1

CARBOHYDRATE-PRESENTING SELF-ASSEMBLED MONOLAYERS: PREPARATION, ANALYSIS, AND APPLICATIONS IN MICROBIOLOGY

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1.1 INTRODUCTION

Carbohydrates are a complex class of essential biomolecules that can be considered as the dark matter of the biological universe as they are greatly understudied yet omnipresent in all kingdoms of life and vital to fully understand biological processes. The structurally diverse carbohydrates are present both on the cell surface and inside cells. They decorate the cell surface to form the so-called glycocalyx, a dense and complex layer of carbohydrates unique for every type of cell or organism, and as such are key to many important biological recognition events by interacting with carbohydrate-binding proteins. Carbohydrate–protein interactions play an important role in various biological events occurring at the cell surface, such as bacterial and viral infections [1,2], cancer metastasis [3,4], and immune response [4]. The study of the interactions between carbohydrates and other biomolecules at biological surfaces
and interfaces is instrumental in the understanding of these processes and contributing to the development of novel diagnostic methods and medicines. The study of carbohydrates, compared to, for example, nucleic acids and proteins, however, poses unique challenges because their structure is nonlinear and their biosynthesis not template driven. The native glycocalyx is too complex, dense, and dynamic for studying these interactions individually, with the current techniques at our disposal. Therefore, a simplified version is often created by the placement of well-defined, synthetic carbohydrates on a surface, so-called glycoarrays or glycosurfaces, to study specific carbohydrate–protein interactions. These fabricated glycosurfaces can also be more readily incorporated in a sensor or a nanostructure and as such used to elicit, detect, or quantify binding events, for example, in diagnostic devices, molecular imaging, and drug delivery applications. Various approaches have been developed to prepare glycosurfaces, each of them with their advantages and drawbacks, and these approaches will be the main focus of this chapter.

We will start the chapter by presenting an overview of the different methods most commonly used to prepare glycosurfaces. These methods will be discussed divided over three sections that each reflect one of the three distinct approaches used to create glycosurfaces: (i) direct formation of carbohydrate-containing self-assembled monolayers (SAMs), (ii) use of secondary (or tertiary) reactions to install a carbohydrate on a preformed SAM, and (iii) noncovalent immobilization of carbohydrates on a surface. The discussion of the secondary reaction approach (ii) is subdivided into two subsections: one addressing the use of unmodified “natural” carbohydrates and the other, the use of synthetic carbohydrate derivatives with a special emphasis on attachment using so-called “click” chemistry. Next, we will focus on several key surface analysis techniques that can be used to characterize a prepared glycosurface and the type of information that can be obtained from each technique. As previously mentioned, carbohydrate–protein interactions are involved in bacterial pathogenesis and symbiosis. A famous example of carbohydrate-mediated bacterial adhesion is between the gut microbiota and the carbohydrates present on the surface of human intestinal cells. Glycosurfaces can be used for the binding, capture, and sensing of gut bacteria. A representative example of this from our own group is the study of interactions between the mannose-specific adhesin of *Lactobacillus plantarum* [5]—a lactic acid bacterium present in various probiotic products, fermented foods, and our gut—and fabricated mannose-terminated glycosurfaces (vide infra) [6]. At the end of this chapter, we will discuss several more applications of glycosurfaces in microbiology, focusing on binding, capture, and sensing of bacteria and bacterial toxins and on the multivalency effects that exert a large influence on the interaction between carbohydrates and proteins in biological systems and on fabricated glycosurfaces.

1.2 PREPARATION OF SAMs CONTAINING CARBOHYDRATES

SAMs are ordered molecular assemblies that spontaneously form on a substrate by chemisorption (or strong interaction) of molecules containing a chemical functionality with a strong affinity for the substrate surface. The chemical structure of
molecules that are used to prepare a SAM is usually subdivided in its constituting parts; the part that adsorbs on the substrate surface can be called the attaching group, the part on the opposing end of the molecule that ends up at the top of the monolayer is called the end group or terminal group, and the intermediate part is called the chain or backbone [7,8]. In this section, we will present an overview of the recent scientific literature on the preparation and properties of SAMs containing carbohydrates as end groups (Table 1.1).

One of the most common combinations of substrate and attaching group is the formation of SAMs of thiols on gold (Table 1.1; entry a), and to our knowledge, this was also the first example of a carbohydrate-presenting SAM. In 1996, Spencer and coworkers reported the formation of SAMs on gold surfaces with a thiol-terminated hexasaccharide. The thiol-terminated hexasaccharide, a truncated amylose derivative consisting of six α-1,4-linked glucopyranosides, was assembled on gold surfaces in its protected (peracetylated) and deprotected form. Both protected and deprotected compounds readily formed SAMs on gold, although the kinetics of SAM formation varied, with the deprotected hexasaccharides achieving an SAM with higher density. The protected hexasaccharide was also successfully deprotected on the surface after the SAM formation: however, the degree of deprotection was slightly lower than when conducted in solution before SAM formation [24]. These early studies already indicate that thiol SAMs on gold are best prepared directly with deprotected carbohydrate derivatives in order to circumvent incomplete deprotection of carbohydrates on the surface and degradation of the unstable thiol on gold SAM itself.

Using a similar approach, Russell and coworkers [9] synthesized protected and deprotected thiol-terminated monosaccharides that were assembled as SAMs on gold-coated glass substrates and afterwards assessed for their interaction with a series of lectins. The SAM formed with a thiol-terminated mannose derivative was exposed to concanavalin A (Con A), a lectin known to bind strongly with mannose, and a lectin from *Tetragonolobus purpureas*, which specifically binds L-fucose. As expected, the mannose-terminated SAM showed selective interaction with Con A, demonstrating that carbohydrate-presenting SAMs can be used to study interactions between carbohydrates and proteins as a simplified version of natural cell surfaces [9].

Houseman and Mrksich [18] were the first to prepare mixed SAMs that consisted of various ratios of a carbohydrate and oligoethylene glycol end group, in which the latter was incorporated to minimize nonspecific interactions. The authors prepared SAMs using N-acetylglucosamine and tri(ethylene glycol) with thiol attaching groups and studied the effect of the concentration of N-acetylglucosamine in the monolayer on an enzymatic reaction [18]. Later in this chapter, we will further discuss the strategy of using molecules to “dilute” the amount of carbohydrate on a surface and thereby tune the carbohydrate presentation and concentration (multivalency effect and optimization of density; page 50).

The relatively easy preparation of thiol SAMs on gold and high tolerance for additional functional groups, including carbohydrate hydroxyls, have made it a popular method to immobilize also other carbohydrates with various levels of complexity: monosaccharides (mannose [10–14], glucose [15–17,32], galactose [13,16,17,37],
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Functional Group</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Gold surface</td>
<td>Thiol</td>
<td></td>
<td>Mannose [9–14], glucose [9,15–17], galactose [13,16,17], N-acetylglycosamine [18], lactose [15], rhamnose [17], maltose [17,19], maltotriose [17], abequose [20], paratose [20], tyvelose [20], globotriose [21], xylose [17], dimethylated maltose [17], GM1 [15], other disaccharides [22,23], hexasaccharide [24]</td>
</tr>
<tr>
<td>(b) Gold surface</td>
<td>Disulfide</td>
<td></td>
<td>Globotriose [25–27], maltose [28], Pκ trisaccharide [29], asialo-GM2 disaccharide [29]</td>
</tr>
<tr>
<td>(c) Gold surface</td>
<td>Disulfide</td>
<td></td>
<td>Mannose [30], glucose [30–32], fucose [30], galactose [30,31], N-acetylglycosamine [30], sialic acid [30], lactose [31]</td>
</tr>
<tr>
<td>Table 1.1</td>
<td>Approaches Used for the Direct Preparation of Carbohydrate-Presenting Substrates</td>
<td></td>
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<td>------------</td>
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<tr>
<td></td>
<td><strong>Functioning Group</strong></td>
<td><strong>Immobilized Product</strong></td>
<td><strong>Immobilized Carbohydrates</strong></td>
</tr>
<tr>
<td>(a) Thiol on gold</td>
<td>SH</td>
<td>Mannose [9–14], glucose [9,15–17], galactose [13,16,17], N-acetylglucosamine [18], lactose [15], rhamnose [17], maltose [17,19], maltotriose [17], abequose [20], paratose [20], tyvelose [20], globotriose [21], xylose [17], dimethylated maltose [17], GM1 [15], other disaccharides [22,23], hexasaccharide [24]</td>
<td></td>
</tr>
<tr>
<td>(b) Thiol on gold (monovalent binding)</td>
<td>SO₂</td>
<td>Mannose [30]</td>
<td></td>
</tr>
<tr>
<td>(c) Thiol on gold (multidentate binding)</td>
<td>SO₂</td>
<td>Mannose, Gb3, globo H [35]</td>
<td></td>
</tr>
<tr>
<td>(d) Alkene on silicon</td>
<td>O₃</td>
<td>Mannose</td>
<td>Lactose [33]</td>
</tr>
<tr>
<td>(e) Alkene on silicon</td>
<td>O₃</td>
<td>Mannose</td>
<td>MANNose [34]</td>
</tr>
<tr>
<td>(f) Phosphonic acid on aluminum oxide</td>
<td>O₃</td>
<td>Mannose, Gb3, globo H</td>
<td>N-Acetylglucosamine, galactose [36]</td>
</tr>
<tr>
<td>(g) Phosphonic acid on aluminum oxide</td>
<td>O₃</td>
<td>Mannose, Gb3, globo H</td>
<td>N-Acetylglucosamine, galactose [36]</td>
</tr>
</tbody>
</table>

(a) Thiol on gold, (b) disulfide on gold (monovalent binding), (c) disulfide on gold (multidentate binding), (d) alkene on silicon, (e) alkyne on silicon, (f) phosphonic acid on aluminum oxide, and (g) silane on silica.
CArboHyDrATe‐PresenTing self‐AsseMbleD MonolAyers

xylose [17], rhamnose [17], disaccharides (lactose [15], maltose [17,19], dimethylated maltose [17]) [20,22,23], and oligosaccharides (GM1 pentasaccharide [15], globotriose [21], maltotriose [17]) [37].

