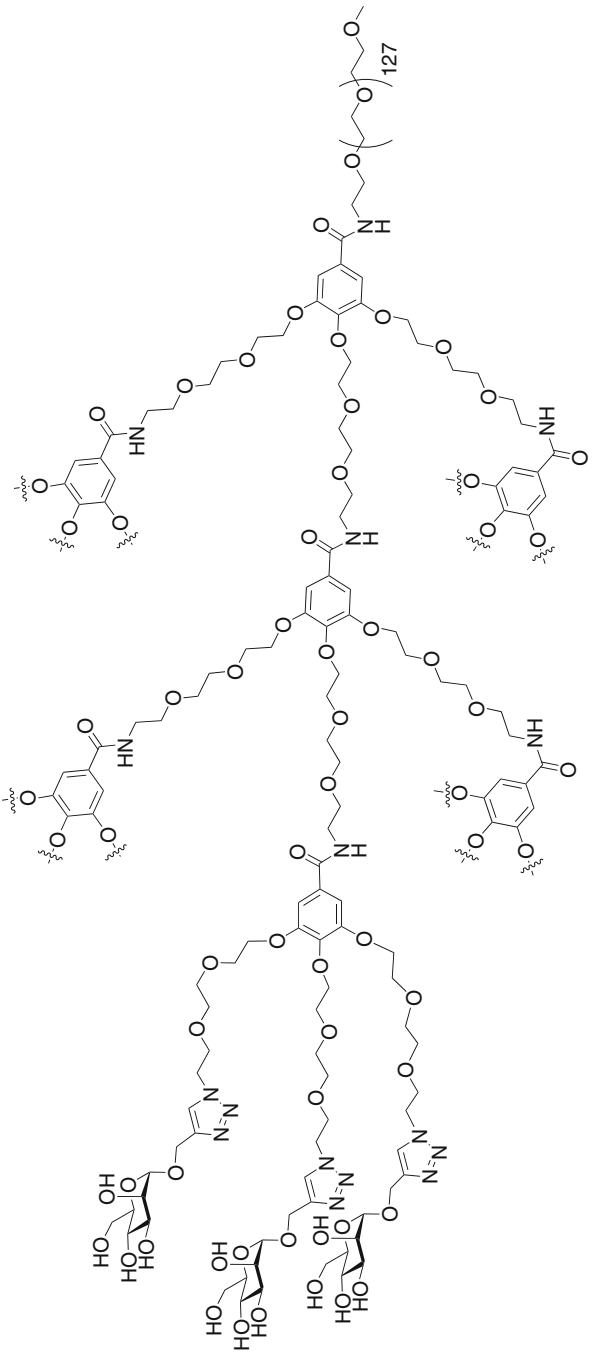


Fig. 27 An unusual design of a multivalent glycoconjugate to be used in carbohydrate–lectin interaction studies [187]

disaccharide-functionalized dendrimers gave faster reactions and improved the yields in general above 93%. Alkyne-terminated scaffolds were reacted with different protected as well as unprotected carbohydrates containing either an anomeric azido group or an azidopropyl group in the presence of sodium ascorbate (0.3 equivalents/alkyne group) and $CuSO_4$ (0.15 equivalents/alkyne group) in a water–DMF mixture. The reactions were exposed to microwave irradiation at 80 °C for 20 min and unreacted material was recovered by column chromatography [189]. Unsymmetrical dendrimers, containing mannosyl residues (16-valent) and two coumarin chromophores as terminating groups, have been prepared using the copper mediated triazole ligation chemistry to assemble wedges constructed on a Boltorn™ scaffold as dendrimer repeating unit (Fig. 28) [190].

Homo- and hetero-bifunctional glycodendrimers terminating with up to 16 fucoside and/or galactoside residues were synthesized in good yields using a convergent approach. Evaluation by turbidimetry was used to study the relative binding and crosslinking abilities of these glycodendrimers with the PA-IL and PA-IIL lectins from *Pseudomonas aeruginosa*. Insoluble complexes were rapidly observed from the first and second generation dendrimers as well as from a mixed glycodendrimer. This hetero-bifunctional glycodendrimer was also evaluated with PA-IL alone and showed potent crosslinking properties [191].

Many examples have been published during the last few years using efficient triazole ligations for the preparation of defined multivalent glycoconjugates. Mannosylated glycoclusters based on pentaerythritol and bis-pentaerythritol scaffolds with nanomolar affinities to the bacterial lectin FimH have been mentioned above [158]. Also tetravalent carbohydrate-centered glycoclusters, constructed on a methyl 2,3,4,6-tetra-*O*-propargyl β-D-galactopyranoside, were prepared efficiently by triazole ligation. As in many other examples, the azido group was introduced into the lactose and *N*-acetyllactosamine moiety via an anomeric 2-azidoethyl aglycon. Recognition of these water-soluble glycoclusters by plant lectin RCA_{120} was examined by capillary affinity electrophoresis using fluorescence-labeled asialoglycans from human α1-acid glycoprotein and this showed a 400-fold stronger inhibitory effect than free lactose, manifesting a strong multivalency effect [136].

4.3.3 Isoxazole Ligation

A 1,3-dipolar cycloaddition between nitrile oxides and alkynes has been reported for the attachment of carbohydrate ligands to different scaffolds [193]. Recently, a one-pot procedure has been published for the preparation of mannosylated and glucosylated glycoclusters by isoxazole ligation. Nitrile oxides were generated in situ in the presence of alkynyl derivatives allowing access to homo- and hetero-multivalent systems containing *O*- and *C*-linked glycosides and isoxazole bridges (Scheme 2). The affinities of some of these neoglyconjugates bearing mannose residues to ConA were evaluated by ELLA [192].

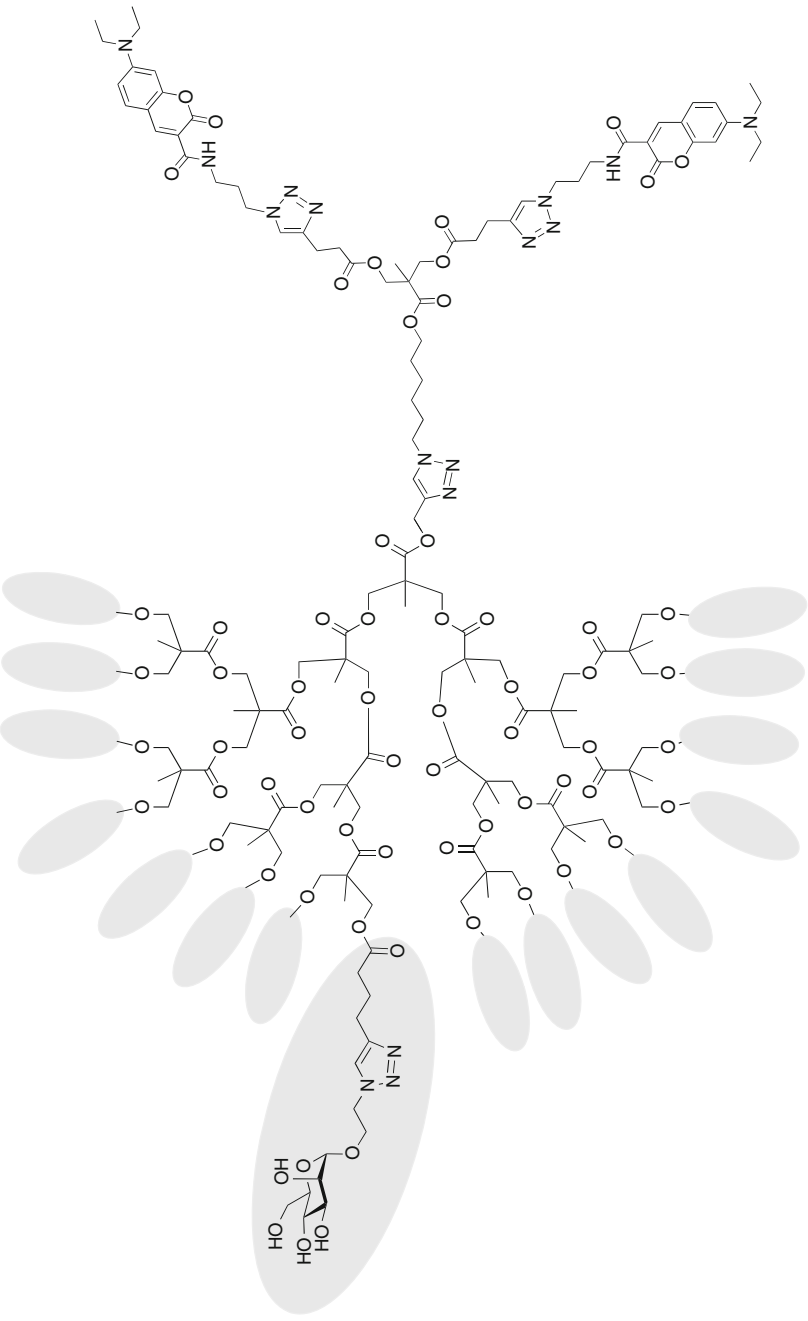
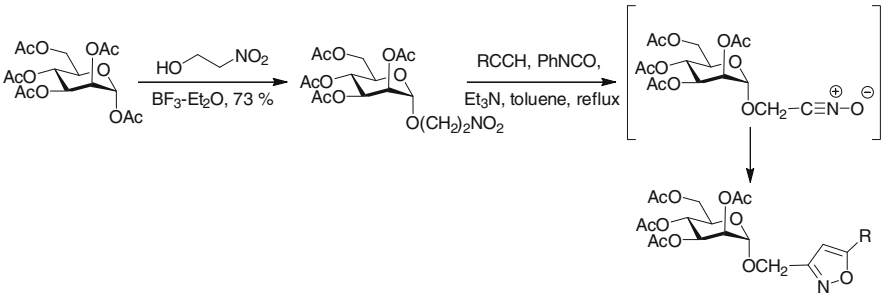


Fig. 28 Unsymmetrical dendrimers, containing both mannose binding units and two coumarin chromophores have been prepared on a Boltorn™ scaffold using "click chemistry" [190]

4.3.4 Traceless Staudinger Ligation

The traceless Staudinger ligation is one of the more recent developments in glycoconjugation by chemoselective ligation (Fig. 29). The original Staudinger reaction is used to convert azido groups into amino groups [194]. For this purpose a trisubstituted phosphine, usually PPh_3, reacts with an azido group under loss of nitrogen. Hydrolysis of the formed aza ylide produces the free amine and the phosphine oxide. The Staudinger reaction has been developed into a chemoselective ligation method to give amide bonds in aqueous solution [195]. This so-called "Staudinger ligation" employs a specially designed phosphine where one of the phosphine substituents carries both the ligation partner and a methyl ester. The latter enables the displacement of the hydrolysis step with an amide bond formation. The Staudinger ligation, a review has been published in 2004 [196], is very efficient but having a



Scheme 2 The preparation of isooxazole glycoclusters [192]

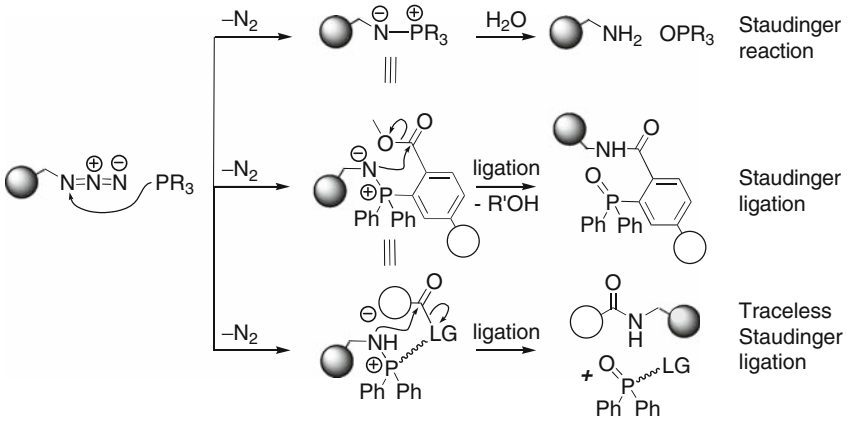


Fig. 29 Comparison between the Staudinger reaction, the Staudinger ligation and the so-called traceless Staudinger ligation

phosphine oxide moiety in the conjugation product is not always practical. This led to the development of the traceless Staudinger ligation [197, 198]. Still an amide bond is formed by attack of the aza ylide nitrogen but in this method the phosphine oxide is released during the amide formation. A drawback is the necessity to completely remove the phosphine oxide derivative, thus the reaction itself is not truly traceless. The other problem is to find good phosphines, which have a reasonable shelf live and a high reactivity in the ligation step. One of the best ligation reagents for this type of reaction has been proposed to be (diphenylphosphino)methanethiol (e.g. *S*-acylated) [199]. NMR studies using ^{13}C labelled reagents suggest that the ligation with this reagent proceeds via a substitution ($N \rightarrow S$) of the iminophosphorane intermediate rather than via an aza-Wittig mechanism. However, less efficient ligation reagents give a phosphonamide by-product as a result of the collapsing iminophosphorane intermediate according to the aza-Wittig mechanism [200]. New phosphino functionalized cross-linkers suitable for protein carrier derivatisation via the traceless Staudinger ligation have also been designed. The ligation was successfully applied to the conjugation of carbohydrate haptens to immunogenic protein carriers using a borane-protected phosphane which was deprotected immediately before ligation to give the best results in terms of stability toward oxidation and reactivity. However, a phosphine that is totally stable toward oxidation, which would allow intramolecular acylation in the absence of an organic co-solvent, is not available yet [201, 202].

The reactions presented above are those most commonly associated with the term chemoselective ligation although there are more reactions which fall into this class. A combination of two orthogonal ligation reactions, hydrazone formation from a hydrazine and an aldehyde (often a glyoxal adduct), and thioether ligation (by substitution of a chloroacetyl group by an in situ formed thiol) in a one-pot procedure have successfully been carried out [203].

4.3.5 Conjugation by Sulfide Formation

Thiols are easily accessible, good nucleophiles and reactive in both ionic and radical reactions, making thioether formation a robust and safe choice for glycoconjugation (Fig. 30). Sulfide conjugation is rather chemoselective and often carried out with unprotected sugar derivatives or in one-pot procedures where the sulfide linkage is established with concurrent removal of base-labile protecting groups (esters) under the often basic conjugation conditions.

The required thiols are prepared either by reduction of a disulfide linkage or by (concomitant) deprotection of suitable thioesters. Conversion of a terminal double bond in a radical reaction can be carried out with both thiols and thioesters and these reactions can be induced by a radical starter or light [37]. Using thiols in the radical step produces directly the wanted functional group while the reaction with thioesters requires an additional deprotection step. The advantage of using a thioester reagent is on the other hand that no troublesome disulfide formation can occur in this step. The free thiols are usually handled under inert atmosphere

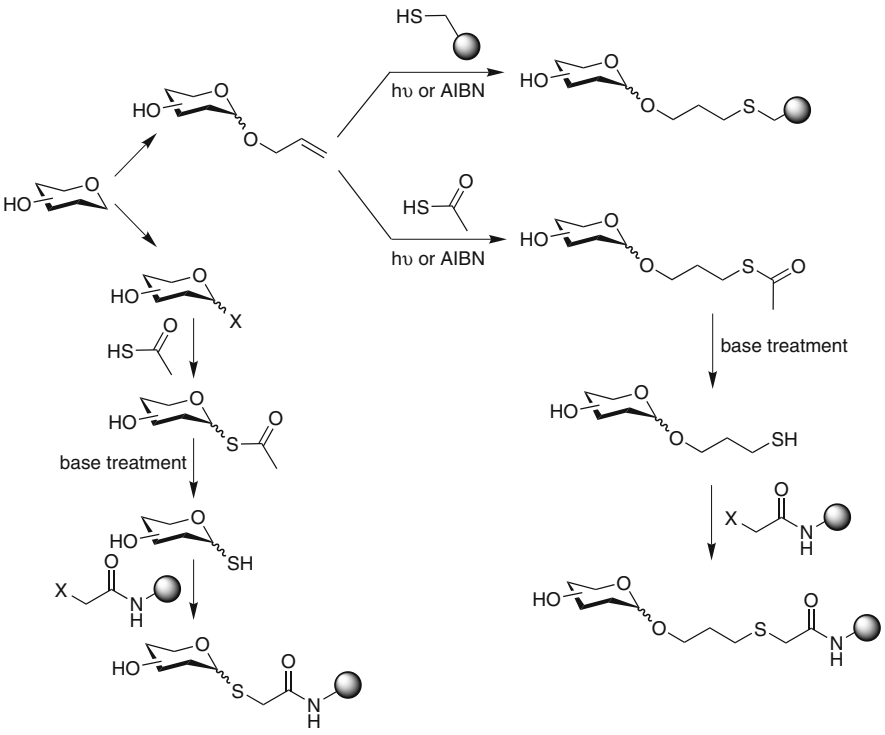


Fig. 30 Some options to produce glycoconjugates via thioether linkage

before and during the conjugation. In ionic-type (substitution) reactions, the conjugation partner is often a chloroacetyl amide and many investigators have applied this methodology. Roy and co-workers have used this technique already in early examples of glycodendrimers (e.g. [37, 204–207]). In a similar way, up to 16-valent α-sialodendrimers have been prepared to study the inhibition of binding of human α_1-acid glycoprotein (Orosomucoid) to *L. flavus* in good yields [205]. Another option is, to use a thiolated carbohydrate species with an acrylamido derivative in a Michael-type reaction. By this means, divalent T-antigen conjugates were obtained in quantitative yield using the thiol sugar derivative together with *N*,*N*′-bis(acrylamido)acetic acid in a 1,4 conjugate addition [208]. A recent example of multivalent α-sialodendrimers has been reported by Sakamoto and collaborators substituting ω-brominated carbosilane scaffolds with thiol telomerized sialic acid derivatives. Preliminary evaluation of the biological responses of the carbasilane dendrimers against influenza virus sialidases showed that some of the dendrimers have inhibitory potency for the sialidase [148]. Three (88%) and nine (36%) globotriosyl epitopes have been linked to a carbasilane scaffold

using benzylether protected sulfides with brominated carbasilane scaffolds in liquid ammonia. Removal of the benzyl group, and subsequent condensation with the thiolate anion was carried out in a one-pot manner [146]. A series of carbasilane dendrimers carrying up to six copies of lacto-*N*-neotetraose has been prepared by mixing the brominated scaffold with the ω-thiolated oligosaccharides in a solution of DMF and MeOH and subsequent addition of sodium methoxide (44–64% yield) [209]. A similar approach has been used with galabiose [149]. Hexavalent glycodendrimers, intended for enzyme-assisted synthesis, have been constructed from the glycosyl thiols on a hexa-allylated polyethylene polymer based scaffold. Radical coupling was carried under 254 nm lamp irradiation using 0.05 M solutions of the scaffold in water and a small excess (1.3 equivalents) of the sugar thiol per allyl group. Purification was accomplished by dialysis [110]. The same approach has been used to introduce six trisaccharide-copies on this scaffold which in a following enzymatic step was converted into lacto-*N*-neotetraose [111]. CB carbohydrate clusters have been synthesized using hexa-allylated cucurbituril derivatives and radical coupling initiated with light produced the glycoconjugates [71]. Sometimes the thiol function is used to introduce an additional linker who in turn is terminated with the functional group required for the final attachment to the scaffold. 3-Thiopropionic acid has been used to introduce the carboxylic function to a T-antigen derivative before coupling up to 32 copies to a PAMAM core [93].

4.3.6 Peptide Chemistry

Amide bond formation is possibly the most frequently used reaction for scaffolding carbohydrates. Usually an amino group-terminated linker, often the aglycon moiety of a glycoside, is reacted with an oligo-carboxylated scaffold. Unproteced or protected carbohydrate derivatives can be employed in the peptide coupling reaction [210]. Since peptide chemistry has been automated for many years, numerous activation reagents and coupling procedures have been developed, of which most work well for the synthesis of multivalent glycoconjugates. Thus, three copies of a ω-aminohexyl 3,6-branched trisaccharide could be attached to a scaffold employing standard peptide coupling conditions such as hydroxybenzotriazole (HOBt), dicyclohexylcarbodiimide (DCC), yielding a nonavalent cluster in about 60% yield [153]. A combination of TBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) and DIPEA (*N*,*N*′-diisopropylethylamine) in DMSO (dimethyl sulfoxide) has been used at ambient temperature to produce up to 32-valent PAMAM dendrimers terminated with *N*-acetyl lactosamine units [93]. Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and DIPEA in DMF has been successful for the synthesis of up to 32-valent mono- and disaccharide terminated poly(lysine) dendrimers (76–91% yield) [211] and also for preparation of an array of galabiosylated glycodendrimers in good to excellent yields (58–99%) [100]. In a similar manner poly(ornithine) scaffolds and poly(lysine) scaffolds [212] have been treated [213]. HBTU and Et_3N were used for

linking of up to eight copies of a GM1 mimic to a 3,5-di-(2-aminoethoxy)-benzoic acid-derived scaffold (n = 2, 60%; n = 4, 40%; n = 8, 14%) [105]. Conjugation of functionalized saccharides to PPI dendrimers was achieved using the peracetylated carbohydrates in acetonitrile, where 1.5 equivalents of the carbohydrate moiety was used per terminal primary amino group [108]. Glucosamine and glucosamine 6-sulfate were attached to half-generations of PAMAM scaffolds terminated with up to 64 carboxylic acid groups. Conjugation was achieved by activating the PAMAM scaffolds with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) at pH 5 to minimize *N*-acyl isourea formation [94]. Acetylated mannosides, equipped with ω-carboxylic acid linkers could be attached to PAMAM and other amino-terminated scaffolds employing BOP or TBTU together with DIPEA (yields up to about 90% were obtained with the divalent cluster, n = 4, 66%; n = 8, 54%; n = 16, 43%) [101]. The preparation of a tetravalent glycocluster according to a convergent strategy has been presented by Takeda and co-workers. The iterative process consisted of a peptide coupling step followed by two deprotecting steps. A protected ω-amino functionalized spacer-equipped trisaccharide related to an antigenic epitope in pectic polysaccharides from the roots of *Bupleurum falcatum L.* was synthesized and linked in the presence of diethyl phosphorocyanidate (DEPC) as coupling agent together with Et_3N in dry DMF to an alanine derivative (92%). After deprotection, the second trisaccharide portion was introduced (89%), followed by an additional deprotection step and linking to the third trisaccharide unit (71%). The tetravalent structure was obtained after another reaction cycle (Fig. 31) [214]. A fivefold excess of mannosyl building block had to be used for the synthesis of comb-like glycodendrimers on solid phase (Fig. 21) [151]. Up to 32-valent fluorescence labeled mannosylated glycodendrimers have been prepared in a peptide synthesizer using PyBOP and *N*-methylmorpholine (NMM) in DMF for the conjugation steps [215]. A recent review article with focus on bioactive multivalent glycostructures based on peptide chemistry has been published in the *Journal of Peptide Science* [216].

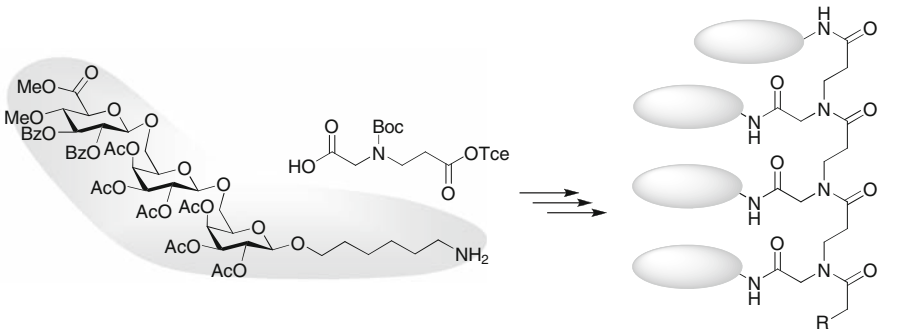


Fig. 31 A comb-like dendrimer produced by sequential introduction of a trisaccharide unit using peptide chemistry [214]

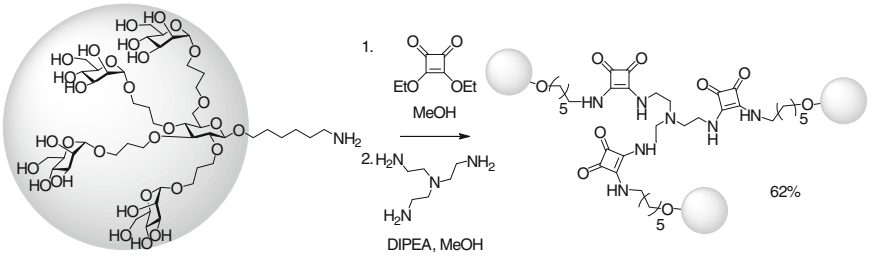
4.3.7 Squarate Chemistry

Although squarate chemistry is well established in glycoconjugate chemistry, only a few examples using this method for the preparation of defined multivalent glycoconjugates have been reported [138]. The conjugation is usually carried out in two steps using a squarate ester, e.g. crystalline dimethyl squaric acid ester, and accessible amino functions in the reaction partners. A ω-amino functionalized spacer-equipped saccharide forms a squaric monoamide ester under slightly basic conditions, then addition of the second amino functionalized reaction partner and increasing pH (usually above 9) produces the linkage. Tetravalent hexylamino-terminated mannosylated wedges were treated with diethyl squarate in methanol, purified by GPC and clustered with tris(2-aminoethyl) amine in methanol containing DIPEA. After 10 days 62% of the dodecavalent glycocluster was isolated (Scheme 3) [138]. A recent finding is that pH and buffer concentration are of crucial importance to obtain reliable and high yielding conjugations [217] which might encourage further use of this chemistry for the synthesis of multivalent glycoconjugates.

4.3.8 Glycosylation Chemistry

Glycosylation chemistry is not the first choice for the synthesis of multivalent glycoconjugates because glycosidation requires fine-tuning of reaction conditions for every individual glycosylation problem. Nevertheless, there are a number of examples in the recent literature using glycosylation mainly for the synthesis of smaller glycoclusters.

Based on pentaerythritol, a nonavalent glycodendrimer bearing terminal α-D-mannoside units has been synthesized in a convergent approach. The terminal trivalent mannoside dendrons were obtained by glycosylation and attached to the core by a triple Sonogashira coupling reaction [156]. Several carbohydrate-based glycoconjugates have been prepared by attaching terminal sugar units using glycosylation conditions. These structures are close mimetics of the natural oligosaccharides. The synthesis of highly branched oligomannoside mimetics (5-mers, 6-mers, a 7-mer, and a 9-mer) have been efficiently synthesized by a blockwise synthetic approach



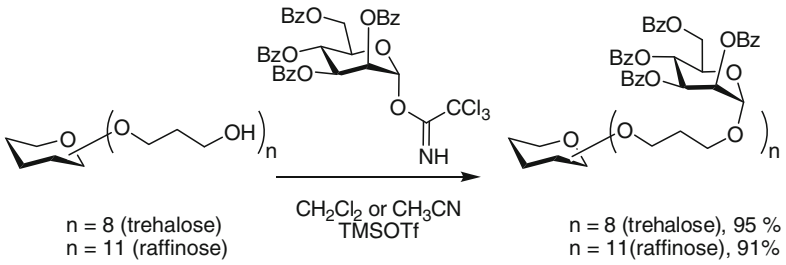
Scheme 3 Conjugation via the squaric ester method [138]

employing cyanoethylidene glycosyl donors and tritylated glycosyl acceptors in triphenylmethylium perchlorate catalyzed condensations, suggesting this route to be useful in the synthesis of more complex dendritic carbohydrate structures [218]. Mannosylated octopus glycosides have been produced employing mannosyl trichloroacetimidates. Up to 11 mannosyl residues were introduced in one glycosylation step (using TMSOTf as promoter and dichloromethane or acetonitrile as solvent) in excellent yield (Scheme 4) [134, 137]. The trichloroacetimidate method has also been successfully used for the preparation of glycodendrimers from polyphenylenes. This new glycodendrimer type, incorporating sugar moieties within a hydrophobic scaffold, is very rare and may remind of an active centre of a hydrophobic pocket situated inside of natural enzymes. The authors of this paper expect that the interior sugar moieties will form inherently stable hydrogen bonds with a guest and may eventually lead to molecular recognition [125].

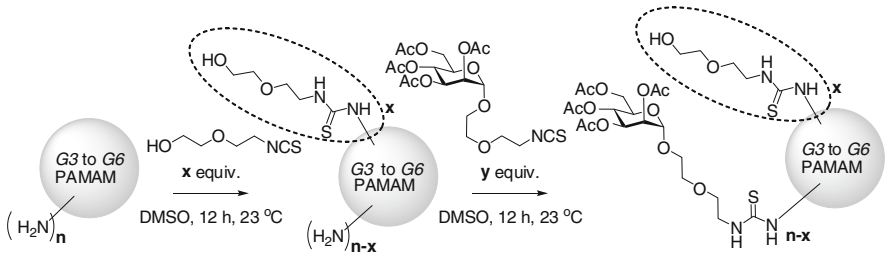
For the synthesis of an array of di- to hexavalent glycoconjugates, initially glycosylation was attempted using the required diols as acceptors and glycosyl donors with acetyl, thiophenyl or thioethyl groups at the anomeric centre. None of the glycosylation reaction were satisfactory, and thus unprotected sugar were reacted in anhydrous methanol with a variety of α,ω-difunctionalised alkanes X–$(CH_2)_n$–X (X = Br, OH, SH, HO–4-[C_6H_4]–, NH_2; n = 0–4, 8), 1,2-propanediamine, tris(2-aminoethyl)amine, pentaerythrityl tetraamine, and a hexaamine dendrimer core. The conjugations reactions producing an array of *N*-glycosides were easy to carry out (35–77% yields) and stable for at least 4 h in an aqueous solution at room temperature [155].

4.3.9 Thiourea Ligation

Conjugation by forming a thiourea linkage has a long tradition in the synthesis of glycodendrimers [219]. In general the ligation between an isothiocyanate and a primary amine proceeds smoothly in the presence of DIPEA in DMF or dichloromethane [220] and sometimes the addition of base is not even necessary (Scheme 5). Mixing and stirring produced octavalent trehalose octopus dendrimers in 41% yield [135]. In a more recent example, a 15-valent cluster was produced in 67% overall



Scheme 4 Up to undecavalent glycocluster were prepared by glycosylation in excellent yield [157]



Scheme 5 Modulated binding has been expected by controlled functionalization of PAMAM scaffolds using thiourea conjugation [224]

yield including deacetylation [139]. Furthermore, differences in reactivity have been used to produce mixed glycoclusters. To allow control of dendrimer formation of a mixed type, the relative reactivity of six saccharides had been determined by competition experiments [221]. Thiourea conjugation has been used to prepare trivalent glycodendrons which consecutively had been linked by the same methodology to βCD scaffolds [133]. Thiourea bridging has also been used to produce pseudooligosaccharides in which the intersaccharide thiourea linkages have been established instead of glycosidic bonds. The presence of the thiourea groups in these oligomers should promote the association with polyphosphates, including nucleic acids, due to the hydrogen-bonding capabilities of thioureas. Moreover, glycooligomers built from thiourea groups should form complex secondary and tertiary structures due to the interplay of hydrogen bonding and rotational restriction at the bonds adjacent to thiocarbonyl groups [222, 223]. Other examples for thiourea-bridged glycoclusters can be found, e.g. in [92, 126, 166, 167, 222, 224].

4.3.10 Reductive Amination

Reductive amination is a common reaction sequence for the synthesis of multivalent neoglycoconjugates. Since no protecting groups are required, this method is generally used for the conjugation of oligosaccharides isolated from a natural source. The reaction occurs between an amine and an aldehyde to form an imine which in turn is reduced to an amine in the presence of a reducing agent such as sodium borohydride. Due to the reaction mechanism the cyclic sugar system is destroyed in the conjugation step, thus it is not useful to conjugate a monosaccharide via this method. Turnbull, Fraser Stoddart and collaborators developed a carbohydrate-based architecture employing a divergent approach for a number of glycodendrimers (Fig. 32), e.g. [119, 225]. The glycosylated dendrons were constructed by linking a maltoside to the 6-OH position of diisopropylidene protected galactoside. The latter served as an open-chain linker obtained by reductive amination [225, 226].

4.3.11 Palladium Chemistry

Metal organic coupling chemistry is rare in glycoconjugate synthesis. One example is the Sonogashira–Heck–Cassar cross-coupling of iodobenzenes (Scheme 6). An array of glycoclusters has been prepared by oxidative homocoupling or palladium catalyzed cross-coupling reactions of alkynyl glycosides with aryl iodides providing an efficient entry into novel "rigid-like" glycoclusters [227]. Sonogashira

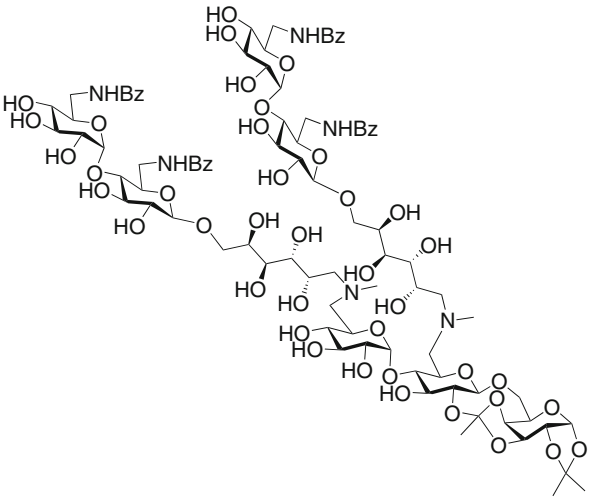
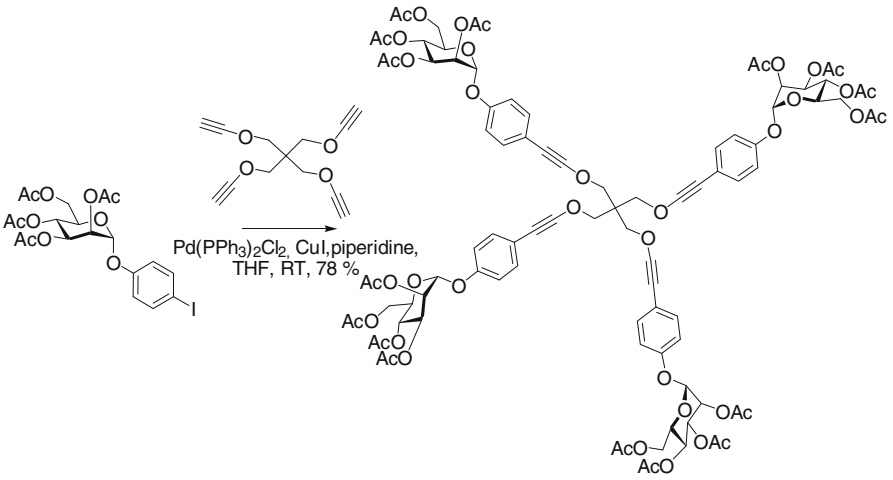


Fig. 32 A basic wedge-structureobtained by reductive amination used by Turbull and coworkers [225]



Scheme 6 Mannosylated glycoclusters prepared by Sonogashira coupling [158]

coupling conditions have also been employed in the synthesis of a nonavalent mannosylated glycodendrimer based on a pentaerythritol scaffold [156]. Tetra- and hexavalent mannoside dendrimers were prepared by combining Sonogashira coupling and triazole ligation chemistry. The obtained series of glycodendrimers were studied on their crosslinking abilities with ConA exhibiting subnanomolar affinities (according to SPR). The clusters were 1,000 times more efficient than mannose in their ability to inhibit the binding of *E. coli* to erythrocytes in vitro, thus representing one of the best ligands known to date towards *E. coli* K12 FimH [158]. Also two rigid divalent galabioside conjugates have been prepared under Sonogashira conditions [100]. The Pd-catalyzed carbon–carbon bond forming reaction between ethynyl *C*-glycosides and poly-iodinated benzenes allowed the introduction of various ethynylene glycoside chains on the phenyl ring thus creating glycoside clusters wherein the sugar moieties were linked to the benzene ring by an all carbon tether [228].

4.3.12 Self-Assembled Glycodendrimers

SAMs presenting carbohydrate structures have been used to study carbohydrate–protein interactions, often by means of SPR measurements [229] and this has recently been reviewed [230]. One standard method is to cover a gold surface with a mixture of alkylsulfides and sugar-decorated alkylsulfides, as has been shown with globotriose derivatives [231]. The specific distribution is obtained during the self assembly process forming a monolayer (Fig. 33a). Another method for preparing carbohydrate-covered SAMs is to use alkylsulfides with an additional functionality. After self-assembly, the second function can be used for attachment of carbohydrate ligands (Fig. 33b). Following this approach, the ligands will react randomly with surface groups. Trivalent mannosylated and fucosylated glycoclusters equipped with a carboxy terminated linker have been prepared to be assembled on gold wafers to serve as glycocalyx mimetics [102]. In a modular approach, trivalent mannosyl clusters equipped with a ω-azido linker were attached to a partially alkyne-terminated SAM, obtained by mixing capped and functionalized alkylsulfides, by triazole bridging after optimization of the reaction conditions for the 1,3-dipolar cycloaddition [73].

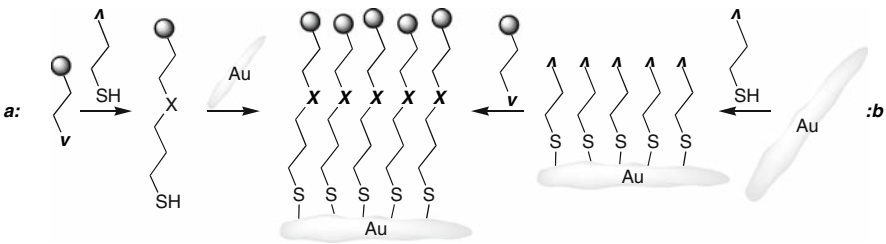


Fig. 33a,b Self-assembling of glycoconjugates is common on Au-surface using either approach

Dendrimer syntheses via self assembling techniques have emerged in the 1990s [232]. Self-assembling glycodendrimers with highly defined stoichiometry can be obtained by mixing smaller scaffolds carrying charges on the shell groups with counter ionic sugar species. This type of structures can serve both as ligands by mimicking the natural ligands of proteins (e.g.: gp 120 of HIV) and drug carriers (Fig. 34) [230].

Cu(II) ions have been used to assemble tetra- and octavalent *N*-acetyl galactosylamide dendrons (Fig. 35). The carbohydrate epitopes were first attached to a bipyridyl system using linker of different length. Tetracoordination of the Cu(II) core produced the glycodendrimers which were characterized using proton and carbon NMR, MALDI-Tof mass spectrometry, and UV–VIS spectroscopy [233].