A general drawback of SAMs created by the adsorption of thiols on gold is their relative limited stability. In order to increase the stability of SAMs on gold, some research groups have prepared SAMs with molecules that can form multiple bonding interactions with the substrate (multidentate adsorbates) (Table 1.1; entry c). The increased stability enables their use under conditions that are not compatible with the monodentated ones [38]. Disulfides can be used to generate more stable SAMs on gold (Fig. 1.1a), and this strategy has been applied to various carbohydrate derivatives: mannose [10,30], galactose [30,31], glucose [30,31], fucose [30], N-acetyl glucosamine [30], sialic acid [30], and lactose [31]. However, some carbohydrate derivatives containing disulfides are designed in a way that does not enable multidentate binding to the surface (Fig. 1.1b and Table 1.1; entry b). Although these molecules also form SAMs on gold, their binding mode and presentation of the carbohydrate are comparable to the binding of single thiol attaching groups [25–29].

As is clear from the previous paragraphs, carbohydrate‐presenting SAMs have up till now been prepared mostly by thiol absorption on gold, but several alternative methods exist, which are discussed next. One of these is the formation of SAMs on hydrogen‐terminated silicon surfaces using terminal alkenes as attaching group (Table 1.1; entry d). In this case, the SAMs can be obtained by thermal or photochemical radical reaction of the alkene, resulting in the formation of a Si–C bond. Acetyl‐protected β-glucose, a mixture of β and α‐sialic acid, and a sialic acid derivative were successfully immobilized on hydrogen‐terminated silicon surfaces using either thermal or photochemical method, depending on the thermal stability of the carbohydrate [39,40].

Using a similar approach, lactose was immobilized as p-vinylbenzylactonoamide on silicon (Fig. 1.2). Through a thermal radical reaction, a silicon‐centered radical, which was formed by the activation of a Si–H bond, reacted with the terminal alkene of the p-vinylbenzylactonoamide molecule in an anti-Markovnikov fashion. After SAM formation, the lactoside‐covered surface was patterned by UV irradiation using a copper grid. The authors showed specific binding of a lactose‐binding lectin (Ricinus communis agglutinin, RCA120) on the regions that were not irradiated with UV light, without nonspecific adsorption of the protein on the SiO2 regions. Compared to the earlier SAMs on gold, this technique offers the advantage that an additional

FIGURE 1.1 Mannose derivatives containing disulfides: (a) disulfide that can form multidentate binding on gold and (b) disulfide that results in monodentate binding on gold.
preparation of SAMs containing carbohydrates

Resistant SAM, such as a polyethylene glycol chain, is not needed to prevent non-specific adsorption of proteins on silicon surfaces [33].

In a similar approach, a mannose derivative containing a terminal alkyne group was used to form SAMs on hydrogen-terminated silicon surfaces by a photochemical radical reaction (Table 1.1; entry e). Hydrosilation of the Si–H surface was achieved by UV/visible light irradiation-generated radicals, which initiate the Si–C bond formation that over time generates the SAM. The mannose-presenting SAM was formed on a patterned substrate and displayed specific protein recognition of fluorescently labeled mannose-binding lectin (Con A) [34].

Another approach to generate covalent SAMs uses carbohydrate derivatives containing a phosphonic acid attaching group that is able to form SAMs on oxide surfaces (Table 1.1; entry f). Using this approach, Wong and coworkers [35] prepared phosphonic acid-presenting derivatives of simple monosaccharides, like mannose, and more complex carbohydrates, like the trisaccharide Glb3 and the hexasaccharide Globo H that were allowed to form SAMs on aluminum oxide-coated glass slides. The glycan arrays generated by this technique were successfully used to study several carbohydrate–protein interactions [35].

Although one of the most common methods to prepare SAMs in general is the modification of surface oxides with alkylsilanes [41], there are not many examples of carbohydrate derivatives containing alkylsilanes to form SAMs, probably due to the reactivity of silanes with the hydroxyls of unprotected carbohydrates and the consequently laborious synthesis routes required to circumvent this. One of the few existing examples is the synthesis of N-acetyl-d-glucosamine and galactose derivatives containing a trialkoxysilane attaching group and their use to form SAMs on silica-coated stainless steel surfaces (Table 1.1; entry g). The presence and availability for biological interactions of the carbohydrates were confirmed by the successful binding of N-acetyl-d-glucosamine- and galactose-binding lectins [36].

In general, there are not many methods for the direct formation of SAMs with carbohydrate derivatives. It is evident that the most well-known and frequently used

**FIGURE 1.2** Immobilization of lactose as p-vinylbenzylactonoamide on silicon.
method is the formation of SAMs of thiols or disulfides on gold surfaces. Although this is an easy and well-established technique for carbohydrate SAMs formation, the limited stability of the thiol SAMs on gold may hamper the scope of their potential applications [42]. However, the formation of thiol SAMs on gold is the most simple method to immobilize carbohydrates on a surface in only one step and is currently still being used successfully, especially to study carbohydrate–protein interactions by surface plasmon resonance (SPR) [14], electrochemical impedance spectroscopy (EIS) [12,13,21], cyclic voltammetry [16], quartz crystal microbalance (QCM) [30], and a cantilever sensor platform [37]. An alternative for the direct formation of SAMs with carbohydrate derivatives is to use a secondary reaction to attach the carbohydrates via the end groups of a previously formed SAM, an approach that is discussed in the following section.

1.3 PREPARATION OF GLYCOSURFACES VIA A SECONDARY REACTION ON SAMs

1.3.1 Glycosurfaces Obtained Stepwise Using Unmodified Carbohydrates

The attachment of unmodified carbohydrates to a reactive surface is the simplest method, because it does not require prior chemical modification of the carbohydrates, which is usually a time-consuming step. For the methods described in this section, in general a preformed SAM presents end groups that react with a functional group of a carbohydrate to form a covalent bond (Table 1.2).

Koberstein and coworkers [43] described a photochemical method for immobilization of a variety of unmodified mono-, oligo-, and polysaccharides on glass, quartz, and silicon substrates. The authors initially synthesized a phthalimide-derivatized silane, which was self-assembled on the substrates to generate phthalimide-terminated surfaces. Upon exposure to UV light, an excited n–π* state was produced that abstracts a hydrogen atom from a nearby molecule (Fig. 1.3a and Table 1.2; entry a). The resulting radicals then recombined and formed a covalent bond that in this case was with a nearby carbohydrate present in the reaction solution. Because of the photochemical nature of the process, this method can be used for direct chemical patterning of surfaces with carbohydrates via a photolithography process. Similar experiments were also successfully performed on benzophenone-terminated surfaces (Fig. 1.3b), which also contain aromatic carbonyls that can photochemically react with natural carbohydrates. However, the thickness of these carbohydrate layers was lower, and the water contact angle was higher than that of the carbohydrates immobilized on the phthalimide-terminated surfaces [43].

Another more recently reported application of a photochemical reaction to immobilize unmodified carbohydrates on surfaces employs perfluorophenylazide-terminated SAMs (Fig. 1.3c and Table 1.2; entry b). Initially, SAMs were formed on gold with perfluorophenylazide-containing thiol groups. Upon irradiation with UV light, the azide moiety yields perfluorophenylnitrene, which is able to insert into C–H bonds (Fig. 1.3c). A series of mono- and oligosaccharides was successfully immobilized in
<table>
<thead>
<tr>
<th>Surface Termination</th>
<th>Unmodified Carbohydrates</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Phthalimide-terminated surface</td>
<td><img src="image" alt="Phthalimide-terminated surface" /></td>
<td><img src="image" alt="Phthalimide-terminated product" /></td>
<td>Galactose, N-acetylgalactosamine, arabinose, rhamnose, mannose, glucose, isomaltotriose, isomaltpentose, isomaltoheptaose [43]</td>
</tr>
<tr>
<td>(b) Perfluorophenyl azide-terminated surface</td>
<td><img src="image" alt="Perfluorophenyl azide-terminated surface" /></td>
<td><img src="image" alt="Perfluorophenyl azide-terminated product" /></td>
<td>Mannose, glucose, galactose [44]</td>
</tr>
<tr>
<td>(c) Hydrazide-terminated surface</td>
<td><img src="image" alt="Hydrazide-terminated surface" /></td>
<td><img src="image" alt="Hydrazide-terminated product" /></td>
<td>N-Acetylglicosamine, mannobiose, methyl mannoypranoside, mannan, sialy Lewis X, isomaltpentaose [45], mannose, heparin decasaccharides [46]</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Surface Termination</th>
<th>Unmodified Carbohydrates</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d) Aminooxy-terminated surface</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><em>N</em>-Acetylglucosamine, mannobiose, methyl mannopyranoside, mannan, sialyl Lewis X, isomaltooltaose [45]</td>
</tr>
<tr>
<td>(e) Vinyl sulfone-terminated surface</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td>Mannose [47], various complex carbohydrates [48]</td>
</tr>
</tbody>
</table>

(a) Phthalimide, (b) perfluorophenyl azide, (c) hydrazide, (d) aminooxy, and (e) vinyl sulfone.
Preparation of Glycosurfaces Via a Secondary Reaction on SAMs

Through these binding studies, it was shown that the surface-bound carbohydrates retained their binding affinities and selectivity. Thus, this technique apparently enables the formation of robust and stable carbohydrate arrays, which can be repeatedly used to study carbohydrate–protein interactions [44]. These photochemical reactions form the basis for convenient methods to immobilize various unmodified carbohydrates onto surfaces, although a major drawback is that the carbohydrates are immobilized in an ill-defined way due to the many reactive sites in the same molecule.

A way to overcome this problem and still use unmodified carbohydrates is to use the anomic hemiacetal present in reducing carbohydrates for the surface immobilization. In solution, this functional group is in equilibrium with the open chain form aldehyde that can undergo various selective reactions. Wang and coworkers [45] used this approach to prepare carbohydrate microarrays on glass slides. They initially immobilized a three-dimensional poly(amidoamine) starburst dendrimer on epoxy-terminated glass, followed by functionalization of the dendrimer with terminal hydrazide (Table 1.2; entry c) and aminooxy (Table 1.2; entry d) groups (Fig. 1.4). These functional groups react with the aldehyde of the reducing carbohydrates, leading to site-specific immobilization via oxime and hydrazine formation. Using these techniques, the authors immobilized various unmodified mono-, oligo-, and polysaccharides with preservation of their specific binding activity [45].

In a similar approach, Zhi and coworkers [46] prepared carbohydrate microarrays by reacting the aldehyde group of a reducing carbohydrate with hydrazide-terminated surfaces. The difference between this approach and the previous one is that the latter uses an additional reduction step of the oligosaccharides to form a reducing end aldehyde moiety, which reacts with the hydrazide groups present on the surface, forming

![Figure 1.3](https://example.com/figure1.3.png)

**Figure 1.3** Photochemical reactions used to immobilize unmodified carbohydrates on surfaces with photoactive end groups: (a) phthalimide, (b) benzophenone, and (c) perfluorophenylazide.
a hydrazone. This hydrazone is then mainly converted into the native \(\beta\)-pyranose form, immobilizing the carbohydrates in a site-specific way [46].

Another approach that leads to a certain degree of site-specific immobilization of unmodified carbohydrates on surfaces makes use of divinyl sulfone as a cross-linking agent between hydroxy-terminated surfaces and the hydroxyl groups of the carbohydrate (Table 1.2; entry e) [47,48]. In the first step, a hydroxy-terminated thiol-based SAM is generated on gold, followed by the immobilization of divinyl sulfone and the unmodified carbohydrate via a Michael addition. The increased nucleophilicity of the anomeric hydroxyl contributes to the immobilization of the carbohydrates mainly via their anomeric center. However, an important drawback of this method is that the carbohydrate may also be immobilized by any of its other multiple hydroxyl groups and can exist as a mixture of \(\alpha\) and \(\beta\) anomers, which is difficult to characterize on a surface and can have an effect on subsequent biological assays. To overcome these problems and to improve the reactivity of the carbohydrates, mannose derivatives containing amine and thiol groups were synthesized and immobilized on these vinyl-terminated surfaces (Table 1.3; entry i). The results indeed showed that the aminated and thiolated mannose derivatives are more efficiently immobilized on the vinyl sulfone-terminated surfaces [47].

**FIGURE 1.4** Chemical process for preparation of 3D aminooxy- and hydrazide functionalized glass slides. *Source:* Reprinted with permission from Ref. 45, Copyright 2009, American Chemical Society.
Although the approaches described in this section are easy and versatile as they can be applied to a variety of natural carbohydrates, their major drawback is the non-specific attachment of carbohydrates onto the surface. The use of chemically modified carbohydrates derivatives for site-selective attachment on surfaces is therefore a more commonly used approach to ensure that all molecules present on the surface are immobilized in a well-defined manner and thus have the same orientation. The reactions that are most frequently used for site-selective attachment of carbohydrates on surfaces are discussed in the following section.

1.3.2 Glycosurfaces Obtained Stepwise Using Synthetic Carbohydrate Derivatives

The most extensively developed method to immobilize carbohydrates on surfaces involves the prior attachment of surface-reactive groups at the anomeric position of carbohydrates, resulting in site-specific immobilization (Table 1.3) [49]. Of course, if one invests the additional time and effort in synthesizing a tailor-made carbohydrate derivative, the subsequent SAM attachment reaction should proceed in a controlled and efficient fashion to allow for a well-defined glycosurface and under mild conditions to allow for a large scope of (complex) carbohydrates.

In view of these desired reaction characteristics, the most frequently used reactions to immobilize carbohydrates on surfaces via this approach belong to the popular so-called “click” reactions. The most used is the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction (Table 1.3; entries a and b), which can be performed in various solvents and tolerates most functionalities. One of the first examples of immobilization of carbohydrates on surfaces using a CuAAC reaction was reported by Wang and coworkers [43]. In their study, azide-containing carbohydrate derivatives (a mannoside, lactoside, and galactose-containing trisaccharide) were successfully immobilized on alkyne-terminated gold surfaces via the CuAAC reaction. The immobilized carbohydrates presented specific binding toward proteins, as analyzed by SPR and QCM [50]. Overall, two different approaches have been used to immobilize carbohydrates on surfaces via CuAAC: Either the alkyne functionality is present on the surface and reacts with azide-containing carbohydrate derivatives [6,51–53,55,100–102], or the azide group is on the surface and reacts with an alkyne-containing carbohydrate [56,57]. While the yield of CuAAC is typically high, a significant drawback of this reaction is the requirement of the toxic copper catalyst, which cannot always be completely removed and might limit the use of the resulting glycosurfaces for diagnostic and other biotechnological applications [103,104].

An interesting alternative to circumvent the toxicity of copper is the use of strained cyclic alkynes that are able to react with azides via a copper-free strain-promoted azide–alkyne cycloaddition (SPAAC) reaction (Table 1.3; entries c and d) [105]. The SPAAC reaction was first described by Bertozzi and coworkers [106] and has been used by our group to attach lactose derivatives containing azide groups on cyclooctyne-terminated Si$_3$N$_4$ surfaces. The bioactivity of the lactoside immobilized on Si$_3$N$_4$ was analyzed by binding studies with a fluorescently labeled lectin [59]. In the same year, Ravoo and coworkers immobilized a mannose derivative containing a
<table>
<thead>
<tr>
<th>Surface Termination</th>
<th>Functionalized Carbohydrates</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
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</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Alkyne-terminated surface</td>
<td></td>
<td>Mannose [6,50–54], galactose [52], glucose [52,55], N-acetylglucosamine [52], sulfo-N-acetylglucosamine [52], sialic acid [52], lactose [50,53], α-Gal trisaccharide [50]</td>
</tr>
<tr>
<td>(b)</td>
<td>Azide-terminated surface</td>
<td></td>
<td>Mucin mimic glycopolymer [56], maltoheptaose [57]</td>
</tr>
<tr>
<td>(c)</td>
<td>Azide-terminated surface</td>
<td></td>
<td>Mannose [58]</td>
</tr>
</tbody>
</table>
Table 1.3

Immobilization of synthetic Carbohydrates Derivatives on surfaces with Different ending group Terminations

<table>
<thead>
<tr>
<th>Surface Termination</th>
<th>Immobilized Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Alkyne-terminated surface</td>
<td>N_3O Azide Cu^{+}</td>
</tr>
<tr>
<td>(b) Azide-terminated surface</td>
<td>O Alkyne Cu^{+}</td>
</tr>
<tr>
<td>(c) Azide-terminated surface</td>
<td>O Cyclooctyne</td>
</tr>
<tr>
<td>(d) Cyclooctyne-terminated surface</td>
<td>N_3O Azide</td>
</tr>
<tr>
<td>(e) Oxime-terminated surface</td>
<td>Norbornene, oxidation</td>
</tr>
<tr>
<td>(f) Alkene-terminated surface</td>
<td>Thiol, h_\text{\text{\tiny{v}}}</td>
</tr>
<tr>
<td>(g) Alkynie-terminated surface</td>
<td>Thiol, h_\text{\text{\tiny{v}}}</td>
</tr>
</tbody>
</table>

(continued)
### Table 1.3 (Continued)

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<th>Surface Termination</th>
<th>Functionalized carbohydrates</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(h) Maleimide-terminated surface</td>
<td>Mannose [65–67], galactose [65], glucose [65,68], N-acetylglucosamine [65]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Vinyl sulfone-terminated surface</td>
<td>Mannose [47,69], galactose [69], complex carbohydrates [69]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(j) Benzoquinone-terminated surface</td>
<td>Mannose, galactose, glucose, fucose, rhamnose, N-acetylglucosamine [70]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Diagram (h):**
- Maleimide-terminated surface
- Maleimide
- SH (Thiol)
- Immobilized product
- Immobilized carbohydrates

**Diagram (i):**
- Vinyl sulfone-terminated surface
- Vinyl sulfone
- R: SH or NH
- Thiol or amine, \( h_v \)
- Immobilized product
- Immobilized carbohydrates

**Diagram (j):**
- Benzoquinone-terminated surface
- Benzoquinone
- Cyclopentadiene
- Immobilized product
- Immobilized carbohydrates
(k) Mannose, galactose, glucose, lactose, maltose [71]

(l) Mannose, lactose, N-acetylglucosamine [72]

(m) Mannose [73], galactose [73,74], maltose [74]

(continued)
### Table 1.3 (Continued)

<table>
<thead>
<tr>
<th>Surface Termination</th>
<th>Functionalized Carbohydrates</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n) Azide-terminated surface</td>
<td><img src="image1.png" alt="Phosphine" /></td>
<td><img src="image2.png" alt="Immobilized Product" /></td>
<td>Galactose, lactose, N-acetyl-lactosamine [75]</td>
</tr>
<tr>
<td>(o) NHS-terminated surface</td>
<td><img src="image3.png" alt="Amine" /></td>
<td><img src="image4.png" alt="Immobilized Product" /></td>
<td>Galactose [76,77], glucose [76,78,79], mannose [76,80], N-acetylglucosamine [76,77], N-acetylgalactosamine [78], sialic acid [77,81], lactose [60,76], N-acetyl-lactosamine [79,81], mannosiose [79], heparin decasaccharide [76], Globo H [82], complex carbohydrates [76,80–84]</td>
</tr>
<tr>
<td>(p) Epoxide-terminated surface</td>
<td><img src="image5.png" alt="Amine" /></td>
<td><img src="image6.png" alt="Immobilized Product" /></td>
<td>Complex carbohydrates [84,85]</td>
</tr>
</tbody>
</table>
(q) Azlactone-terminated surface

(r) Aldehyde-terminated surface

(s) Amine-terminated surface

(t) Isocyanate-terminated surface

Glucamine [86]

Mannose, galactose, glucose [87]

Mannose, galactose, glucose, glucosamine, cellobiose, lactose, lactosamine [88]