Dendrons constructed on an "aromatic-scaffold" by a convergent approach were capped with up to 64 copies of lactose and up to eight copies of Galα1–3Galβ1–4GlcNAc. No satisfactory MALDI-Tof data could be obtained for the larger dendrimers, and line broadening observed in the NMR spectra indicated the formation of superstructures by aggregation in water which could be confirmed and quantified by multiangle light scattering (MALS). As the driving force, pre-organization by intramolecular π–π stacking of the aromatic systems and subsequent hydrophobic core-core contact reducing the conformational flexibility of the glycodendrimer has been suggested. The size of these aggregated glycodendrons – the largest contained about 1,500 individual molecules – was measured using atomic force microscopy

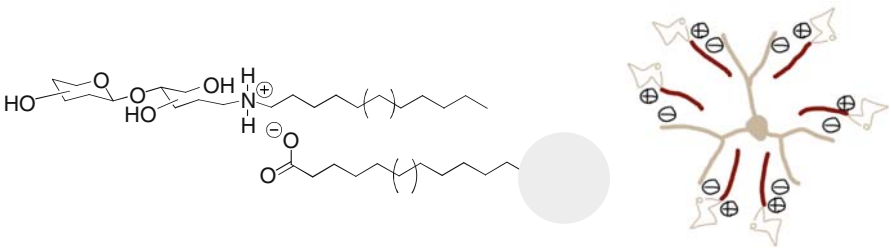


Fig. 34 Electrostatic interactions using charged scaffolds and ligands [230]

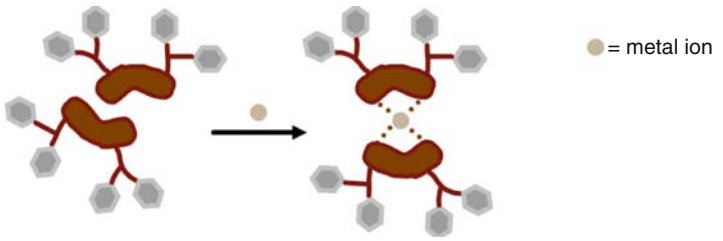


Fig. 35 Schematic presentation of a self-assembling glycodendrimer via metal coordination [233]

(AFM) and approximated to be between 10 and 60 nm [234]. These non-covalent glyconanoparticles formed in water showed a remarkable homogeneity in their size and could be deposited on different surfaces and the aggregation was shown to occur both in vitro and in vivo [235].

Recently, a series of galactosylated, glucosylated and mannosylated glycodendrons assembled on a hydroxyquinoline initiator core has been reported. This fluorescent core is able to bind transition and lanthanide metal complexes by self-assembly leading to high nuclear glycodendrimers. Both optical and protein-binding properties were investigated and it was found that high sugar density of the metallo-glycodendrimer was essential for lectin binding with ConA [236].

5 Perspectives

Since Vögtle's iterative polymers and Roy's first glycodendrimer, a vast number of bioactive multivalent molecules have been synthesized. Many have been studied in biological systems and some have found their way towards more general applications. While the benefit of multivalent interactions is well appreciated, it is still not possible to foresee a perfect multivalent architecture for a given interaction. Various types of scaffolds, linkers and ligands have been tried and combined in many imaginable ways and new ones are to come. The conjugation chemistry has been developed to a point that researchers dare to link valuable oligosaccharide ligands onto multivalent scaffolds, and linkers are getting longer and more sophesticated. However, as colorful as the architecture of multivalent structures is, the field of applications is as broad and widens the scope for biomedical science. Biocompatibility has been elucidated for many multivalent glycoconjugates and powerful ligands have been found for their receptors, although multivalent ligand–receptor interactions have not been understood down to all details. Initial studies with amino-terminated PAMAM scaffolds showed a decreased cytotoxicity compared to early polylysine-based dendrimers but this was later revised into a dependency on overall size and surface functionality. Glycodendrimers have been made biocompatible and now there are non-toxic, cytotoxic, immunogenic, and non-immunogenic compounds available some, with the ability to traverse biological barriers. After more than two decades of diligent production of more or less symmetrical glycodendrimers, the theme seems currently almost to turn back to the point from where the idea of glycodendrimers was first born. Many of the highly symmetrical, higher generation glycodendrimers are a pleasure to look at and beautiful objects for the gallery of the glycochemical art. These densely packed structures have, however, almost lost all the advantages of multivalent systems. Their bioavailability is in general rather lowered and the binding efficiency and plasticity diminished. Recent multivalent structure design tries to account for the nature of the receptor sites in order to achieve a better fit of the multivalent glycoconjugate ligands, e.g. by producing well defined polymers with distinct distance between the carbohydrate epitopes or by using globular shaped nanoparticles with defined ligand density.

In order to study the inhibition of the adhesion of uropathogenic *E. coli* via the FimH lectin, up to 16-valent mannose-carrying glycodendrimers and 21-valent glycopolymers were synthesized using different aromatic cores for the smaller clusters and PAMAM scaffolds for structures with higher valency [101]. It has been stated that complete inhibition of the adhesion process might require extremely large systems [237, 238]. However, there is no general agreement whether very big or well-designed and rather small will lead to an ideal ligand.

More knowledge about the molecular concert operating in glycobiology is required before the design of multivalent glycoconjugates can be optimized accordingly.

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Structure, Function, and Assembly of Type 1 Fimbriae

Stefan D. Knight and Julie Bouckaert

Abstract Bacterial infections constitute a major global health problem, acutely accentuated by the rapid spread of antibiotic resistant bacterial strains. The widespread need for bacteria to attach – adhere – to target cells before they can initiate an infection may be used to advantage by targeting the bacterial adhesion tools such as pili and fimbriae for development of novel anti-bacterial vaccines and drugs. Type 1 fimbriae are widely expressed by *Escherichia coli* and are used by uropathogenic strains to mediate attachment to specific niches in the urinary tract. These fimbriae belong to a class of fibrillar adhesion organelles assembled through the chaperone/usher pathway, one of the terminal branches of the general secretion pathway in Gram-negative bacteria. Our understanding of the assembly, structure and function of these structures has evolved significantly over the last decade. Here, we summarize current understanding of the function and biogenesis of fibrillar adhesion organelles, and provide some examples of recent progress towards interfering with bacterial adhesion as a means to prevent infection.

Keywords Assembly, Bacterial adhesion, Chaperone/Usher pathway, Fimbriae/Pili, Lectin

Contents

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Abbreviations

C-HEGA	cyclohexylbutanoyl-*N*-hydroxyethyl glucamide
DSC	donor stand complementation
DSE	donor strand exchange
ETEC	enterotoxigenic *E. coli*
H-NS	histone-like nucleoid-structuring protein
IBC	intracellular bacterial community
Ig	immunoglobulin
IHF	integration host factor
IM	inner membrane
Lrp	leucine-responsive regulatory protein
OM	outer membrane
SAM	self-assembled monolayer
SDA	single-domain adhesin
TDA	two-domain adhesin
THP	Tamm-Horsfall protein
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection

1 Introduction

Infectious diseases constitute a major global health and health cost burden. The World Health Report 2004 [1] estimates that infectious and parasitic diseases caused almost 11 million deaths in 2002. The same report estimates that respiratory and diarrhoeal infections were responsible for 4.0 and 1.8 million deaths, respectively, in 2002, to be compared to the toll of diseases such as AIDS (2.8 million deaths), tuberculosis (1.6 million deaths), and malaria (1.3 million deaths). A significant proportion of respiratory and diarrhoeal infections are caused by Gram-negative pathogens such as *Salmonella enterica* (typhoid fever, enterocolitis), *Haemophilus influenzae* (pneumonia), *Bordetella pertussis* (whooping cough), and *Escherichia coli* (diarrhoea). The overwhelming majority of the world's annual 4 million neonatal deaths occur in developing countries. Bacterial infection is the major cause of neonatal admissions to hospitals, and probably the

biggest cause of morbidity in the community. The most common serious neonatal infections involve bacteraemia, meningitis, and respiratory tract infections, and case fatality rates may be as high as 45%. Key pathogens in these infections are *E. coli*, *Klebsiella* sp., *Staphylococcus aureus* and *Streptococcus pyogenes* [2].

Although it remains true that bacterial infections can often be efficiently treated with antibiotics, the emergence of antibiotic resistant bacterial strains is a serious problem [3, 4]. For example, treatment of typhoid fever relies on prompt administration of antibiotics, but strains of *S. typhi* resistant to one or more of the commonly used antibiotics have emerged worldwide [5]. *E. coli* infections in humans (mostly urinary tract infections (UTIs)) can normally be treated with antibiotics, but again, the emergence of antibiotic resistant bacterial strains poses a serious and growing threat [6–8]. In light of this, the use of antibiotics in the treatment and control of human as well as of animal infections should be minimized, and development of novel strategies to treat and prevent bacterial infections are urgently needed [3].

One aspect of the pathogen-host interaction that shows great promise as a target for development of novel means of interfering with bacterial infections is the early establishment of physical contact between pathogen and host. Adhesion of bacteria to target tissues is frequently a necessary first step in pathogenesis [9–11]. For example, uropathogenic *E. coli* (UPEC) depend on specific binding to mannose-containing receptors on the luminal surface of the bladder epithelium for the establishment of cystitis (bladder infection) [12–16], whereas binding to Galα1–4Gal-containing receptors in the upper urinary tract is a prerequisite for the establishment of pyelonephritis (kidney infection) [17]. Blocking of adhesion can provide an efficient way of interfering with bacterial infections. For example, blocking binding to mannose-containing receptors has been shown to prevent UPEC adhesion to the bladder uroepithelium and cystitis [13, 14, 18, 19]. Similarly, blocking the binding of UPEC to Galα1–4Gal-containing receptors can prevent adhesion to and infection of kidney epithelium [20, 21].

Most bacteria depend on expression of specialized adhesive organelles on the bacterial cell surface to mediate attachment to target tissues. Gram-negative bacteria can grow hair-like adhesive organelles referred to as pili (from the Latin word for 'hair') or fimbriae (from the Latin word for 'thread') [22] arranged in a multitude of 'hairstyles' ranging from soft, long, wavy hair, to afro style (Fig. 1). In one common class of such fibrillar organelles [25], many copies of a receptor binding 'single-domain adhesin' (SDA) are incorporated in a thin (2–5 nm wide) and flexible fibre. Examples of such 'polyadhesins' include the Dr adhesins expressed by many UPEC strains, SEF14 fimbriae of *Salmonella enteritidis*, Myf fimbriae of *Yersinia enterocolitica*, and the pH6 and F1 antigens expressed by *Yersinia pestis*. A second class of fibrillar adhesive organelle commonly expressed by Gram-negative pathogens display a carbohydrate-binding 'two-domain adhesin' (TDA) at the tip of complex pili or fimbriae. These composite structures frequently incorporate the TDA at the tip of a thin (~2 nm) and flexible 'tip fibrillum' linked to a relatively rigid 1–2 μm long and ~8 nm wide 'stalk' or 'rod', consisting of a helically wound fibre of fimbrial subunits ('pilins'). Examples of TDA-displaying adhesive organelles include the much studied P pili, and type 1 fimbriae (the main focus of this chapter) that provide UPEC with the ability to bind to Galα1–4Gal-containing and mannose-containing receptors

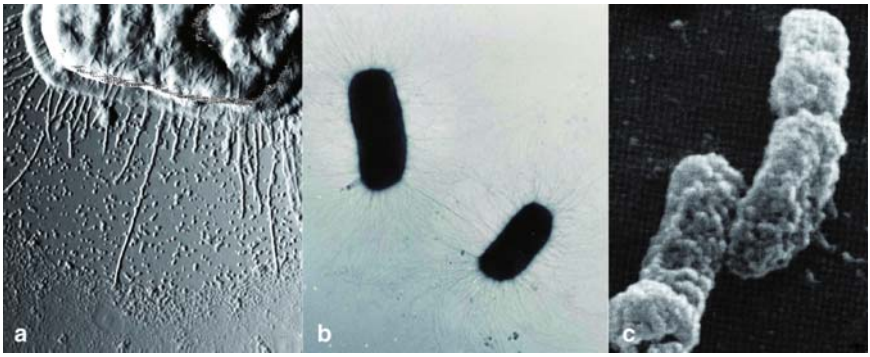

Fig. 1 Examples of bacterial 'hair styles'. **a** Thick and straight: type 1 fimbriated *E. coli*. Type 1 fimbriae are composite structures with a relatively rigid 8 nm wide rod tipped by a thin (2.5 nm) more flexible tip fibrillum. **b** Thin and wavy: F17 fimbriated *E. coli* covered with 1–3 μm long, flexible, 2–3 nm wide fimbriae. **c** Tangled (capsules and sheaths): *Y. pestis* F1 capsular antigen. No individual fibres are visible, but the capsule consists of a tangle of thin (~2 nm) flexible fibres. AFM amplitude picture (**a**) kindly provided by Dr R. Willaert, Ultrastructure, VU Brussels; TEM picture (**b**) kindly provided by I. Caplier, Protein Chemistry, VU Brussels and reprinted from [23] with permission from the publisher; SEM picture (**c**) reprinted from [24] with permission from the publisher

respectively, and that are important for UPEC colonization of the urinary tract. The specialized SDA and TDA bacterial adhesive organelles provide promising targets for development of new classes of anti-bacterial drugs that may either directly block adhesion or interfere with the biogenesis of adhesive organelles, and for the development of novel vaccines to prevent bacterial infections.

2 SDA and TDA Fimbrial Adhesins: Ultrastructure and Assembly

Results from structure-based studies of components of the P pilus, type 1 fimbriae, and F1 antigen systems over the last 8 or so years have led to a relatively refined understanding of how SDA and TDA fimbrial adhesins are constructed, and of how periplasmic chaperones direct and promote their assembly. Key to the success of these studies has been the realization that the fimbrial subunits, although very unstable on their own, can be isolated as stable complexes with their cognate periplasmic chaperone, and that assembly can be blocked by mutations in the N-terminal region of the subunits, allowing isolation and characterization of assembly intermediates. These studies have shown that, in spite of a great deal of variation in appearance and binding specificity, a large group of bacterial pili/fimbriae share the same underlying fibre structure, consisting of a string of non-covalently linked immunoglobulin (Ig)-like modules. This linear fibre structure is assembled from monomeric, incomplete Ig subunits by a periplasmic chaperone together with an outer

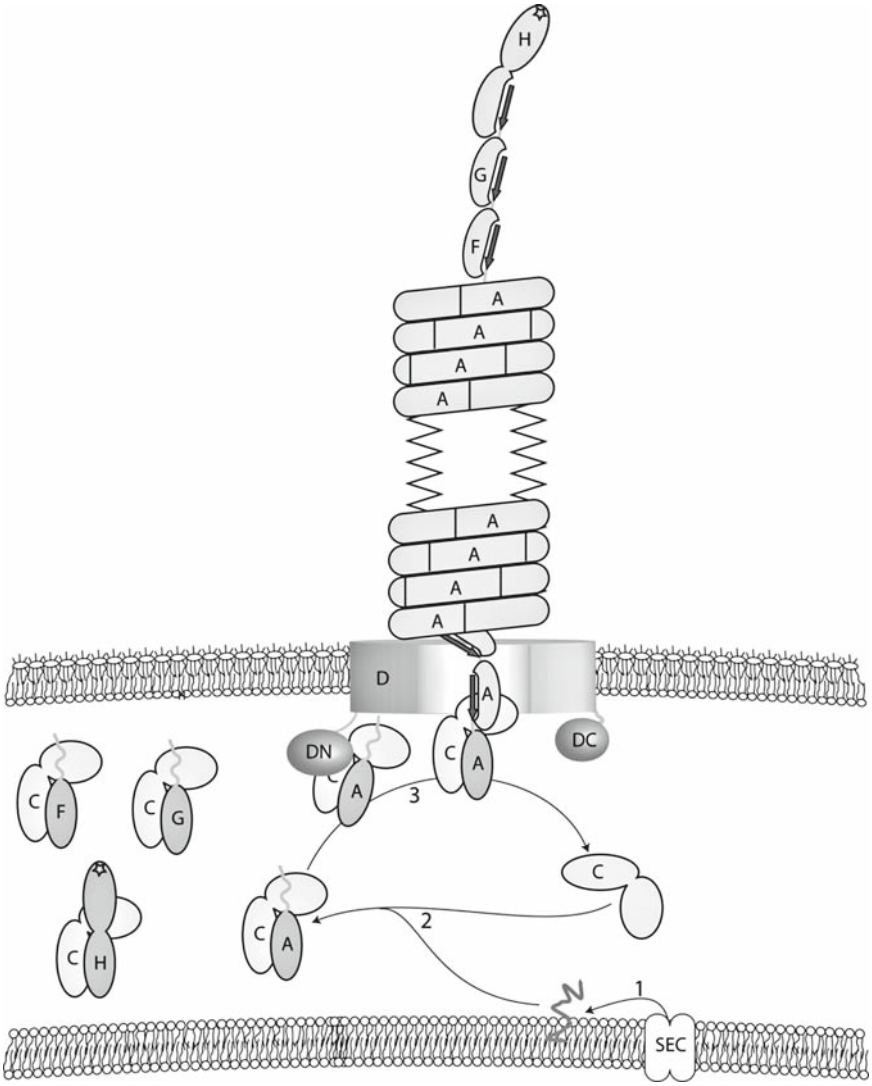

Fig. 2 Schematic illustration of chaperone/usher-assisted assembly of type 1 fimbriae. Subunits (A, F, G, H) enter the periplasm via the Sec system and transiently remain associated with the inner membrane (step 1). FimC chaperone (C) binds newly translocated, partially unfolded subunits to form soluble and stable chaperone:subunit complexes (step 2). Targeting of the binary FimC:FimH pre-assembly complex to an empty FimD usher (D) initiates assembly. At the usher, an incoming chaperone:subunit complex attacks the chaperone:subunit complex capping the base of the growing fibre (step 3). The usher catalyses DSE in which the capping chaperone at the base is released, the N-terminal G_d donor strand of the attacking subunit is inserted into the polymerization cleft of the subunit at the base to form a new fibre module, and a new chaperone-capped subunit is added at the base. For further details of the assembly process, see text

membrane usher (Fig. 2) [9, 11, 26–31]. The final fibre morphology is determined by additional interactions between non-adjacent pilin subunits in the fibre, allowing some fibres to coil into rigid helical structures such as the fimbrial rods, while some remain as relatively flexible extended linear fibres that sometimes collapse into amorphous 'sheaths' or 'capsules'. The structural pilin subunits of complex fimbriae, and the SDA subunits of polyadhesins, have the same basic architecture and are assembled in the same manner.

2.1 Fibre Structure

The subunits of fimbriae are constructed essentially as Ig-like β-sandwiches, but with a circular permutation that positions the sequence corresponding to the seventh, C-terminal, Ig β-strand (strand G of a canonical Ig domain) at the N-terminus of the polypeptide sequence [32–35] (Figs. 3a and 4a). In a typical Ig fold, the 'top' edge of the sandwich, defined by the A and F strands, is capped by the C-terminal G strand, which is hydrogen bonded to the F strand and provides hydrophobic residues to the core of the fold. Free pilin subunits are only marginally stable, and no structure for a monomeric pilin in the absence of chaperone has been obtained. Sometimes, in the absence of the chaperone, soluble, domain-swapped pilin dimers [36] or trimers [37, 38] are formed and have been reported in their crystal structures. However, the oligomerization of pilins in this manner is a dead-end process, and the pilins in these oligomers are not able to assemble into fibrillar structures.

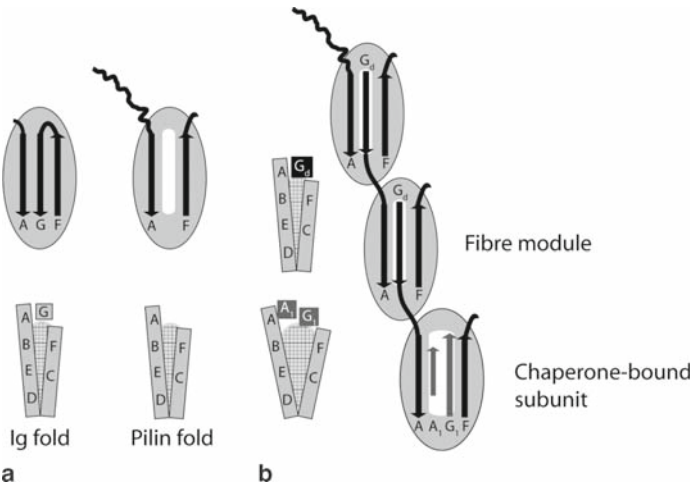

Fig. 3 Schematic illustration of: **a** relation between the Ig and the pilin fold; **b** DSC before (subunit bound to chaperone; *below*) and after (in fibre module; *above*) DSE. The ellipsoids represent the β-sandwich of the Ig/pilin fold as viewed down at the AF edge of the sandwich; the rectangles represent β sheets and strands (*as labelled*) viewed edge on

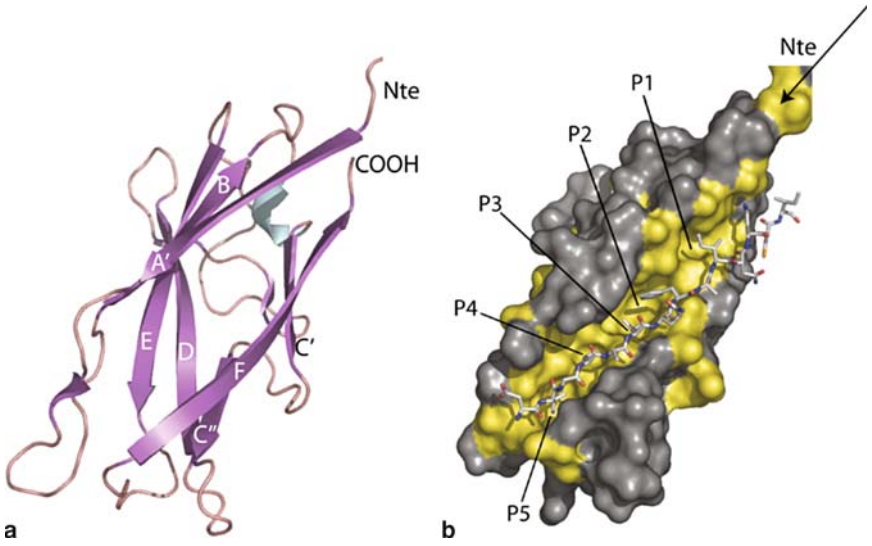

Fig. 4 Pilin structure: **a** ribbon diagram of a Caf1 subunit illustrating the Ig-like pilin fold. Main secondary structural elements are labelled; Nte denotes the N-terminal extension (disordered unless used for DSC of a neighbouring subunit in a fibre); **b** surface representation of Caf1 illustrating the hydrophobic acceptor cleft (hydrophobic residues Ala, Val, Leu, Ile, Phe, Met *coloured yellow*) with the G_d strand from the Caf1M chaperone (*stick model*) bound; sub-pockets of the acceptor cleft, designated P1-P5 in the nomenclature of Sauer et al. [34], are labelled

Various structures of pilins bound to chaperone or peptides [32–34, 39, 40], or incorporated into fibres [35, 41], show that in the absence of a seventh β-strand, a pilin subunit cannot cap its AF edge, and a closed hydrophobic core is not formed. Instead, a deep hydrophobic cleft is created on the surface of the subunit (Fig. 4b). The hydrophobic effect drives folding of globular proteins by favouring the packing of hydrophobic side chains together in a hydrophobic core, shielded from the surrounding water. In pilins, however, owing to the absence of a seventh C-terminal (G) strand, the polypeptide chain simply cannot fold in such a way as to create a shielded hydrophobic core, explaining the instability of free pilin subunits.

The N-terminal extension of pilins, which is flexible in solution and does not contribute to the subunit's globular fold, carries a β-strand motif of alternating hydrophobic and hydrophilic residues. Deletions or mutations in this N-terminal region block assembly of pilin subunits into fibres [34, 35, 39, 41–44]. The structure of a Caf1M:$(Caf1)_2$ ternary complex [35, 41], with the minimal *Y. pestis* F1-antigen fibre ($(Caf1)_2$) bound to the Caf1M chaperone, revealed that fibre subunits are linked together by insertion of the N-terminal extension of one subunit into the hydrophobic cleft of the second subunit (Fig. 3b). The inserted N-terminal segment adopts a β-strand conformation running antiparallel to the F strand, with hydrophobic side chains bound in three (TDA systems) or five (SDA systems) acceptor cleft sub-pockets (Fig. 4b), hence completing the Ig fold of the subunit. This mode of binding, termed 'donor strand complementation' (DSC), had previously been predicted for

type 1 and P pilus fibres [32–34], and is likely to be present in all surface polymers assembled through the chaperone/usher pathway. The resulting linear fibre is composed of globular modules each having an intact Ig topology generated by DSC (Fig. 3b). In this fibre, each Ig module is made from two polypeptide chains, with the G strand being provided in trans (because the N-terminal segment of one subunit is donated to fulfil the role of the Ig-fold G strand in a second subunit, the N-terminal sequence is also referred to as the G_d (d for donor) sequence). TDAs lack the N-terminal G_d polymerization sequence and instead have an entire receptor-binding domain coupled to the N-terminus of the pilin domain. As a consequence, TDAs can only be incorporated in a single copy at the tip of fimbriae. In earlier work based on immunogold labelling, the FimH adhesin was reported to be laterally located along the type 1 fimbrial shaft [45]. However this is contradicted by later findings, using quick-freeze deep-etch electron microscopy [46], or gold-coupled antibodies in combination with transmission electron microscopy [47], and is incongruent with current understanding of fimbrial structure and assembly.

In spite of their non-covalent nature, and in contrast to free pilins, or pilin subunits bound to their chaperone, studies of the *Y. pestis* F1 antigen system have shown that the fibre Ig modules can be extremely stable. The folding free energy estimated from reversible unfolding of an engineered monomeric F1 fibre module (Caf1-SC) in GdmCl at 37 °C is in the range 70–80 kJ mol^{-1} [41]. This should be compared to the typical range of 20–60 kJ mol^{-1} maximum stability for stable proteins in physiological conditions [48, 49]. This apparently narrow range of optimal stability for globular proteins might be explained by evolutionary pressure to adapt to functional constraints, such as the need for enough conformational flexibility to allow, e.g., induced-fit binding or natural protein turnover, rather than by any intrinsic physical limitations on protein stability. In contrast, the function of adhesive surface fibres requires them to be mechanically resilient, and in the absence of a counteracting evolutionary pressure (e.g., there is no need for protein turnover outside of the cell) no functional constraints on maximum stability apply.

2.2 Fibre Biogenesis

Biogenesis of stable polymeric surface fibres such as those of pili, fimbriae, or capsules, poses many challenges to the Gram-negative bacterial cell. It must be able to protect the unstable and highly aggregative fibre subunits from aggregation and proteolytic degradation during their transport from the site of production in the cytoplasm, across the inner membrane (IM) and the periplasm, to the site of assembly at the outer membrane (OM) of the cell. Having reached the OM, subunit assembly must be controlled to form the desired polymeric structure, which must then be secreted to the cell surface. In the cytoplasm, pilin subunits are expressed as preproteins with an N-terminal export signal that targets them for export via the general secretion (Sec) pathway [50]. In the periplasm, a periplasmic chaperone,

together with an OM usher, handle the subsequent events that lead to assembly of pilin monomers into surface located fibrillar structures (Fig. 2).

The periplasmic chaperones are steric chaperones that bind to fibre subunits as they emerge in the periplasm, ensure their correct folding, and deliver the folded subunits to the usher where they are assembled into fibrillar polymeric structures. For TDA-carrying structures, binding of a chaperone:TDA complex to an empty usher initiates assembly [51–53] which then proceeds by sequential addition of pilin subunits to the base of the growing fibre and simultaneous secretion of the fibre through the usher pore [54]. In the absence of chaperone, subunit folding is slow and leads to a marginally stable and aggregation prone structure [55], whereas in the presence of chaperone, stable and soluble chaperone:subunit complexes are rapidly formed. In contrast to the many ATP-dependent cellular chaperones, the periplasmic chaperones do not require an input of external energy for subunit release (there is no ATP in the periplasm), and organelle assembly is independent of cellular energy [56].

The L-shaped periplasmic chaperones comprise two Ig-like domains joined at ~90° angle, with a large cleft between the two domains (Fig. 5). The F_1 and G_1 β-strands in the 1st, N-terminal, domain are connected by a long and flexible loop that protrudes like a handle from the body of the domain. This loop harbours a conserved motif of hydrophobic residues that is critical for subunit binding. FGL chaperones, used for assembly of SDA polyadhesins such as F1 antigen (Caf1M chaperone), have a relatively long F_1–G_1 loop (hence FGL for F_1–G_1 Long), whereas the FGS chaperones used for assembly of composite structures such as type 1 fimbriae (FimC chaperone) or P pili (PapD chaperone) have a relatively short F_1–G_1 loop (hence FGS for F_1–G_1 Short) [25]. FGL chaperones are also distinguished from FGS chaperones by a longer A_1 strand and a disulphide link bridging the F_1 and G_1 strands.

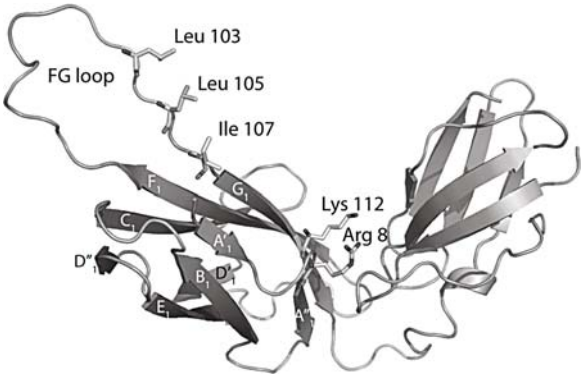

Fig. 5 Chaperone structure. Ribbon diagram of FimC chaperone from the structure of the FimC:FimH complex [32]. β-strands in the N-terminal domain are labelled. The hydrophobic residues in the G_d donor strand are shown as stick models and labelled. Also shown are the two invariant residues in the subunit binding cleft between the two Ig-fold domains that are crucial for subunit binding and chaperone function

Subunits bind in the cleft between the two chaperone domains (Fig. 6) with the subunit C-terminal carboxyl group anchored by two positively charged residues (Arg8 and Lys112 in FimC) at the bottom of the subunit-binding cleft (Fig. 5). These two residues are strictly conserved in all the periplasmic chaperones and are crucial for chaperone function [25, 58, 59]. As described above, the incomplete fold of pilin subunits precludes the formation of a closed hydrophobic core, leaving a surface-exposed hydrophobic cleft between the two sheets at the AF edge of the subunit (Figs. 3 and 4). In chaperone:subunit complexes, the A and F edge strands are hydrogen bonded to the A_1 and G_1 strands of the chaperone, which creates a super-barrel of β-strands from both the subunit and the chaperone [35]. In the super-barrel, large hydrophobic residues from the G_1 donor strand of the chaperone are inserted between the two sheets of the subunit β-sandwich and become an integral part of the bound subunit's hydrophobic core (Fig. 4b). The large hydrophobic donor side chains of FGS chaperones occupy sub-pockets P1–P3; a conserved asparagine side chain binds in the P4 pocket [32, 33]. FGL chaperones donate small hydrophobic side chains to all five sub-pockets in the acceptor cleft [35, 39]. The chaperones thus cap the hydrophobic polymerization cleft, thereby preventing both premature assembly and unspecific subunit aggregation, and protecting subunits from proteolytic degradation. It should be noted that, in contrast to the DSC interaction between subunits in an assembled fibre, where the subunit G_d donor strand is inserted antiparallel to the F strand to create a complete canonical Ig module, the chaperone G_1 donor strand is inserted parallel to the F strand.

Periplasmic chaperones deliver folded pilin subunits to the OM usher. At the usher, chaperone:subunit interactions must be replaced by subunit:subunit interactions. Hence, the A_1 and G_1 strands of the chaperone capping the subunit at the base of the growing structure, on the periplasmic side of the usher, must dissociate to allow the N-terminal sequence of the next subunit to be bound in the polymerization cleft (Fig. 2). This exchange process, called 'donor strand exchange' (DSE) [32–35], can occur even in the absence of usher as evidenced, e.g. by the accumulation of low molecular weight Caf1 polymers in the periplasm of Caf1A usher negative bacteria expressing Caf1M chaperone and Caf1 subunit, or in vitro following incubation of Caf1M:Caf1 complex [43]. However, compared to usher mediated assembly, this process is slow and inefficient, suggesting that DSE assembly is catalyzed by the usher.

Two basic models for DSE have been suggested [35]. In the first model, the chaperone bound to the subunit at the base of a growing fibre is first released, followed by ordering and insertion of the N-terminal G_d donor sequence of the next chaperone:subunit complex into the unoccupied polymerization cleft. The second model involves concerted release of the chaperone G_1 donor strand and insertion of the subunit G_d donor strand in a zip-out-zip-in mechanism. The observation of a transient quaternary complex between two chaperone:subunit complexes during assembly of type 1 fimbriae [39, 60] strongly argues for the second model. Interactions with the P5 acceptor cleft sub-pocket have been shown to be critical for initiation of DSE [39].

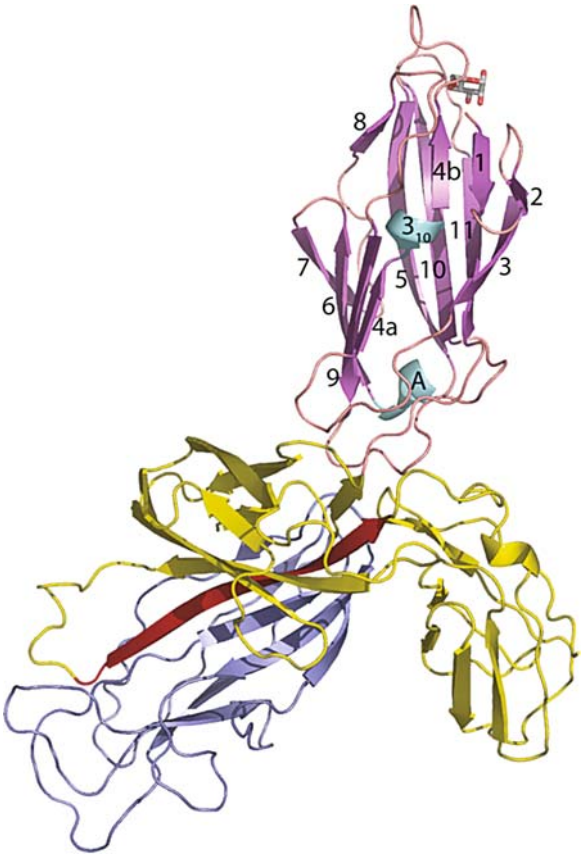

Fig. 6 Crystal structure of FimC:FimH chaperone:adhesin complex bound to α-D-mannose [57], showing the chaperone FimC (*yellow; G_d strand red*), the pilin domain of FimH (*steel-blue*) that is donor-strand complemented by the chaperone G_d strand, and the receptor-binding domain (*violet with blue-green helices*, secondary structure labelled as described earlier [32]). D-Mannose bound to the mannose-binding pocket is shown as a *ball-and-stick model*

No energy input from external sources is required to convert periplasmic chaperone:subunit pre-assembly complexes into free chaperones and secreted fibres [56]. Instead, assembly is driven by subunit folding energy conserved by the chaperone [35, 41]. Comparison of the structure of Caf1 bound to Caf1M, and its structure in the F1 fibre module, revealed a large conformational difference. In the Caf1M:Caf1 super-barrel, the chaperone G_1 donor strand occupies the polymerization cleft, with large hydrophobic residues from the G_1 donor strand inserted between the two sheets of the subunit β-sandwich, preventing them from contacting each other (Figs. 3b and 4b). Molecular dynamics simulations predict that this open, partially folded molten-globule like conformation is not stable and would not be maintained in solution. In contrast to the chaperone donor residues, the much

smaller donor residues in the subunit N-terminal G_d donor segment do not intercalate between the two sheets of the subunit β-sandwich, allowing close contact between them in the fibre module (Fig. 3b). For FGS assembly systems, a similar change accompanies DSE, but now involving a register shift from P1–P4 sub-site binding in the chaperone complex to P2–P5 sub-site binding in the fibre module [34]. A huge difference in stability between the chaperone-bound, partially folded conformation, and the fully folded native fibre conformation of pilins creates a free energy potential that drives fibre formation.

Several observations suggest that periplasmic chaperones target and bind subunits in an unfolded or at least partially unfolded state. The high efficiency of chaperone/usher-mediated assembly in vivo [56] suggests that this process cannot rely on the slow self-folding of subunits [55]. Recently, Vetsch et al. [61] verified that the FimC chaperone indeed binds to unfolded pilin subunits. Chaperone binding was shown to increase the rate of folding by a factor of 100. To bind unfolded subunits, the periplasmic chaperone, presumably, has to recognize some common feature of the (partially) unfolded conformations. The extensive interactions between the hydrophobic cores of the N-terminal domain of the chaperone and the subunit observed in the structures of chaperone:subunit complexes suggest that the chaperone might recognize and attract hydrophobic core residues that are exposed in unfolded subunits. The surface exposed hydrophobic patch created by the bulky hydrophobic side chains in the G_1 donor strand of free chaperones [25, 42] (Fig. 5) might attract (partially) unfolded subunits and provide a template onto which the subunit core can condense, facilitating folding. At the same time, however, because of intercalation of the large chaperone donor residues in the subunit hydrophobic core, subunit folding coupled to chaperone binding does not reach the native state, and the subunit is trapped in an open, activated, high-energy conformation. The resulting meta-stable complex provides a convenient substrate for fibre assembly.

For DSE and secretion to proceed, the usher must interact with chaperone:subunit complexes in the periplasm to facilitate dissociation of the chaperone and polymerization of subunits. The growing polymer must then be translocated across the OM to the cell surface. The ushers are large (80–90 kDa) porin-like integral OM proteins [62–64]. Recent results [52, 65] show that both the PapC (P pilus) and the FimD (type 1 fimbrial) ushers form 7×10 nm^2 homodimers with a ~2 nm pore in the middle area of each monomer. Such a pore is wide enough to allow translocation of folded structural subunits or their polymers through the OM. Functional studies of the PapC and FimD ushers have shown that in addition to a central trans-membrane β-barrel they contain N- and C-terminal periplasmic domains important for initial binding of chaperone:subunit complexes, and subsequent assembly steps respectively [52, 63, 64, 66–68]. Based on an analysis of usher sequences, a third conserved domain located in the central β-barrel region of the ushers has recently been proposed [69]. This 'predicted middle domain' is predicted to have a β-sandwich fold and to be located in the periplasm.

Recently, the structure of a FimC:$FimH_{pilin}$ complex ($FimH_{pilin}$ is the C-terminal pilin domain of FimH) bound to the N-terminal domain of the type 1 pilus usher

($FimD_N$) was reported [68]. The structure of this complex, as well as that of a similar complex from the F1 antigen system (A. Dubnovitsky, A. Zavialov, S.D. Knight, unpublished), shows that the ushers have a chaperone binding surface formed by the folded core of the usher domain and by an extended N-terminal 'tail' of the usher. As we had already predicted in 1996 [25], the usher recognizes a patch of conserved hydrophobic residues on the 'back' of the chaperone N-terminal domain. Whereas in the FimC:$FimH_{pilin}$:$FimD_N$ complex there are also significant interactions between the usher N-terminal tail and the chaperone-bound subunit, very few such interactions are present in the F1 antigen complex. This might reflect the need to distinguish several different subunit types and to assemble these in a particular sequence in the more complex type 1 fimbrial (TDA) system but not in the F1 antigen (SDA) system.

3 Type 1 Fimbriae

Studies of fimbriae have been ongoing since the 1950s. Duguid et al. [70] reported seven different fibrillar structures and named these types 1–6 and F fimbriae. Brinton [71] recognized six different types and named these types I–V and F pili. Duguid's type 1 fimbriae and Brinton's type 1 pili refer to the same fibrillar organelle [22]. Generally, the terms 'type 1 fimbriae' or 'type 1 pili' refer to any 6–8 nm-wide fimbrial structure that mediates agglutination of guinea pig red blood cells in a mannose-inhibitable manner. Such fimbriae are also referred to as 'common', 'somatic', or 'mannose-sensitive' fimbriae. At least two genetically distinct type 1 fimbriae with distinct molecular compositions and receptor binding profiles exist, the *E. coli* type 1 fimbriae (type 1^E) and *Salmonella* type 1 (SEF21) fimbriae (type 1^S) [72]. The focus of this chapter is on the *E. coli* type 1^E fimbriae, which in the following will be referred to simply as type 1 fimbriae.

Type 1 fimbriae are common throughout the *Enterobacteriaceae*. They can easily be visualized in the electron microscope as 1–2 μm long and ~7 nm wide structures extending peritrichiously from the bacterial cell surface (Fig. 1a). They mediate binding to a wide range of glycoproteins carrying one or more N-linked high-mannose structures. Binding can be inhibited by D-mannose and a variety of natural and synthetic oligosaccharides containing terminal mannose residues. High-resolution images show that type 1 fimbriae are composite structures with a short (~20 nm) and thin (2 nm) tip fibrillum at the distal end of the 7-nm fimbrial rod [46]. Type 1 fimbriae are relatively stiff structures (very little bending is observed in electron micrographs of isolated type 1 fimbriae (see, e.g. [47, 71])), but are not rigidly cemented to the cell surface since individual fimbriae can be seen extending at various angles from the cell surface in electron micrographs of type 1 fimbriated bacteria. A structural reconstruction based on electron microscopy and fibre diffraction data [47] shows that the rod of type 1 fimbriae is a right-handed helical structure with 27 FimA subunits in the 19.3 nm helical repeat (eight turns) and a 2.1–2.5 nm-wide central axial hole.

3.1 Genetic Organization and Regulation

The production of type 1 fimbriae requires at least eight genes localized within the *fim* gene cluster and is subject to phase variation (Fig. 7). *fimA*, *fimF*, *fimG*, and *fimH* code for the four protein components of type 1 fimbriae, with a FimA polymer forming the helical rod, and a single FimH TDA strategically located at the distal tip of each fimbriae (Fig. 2) [46]. FimH is attached to the rod via single copies of FimG and FimF, forming the short and stubby tip fibrillum. Assembly of type 1 fimbriae is mediated by the FimC chaperone together with the OM usher FimD (Fig. 2). *fimB* and *fimE* encode regulatory proteins that control the expression of type 1 fimbriae as outlined below. It is currently not clear whether FimI constitutes a structural subunit of type 1 fimbriae or is a regulatory protein, although *fimI* certainly is required for normal fimbrial biosynthesis [74]. Site-directed mutagenesis employed to create lesions in *fimI*, and chromosomal *fimI* mutations, produced fimbriation-negative phenotypes. Minicell analysis associated *fimI* with a 16.4-kDa non-cytoplasmic protein product. It has been suggested that FimI could have a similar function as the PapH protein involved in cell anchoring and length modulation of *E. coli* P pili [75], but this seems unlikely since no effect on fimbrial length or anchoring was observed in the studies by Valenski et al. [74].

Phase variation allows individual bacteria to turn the expression of specific virulence factors on or off. Most of the fimbrial adhesins expressed by *E. coli* are controlled by phase variation and hence only a subset of a bacterial population will express a particular adhesin at any given time. A high level of fimbriation triggers inflammatory host responses, putting the entire bacterial colony at risk of being eliminated. Lowering the proportion of fimbriated bacteria decreases the inflammatory response to levels where it may even be beneficial for the pathogen colony [76]. The proportion of bacteria expressing a particular fimbrial adhesin can be influenced by a number of environmental factors such as temperature, pH, osmorality, or the presence of specific ligands in the surrounding medium. Phase variation of type 1 fimbrial expression in *E. coli* is controlled by the site-specific recombination of a 314-base pair invertible element [77], or *fim* switch (Fig. 7b). The *fim* switch is a transcriptional control element of type 1 fimbrial expression. It contains the promoter for the *fimA* gene encoding the major subunit of type 1 fimbriae. The site-specific recombinases FimB and FimE recognize the 9-base pair inverted repeats

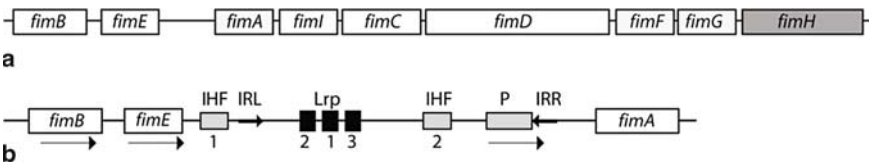

Fig. 7 **a** *fim* gene cluster. **b** Schematic drawing of the *fim* locus with the *fim* switch in the ON (*fimbriate*) orientation and recombinase genes. The direction of transcription of the genes is shown (*arrows*). Lrp, leucine-responsive regulatory protein; IHF, integration host factor; IRL, inverted repeat left; IRR, inverted repeat right; P, *fimA* promoter [73]

flanking the *fim* switch. FimB facilitates inversions in both directions, while FimE can only promote switching from the ON to the OFF phase [78]. In the ON phase orientation, the promoter is correctly oriented for transcription of *fimA* and the rest of the *fim* gene cluster, whereas in the OFF orientation there is no transcription [79]. The predominance of FimE results in the preferential rearrangement of the element in the OFF phase orientation [80]. The genome of the pyelonephritis isolate CFT073 [81] contains genes for three unlinked, FimB- and FimE-like (~50% amino acid sequence identity) tyrosine site-specific recombinases [82]. At least one of these, IpuA, is active at its native chromosome location and can switch *fim* both ways independently of FimB and FimE. A second recombinase, IpbA/HbiF, which appears to be relatively common in commensal, UPEC, and meningitis-associated *E. coli* strains [82, 83], promotes OFF to ON switching to maintain a locked ON phenotype when overexpressed in trans. The suppression of HbiF activity (preventing a locked ON phenotype) in the meningitis isolate RS218 appears to be required to obtain a high degree of bacteraemia in neonatal rats [83].

In addition to the *fim* recombinases, efficient inversion of the *fim* switch requires accessory global regulators like integration host factor (IHF), leucine-responsive regulatory protein (Lrp), and histone-like nucleoid-structuring protein (H-NS). IHF binds with high affinity to two sites, one upstream of and one inside the *fim* switch, to promote switching by FimB (100-fold) and FimE (15,000-fold) [84]. Mutations in the IHF binding sites lower the affinity of IHF binding in vitro and the frequency of FimB and/or FimE recombination in vivo. The presence of the global regulator Lrp is required for normal inversion of the *fim* switch in vivo. Lrp binding increases both FimB and FimE recombination in vivo by binding with high affinity to two of the three sites (1 and 2) within the *fim* switch [85]. This is a rare example of positive Lrp-mediated regulation. Lrp-mediated activation is enhanced by branched side chain aliphatic amino acids, in particular leucine, and also by alanine. Binding of Lrp to its third site inhibits recombination [86]. H-NS down-regulates expression of *fimB* and *fimE* in a temperature-dependent manner, with opposite effects at 30 and 37 °C, by binding to the regions containing the *fimB* and *fimE* promoters respectively [87]. The temperature-dependent H-NS activity modulates type 1 fimbrial expression to favour a fimbriated state at the mammalian body temperature.

As a consequence of host defences against bacterial infection, both sialic acid and GlcNAc release from the host is enhanced. These two potentially key indicators of host inflammation regulate type 1 fimbrial phase variation to inhibit fimbriation through the NanR (sialic acid responsive) and NagC (GlcNAc-6P responsive) regulators [88–90]. NanR targets an operator located over 600 bp upstream of the *fimB* promoter. Binding of NagC to its two operator regions, one close to the NanR binding site and one 212 bp closer to the *fimB* promoter, is enhanced by IHF binding to a site located between the two NagC binding sites. In the absence of sialic acid and GlcNAc, NanR and NagC bind to their operator sites to stimulate FimB expression and promote switching to the fimbriated state. In the presence of sialic acid or GlcNAc/GlcNAc-6P, binding is lost, favouring the non-fimbriated state. Possibly, the ability of *E. coli* to sense the inflammatory host response and to in turn respond by down-regulating expression of type 1 fimbriae can help the infecting bacteria to circumvent host defences by balancing host-parasite interactions.

Numerous examples from recent work demonstrate that the expression of different cell surface structures by bacteria is co-ordinately controlled and often mutually inhibitory [91–93]. Bacteria coordinate gene expression at the single cell level to prevent co-expression of different types of fimbriae. P or S fimbrial expression in clinical isolates leads to type 1 fimbrial repression, through the activity of PapB and SfaB respectively [94, 95]. A mutant of the CFT073 *E. coli* strain incapable of expressing either type 1 or P fimbriae compensated by synthesizing F1C fimbriae [96]. The study by Snyder et al. [96] also showed for the first time that the type 1 vs P fimbrial cross-talk works in both directions and that inversely coordinated expression of adhesin gene clusters also occurs in vivo. Phase variation and coordinated regulation are presumably employed by the pathogenic bacteria to adapt to sequentially changing environments during infection, colonization, and/or invasion [97].

3.2 Role in Disease and Biofilm Formation

Type 1 fimbriae have a well established role in UTI [98], and have also been implicated in neonatal meningitis [99], Crohn's disease [100, 101], and bovine mastitis [102–106]. Their involvement in UTI pathogenesis has been extensively studied. UTIs are believed to affect at least 50% of women in the western world over their lifetime. Each year, 150 million UTIs have been estimated to occur worldwide. The most common cause of UTI in humans is UPEC infection, accounting for about 80% of reported cases. UPEC-caused UTI is also common in animals, e.g. cats and dogs [107].

The expression of mannose-specific type 1 fimbriae on the cell envelope of UPEC allows for the attachment to the epithelium of the bladder and the lower urinary tract. This leads to fast colonization rates, resulting in lower urinary tract infections (cystitis). Binding is crucial for efficient infection, and blocking adhesion has been shown to prevent cystitis [12–14, 16, 108]. However, type 1 fimbriae trigger inflammatory host responses and stimulate the production of proinflammatory cytokines including IL-6, IL-8, and TNF-α [109–111]. Hence, the ability to control the degree of fimbriation in a colony through phase variation (see Sect. 3.1) is important for successful colonization. In general, UTI can be treated with antibiotics, such as trimethoprim sulfamethoxazole, fluoroquinolone, nitrofurantoin and fosfomycin. However, both the rise of antibiotics resistance [6–8] and the recurrent nature of UTIs [112] are worrisome. Approximately 25–35% of initial UTI episodes recur within 3–6 months, and in about two thirds of the cases the recurrent infection involves reinfection by the same bacterial strain that caused the primary infection. During the past decade, Hultgren's group unravelled the mechanisms responsible for recurrent UTI [113]. Contrary to what was previously believed, UPEC can invade into the epithelium lining the bladder and lower urinary tract, the urothelium [15, 114]. Invasion is triggered by binding of type 1 fimbriae to the uroplakin complexes that cover the luminal surface of the urothelium (see Sect. 3.3). Following invasion, most UPEC strains are able to form large intracellular bacterial

communities (IBCs) that eventually mature into a slow-growing, biofilm-like organisation of coccoid-shaped bacteria [115, 116]. Bacteria in these IBCs are protected from antibiotics and the immune system of the host, and in some hosts can persist for months in a dormant and antibiotic-insensitive state [117]. Despite invasion and intracellular replication being relatively rare events, the successfully invasive bacteria rapidly produce progeny of about 500 bacteria per IBC. Within a few hours, the progeny bacteria escape from the matured IBC [118], (re)invade host cells, and reinitiate the IBC cascade [98]. Very little is known about type 1 fimbrial gene regulation during the different stages in the pathogenic cascade of recurrent UTIs. However, since initiation of UPEC biofilm formation on abiotic surfaces is dependent on type 1 fimbriae [119], it remains an intriguing question whether they are also expressed in the IBCs.

Biofilms consist of surface-associated colonies of bacteria and are a major concern for implanted medical devices and in many diseases [120–122]. Bacterial biofilms are highly organized communities embedded in an exopolysaccharide matrix, and contain several layers of bacteria with distinct functions. Biofilms grow slowly but efficiently to coat abiotic surfaces, and can eventually almost completely fill up narrow tubing. Because of the entrenched structure of biofilms, an antibiotic cannot penetrate deeply enough into the distant parts of the biofilm to kill all bacteria. Therefore, biofilms are difficult to remove mechanically or by means of antibiotics or disinfectants.

Biofilm formation is a complex process involving adhesion, aggregation, and expansion of the bacterial community, and requires the careful orchestration of gene expression to co-ordinately activate the multiple cellular mechanisms involved in establishment, development, and maintenance of the bacterial community [121]. The first crucial step in biofilm formation is adhesion. Type 1 fimbriae are critical for formation of *E. coli* biofilm on abiotic surfaces, including polyvinyl chloride, polypropylene, polycarbonate, polystyrene, and borosilicate glass, and the initial attachment is mannose-inhibitable [119]. On the other hand, the adhesin appears not to be strictly necessary for biofilm formation [123]. Type 1 fimbriae without the FimH TDA also facilitate immunoglobulin-mediated (secretory immunoglobulin A and IgM) biofilm formation by *E. coli* on polystyrene. Nevertheless, biofilm formation is more pronounced for bacteria with type 1 fimbriae that carry the adhesin, and it is arguable whether FimH-lacking type 1 fimbriae are really expressed on the cell envelope of clinical isolates of *E. coli*.

Following adhesion and establishment of an adhered bacterial community, further biofilm development requires tight interactions between individual cells in the community. In *E. coli*, the product of the *flu* gene, Ag43, mediates auto-aggregation of bacteria and micro-colony formation [124, 125]. Ag43-induced auto-aggregation cannot take place in the presence of type 1 fimbriae [124], and the expression of fimbriae and Ag43 are co-ordinately controlled [126].

The extent to which the factors that are important for biofilm formation on abiotic surfaces are also important for biofilm formation on biotic surfaces (e.g. in uroepithelial IBCs) is not known, and needs to be studied further. The observation of bacterial biofilm formation on abiotic surfaces mediated by (glyco)proteins [123] is highly

relevant in view of the use of medical implants such as urinary catheters. In particular, the mannosylated Tamm-Horsfall protein (THP) (previously called uromodulin) is secreted in urine as a natural inhibitor of type 1 mediated bacterial adhesion [127] (see Sect. 3.3). Glycoproteins such as THP could coat the catheter walls and serve as glue for type 1 fimbriated *E. coli* to initiate biofilm growth.

3.3 Receptors in the Urinary Tract

The urothelium is a highly specialized endothelium covered almost completely with uroplakins. The most abundant glycoprotein receptors for FimH in the lower urinary tract are uroplakins UPIa and UPIb [128, 129]. These are integral membrane proteins that, together with UPII and UPIII, form ring-shaped complexes with a central 3.7 nm wide cavity in the highly differentiated superficial cell layer of the bladder [130, 131]. Electron microscopy of FimH added to asymmetric unit membranes of the bladder provided evidence that FimH binds to the inner six sub-domains of the 16-nm uroplakin particles, at the sites where UPIa is located [132]. Under quick-freeze, deep-etch electron microscopy, the type 1 tip fibrillae appear to be at least partially buried in the central region of the uroplakin complexes [15]. This suggests that the high-mannose chains on UPIa are exposed on the surface of the central cavity of uroplakin complexes, and that binding is accompanied by (partial) insertion of the tip fibrillum into the cavity. The interaction of FimH with terminally exposed mannose residues on UPIa can lead to severe consequences for the targeted host cells, including bacterial uptake and internalization (see Sect. 3.2).

Recent mass-spectrometric determinations revealed that, like mouse UPIa, human UPIa also carries high-mannose chains while human UPIb only carries complex type glycans [133]. These findings established the concept that UPIa is the major urothelial receptor in humans and other mammals. The determination of uroplakin glycans has been technically challenging due to the difficulty in isolating the highly insoluble uroplakins individually (the uroplakins form a tight complex with each other in the epithelial membrane) and because of the complexity and heterogeneity of the carbohydrates. Mouse and human UPIa are very similar in their glycosylation and hence FimH binding, validating the use of mice as an in vivo UTI model [116]. Interestingly, small differences in the glycosylation of UPIb from bovine vs mouse or human leads to detectable FimH binding of the bovine UPIb, but not to human UPIb [133]. It remains to be seen whether the glycosylation of human UPIa, determined here from cells of 14–16 week old embryonic bladders, is age-dependent. It is interesting to note that β-galactosidase activity is a marker for senescent human cells in culture and aged skin cells in vivo [134], and also marks the senescence, increasing sharply during 25–40 days of age, of normal human urothelium in an organ-like culture [135]. A 40-week period typically represents the turnover rate of differentiated cells in the bladder of mice [136]. The enzyme β-galactosidase cleaves terminal galactose residues from complex glycans, which together with *N*-acetyl glucosaminidase activity would lead to exposure of terminal mannose

residues. Such activity could potentially make UPIb on aged human cells susceptible to FimH binding and adherence of uropathogenic bacteria.

Binding of type 1-fimbriated UPEC to high-mannose moieties on THP inhibits uroplakin binding and it has been suggested that it is an important host defence mechanism [137]. THP is a heavily glycosylated GPI-anchored protein located on the cells lining the thick ascending limb of Henle's loop in the kidney. A specific protease cleaves THP and releases large amounts into the urine (~50 mg day^{-1} in urine from healthy human individuals), making it the most abundant protein in normal human urine. THP knock-out mice have been shown to be more prone to bladder infection by type 1 fimbriated UPEC [138, 139]. There are seven *N*-glycosylation sites in THP, of which one, Asn251, carries high-mannose carbohydrate chains [140]. In humans, the most abundant glycoform is Man6 (75%), followed by Man7 (17%) and Man5 (8%) [127]. Man5 is the only one of these glycoforms that exposes Manα1–3Manβ1–4GlcNAc at the non-reducing end of its high-mannose D1 arm. In pigs, the most abundant glycoform is also Man6 (53%), but the Man5 content is much higher (47%) than in human THP. Pig THP binds about threefold better than human THP to type-1 fimbriated *E. coli* [127], consistent with our finding that glycan structures exposing the Manα1–3Manβ1–4GlcNAc epitope provide the tightest FimH binding [141].

3.4 The FimH Adhesin

Adhesion of UPEC as well as other strains of *E. coli* to physiological receptors or other surfaces displaying mannose-containing receptors depends on the FimH TDA located at the tip of type 1 fimbriae. The first crystal structure of FimH was determined in complex with its periplasmic chaperone, FimC [32]. This structure clearly demonstrated that FimH is divided into two domains; a receptor-binding, or lectin, domain, and a pilin domain (Fig. 6). The latter is needed to connect FimH to FimG, which is incorporated in the tip fibrillum of type 1 fimbriae after FimH. The two domains of FimH are coupled through a very flexible linker (J. Bouckaert, unpublished results) made of two glycine residues, Gly159 and Gly160. These precede the third conserved cysteine (Cys161) that makes a disulphide bond (to Cys187) within the pilin domain.

The FimH receptor-binding domain was originally described as an 11-stranded β-barrel, with a fold unrelated to any other protein fold known at that time [32]. With the second and third three-dimensional structures of TDA receptor-binding domains, from PapG-II [142] and F17G [143], becoming revealed, the overall similar fold among these structures became apparent (Fig. 8). From then on, the receptor-binding domains have been characterized as Ig folds, with large structural variation in the loops joining the Ig core made up of strands B, C, E, and F [143]. The pilin domain of TDAs has the same fold as pilin subunits with an incomplete Ig sandwich lacking the 7th, G, strand. Hence, FimH comprises one complete and one incomplete Ig fold. In the receptor-binding domain, the G strand leads directly into

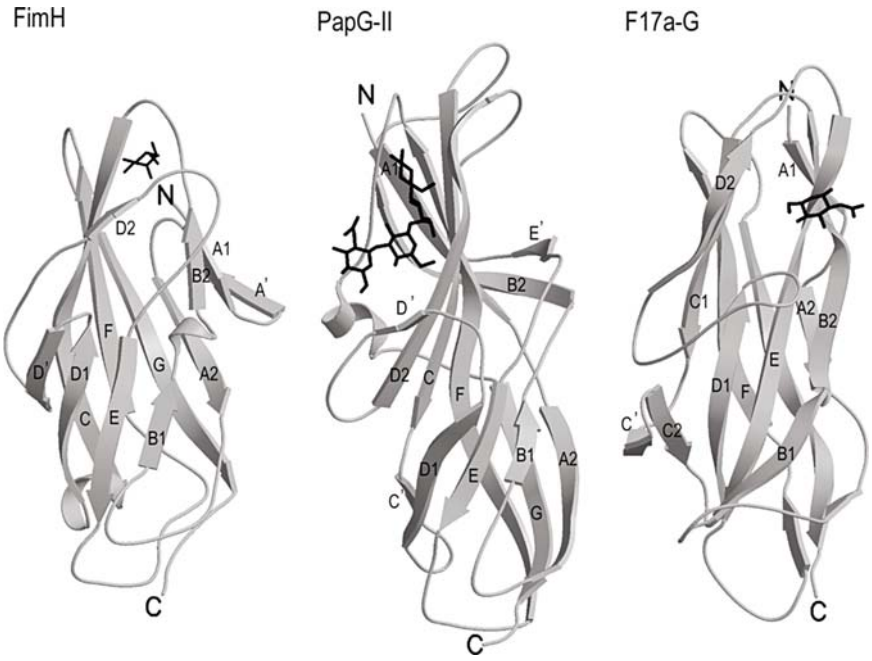

Fig. 8 The receptor-binding domains of three TDAs, FimH [57], PapG-II [142], and F17a-G [143], viewed in the same orientation (obtained by superimpositioning of the structural core of their Ig-fold). The receptor binding sites are in complex with α-D-mannose, globotetraoside, and *N*-acetyl glucosamine, respectively, shown as *black ball-and-stick models*. Structurally equivalent β-strands are labelled with their Ig-fold names

the double-glycine linker (Gly159 and Gly160) that joins the receptor-binding domain to the FimH pilin domain, and is the structural equivalent of the donor strand in the pilin fibre modules. SDA polyadhesins sometimes incorporate a specialised 'invasin' at the very tip of the fibrillar structure [36, 144, 145]. To trigger invasion, an invasin must bind to and interact with target receptors, and so is also an adhesin. Invasins have the same basic fold as pilins but lack an N-terminal polymerization sequence and hence, like the TDAs, can only be located at the tip of the fibre. FimH, which has the dual role of binding and triggering invasion (see Sect. 3.2), might then be thought of as a special case of invasin molecule where a binding/invasin domain is covalently coupled to a pilin domain, rather than linked via DSC as in the polyadhesins.

3.4.1 The Mannose-Binding Pocket

In the FimC:FimH crystals, the receptor-binding domain of FimH was found to have bound to the open ring sugar glucamide of cyclohexylbutanoyl-*N*-hydroxyethyl glucamide (C-HEGA) [32]. C-HEGA had been added at 300 mM to the crystallization

medium in order to improve crystal quality, but was not known to bind FimH. Its binding led to the identification of the mannose-binding pocket, delineated by the loops between β-strands 10 and 11, and between 4 and 5, forming an upper ridge around the binding site, and the β2-β3 loop forming the lower ridge (Fig. 9a). In the crystal structure of the FimC:FimH complex, the glucamide part of C-HEGA was bent in a way to approximate closely the cyclic pyranose ring. Later, the structure of the FimC:FimH complex bound to d-mannose confirmed that C-HEGA had bound in a way that mimicked binding of mannose [57].

Mutation of the amino acids interacting with C-HEGA to alanine or closely resembling amino acids almost uniformly led to complete abolishment of type 1 mediated bacterial haemagglutination, bladder cell binding and bladder tissue colonization [57]. The mutagenesis study gave the insight that the contribution of each single amino acid in the binding pocket is almost uniformly crucial for mannose binding, implying that the recognition by FimH is highly fine-tuned and specific. Furthermore, it showed that modification of the FimH binding pocket directly affected type 1 mediated bacterial adherence to its physiological target, the urothelial mannosylated receptors.

The mannose-binding pocket is a small, deep and negatively charged pocket at the tip of the FimH adhesin (Fig. 9b). Bound mannose makes direct hydrogen bonds to the side chains of residues Asp54, Gln133, Asn135, and Asp140, to the positively charged amino terminus, and to the main chain of Asp47 (Fig. 9c). There are also indirect water-mediated hydrogen bonds from O2 of mannose to the side chain of Gln133 and to the main chain oxygen of Phe1 and Gly14. The water molecule mediating these contacts fills up the space between O2 of mannose and the Phe144 side chain that together with the side chain of Ile13 defines the bottom of the binding site. A collar of hydrophobic residues extends from the mannose-binding pocket towards the tip of the FimH molecule. The high ridge of the collar is bordered by two tyrosine residues, Tyr48 and Tyr137, referred to as the tyrosine gate [147]. These structural features of FimH explain the relatively strong binding of aromatically substituted mannose residues, such as *p*-nitrophenyl mannopyranoside and methyl umbelliferyl mannopyranoside. The hydrophobic collar around the binding site directs the electrostatic attraction forces for binding of the polar mannose residue into the small and charged mannose-binding pocket. The aromatic groups of ligands can pack on the broad hydrophobic platform between the tyrosine rings. This feature is also important for tight binding of alkyl mannosides. In a study based on the unexpected finding of a tight-binding butyl mannoside in the mannose-binding pocket of FimH [147], it was shown that linear alkyl chains on mannose increase the affinity for FimH beyond these for the aromatically substituted mannose. In addition to a strongly hydrophobic nature, the alkyl chains retain significant conformational freedom while interacting to the broad hydrophobic platform of the tyrosine gate, as illustrated by the two alternative bound conformations observed in the two independent crystal structures obtained for the FimH:butyl mannoside complex [147]. This conformational freedom could counteract the entropic cost upon binding of these relatively flexible ligands, thereby significantly enhancing affinity.

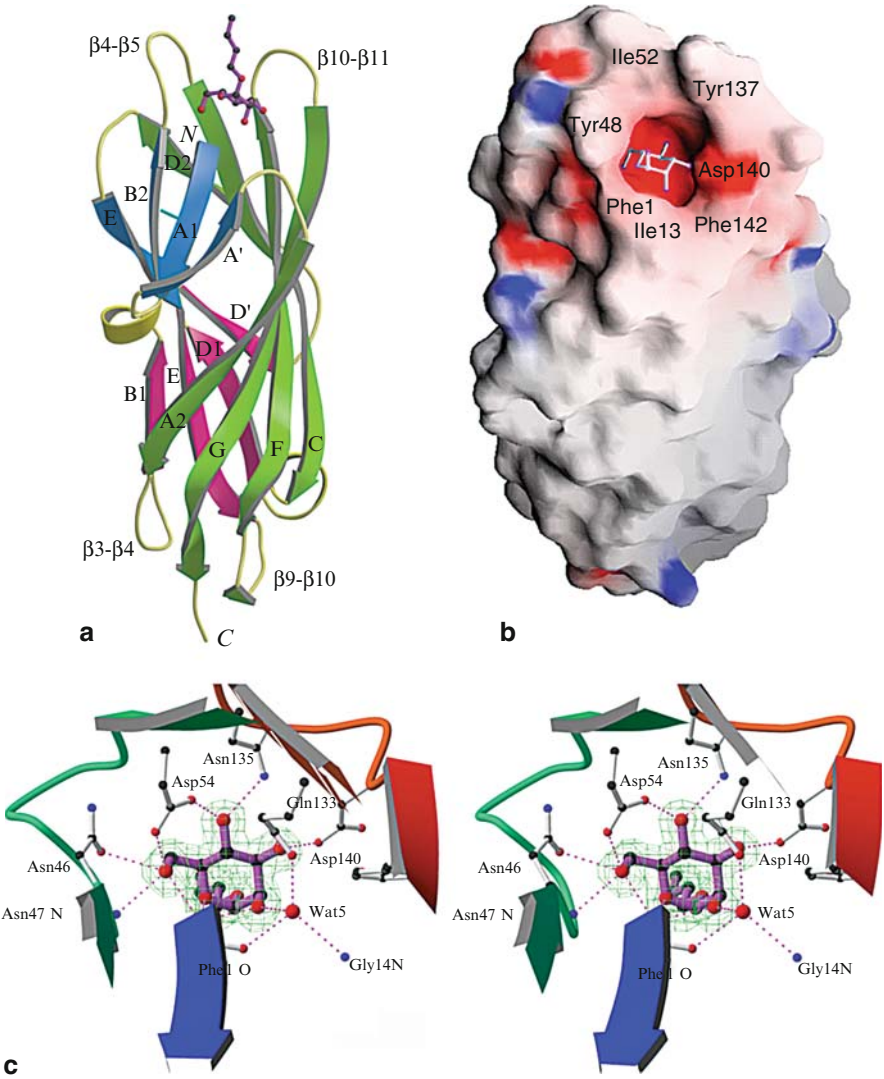

Fig. 9 **a** The three-dimensional structure of the FimH receptor-binding domain is an elongated 11-stranded β-barrel with an Ig-fold. Numbering is according to conventions established for antibody domains [146] and for PapD [33] and loop identifications according to previous descriptions (reference strand numbers A1 = 1, A' = 2, A2 = 3, B1 = 4, B2 = 4', C = 5, D1 = 6, D' = 7, D2 = 8, E = 9, F = 10, G = 11) [32, 57]. The bound saccharide is butyl α-D-mannose [147]. **b** The receptor-binding domain of FimH displaying the electrostatic potential surface [148], with positively charged residues shown in blue, negatively charged residues in red and neutral and hydrophobic residues in white. The residues of the hydrophobic ridge around the mannose-binding pocket are labelled. **c** Stereo image of the mannose binding pocket, viewed 90° away from the orientation in Fig. 9a, and seen from the inside of FimH. The $2F_o–F_c$ electron density for butyl α-D-mannoside is shown. FimH can make 14 possible hydrogen bonds (*purple broken lines*) with the non-reducing mannose. The only oxygen of mannose that is not involved in direct interaction with FimH is the axially oriented O1, sticking outwards of the pocket and in this structure linked to butyl. This agrees with the receptor binding site of FimH being able to bind only to terminally exposed mannose residues on high-mannose glycans [149–153]. A reprinted from [147] with permission from the publisher. B reprinted from [57] with permission from the publisher

3.4.2 Mannose Specificity and Affinity

Analyses of FimH receptor specificity have mostly been performed by using whole fimbriated bacteria. For a few years now it has been possible to compare these results with those of the isolated and soluble receptor-binding domain of FimH (residues 1–158). The same solution affinity equilibrium constants for FimH binding to a series of carbohydrates were obtained in both a surface plasmon resonance (SPR) assay using a monoclonal antibody directed against the mannose binding pocket of FimH, and a tritiated mannose displacement assay [147]. In these binding studies, FimH was confirmed not only to be highly specific, but also to have an unusual high affinity (K_d = 2.3 μM) for mannose. This result had already been indicated from the crystal structure of the FimH-mannose complex, because of the very tight network of hydrogen bonds involved in mannose binding, with in total 14 potential hydrogen bonds involving all of the mannose's oxygen atoms except for the α-anomeric O1 atom [57]. Of all the tested mono- and disaccharides other than mannose (methyl 2-deoxy-α-d-mannopyranoside, glucose, galactose, fructose, sucrose, and turanose), only fructose has an affinity approaching that of FimH for mannose, binding with only 15-fold lower affinity. The relatively tight binding of fructose is presumably due to ring opening and conversion to the pyranose form of fructose (Fru_p). Modelling predicts that Fru_p is differently oriented in the binding site compared to mannose (Fig. 10), allowing it to bind with only one hydrogen bond less than mannose. This hydrogen bond is replaced by a hydroxyl-methyl group interaction. Similar observations of the compatibility between mannose and fructose binding have been made in crystal structures of the bacterial lectin LecB, also named PALII, from *Pseudomonas aeruginosa* [154], the legume lectin from *Pterocarpus angolensis* [155], and the HIV gp120 antibody 2G12 [156].

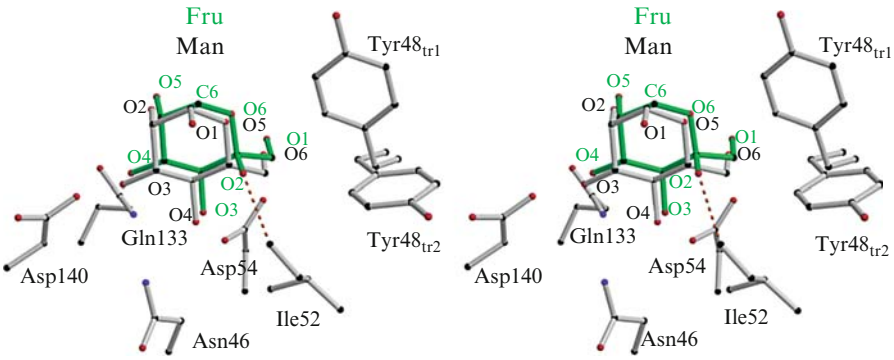

Fig. 10 Fructose superimposed on mannose in the FimH binding pocket. Fructose in *green* (for clarity of the picture, each atom was shifted 0.3 Å from the ideal superposition), mannose in *grey*. The two different Tyr48 side chain conformations are shown. Note the lack of O1 of mannose (anomeric oxygen) and the presence of (an extra) hydroxyl on C2 (the equivalent of C5 of mannose) that is in close contact (2.7 Å) with Ile52 (*orange dashed bond*)

3.4.3 Recognition of Oligosaccharides and Fine Specificity

The physiological receptors for FimH are *N*-glycosylated proteins carrying high-mannose structures. The largest high-mannose glycan, oligomannose-9 (Fig. 11), is fully substituted with terminal α1–2 linked mannosides on its D1, D2, and D3 arms. It has long been known that only mannose at the end of such an arm can bind in the mannose binding pocket of FimH. In oligomannose-9, all three terminal mannose residues at its non-reducing ends are potential candidates for binding in the monosaccharide-binding pocket of FimH. The affinity indeed increases with a factor of three for oligomannose-9 over Manα1–2Man, which at the same time indicates that the binding of oligomannose-9 is not polyvalent. Thus not all three terminal residues are bound simultaneously by one FimH molecule, but the probability to encounter Manα1–2Man has been increased threefold. Prolongation of Manα1–2Man at its reducing end, as in Manα1–2Manα1–2Man, increases affinity only moderately and is glycosidic linkage-independent. These findings suggest that a complementary fit of the larger trisaccharides, providing more hydrogen bonds, more van der Waals interactions and burial of hydrophobic surface, is important for generating tighter binding.

The group of Sharon first observed that other, higher-affinity epitopes, are hidden in oligomannose-9 and are only accessible in high-mannose substructures much smaller than oligomannose-9 [149, 150, 157]. Probing the fine specificity of FimH for high-mannose epitopes using a series of oligomannosides, corresponding to substructures of high-mannose N-linked glycans on proteins, revealed that for those oligomannosides (oligomannose-3 and -5) where the D1 arm is not capped at the non-reducing end by an α1–2 linked mannose residue (Fig. 11) [158], the affinity is very high (K_d = 20 nM) [147]. This affinity parallels the affinity of FimH for aryl (*p*-nitrophenyl α-D-mannose K_d = 44 nM and methyl umbelliferyl α-D-mannose K_d = 20 nM), and alkyl mannosides (pentyl α-D-mannose, K_d = 25 nM, hexyl α-D-mannose, K_d = 10 nM, and heptyl α-D-mannose, K_d = 5 nM) [147].

The highest-affinity epitope for FimH, Manα1–3Manβ1–4GlcNAc, is not free for binding to FimH in any of the recently elucidated glycan structures on mouse UPIa [133]. However, a change in the glycosylation of UPIa or other urothelial surface proteins may alter the host susceptibility to UTIs. Interactions of FimH with monomannose in vitro, but not with trimannose (Manα1–3(Manα1–6)Man), correlate with binding to human bladder epithelium, despite the 10-fold higher affinity of FimH for the latter ligand [57]. This may be understood from the fact that mannose residues that can function as FimH mono-mannose receptors are always present at the non-reducing termini (D1, D2 or D3 arm) of any high-mannose glycan substructure. In contrast, trimannose is only accessible at the non-reducing end of oligomannose 7D1, 6, 5, and 3 (Fig. 11). Only very low amounts of oligomannose 6 are found on mouse UPIa, whereas oligomannose 7, 8, and 9 occur in significantly higher amounts [133]. Hence trimannose is available for FimH binding to a much smaller extent than mono-mannose. This is congruent with our previous findings for binding to human bladder tissue of FimH variants with engineered point mutations in the mannose-binding pocket [143]. The binding of recombinant type 1 fimbriated *E. coli* strains to human bladder tissue sections was correlated with mono-mannose binding

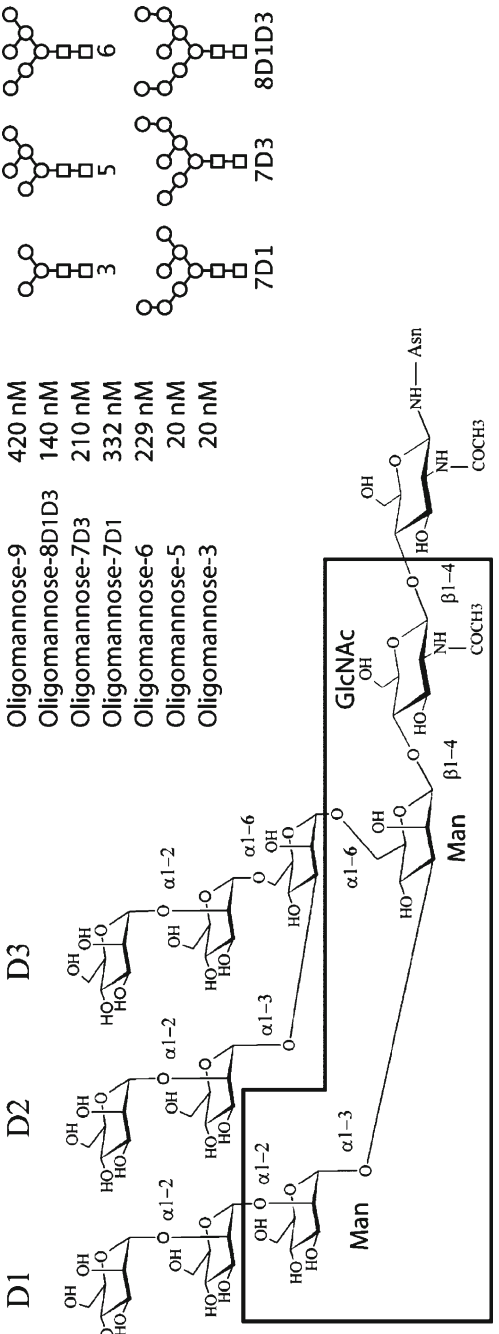

Fig. 11 Epitope mapping on the N-linked high-mannose glycan with FimH on Biacore*3000* reveals the highest affinity (K_d = 20 nM) for the Manα1–3Manβ1–4GlcNAc epitope (boxed) on oligomannose-3 and oligomannose-5, due to the presence of an unsubstituted D1 arm. Structures of the different oligomannoses are illustrated schematically to the right (*squares* = GlcNAc; *circles* = Man)

of the corresponding mutant FimC:FimH complexes, but not with their (tighter) trimannose binding.