N-Acetylglucosamine, N-acetyl-lactosamine [89]
<table>
<thead>
<tr>
<th>Surface Termination</th>
<th>Functionalized Carbohydrates</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(u)</td>
<td><img src="image" alt="Amine-terminated surface" /></td>
<td><img src="image" alt="Immobilized Product" /></td>
<td>Mannose [90]</td>
</tr>
<tr>
<td>(v)</td>
<td><img src="image" alt="Amine-terminated surface" /></td>
<td><img src="image" alt="Immobilized Product" /></td>
<td>Mannose, glucose, N-acetylglucosamine, fucose, lactose, maltose, melibiose [91]</td>
</tr>
<tr>
<td>(w)</td>
<td><img src="image" alt="Hydroquinone-terminated surface" /></td>
<td><img src="image" alt="Immobilized Product" /></td>
<td>Galactose, glucose [92]</td>
</tr>
</tbody>
</table>
(a) CuAAC on alkyne-terminated surfaces, (b) CuAAC on azide-terminated surfaces, (c) SPAAC on azide-terminated surfaces, (d) SPAAC on cyclooctyne-terminated surfaces, (e) nitrile oxide–alkene cycloaddition, (f) thiol–ene, (g) thiol–yne, (h) thiol–maleimide coupling, (i) Michael addition, (j) Diels–Alder on benzoquinone, and (k) maleimide-terminated surfaces, (l) Diels–Alder with inverse electron demand, (m) disulfide formation, (n) Staudinger ligation, (o) amide coupling on NHS-terminated surfaces, (p) epoxide–amine addition, (q) azlactone–amine coupling, (r) imine formation on aldehyde-terminated surfaces, (s) reductive amination on amine-terminated surfaces, (t) isocyanate–amine coupling on isocyanate-terminated surfaces, (u) isothiocyanate–amine coupling on amine-terminated surfaces, (v) Michael addition on amine-terminated surfaces, (w) benzoquinone–aminooxy reaction, (x) enzymatic transglycosylation, (y) surface-initiated atom transfer radical polymerization (SI-ATRP), and (z) UV-induced graft polymerization on polypropylene membranes.
Cyclooctyne group on azide-terminated surfaces [58]. The main disadvantage of the SPAAC reaction is the more challenging synthesis of carbohydrate derivatives containing cyclooctynes when compared with the ones containing linear terminal alkynes. The limited stability of cyclooctyne-terminated monolayers also needs to be considered when using the SPAAC reaction to immobilize biomolecules on surfaces.

Another reaction used by Ravoo and coworkers [58] to immobilize carbohydrates on surfaces involves in situ generation of nitrile oxide moieties on the surface, which react with alkenes or alkynes, forming isoxazolines (Table 1.3; entry e). The nitrile oxide groups on the surface were generated by oxidation of oxime-terminated surfaces using diacetoxyiodobenzene as the oxidizing agent. Using this approach, the authors immobilized a galactose derivative containing a norbornene group, and the galactose-terminated surfaces were shown to bind fluorescently labeled peanut agglutinin (PNA) [58].

Another approach immobilizes carbohydrates on surfaces using a “click” reaction between thiols and alkenes or alkynes, called the thiol–ene or thiol–yne reaction (Table 1.3; entries f and g), respectively. The radical thiol–ene and thiol–yne reactions are usually carried out under UV light using a photoinitiator such as 2,2-dimethoxy-2-phenylacetophenone (DMPA). Ravoo and coworkers [58] used the thiol–ene photochemical reaction to attach a thiol-containing mannose derivative on alkeneterminated surfaces [60]. The same type of reaction was also used by Ramström and coworkers [61] to attach mannose and galactose derivatives on polymer-coated quartz surfaces functionalized with alkenes or alkynes. The authors carried out the reactions at room temperature and in aqueous solutions of the thiol-containing carbohydrates, in contrast with the reported reaction conditions for both thiol–ene and thiol–yne reactions, that is, using nonpolar solvents. The addition of a radical initiator was not necessary for the reactions, and as expected, the thiol–ene reaction proceeded at a higher rate than the thiol–yne. The carbohydrate-terminated surfaces were then tested for their binding affinity of various lectins, showing good selectivity [61]. The thiol–yne reaction was also used to immobilize glucose derivatives on an alkyne-presenting microporous polypropylene membrane [63,64] and on an alkyne-terminated silicon surface [107]. In the latter example, the consecutive double addition of thiol-terminated carbohydrates onto the alkyne in the thiol–yne reaction resulted in a higher surface density of carbohydrates when compared with the thiol–ene reaction.

Thiol-containing carbohydrate derivatives can also be used for immobilization onto maleimide-terminated surfaces (Table 1.3; entry h). Mrksich and coworkers used thiol–maleimide coupling to attach mannose, galactose, glucose, and N-acetylgalcosamine derivatives on mixed disulfide monolayers on gold [65]. The same approach was also used later to prepare mannose-terminated surfaces, which were used to compare the binding responses with Con A of propagating SPR and localized SPR sensors [66]. Glucose [68]- and mannose [67]-terminated surfaces were also prepared using the same reaction.

Another reaction used to immobilize carbohydrates on surfaces is the Diels–Alder reaction. Diels–Alder was used to attach various carbohydrate–diene conjugates on
benzoquinone- (Table 1.3; entry j) [70] and maleimide- (Table 1.3; entry k) [71]
terminated surfaces. The Diels–Alder reaction with inverse electron demand
(Table 1.3; entry l) was also successfully used to immobilize alkene-containing car­
bohydrate derivatives on tetrazine-terminated surfaces [72].

Thiol-containing carbohydrate derivatives have also been used for immobilization
on thiol-terminated monolayers by formation of disulfides (Table 1.3; entry m).
Based on this method, Corn and coworkers attached mannose and galactose deri­
vatives on gold surfaces to fabricate carbohydrate arrays for imaging SPR [73].
Badyal and coworkers also used the formation of disulfides as a method to immobilize
galactose and maltose on thiol-terminated surfaces [74]. An advantage of this
immobilization method is the possibility of reducing the disulfide bonds and reusing
the same thiol-terminated surfaces to attach other carbohydrates, if the initial
monolayer is stable enough to survive the reduction step.

Cairo and coworkers prepared galactose, lactose, and N-acetyl-lactosamine-
terminated surfaces using the Staudinger ligation as immobilization method
(Table 1.3; entry n). For this technique, the authors initially introduced an azide
functionality to a carboxymethyldextran surface, which reacted with phosphane-
containing carbohydrate derivatives. The modified surfaces were then used to
analyze carbohydrate–lectin interactions by SPR [75].

Another functional group frequently used for attachment of carbohydrates on
surfaces is the amine group, which can either be present on the surface or in the car­
bohydrate derivative. Amine-containing carbohydrate derivatives can be attached to
carboxy-terminated surfaces using carbodiimide chemistry (e.g., dicyclohexylcar­
bodiimide, DCC) or N-hydroxysuccinimide (NHS) coupling chemistry (Table 1.3;
entry o). Using this latter technique, a series of mono- and disaccharide derivatives
containing an amine group was synthesized and attached on NHS-terminated mono­
layers on gold surfaces. The specific binding of proteins on the carbohydrate arrays
was analyzed by SPR, matrix-assisted laser desorption ionization time-of-flight
mass spectrometry (MALDI-ToF-MS), and on-chip enzymatic modification [76].
This NHS approach has also been used to immobilize a variety of monosaccharides
[77,79,80], disaccharides, [60,77,81], and also more complex carbohydrates [80–84]
and has been used by the Consortium for Functional Glycomics (CFG) for the
surface immobilization of carbohydrates for years [108]. The resulting surfaces
were used to prepare arrays used for various biotechnological applications such as
profiling diverse glycan-binding proteins [83] and studying carbohydrate-specific
cell adhesion [77].

Carbohydrate derivatives containing amine groups have also been immobilized
on epoxy-terminated surfaces (Table 1.3; entry p). De Boer and coworkers used
this technique to immobilize a series of complex carbohydrates and prepare an
array to study carbohydrate–protein interactions [85]. Cummings and coworkers
[84] compared the immobilization of amine-containing complex carbohydrate
derivatives on epoxy and NHS-terminated surfaces. The authors observed that the
minimum detectable printing concentrations are lower on the epoxy slides, but
that this reaction is less specific when compared with printing on NHS-terminated
surfaces [84].
Amine-containing molecules can also be immobilized on azlactone-terminated surfaces (Table 1.3; entry q). Using this approach, Lynn and coworkers immobilized \( \alpha \)-glucamine on polymeric films with terminal azlactones, and the resulting surfaces prevented the adhesion and growth of mammalian cells \textit{in vitro} [86]. Another technique to immobilize amine-containing carbohydrate derivatives is by reacting with aldehyde-terminated surfaces, forming an imine bond (Table 1.3; entry r). Wang and coworkers used this approach to attach monosaccharides onto a polyacrylamide hydrogel activated with glutaraldehyde and employed the resulting surfaces for bacterial capture [87]. The use of carbohydrate-terminated surfaces to capture microorganisms will be discussed in more detail at the end of this chapter. The formation of an imine bond can also be used to attach aldehyde-functionalized carbohydrates on amine-terminated surfaces. The imine bond can then be reduced with mild reducing agents such as sodium cyanoborohydride to obtain irreversible immobilization (Table 1.3; entry s) [88]. The reaction between amines and isocyanate or isothiocyanate groups (Table 1.3; entries t and u) has also been used to immobilize carbohydrate derivatives on surfaces. Amine-functionalized carbohydrates were successfully immobilized onto 96-well plates coated with an isocyanate-presenting polymer [89]. Using a similar reaction, a mannose derivative containing an isothiocyanate group was attached to amine-terminated surfaces, and the resulting surfaces were used for bacterial capture [90]. Another strategy that has been used to prepare glycosurfaces involves the reaction between amines and vinyl sulfones (Table 1.3; entry v). Ratner and coworkers used this reaction to immobilize carbohydrates on the surface of silicon photonic microring resonators, used for label-free detection of glycan–protein and glycan–virus interactions [69]. Another possibility is to synthesize carbohydrate derivatives with a vinyl sulfone group, which then reacts with amine-terminated surfaces such as commercially available amine-functionalized glass slides or microwell plates [91].

Another strategy used to prepare glycosurfaces is to synthesize carbohydrates presenting an oxyamine group, which reacts with the ketone group in electroactive quinone-terminated monolayers (Table 1.3; entry w). A remarkable advantage of this approach is the possibility to release the immobilized carbohydrate by applying a constant potential under reducing conditions to regenerate the original surface. Using this technique, glucose, galactose, and mannose derivatives were immobilized on gold surfaces, which were used to prepare microarrays to analyze binding of lectins [92].

Carbohydrates can also be attached on surfaces via enzymatic reactions. In a recent work, Xu and coworkers [93] immobilized lactose on QCM chips modified with hydroxy-terminated poly(ethylene glycol) (PEG) brushes via enzymatic trans-glycosylation (Table 1.3; entry x). The \( \beta \)-galactosidase enzyme catalyzed the transfer of the galactose unit from lactose to the hydroxyl acceptor, yielding the glycoside compound with a \( \beta \)-1,4-glycosidic bond. The modified surface was used to selectively bind a lectin, while it displayed minimal nonspecific adsorption of bovine serum albumin (BSA) [93].

Finally, another quite distinct method to prepare glycosurfaces is the use of polymerization reactions with carbohydrate-containing monomers. The most commonly used polymerization method is the surface-initiated atom transfer radical
polymerization (SI-ATRP) (Table 1.3; entry y), a powerful and versatile controlled radical polymerization method that enables control over polymer thickness and functionality [109]. Using this method, Fukuda and coworkers [94] prepared glucose-terminated surfaces with protected glucose-functionalized monomers. The glucose units were subsequently deprotected quantitatively by removing the isopropylidenyl protecting groups, which did not result in degrafting or chain breaking of the glycopolymers [94]. The same monomer was later also used by Ayres and coworkers and Yoon and coworkers, but with different surface-bound initiators [95,96]. Additionally, Ayres and coworkers performed a sulfonation reaction to also obtain glycosurfaces that may mimic heparin, a naturally occurring sulfonated glycosaminoglycan [95]. Another example using SI-ATRP to functionalize surfaces with glycopolymers employs D-glucosamidoethyl methacrylate as the glycomonomer used for the polymerization. The capability of preventing the binding of nonspecific proteins, such as lysozyme, BSA, and fibrinogen, on this glycopolymer was shown by SPR studies [110]. The property of certain glycopolymers to prevent nonspecific protein adsorption on surfaces was also explored by Yu and Kizhakkedathu [97]. In their work, the authors prepared glycopolymer brushes by SI-ATRP using unprotected mannoside, glucoside, and galactoside N-substituted acrylamide derivatives as the monomers. The surfaces modified with the glycopolymers showed low BSA and fibrinogen adsorption, but also preserved the specific protein interaction as shown in the binding study with Con A [97]. Another example of a mannose-containing polymer was prepared by free radical polymerization on a gold surface, and binding with Con A was studied by QCM, atomic force microscopy (AFM), and SPR techniques [98]. A galactose-containing monomer was synthesized by Ulbricht and coworkers [99] and grafted on a polypropylene microfiltration membrane surface by UV-induced graft polymerization (Table 1.3; entry z). The galactose-presenting glycopolymer was successfully recognized by the bacterium Enterococcus faecalis, increasing the adhesion of bacteria (by a factor of 39) when compared with the unmodified polypropylene surfaces [99]. The main advantage of preparing glycopolymers on surfaces is that the orientations in which they are presented probably better resemble the complex natural presentation of carbohydrates when compared to SAMs. However, one current challenge is to limit the polydispersity in order to obtain well-defined glycosurfaces.

The chemoselective reactions discussed in this section are the most frequently used approach to immobilize carbohydrates on surfaces in a well-defined and controlled manner. In general, the main benefit of this approach is the possibility of obtaining a homogeneous carbohydrate layer, in which all the carbohydrate units are presented in the same orientation. They are usually bound to the surface via a linker connected to the anomeric carbon of the carbohydrate, yielding a carbohydrate layer with defined anomeric centers and thus hopefully similar properties compared to the glycosurfaces present in nature. However, this approach also has the disadvantage of requiring chemical modification of the carbohydrates for the immobilization on the previously prepared initial SAM. The synthetic methods required for the modification of the carbohydrates might limit the wider applicability of these chemoselective reactions in the carbohydrate nanotechnology scientific community.
1.3.3 Noncovalent Immobilization of Carbohydrates to Prepare Glycosurfaces

The preparation of glycosurfaces using secondary reactions, either using unmodified carbohydrates or in a chemoselective way, usually has the advantage of yielding stable modifications due to the covalent nature of the bond used for the immobilization of the carbohydrate. Noncovalent immobilization is a quick and easy alternative to immobilize carbohydrates on surfaces, although it usually yields less stable glycosurfaces.

An interesting approach to prepare glycosurfaces using noncovalent immobilization methods is using glycolipids (Table 1.4; entry a), which can interact with surfaces presenting hydrophobic monolayers or also with surfaces that are intrinsically hydrophobic [115,126]. Using this approach, Kiessling and coworkers [111] prepared a mannose-presenting glycolipid, which interacted with a previously prepared hydrophobic SAM. The carbohydrate density on the glycolipid bilayer was varied by using different percentages of the mannose glycolipid relative to phosphatidylcholine. The modified surfaces were then used to investigate the specific binding of Con A by SPR [111]. Mannose glycolipids with two hydrophobic alkyl chains were also immobilized on surfaces by a similar technique [113–115]. More complex carbohydrates such as gangliosides were also immobilized on surfaces using a similar approach and used to investigate the binding of cholera toxin [112,119,127]. The same technique was also used to prepare glycosurfaces with C-lactose and C-galactose glycolipids on a commercially available HPA hydrophobic chip (Biacore) [117]. Natural glycolipids secreted by Pseudozyma yeast, known as mannosylerythritol lipids, were also immobilized on the HPA hydrophobic chip [128]. Using a slightly different technique, but also with a carbohydrate derivative containing hydrophobic chains, Liu and coworkers [118] prepared N-acetylglucosamine-terminated surfaces. In their approach (Fig. 1.5), a glass slide was initially functionalized to present amino groups. The amino-terminated glass slide was then covered with gold nanoparticles, and these nanoparticles were then functionalized with thiols containing hydrophobic chains, which were used to interact with the glycolipids [118].

Noncovalent immobilization of carbohydrates by hydrophobic interactions is not restricted to long alkyl chains. Other groups that provide hydrophobic or fluorophase interactions can be bound to carbohydrates and used to prepare glycosurfaces, such as aromatic rings and perfluorinated hydrocarbon chains (Table 1.4; entry b). Lindhorst and coworkers prepared mannose-terminated surfaces using trityl-containing mannose derivatives, which were immobilized onto polystyrene microplates [67,116]. Complex carbohydrates were also immobilized on aluminum oxide-coated glass surfaces via interaction between perfluorinated hydrocarbon chains immobilized on the surface and the ones present on the carbohydrate derivative [35].

Another type of interaction that can be used to prepare glycosurfaces via noncovalent immobilization is ionic interaction between surfaces and molecules with opposite charges (Table 1.4; entries c and d). The anionic polysaccharide heparin was immobilized on a positively charged amine-terminated surface [120]. Coullerez and coworkers also prepared a glycosurface based on the polycationic graft copolymer poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG), which was modified to present mannose units and immobilized via ionic interactions on a negatively charged surface [121].
### Table 1.4  Methods of Noncovalent Immobilization of Carbohydrates On Surfaces

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Functional Group</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Glycolipids-</td>
<td><img src="image" alt="Glycolipid" /></td>
<td>Mannose [111–116], galactose [117], N-acetylglucosamine [116,118], lactose [117], cellobiose [117], maltose [117], GM1 [112,119], other complex carbohydrates [112]</td>
</tr>
<tr>
<td></td>
<td>terminated or</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>other hydrophobic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>Fluoroalkyl-</td>
<td><img src="image" alt="Fluorous-tagged carbohydrate" /></td>
<td>Mannose, lactose, cellobiose, cellotriose, cellotetraose, Gb5, Globo H [35]</td>
</tr>
<tr>
<td></td>
<td>terminated surface</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c)</td>
<td>Amine-terminated</td>
<td><img src="image" alt="Heparin" /></td>
<td>Heparin [120]</td>
</tr>
<tr>
<td></td>
<td>surface</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d)</td>
<td>Nb$_2$O$_5$-coated</td>
<td><img src="image" alt="PLL-g-PEG/PEG-mannoside" /></td>
<td>Mannose [121]</td>
</tr>
</tbody>
</table>

(continued)
### TABLE 1.4 (Continued)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Functional Group</th>
<th>Immobilized Product</th>
<th>Immobilized Carbohydrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(e)</td>
<td>Streptavidin-terminated surface</td>
<td>Biotin-conjugated carbohydrates</td>
<td>Complex carbohydrates [122–124]</td>
</tr>
</tbody>
</table>

- (a) Glycolipids, (b) perfluorinated hydrocarbon chains, ionic interactions on (c) positively and (d) negatively charged surfaces, (e) biotin–streptavidin interaction, and (f) DNA-directed immobilization.
More specific noncovalent interactions, as between biotin and its protein binding partners (avidin, neutravidin, or streptavidin), have also been used to prepare glycosurfaces for biotin-linked carbohydrates (Table 1.4; entry e) [129]. For the carbohydrate immobilization, biotin-terminated layers were initially prepared, followed by binding of neutravidin on the biotin-terminated surfaces (Fig. 1.6). Finally, the biotin group linked to sialoside derivatives could then interact with the remaining free binding sites of the neutravidin units. The surface-bound sialosides were used to study carbohydrate–protein interactions [124,130].

Additionally, DNA-directed immobilization can also be used to prepare glycosurfaces (Table 1.4; entry f). Chevolot and coworkers successfully immobilized galactose derivatives on borosilicate glass slides using this technique [125].

The carbohydrates present on the cell surface in nature are usually present in the fluid lipid bilayer of membranes as glycoconjugates of proteins or lipids and, consequently, are exposed to their interaction partner in a dynamic fashion. In order to better mimic this aspect of natural glycosurfaces, some noncovalent immobilization techniques can be used, such as solid-supported lipid bilayers [115,126]. In this case, the carbohydrates may be exposed in a more dynamic way and may behave more similarly to the natural glycosurfaces.

In general, various techniques to prepare glycosurfaces have been developed in the past few decades. These techniques can be divided in four main groups: SAMs of molecules containing carbohydrates, covalent immobilization of unmodified carbohydrates by secondary reactions, chemoselective covalent immobilization of carbohydrate derivatives, and noncovalent immobilization of carbohydrates. All the approaches discussed have their advantages and their drawbacks, and the optimal technique usually depends on the application of the
glycosurfaces and the expertise of the scientists responsible for their design and preparation. However, what all these techniques have in common is the need to comprehensively characterize the obtained glycosurfaces. The main techniques used for characterization of glycosurfaces, including a few examples, will be discussed in the following section.