There is ample evidence that the carbohydrate expression profile of eukaryotic cells can be subject to variation in certain disease states, but also under normal physiological conditions such as menopause and aging (for a review, see [159]). Interestingly in this regard, diabetic patients are more prone to UTI than otherwise healthy individuals [160]. Twice as many type 1 fimbriated *E. coli* adhere to urothelial cells of diabetic patients than of healthy individuals, even taking into consideration the presence of various substances excreted in the urine, such as albumin, glucose and THP [161]. Unfortunately, it is currently unknown whether this can be attributed to altered glycosylation of FimH receptors in the urinary tract. A better understanding of the fundamental relationships between physiological conditions of the host and carbohydrate expression on urothelial cells is thus needed.

The (limited) allelic variation in FimH [57] between *E. coli* clinical isolates from intestinal or extra-intestinal origin, mainly from the urinary tract, has long been though to affect directly the structure and function of the FimH receptor-binding domain. Type 1 fimbriated bacteria from different *E. coli* clinical isolates display altered mannoside binding and adhesion profiles. Recombinant *E. coli* expressing the CI#4 (UPEC) FimH variant adhere tightly to yeast mannan, A498 human kidney cells, and J82 human bladder cells, whereas bacteria expressing FimH from the fecal *E. coli* F-18 strain show poor adhesion to all three substrates [162]. The F-18 strain only adheres under shear conditions in parallel flow chambers, and its cells do not agglutinate guinea pig red blood cells under static conditions but only co-aggregate under rocking [163]. Minor sequence variations in FimH have been held responsible for these functional differences [162, 164–166].

Recent affinity measurements using the isolated FimH receptor-binding domain originating from a number of different strains, including UPEC, commensal *E. coli*, and EHEC strains, contradict this hypothesis [141]. The FimH receptor-binding domains from two UPEC, one fecal, and three EHEC strains have identical affinities for mannose and a series of high-mannose glycans and high-mannose substructures (the only exception to this is the FimH domain from some EHEC strains that have a Lys instead of an Asn residue at position 135 in the binding pocket, abolishing all binding). As an example, our data show that there are no significant differences in the affinities for d-mannose or trimannoside between the FimH from *E. coli* F-18 and from *E. coli* CI#4. Hence, the single Gly73Glu amino acid difference between these two FimH variants does not have a direct effect on affinity. This suggests that factors other than differences in mannose binding of FimH per se cause the different adhesion phenotypes.

Factors that might influence adhesion properties include the number of fimbriae per cell, the number of fimbriae that are functional for receptor binding, the fraction of cells that are fimbriated, the length and the flexibility of fimbriae, and the ability of fimbriae to deform under the influence of mechanical force. For example, it is up to date not known to what extent variant residues in the FimH receptor-binding domain affect assembly and thus the number of fimbriae on the bacterial cell envelope of bacteria expressing mutant FimH receptor-binding domains. The receptor-binding domain is necessary to initiate pilus assembly by interactions between

the FimH receptor-binding domain and the usher OM export channel [51, 66, 167], and it is not impossible that some of the mutations that affect adhesion target (or indirectly influence the conformation of) residues that interact with the export channel of the FimD usher. For example, the natural variant residue Ala/Val27, a double mutation engineered to inhibit FimH linker strand extension, Gln32Ala/Ser124Ala [163], and the point mutation Val156Pro, engineered to facilitate linker strand extension [163], are all located on the surface of FimH. Hence, the effect of these variations on assembly is worthy of further investigation. Reduced assembly efficiency, resulting in a smaller number of fimbriae, could significantly reduce the possibilities for multivalent binding, as described earlier [168].

The type 1 fimbrial shaft has a strong influence on the affinity and specificity of adhesion [169–171]. For example, type 1 fimbriae from *S. typhimurium*, *K. pneumoniae*, and *E. coli*, exhibit distinct adhesion and haemagglutination profiles. In contrast, FimH molecules from any of these three species bind to a broad range of mannose-containing compounds when expressed as MalE fusion proteins. Intriguingly, the adhesion profile of bacteria expressing hybrid type 1 fimbriae (e.g. *K. pneumoniae* FimH at the tip of *E. coli* type 1 fimbriae or vice versa) resembles the adhesion profile of the bacteria from which the fimbrial rod was derived [171]. Electron microscopic pictures have revealed that the helical rods of type 1 fimbriae and P pili can extend significantly as a result of either mechanical shear during preparation [172], or from exposure to 50% glycerol [173]. The extension is due to breaking of the quaternary interactions between non-adjacent pilin subunits within the right-handed helical rod, leading to its uncoiling. Extension of fimbriae in response to shear was suggested to allow an increased number of fimbriae of a bacterium to adhere simultaneously, thereby increasing the strength of adhesion [174]. Recent work using optical tweezers demonstrates that the unfolding of the helical structure of P and type 1 fimbriae to a linear conformation is fully reversible and that the fimbrial rod can work as a spring under shear forces [175–177]. The spring-like qualities of the rods have been suggested to be fine tuned to support the formation of long-lived catch bonds that promote tight adhesion under conditions of high shear stress [177] (see Sect. 3.5.2). Since there is much more sequence variability between structural subunits in different *E. coli* strains than between TDAs, rod properties can be expected to vary considerably. In contrast, different FimH variants have been shown to have indistinguishable binding properties [141]. Hence, differences in adhesive properties are most likely due to different rod properties caused by sequence differences between rod subunits (FimA in the case of type 1 fimbriae).

3.5 Factors that Enhance Adhesion

3.5.1 Multivalency

The presence of multiple fimbriae on the cell surface allows bacteria to adhere to target surfaces by simultaneously using multiple and often weak, non-covalent protein-carbohydrate interactions between the adhesin and its receptor. Such

multivalent binding significantly increases the adhesive forces and is the reason why even relatively weak adhesin-receptor affinities can provide considerable binding strength. Studies of adhesion of type 1 fimbriated *E. coli* to self-assembled monolayers (SAMs) displaying α-C-mannoside ligands have revealed that both the relative orientation of bacteria towards the binding surface, and the density of receptors on the surface, strongly influence the number of fimbriae that are involved in bacterial attachment and hence the strength of adhesion [168]. Side-on attachment of the elongated *E. coli* bacteria leads to strong polyvalent interactions mediated by multiple fimbriae, whereas in end-on attachment, achieved by manipulating the bacteria with optical tweezers, only one or two fimbriae simultaneously are able to find an accessible mannose ligand, resulting in relatively weak adhesion. This effect is much more pronounced for binding to a high-density SAM (4×10^5 ligands μm^{-2}) compared to a SAM with a low (4 ligands μm^{-2}) surface density of mannose. In the experimental set up of Liang et al. [168], the force required to remove a bacterium attached side-on to the SAM varied between 3.5 to ≥18 pN (high-density SAM) and between 3.8 to 7.8 pN (low-density SAM), suggesting that the number of fimbriae involved in adhesion differed from bacterium to bacterium. In some cases, the maximum force available in the apparatus used (18 pN) was not enough to immediately detach bacteria. However, sweeping the position of the optical tweezers across the entire length of the bacterium eventually led to detachment, presumably through sequential dissociation of individual interactions between the α-C-mannoside ligands and FimH receptors in a 'Velcro-like' manner. From repeated measurements of detachment of bacteria attached end-on, the force required to break the interaction between a single type 1 fimbriae and a surface-bound α-mannoside was estimated at 1.7 pN.

3.5.2 Shear-Enhanced Adhesion

Bacterial adhesion often occurs under conditions of shear stress exerted by the flow of liquids (e.g. urine, saliva, mucus) over the binding surface. Although in many cases shear stress counteracts adhesion by limiting binding and washing away unbound bacteria, cases where shear stress increases adhesion have also been observed and might constitute an important factor in bacterial colonization of target tissues. The groups of Vogel and Sokurenko jointly investigated shear-dependent 'stick-and-roll' adhesion of type 1 fimbriated *E. coli* [163, 177–182]. In their parallel flow chamber experiments, bacteria in PBS-BSA solutions were pumped at various flow rates through parallel plates coated with mono-mannosylated BSA [182] or with RNase B (an *N*-glycosylated protein carrying high-mannose structures) [178–180], and video-imaged to follow attachment and detachment from the surfaces. At low flow rates, bacteria were observed to roll along the surface. Presumably, rolling adhesion is caused by the transient formation and breaking of individual FimH-mannose interactions as the bacteria are transported along the glycosylated surface. Moderately high shear was found to enhance the adhesion of type 1 fimbriated *E. coli* to mannose-containing surfaces. With increased flow rate or viscosity, the

bacteria switched from rolling attachment to stationary binding. As expected, at even higher levels of shear stress, the bacteria eventually detached and were washed away. Shear-enhanced adhesion was more pronounced for binding to mono-mannose-coated surfaces than for binding to surfaces displaying trimannose or high-mannose structures. The observed effect of shear on adhesion was also different for different type 1 fimbriated *E. coli* strains expressing different FimH variants, being more pronounced for fecal isolates [163]. In summary, the less specific and hence the lower the affinity is for the receptor, the larger the effect of shear force is on adhesion. Shear-enhanced adhesion was proposed to depend on the formation of so-called 'catch bonds', i.e. bonds that become stronger under application of force [183]. Conformational changes in the FimH mannose-binding pocket caused by shear force-induced extension of the FimH interdomain linker were suggested to promote the formation of catch bonds.

Shear-dependent adhesion may be physiologically relevant, allowing bacteria to move and spread over surfaces at low shear stress levels while keeping them firmly attached at higher shear stress, e.g. during voiding of urine. In particular, the weak, rolling, type of adhesion appears to allow bacteria to more rapidly spread and colonize target surfaces in the presence of moderate fluid flow [178]. In addition, shear-activated adhesion may help protect bacteria from soluble inhibitors present in, e.g. urine (see Sect. 3.3) or administrated as anti-adhesive drugs. Nilsson et al. [179, 180] studied the inhibitory effect of methyl α-D-mannose on binding of type 1 fimbriated *E. coli* to mannosylated surfaces under different shear stress conditions. Type 1 fimbriated *E. coli* were allowed to bind to RNase B-coated flow cell surfaces for 6 min at low (0.25 Pa) shear stress before addition of 400 mM methyl α-D-mannose to the flow solution. At low shear stress levels (≤0.5 Pa), where bacteria exhibit weak rolling adhesion behaviour, the presence of methyl α-D-mannose dramatically increased the rate of bacterial release from the surface. In contrast, almost no detachment was observed when high (4 Pa) shear stress levels, promoting tight stationary adhesion, were applied. The reason for this bimodal behaviour is presumably that during rolling, FimH molecules transiently bind and detach from surface receptors, allowing a soluble inhibitor such as methyl α-D-mannose to eventually bind to all FimH molecules, thereby preventing further adhesion. In contrast, stationary adhesion is mediated by long lived adhesin-receptor bonds that prevent inhibitor binding during the time course of the experiments (~10 min). Nevertheless, the presence of a soluble inhibitor such as methyl α-D-mannose during a longer period of time (hours) can sometimes promote biofilm formation under conditions of low shear by loosening the interaction between statically adhering bacteria and the surface, allowing them to spread by rolling [178]. In conclusion, for a free small-molecule ligand to be an efficient FimH anti-adhesive even under conditions of fluid flow, it must bind significantly tighter to FimH than the receptor molecules to overcome the shear-enhanced affinity. In this context it is interesting to note that whereas human uroplakin UP1a exposes mainly mono-mannose receptors [133], human THP (see Sect. 3.3) displays significant amounts of the high-affinity Man6 (75%) and Man5 (8%) receptors [127].

Steered molecular dynamics simulations predicted that application of tensile force along the long axis of FimH (as might result, e.g. from shear forces) would result in extension of the linker region between the receptor-binding and pilin domains [163]. As mentioned above, extension of the linker was proposed to facilitate the formation of catch bonds. Congruent with this idea, bacteria expressing type 1 fimbriae with either naturally occurring or engineered FimH variants with mutations predicted to destabilize the linker region and facilitate linker extension were shown to exhibit decreased shear-dependent adhesion. Recently, a two-state catch-bond model derived from allostery that beautifully explains and predicts the data observed for stick-and-roll adhesion of type 1 fimbriated *E. coli* to mannose-coated surfaces was proposed [181]. In contrast to the sliding-rebinding mechanism proposed for catch-bond formation in P and L selectins [184], this model involves two interchangeable conformational states of the mannose-binding pocket. Extension of the FimH linker is proposed to allosterically change the binding pocket from a weak to a tight-binding conformation. The crystal structures of mannose and of butyl-mannose bound to FimH [57, 147] show that mannose binds snugly into a highly specific mannose-binding pocket at the tip of the FimH receptor-binding domain (see Sect. 3.4). As indicated by the unusually high monosaccharide affinity of FimH for mannose (K_d = 2.3 μM), the pocket appears near-optimal for mannose binding with almost all of the hydrogen-bonding potential satisfied. Hence it is difficult to envision how conformational changes could additionally enhance mannose binding. It is possible that the observed structures correspond to the tight-binding conformation, and that the weak-binding conformation is not favoured by the crystallization conditions used to grow crystals. In this context it is worth noting that automated docking studies of mannose binding to FimH predicts two main binding modes, one corresponding to that observed in the crystal structures, and a second binding mode in which the mannose residue is rotated within the binding pocket (J. Berglund, D. Choudhury, S.D. Knight, unpublished).

3.6 Medical Applications

3.6.1 Vaccines

Vaccines targeting some bacterial infections (e.g. pertussis, typhoid fever, ETEC diarrhoea and bovine mastitis, e.g. [185–187], and glycoconjugate vaccines targeting *Haemophilus*, *Neisseria* and *Streptococcus*, e.g. [188–190]) are available or are being developed. With the exception of the highly successful glycoconjugate vaccines, many vaccines have been formulated using either killed whole bacteria or live attenuated bacterial strains. Such whole cell vaccines are associated with a number of problems such as more or less severe side effects (severe local reactions, fever), the need for rigorous safety measures to ensure that live pathogenic bacteria are not spread from the production plant or transferred with the vaccine, or problems of controlling the stability, strength, and nature of immune response owing to variations in

antigen presentation and in the amount of antigen present in the vaccine. Additionally, whole cell antigen presentation may shield potentially efficient broad-range antigens from the immune system. This is the case with TDA fimbriae, where a conserved (e.g. [57]) and intrinsically highly antigenic receptor-binding adhesin is incorporated as a minor component of a large complex protein structure on the bacterial cell surface, leading to efficient production of antibodies directed against the much less conserved bulk components of the organelle but not against the adhesin.

Because of their critical role in pathogenesis and because they are naturally expressed on the surface of bacteria, bacterial adhesins have long been considered as attractive components of vaccines [191], so far with only limited success. Many adhesin vaccine formulations have been based on intact adhesive organelles (e.g. fimbriae), which are antigenically highly variable and hence induce protection limited to bacteria expressing the same fimbrial variant. For example, antibodies directed against purified whole type 1 fimbriae or P pili protect against cystitis and pyelonephritis respectively, in both murine and primate models for these diseases [14, 17, 192–194]. However, protection is limited to either *E. coli* strains homologous to that from which the fimbriae used for immunization were derived, or to a small subset of serologically cross-reactive heterologous strains. Therefore, any vaccine composed predominantly of the major structural proteins of fimbriae (e.g. FimA or PapA) will be of limited value because antibodies developed against these highly variable proteins are specific for the strains from which the protein used for immunization was derived.

The realization that TDAs could be obtained as stable and soluble complexes with their cognate chaperone inspired new hope for the development of adhesin-based vaccines. Promising preclinical trials with UTI vaccines based on the FimC:FimH chaperone:adhesin complex [195] and on the PapD:PapG chaperone:adhesin complex [21, 196] have been reported. Both vaccine candidates were shown to protect against UPEC mucosal infection in murine and primate models. In vitro binding data suggested that the ability of anti-FimH antibodies to block type 1 fimbrial adhesion contributed significantly to FimC:FimH-induced protection. No clinical trials with the PapD:PapG vaccine have as of yet been reported. Development of the FimC:FimH vaccine candidate was dropped during phase II clinical trials because of limited protection (MedImmune, Inc., Annual Report 2002).

One possible reason for the limited success with adhesin subunit vaccines might relate to how these vaccines have been formulated. Monomeric presentation of antigens such as that used for the FimC:FimH and PapD:PapG UTI vaccines often gives poor immunogenic response. Multivalent antigen presentation generally results in a significantly improved response [197]. Multivalent antigen presentation may be achieved by coupling subunit antigens to a suitable carrier particle. For multivalent presentation to be efficient, antigens must be coupled without disturbing their antigenic capacity. Ideally, antigens should be presented on the carrier particle in the same way as they would be on the surface of the pathogen from which they have been derived, so as to present the immune system with a good mimic of the pathogen surface. In the case of adhesins, the receptor binding function must not be destroyed, or masked, if blocking antibodies are to be raised. Several recent examples show that the receptor-binding domain of TDAs such as

FimH or PapG may be expressed on its own without affecting the structure or binding properties [142, 143, 147, 198–200]. Since the carbohydrate binding site is located in the top half of the receptor-binding domains, distal from the C-terminal linker region that connects it to the pilin domain in the full length TDAs, modified proteins consisting of the isolated receptor-binding domain coupled to a suitable C-terminal tag could be used to incorporate functional adhesins into multivalent antigen-carrying particles for use as vaccines. Using this approach, we recently produced FimH-decorated ISCOM [197, 201, 202] particles, and are investigating the immunogenic properties of these particles (G. Askarieh, J.-I. Flock, S.D. Knight, unpublished).

3.6.2 Pilicides

Pilicides are small organic molecules designed to interfere with pilus biogenesis [203, 204]. The activity of a family of bicyclic 2-pyridones, termed pilicides, has been evaluated in two different UPEC fimbrial systems, and its interactions have been examined in detail in a crystal structure of the FimC:FimH chaperone-adhesin complex with a bound pilicide [205]. The binding of the pilicide would interfere with the binding of the N-terminal periplasmic domain of the usher to the chaperone-pilin interface, particularly of the bulky hydrophobic usher residues Phe4, Leu19, and Phe22 [68]. Note that this was an unexpected result since the pilicide had been designed to bind to the chaperone cleft and block subunit binding.

3.6.3 Anti-Adhesives

An alternative to adhesin-based vaccines as a means to block bacterial adhesion is the use of small compounds that interact tightly with target adhesins [206]. Aromatically substituted mannosides have long been known to be particularly potent inhibitors (nanomolar binding constants) of FimH-mediated bacterial adhesion [207]. Fruit juice, in particular cranberry juice [208], has traditionally been considered to be useful in the treatment of UTIs, and a positive effect of cranberry juice consumption has been observed in controlled clinical trials (e.g. [209–211]). Fructose has been shown to be the active compound in fruit juices that inhibits adhesion of type 1 fimbriated *E. coli* [208]. Fructose binding to FimH is only ~15 times weaker than mannose binding, and fructose binds more tightly to FimH than the physiological P pilus globotetraoside receptor does to PapG-II [147].

We recently reported two independent crystal structures for the FimH receptor-binding domain [147]. In both structures we found a butyl mannoside, derived from the yeast extract used to grow bacteria for protein expression, bound in the mannose-binding site. The serendipitous discovery of this ‘sticky’ ligand led to the identification of alkyl mannosides as a new class of high-affinity FimH ligands. The mannose group of the butyl mannoside binds identically to D-mannose (Fig. 9c). The alkyl chain extends out of the pocket towards Tyr48 and Tyr137, making van

der Waals contacts to both tyrosine rings. We discovered that butyl α-D-mannoside binds to FimH significantly better (K_d ~150 nM) than mannose (K_d ~2.3 μM). To investigate the effect of sequential addition of methyl groups to the O1 oxygen of D-mannose, a series of alkyl mannosides were synthesized and the dissociation constants determined using two different binding assays. We discovered a linear correlation between the binding free energy, as calculated from the measured dissociation constants, and the number of methyl groups (between 1 and 7) in the alkyl mannoside, with each additional methyl group contributing on average –0.6 kcal mol^{-1} of binding energy. The best binding alkyl mannoside, heptyl mannoside, binds a few hundred times stronger than mannose, equivalent in affinity to the most tightly-binding aromatically substituted mannosides for FimH and of mannose dendrimers for type 1 fimbriated *E. coli* [207, 212]. Alkyl mannosides have not previously been recognized as strong binders to FimH. Since they are easily synthesized and highly soluble in water, they may be interesting as potential blocking agents for FimH-mediated adhesion.

Just as polyvalent binding is used by bacteria to enhance adhesion, polyvalency can potentially be used to create potent adhesion inhibitors. Because small molecule adhesin inhibitors with sufficient affinity to block bacterial adhesion might be hard to accomplish, a lot of interest lies in the design of multivalent anti-adhesives that target several fimbrial adhesins simultaneously. However, the design of multivalent inhibitors for fimbrial adhesins is much more complicated than for multimeric multivalent proteins. For the latter, a relatively constant distance between the binding sites can be predicted, depending on the quaternary organization of the multimeric protein. For fimbriated bacteria on the other hand, the spacing between the fimbrial adhesins is not constant and difficult to estimate. The length of type 1 fimbriae can vary considerably. Also the number of fimbriae expressed on the cell envelope and hence the average spacing between them can vary between strains and moreover depends on the growth conditions favouring or disfavouring expression. Because type 1 and P fimbrial expression are known to be subject to phase regulation (see Sect. 3.1), normally only a percentage of *E. coli* cells in a population are fimbriated. The fimbrial tip, including the FimH adhesin, may break off under shear force for example during preparation steps prior to the analysis of the bacterial cells, making it difficult to asses the number of functional fimbriae present. Finally, although type 1 fimbriae are relatively rigid, their extended shape allows them some freedom to wave around in the solvent. The short, stubby tip fibrillum is flexible. All of these factors increase the uncertainty in distances between the targeted FimH receptor-binding domains making both the design process and evaluation of results more difficult.

The plasticity of the fimbrial rod and the flexibility of the tip fibrillum help to increase the number of possible encounters of the fimbrial adhesins with the sugar epitopes on the host cells. In this regard, the distance between the sugar receptors on a surface to be bridged by the fimbrial adhesins is not fixed and does not really have a strict upper limit. A minimal distance between two mannose epitopes can however easily be imagined, as two FimH receptor-binding domains on two fimbriae cannot be brought together too closely. A first idea of a minimal

distance between two sugar epitopes can be obtained by considering binding of alkyl mannosides to the mannose-binding site of FimH. The length of an alkyl tail O-linked to α-D-mannose that is optimal for interaction with FimH is seven carbon atoms (K_d = 5 nM for heptyl α-D-mannose) [147]. This suggests that a linker between two mannose residues that are both meant for interaction with FimH theoretically should be at least 14–16 atoms long for the two binding surfaces of FimH not to sterically interfere with each other. The enhanced binding of alkyl and aryl mannosides demonstrates that the first part of the aglycon in a polyvalent mannose compound should in fact not even be considered as a linker, but as part of the ligand.

Type 1 fimbriated UPEC have probably been the most extensively studied target of glycodendrimer chemistry [212, 213]. Nevertheless, glycodendrimer chemistry designed to inhibit type 1 mediated bacterial adherence has proven excruciatingly difficult. The most successful example of a large glycodendrimer FimH inhibitor is the DP16 dendrimer designed by Nagahori et al. [212]. This inhibitor inhibits type 1 fimbrial adhesion considerably better (sub-nanomolar IC_{50} values) than monovalent mannose but still not significantly better than the best-known small-molecule inhibitors. The difficulties in finding strong multivalent adhesion inhibitors could be solely due to incorrect linkage of the mannose residue in the dendrimer, preventing mannose from binding in the mannose binding pocket of FimH [214], but most often reflects the difficulties in trying to use molecular ligands to simulate the complexity of multivalent cellular host-pathogen interactions.

4 Conclusion and Perspectives

During the last decade, considerable progress has been made in understanding chaperone/usher-mediated fimbrial assembly, and the contribution of fimbriae to bacterial adhesion and disease. By providing a folding platform consisting of a pair of template β-strands (A_1 and G_1), and large hydrophobic donor residues, periplasmic chaperones promote subunit folding and partition intrinsically aggregative protein subunits away from non-productive aggregation pathways. Subunit folding onto this platform results in chaperone donor residues being incorporated into the core of the subunit and formation of a fused super-barrel with the subunit in an open, activated high-energy conformation. Following dissociation of this activated subunit from the chaperone, folding is completed to form a condensed hydrophobic core. By arresting subunit folding and trapping subunits in a molten globule-like high-energy conformation, the chaperones preserve folding energy that can drive assembly even when chaperone:subunit interactions are more extensive than subunit:subunit interactions in the fibre. In contrast to the rather detailed understanding of the periplasmic chaperones, very little is still known about the assembly process per se and the workings of the outer membrane usher. A first glimpse of how the usher recognizes chaperone:subunit complexes has provided some hints about how specificity and ordered assembly of complex structures is achieved. In the following years,

hopefully new structures of ushers, and of complexes between ushers and chaperones and subunits will allow us to begin to fill in the remaining gaps in our understanding of fimbrial assembly. Understanding the molecular details of fimbrial biogenesis will continue to contribute to our ability to invent and/or discover new agents that interfere with the process (exemplified by the recent success with pilicides) and that may become useful in the treatment of bacterial infections.

Our understanding of bacterial adhesion has now reached a level where we can begin to exploit it for the development of novel anti-adhesive compounds for use in, e.g. medicine. Structural insight into the host-pathogen fimbrial interactions can significantly facilitate rational design of highly potent, small, and monovalent sugar-derived inhibitors that are relatively easy to synthesize; and finally truly polyvalent, dendrimeric inhibitors could be derived from these. Other future anti-adhesive therapies will focus on the cellular biology of the bacterial attachment, invasion, and reproduction processes. A first step in that direction has been made by the treatment of mouse bladder with an exfoliation agent, protamine sulphate, to remove cells containing bacterial reproduction factories from the superficial endothelial cell barrier of the bladder [215].

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Carbohydrate Receptors of Bacterial Adhesins: Implications and Reflections

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Abstract Bacteria entering a host depend on adhesins to achieve colonization. Adhesins are bacterial surface structures mediating binding to host surficial areas. Most adhesins are composed of one or several proteins. Usually a single bacterial strain is able to express various adhesins. The adhesion type expressed may influence host-, tissue or even cell tropism of Gram-negative and of Gram-positive bacteria. The binding of fimbrial as well as of afimbrial adhesins of Gram-negative bacteria to host carbohydrate structures (=receptors) has been elucidated in great detail. In contrast, in Gram-positives, most well studied adhesins bind to proteinaceous partners. Nevertheless, for both bacterial groups the binding of bacterial adhesins to eukaryotic carbohydrate receptors is essential for establishing colonization or infection. The characterization of this interaction down to the submolecular level provides the basis for strategies to interfere with this early step of infection which should lead to the prevention of subsequent disease. However, this goal will not be achieved easily because bacterial adherence is not a monocausal event but rather mediated by a variety of adhesins.

Keywords Adhesins, (A)fimbrial, Pili, Receptors, Anti-adhesion strategies

Contents

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1 Introduction

Microorganisms, such as the bacteria which colonize the gut, are known to be beneficial for host fitness (commensal). However, under certain conditions some of them acquire virulence-associated genes by horizontal gene transfer, become pathogenic and cause disease [1, 2]. In order to cause disease, bacteria must gain access into the host body and must be able to colonize the appropriate niche [3]. To colonize host mucosal surfaces, microorganisms have developed the capacity to produce surface molecules which enable them to adhere to the host cells and tissues [4]. Adhesion protects bacteria against natural cleaning mechanisms of the host, such as peristalsis of the intestine, coughing, airflow in the respiratory tract or the flow of urine through the urinary tract, and provides better access to the sources of nutrition [5]. It also facilitates the delivery of toxic agents and invasion of the bacteria into host tissues and cells. Many of the adhesive surface molecules expressed by bacteria are carbohydrate-binding proteins (lectins) called adhesins. Adhesins bind to the complementary carbohydrate receptors of the host cell membrane glycoproteins or glycolipids and determine the species specificity of pathogens [6, 7] and their preference for certain host cell or tissue types (tropism). For example, 987P and K99 fimbriated strains of enterotoxigenic *E. coli* can cause diarrhea in piglets, but not in adult pigs or humans [8–10]. Alternatively, host cells also express various cell surface molecules (lectins) that bind carbohydrates present on the surface of other cells or bacteria. The specific interactions between adhesins (lectins) and glycans are also crucial in cell–cell and cell–matrix interactions, signaling, differentiation and development [11]. Several other types of infectious agents such as viruses [12], fungi, e.g. *Candida albicans* [13] and amoebal parasite, e.g. *Acanthamoeba* [14] also require carbohydrate-mediated adherence for infection.

2 Bacterial Adhesins and Carbohydrate Host Receptors

Bacteria are capable to avoid successfully the mechanical defences of the host by specifically adhering to host cell surface carbohydrates linked to glycoproteins or to glycolipids. Adhesion is mediated either by hair-like hetero- or homopolymeric bacterial surface appendages called fimbriae, fibrils or pili, or by nonfimbrial adhesins. The adhesive subunit of heteropolymeric adhesins is in most cases located at the tip of these hair like structures [15, 16]. The expression of these adhesion determinants is not constitutive but dependent on, e.g. growth conditions. Thus, the bacteria may shift periodically back and forth between a fimbriated and nonfimbriated state. This phenomenon is known as phase variation. Moreover, an individual bacterium is capable to express either various types of fimbrial adhesins or different alleles of the same adhesin with different carbohydrate receptor specificities or affinities. For example, uropathogenic *E. coli* strain 536 (O6:K15:H31] has been shown to produce various types of fimbrial adhesins, including type 1,

Table 1 Carbohydrates as attachment sites for bacterial pathogens on animal tissues (Adapted from [19])

Organism	Target tissue	Carbohydrate	Structure
E. coli Type 1	Urinary	Manα3Manα6Man	GP
E. coli P	Urinary	Galα4Gal	GL
E. coli S	Neural	NeuAc (α2-3)Galβ3GalNAc	GL
E. coli CFA/1	Intestinal	NeuAc (α2-8)	GP
E. coli F1C	Urinary	GalNAcβ4Galβ	GL
E. coli F17	Urinary	GlcNAc	GP
E. coli K1	Endothelial	GlcNAcβ4GlcNAc	GP
E. coli K99	Intestinal	NeuAc(α2-3)Galβ4Glc	GL
C. jejuni	Intestinal	Fucα2GalβGlcNAc	GP
H. pylori	Stomach	NeuAc(α2-3)Galβ4GlcNAc	GP
		Fucα2Galβ3(Fucα4)Gal	GP
K. pneumoniae	Respiratory	Man	GP
N. gonorrhoea	Genital	Galβ4Glc(NAc)	GL
N. meningitidis	Respiratory	[NeuAc(α2–3)] Galβ4GlcNAcβ3Galβ4GlcNAc	GL
P. aeruginosa	Respiratory	L-Fuc	GP
	Respiratory	Galβ3Glc(NAc)β3Galβ4Glc	GL
S. typhimurium	Intestinal	Man	GP
S. pneumoniae	Respiratory	NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc	GL
S. suis	Respiratory	Galα4Galβ4Glc	GL

GP = glycoprotein, GL = glycolipids

P-related (Prf) and S-fimbriae [17]. P fimbriae of uropathogenic *E. coli* strains present three known classes of adhesin variants namely PapGI, PapGII, and PapGIII with different binding properties [18].

Although various adhesins of pathogenic bacteria have been identified (Table 1), only a small number of bacterial adhesins together with their host carbohydrate receptors has been well studied at the molecular level. For example, two fimbrial adhesins namely FimH and PapG of uropathogenic *E. coli* (UPEC) strains and adhesin F17-G of enterotoxigenic *E. coli* [20] have been crystallized and their three-dimensional structures have been elucidated [15, 21, 22]. Several other frequently expressed fimbrial adhesins of UPEC such as members of the S-fimbrial family which includes among others S-fimbria subtypes SfaI and SfaII and F1C fimbriae have also been well characterized and their host receptors were identified [23].

3 Type 1 Pili

FimH is the mannose-specific adhesive subunit of type 1 fimbriae and located at the tip of this organelle [24]. However, its presence in the fimbrial shaft has also been described which only becomes active upon fragmentation of the fimbrial shaft which in turn leads to the exposure of the binding site [25]. In addition to UPEC strains, type 1 fimbriae are expressed by a large number of other *E. coli* isolates,

e.g. from intestinal infections [4, 18]. They are also produced by other enterobacterial species such as *K. pneumoniae, Salmonella typhimurium* and *S. enteritidis*. On the basis of different affinities towards its natural receptor mannose or methyl α-mannoside, phenotypes of *E. coli* type 1 can be functionally subdivided into either high mannose-binding (MIH) or low mannose-binding (MIL) phenotypes [26]. Most isolates from the large intestine of healthy humans (around 80%) express a distinct MIL phenotype, whereas most isolates from urinary tract infections (more than 70%) express MIH variants [19]. Minor sequence variation in the FimH protein from different clinical isolates has been shown to be responsible for this altered carbohydrate-binding profile of the fimbriae [26].

Although the monosaccharide mannose linked to glycoproteins is the receptor for type 1 fimbriae of *E. coli* these fimbriae show a 40-fold higher affinity for oligosaccharides such as Manα3Manβ4GlcNAc or Manα6(Manα3) Manα6(Manα3)Man which are constituents of cell surface glycoproteins [27]. Similarly, the FimH subunits of *E. coli* and *K. pneumoniae* while being 88% homologous differ in their relative affinity for Manα3Manβ4GlcNAc and *p*-nitrophenyl α-mannosides [28, 29]. In addition, type 1 fimbriae of *Salmonella* species do not exhibit an enhanced affinity for mannose with hydrophobic substituents or for Manα3Manβ4GlcNAc [19].

X-ray analysis of the three-dimensional structure of FimC (this is the chaperon)–FimH (the adhesin) in complex with C-HEGA an analog of α-mannoside [15], with α-mannoside [30] and with α-butyl-mannoside [31] showed that a large portion of mannose surface is buried in the negatively charged binding cavity of the FimH lectin and it is involved in hydrogen bonding with combining site residues. The binding cavity is surrounded by a hydrophobic ridge which is responsible for the enhanced binding of hydrophobic mannose conjugates [30, 31]. It could be that the binding site of *Salmonella* FimH is smaller than that of *E. coli* and *K. pneumoniae* and is devoid of the hydrophobic ridge and hence does not exhibit an enhanced affinity for mannose with hydrophobic substituents or for Manα3Manβ4GlcNAc. Furthermore, recent studies involving the type 1 fimbriae of *E. coli, K. pneumoniae* and *Salmonella* showed that the fimbrial shaft imposes conformational constraints and thus modulates the binding specificity of fimbriae [32]. FimH adhesin alone is capable of binding to a broad range of mannosides. This observation may be responsible for the tissue tropism observed for the various type 1 fimbriated pathogens [33].

4 P Fimbriae

P-fimbriated uropathogenic *E. coli* strains are linked to the more serious urinary tract infections. The adhesin PapG of P-fimbriated uropathogenic *E. coli* strains in contrast to the adhesin FimH of type 1 fimbriae is specific for glycosphingolipids of the globo series namely globotriaosylceramide (GbO3), globotetraosylceramide (GbO4) and Forssman antigen (GbO5). These bacteria bind to the galabiose Galα4Gal disaccharide when it is present either at the nonreducing position or at an internal one of such glycolipids. There are three different alleles of PapG

(PapGI, II and III) which bind with different specificities to different receptor isotypes. PapG1 binds favourably to the human urinary bladder whereas PapGII and GIII favour colonization of the human and dog kidney, respectively [18, 21]. The recently determined crystal structure of PapGII adhesin bound to a galabiose-containing ligand not only reveals the molecular basis for the tropism conferred by the PapGII adhesin but also suggests a plausible model that accounts for the tropism conferred by PapGII [21].

5 S Fimbriae

The members of the S-fimbrial family differ in their receptor specificities. S-fimbrial adhesin Sfa-S recognize α-sialyl-2-3-β-lactose-containing receptors and are predominantly expressed by strains which cause sepsis and meningitis but also by urinary tract infection (UTI) isolates [34, 35]. Their binding to brain glycolipids has also been demonstrated [36]. In contrast, the F1C-fimbrial adhesin binds with a high affinity to β-GalNAc-1, 4-β-Gal containing glycolipid and is preferentially expressed by urinary tract infection (UTI) isolates [23].

6 Other Adhesins of Gram-Negative Bacteria

Furthermore, bacteria specifically binding to other carbohydrate receptors have also been described (Table 1). For example, *Neisseria gonorrhoeae*, a genital pathogen binds to *N*-acetyllactosamine (Galβ4GlcNAc, LacNAc). *Helicobacter pylori*, the causative agent of peptic ulcer, expresses several lectins with distinct binding specificities [37, 38]. Some of them recognize NeuAc(α2–3)Galβ4Glc(Sia3Lac) and its *N*-acetylglucosamine analog (Sia3LacNAc) while others are specific for the Le^b determinant Fucα2Galβ3[Fucα4]GlcNAc. The adhesins of enterotoxigenic *E. coli* K99 and F17G of F17 fimbriae bind to glycolipids containing *N*-glycolylneuraminic acid (NeuGc) and *N*-acetylglucosamine receptors on the microvilli of the intestine of ruminants, respectively. The high resolution crystal structure of the lectin domain of F17-G in complex with *N*-acetylglucosamine revealed that the monosaccharide is bound on the side of the ellipsoid-shaped protein in a site conserved in all natural variations of F17-G [20].