**FIGURE 1.6** Schematic representation of the carbohydrate immobilization using the noncovalent interaction between NeutrAvidin and biotin. Source: Reprinted with permission from Ref. 124, Copyright 2009, American Chemical Society.
1.4 CHARACTERIZATION OF GLYCOSURFACES

Various methods are reported in the literature for preparation of glycosurfaces, as described in the previous sections. Glycosurfaces generated via all these methods need to be characterized to prove the successful modification of the surface. In this section, we provide an overview of the most frequently used techniques used to characterize glycosurfaces (Fig. 1.7), including the type of information that can possibly be obtained with each of these techniques.

1.4.1 Contact Angle Measurements (Wettability)

One of the most often used techniques for characterization of modified surfaces, including glycosurfaces, is the measurement of the surface wettability. The surface wettability is measured statically by placing a small drop of liquid on a surface and measuring the angle formed between the liquid and the solid surface, named contact angle. Nowadays, these measurements are obtained using a camera to record the image of the drop and a software that provides different fitting models to calculate the contact angle [131,132]. The main solvent used for contact angle measurements is water, but more apolar solvents can also be used. In general, according to the values of the static water contact angle, the surfaces can be divided in hydrophilic ($\theta_{CA} < 90^\circ$) or hydrophobic ($\theta_{CA} > 90^\circ$) [132]. A surface after functionalization with carbohydrates typically displays a low water contact angle due to the hydrophilicity.
of the unprotected carbohydrates. The most frequently used contact angle measurement is the static contact angle; however, advancing and receding contact angles can also be measured. The difference between the advancing and receding contact angles, called contact angle hysteresis, represents the “nonideality” of a surface and is related to the adhesion of materials on the surface [132].

Water contact angle measurements have been used by many researchers as the first technique to quickly and easily follow reactions with carbohydrates on surfaces. Yu and Kizhakkedathu [97], for example, compared the water contact angles of glycopolymer on surface generated both with protected carbohydrate derivatives and deprotected ones. The surfaces generated with the protected carbohydrates presented a contact angle of 53.8°, whereas the ones generated with the deprotected carbohydrates had a contact angle of 7.2°. When the deprotection step was performed on the surface after generation of the glycopolymer, the contact angle of the resulting surfaces was 33.5°, showing that the deprotection on the surface was incomplete [97]. Xu and coworkers [64] used water contact angle measurements to evaluate the effect of glycosyl density on the hydrophilicity of a polypropylene membrane. The authors observed a significant decrease of the contact angle with increase of the glycosyl density [64].

Although other techniques need to be used to analyze the functionalization of a surface with carbohydrates at the molecular/structural level, the contact angle measurement is an easy and quick method that provides a good indication about whether a functionalization step succeeded and the overall surface wettability.

1.4.2 X‐Ray Photoelectron Spectroscopy (XPS)

Another technique that has been frequently used for characterization of glycosurfaces is X‐ray photoelectron spectroscopy (XPS), which provides quantitative information on the elemental and chemical composition of a surface to a depth of a few nanometers [133]. In XPS, an X‐ray source induces the emission of photoelectrons from the surface. The emitted photoelectrons are collected, identified, and quantified according to their energy. The method is quantitative because the binding energies of the core levels that are analyzed are element specific, while the signal intensities are not influenced significantly by the precise nature of chemical bonding [134]. An important advantage of XPS when compared to other techniques that provide elemental and chemical composition information is that it is essentially a nondestructive technique [135]. Two different types of surface analysis can be performed in XPS: wide‐scan analysis, which provides (quantitative) information on the elemental composition, and high‐resolution analysis (yielding a narrow‐scan spectrum), which provides chemical bond information [136].

Many authors have used XPS to characterize glycosurfaces made with mixed SAMs of molecules containing carbohydrates and oligoethylene glycol or hydrocarbon units. In this case, the elemental analysis (wide‐scan spectrum) is not sufficient to distinguish between the carbon and oxygen atoms of the carbohydrates and those of the ethylene glycol or hydrocarbon units. However, in the C1s narrow scan spectrum, it is possible to obtain quantitative information about the chemical bonds that are present on the glycosurface. One can conveniently identify an XPS signal that is characteristic of carbohydrates, corresponding to the anomeric acetal carbon
Characterization of glycosurfaces

(O–C–O), that is present around 288 eV [6,137–139]. Dhayal and Ratner, for example, used this signal to quantify the relative coverage of carbohydrate in mixed monolayers with ethylene glycol units [137].

Overall, XPS provides very useful information on the composition and chemical state of elements present on surfaces. Although it has been used for the characterization of glycosurfaces, its more restricted availability to laboratories specialized in surface chemistry has limited its widespread application.

1.4.3 Infrared Reflection–Absorption Spectroscopy (IRRAS)

Infrared reflection–absorption spectroscopy (IRRAS) is another technique that has been used for characterization of glycosurfaces. IRRAS is a truly nondestructive technique that provides information about the chemical composition of surfaces, based on frequencies and intensities of molecular vibrations [140]. IRRAS measurements can, in principle, also provide information about the molecular orientation within the layer, using polarized light [141,142], but the short-range disorder of glycosurfaces will typically hamper providing such detailed information.

An advantage of infrared spectroscopy is that it does not require ultrahigh vacuum conditions, as opposed to XPS. Most IRRAS analysis of SAMs can be performed in air or inert atmosphere [141]. Depending on the substrate, transmission infrared spectroscopy can be used instead of IRRAS, for example, for glass [143] and porous aluminum oxide [6,101].

The characteristic bands of carbohydrates are present at around 3300–3500 cm\(^{-1}\), related to the stretching of intramolecular and intermolecular OH groups, and as three shoulders at around 1147, 1090, and 1045 cm\(^{-1}\), related to the hemiacetal of carbohydrates (O–C–O) [55,141]. Some other IR bands that can often be found on prepared glycosurfaces are found at around 2850 and 2920 cm\(^{-1}\). They relate to the symmetric and antisymmetric stretching vibrations of the CH\(_2\) chain, respectively, which are present when the carbohydrates are attached to a hydrocarbon chain [55,141]. The position of these bands may give information about how ordered the monolayer is [141,144].

IRRAS is a useful and accessible technique for characterization of glycosurfaces. However, quantification of the carbohydrate density is challenging, and another technique is probably required for more comprehensive characterization of a glycosurface.

1.4.4 Mass Spectrometry

Mass spectrometry techniques, such as time-of-flight secondary ion mass spectrometry (ToF-SIMS) and direct analysis in real-time high-resolution mass spectrometry (DART-HRMS), have also been used to obtain information about the composition of glycosurfaces. ToF-SIMS is a highly sensitive technique that can be used for surface chemical mapping and also enables the elucidation of molecular orientation [145]. In SIMS, a pulsed ion beam removes molecular fragments from the surface, and the secondary ion fragments are accelerated by an electrical field. The ToF detector measures the velocity of the ions, which depends on the mass-to-charge ratio [146].
Some characteristic peaks that can be observed in ToF-SIMS analysis of glycosurfaces are related to oxygen-rich anions, namely, $C_3H_5O_2^-$, $C_2H_3O_2^-$, $C_2H_2O_2^-$, $CHO^-$, $C_2HO^-$ [71], and $C_3H_5O_2^+$ [47]. Other signals mostly depend on the type of SAM present on the surface, such as $CH_2AuS$, $C_2H_4AuS$, and $C_4H_8AuS$ for alkanethiol SAMs on gold, for example [138]. ToF-SIMS has also been used for characterization of patterned glycosurfaces, with detection of characteristic carbohydrate ions depending on the surface localization [71, 141].

Another mass spectrometry technique, which has recently been studied in detail in our group, for the characterization of glycosurfaces is DART-HRMS. In DART-HRMS, excited He gas is impinged onto the surface under ambient conditions. This effects an interaction between such excited He and the surface or between atmospheric species obtained from ionization by excited He and the surface. Surface-bound groups are in this process removed from the surface and ionized [147]. We used DART-HRMS to characterize lactose-, mannose-, and fucose-functionalized porous aluminum oxide surfaces. Using the tremendous structural power of HRMS, it is thus easily possible to discriminate between the immobilized monosaccharides and between various disaccharides [100]. In this way, DART-HRMS provides structural information about glycosurfaces that is not easy to obtain otherwise.

Mass spectrometry techniques, such as ToF-SIMS and DART-HRMS, are useful to obtain information about chemical composition of glycosurfaces and also to prove the localization of carbohydrates on patterned surfaces. A drawback is that these techniques are destructive, so the glycosurfaces that are analyzed cannot be further used for biological tests.

### 1.4.5 Ellipsometry

Ellipsometry is a powerful optical tool to measure the thickness of thin films, including glycosurfaces. Ellipsometric measurements are usually performed with a light beam that propagates in air (or vacuum) and is reflected by (or transmitted through) the sample, eventually arriving at the detector. This technique can determine the thickness of monolayers or glycopolymers on surfaces, because the light reflected by the interface of an initial layer interferes with the light reflected by the interface of a second layer on top of it [148].

Ellipsometry has usually been used in combination with other techniques to characterize glycosurfaces, because it does not provide information on chemical composition. An interesting characteristic of ellipsometry is the possibility of measuring layer thickness in various conditions, including the presence of different solvents. This technique is useful to measure SAMs directly prepared with carbohydrate derivatives [17, 25, 34], glycosurfaces in which the carbohydrate is immobilized via a secondary (or tertiary reaction) [90], and also for glycopolymers on surfaces [96, 97, 110].

### 1.4.6 AFM

AFM is a scanning probe microscopy imaging technique used to investigate roughness of surfaces, size, shape, structure, dispersion, and aggregation of nanomaterials. The equipment consists of a micro-machined cantilever, usually made of silicon or
silicon nitride, with a sharp tip at one end. This tip detects the deflection of the cantilever caused by repulsion or attraction between the tip and the analyzed surface. It presents different scanning modes: noncontact or static mode, contact mode, and intermittent sample contact mode (or dynamic and tapping mode) [149].