7 Adhesins of Gram-Positive Bacteria

Similar to the Gram-negative bacteria, Gram-positive bacteria also express a great variety of factors that enable these micro-organisms to adhere to host cell structures on cell surfaces. Most of these are proteins binding to proteinacous partners.

Prominent examples are matrix-binding proteins called MSCRAMMS (microbial surface components recognizing adhesive matrix molecules). These proteins bind to extracellular matrix proteins such as fibronectin, collagen, vitronectin, laminin and others [39]. Many of these adhesins play an important role in pathogenesis of Gram-positive pathogens. In contrast to the extensive work on protein–protein interactions between adhesins of Gram-positive bacteria and host surfaces, less is known about binding of Gram-positive bacterial adhesins to carbohydrate structures.

8 Adhesins of Streptococcus

One of the best studied carbohydrate binding adhesins of Gram-positive bacteria is expressed by *Streptococcus suis* strains. This bacterium causes meningitis, septicemia, and pneumonia in pigs and also in humans [40]. *S. suis* recognizes galactose containing glycolipids with specificity to the disaccharide Galα1–4Gal sequences present, e.g. in P blood group antigens. Moreover, experimental binding to pharyngeal tissue of pigs could be inhibited by trihexosylceramide (GbO3) indicating that the glycolipid may function as a receptor for galactose-binding strains of *S. suis* in pig pharyngeal epithelium [41, 42]. Two variant adhesins, inhibitable by galactose and *N*-acetylgalactosamine (type PN) or galactose only (type Po) have been described. Both prefer binding of the disaccharide in a terminal position [43, 44]. Surprisingly, on *S. suis*, no fimbriae have been observed, so it is likely that the adhesins of *S. suis* are afimbrial ones.

Streptococcus gordonii and related species of the viridans group are major components of the human oral microflora. These organisms play prominent roles as pioneer colonizers in the development of dental plaque [45]. In addition to colonizing surfaces in the human oral cavity, viridans streptococci cause infective endocarditis. The sialic acid-binding adhesins Hsa and GspB of *S. gordonii* have been shown to bind sialoglycoproteins on the platelet surface. The sialoglycoprotein receptors were identified as platelet glycoprotein Ib α (GPIbα) and glycoprotein IIb (GPIIb) [46, 47]. GspB and Hsa are serine-rich surface glycoproteins that consist of an *N*-terminal putative signal peptide, a short basic amino acid-rich region, a longer serine-rich region and a *C*-terminal cell wall anchoring domain. Binding assays with fusion proteins of the basic amino acid-rich region to a panel of oligosaccharides revealed that the basic amino acid-rich region BR of Hsa can bind both α(2-3) sialyllactosamine [NeuAcα(2-3)Galβ(1-4)GlcNAc] and sialyl-T antigen [NeuAcα(2-3)Galβ(1-3)GalNAc], whereas the BR of GspB only binds sialyl-T antigen [47]. GspB facilitates binding to carbohydrates bearing sialic acid in either α[2–3) or α(2–6) linkages, with a slight preference for α(2–3) linkages [46].

Another member of the viridans streptococci, *S. mutans*, plays an important role in the formation of dental caries and binds tightly to tooth surfaces. The bacterium is capable of binding salivary components such as salivary agglutinin. It has been shown that *S. mutans* can bind to Lewis antigen carbohydrate epitopes containing fucose residues that are present on salivary agglutinin [48].

Streptococcus pneumoniae is a major cause of pneumonia, otitis media, meningitis, and septicemia resulting in the death of more than 1 million people every year. Various virulence determinants of pneumococci have been described including the highly variable polysaccharide capsule, pneumolysin toxin, and carbohydrate binding proteins. For example, binding of *S. pneumoniae* to respiratory cells can be inhibited by sialyted oligosaccharides containing the pentasaccharide NeuAc(α-3) Galβ4GlcNAcβ3Galβ4Glc, as well as with the corresponding internal tetra- and trisaccharides Galβ4GlcNAcβ3Galβ4Glc and GlcNAcβ3Galβ4Glc, respectively [49]. Interestingly, sialylated oligosaccharides and glycoproteins at concentrations in the millimolar range block adherence of *S. pneumoniae* to epithelial cells of the upper respiratory tract, thereby reducing the load of colonizing organisms and diminishing the risk of infection. Moreover, in a rabbit model of pneumonia, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc protected against infection by *S. pneumoniae*, and in an infant rat model, also reduced nasopharyngeal colonization by *S. pneumoniae* [50]. Thus, the use of orally or nasally administered oligosaccharides as prophylactic and/or therapeutic agents to promote clearance of *S. pneumoniae* from the nasopharyngeal mucosa may be applied as a strategy of reducing the risk of developing otitis media, meningitis or pneumonia.

Older reports deal with the binding of Gram-positive and Gram-negative pathogens involved in lung infections of cystic fibrosis patients and pneumonia such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, Haemophilus influenzae, Streptococcus pneumoniae*, and *Klebsiella pneumoniae.* These pathogens bind specifically to fucosylasialo-GM1 (Fucα1-2Galβ1-3GalNAcβ1-4Galβ1-1Cer), asialo-GM1 (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer), and asialo-GM2 (GalNAcβ1-4Galβ1-4Glcβ1-1Cer) present on the surface of lung tissues [51]. The investigated pathogens do not bind to galactosylceramide, glucosylceramide, lactosylceramide, trihexosylceramide, globoside, paragloboside, or glycosphingolipids including the gangliosides GM1, GM2, GM3, GD1a, GD1b, GT1b, and Cad. The authors concluded that the tested bacteria require at least terminal or internal asialo GalNAcβ1–4Gal sequences. This observation may be of biological relevance as substantial amounts of asialo-GM1 occur in human lung tissue [51]. However, additional work has to be done to characterize the significance of these binding properties for the pathogenesis of lung infections. Furthermore, it is not known which adhesive proteins are involved in the described Gram-positive pathogen–carbohydrate interaction.

9 Adhesins of *Staphylococcus*

Gram-positive opportunistic pathogens such as *Staphylococcus epidermidis, S. aureus, E. faecalis and E. faecium* have the capacity to form biofilms on foreign medical devices such as catheters, and surgical implants [52, 53]. These microorganisms are normal inhabitants of healthy humans, in recent years, however, the bacteria emerged as a common cause of nosocomial infections [54]. Interestingly,

the capacity of staphylococci to form thick multilayered biofilms on polymer and metal surfaces is associated mostly with the synthesis of an extracellular polysaccharide the so-called polysaccharide intercellular adhesin (PIA) [55]. The enzymes involved in PIA production are encoded by the *icaADBC* operon [56]. The *icaADBC* genes are more prevalent in *S. epidermidis* strains from device-associated infections than in commensal isolates suggesting an important role of polysaccharide interactions in pathogenic *S. epidermidis* strains [57]. Biofilm-positive bacteria are less susceptible against the action of antibiotics and are shielded from the host immune system. The polysaccharide PIA is a linear β-1,6-linked glucosaminoglycan that is essential for intercellular adhesin and biofilm accumulation of many *S. epidermidis* clinical isolates [58]. That PIA mediates also adherence to host structures is not known, however, it is tempting to speculate about this.

10 Implication in Antimicrobial Activity

The examples discussed above impressively show the diversity and importance of interaction between bacterial adhesion determinants and carbohydrate-containing receptor molecules. Pathogenic bacteria use binding to carbohydrates in different disease stages. However, many questions remain to be answered to unravel the full spectrum of bacteria–host interactions via carbohydrate binding.

Nevertheless, anti-adhesion strategies based on interference of pathogen–host interaction by carbohydrates represents an attractive approach in antimicrobial therapy. This concept may especially be promising as antimicrobial resistance is becoming a global health problem. Specific inhibition of adhesion properties should apply less selective pressure on pathogens therefore avoiding rapid resistance development. However, microorganisms use multiple adhesion factors, thus several structures have to be targeted for efficient inhibition of adhesion to host tissues. Moreover, many inhibitors of protein–carbohydrate interaction show only relatively low affinity, this is true at least for monovalent inhibitors. The development of improved multivalent ligands with higher affinity is one principle to increase the efficacy of anti-adhesion therapy. For in vivo application the size and polarity of such ligands may be problematic, therefore topical applications such as in the intestine or lung may have the highest potential for therapeutical use.

11 Final Reflections

Fimbrial adhesins may be visualized by electron microscopy. However, the processing for analysis by electron microscopy might cause alterations of the specimens, e.g. by dehydration. This might result in the collapse of certain fimbriae. Therefore, despite no fimbriae are visible by electron microscopy the “afimbrial” adhesins of the Dr family, e.g. AfaE of diarrheagenic and uropathogenic *E. coli* in fact assemble

into flexible fibers with the AfaD at the tip, which is an invasin [59]. This example also shows that fimbriae are often multi purpose tools. Obviously, they not only mediate adherence but function also as invasins for uptake into host cells and activate host proteases in order to cross host barriers, i.e. the blood-brain-barrier [60]. The later might be achieved by immobilization of plasminogen and tissue-type plasminogen activator on S and type 1 fimbriae [61].

Other adhesins of *E. coli* as antigen 43, AIDA-I, TibA and intimin of enteropathogenic and enterohemorrhagic *E. coli* are true afimbrial adhesins i.e. they are integral outer membrane proteins. However, also intimin seems to be involved in invasion of host cells [62, 63]. Intimin, which is actually a whole family of adhesins, is the only example of an adhesion that uses a protein (Tir: translocated intimin receptor) in the host cell membrane, that is a bacterial protein inserted into the host by the bacterial type 3 protein secretion system [64].

At least some adhesins bind to more than one receptor. Intimin might not only bind to Tir but also to β1 containing integrins as well as to nucleolin [65]. The Dr adhesin recognizes not just the short consensus repeat domain as all members of the Dr family of adhesins but also type IV collagen, which is a critical step in renal persistence [66].

Besides fimbrial and afimbrial adhesins, there are other surface structures that act as adhesins. For example, in *Pseudomonas aeruginosa* the major flagellar protein flagellin was identified as the adhesin responsible for binding to Muc 1 mucin [67].

In summary, bacterial adherence is not just mediated by one bacterial surface structure interacting with one host receptor. Rather, adherence is a process involving several bacterial and host cell components which interact in a temporal and special order. Interference with these processes in order to block infection will not be an easy task [68].

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The Multiple Carbohydrate Binding Specificities of *Helicobacter pylori*

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Abstract Persistent colonization of the human stomach by *Helicobacter pylori* is a risk factor for the development of peptic ulcer disease and gastric cancer. Adhesion of microbes to the target tissue is an important determinant for successful initiation, establishment and maintenance of infection, and a variety of different candidate carbohydrate receptors for *H. pylori* have been identified. Here the different the binding specifities, and their potential role in adhesion to human gastric epithelium are described. Finally, recent findings on the roles of sialic acid binding SabA adhesin in interactions with human neutrophils and erythrocytes are discussed.

Keywords Adhesin, Carbohydrate binding, Glycoconjugate receptor, *Helicobacter pylori*, Microbial adhesion

Contents

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Abbreviations

BabA	blood group antigen binding adhesin
HpaA	*H. pylori* adhesin A
HP-NAP	neutrophil-activating protein of *pylori*
SabA	sialic acid binding adhesin.

The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: Eur J Biochem 257:293, 1998).

1 Introduction

Half of the world's population is estimated to be colonized with the gastric pathogen *Helicobacter pylori.* However, although infection with *H. pylori* and the associated chronic gastritis are common, only a small fraction of those infected develops any further consequences such as peptic ulcer or gastric adenocarcinoma [1]. Thus, the infection is by itself not sufficient to cause disease and, since the discovery of *H. pylori* by Warren and Marshall in 1983 [2], this bacterium has been subjected to intense studies aimed at characterizing host and bacterial factors associated with pathological sequelae of the infection. The most well-recognized virulence factors identified to date are the urease, the flagellae, the cytotoxin-associated pathogenicity island (*cag*-PAI), the vacuolating cytotoxin (VacA), and adhesins (reviewed in [3]).

Bacterial adherence to mucosal cells of the target tissue is an important virulence trait of pathogenic bacteria. The majority of known microbial attachment sites on host cells and tissues are glycoconjugates. A number of different approaches have been employed for elucidation of carbohydrate receptors for *H. pylori*, such as, e.g., hemagglutination and hemagglutination-inhibition [4], binding to glycosphingolipids separated on thin-layer plates [5, 6], or in situ analysis of the binding of *H. pylori* to human gastric surface mucosal cells [7]. Thereby, at least nine separate carbohydrate binding specificities have been identified, as summarized in Table 1.

It should, however, be noted that despite this multitude of potential carbohydrate receptors, only two *H. pylori* adhesins have been identified to date, i.e., the Le^b-binding adhesin BabA [16], and the sialic acid-binding adhesin SabA [8]. An additional *H. pylori* carbohydrate binding protein is the soluble neutrophil-activating protein

Table 1 Potential *H. pylori* carbohydrate receptors

1. Sialic acid-terminated glycoconjugates	[4, 8, 9]
2. Sulfate-containing carbohydrates	[5, 10]
3. Ganglioseries glycosphingolipids	[6, 11]
4. Fucosylated blood group antigens	[7]
5. NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAc-terminated glycoconjugates	[12]
6. Lactosylceramide	[6]
7. Galactosylceramide/glucosylceramide	[13]
8. Lactotetraosylceramide	[14]
9. Neolacto-glycolipids	[15]

(HP-NAP), which interacts with NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ-terminated glycoconjugates [12].

In the following the various *H. pylori* carbohydrate binding specificities, and identified bacterial adhesins, are first described, followed by a discussion of their role(s) in adhesion of the bacteria to the two target tissues: human gastric epithelium and human neutrophil granulocytes. Finally, recent findings on the roles of sialic acid binding in interactions of *H. pylori* with human neutrophils and erythrocytes are highlighted.

2 Fucosylated Blood Group Antigens

The recognition of the Le^b blood group antigen (Fucα2Galβ3(Fucα4)GlcNAc) by certain strains of *H. pylori* was reported over a decade ago [7]. A combination of in situ analysis of the binding of *H. pylori* to human gastric surface mucosal cells, blocking of binding by pre-incubation of bacteria with neoglycoconjugates, and binding of *H. pylori* to glycoconjugates immobilized on protein blots, was used to elucidate this binding specificity. Subsequently, the cognate *H. pylori* Le^b-binding adhesin BabA (blood group antigen binding adhesin), a 78-kDa protein belonging to the hop family of *H. pylori* outer membrane proteins, was identified by the retagging technique based on its affinity for Le^b [16]. Two genes, *babA1* and *babA2*, both potentially encoding the BabA adhesin were identified. Only the *babA2* gene gives rise to a functional adhesin, since in *babA1* the initiation codon is missing. However, several Le^b non-binding strains possessing silent *babA* gene sequences may become active by recombination into the partially homologous *babB* locus forming chimeric *babB/A* genes [17]. *H. pylori* strains expressing BabA together with the vacuolating cytotoxin VacA and the cytotoxin associated antigen CagA (triple positive strains) are highly associated with severe gastrointestinal diseases, as peptic ulcer or gastric adenocarcinoma [18, 19].

The initial studies were performed using the South American *H. pylori* strain P466, which binds to Le^b (Fucα2Galβ3(Fucα4)GlcNAc-) and H type 1 (Fucα2Galβ3GlcNAc-) blood group determinants, related to the blood group O phenotype [7]. However, investigation of the binding characteristics of a large number of *H. pylori* strains of different geographical origins revealed a different pattern [20]. Of the strains from the South American Amerindian population, 40% bind only to Le^b/H type 1 blood group determinants, which correlates with the unusual predominance of the blood group O phenotype among the Amerindians. In contrast, 95% of the *H. pylori* strains from Europe, Asia and Alaska also recognize the ALe^b (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAc-) and BLe^b (Galα3(Fucα2)Galβ3(Fucα4)GlcNAc-) blood group determinants (exemplified in Table 2), demonstrating an adaption of the BabA adhesin to the receptor availability in the local host population.

The BabA adhesins are thus divided into "specialist" BabA adhesins which preferentially bind the Le^b determinant, and "generalist" BabA which also bind the ALe^b and BLe^b determinants, i.e., the "generalist" adhesins tolerate the substitution by an α-linked GalNAc or Gal at the 3-position of the Gal, indicating a variant architecture of the carbohydrate binding site of the BabA adhesin.

Table 2 Comparison of binding of *H. pylori* strains CCUG 17875 and P466 to glycosphingolpids on thin-layer chomatograms

No. trivial name	Glycosphingolipid structure	CCUG 17875	P466
1. H5 type 1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+[a]	+
2. H5 type 2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
3. Le[a]-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	–	–
4. X-5	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	–	–
5. Le[b]-6	Fucα2Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	+++	+++
6. Y-6	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4G	–	–
7. B6 type 1	Galα3(Fucα2) Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	–
8. B6 type 2	Galα3(Fucα2) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
9. A6 type 1	GalNAcα3(Fucα2) Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	–
10. B7 type 1	Galα3(Fucα2)Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	+++	–
11. B7 type 2	Galα3(Fucα2)Galβ4(Fucα3) GlcNAcβ3Galβ4Glcβ1Cer	–	–
12. A7 type 1	GalNAcα3(Fucα2)Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	+++	–

[a]Binding is defined as follows: + denotes a weak and +++ a significant darkening on the autoradiogram when 4 μg is applied on the thin-layer plate, while – denotes no binding

3 Lactosylceramide and Ganglioseries Glycosphingolipids

Many *H. pylori* strains, like several other bacteria – both pathogens and commensals [21], also bind to gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) [11], with a concomitant binding to lactosylceramide (Galβ4Glcβ1Cer), isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer) and gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer) [6].

There are conflicting suggestions concerning which part of the gangliotriaosylceramide/gangliotetraosylceramide structures that is recognized by the lactosylceramide-binding bacteria. One alternative is that the lactose saccharide (Galβ4Glcβ) is primarily recognized by the adhesin, and that the extensions specifying the ganglio-series (i.e., the β4-linked GalNAc and Galβ3GalNAc) are tolerated for steric reasons, while other extensions make the lactose epitope inaccessible [21]. The alternate suggestion is that the binding to lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide represents two separate binding specificities [22].

The gangliotriaosylceramide/gangliotetraosylceramide binding of *H. pylori* was abolished by conversion of the acetamido group of the *N*-acetylgalactosamine to an amine [6]. These results are most compatible with the interpretation that lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide represent two separate binding specificities. However, binding of *H. pylori* to lactosylceramide is

frequently accompanied by binding to gangliotriaosylceramide and gangliotetraosylceramide, suggesting that the factors controlling the expression of the lactosylceramide-binding adhesin are similar or identical to those which regulate the expression of the gangliotriaosylceramide/gangliotetraosylceramide-binding adhesin.

Lingwood et al. have reported that the gangliotriaosylceramide/gangliotetraosylceramide binding of *H. pylori* also involves a concomitant binding to phosphatidylethanolamine [11], and this glycerolipid was used for affinity isolation of a 63-kDa protein from *H. pylori* with phosphatidylethanolamine and gangliotriaosylceramide/gangliotetraosylceramide binding properties [23]. However, subsequent studies identified this protein as a catalase [24], and thus, most likely, not an adhesin.

Interestingly, by structure similarity searches Fantini et al. recently identified a glycosphingolipid-binding motif in HpaA (*H. pylori* adhesin A; see below) [25]. A synthetic peptide corresponding to this proposed glycosphingolipid-binding motif interacted with lactosylceramide but not with globotriaosylceramide, in line with the glycosphingolipid binding pattern of the whole bacterial cells [6]. The identification of this glycosphingolipid binding motif may allow further dissection of the lactosylceramide binding properties of *H. pylori* and other lactosylceramide-binding bacteria.

The predominant diglycosylceramide of the human gastric epithelium was recently identified as galabiaosylceramide (Galα4Galβ1Cer), a glycosphingolipid not recognized by *H. pylori* bacterial cells [26]. Still, a distinct binding of *H. pylori* in the diglycosylceramide region in one out of seven samples was found upon assaying binding to non-acid glycosphingolipids of human gastric epithelium [6]. By mass spectrometry of the binding-active glycosphingolipid sample dihexosylceramides both sphingosine and phytosphingosine and both hydroxy and non-hydroxy fatty acids were identified. This *H. pylori*-binding non-acid glycosphingolipid fraction most likely contained lactosylceramide, indicating that there are individual differences in the composition of diglycosylceramides in the human gastric epithelium. Galabiosylceramide is present in all individuals, but the relative amount of lactosylceramide varies, and only a few individuals have enough lactosylceramide to allow detection of *H. pylori* binding in the chromatogram binding assay.

4 Sulfated Carbohydrates

In the first report of *H. pylori* glycosphingolipid binding, Saitoh et al. demonstrated binding of the bacteria to the two major acid glycosphingolipids of human stomach, i.e., the GM3 ganglioside (NeuAcα3Galβ4Glcβ1Cer) and sulfatide (SO_3–3Galβ1Cer), by binding of *H. pylori* to glycosphingolipids separated on thin-layer chromatograms [5]. However, the GM3 binding was later in refuted in a study using KATO III cells, where only sulfatide binding was obtained [27]. The heat-shock protein Hsp 70, which can be induced at low pH, has been proposed to be the adhesin involved in the binding of *H. pylori* to sulfatide [28, 29]. The neutrophil-activating protein HP-NAP

is to some extent associated with the bacterial cell surface [10], and may also be involved in binding of *H. pylori* to sulfated carbohydrates, as described below.

In addition, a majority of *H. pylori* strains bind to heparan sulfate with high affinity [30], and a battery of heparan sulfate binding bacterial proteins have been described [14, 31].

5 Lactotetraosylceramide

An *H. pylori*-binding non-acid glycosphingolipid of human meconium was initially isolated, and characterized by mass spectrometry and proton NMR as lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) [32]. Subsequently, lactotetraosylceramide was also identified in human gastric epithelial cells, in one out of seven samples. Enzymatic removal of the terminal galactose of lactotetraosylceramide abolished the binding, as did hydrazinolysis of the acetamido group of the *N*-acetylglucosamine (Table 3), indicating that the *H. pylori* binding epitope is the Galβ3GlcNAcβ sequence.

The Le^b determinant (Fucα2Galβ3(Fucα4)GlcNAcβ) is based on the type 1 disaccharide unit, which is the terminal part of lactotetraosylceramide. However, *H. pylori* strains devoid of Le^b-binding BabA (e.g., the 26695 strain and the MO19 strain) recognized lactotetraosylceramide, and inactivation of the *bab*A gene had no influence on lactotetraosylceramide binding (Fig. 1). Thus, the binding of *H. pylori* to the Le^b determinant and to lactotetraosylceramide are two separate binding specificities.

The blood group status of the individual with *H. pylori*-binding gastric lactotetraosylceramide was ALe(a+b−)non-secretor, in agreement with the presence of unsubstituted lactotetraosylceramide in this individual. This non-secretor status is interesting in view of the increased prevalence of duodenal ulcer among non-secretors [33]. Non-secretion is not associated with increased susceptibility to infection with *H. pylori* [13]. However, a speculative theory is that the secretor status determines the outcome of the colonization, i.e., that the increased liability of non-secretors to develop peptic ulcer disease is due to the presence of the *H. pylori*-binding lactotetraosylceramide on the gastric epithelium of these individuals.

Table 3 Comparison of lactotetraosylceramide-related binding preferences of *H. pylori*

No. trivial name	Glycosphingolipid structure	Binding
1. Lactotri	GlcNAcβ3Galβ4Glcβ1Cer	–[a]
2. Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+
3.	$Gal\beta3GlcNH_2\beta3Gal\beta4Glc\beta1Cer$	–
4. Le^a-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	–
5.	Galα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–
6.	NeuGcα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–
7.	NeuAcα6Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–

[a]Binding is defined as follows: + denotes a binding when 2 μg of the glycosphingolipid is applied on the thin-layer chromatogram, while – denotes no binding even at 4 μg

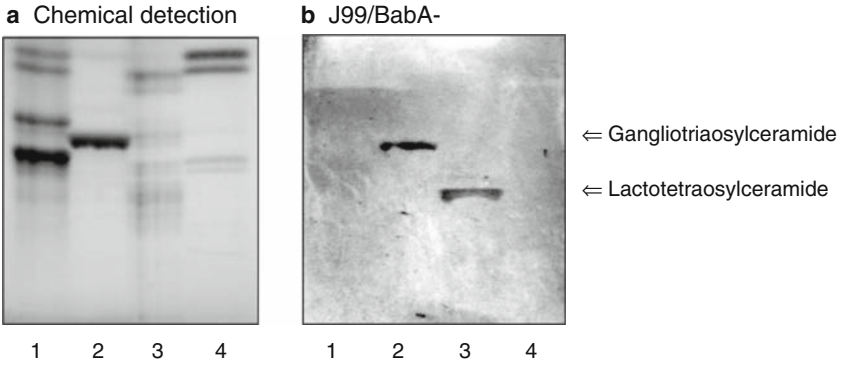

Fig. 1 Binding of a mutant *Helicobacter pylori* strain with deletion of the BabA adhesin (J99/BabA-) to glycosphingolipids on thin-layer chromatograms. Thin-layer chromatogram with separated glycosphingolipids after chemical detection with anisaldehyde (**a**), and autoradiogram after binding of ^{35}S-labeled *H. pylori* strain J99/BabA- (**b**). The lanes were: Lane 1, non-acid glycosphingolipids of human blood group O erythrocytes, 40 μg; Lane 2, non-acid glycosphingolipids of guinea pig erythrocytes, 40 μg; Lane 3, non-acid glycosphingolipids of human meconium, 40 μg; Lane 4, non-acid glycosphingolipids of human neutrophils, 40 μg. The *arrows* denote the relative mobilities of gangliotriaosylceramide (present in lane 2) and lactotetraosylceramide (present in lane 3)

The lactotetraosylceramide binding property was present in the majority (88%) of *H. pylori* isolates tested, and thus a conserved property of this gastric pathogen, indicating that it is an important virulence factor. However, further definition of the role of the lactotetraosylceramide binding capacity in colonization and disease development must await the identification of the corresponding adhesin.

6 Galactosylceramide and Glucosylceramide

Abul-Milh et al. have reported that *H. pylori* bacterial cells bind to galactosylceramide with sphingosine and both hydroxy and non-hydroxy fatty acids [15]. They also found that when the chromatogram binding assay was performed under microaerobic conditions, intended to keep the bacteria viable, *H. pylori* bound to glucosylceramide with sphingosine and hydroxy fatty acids. The galactosylceramide/glucosylceramide adhesin(s) has not yet been identified.

7 Neolacto Glycosphingolipids

A majority of *H. pylori* strains also binds to the neolacto (Galβ4GlcNAcβ) core structure, with a preferential binding to carbohydrate chains with repetitive neolacto elements [34]. Enzymatic and chemical degradation of sialylneolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) showed that the preferred binding epitope in this context is the GlcNAcβ3Galβ4GlcNAcβ sequence.

The terminal β3-linked GlcNAc can be exchanged for GalNAcβ3, GalNAcα3 or Galα3 without loss of *H. pylori* binding. Calculated minimum energy conformations demonstrated topographical similarities in the spatial orientation of these trisaccharides, despite the different terminal substituents and anomeric configuration, which makes it reasonable that they may be accommodated within the same binding site. However, the adhesin mediating neolacto binding still awaits identification.

8 Sialic Acid-Terminated Glycoconjugates

Binding of *H. pylori* to sialylated glycoconjugates was initially identified by Evans et al. by hemagglutination studies [4], and a potential sialyllactose-binding protein, denoted HpaA (*H. pylori* adhesin A), was later characterized [35]. However, subsequent studies showed that HpaA is a lipoprotein, that deletion of the *hpaA* gene did not eliminate cell binding or hemagglutination, and that the HpaA protein was either located in the cytosol [36] or on the flagellar sheath [37]. The role of HpaA as a sialic acid binding adhesin has therefore been questioned.

A sialic acid dependent binding of *H. pylori* to the extracellular matrix protein laminin, mediated by a 25 kDa outer membrane protein, has also been described [38].

8.1 The Sialic Acid Binding Adhesin (SabA)

Following the identification of the BabA adhesin it was observed that deletion of *babA* did not abolish all *H. pylori* binding to human gastric tissue sections [8]. A further observation made was that this *babA* deletion strain bound to gangliosides of human neutrophils and human adenocarcinomas. Subsequently, a novel high affinity receptor for *H. pylori* was isolated from a human gall bladder adenocarcinoma and characterized as sialyl-dimeric-Le^x (NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer). The sialic acid binding adhesin of *H. pylori* (SabA) was identified by re-tagging and proteomics-based mass spectrometry techniques using sialyl-Le^x-BSA as probe. SabA, like BabA, belongs to the hop family of *H. pylori* outer membrane protein. The *sabA* gene is frequent among *H. pylori* isolates, but the expression of the SabA adhesin is highly subjected to phase-variation [8, 39].

The structural requirements for SabA-mediated *H. pylori* ganglioside binding was investigated by using a library of variant gangliosides [9]. The wild type SabA-expressing bacteria bound to *N*-acetyllactosamine-based gangliosides with terminal α3-linked NeuAc, while gangliosides of the ganglioseries, or *N*-acetyllactosamine-based gangliosides with terminal NeuAcα6, NeuGcα3, or NeuAcα8NeuAcα3 were not recognized (summarized in Table 4).

Table 4 Comparison of ganglioside binding of SabA-expressing *H. pylori* strains and *H. pylori* neutrophil-activating protein HP-NAP

No. trivial name	Structure	SabA+ *H. pylori*	HP-NAP
1. NeuAc-GM3	NeuAcα3Galβ4Glcβ1Cer	–[a]	–
2. NeuAc-GM1	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	–	–
3. NeuAc-GD1a	NeuAcα3Galβ3GalNAcβ4 (NeuAcα3)Galβ4Glcβ1Cer	–	–
4. NeuAc-GD1b	Galβ3GalNAcβ4(NeuAcα8 NeuAcα3)Galβ4Glcβ1Cer	–	–
5. NeuAc-GT1b	NeuAcα3Galβ3GalNAcβ4 (NeuAcα8NeuAcα3)Galβ4Glcβ1Cer	–	–
6. NeuAcα3SPG	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	–
7. NeuAcα6SPG	NeuAcα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
8. NeuGcα3SPG	NeuGcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
9. NeuAc-DPG	NeuAcα8NeuAcα3Galβ4Glc NAcβ3Galβ4Glcβ1Cer	–	–
10. NeuAcα3-Le[a]	NeuAcα3Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	–/+[b]	–
11. NeuAcα3-Le[x]	NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galβ4Glcβ1Cer	+	–
12. NeuAcα3-nLc$_6$	NeuAcα3Galβ4GlcNAcβ3Galβ 4GlcNAcβ3Galβ4Glcβ1Cer	+	+
13. NeuGcα3-nLc$_6$	NeuGcα3Galβ4GlcNAcβ3Galβ 4GlcNAcβ3Galβ4Glcβ1Cer	–	+
14. NeuAcα3-nLc$_8$	NeuAcα3Galβ4GlcNAcβ3Galβ4Glc NAcβ3Galβ4GlcNAcβ 3Galβ4Glcβ1Cer	+	+
15. NeuGcα3-nLc$_8$	NeuGcα3Galβ4GlcNAcβ 3Galβ4Glc NAcβ3Galβ4GlcNAcβ 3Galβ4Glcβ1Cer	–	+
16. VIM-2	NeuAcα3Galβ4GlcNAcβ 3Galβ4(Fucα3) GlcNAcβ3Galβ4Glcβ1Cer	+	–
17. NeuAcα3-dimer-Le[x]	NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	+	–
18. NeuAc-G-10	NeuAcα3Galβ4GlcNAcβ6(NeuAcα3 Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	–
19. NeuGc-G-10	NeuGcα3Galβ4GlcNAcβ 6(NeuGcα3Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
20.	NeuGcα3Galβ4GlcNAcβ 6(NeuGcα3Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
21. G9-B	Galα3(Fucα2)Galβ4GlcNAcβ 6(NeuAcα3Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	–

[a]Binding is defined as follows: + denotes a binding when 2 μg of the glycosphingolipid is applied on the thin-layer chromatogram, while – denotes no binding even at 4 μg

[b]Binding to NeuAcα3-Le[a] (No. 10) is variable, i.e., dependent on the *H. pylori* strain used [39, 63]

Several studies have shown that the minimal epitope for SabA-mediated *H. pylori* binding to gangliosides is NeuAcα3Gal [4, 40, 41]. However, comparative binding studies [39] showed that an increased binding affinity is obtained by:

1. Increased length of *N*-acetyllactosamine core chain: NeuAcα3nLc8 (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) >> NeuAcα3nLc6 (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) > NeuAcα3SPG (NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). This effect is most likely due to an improved accessibility of NeuAcα3Gal epitope when presented on a longer core chain.
2. Branches of the carbohydrate chain: NeuAc-G-10 (NeuAcα3Galβ4GlcNAcβ6(NeuAcα3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer) >> NeuAcα3nLc6), giving a divalent presentation of the binding epitope.
3. Fucose substitution of the *N*-acetyllactosamine core chain: NeuAcα3-dimeric-Le^x (NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) > VIM-2 (NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) >> NeuAcα3nLc6). The fucose residues of NeuAcα3-dimeric-Le^x and the VIM-2 ganglioside may either interact with the carbohydrate binding site of the SabA adhesin, or affect the conformation of the ganglioside providing an optimal presentation of the NeuAcα3Gal head group.

8.2 H. Pylori Neutrophil-Activating Protein (HP-NAP)

The *H. pylori* neutrophil-activating protein HP-NAP was first isolated from water extracts of bacterial cells, and by PCR analyses the gene for this protein was found in all strains analyzed [42]. The name neutrophil-activating derives from the capacity of HP-NAP to promote production of reactive oxygen radicals in human neutrophil granulocytes, and upregulate CD11b/CD18 on the neutrophil surface leading to increased adhesion of the neutrophils to endothelial cells. In addition, HP-NAP is an iron-binding protein [43], and has a role in protection of bacterial DNA against oxidative stress [44].

The potential carbohydrate recognition by HP-NAP was investigated by binding to glycosphingolipid fractions from various sources on thin-layer chromatograms [12]. A distinct binding to two compounds in the acid fraction of human neutrophil granulocytes (the target cells of HP-NAP) was thereby obtained, and the binding-active gangliosides were subsequently identified as NeuAcα3neolactohexaosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and NeuAcα 3neolactooctaosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) (Table 4).

Other HP-NAP binding compounds, not found in the human target white cells, were NeuGcα3neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), NeuGcα3neolactooctaosylceramide (NeuGcα3Gal

β4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), and sulfate-containing glycosphingolipids, as sulfatide (SO_3–3Galβ1Cer) and sulfated gangliotetraosylceramide (SO_3–3Galβ3GalNAcβ4Galβ4Glcβ1Cer). The interaction with sulfated glycosphingolipids was dependent on the sulfate group, since no binding to the corresponding non-sulfated compounds (galactosylceramide and gangliotetraosylceramide, respectively) occurred. Binding of HP-NAP to sulfated oligosaccharides, as SO_3–3Gal, SO_3–3GlcNAc and sulfated Lewis^a of human high-molecular weight salivary mucins, was subsequently demonstrated by Namavar et al. [45].

To delineate further the structural requirements for HP-NAP carbohydrate binding, NeuGcα3neolactohexaosylceramide has been chemically and enzymatically modified (Fig. 2) (ST, unpublished data). First, the terminal sialic acid is necessary for the interaction, since no binding to the de-sialylated compound was obtained (lane 6). Binding was also lost upon converting C(1) of NeuGc into an alcohol (lane 2). However, the C(1)-amide and C(1)-benzylamide were binding-active (lanes 3 and 4), indicating that the hydrogen binding capacity of a carboxyl or amide group at C(1) is necessary for binding to occur.

The partial de-N-acetylation (lane 5) gave rise to at least two band migrating below NeuGca3neolactohexaosylceramide, corresponding to variants with modified sialic acid and/or *N*-acetylglucosamines [46]. HP-NAP bound only to the remaining unmodified NeuGcα3neolactohexaosylceramide, indicating a role for the *N*-acetylglucosamine(s) in the interaction.

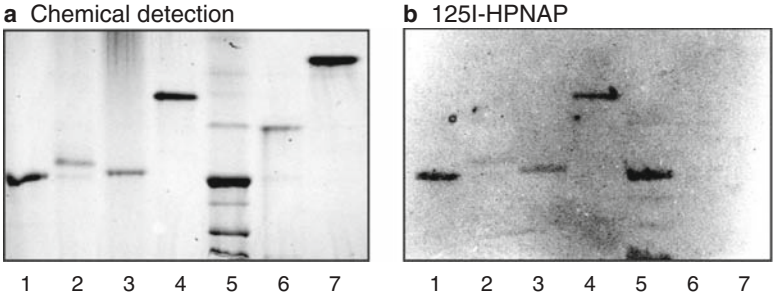

Fig. 2 Binding of ^{125}I-labeled neutrophil-activating protein from *Helicobacter pylori* (HP-NAP) to derivatized NeuGcα3neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) on thin-layer chromatograms. Glycosphingolipids detected with anisaldehyde (**a**) and autoradiogram obtained after binding of ^{125}I-labeled neutrophil-activating protein HP-NAP (**b**). Lanes: Lane 1, No. 1 NeuGcα3neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; Lane 2, alcohol produced from No. 1 (R–COOH → R–CH_2OH), 2 μg; Lane 3, primary amide produced from No. 1 (R–COOH → R–$CONH_2$), 2 μg; Lane 4, benzylamide produced from No. 1 (R–COOH → R–CONH–$CH_2C_6H_5$), 2 μg; Lane 5, products from de-N-acetylation of No. 1, 2 μg; Lane 6, de-sialylated No. 1 (Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; Lane 7, reference globoside (GalNAcβ3Galα4Galβ4Glcβ1Cer), 2 μg

HP-NAP is to date the only *H. pylori* carbohydrate binding protein that has been subjected to structural studies, although not in complex with carbohydrates. The neutrophil-activating protein forms a dodecameric structure arranged in a nearly spherical shell with a central iron-containing cavity [47]. The structure of HP-NAP has similarities to dodecameric bacterial ferritins (Dps-like family). However, unlike the Dps proteins, a large number of positively charged residues are found on the surface of HP-NAP.

9 Potential Roles of the Carbohydrate Binding Specificities in Adhesion to Target Cells

9.1 The Human Gastric Epithelium

H. pylori has a very narrow host and tissue range and has only been found in connection with gastric epithelium from humans and monkeys [48]. In the *H. pylori*-infected stomach the majority of the bacteria are found within the gastric mucus layer, while approximately 20% of the *H. pylori* population are found attached to the gastric epithelial cells.

While some of the putative *H. pylori* carbohydrate receptor sequences are found in a variety of human cells, some have hitherto not been identified in human tissues. An example of the latter group is isoglobotriaosylceramide, which has been characterized in, e.g., dogs, cats, and rats, but not in humans. Similarly, gangliotriaosylceramide or gangliotetraosylceramide have not been chemically identified in peripheral human tissues.

In contrast, galactosylceramide, glucosylceramide, sulfatide and lactosylceramide are almost ubiquitous glycosphingolipids, and expressed in a large variety of cells [49]. This broad distribution makes these glycosphingolipids unlikely as determinants of target tissue specificity. However, once the tissue specific binding has been established, a second-step binding to these relatively short glycosphingolipids may confer a more membrane-close attachment, and lead to increased concentrations of toxins and other effector molecules at the cell membrane.

Neolacto-containing glycosphingolipids are also found in several human tissues, such as erythrocytes [49], granulocytes [50–52], placenta [53] and semen [54], and the terminal neolacto sequence is also a common core structure in carbohydrate chains of glycoproteins [55]. However, the *H. pylori*-binding neolacto epitope has not been found in human gastric epithelial cells, and although polylactosamine chains are present in both non-acid glycosphingolipids and in glycoproteins of human neutrophil granulocytes no binding of the bacteria to these compounds occurs, most likely due to steric hindrance from branches [34]. Further studies are thus needed to establish the function for *H. pylori* neolacto binding.

Lactotetraosylceramide, on the other hand, has only been chemically identified in the human gastrointestinal tract, i.e., in the gastric epithelium [32], in human meconium [56], and in the small intestine of one individual after surgery for peptic ulcer disease [57].

The Le^b/ALe^b/BLe^b determinants are also expressed in the human gastrointestinal tract [58, and references therein], and in the human stomach present on the surface mucous cells [7], and on the human gastric mucin MUC5AC [59], both of which are recognized by BabA-expressing strains in a Le^b-dependent manner.

Thus, both the Le^b epitope and lactotetraosylceramide are human specific and present in the gastric epithelial cells, and are candidate determinants of target tissue tropism.

The normal human gastric epithelium has a low content of sialylated glycoconjugates [60]. However, upon inflammation a shift in glycosylation occurs with an upregulated expression of *H. pylori*-binding sialyl-Le^x-containing glycoconjugates [8]. Induction of sialyl-Le^x-containing glycoconjugates in the gastric epithelium also occurs in experimental *H. pylori*-infection of Rhesus monkeys. This glycosylation shift may be relevant for the maintenance of a chronic infection by mediating the adhesion of bacteria to the epithelium in the already inflamed stomach.

9.2 Role of SabA in Activation of Human Neutrophils

As described above, two sialic acid-binding proteins of *H. pylori* were identified, i.e., HP-NAP and the SabA adhesin. Since both bind to NeuAcα3-neolactohexaosylceramide and NeuAca3neolactooctaosylceramide, their binding patterns are overlapping (Table 4). Their relative roles in ganglioside binding were investigated by using knock-out mutant strains deleted of the sialic acid binding adhesin SabA, or the NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ-binding neutrophil activating protein HPNAP [9]. No ganglioside binding was obtained with the mutant strain with deletion of the *sabA* gene, while the HP-NAP knock-out strain had a ganglioside binding capacity identical to the parent wild type strain. Thus the binding of *H. pylori* bacterial cells to gangliosides is mediated by the SabA adhesin only.

Upon *H. pylori* infection, inflammatory infiltrates consisting mainly of neutrophils and monocytes are found in the gastric mucosa, and certain *H. pylori* strains nonopsonized stimulate neutrophils to production of reactive oxygen species causing oxidative damage of the gastric epithelium [61]. Several of the *H. pylori* binding gangliosides, as sialylα3-neolactotetraosylceramide, sialylα3-neolactohexaosylceramide, sialylα3-neolactooctaosylceramide, the VIM-2 ganglioside and sialyl-dimeric-Le^x glycosphingolipid, are found in human neutrophils [51, 52], suggesting that the ganglioside binding capacity might be involved in neutrophil activation. Indeed, the *H. pylori*-induced neutrophil oxidative burst reaction was partly inhibited by preincubation with sialylated oligosaccharides [62]. Thus, nonopsonic *H. pylori*-induced activation of human neutrophils occurs by lectinophagocytosis, i.e., recognition of sialylated glycoconjugates on the neutrophil cell surface by a bacterial adhesin leads to the phagocytosis and the oxidative burst reaction.

The relative contributions of SabA and HP-NAP in neutrophil activation was investigated using a battery of *H. pylori* deletion strains [63].

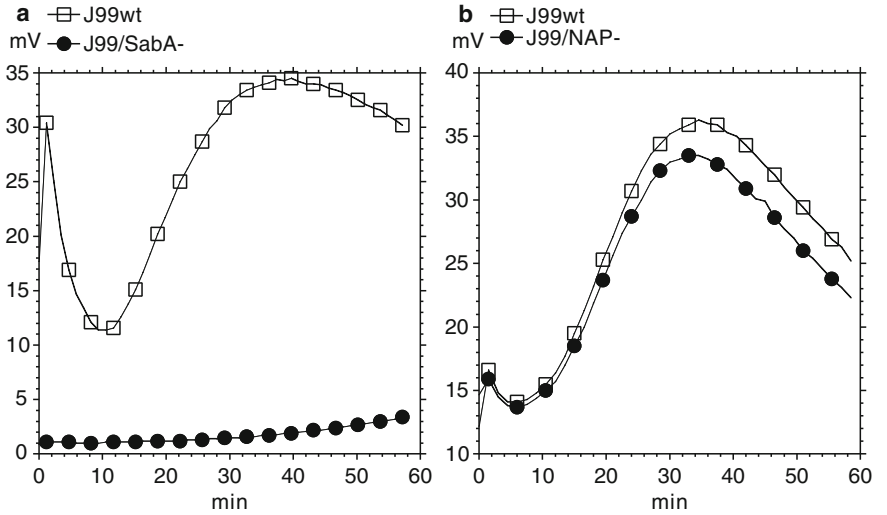

Fig. 3 Luminol-enhanced chemiluminescence of human neutrophils challenged by nonopsonized wild type *Helicobacter pylori* strain J99 (J99 wt), and its isogenic mutants with deletions of the SabA adhesin (J99/SabA-) and HPNAP (J99/NAP-)

Mutant and wild type strains lacking SabA had no neutrophil-activating capacity (Fig. 3a), while the absence of the neutrophil-activating protein HP-NAP (Fig. 3b), or the Le^b-binding BabA had no effect on the neutrophil activation. Thus, SabA-mediated binding of *H. pylori* bacterial cells to sialylated neutrophil receptors plays an important initial role in the adherence and phagocytosis of the bacteria and the induction of the oxidative burst. The events following binding of *H. pylori* to sialylated neutrophil cell surface glycoconjugates leading to NADPH oxidase activation involve a G-protein-linked signaling pathway and downstream activation of PI3-kinase, as shown by experiments using inhibitors of intracellular signaling.

9.3 SabA: The Sialic Acid Dependent Hemagglutinin

The *H. pylori* sialic acid binding was initially discovered by hemagglutination studies in 1988 [4]. Still, the sialic binding hemagglutinin has remained elusive throughout the years. However, recently hemagglutination studies using *sabA* deletion mutants demonstrated that SabA is the sialic acid-dependent hemagglutinin of *H. pylori* [64]. A polymorphic binding of clinical isolates so sialylated glycans was found with variant preferential binding to sialyl-dimeric-Le^x (NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galb4(Fucα3)GlcNAcβ), sialyl-Le^a (NeuAcα3Galβ3(Fucα4)GlcNAcβ) and sialyl-*N*-acetyllactosamine (NeuAcα3Galβ4GlcNAcβ). Protease treatment of the erythrocytes showed that *H. pylori*-mediated hemagglutination mainly occurs through interactions with erythrocyte glycosphingolipids.

Interestingly, in situ hybridization demonstrated *H. pylori* bacterial cells associated with erythrocytes in capillaries of the gastric mucosa in infected humans and in Rhesus monkeys, suggesting that the bacteria can reach the gastric mucosal capillaries, attach to the erythrocytes, and may ultimately disseminate into the circulation.

10 Concluding Remarks

The carbohydrate binding of *H. pylori* is still a complex situation with many postulated receptors. The picture is further confounded by the use of different strains, phase-variation, and use of different methods. The complex carbohydrate recognition pattern identified might partly be a result of the intense research efforts directed towards *H. pylori*. A parallel case is the multiple adhesive mechanisms described for *Pseudomonas aeruginosa*, another bacterium subjected to intense studies [65, and references therein].

The major part of the potential *H. pylori* carbohydrate receptors are orphans in the sense that no corresponding adhesin has yet been identified. An unusually high proportion (1%) of identified open reading frames of the genome sequences of *H. pylori* strains 26695 and J99 is predicted to encode outer membrane proteins (OMPs) [66], which may represent hitherto unidentified adhesins. Characterization of the adhesins corresponding to the orphan carbohydrate receptors, along with studies with deletion mutants, are urgently needed in order to establish the biological role(s) of these candidate carbohydrate receptors.

H. pylori infection tends to persist for life despite the gastric inflammatory response. This persistent infection requires a balanced host-bacterial interaction, with continuous adaption of bacterial binding properties to match the dynamic changes of glycosylation of the gastric epithelium. This may be achieved by phase variation as described for SabA [8], or by recombination events as described for BabA [17].

While patients with higher densities of Le^b in the gastric epithelium have higher *H. pylori* loads [67, 68], the presence of sialyl-Le^x is correlated with higher colonization density in patients lacking gastric Le^b [69]. Thus, the sialic acid binding capacity of *H. pylori* may have multiple roles. First, it mediates adhesion of bacteria to the gastric epithelium, and the binding capacity is augmented by the inflammation-induced up-regulation of sialylated glycoconjugates in the gastric epithelium. Second, binding of *H. pylori* to sialylated neutrophil receptors leads to neutrophil activation to an oxidative burst reaction, with production of reactive oxygen metabolites, and release of biologically active enzymes, giving rise to further tissue damage. Third, the sialic acid dependent hemagglutinating capacity of *H. pylori* may be related to the intriguing finding of the bacteria associated with erythrocytes in capillaries of the human gastric mucosa. These findings merit further investigations in view of the suggested association of *H. pylori* infections and extra-gastrointestinal diseases, such as, e.g., coronary heart disease [70].

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Bitter Sweetness of Complexity

A. K. Horst and C. Wagener

Abstract Glycosylation of proteins, lipids and mucins has gained increasing complexity in the course of evolution. Metazoans and mammals exhibit extensively exploited pathways of *N*-glycan biosynthesis, with unique features that are not found in plants or protozoans.

Paralleling the complexity of *N*-glycan structure, their impact on regulatory processes has become very diverse and has evolved into a multidimensional lattice imprinting modes of cellular communication. Processes that are regulated by *N*-glycans are cellular adhesion and motility, growth factor and cytokine signalling, metabolic homeostasis, and binding of certain pathogens. Consequently, alterations in *N*-glycan biosynthesis interfere with cellular proliferation and differentiation and may produce disturbances in embryonic development, trigger inflammatory processes, favour tumour development and enhance the metastastic dissemination of primary tumours. Particular *N*-glycans that have been causally related to these pathological scenarios are the complex-type *N*-glycans, branching from oligomannosidic core structures into β-glycosidic linkages, connected to acetylated glucosamine and galactose, and yield extended lactosamine chains of variable length. These *N*-acetyllactosamines are preferred building blocks for further modification by fucosylation, sialylation, and sulphation, thus creating binding sites for different galectins or selectins. The focus of this review will be on the β1,6-*N*-acetylglucosaminyltransferase-V/GnT-V/Mgat5, a phylogenically conserved enzyme that is required for the synthesis of β1,6-branched complex-type oligosaccharides in the medial Golgi compartment, and its implications in metabolism and cancer progression.

Keywords Complex-type *N*-glycans, Adhesion, Cellular Signalling, Cancer, Metastasis

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Abbreviations: Asn: Asparagine; CEA/CEACAM5: Carcinoembryonic antigen/CEA-related cell adhesion molecule 5; CRD: Carbohydrate-recognition domain; ECM: Extracellular matrix; EGF/-R: Epidermal growth factor: EGF-receptor; EMT: Epithelial-mesenchymal transition; ER: Endoplasmic reticulum; ERK: Extracellular signal regulated kinase; FAK: Focal adhesion kinase; FGF/-R: fibroblast growth factor; FGF-receptor; Fn: Fibronectin; Gal: galactose; Glc: Glucose; GnT: *N*-acetylglucosaminyltransferase; ICAM1: Intercellular adhesion molecule-1 (CD54); IGF/-R: insulin-like growth factor; IGF- receptor; LAMP-1: Lysosome-associated membrane protein-1; Ln: Laminin; L-PHA: Phaseolus vulgaris leukoagglutinating lectin; MelCAM: Melanoma cell adhesion molecule (CD146); Mgat5: Protein: GnT-V; *mgat5*: Gene coding for GnT-V; *mgat1*$^{-/-}$ or *mgat5*$^{-/-}$ mice: Mgat1 or 5 knockout mice; PDGF/-R: platelet-derived growth factor; PDGF-receptor; PI3K: Phosphatidylinositol-3-kinase; PKB: Protein kinase B; Pro: Proline; PyMT: polyoma virus encoded middle T-antigen; Ser: Serine; Smad: Proteins with sequence similarity to the SMA and MAD proteins; v-src/pp60^{src}: Rous sarcoma virus-encoded protein kinase; TDP: thymidine diphosphate; Thr: Threonine; TGFβ: transforming growth factor-beta; TβR: TGFβ-receptors I and II; UDP: Uridine diphosphate

Contents

1 Introduction

The complexity of combinations to build glycosylated structures on protein and lipid backbones is extensive, since the individual modifications are not encrypted immediately by the genetic code. A hexose backbone offers up to five alternate sides for modifications via either α- or β-glycosidic bonds. The extent to which nature uses every one of these sites is determined by the actual environment of the hexose within a sugar chain or its localization and proximity to modifying and metabolising enzymes. The pathway of *N*-linked glycosylation reactions reflects distinct evolutionary steps in its sequence of reactions which also becomes evident by the distinct compartimentalization of the modifying enzymes within the cell. In bacteria, polypeptide backbones with L-amino acid chains are not being modified by *N*-linked oligosaccharides, whereas in lower and higher eukaryotes, basic glycosylation patterns are present that lead to the synthesis of high-mannose

oligosaccharides. A pentasaccharidic core structure is shared by all lower eukaryotes, such as yeast and fungi, and other unicellular organisms. In metazoans and mammals, this evolutionary precursor is further modified by a complex set of glycosidases and glycosyltransferases that render hybrid-type and complex-type oligosaccharide chains.

The motif in proteins for *N*-glycan attachment in the dolichol cycle in the endoplasmic reticulum (ER) is Asn-X-Ser or Asn-X-Thr, with X resembling any amino acid but Pro. *N*-glycan processing of glycoproteins in the Golgi affects stability of proteins and supports protein maturation and activity and controls their ultimate location and trafficking. Once targeted to the cell surface, glycosylation protects the proteins against cleavage by proteases, and increases hydrophilicity, in case the proteins are shed or specifically cleaved and remain solute in the extracellular space or serum. The extent of protein glycosylation, in turn, is controlled by the metabolic availability of the respective donor substrates and may reflect alterations in hexose metabolism, for example in diabetes or during cancer progression, as a result of shifts in the metabolic balance and the Warburg effect, i.e. the predominant production of energy by glycolysis in cancer cells rather than by oxidation of pyruvate in mitochondria [1]. Also, the three-dimensional conformation of the polypeptide within the Asn-X-Ser/Thr motif controls the attachment of glycans to the Asn of a growing polypeptide backbone [2, 3]. There are a myriad of theoretical possibilities to create different glycoforms of differentially glycosylated proteins, but only a fraction of these is actually synthesized and even less are participating in recognition or regulatory processes. Additionally, the relative amounts of *N*-linked glycans and their degree of branching balance the cellular signalling that is elicited through glycosylated cell surface receptors. Thus, dysregulation of cellular communication by alterations in glycosylation patterns and their complexity is causally related to inflammatory and malignant diseases. Ectopic or enhanced expression of glycosyltransferases may lead to reprogramming of the proliferation pattern of cancer cells so that they gain motility or invasive properties and spawn dissemination. Three different β-*N*-acetylglucosaminyltranfserases, GnT-III, -IV and -V, are correlated with tumour-related alterations of *N*-glycan branching and complexity. Especially the tri- and tetra-antennary β1,6-*N*-acetylglucosamine structures are related to invasive malignant disease.

After transfer of ER-borne dolichol-linked precursor oligosaccharides to Asn-residues of polypeptides, the core oligosaccharide structure is modified by ER-resident glucosidases and mannosidases and is travelling to the Golgi compartment, where they undergo further modifications and branching, yielding the hybrid and complex-type bi-, tri- and tetra-antennary oligosaccharide structures (summarized in Fig. 1a). Branching of the oligosaccharide chains is usually initiated by the addition of a bisecting GlcNAc (*N*-acetylglucosamine) to a core mannoside residue by an enzyme called mannosyl(alpha-1,3-)glycoprotein beta-1,2-*N*-acetylglucosaminyltransferase 1, referred to as Mgat1 (Fig. 1a). For the synthesis of complex type oligosaccharides, Mgat1-activity is essential, since other sugar modifying enzymes only become active once Mgat1 has been in play: Mgat1 products yield high affinity substrates for α-Mannosidase II, paving the way for further modifications by different Mgat-isoforms

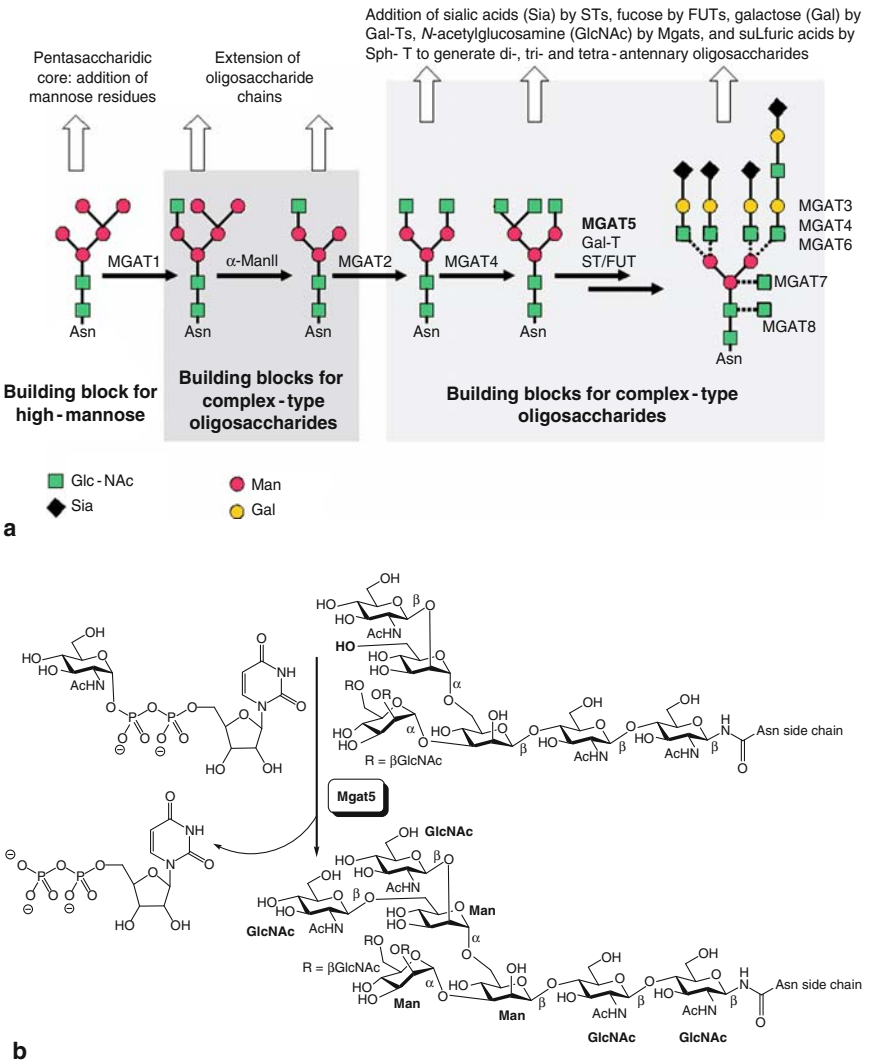

Fig. 1 **a** Schematic representation of key enzymatic reactions in the biosynthesis of hybrid- and complex-type N-linked oligosaccharides. This figure depicts some of the biosynthesis pathways and activities of enzymes crucial for the synthesis of hybrid- (*boxed in dark grey*) and complex-type *N*-linked glycan chains (*boxed in light grey*) based on modifications of a high mannose core. The *white arrows* indicate that the basic building blocks of different oligosaccharides are subject to further modifications. They represent precursors for the synthesis of more complex-type oligosaccharides. The individual activity of the different transferases or glycosidases yields products that, in turn, render poor or high-affinity substrates that determine the path taken in oligosaccharide biosynthesis. The bi-, tri- and tetra-antennary *N*-glycans are subject to further elongation by β-*N*-acetylgalactosyltransferases or galactosyltransferases and can be modified by the terminal addition of sialic acids, fucose residues and sulphate groups, giving rise to the Lewis antigens. Similarly, the selectin ligands can be synthesized after further branching following extension of the tri- and tetra-antennary branches. *Dotted lines* indicate optional routes for *N*-glycan modification

and β-*N*-galactosyltransferases, as well as termination of *N*-acetyllactosaminoglycan chains by sialic acid, and the addition of terminal fucose, or sulphates.

MGAT5 (α-1,6-mannosylglycoprotein 6-β-*N*-acetylglucosaminyltransferase A; EC: 2.4.1.155; AF113921.1 [4]; AF474154.1 [5]) is a single-pass type II membrane protein, residing in the medial Golgi compartment. In human and rodents, Mgat5 shares a high sequence homology on amino acid level, and it is encoded by a single gene. It catalyzes the addition of *N*-acetylglucosamine in β1,6 linkage to the α-linked mannose of bi-antennary *N*-linked oligosaccharides. More specifically, it uses UDP-(uridine-diphosphate-)-*N*-acetyl-D-glucosamine and 6-(2-(*N*-acetyl-β-D-glucosaminyl)-α-D-mannosyl)-β-D-mannosyl-residues as substrates to create peptide-boundUDP-6-(2,6-bis(*N*-acetyl-β-D-glucosaminyl)-α-D-mannosyl)-β-D-mannosyl-oligosaccharides.

The reaction catalyzed by Mgat5 is depicted in Fig. 1b. Both hybrid and complex-type oligosaccharide chains may be extended further by addition of galactose to build *N*-acetyllactosamines, catalyzed by the Gal-T. Additionally, terminal fucose, sialic acid residues or sulphate groups can be added to create the different blood group or Lewis antigens, for example. Synthesis of di-, tri- and tetra-antennary structures is accomplished by the action of the different Mgat-isoforms (Fig. 1a). Mgat5 catalyzes the a key step in the sequential action of different monosaccharide transferases that yield complex oligosaccharide side chains on glycoproteins. The extent of cell surface glycosylation regulates the expression and function of several proteins involved in inflammation, or tumour metastasis. Conversely, abrogation of Mgat5 expression reverts disease-related phenotypes which make Mgat5 an attractive target for therapies. The consequences of Mgat5 expression and lack thereof will be discussed in detail below.

2 *Mgat*-expression in Embryonic Development and Consequences on Aging

The equilibrium between the synthesis of hybrid-type and bisecting versus tri-, and tetra-antennary complex-type *N*-linked glycans is regulated by the sequential order specific enzymes become active in and by the relative activities of the eight different isoforms of Mgats, respectively. The sequence of reactions shown in Fig. 1a not only present a set of general options for the synthesis of *N*-linked glycans. They also

Fig. 1 (Continued) by different isoforms of MGAT enzymes. MGAT1-8: β-*N*-acetylglucosaminyltransferases 1–8; ST: α-Sialyltransferases; FUT: α-Fucosyltransferases; Gal-T: β1,4Galactosy ltransferases; Sph-T: Sulphotransferases. β Schematic representation of the reaction catalyzed by MGAT5. MGAT5 uses UDP-(uridine-diphosphate-)-*N*-acetyl-D-glucosamine as a substrate that is added to a peptide-bound *N*-acetyl-β-D-glucosaminyl-(α-D-mannosyl)-β-D-mannosyl-oligosaccharide. The 6'hydroxyl group of the mannose that serves as an acceptor for the UDP-*N*-acetyl-D-glucosamine residue is highlighted in bold print. This figure illustrates branching of an oligosaccharide chain on the 2′and 6′ position of a mannose residue. The *N*-acetyl-D-glucosamine residues may serve as a primer for the synthesis of lactosaminoglycans

dictate a certain hierarchy in the order of synthesis paths taken. The products of MgatIII-activity yield low-affinity substrates for α-mannosidase II, and thus pave the way for hybrid type oligosaccharide synthesis. In contrast, Mgat2-, 4- and 5-derived products are higher-affinity substrates for α-mannosidase II, and subsequently, their activity leads to an increase of the ratio complex- over hybrid-type oligosaccharides [6] (Fig. 1a). The importance of the expression of Mgats is emphasized by observations that abrogation of the ability to synthesize hybrid or complex-type glycans leads to embryonic lethality in mice: in spite of their homozygous deficiency in Mgat1-expression, these animals are able to initiate and regulate basic, but yet limited cellular differentiation processes. Mgat1-knockout (*mgat1*$^{-/-}$) animals die in utero between days E9.5 and E10.5 post implantation due to neural defects. Often, they exhibit a situs inversus phenotype [7, 8]. Furthermore, in spite of the generation of hemangiogenic progenitor cells, functional vascularization is compromised in *mgat1*$^{-/-}$ embryos, with severe vascular dysfunctions, such as vascular hypertrophy that also affects the heart and haemorrhage [8, 9]. Interestingly, impairment of the biosynthesis of the core 1 *O*-glycan, also known as T-antigen (Galβ1-3GalNAcα1-Ser/Thr) by systemic deletion of the core 1 b1,3-galactosyltransferase (T-synthase), is equally embryonic lethal due to defective angiogenesis. The T antigen is a precursor for extended and branched *O*-glycans of largely unknown function. However, in analogy to the precursor for complex type *N*-glycans, the core 1 *O*-glycan is a basic building block giving rise to extended *O*-glycans that are attached to Ser/Thr residues on glycopeptides and that may carry sialylated selectin ligands, for example. Defects in T-antigen sialylation compromise functional vascular network formation ([9, 10] and references therein). This indicates that loss of functional expression of oligosaccharide modifying enzymes is the more severe the earlier the defective enzymes would normally act on the sequential modification of principal building blocks. In later stages of glycan biosynthesis, a certain redundancy of different transferase or glycosidase isoforms may offer a limited compensation for functional defects, depending on the individual spatiotemporal expression of these enzymes.

In contrast to Mgat1-knockouts, Mgat5-knockout (*mgat5*$^{-/-}$) embryos are viable and born after full-term pregnancies, though they exhibit some tissue defects and the overall amount of *N*-linked glycosylation displayed on polypeptides is reduced [11, 12]. Mgat5-expression was found to commence on embryonic day 7, with a broad expression pattern, including the central nervous system, basal layers of certain skin epithelia, intestines, kidneys, endocrine tissues and in the respiratory tract [12]. Adult Mgat5$^{-/-}$ mice develop resistance to weight gain in spite of high-caloric feeding, paralleled by increased oxidative respiration and sensitivity towards fasting [7, 13]. Mutant cells that render defects in the Golgi-UDP-transporter or in Mgat5-activity exhibit increased sensitivity to growth factor deprivation [14, 15]. In this context, it is worth mentioning that the C57BL/6 mouse strain used to examine the consequences of the *mgat5*-knockout exhibits high susceptibility to diet-induced obesity, type 2 diabetes, and atherosclerosis [16]. Additionally, osteal cellular turnover is reduced in the Mgat-deficient mice, manifesting as osteoporosis and reduced osteogenesis, and is accompanied with progressive loss of muscle

mass [13, 17]. Ultimately, Mgat5$^{-/-}$ mice with homozygous deletion of the *mgat* gene exhibit a significantly shorter life span than heterozygous or WT littermates [13]. Furthermore, homozygous *mgat5* $^{-/-}$ mice exhibit a higher susceptibility to autoimmune diseases, reduced cancer progression and behavioural abnormalities, with the latter depending on the mouse strain used [12].

3 Mgat5: Interdependent Regulation of Metabolism and Cytokine and Growth Factor Responses

As indicated above, the relative Mgat5 activities and the individual's metabolism are not independent of one another. High activity in the hexose catabolizing pathways yield substrates for the generation of *N*-glycans, for example *N*-glucosamine, and utilized as a desoxyribonucleotide derivative (UDP-GlcNAc as glycosyl donor) to generate hybrid-type and complex-type *N*-glycans attached to transmembrane cell surface signalling receptors. Previous studies have confirmed that Mgat5 exhibits exceptionally high K_m values in comparison to other GnTs for UDP-GlcNAc, so that the intracellular concentrations of the substrate largely determine the amount of complex-type *N*-glycans found on glycoproteins [18, 19]. Besides UDP-Glc-NAc, Mgat5 is also utilizing thymidine diphosphate-(TDP)-Glc and UDP-Glc as substrate. This indicates that the amount of catabolic substrates and the total synthesis of branched *N*-glycans not only provide a sugar reservoir for decoration of receptors engaged in cellular signalling: the balance in hexose metabolism also encodes specific signalling thresholds that are determined by the overall metabolite concentrations as well as the specific compositions of *N*-glycans on the cell surface (Fig. 2).

Mgat5-deficient mice are prone to develop a metabolic syndrome that is caused by inadequate utilization of growth factors, which is a consequence of inefficient growth factor receptor internalization and recycling. Consequently, membrane plasticity is changed and communication processes that are dependent on caveolin- as well as clathrin-dependent receptor recycling or phagocytosis become dysregulated [17, 20]. Therefore, shifts in the subcellular distribution of receptor populations between the lysosomal and endosomal compartments may occur that result in impairment of appropriate growth factor release and explain why embryonic fibroblasts isolated from *mgat5* $^{-/-}$ mice, for example, are less sensitive to anabolic cytokines and show reduction in cellular glucose uptake [13, 20].

As mentioned above, the alterations in signal transduction intensities of specific growth-factor receptor tyrosine kinases or adhesion molecules are based on changes in their spatio-temporal distribution, and result in either sustained exposure on the cell surface and/or inadequate clustering within cell surface microdomains (Fig. 3). Once expressed on the cell surface, signalling receptors carrying complex-type *N*-glycans offer binding sites for multivalent galectins that engage cellular signalling receptors into a multimeric lattice or bundles in specific membrane microdomains, such as lipid rafts. Complex-type *N*-glycan cross-linking by galectins is proportional to the relative *N*-acetyllactosamine content in the *N*-glycans, profiting from

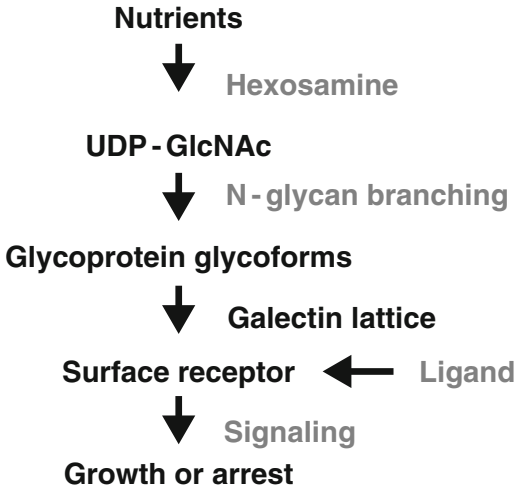

Fig. 2 Schematic representation summarizing the interdependence between supply with hexosamines and the consequences on molecular mechanisms that alter cellular signalling. Rich nutrient supply modulates the biosynthesis of complex-type *N*-glycans and affects signal transduction cascades that control cell growth and arrest. The individual levels within the metabolic network are integrated into a hierarchy. This flow chart shows that the initial input of hexosamines determines the synthesis of different glycoforms of glycoproteins that are targeted to the cell surface. Once exposed on the cell, galectins may bind to the glycosylated receptors; after ligand binding to the receptors, cellular signalling is activated and may lead to either cellular growth or growth arrest. Reprinted and adapted from [20], with permission from Elsevier

increases in the overall avidity the more ligands become available [21, 22]. Sustained surface exposure and clustering of signalling receptors creates a platform to multiply their signalling intensities caused by facilitated access of their respective ligands (Fig. 3). Fine-tuning of these galectin-*N*-acetyllactosamine interactions is elicited by terminal modifications of the *N*-glycans by terminal sialic acids or fucose residues, for example, which restrict galectin-mediated *N*-glycan cross-linking or regulate complex-formation with signalling co-receptors [23]. *N*-Acetyllactosaminoglycans in tetra-antennary structures are preferentially bound by galectins-3 and -9 and render modulation of signalling through receptor-unrelated ligands. Galectins-3 and -9 have multimerization domains distinct from their CRDs (carbohydrate recognition domains; Gal-3) or possess two CRDs (Gal-9). The structure and functions of the different galectins and their individual implications in physiological and pathological processes have been reviewed elsewhere [24, 25].

Mgat5-mediated modulation of signal transduction through appropriately glycosylated cell surface receptors and the consequences of galectin-induced clustering were investigated in greater detail for the T-cell receptor complex, the TβRs (transforming growth factor-β receptors I and II), the N- and E-cadherins, diverse integrins and chemokine/growth factor receptors. Enhanced clustering of the T-cell receptors within the T-cell receptor/-co-receptor signalling complexes, for example, is observed in the absence of Mgat5-mediated glycosylation, inducing higher sensitivity towards

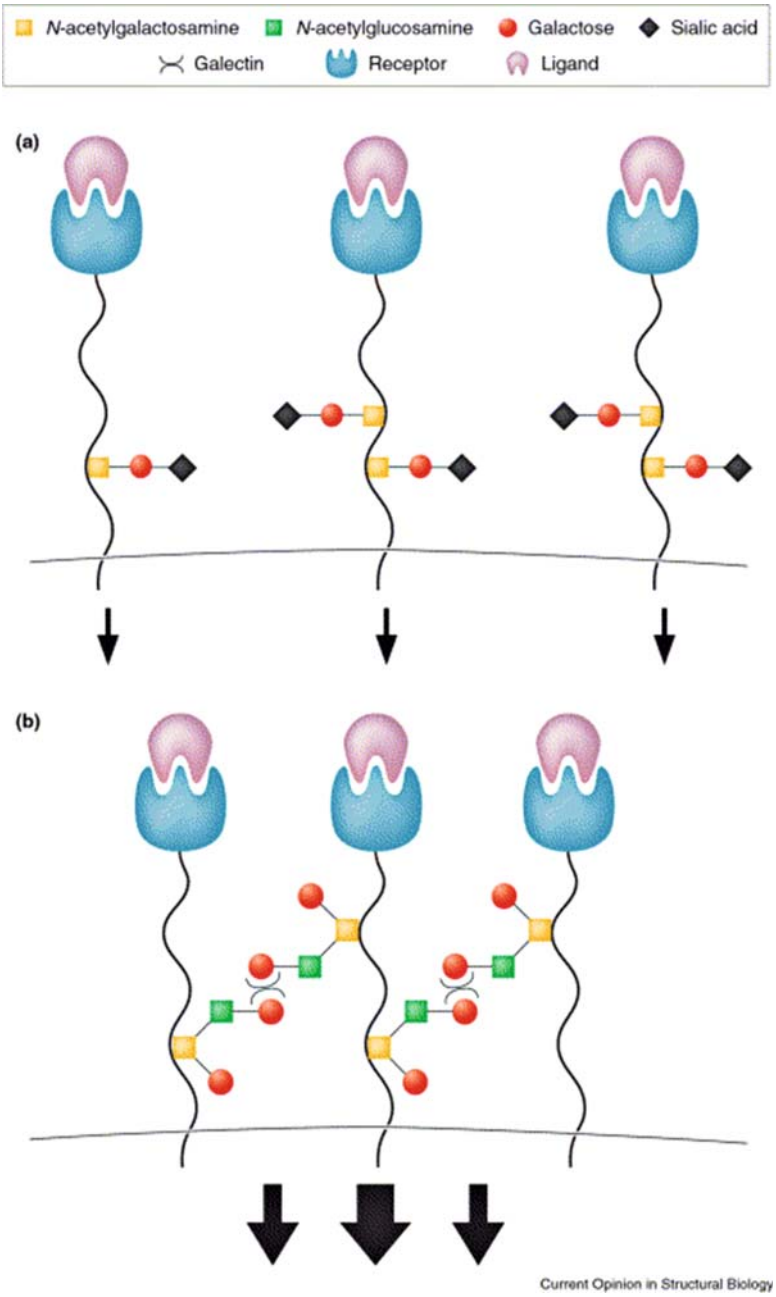

Fig. 3 Regulation of transmembrane receptor signalling by galectin-induced lattice formation. Model of galectin-mediated glycoprotein lattices enhancing signalling. **a** Individual receptors engage individual ligands and each sends a weak signal. The presence of sialic acids, for example, inhibits galectin-mediated cross-linking of the receptors. **b** Appropriate glycosylation allows galectin-mediated cross-linking of the receptors. Subsequent engagement by ligands sends signals that are temporally and spatially concentrated to result in an overall increase in signal strength and/or duration. The relative strength or duration in downstream signal transduction is represented by the thickness of the black arrows. Adapted from [23], with permission from Elsevier

its ligands. This produces a hypersensitivity phenotype in Mgat5-deficient mice, rendering increased susceptibility for experimental autoimmune encephalitis, which is the analogous mouse model for multiple sclerosis in humans [26].

4 Mgat5 Expression is Affected by Cellular Signalling

Besides these immunological effects, enhanced Mgat5-expression and the cell surface exposure of proteins bearing β1,6-branched tri- and tetra antennary *N*-glycans are determinants of the malignant phenotype. Enhanced Mgat5-activity is observed after malignant transformation, and the expression of β-1,6-branched *N*-glycans correlates with metastasis formation of various tumour cell lines in vitro and in vivo [11, 27].