AFM can also be used as a highly sensitive force machine that can measure forces as small as 10–20 pN. This property enables the use of AFM to measure nano-adhesive properties of samples. By using a (bio)functionalized cantilever tip, it is even possible to measure the attraction or binding event, between a (bio)molecule and a (bio)functionalized surface [150,151].

For the characterization of glycosurfaces, AFM has mainly been used to investigate surface roughness, especially for glycopolymers on surfaces [28,32,97,124]. AFM is an interesting technique to investigate how the carbohydrates are distributed on a surface; however, it provides limited information on the nature of the carbohydrate molecule. One way to increase the information provided by AFM is the use of specific lectins at its cantilever tip. In addition, AFM can be combined with other characterization techniques, such as XPS, ToF-SIMS, and IR, for confirmation of the biofunctionalization of the surface with carbohydrates.

Various techniques have been used for characterization of glycosurfaces, and each of them provides different types of information. It is usually necessary to apply a combination of two or three techniques to properly characterize a glycosurface. The best selection of characterization methods often depends on the material of the modified substrate, the type of modification (SAMs, glycopolymers, or noncovalent immobilization), and the intended final application of the glycosurface.

1.5 APPLICATION OF GLYCO SURFACES IN MICROBIOLOGY

As mentioned in the introduction, carbohydrates are present on the surface of most multicellular and single cell organisms and their interaction with other biomolecules, especially receptor proteins are critical in many biological processes, but currently only poorly understood. Glycosylated surfaces are an emerging type of biomaterials that can be used in the investigation of interactions between carbohydrates and other biomolecules, mainly proteins, either in isolation or on microbial cells. The study of these interactions can contribute to the discovery of novel carbohydrate–protein (usually lectins)-binding partners, which can for instance be useful to identify disease biomarkers or to better characterize certain microorganisms [152]. The main application of prepared glycosurfaces is currently for carbohydrate microarrays [49,153], which will be discussed in details in Chapter 9 (carbohydrate nanotechnology and its application to microarray technology). When compared to antibodies or DNA-based sensors, the carbohydrate-based sensors have the advantages of being more stable under various conditions [154]. However, the main drawbacks are the lower selectivity and lower binding affinity of the carbohydrate-based sensors.

Glycosurfaces have also been used for binding, capture, and sensing of bacteria via the interaction with the lectins present on the surface of many bacterial species. In bacterial infections, a well-known carbohydrate–protein interaction is the one between mannoses and the type 1 fimbrial lectin (FimH) present on the cell surface of
Enterobacteriaceae species, including the uropathogenic Escherichia coli (UPEC). This interaction is responsible for the adhesion of UPEC on the luminal surface of the bladder epithelium, causing bladder infection and inflammation. In this case, mannose-presenting surfaces can be used to detect these pathogenic bacteria [1].

In this section, we will focus on some examples of applications that use glycosurfaces for binding, capture and sensing of bacteria and bacterial toxins, as well as the multivalency effect that plays an important part in carbohydrate–protein interactions on glycosurfaces.

1.5.1 Binding, Capture, and Sensing of Bacteria

The genetic model bacterium E. coli has been frequently used as a model for microbial capture using glycosurfaces due to the well-known interaction between their fimbrial adhesins and the glycocalyx of their host cells. The interaction between α-d-mannoside residues and the FimH adhesin present on the type 1 fimbriae of many strains of E. coli is the most investigated bacterial adhesion process so far [1]. Wang and coworkers [11] used two different approaches for E. coli detection via carbohydrate–adhesin interactions: direct detection and Con A-mediated detection (Fig. 1.8). The direct detection involved detecting the interaction between a mannose-terminated SAM and the adhesin units present on the fimbriae of the bacteria. The Con A-mediated detection, on the other hand, involved the initial binding of the lipopolysaccharide present on the bacterial surface to Con A that in turn binds to the mannose-terminated SAM. The signal response was around eight times larger when the Con A-mediated detection was used. This is attributed to the formation of bridges between E. coli and the mannose-presenting surface, leading to a relatively rigid and strong attachment [11]. Most studies using glycosurfaces for binding, capture, or sensing of E. coli however use the direct detection approach [13,90,121,155–157], as the use of the multivalent binding capacity of Con A as an intermediate adds additional complexity to interpreting the already complex carbohydrate–protein interaction of interest [87]. In general, the bacterial binding can be detected and/or quantified by, for example, (fluorescence) microscopy [90,121], faradaic EIS [13], metal mesh device (MMD) sensors [155], or quartz crystal microbalance with dissipation (QCM-D) techniques [156].

**FIGURE 1.8** Schematic representation of direct E. coli detection and Con A-mediated E. coli detection. Source: Reprinted with permission from Ref. 11, Copyright 2007, American Chemical Society.
Different *E. coli* strains have been used as a model for investigating this binding, such as the ORN178 [13,115,155] and DH5α [157] strains—which both possess the wild-type FimH domain—or a fluorescently GFP-expressing strain (*E. coli* pPKL1162) (Fig. 1.9) that also overexpresses the type 1 fimbriae at its surface [90]. The interaction between bacteria and glycosurfaces is a complex process, and proper negative controls are therefore required. Some *E. coli* strains that do not present the type 1 fimbriae have been used as such a negative control, such as AAEC191A [121] and HB101 strains (nonfimbriated *E. coli*), a strain lacking the plasmid for FimH expression (but expressing the rest of the *fim* gene cluster) [121], and the ORN 208 strain (expresses abnormal type 1 pili that fail to mediate mannose-specific binding) [13,115,155]. The use of these strains as negative controls is important to show that the binding process is mostly due to the interaction between the carbohydrates and the adhesins, partially excluding nonspecific interactions as the source for any observed binding. Another option for a negative control is the addition of soluble carbohydrates to the suspension of bacteria before the binding to the glycosurface. If the soluble carbohydrate is the same as the one present on the glycosurface, it will saturate the carbohydrate-binding sites of any present bacterial adhesins and thereby strongly decreases the bacterial glycosurface binding when compared with the experiments in the absence of the soluble carbohydrate [6,87].

Another bacterium known to have adhesins that are related to its binding of natural glycosurfaces and the formation of biofilms is *Pseudomonas aeruginosa*. *P. aeruginosa* synthesizes two surface adhesins, named PA-IL (or LecA), which specifically binds galactose, and PA-III (or LecB), which specifically binds fucose [158,159]. Wei and coworkers [160] studied the binding of *P. aeruginosa* on glycosurfaces with trisaccharides containing β-D-N-acetylgalactosamine or α-L-fucose. The bacteria adhered to both glycosurfaces; however, they displayed a lower adhesion to the surface with the fucose-containing trisaccharide than on the one containing N-acetylgalactosamine, which naturally occurs on the epithelium of the pulmonary tract. This difference might be due to the fact that the fucose-binding adhesin PA-III is regulated in an environmentally dependent fashion, requiring certain compounds in the culture medium for proper expression [160]. More recently, Liu and coworkers [156] investigated the binding of *P. aeruginosa* by QCM-D using surfaces coated with glycopolymers generated with glucose and lactose-containing monomers. The bacteria showed an increased, and calcium-dependent, adhesion to the lactose-presenting surface, probably due to the interaction between PA-IL and the galactose unit [156].

Surfaces coated with glycopolymers containing lactose were also used to increase the binding of *E. faecalis* via interaction between an adhesin on its surface and the galactose unit [99]. It was shown that the presence of soluble galactose decreased the bacterial binding, whereas a soluble glucose negative control did not influence the binding. The binding of *E. faecalis* was also significantly affected by the blocking of the carbohydrates with peanut agglutinin (PNA), a lectin that specifically binds galactose. Prior exposure of the glycosurface to BSA or Con A had considerably less influence on the bacterial binding when compared with PNA [99].

Glycosurfaces were also recently used by us for increased binding of *L. plantarum*, a probiotic bacterium that presents a mannose-binding adhesin on the cell surface [161]. Mannose-terminated porous aluminum oxide (PAO) was used for binding
FIGURE 1.9  Adhesion of fluorescent bacteria to the different stages of the SAM during the ‘dual click’ approach. The GFP-transformed *E. coli* bacteria (pPKL1162) enable a fast, direct fluorescence readout to investigate bacterial adhesion on surfaces. The native gold surface (I) was used as reference in each of the other experiments. As can be seen in the epifluorescence micrographs, the (non-specific) adhesivity of the alkyne-terminated SAM II is comparable to the one of the native Au surface. Introduction of the OEG chain reduces the adhesion significantly, while the α-mannosyl-terminated SAM is effectively recognized by the *E. coli* leading to heavy adhesion. Source: Reproduced from Ref. 90 with permission from The Royal Society of Chemistry.
and subsequent growth of *L. plantarum* on the same surface. PAO is a cheap nanoporous surface that has been used for various biotechnological applications. Bacterial growth is possible on PAO because nutrients can diffuse from the medium on which PAO is placed to the top surface via the pores present on the surface [162]. We also showed in a control experiment that a soluble mannose derivative was able to reduce the binding of *L. plantarum* to the mannose-terminated glycosurface by blocking the mannose-binding adhesin [6].

### 1.5.2 Binding and Sensing of Bacterial Toxins

Although the use of glycosurfaces for detection of bacterial cells is still limited, there are various examples of detection of bacterial toxins using glycosurfaces. This is probably because the interaction between carbohydrates and whole cells is more complex and challenging to characterize than the interaction between carbohydrates and toxins. The detection of bacterial toxins using glycosurfaces is possible because a large number of bacterial toxins target carbohydrate derivatives on host cell surfaces to enter into the cells. Some examples of bacterial toxins that target host cell surface carbohydrates include cholera toxin, shiga and shiga-like toxins, tetanus toxin, botulinum toxin, and pertussis toxin [163].