The imbalances in cellular signalling that ultimately lead to a malignant phenotype are a result of quantitative and qualitative shifts in signalling by growth-promoting (e.g. EGFR) or growth-inhibiting (e.g. TβRs) receptor tyrosine kinases and their downstream signalling pathways (Fig. 4; [20, 28] and references therein). Mgat5-expression is up-regulated by oncogene-encoded proteins, inducing the activation of key regulatory pathways that enhance cellular proliferation, such as the Ras/extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt/protein kinase B (PKB) pathways, and that are involved in the formation and degradation of focal adhesion complexes [12, 29–33]. In a mouse model of mammary tumorigenesis, expression the polyoma virus encoded polyoma middle T-antigen (PyMT) in the mammary glands induces tumour growth and metastasis because it acts as a substrate for Src-kinase ($pp60^{src}$), and subsequently becomes a scaffold and positive regulator for the regulatory signalling proteins that are modified by Ras or PI3K. Additionally, Mgat5 activity is enhanced in PyMT-transgenic mice [12]. In contrast to these proliferation-promoting activities, the decline of the β1,6-glycan contents by suppression or deletion of Mgat5 activity attenuates growth factor receptor mediated signalling for example, through the EGFR, and thus impedes processes that are dependent on growth factor and ERK-regulation (see above). The consequences of these deficiencies in *N*-glycan-dependent signal regulation were shown in *mgat5*$^{-/-}$ mice: mice bearing endogenously induced mammary tumours exhibited a significant reduction of cancer growth and metastasis in contrast to their Mgat5 expressing littermates [11].

5 Mgat5-Activity Regulates Cellular Adhesion and Signalling

Besides regulatory effects on signalling through cellular cytokine and growth factor receptors, adhesion processes are controlled by the individual *N*-glycan content of particular adhesion molecules. A critical step in cellular invasiveness is the fine-tuned regulation of cell adhesion to the extracellular matrix, executed by focal cell adhesion synapses (focal adhesion complexes) that are regulated by the focal adhesion kinase

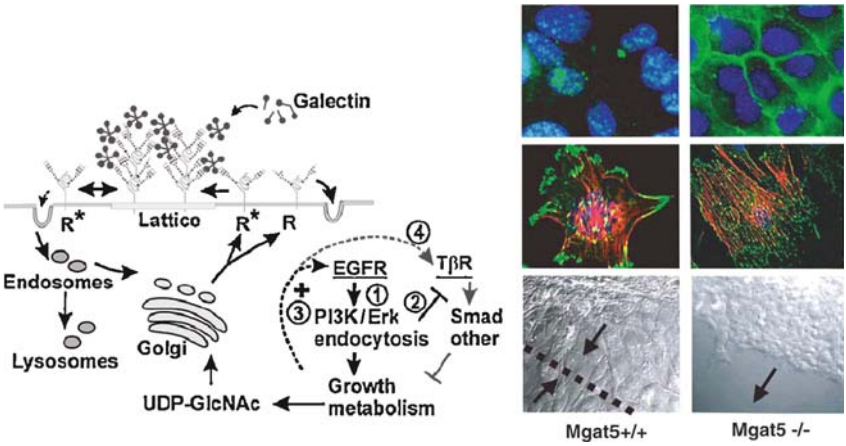

Fig. 4 **a** Hexosamine regulation of surface glycoproteins and responsiveness to growth and arrest cues. Schematic representation of Mgat5-regulated signalling of the growth-promoting epidermal growth factor receptor (EGFR) and the growth-arresting TGF-β-receptors (TGFβR1 and TGFβ-R2, both referred to as TβR). Differential glycoforms of cell surface receptors (R and R*) are engaged into galectin-lattices or are subject to internalization and receptor recycling, depending on the degree of their individual *N*-glycan content. Receptors with high *N*-glycan content are subject to prolonged growth inducing signalling (e.g. EGFR, ①) and cell surface exposure whereas receptors with few *N*-linked glycans are routed for internalisation and thus suppressed in their inhibitory signalling activities (②,TβRs). In WT cells, stimulation of EGFR- tyrosine kinase activity enhances PI3K-mediated signalling (③) and promotes F-actin remodelling, as well as internalization of receptors with low *N*-glycan content (④). Subsequently, internalized receptors become glycosylated, leading to their surface exposure, and galectin engagement. Reprinted from [20], with permission from Elsevier. **b** Impact of Mgat5-expression cellular behaviour of tumour cells. Mgat5-expression controls the expression and localization of cell surface receptors (*upper panels*), the formation of focal adhesions (*middle panel*), and cellular motility (*lower panels*), demonstrated in Mgat5-positive (Mgat5+/+) and –negative epithelial tumour cells ($Mgat5^{-/-}$). In brief, this figure summarizes the sequence of events leading to the so-called epithelial-mesenchymal transition (EMT): alterations in the expression of cell surface receptors and adhesion molecules (*here*: anti-E-cadherin, *green staining*, *upper panels*; nuclei are stained in *blue*) produced by changes in receptor glycosylation lead to alterations in their localization. The EMT is considered as a key event that describes the molecular processes that induce an invasive, spindle-shaped cellular morphology in epithelial cells. E-cadherin is hardly expressed on the cell surface of Mgat5-expressing cells, whereas it localizes to tight junctions in *mgat5*$^{-/-}$ cells and exerts homotypic cell adhesion. Changes in cellular adhesive properties (*middle panels*) are determined by alterations of the localization and expression of adhesion molecules and the turnover of focal adhesion complexes, visualized by anti-vinculin-staining (*green fluorescence*). In $Mgat5^{-/-}$ cells, the formation of cortical actin stress fibres (actin filaments, *red fluorescence*) is enhanced, whereas Mgat5-expressing cells contain multiple lamellipodia (anti-vinculin-staining, *green fluorescence*). As a result, Mgat5-expressing cells exhibit increased cellular motility and adopt a spindle-shaped phenotype. This is shown in the *lower panels* of (**b**) in the phase contrast photographs. The *arrows* indicate the direction of cellular movement and the *dotted line* indicates a previously cell-free area. This "wound" was scratched into a confluent layer of tumour cells and cellular migration was assessed 24 h post scratching. $Mgat5^{+/+}$ cells (*lower left panel*) migrated into the cell-free area whereas Mgat5-negative cells (*lower right panel*) maintained their morphology and did not migrate into the wounded area. From [17], reprinted with permission from AAAS

(FAK) and contain integrins, cadherins or other cell surface receptors and that are connected to cytoskeletal components in a phosphorylation-dependent manner. In turn, cellular adhesion is regulated by the overall affinity of adhesion molecules to their ligands, which is subject to modulation by the expression of specific glycoforms of these receptors and creates a feedback loop to the hexosamine-dependent signalling–threshold buffered by receptor clustering and galectin-dependent lattice formation. The capability of the different receptor glycoforms to engage into galectin-mediated clustering induces their so-called *multiplicity* that leads to enhanced downstream signalling (see above; Fig. 3). The connection between Mgat5-expression and the regulation of cellular adhesion and motility is described in Fig. 4a, b. Based on their *N*-glycan content, surface signalling receptors with high numbers of *N*-linked β1,6-branched glycans present high multiplicity receptors that respond immediately to the given hexosamine supplies, such as the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR), the insulin-like growth factor receptors (IGFR) and the fibroblast growth factor receptors (FGFRs). Signalling through these receptors induce cellular growth and is required for enhanced motility, targeting pathways through PI3K/Akt/PKB/PTEN, ras/raf, and $pp60^{src}$, for example, as mentioned above [11, 34]. In contrast, receptors and other surface molecules that present only few target sites for *N*-linked glycosylation are subject to rapid internalization via endocytosis, such as TβRs or the GLUT4 transporter ("low multiplicity receptors" [20]). Interestingly, TβRs elicit growth inhibition when expressed in high densities on the cell surface. Conversely, they support synthesis of extracellular matrix components in case their expression levels are low, and may therefore rather enhance invasive processes [28, 35] (Fig. 4a).

Growth factor-induced hexosamine catabolism ultimately enforces glycosylation and cell surface presentation of these previously sparsely expressed *low multiplicity* receptors. The association of the low-multiplicity receptors with galectins increases their presence and activity on the cell surface. Subsequently, their autocrine, negative feedback regulation ceases forward signalling and would ensure check and balance of cellular proliferation under physiological conditions (Fig. 4a) [20].

6 Complex *N*-Linked Glycans in Malignancy

The observations detailed above summarize the context between malignant traits in cellular behaviour and the complex *N*-glycan content of cell surface signalling receptors and adhesion molecules. The causative correlation between Mgat5 expression and malignant disease becomes evident following re-expression of Mgat5 in previously $Mgat5^{-/-}$ tumour cells, since sensitivity towards EGF, IGF, PDGF, TGF-b and bFGF, autonomous growth control, as well as the invasive phenotype are restored in vitro and in vivo [17, 20]. A malignant phenotype is not only based on the alterations in the localization of specific cell surface receptors, as described before (Fig. 4b,), but also due to direct modulation of N-and E-cadherin-controlled cell-to-cell adhesion or integrin-mediated cellular adhesion processes to

extracellular matrix (ECM) components, such as fibronectin (Fn), laminin (Lm) or collagen IV [36, 37]. Deletion of the *mgat5* gene in murine embryonic fibroblasts hampers the expression of the mature form of integrinβ1 on the cell surface, for example, as a consequence of suppression glycosyltransferases that would normally act in tandem (β1,4-GalT and Mgat5) [38]. Furthermore, analyses of different invasive vs non-invasive murine and human melanoma cell lines or fibroblasts revealed enrichment of β1,6 *N*-acetylglucosamines in the malignant cells. The attachment of human or murine fibrosarcoma cell lines to the substratum and their motility is regulated by N-cadherin; the regulatory impact of N-cadherin on cell adhesion depends on its homophilic adhesion qualities that are fine-tuned by its *N*-glycan contents. Treatment with swainsonine, an indolizidine alkaloid that inhibits α-mannosidase activity [39], leads to an increase in local N-cadherin clustering and thus enforces adhesion; conversely, enhanced branching of poly-*N*-lactosaminoglycans by Mgat5 ultimately reduces the clustering potential of N-cadherin on the cell surface, leading to increased catenin-phosphorylation and expression of src kinase. Catenin phosphorylation induces an increase in cellular motility [37, 40].

The observation that key adhesive and signalling proteins are subject to regulation by *N*-glycan modifications prompted the identification of additional candidate substrate molecules for Mgat5; more specifically, Mgat5 products were identified on more glycosylated regulatory proteins that often become dysregulated during tumour progression. Amongst those are the β1 integrins ($\alpha_3\beta_1$ and α_5 [23], the adhesion molecule L1, Mac-2 binding protein, melanoma cell adhesion molecule (MelCAM, CD146), intercellular adhesion molecule-1 (ICAM-1; CD54), melanoma-associated antigen, tumour-rejection antigen-1, melanoma-associated chondroitin sulphate proteoglycan-4, or carcinoembryonic antigen/carcinoembryonic antigen-related cell adhesion molecule-5 (CEA; CEACAM5), and the galectin-binding intracellular lysosome-associated membrane protein-1 (LAMP1). Similar to the aforementioned consequences of differential *N*-linked glycosyation of the cadherins or growth factor receptors, these cell adhesion molecules or intracellular galectin binders carry polylactosamine structures and complex-type *N*-glycans that alter their stability, cellular localization, and adhesive properties.

However, the relative expression levels of these adhesion proteins appear rather secondary to the impact that their actual *N*-glycan context has on their specific metastatic potential [41–44].

In agreement with the observations in different cell lines and animal models, there is also a correlation between the β1,6-linked glycosylation/Mgat5-activity and the invasive phenotypes of human cancers [45]. High Mgat5 activity in tumours or ectopic Mgat5-expression usually correlates with an increase in the metastatic dissemination of a primary tumour and provides and independent prognostic parameter. For human mammary carcinomas and gliomas, for example, the inappropriate β1,6-linked glycosylation of cell surface molecules is indicative of invasive disease [46, 47]. In the case of human breast cancers, increased complex-type *N*-glycan contents on the tumour cells directly correlates with tumour invasiveness and the presence of lymph node metastases and thus predicts poor prognosis. Hence, the expression of the β1,6-linked *N*-glycans has been established as an independent

prognostic marker. In specimens from human tumours, the β1,6-*N*-glycan content can be probed with the β1,6-*N*-glycan branch-specific *Phaseolus vulgaris* leukoagglutinating lectin (L-PHA). The use of L-PHA-staining on collections of tumour specimens, for example, on tissue microarrays, can be employed a predictor for disease outcome and patient survival [46].

Besides the gain in tumour cell motility or invasiveness, the attachment of disseminating cancer cells to vascular beds in metastasis-prone organs is facilitated by tumour cells that express Mgat5, since Mgat5 activity produces precursors for the synthesis of selectin ligands. The expression of selectin ligands is crucial for the extravasation and metastatic seeding of tumour cells and enhances the adhesion to endothelia of organs that render target sites for metastasis formation [48]. Human colon cancer cells, for example, exhibit increased adhesion to endothelial E-selectin [49]. E-selectin-expression on endothelia is induced by inflammatory and malignant processes, and renders facilitated hematogenous metastasis and poor prognosis of the primary colon tumours, and thus presents an independent risk factor for their metastatic spread [49]. Attachment of disseminated tumour cells to endothelia in metastatic niches is a result of reduction in the cellular adhesion to Fn, accompanied by enhanced binding of the endothelial selectins [49]. In addition to the generation of selectin ligands on colonic tumour cells, Mgat5 activity also directly modulates their invasive potential by modification of the tissue inhibitor of matrix metalloprotease-1 (TIMP-1) and protein tyrosine phosphatase kappa. As a consequence of the changes in the *N*-glycan decoration of these targets on tumour cells, cellular motility and degradation of the ECM are enhanced [50–53].

Furthermore, Mgat5 activity is functionally implicated in pathological angiogenesis, as a critical determinant in tumour growth and metastasis, even though its immediate enzymatic activity does not appear to be relevant in this context: the secreted, soluble form of Mgat5 is a substrate for the γ-secretase (presinilin-1). The angiogenic activity of soluble Mgat5 is based on its function in the release of ECM-bound bFGF2, and thus yielding higher concentrations of the angiotrophic bFGF2 [54–57].

7 Glycan-based Therapeutic Approaches for Malignant Diseases

As indicated by the diverse activities of Mgat5 that are involved in producing a metastatic phenotype, Mgat5 offers an attractive target for therapeutic intervention. Most importantly, as demonstrated in different animal models in vivo, alterations of abrogation of Mgat5 activity, or the inhibition of precursor-processing into high-mannose to complex-type oligosaccharides by swainsonine, show that targeting glycosylation-based malignancy harbours therapeutic benefits for human cancers. Swainsonine is used as a therapeutic for renal cancer [11, 58–61]. As suggested in a recent reflection on "*the method to the madness of N-glycan complexity*" [62], manipulating the different adhesion mediated by galectins involved in lattice formation might offer novel strategies towards glycan-targeted therapies; so far, anti-galectin-3 approaches reached clinical trials for pancreatic and colon carcinomas [58, 62]. Additionally,

putative substrate mimetics for the different glycosyl transferases or lectin ligands have been subject of investigation with respect to their potential therapeutic implications. It remains a challenge for the future, however, to gauge whether or not broad-spectrum inhibitors that target common glycosylation pathways or specific carriers of complex-type glycans may render the most beneficial targets.

8 Conclusion

N-Linked glycans resemble key components regulating cellular communication. Additionally, they connect the catabolic metabolite reservoir for glycan biosynthesis with the ability of eukaryotic cells to respond to alterations in their environment. Cellular hexose uptake and the activity of particular cell signalling receptors that regulate cellular growth or motility are not independent of one another, and they are coupled via long-distance feed back loops. The long-distance loops are embedded in a complex regulatory network that contains additional tools for glycan-mediated communication, e.g. saccharide processing enzymes or intra- and extracellular carbohydrate binding molecules. Shifts in the balance of hexose metabolism and differential glycosylation of cell surface molecules are frequently observed in human diseases, e.g. diabetes types 1 and 2, autoimmune diseases, infectious diseases, and cancer. In the case of malignant diseases, specific glycoforms of adhesion molecules or signalling receptors enhance the metastatic dissemination of tumour cells and thus deteriorate the patient's prognosis. The expression of particular oligosaccharides can be used as a prognostic marker for the prediction of metastatis formation. Complex-type oligosaccharides, such as Mgat5 products, are associated with the development and progression malignant disease and, conversely, inhibition of complex-type oligosaccharide biosynthesis can revert the malignant phenotype in different experimental setups. Therefore, the therapeutic targeting of complex-type oligosaccharide biosynthesis offers attractive prospects.

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Staphylococcus epidermidis Biofilms: Functional Molecules, Relation to Virulence, and Vaccine Potential

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Abstract Medical device-associated infections, most frequently caused by *Staphylococcus epidermidis* and *Staphylococcus aureus*, are of increasing importance in modern medicine. The formation of adherent, multilayered bacterial biofilms is crucial in the pathogenesis of these infections. Polysaccharide intercellular adhesin (PIA), a homoglycan of β-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues, of which about 15% are non-*N*-acetylated, is central to biofilm accumulation in staphylococci. It transpires that polysaccharides – structurally very similar to PIA – are also key to biofilm formation in a number of other organisms including the important human pathogens *Escherichia coli*, *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, *Yersinia pestis*, and *Bordetella spp*.. Apparently, synthesis of PIA and related polysaccharides is a general feature important for biofilm formation in diverse bacterial genera. Current knowledge about the structure and biosynthesis of PIA and related polysaccharides is reviewed. Additionally, information on their role in pathogenesis of biomaterial-related and other type of infections and the potential use of PIA and related compounds for prevention of infection is evaluated.

Keywords Adhesion, Biofilm, *Escherichia coli*, Foreign body-related infection, Polysaccharide intercellular adhesin, *Staphylococcus epidermidis*, Vaccines

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Contents

1 Introduction

The study of biofilms is gaining in importance in many areas of microbiology, including the industrial and clinical arenas. This review concentrates on aspects of biofilms in a clinical context and specifically considers those produced by the coagulase-negative staphylococci, mostly *Staphylococcus epidermidis*, and other medically important microorganisms.

A biofilm is a complex consortium of adherent microorganisms encased by a polymeric matrix [1]. Organisms in the biofilm state behave differently from their 'free' planktonic counterparts, and inter-bacterial communication occurs within the community (a process known as quorum sensing). Many pathogenic bacteria can produce biofilms, but the archetypal examples are the staphylococci, which normally colonize human epithelium and mucous membranes [2]. *Staphylococcus aureus* causes many important syndromes of infection and is highly pathogenic [3, 4], whereas *S. epidermidis*, which is ubiquitous on human skin, was for many years considered to be a harmless commensal [2, 5]. However, *S. epidermidis* has in recent years also emerged as an important pathogen, and is now among the five most frequent organisms causing nosocomial infection [6, 7]. Its clinical importance is directly related to its propensity to form biofilms, which attach to biomaterials such as those used in medical devices [8–10]. Such devices include intravenous catheters, joint prostheses, cardiac valve prostheses, pacemakers, cerebrospinal fluid shunts and peritoneal dialysis catheters. The increasing use of all these in modern medicine has propelled *S. epidermidis* into clinical prominence.

A four-stage model has been used to describe the development of a bacterial biofilm. The stages comprise, first, primary attachment of cells to the underlying biomaterial, second, accumulation in multiple bacterial layers, third, maturation of the biofilm, and fourth, detachment of planktonic cells from the biofilm, which may then initiate a new cycle of biofilm formation elsewhere [11]. In the staphylococci clear evidence exists for the attachment and accumulation/maturation stages of the biofilm model [12] (Fig. 1), while active detachment mechanisms are not well

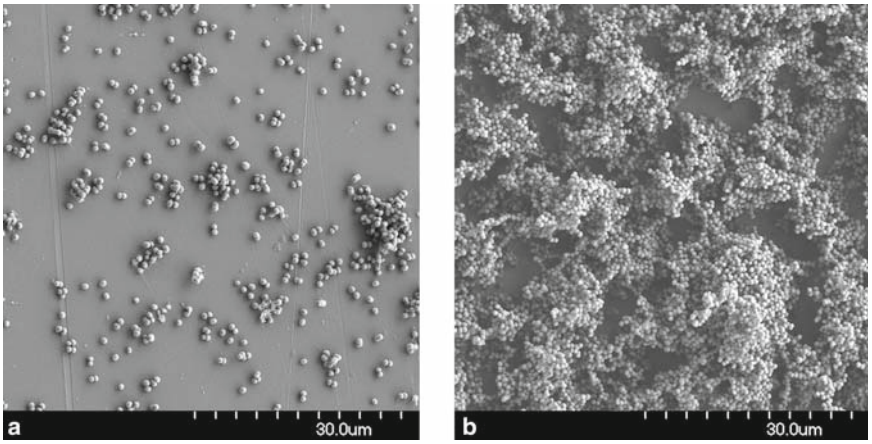

Fig. 1 Scanning electron microscopy of *S. epidermidis* 1457 biofilm formed on stainless steel after 2 h (**a**) and 18 h (**b**) of incubation. Scanning electron microscopy was performed as described previously [12]

characterized [13]. Antibiotic treatment of organisms in a biofilm is extremely difficult. Various explanations have been put forward for this, including poor antibiotic penetration of the biofilm, enzyme inactivation of antimicrobials, efflux, and variation in oxygen tension gradients, but the major factor is likely to be a result of poorly-understood metabolic changes in the bacteria which render them less susceptible [14]. In addition, release of planktonic bacteria into the bloodstream may lead to persistent bacteraemia and seeding to other indwelling devices, as described in the four stage model. These difficulties mean that it is often necessary to remove or replace infected devices, which may place the patient at considerable risk, and in some cases be devastating as well as financially costly.

2 Primary Attachment

Primary attachment occurs by both specific and non-specific mechanisms, and to the native surfaces of implanted biomaterials as well as to surfaces primed by adsorption of host-derived plasma proteins, extracellular matrix (ECM) proteins and coagulation products (platelets and thrombi) (Fig. 2) [20, 21].

2.1 Native Surface Attachment

The level of attachment is usually greatest to a native surface, aided in part by the hydrophobicity of the staphylococcal cell surface and the surface of the biomaterial [22–30].

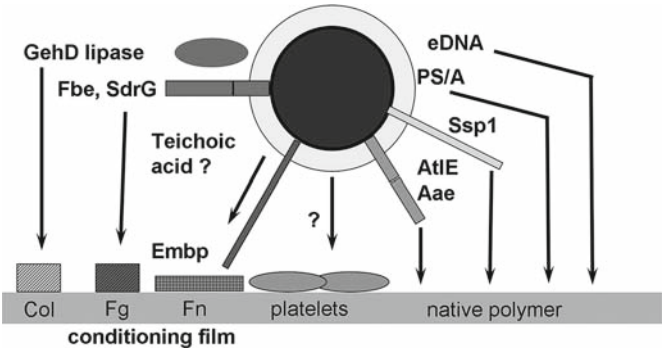

Fig. 2 Scheme of attachment mechanisms employed by *S. epidermidis* on native and conditioned polymer surfaces [2]. After implantation biomaterial surfaces are rapidly covered by a conditioning film composed of extracellular matrix proteins, thrombi, and activated platelets. *S. epidermidis* will attach to either native or conditioned surfaces employing a variety of different adhesive molecules. Fbe, SdrG: *S. epidermidis* fibrinogen binding protein [15, 16]; Embp: *S. epidermidis* fibronectin binding protein [17]; AtlE: *S. epidermidis* autolysin; Aae: *S. epidermidis* autolysin/adhesin; Ssp1: staphylococcal surface protein 1 [18, 19]; GehD: *S. epidermidis* lipase; PS/A: *S. epidermidis* polysaccharide/adhesin; eDNA: extracellular DNA; Col: collagen; Fg: fibrinogen; Fn: fibronectin

The most important adhesion molecule for native surfaces is *S. epidermidis* autolysin AtlE. This was identified by transposon mutagenesis in a mutant defective in attachment to polystyrene but which could still attach to glass [31, 32]. An AtlE-defective mutant was attenuated in virulence in a rat central venous catheter infection model [33], and in fact AtlE seems to have a dual role in attachment, as it also binds specifically to the ECM protein vitronectin [32, 34].

The 220 kDa cell-wall associated protein Ssp1, which is organised in a fimbria-like structure, mediates attachment to polystyrene in an *S. epidermidis* strain [18, 19] but has not been further studied. In addition, a capsular polysaccharide adhesin, PS/A, has been identified in *S. epidermidis* RP62A [35]. This mediates primary attachment to unmodified silastic catheter surfaces, but not to other types of polymers such as polyethylene [36]. Later studies have shown that PS/A is structurally very similar if not identical to polysaccharide intercellular adhesin (PIA) of *S. epidermidis* [37–42]. This will be discussed in more detail in the next section of this review.

Interestingly, chromosomal DNA released from bacterial cells (extracellular DNA/eDNA) has been shown to support *Pseudomonas aeruginosa* biofilm development [43]. DNAse I inhibited nascent biofilm formation, and when added to established but still young biofilms disintegrated them to some extent, whilst the effect on mature (>82 h) biofilms was not as marked. The findings indicate a role for eDNA in early *P. aeruginosa* biofilm development [43].

Recently published work has described how eDNA released by *S. epidermidis* and *S. aureus* also contributes to biofilm formation [44–47]. Again DNase I was found to degrade eDNA and thereby inhibit nascent biofilm formation as well as disintegrating pre-formed biofilms on some occasions [44–47]. However, analysis of primary attachment of DNAse I treated *S. epidermidis* cells indicated that eDNA acted predominantly in the early attachment phase of biofilm formation [47].

Release of eDNA from the staphylococci involved murein hydrolyses like CidA of *S. aureus* and AtlE of *S. epidermidis* [46, 47]. Whether the previously discovered role of *S. epidermidis* AtlE in primary attachment [32, 48] relies entirely on its effect on release of eDNA [47] remains an intriguing question.

2.2 Attachment to Extracellular Matrix Proteins

Specific binding to surface ECM proteins (such as fibronectin, fibrinogen, collagen, thrombospondin, and vitronectin) involves cell-wall associated adhesins known as MSCRAMMs (*m*icrobial *s*urface *c*omponents *r*ecognising *a*dhesive *m*atrix *m*olecules) [49]. As well as the autolysin/adhesin AtlE, mentioned above, another *S. epidermidis* surface-associated autolysin, Aae, has been described, which also binds to vitronectin, though not to polystyrene [50]. The gene encoding the fibrinogen-binding protein Fbe/SdrG [15, 16] is common in clinical *S. epidermidis* isolates [15, 51–53], but its expression must be highly variable as many strains do not bind avidly to fibrinogen [15]. Fbe-specific antibodies block adherence of *S. epidermidis* to fibrinogen-coated catheters [54] and attenuate infection in animal models [55, 56]; hence Fbe has potential as a vaccine candidate.

The giant 1 MDa fibronectin-binding protein Embp [17] is also present in the majority of clinical *S. epidermidis* isolates [51, 52] but antibodies directed against it were not sufficiently opsonic to enhance phagocytosis [55]. Binding of *S. epidermidis* to fibronectin-modified surfaces is also enhanced by purified teichoic acids [57]. Force dynamics of *S. epidermidis* binding to fibronectin-coated surfaces have been studied using scanning force microscopy, however, the fibronectin binding receptor employed by the respective isolate remained undefined [58].

The lipase GehD is secreted by *S. epidermidis* and specifically binds collagen [59]. Like AtlE, it displays not only enzymatic activity but also functions as an adhesin. The factor(s) responsible for *S. epidermidis* binding to thrombospondin have yet to be identified.

3 Biofilm Accumulation

The predominant mechanism of biofilm accumulation in staphylococci involves polysaccharide intercellular adhesin (PIA) [60–66]. *S. epidermidis* strains lacking this adhesin are also regularly isolated from biomaterial-related infections, a fact which prompted a search for an alternative, PIA-independent accumulation mechanism [51, 52, 60–62, 67, 68]. The responsible molecule was identified as accumulation associated protein, Aap [69–71], and there may also be a role for additional proteinaceous intercellular adhesins [52]. Aap has a similar-acting homolog, SasG, in *S. aureus* [72], adding to the accumulating evidence that proteinaceous intercellular adhesins are also of importance in *S. aureus* biofilm formation and device-related infection [52, 72–74].

3.1 Polysaccharide Intercellular Adhesin (PIA)

PIA is the major functional component for intercellular adhesion in *S. epidermidis* biofilms [60–62]. The enzymes required by *S. epidermidis* to synthesize PIA are encoded by the *icaADBC* locus [31, 75], which is also found in *S. aureus* [64, 65], *S. caprae* [76], and *S. lugdunensis* [77], while homologous DNA sequences have been detected in other coagulase-negative staphylococci (Table 1) [64, 78].

Interestingly, orthologous gene loci, *pgaABCD* or *hmsHFRS*, involved in synthesis of polysaccharides used in biofilm formation occur in a wide variety of eubacteria, including important human pathogens such as *Escherichia coli* [79], *Aggregatibacter (Actinobacillus) actinomycetemcomitans* [80], *Actinobacillus pleuropneumoniae* [80], *Yersinia pestis* [84–86], and *Bordetella spp.* [82, 83]. This suggests that PIA synthesis is a general mechanism employed in biofilm formation by a diverse group of Gram-positive and Gram-negative eubacteria (Table 1; Fig. 3).

3.1.1 Structural Analysis of PIA and Related Polysaccharides of Staphylococci

The structural analysis of polysaccharides involved in biofilm formation of *S. epidermidis* has been hampered repeatedly by difficulties with the specific identification and purification of the molecules involved in biofilm formation. Not infrequently, components from the media used to grow the bacteria have been purified and analyzed leading to erroneous conclusions [41, 87]; for a review see also [88]. A significant step forward in the purification and elucidation of composition and structure of the functionally important polysaccharides was the isolation of isogenic biofilm-negative transposon

Table 1 Presence of *icaADBC* homologous or orthologous loci in various Gram-positive and Gram-negative bacteria

Gram-positive	Gram-negative
Staphylococcus epidermidis [31]	*Escherichia coli* [79]
Staphylococcus aureus[64, 65]	*Aggregatibacter actinomycetemcomitans* [80]
Staphylococcus caprae[76]	*Actinobacillus pleuropneumoniae* [81]
Staphylococcus saprophyticus [78]	*Bordetella pertussis* [82]
Staphylococcus lugdunensis [64, 77]	*Bordetella bronchiseptica* [82, 83]
Staphylococcus capitis [64, 78]	*Bordetella parapertussis* [82]
Staphylococcus sciuri [78]	*Yersinia pestis* [84–86]
Staphylococcus caseolyticus [78]	
Staphylococcus cohnii [78]	
Staphylococcus condimenti [78]	
Staphylococcus simulans [78]	
Staphylcoccus pasteurii [64]	
Staphylococcus auricularis [64]	
Staphylococcus intermedius [64]	
Staphylococcus piscifermentans [64]	

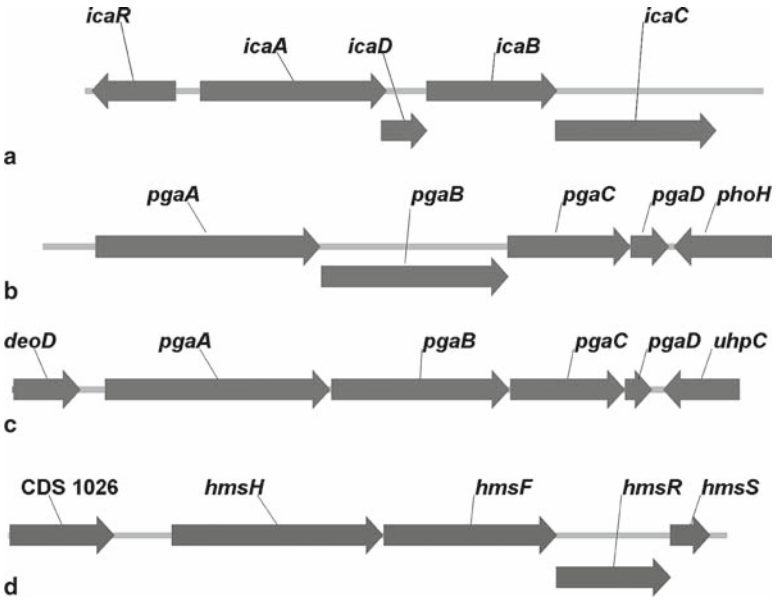

Fig. 3 Chromosomal regions containing gene loci involved in PIA synthesis of *S. epidermidis* RP62A (4500 bp, (**a**)), *E. coli* K12 (7866 bp, (**b**)), *A. actinomycetemcomitans* (7824 bp, (**c**)), and *Y. pestis* (8380 bp, (**d**)). *icaB, pgaB, and hmsF* and *icaA, pgaC, and hmsR* show close homology and represent *N*-deacetylase and glucosaminyl-transferase, respectively. For detailed description of the respective activity see text

insertion mutants from biofilm-positive wild-type strains. Comparison of parallel extracts of wild-type and mutant bacteria ascertained that detected sugars were indeed part of the functionally important molecules [40, 61].

The structure of PIA was originally determined for biofilm-forming *S. epidermidis* 1457 and RP62A grown in trypticase soy broth. PIA was extracted from the cells by sonication [40]. The polysaccharide was purified to homogeneity by gel filtration and anion-exchange chromatography [40] and separated by Q-Sepharose chromatography into a major polysaccharide I (>80%), which did not bind to Q-Sepharose, and a minor polysaccharide II (<20%), which was moderately anionic. As shown by chemical analyses and NMR spectroscopy, polysaccharide I is a linear homoglycan of β-1,6-linked 2-amino-2-deoxy-D-glucopyranosyl residues. On average 80–85% of them are *N*-acetylated, while the rest are non-*N*-acetylated and positively charged. Cation exchange chromatography separated molecular species whose content of non-*N*-acetylated glucosaminyl residues varied between 2% and 26%. Chain cleavage of PIA polysaccharide I by deamination with HNO_2 revealed a more or less random distribution of the non-*N*-acetylated glucosaminyl residues, with some preponderance of glucosaminyl-rich sequences. Polysaccharide II is structurally related to polysaccharide I but has a lower content of non-*N*-acetylated D-glucosaminyl residues. By colorimetric assay it was shown that polysaccharide II contains phosphate (see also discussion below), and ester-linked

succinate, rendering it anionic. Due to its function in biofilm accumulation of *S. epidermidis* it is referred to as polysaccharide intercellular adhesin (PIA) [40, 61]. A high apparent molecular weight was determined for PIA eluting in the void volume of Sephadex G200 [40, 61] or Sephacryl S300 columns (C. Fischer and D. Mack, unpublished results). However, methylation analysis revealed a ratio of reducing terminal sugar residues to total sugar residues of 1:130 and therefore an implied average M_r of 30,000 for PIA polysaccharide chains [40].

PIA turned out to be the hemagglutinin of *S. epidermidis* [89–91], and purified polysaccharide I of PIA inhibited hemagglutination [90]. The same preparations of PIA did not however promote hemagglutination of *S. epidermidis* on its own [90].

Raman spectra obtained from colonies grown on agar differentiated clonally distinct strains of *S. epidermidis*, but generated almost indistinguishable spectra for PIA-positive wild-type strains *S. epidermidis* 1457 and 8400 and their respective isogenic PIA-negative *icaA*-insertion mutants – indicating that the polysaccharide did not grossly influence the overall spectrum generated by whole bacterial cells [92].

Joyce and co-workers [38] used NMR spectroscopy and chemical analysis to analyse in detail polysaccharide produced by biofilm-producing *S. aureus* MN8m, a spontaneous mucoid mutant of *S. aureus* MN8 overexpressing the polysaccharide because of a five base pair deletion in the promoter region of the synthetic *icaADBC* locus [65, 93]. In their study they referred to the polysaccharide as *Staphylococcus aureus* exopolysaccharide (SAE) [38]. *S. aureus* MN8m was grown in a fermenter using soy peptone, yeast extract medium and purified from the supernatant of the fermentation medium [38]. The basic structure of a β-1,6-linked *N*-acetylglucosamine (GlcNAc) homopolymer was confirmed and the presence of *O*-succinoylation at the 3- and 4-hydroxyl groups of the GlcNAc residues in the polysaccharide was demonstrated for the first time by NMR spectroscopy [38]. However, the presence of phosphate in SAE was not detected [38]. SAE preparations from *S. aureus* MN8m had a higher proportion of non-*N*-acetylated glucosamine residues (43%) as compared to *S. epidermidis* 1457 PIA (15–20%) [38, 40], but contained comparable amounts of ester-linked succinate (8–9% as compared to 6% in *S. epidermidis* 1457 PIA polysaccharide II). An apparent molecular weight for SAE of 346,000 was determined by size-exclusion chromatography. In contrast to the PIA polysaccharide I preparations [90], the exopolysaccharide isolated (SAE) was active in in vitro hemagglutination assays [38]. It was concluded that *S. aureus* strain MN8m produces a polymer that is chemically and biologically closely related to the PIA produced by *S. epidermidis* [38, 40].

Initially Tojo and colleagues had isolated and characterised a polysaccharide promoting attachment of *S. epidermidis* to silastic catheter surfaces referred to as PS/A [35]. After discovery of PIA and its synthesis by enzymes encoded by the *icaADBC* locus [31, 40, 48, 60, 61] it appeared that PS/A production also depended on the *icaADBC* locus [35, 37]. Isolation of PS/A by McKenney and co-workers revealed a polysaccharide of high molecular mass composed of β-1,6-linked glucosamine residues with a high degree of *N*-succinoyl and *N*-acetyl modification at the 2-position of the sugar ring [37]. *IcaADBC* was also found to be present in *S. aureus* [64, 65] and, following analysis of polysaccharide purified from *S. aureus* MN8m, a similar structure of β-1,6-linked GlcNAc with a high

degree of *N*-acetylation and *N*-succinoylation was proposed [65]. However, Maira-Litran and colleagues later found no *N*-succinoylglucosamine in the polysaccharide of the same strain [39]. The material was henceforth referred to as PNAG and was produced with a wide range of molecular masses that could be divided into three major fractions with average molecular masses of 460 kDa (PNAG-I), 100 kDa (PNAG-II), and 21 kDa (PNAG-III) according to gel filtration analysis using Sephacryl S300 [39]. It turned out that the *N*-succinoylation was an artefact generated through use of strong acids during the purification process [37–39, 41, 65].

Recently, Sadovskaya and colleagues investigated the structure of PIA produced by *S. epidermidis* RP62A and polysaccharide produced by *S. aureus* MN8m using tryptic soy broth, brain-heart infusion broth, and a variety of different culture conditions prior to extraction of the polysaccharide by sonication [42]. PIA was purified by Sephacryl S300 gel filtration eluting at the void volume (PIA-1) with further polysaccharide eluting later (PIA-2). NMR spectroscopy confirmed the basic structure of PIA and provided direct NMR-evidence for the presence of *O*-succinoylation in PIA from *S. epidermidis* RP62A [42]. Using Q-Sepharose anion-exchange chromatography the observed ratios of neutral and negatively charged PIA I and PIA II were similar to those previously described for *S. epidermidis* 1457 [40]. Importantly, Sadovskaya and colleagues compared the PIA and SAE prepared from *S. epidermidis* 1457 and *S. aureus* MN8m using identical conditions of extraction by sonication, Sephacryl S300 purification, and NMR spectroscopy [42]. They found that the polysaccharides from the column had similar elution profiles and virtually identical ^{1}H NMR spectra. Polysaccharide from *S. aureus* MN8m showed more pronounced peaks at 5.1, 2.7, and 3.0 ppm, indicating a higher proportion of charged groups, succinoyl residues and free amino groups, as compared to PIA from *S. epidermidis* RP62A [42]. When substituting 0.9% NaCl for phosphate buffered saline during purification of PIA no evidence for the presence of polysaccharide-bound phosphate was obtained by colorimetric analysis or NMR spectroscopy, suggesting that the initial observation of the presence of phosphate may have been due to carryover from the buffers used [40, 42].

In the future it will be interesting to determine whether the differences in molecular weights of PIA as calculated from the ratio of reducing sugar residues to total sugar by methylation analysis (approx. 130 sugar residues and M_r 30,000) [40] and the apparent molecular weights of PIA, PNAG, and SAE as determined by gel filtration analysis [38–40, 42], have any biologic significance or are solely due to inherent properties of the analytic methodology used [38, 40, 42]. A possible explanation could be that PIA forms aggregates in solution, which result in a high apparent molecular weight on gel filtration. If so, PIA might act simply by stably linking different cells within a biofilm by ionic interactions between positive and negative charges within the polysaccharide. However, occurrence of a specific proteinaceous receptor specifically binding to PIA remains another possibility.

In conclusion, PIA is a homoglycan of β-1,6-linked *N*-acetylglucosamine, containing positive charges due to a fraction of free 2-amino groups (no *N*-acetylation) and negative charges due to *O*-succinoyl ester residues (Fig. 4). There is now wide consensus that, despite possible variation in the degree of non-acetylated, free amino groups, *O*-succinoylation and possibly molecular size of PIA, P/SA, SAE, and PNAG they are the same chemical entity, namely PIA [8, 10, 41].

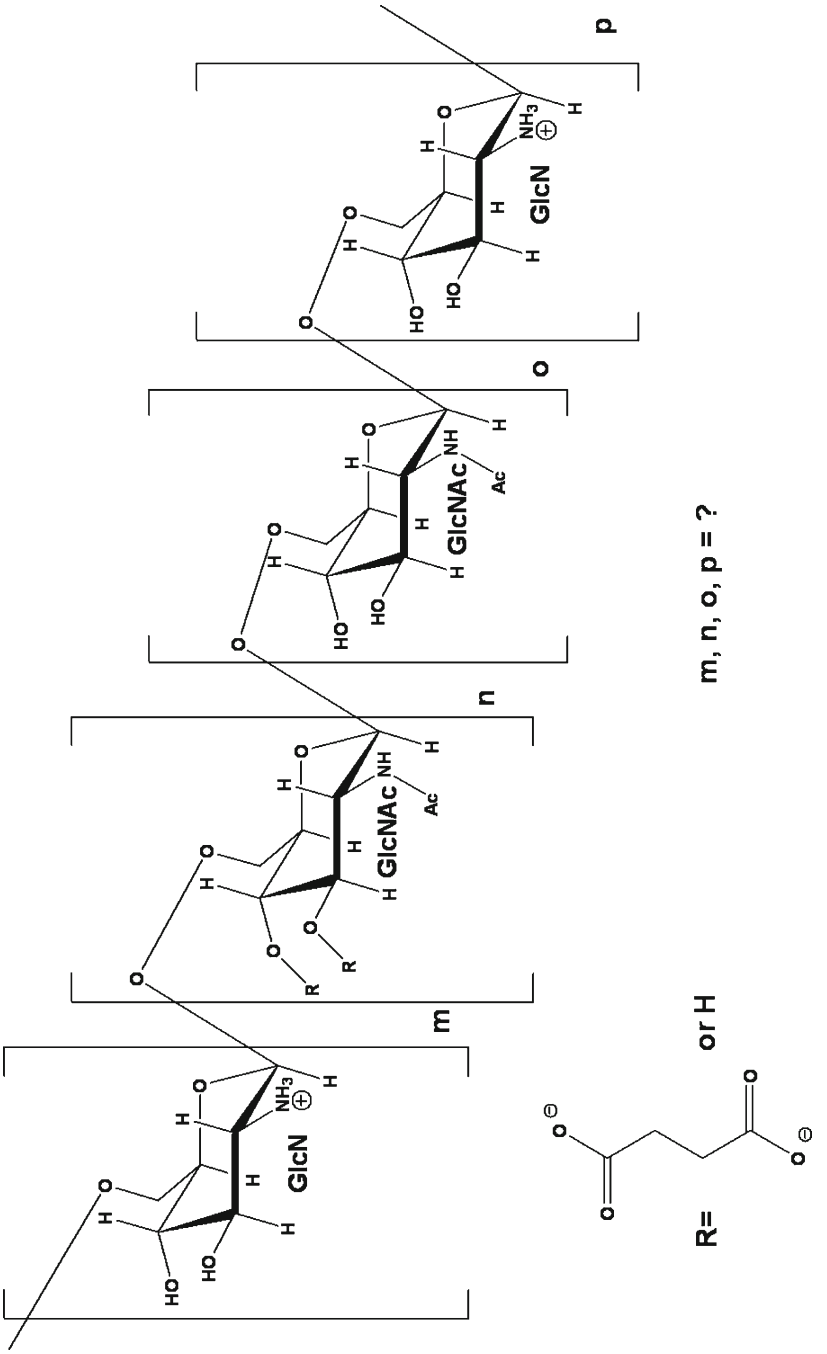

Fig. 4 Schematic representation of polysaccharide intercellular adhesin (PIA). The backbone of the unbranched polysaccharide consists of β-1,6-linked *N*-acetylglucosamine residues. PIA carries 15% non-*N*-acetylated, free 2-amino groups and *O*-succinoyl ester residues. The presence of negative and positive charges is apparently of functional relevance for intercellular adhesive properties of PIA

3.1.2 Structural Analysis of PIA-Related Polysaccharides of Gram-Negative Bacteria

Polysaccharides of bacteria other than staphylococci which require *icaADBC*-orthologous genes like *pgaABCD* for synthesis have been best characterised in *E. coli* [79, 94, 95]. Extracts of *E. coli* TRXWEC expressing either cloned *pgaABCD* or the same strain with a control plasmid, respectively, were analysed by gel filtration. A hexosamine containing polymer with apparent molecular weight >400,000 was consistently identified in the *pgaABCD* expressing strain [79]. NMR spectroscopy of the purified polysaccharide revealed β-1,6-linkage of GlcNAc moieties. The same analysis showed only low amounts of non-*N*-acetylated GlcNAc (≤3%) and also suggested the presence of signals consistent with presence of low amounts of succinoylation [79]. Expression of PIA-related polysaccharide, referred to as PGA, was confirmed using PIA-specific antiserum [29, 94]. Ninhydrin analysis of free amino groups in purified PGA revealed 5% of free amino groups [95].

Direct evidence for production of PGA has been inferred from observation of immunoreactive polysaccharide in *pgaABCD*-positive *A. actinomycetemcomitans* using immunofluorescence and specific anti-PIA antiserum [80]. Additional indirect evidence was obtained through the activity of dispersin B, a β-1,6-GlcNAc-specific hexosaminidase produced by *A. actinomycetemcomitans* which specifically degrades PIA and structurally closely related polysaccharides [85, 96, 97], which was also found to disintegrate *A. actinomycetemcomitans* and *A. pleuropneumoniae* biofilms [80].

Purified PGA of *A. pleuropneumoniae* serotypes 1 and 5 was further studied using NMR spectroscopy and chemical analysis, which confirmed the basic structure of β-1,6-linked GlcNAc polymers [81]. Preparations of PGA from different *A. pleuropneumoniae* strains had between 1% and 22% non-*N*-acetylated amino groups, while there was no evidence for any covalent succinate modification [81].

PGA was firmly attached to *A. actinomycetemcomitans* and could only be extracted by hot phenol extraction. These polysaccharide preparations remained contaminated by lipopolysaccharide, however, some analysis by NMR was possible confirming a β-1,6-linked GlcNAc backbone for PGA in this species. No information was obtained regarding the degree of non-*N*-acetylated amino groups and succinoylation of PGA of *A. actinomycetemcomitans* [98].

Evidence that the *hmsHFRS* locus of *Y. pestis* is responsible for synthesis of a polysaccharide involving the basic β-1,6-glycosidic linkages of GlcNAc was first obtained indirectly by the observation that *Y. pestis* biofilms were inhibited by the specific hexosaminidase dispersin B of *A. actinomycetemcomitans* mentioned above [85]. Interestingly, preformed *Y. pestis* biofilms were not disintegrated by dispersin B, indicating that either these biofilms are stabilised by additional mechanisms or access of the glycosidase is prevented by unknown means in mature biofilms [85]. Direct evidence for *hmsHFRS*-dependent synthesis of a PIA-homologous polysaccharide was obtained recently by immunochemical assay using PIA-specific antiserum [86].

Similarly, a number of *Bordetella spp.* including *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* synthesise a polysaccharide cross-reactive with *S. aureus* PNAG-

specific antiserum [99] in immunodot-blot assays [82]. This was shown to be dependent on activity of the *bpsABCD* locus [82, 83]. *B. brochiseptica* and *B. parapertussis* biofilm formation was also inhibited by the specific glucosaminidase dispersin B, providing further evidence for the production of a polysaccharide with similar basic structure to PIA [82]. Information about non-*N*-acetylated GlcNAc content or succinoylation of the *Bordetella spp.* polysaccharides has not yet been reported.

3.1.3 Biosynthesis of PIA and Related Polysaccharides

PIA synthesis depends on the activity of the *icaADBC* locus of *S. epidermidis* (Fig. 3) [31]. The operon comprises four open reading frames, *icaA, icaD*, *icaB* and *icaC* [75]. Upstream of the *icaA* start codon is *icaR*, encoding a 185 amino acid (aa) tetR-type transcriptional regulator with a proposed HTH DNA binding motif at its *N*-terminus. IcaR negatively controls *icaADBC* transcription and is part of a regulatory network governing transcription of *icaADBC* including the alternative sigma factor σ_B, *sarA*, *sarZ*, ClpP, and quorum sensing regulator *luxS* [100–108]. IcaR has a similar function in *S. aureus* [93, 109], in which the regulatory network of *icaADBC* expression is similarly complex. In *S. lugdunensis icaR* is missing and PIA is apparently not regularly synthesised in *S. lugdunensis* biofilms [77, 110] . Regulation of *icaADBC* expression is significantly different in *S. aureus* and *S. epidermidis* [111] and is beyond the scope of this article; however, there are a number of recent reviews covering this important topic [112–115].

Expression of all four *icaADBC* genes is required for synthesis of a functionally active PIA molecule (Fig. 5a) [10]. PIA synthesis was studied in detail in a reconstituted in vitro polysaccharide synthesis system composed of membrane fractions of recombinant *S. carnosus* expressing various combinations of the *icaADBC* genes and with UDP-GlcNAc as a sugar donor [75]. IcaA belonging to the glycosyltransferase 2 family is an integral membrane protein comprising 412 aa and 4 predicted transmembrane domains [31, 75, 116]. IcaA alone directs the synthesis of β-1,6-linked GlcNAc oligosaccharides of up to 20 GlcNAc units. IcaD, a 101 aa integral membrane protein with two potential membrane spanning domains, is required for full activity of IcaA in vitro. It may be a chaperone directing folding and membrane insertion of IcaA and may act as a link between IcaA and IcaC [75]. IcaC, a 355 aa integral membrane protein with 10 predicted transmembrane domains, is essential for the synthesis of intact, immunoreactive PIA, and may be involved in externalisation and elongation of the growing polysaccharide [75].

IcaB, in its mature form, is a 259 aa secreted protein with a predicted signal sequence, and belongs to the polysaccharide deacetylase family including, for example, chitin deacetylases or the chitooligosaccharide deacetylase NodB of *Rhizobium melioti*. IcaB is the deacetylase responsible for the de-*N*-acetylation of PIA, and is crucial for activity of PIA in biofilm formation and for virulence both in *S. epidermidis* [117] and *S. aureus* [118]. PIA synthesised by *S. epidermidis* and *S. aureus* Δ*icaB*-mutants, where the *icaB* genes have been deleted by allelic gene

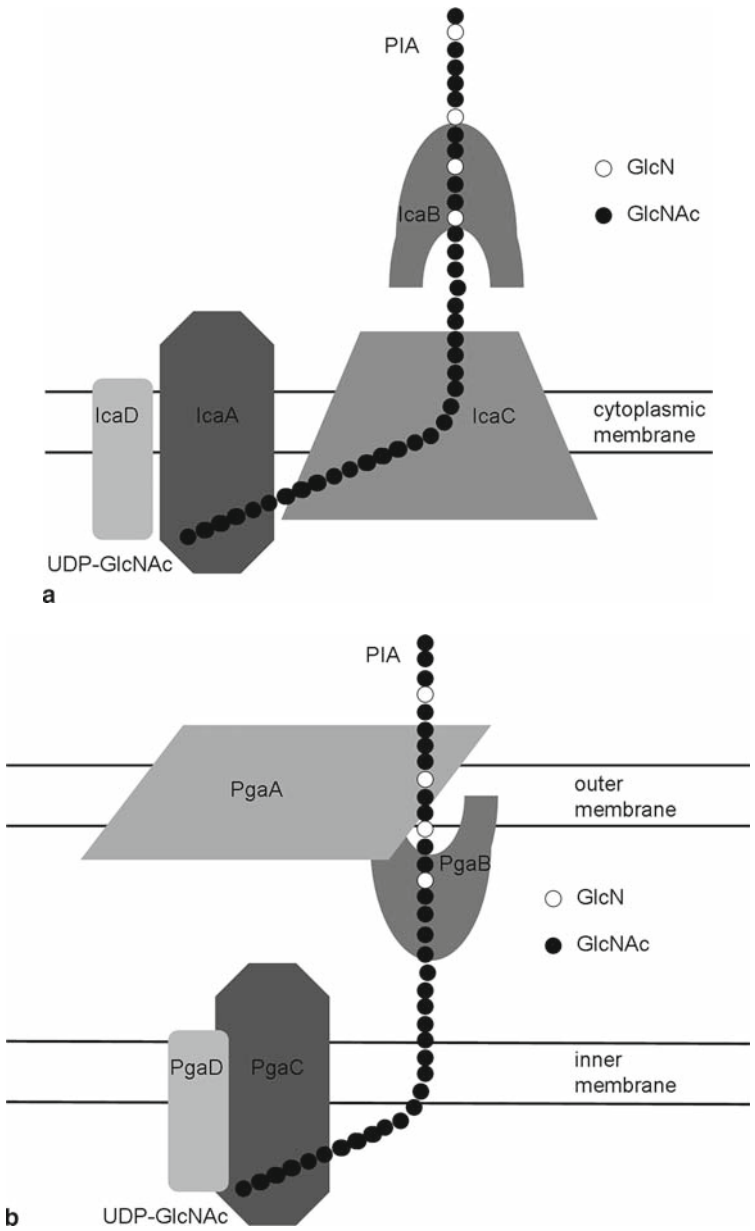

Fig. 5 Scheme of the biosynthetic machinery for PIA synthesis in *S. epidermidis* as proposed by Götz [10] (**a**) and for *E. coli* as proposed by Romeo and colleagues [95] (**b**). **a**: IcaA and IcaD proteins are integral membrane proteins in the cytoplasmic membrane of *S. epidermidis* using UDP-GlcNAc to synthesise β-1,6-linked GlcNAc oligomers, which are elongated and translocated across the cytoplasmic membrane supported by IcaC. IcaB is a secreted protein removing some (approx. 15%) of acetyl groups from GlcNAc residues. It is currently unknown if there are explicit protein-protein interactions between any of the four proteins encoded by *icaADBC*. **b**: PgaC, the homolog of IcaA in *E. coli*, is located together with PgaD in the inner membrane synthesising PIA from UDP-GlcNAc. PgaB, an outer membrane protein, is believed to bind PIA in the periplasmic space, de-acetylating some of the amino groups of GlcNAc residues, and directing the polysaccharide to the pore formed by PgaA in the outer membrane, which then translocates PIA to the bacterial exterior (for details see text)

replacement, does not contain non-*N*-acetylated GlcNAc and as a consequence is poorly retained on the cell surface [117, 118]. It is not currently known whether IcaB acts only on nascent PIA during synthesis or whether secreted IcaB in purified form might also increase the degree of de-*N*-acetylation of PIA after its synthesis or in purified form.

Interestingly, membrane preparations of *icaADBC*-positive *S. epidermidis* 1457 grown in TSB lacking glucose promoted production of immunoreactive PIA in vitro, when UDP-GlcNAc was provided, whilst in vivo bacteria grown under the same conditions did not synthesize PIA due to the essential requirement of an as yet uncharacterized glucose-dependent protein factor [119].

Which biosynthetic steps are required for *O*-succinoylation of PIA is currently unresolved. Recently, the chemical synthesis of oligosaccharides comprising up to 11 β-1,6-linked glucosamine residues and their respective per-*N*-acetylated counterparts has been accomplished [120]. This might be of value in antigen preparation for specific immunochemical assays or as haptens for generation of specific antibodies.

In Gram-negative bacteria PGA biosynthesis has been best characterized for *E. coli* and *Y. pestis* [79, 86, 95]. Only two of the *pgaABCD* or *hmsHFRS* genes have homology to genes of the *icaADBC* locus, namely the glycosyltransferases *pgaC*, *hmsR* and *icaA* and the *N*-deacetylases *pgaB*, *hmsF* and *icaB*, respectively. This is to be expected as the cell wall architecture of Gram-positive and Gram-negative bacteria is fundamentally different and therefore has very different requirements for the transport of polysaccharide to the cell exterior. Interestingly, while it is assumed that in the synthesis of PIA by staphylococci IcaC somehow is involved in the translocation of the growing polysaccharide chain over the cytoplasmic membrane [10], in the Gram-negative synthetic machinery no equivalent of IcaC is present in the cytoplasmic membrane unless PgaD would function in this respect, while translocation of the polysaccharide via the outer membrane is thought to involve PgaA [95] (Fig. 5b). Comparison of the biosynthesis of PIA in Gram-positive and Gram-negative organisms may reveal important clues how the organisms deal with this important task to translocate these large hydrophilic molecules across membranes.

All proteins of the *pgaABCD* and the *hmsHFRS* loci are required for *E. coli* and *Y. pestis* biofilm formation, respectively [79, 95, 121]. PgaC is a 441 aa inner membrane protein including a single glycosyltransferase 2 domain and five predicted transmembrane domains. Its *N*-terminus is predicted to be in the periplasm and its *C*-terminus in the cytoplasm. However, the homologous glycosyltransferase HmsR of *Y. pestis* involved in PIA synthesis has been shown by fusion-protein analysis and two-hybrid systems to have only four transmembrane domains, with the *N*- and *C*-terminal sequences in the cytoplasm, despite prediction of a similar domain structure to PgaC [86]. This serves to highlight that though these predictions are valuable models, they require experimental confirmation.

PgaD is a 137 aa inner membrane protein with two predicted transmembrane regions and *N*- and *C*-terminal regions predicted to reside in the cytoplasm. Fusion-protein analysis and two-hybrid systems confirmed this prediction for

HmsS, the homologous protein of *Y. pestis* [86]. In both Δ*pgaC*- and Δ*pgaD*-deletion mutants of PGA-overproducing *E. coli* TRMG, accumulation of immunoreactive PGA was not detected indicating that both are required for efficient synthesis of PGA [95].

PgaA is a 791 aa protein with an *N*-terminal signal sequence and no obvious homologous genes of known structure and function. In a Δ*pgaA*-mutant PGA was still synthesised, but rather than being partially released into the culture medium under shaking culture conditions it was retained on the cell surface. In immunoelectronmicroscopy the periplasm of mutant cells was dramatically expanded and PGA accumulated at the cell poles [95]. Homology HHpred server (http://toolkit.tuebingen.mpg.de/hhpred) identified a similarity at the *N*-terminus of PgaA with a tetratricopeptide repeat domain of human nucleoporin *O*-linked GlcNAc transferase, yeast mitochondrial outer membrane translocon protein Tom70p, and human peroxisomal targeting signal-1 receptor PEX5 [95]. At the *C*-terminus there was similarity with *E. coli* long-chain fatty acid transporter protein FadL, forming a membrane pore composed of 14 antiparallel ß strands [122]. Therefore PgaA might also form a pore structure essential for transporting PGA from the periplasm through the outer membrane to the cell exterior [95].

PgaB is a 652 aa outer membrane lipoprotein with an *N*-terminal signal sequence and a predicted lipidation site at Cys_{21}. In addition to the *N*-deacetylase domain *pgaB* contains a *C*-terminal domain of unknown function, which according to HHpred has homology with carbohydrate binding and modifying domains [95]. In a Δ*pgaB*-mutant PGA also accumulated on the cell surface and in the periplasmic space, similar to the Δ*pgaA*-mutant. Release of PGA into the culture medium at 37 °C was severely diminished, but not completely abolished as in the Δ*pgaA*-mutant [95]. Analysis of the PGA for free amino groups in the Δ*pgaB*-mutant revealed a significant decrease (0%) as compared to the wild-type (5%) and a *pgaB* overexpressing strain (22%), directly confirming the function of PgaB as the responsible de-*N*-acetylase [95].

A model has been proposed for PGA synthesis whereby PGA is assembled from UDP-GlcNAc by PgaC and PgaD residing in the inner membrane of *E. coli* [95] (Fig. 5b). It appears there is no equivalent of IcaC for transport of growing PIA across the inner membrane. In the periplasmic space the nascent polysaccharide is bound by the proposed PGA-binding domain of PgaB, consecutively de-*N*-acetylated by its *N*-deacetylase activity and handed over to PgaA, forming a β-barrel pore in the outer membrane which allows export of the polysaccharide to the cell surface [95]. In support of this model, experimental evidence suggests that HmsR and HmsS involved in PIA synthesis of *Y. pestis* do physically interact in a complex also involving regulators HmsP and HmsT in the inner membrane [86]. Additionally, the putative β-barrel protein HmsH and HmsF are localised in the outer membrane of *Y. pestis* [123, 124].

Similar to the situation in staphylococci, information has not yet been obtained about the catalytic enzymes involved in succinoylation of PIA-related polysaccharides produced by Gram-negative bacteria, as far it has been substantiated as a component of those polysaccharides.

3.2 Importance of PIA and Biofilm Formation for Virulence of Staphylococci

The contribution of biofilm formation to virulence of coagulase-negative staphylococci, mostly *S. epidermidis*, has attracted considerable attention due to the epidemiological observation of frequent occurrence of biofilm-positive *S. epidermidis* strains in significant clinical infection (reviewed in [125]). However, early studies into the pathogenicity of clinical *S. epidermidis* isolates using a number of different foreign-body infection models, such as the mouse or rat subcutaneous catheter infection models or rabbit catheter-induced endocarditis models, failed to demonstrate conclusively that biofilm-producing *S. epidermidis* clinical isolates were more virulent (for a review see [2]).

Later, isogenic pairs of biofilm-positive wild-type and biofilm-negative mutants were used to study the contribution of PIA to virulence in *S. epidermidis* in several infection models. In a subcutaneous catheter infection model in mice and a central venous catheter infection model in rats [126–128], biofilm-positive, PIA-producing *S. epidermidis* 1457 was more virulent than its isogenic biofilm-negative transductant 1457-M10 which contained a Tn917 insertion in *icaA* [90]. This result was confirmed with an independent isogenic strain pair obtained from biofilm-positive *S. epidermidis* O-47 using the same models [33]. Furthermore, expression of *icaRADBC* in *icaADBC*-negative *S. epidermidis* strains also led to increased virulence in a rat central venous catheter model [129]. In a *Caenorhabditis elegans* infection model biofilm-positive *S. epidermidis* 9142 killed the worms more rapidly and accumulated to higher intestinal concentrations than its isogenic *icaA*-insertion mutant 9142-M10 [130]. Complementation of the biofilm-negative mutant with cloned *icaADBC* restored virulence parameters to wild-type levels [130].

In line with these observations, biofilm-positive, PIA-positive *S. epidermidis* 1457 displayed decreased susceptibility to killing by antimicrobial peptides like LL-37, dermcidin and human β-defensin 3 as well as decreased phagocytosis and killing by polymorphonuclear granulocytes (PMNs) as compared to its isogenic *icaA*-insertion mutant 1457-M10 [131]. *S. epidermidis* 1457 grown as a biofilm was significantly protected from phagocytic killing after opsonisation with normal human serum as compared to the same strain grown planktonically, which was killed to the same extent as planktonic cells of its isogenic biofilm-negative *icaA*-insertion mutant [132]. Biofilm-positive wild-type bacteria pre-opsonised with normal human serum were killed less efficiently than the isogenic biofilm-negative cells [132]. Killing depended significantly on activation of classic or lectin-mediated complement pathways. Biofilm-positive *S. epidermidis* 1457 induced more C3a, but C3b and human IgG surface deposition was decreased compared to the isogenic biofilm-negative mutant. In bacteria derived from explanted infected catheters from a mouse catheter infection model, which reproduced the previously observed virulence differences in the isogenic strain pair, ex vivo phagocytic killing by PMNs of biofilm-positive wild-type bacteria was decreased, probably due

to less sufficient opsonisation of biofilm bacteria in vivo [132]. Biofilm-grown cells of *S. epidermidis* 9142 were killed less efficiently than planktonically grown cells opsonised with antibodies raised against de-*N*-acetylated PIA/PNAG [99, 133]. Confocal microscopy indicated that anti-PIA/PNAG antibodies diffused into *S. epidermidis* biofilms sufficiently to allow opsonisation [133, 134].

A Δ*icaB*-mutant of *S. epidermidis*, synthesizing only fully *N*-acetylated polymer, and hence displaying a biofilm-negative phenotype, had decreased resistance against phagocytosis, increased susceptibility to antimicrobial peptides LL-37 and human β-defensin 3 as well as attenuated virulence in a mouse catheter infection model [117].

However, it has to be borne in mind that *S. epidermidis* is an opportunistic pathogen and that virulence mechanisms important in one type of device-related infection might be less crucial in others. This is apparent in the guinea-pig tissue cage model [135], which showed no difference in virulence between a biofilm-positive wild-type *S. epidermidis* 1457 and its isogenic *icaA*-insertion mutant when animals were infected with both strains separately, or between *icaADBC*-positive and -negative clinical isolates [136, 137]. However, PIA was expressed in vivo in the tissue cages and, when both strains were used together, the wild-type out-competed the insertion mutant [138]. The explanation may lie in the fact that phagocytes are severely impaired in tissue cages [139], so that the expected advantage of the wild-type was not so clearly apparent in this context. Interestingly, *icaADBC* transcription becomes continuously down-regulated in the course of a chronic catheter infection in rats, indicating that initially synthesized PIA might have a long half-time in vivo, consistent with the fact that no PIA degrading enzymatic activity has yet been detected in staphylococci, in contrast to dispersin B of *A. actinomycetemcomitans* [96, 97, 138, 140].

Similarly, virulence of biofilm-positive *S. aureus* Sa113 was not significantly different from that of its isogenic *icaADBC*-deletion mutant in the guinea-pig tissue cage infection model and a variation of this model in mice [136, 141]. However, in murine models of systemic infection including mouse bacteraemia model, renal abscess model, and mouse lethality model, significant virulence differences of *S. aureus* wild-type strains and isogenic Δ*icaADBC*-mutants were demonstrated [142]. Clearing from blood after intravenous challenge 2 and 4 h post infection, bacterial load in kidneys 5 days after i.v. challenge, and lethality after intraperitoneal injection of bacteria was significantly different with three isogenic Δ*icaADBC*-mutants of *S. aureus* strains 10833, Newman and MN8 [142]. All three Δ*icaADBC*-mutants were also significantly more rapidly killed in an antibody-independent phagocytosis assay with infant rabbit serum as the complement source [142].

Y. pestis transmission from the flea to mammals depends upon blockage of the proventriculus of the flea, which is accomplished by a biofilm of dense aggregates of plague bacteria. This blockage prevents the flea from feeding and provokes repeated feeding attempts, thereby facilitating the transmission of *Y. pestis* [123, 143]. Biofilm formation of *Y. pestis* depends on the *hmsHFRS* locus and synthesis of PIA-related polysaccharide, indicating a role of these molecules in transmission of plague [84, 86, 124].

3.3 Proteinaceous Intercellular Adhesins

3.3.1 Accumulation Associated Protein

Despite PIA being a major mechanism of biofilm accumulation, strains of *S. epidermidis* lacking *icaADBC* may also produce biofilm, and biofilm-positive PIA-negative strains have been isolated in clinical contexts [51, 52, 63, 67, 68, 144–146]. It was therefore apparent for some time that another means of accumulation must exist, prior to the discovery that the cell-wall-linked accumulation associated protein, Aap, could mediate biofilm formation independently of PIA/polysaccharide [70]. Aap is anchored via an LPXTG motif and comprises a domain A, which may itself contain a lectin-like domain [147], and a repetitive domain B, composed of a variable number of 128 amino acid repeats [70]. These repeats are also called 'G5 domains' which hypothetically possess *N*-acetylglucosamine binding ability [147]. Proteolytic processing of Aap leads to removal of the *N*-terminal domain A, exposing domain B, which gives Aap intercellular adhesive properties causing biofilm accumulation [70]. The proteolytic activation may be mediated either by staphylococcal exoproteases or by the host, as part of the early immune response against *S. epidermidis* [4, 70]. Clearly the ability to respond to host proteases by forming a biofilm is in the organism's favour and enables it to evade phagocytic clearance, as indicated by the less efficient killing of Aap-aggregated *S. epidermidis* 5179-R1 as compared to its biofilm-negative, non-aggregated wild-type [70].

Aap is widespread in clinical isolates [51, 52, 148], and Aap-dependent biofilm formation can be directly demonstrated in clinical isolates [52, 70], so it seems likely that it is, like PIA, an important virulence factor. Aap has significant homology with cell wall protein SasG of *S. aureus* [149, 150], in which SasG-mediated biofilm accumulation has recently been demonstrated using the same mechanisms [72].

3.3.2 Other Surface Proteins

S. aureus strains of bovine origin have been found to produce a surface protein known as biofilm associated protein (Bap) which supports biofilm formation [151]. It remains uncertain whether Bap is important in the attachment or the accumulative phases of biofilm formation. A homologous protein was found in a few (4/38) biofilm positive *S. epidermidis* strains from ovine or caprine mastitis [152], but not in strains from humans [52]. Another surface protein, Bhp, which is related to Bap, is present in 10–19% of *S. epidermidis* isolates from human infections, so is also likely to be of only minor clinical importance [51, 151]. Recently, it has been shown that fibronectin-binding proteins FnbPA and FnbPB of *S. aureus* support biofilm accumulation of *S. aureus* independently of PIA-related mechanisms [74].

4 Use of Polysaccharide-Mediated Biofilm Accumulation Mechanisms in Prevention of Infection and Colonisation

4.1 Vaccine Potential of PIA-Related Polysaccharides

Bacterial capsular polysaccharides have been most successful in the generation of effective vaccines against major human pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* by conjugating the polysaccharides to protein carriers like diphtheria toxoid. Therefore, Pier and colleagues have investigated this route systematically for staphylococcal polysaccharides involved in biofilm formation, from the inital stages of PS/A over PNSG to PIA and PNAG, respectively [37, 39, 65, 99, 153].

In rabbits and mice purified PNAG was immunogenic and the polysaccharide fractions with the highest apparent molecular weight induced sera with the highest titres, while fractions with apparent M_r <100,000 were not immunogenic [39]. Rabbit immune sera induced opsonophagocytic killing of the biofilm-positive, PNAG-positive *S. epidermidis* M187 and three *S. aureus* wild-type strains expressing PNAG at various levels. The phagocytosis assay used was composed of human PMNs and baby rabbit serum as a complement source absorbed with *S. aureus* MN8m to remove antibodies reactive with the target strain, while the rabbit pre- and post-immune sera were absorbed with an *S. aureus* MN8m Δ*icaADBC*-mutant to provide the reaction with specificity to PNAG antigen [39]. Killing with immune serum was about 55–85%, while pre-immune serum never led to more than 15% killing [39]. It is not clear why *S. aureus* MN8m did not absorb all IgG antibodies from the antisera used, as *S. aureus* strains usually express protein A [39].

Maira-Litran and colleagues [99] compared vaccines comprising diphtheria toxoid (DT) conjugated either with native PNAG or with chemically modified dPNAG in which the *N*-acetyl groups had been removed to a degree of about 15% *N*-acetylation. Both conjugate vaccines were immunogenic in mice and rabbits; however, PNAG-DT primarily elicited antibodies with specificity to highly acetylated PNAG while dPNAG-DT elicited antibodies reactive with both PNAG and dPNAG. The opsonophagocytic killing of both antisera were tested in a system composed of PMNs, baby rabbit serum as a complement source pre-absorbed with the respective *S. epidermidis* or *S. aureus* target strains, and sera pre-absorbed with the *S. aureus* MN8m Δ*icaADBC*-mutant, as above [99]. Sera raised against dPNAG-DT had a more pronounced killing effect than those raised against PNAG-DT with several *S. aureus* strains and *S. epidermidis* M187 as targets. Opsonophagocytic killing by anti-dPNAG-DT was more strongly inhibited by dPNAG than PNAG absorption, leading to the hypothesis that the antibodies with the strongest biological activity are directed against the non-*N*-acetylated sugars in the PNAG polysaccharide [99]. Passive immunisation with anti-dPNAG-DT and anti-PNAG-DT enhanced bacterial clearance in a mouse bacteraemia model 2 h after i.v. challenge with four different *S. aureus* strains, while passive immunisation with a goat anti-dPNAG-DT serum protected mice against lethal challenge with *S. aureus* [99].

Human sera from children with cystic fibrosis have been found to contain antibodies against PNAG [154]. Patients colonised with *S. aureus* had higher PNAG-specific opsonophagocytic killing activity in their sera than non-colonised patients but average antibody titre levels were not significantly different [154]. Here antibodies directed against the non-*N*-acetylated backbone of the PNAG polysaccharide appeared to have superior opsonophagocytic activity. This notion was supported by the observation that a humanised monoclonal antibody F598 had the highest opsonophagocytic killing activity in a PNAG specific killing assay as described above as compared to two other mAbs [155]. F598 had a high amount of binding activity to both PNAG and dPNAG, while the inferior mAbs had much lower binding activity to dPNAG [155]. F598 was active against a variety of different *S. aureus* strains and *S. epidermidis* M187 and was also protective in a murine lethality model after intraperitoneal infection with *S. aureus* strain Reynolds [155].

As already discussed, deletion of *icaB* in both *icaADBC*-positive *S. aureus* and *S. epidermidis* leads to the synthesis of fully *N*-acetylated PIA, which is poorly retained on the cell surface rendering the respective mutants biofilm-negative and less virulent [117, 118]. Overexpression of *icaB* in *S. aureus* 10833 leads to increased biofilm formation resulting potentially from higher intercellular adhesion activity of PIA/PNAG containing higher amounts of non-*N*-acetylated amino groups, although the latter has not been shown directly [118]. Support for a prominent role for dPNAG specific antibodies in opsonophagocytosis comes from observations of the IcaB overexpressing strain, which was killed much better than wild-type in a PNAG-specific opsonophagocytosis assay using mAb F598 with specificity for both PNAG and dPNAG [118]. Interestingly, goat anti-dPNAG and anti-PNAG serum led to opsonophagocytic killing of PNAG-expressing *E. coli* strains isolated from urinary tract infection and bacteraemia [156]. Passive immunisation with the respective antisera also protected mice from lethality on intraperitoneal challenge with different *E. coli* strains in a murine lethality model [156].

Taken together, these are exciting results suggesting the possible use of PIA/PNAG as a vaccination target for active or passive immunisation, not only against staphylococcal infections, where it is urgently needed, but potentially also against other PIA/PNAG-expressing human pathogens like *E. coli*, *A. actinomycetemcomitans*, *Y. pestis* and *Bordetella spp*.. However, much remains to be learned. It is apparent that anti-PNAG antibodies have a clear stimulating effect on opsonophagocytosis in assays designed to show the specificity of these activities to PIA/PNAG. However, normal human sera contain already significant titres of anti-PIA/PNAG antibodies and have additional opsonic activity against staphylococci [4, 154, 157]. So it remains to be seen whether the generation of additional specific antibodies, eventually directed against fully de-*N*-acetylated PIA, can add any significant protective effect in those patients who typically come down with staphylococcal infections, such as immunocompromised patients with indwelling medical devices. The eventual prospect of full chemical synthesis of the respective antigens may aid in the evaluation of these important goals [120].

4.2 Specific Disruption of PIA-Dependent Biofilms

Dispersin B, as previously mentioned, is a specific glycosidase produced by *A. actinomycetemcomitans* and involved in this organism in biofilm detachment [96]. The 361 aa long protein belongs to the family 20 of glycosid hydrolases (GH-20) comprising enzymes such as β-hexosaminidase with different substrate specificities and activities [158]. In contrast to other enzymes of this family, which usually cleave β-1,4-glycosidic linkages between GlcNAc residues, dispersin B has specificity for β-1,6-linked GlcNAc and therefore degrades PIA produced by various other organisms including *S. epidermidis* [52, 85, 97], *S. aureus* [45, 52], *E. coli* [85], *Y. pestis* [85] and *Bordetella spp.* [82] as well as *A. actinomycetemcomitans* and *A. pleuropneumoniae* [80, 81, 98]. By degrading PIA into oligosaccharides down to monomers of GlcNAc [85], dispersin B interferes with biofilm formation of all the organisms mentioned above. For many of those, especially the staphylococci, it has also been shown that established biofilms are efficiently disintegrated [45, 52, 85, 97]. Coating of surfaces of polyurethane and teflon catheters with dispersin B prevented biofilm formation by *S. epidermidis* thereon, indicating that this enzyme might be useful in the prevention of staphylococcal infection of biomaterial surfaces [97].

An elegant approach has been used recently to deliver dispersin B to established *E. coli* biofilms. Dispersin B was cloned into bacteriophage T7 in a way which allowed expression of biologically active dispersin B when the recombinant phage infected *E. coli* biofilms [159]. The recombinant phage enhanced the biofilm-removing effect by 4.5 log10 units as compared to the wild-type phage and might therefore be useful for reducing bacterial biofilms in important medical and biotechnological applications.

5 Conclusion and Outlook

This review has attempted to summarise some aspects of what is known about the structure and biosynthesis of bacterial biofilm components, their role in the pathogenetic process of biomaterial-related infection and possible future applications of this knowledge. As is evident, much more research is needed before we can achieve effective prevention and treatment of biofilm-related infection, which is a major goal in light of the millions of patients affected in the developed world. Most excitingly, if these approaches related to PIA/PNAG polysaccharides are successful, they may also lead to solutions of infection problems with some of the most important human pathogens in hospital as well as the community like *S. aureus* and *E. coli*.

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