Cholera toxin, secreted by *Vibrio cholerae*, is the cause of the pathology observed in cholera, mainly watery diarrhea and vomiting, that can lead to lethal dehydration and still is a common illness in many developing countries. The natural ligand of cholera toxin is the glycosphingolipid ganglioside GM1, which is present on the cellular membrane of the intestinal epithelial surface of the host. The major contributors to the binding are the galactose and the N-acetylneuraminic acid units of the GM1 ganglioside [164]. Glycosurfaces containing these carbohydrates may therefore be useful for detection of cholera toxin. GM1-terminated surfaces have been used for detection of cholera toxin [112,119,165–167] and also to study the inhibition of cholera toxin binding by soluble carbohydrate derivatives [127]. Ligler and coworkers [163] detected cholera toxin on both N-acetylneuraminic acid and N-acetylgalactosamine-terminated surfaces, with a limit of detection of 100 ng ml⁻¹. As expected, the binding of the toxin to the N-acetylneuraminic acid-terminated surface was more intense than to the N-acetylgalactosamine-terminated one [163]. Even more simple carbohydrates, such as galactose, showed interaction with cholera toxin when immobilized on a surface used to generate carbohydrate microarrays [168].

Shiga toxin and shiga-like toxins have also been detected using glycosurfaces. These toxins are known to cause gastrointestinal diseases after invading mammalian cells through binding to the carbohydrate portion of glycolipids on the cell surface. A shiga-like toxin produced by *E. coli* O157:H7 presents specificity toward globotriaosylceramide (Gb3), which contains the trisaccharide αGal(1→4)βGal(1→4)βGlc [169]. Consequently, Gb3 derivatives and more simple carbohydrates that are part of Gb3 have been immobilized on surfaces and used for the binding of shiga-like toxins as detected by QCM [26,170] and SPR [29,171–173].

Other glycosurfaces have been used to investigate the interactions between carbohydrates and other bacterial toxins [108,174], such as *E. coli* heat-labile enterotoxin [129], and tetanus toxin [163].
1.5.3 Multivalency Effect

Although single interactions between carbohydrates and proteins are in general very weak, multiple interactions can reinforce one another and together achieve avidities that result in strong binding. Almost all carbohydrate–protein interactions in nature are multivalent, including the interactions between carbohydrates and bacterial adhesins or toxins discussed in the previous sections. The avidity and even the selectivity of carbohydrate–protein interactions clearly depend on the density of the carbohydrate units on a studied surface [175]. In this case, for an optimal binding of a protein on a glycosurface, it is important that the carbohydrate is homogeneously distributed on the surface and present in a suitable density. Obviously, even when surfaces are functionalized with monovalent ligands, they can act as a multivalent system based only on the immobilization of the monovalent agents on the surface [176].

Kahne and coworkers [22] investigated the influence of carbohydrate surface density on protein-binding selectivity. They showed that the Bauhinia purpurea lectin switches its selectivity from one carbohydrate to another depending on the surface density of the ligands in a mixed SAM [22]. Houseman and Mrksich [18] investigated the influence of the surface density of carbohydrates on an enzymatic glycosylation reaction. In their system, the optimal density of carbohydrate for the enzymatic glycosylation was an intermediate density, around 70%, with significant decrease of glycosylation rates at higher densities of carbohydrates [18]. The influence of the carbohydrate density on antibody binding was investigated by SPR. When the carbohydrate was present on the SAM at a high density, the antibody binding was minimal. The carbohydrate density needed to be reduced to around 25% to obtain an increase of antibody binding [20]. Similar results were found for binding of Con A on maltose-terminated surfaces, with increase of the binding being observed when the carbohydrate density decreased (Fig. 1.10). The binding also changed from monovalent recognition to bivalent recognition when the carbohydrate was diluted to 10% [177]. One explanation for the low binding of proteins to glycosurfaces with a high carbohydrate density is attributed to the tight packing of the end groups in the SAM, resulting in limited access of the carbohydrates to the protein binding pocket [20].

Ratner and coworkers [137] showed that the effect of the carbohydrate density in the binding of proteins also depends on the type of molecule used for the dilution (Fig. 1.11). When a molecule with a long chain (longer than the carbohydrate) was used for dilution, the increase of carbohydrate density increased the lectin binding. On the other hand, when a short molecule was used for the dilution, the maximum lectin binding was found around 40–60% carbohydrate density, followed by a plateau and a slight decrease of binding [137].

Stine and coworkers [12] observed a difference in the optimal mannose density for Con A binding on flat gold and nanoporous gold. Although the dilution of the carbohydrate in the SAM increased the Con A binding for both surfaces, the optimal mannose density proved lower for nanoporous gold. Whereas the optimal mannose density for Con A binding was 1:9 ratio (mannoside/alkane thiol) for flat gold, the best Con A binding was found to be 1:19 for nanoporous gold [12].

Buriak and coworkers [36] did not find a decrease of protein binding with an increase of carbohydrate density, but rather an optimal protein binding when the
carbohydrate is present at 30% density, followed by a plateau when the carbohydrate density was increased [36]. Szunerits and coworkers obtained similar results for binding of \textit{Lens culinaris} lectin on mannose-terminated surfaces, with a maximum response for carbohydrate density of 60%, with a plateau of response when increasing the mannose density [53].

Another way of studying the effect of multivalency on the binding of lectins to glycosurfaces is using carbohydrate derivatives with different valencies. Pieters and coworkers [168] investigated the binding of a series of lectins to five different carbohydrates presenting valencies ranging from one to eight. Using a microarray, it was possible to investigate the lectin specificity and the multivalency effect for each carbohydrate in a single experiment. The authors found various profiles, depending on the carbohydrate and the lectin, but with maintenance of specificity [168].

Changes in the carbohydrate density of SAMs or in the way of presenting the carbohydrate units can also interfere in the bacterial binding to glycosurfaces. Carbohydrate derivatives with different valencies were immobilized on a surface, and the binding of \textit{E. coli} was investigated. The bacterial binding increased when the amount of carbohydrate units per molecule increased from one to three. However, when the carbohydrate units further increased to six and nine, there was a decrease in bacterial binding [121]. The binding of \textit{E. coli} to dynamic glycosurfaces with different densities showed a change in the avidity from monovalent to multivalent as the mannose density increased (Fig. 1.12) [115]. Recently, Lindhorst and coworkers [178] investigated the influence on bacterial binding to mannose-presenting surfaces of both the carbohydrate density and of different ways of presenting mannose. The authors showed that at high densities of carbohydrate, the simple monovalent mannoside provided a more adhesive surface for \textit{E. coli} than the bivalent and trivalent ones. However, at lower densities, the binding of \textit{E. coli} was higher on the surfaces with bivalent and trivalent mannosides when compared to the monovalent one. The highest binding affinity was found for the lowest concentration tested with the trivalent mannoside (Fig. 1.12) [178].

It is interesting to notice that a higher carbohydrate density on a SAM does not necessarily result in greater binding of lectins or bacteria. It can either not provide
any improvement in binding, resulting in waste of the excess of carbohydrate or, in the worst case, decrease the binding [36]. For this reason, it is important to determine the optimal carbohydrate density in a glycosurface to maximize the biological effect and also to reduce the waste of carbohydrate derivative.

**FIGURE 1.11** Assembly of mixed sugar/oligoethylene glycol (OEG) SAMs on gold. *Source:* Reprinted with permission from Ref. 137, Copyright 2009, American Chemical Society.

**FIGURE 1.12** (Upper) Formation of a glycan presenting supported lipid bilayer (SLB) surface from a small unilamellar vesicle (SUV) solution. (Lower) Schematic illustration of a glycan density gradient microarray for pathogen adhesion. *Source:* Reprinted with permission from Ref. 115, Copyright 2009, American Chemical Society.
1.6 OUTLOOK

In the previous sections, we presented an overview of the methods frequently used to prepare glycosurfaces via three different general approaches: (i) direct formation of SAMs containing carbohydrates, (ii) secondary reactions (with unmodified and modified carbohydrates), and (iii) noncovalent immobilization. The main challenge in the modification of surfaces with carbohydrates is to develop an approach that yields well-defined and stable glycosurfaces using the minimal number of steps. These steps should ideally be reproducible, simple, cheap, and available to a great number of scientists. Additionally, the ideal glycosurface should be stable to a wide range of conditions, and for certain applications, it should be resistant to multiple cycles of regeneration, without loss of its original properties. For industrial applications, it would also be important to consider the possibilities of scaling up the production process. The most currently used approach for straightforward glycosurfaces is still the method that was first developed, formation of thiol-based SAMs of carbohydrate derivatives on gold, mainly because of their easy preparation. As the study and utilization of the biological role of carbohydrates has been rapidly increasing over the past decades, glycosurfaces are required that enable an ever increasing amount of sensitivity, complexity, and precise control over their composition. In general, the trend is therefore to explore other approaches that enable stable and controlled site-specific immobilization of unprotected carbohydrates via an attaching group on their anomeric position.

Besides the often applied copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), an increasing range of other “click” reactions [179,180] have become available also for use in surface attachment of carbohydrates. The full potential of these reactions for the preparation of glycosurfaces has not yet been reached, but over the coming years will allow for more advanced glycosurfaces and perhaps the ideal one. When preparing a glycosurface, it is of course essential to know what it looks like before using it. Various characterization techniques have been applied for glycosurfaces, namely, contact angle measurements, XPS, IRRAS, mass spectrometry, ellipsometry, and AFM. All these methods provide different types of information, and the main challenge is to select what is the best combination of techniques to characterize a glycosurface. This selection usually depends on the substrate used to prepare the glycosurface, the type of modification, and the application of the glycosurface. It should give the highest level of information in the shortest time possible, ideally without destroying the glycosurface.

Glycosurfaces have already proven to be very useful in increasing our understanding of the binding between carbohydrates and other biomolecules, mainly proteins, and have been successfully used for detection of known carbohydrate–protein interactions and helping the discovery of new binding partners and the development of novel therapies. Because of the enormous number and diversity of carbohydrate-containing biomolecules and the fact that most are still uncharted, there is a great need for reliable and sensitive methods for their high-throughput characterization, and it is evident that the next-generation glycosurfaces will be instrumental in this [181]. What is needed now is a focus on developing and optimizing immobilization techniques that yield well-defined and stable complex glycosurfaces and also on developing and optimizing automated (chemo-enzymatic) synthetic methods [182–184] to prepare the required
complex carbohydrates with suitable reactive functional groups at the anomeric position for surface immobilization. Well-established automated synthetic methods may eventually make the synthesis and application of complex carbohydrate derivatives a routine procedure that is more accessible to non-specialists and thereby widen their application to slowly but surely illuminate this complex dark matter-world.

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