

# **Multivalent Glycoarchitectures of Different Dimensions and their Biological Evaluation**

DISSERTATION

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The work presented herein was carried out in the research group of Prof. Dr. Rainer Haag from May 2006 until April 2011 at the Institute of Chemistry and Biochemistry of the Freie Universität Berlin. This time also includes a three-month research visit in the research group of Prof. Dr. René Roy, at the University of Québec á Montréal (UQAM), Canada from October 2007 until January 2008.

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## 1. GENERAL INTRODUCTION

### 1.1 Carbohydrates (Saccharides)

Carbohydrates (mono-, di-, oligo-, and polysaccharides) are the most abundant group of natural products. They play a vital role in biological recognition processes, as they are fundamental constituents of every cell surface. Like nucleic acids and proteins, they are well-known biological information carriers. Poly- and oligosaccharides can be highly branched with various stereo centers to form a variety of linkage types that result in complex structures and an extremely high amount of structural variation.

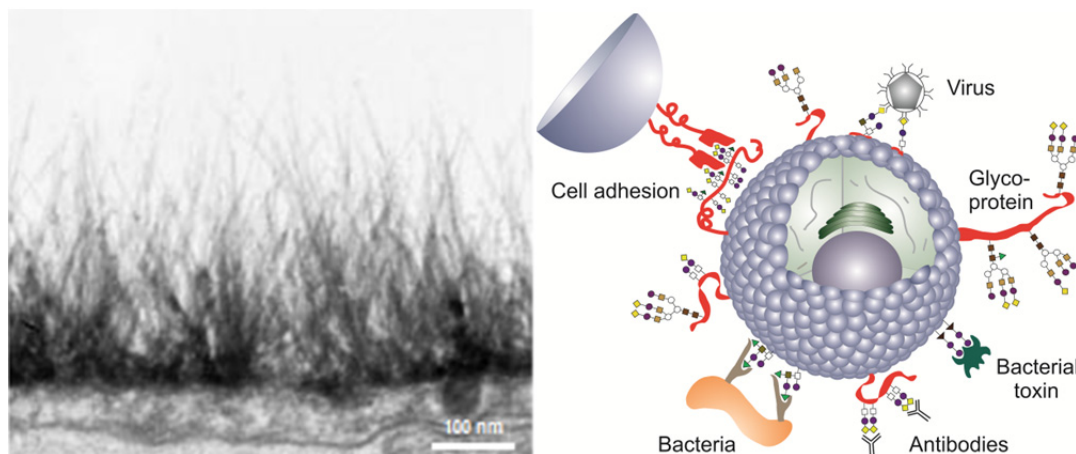
Polysaccharides are macromolecules consisting of a large number of monosaccharide residues, which are linked together by glycosidic bonds. The most well-known polysaccharides can be divided into two broad groups: structural polysaccharides (cellulose and chitin) and storage polysaccharides (glycogen and starch). Cellulose is a major structural component of plant cell walls. It is an unbranched polysaccharide with about ten thousand  $\beta$ -1,4-linked D-glucose (Glc) units per chain. Chitin is closely related in structure to cellulose and is an unbranched polysaccharide consisting of  $\beta$ -1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues. Large amounts of chitin are found in the cuticles of arthropods and in the cell walls of most fungi. Glycogen is a hyperbranched polysaccharide found in all animal cells. In humans and other vertebrates it is principally stored in the liver and muscles and is the main form of stored carbohydrate in the body, acting as a reservoir of glucose. Starch is similar to glycogen but is found in plant cells. The starch granules are made up of two polysaccharides, amylose (an unbranched molecule made up of several thousand  $\alpha$ -1,4-linked Glc units, coiled helically into a more compact shape) and amylopectin (a branched structure that contains twice as many Glc units as amylose).<sup>[1]</sup>

The surface of mammalian cells is covered by a dense coating of complex carbohydrates named glycocalyx, wherein carbohydrates appear mainly conjugated to proteins (glycoproteins, proteoglycans) and lipids (glycolipids).<sup>[2]</sup> It was found that only selected groups of monosaccharide residues are present in glycoproteins and glycolipids. These include: *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), D-glucose (Glc), D-galactose (Gal), D-mannose (Man), L-fucose (Fuc), D-xylose (Xyl), and sialic acids (SA).<sup>[3]</sup> Sialic acid terminated glycans (sialooligosaccharides) which are present on cell surface glycoproteins are especially involved in a large variety of biological events. As boundary residues, they are ideally positioned to participate in various carbohydrate-protein interactions (see Table 1, Section 1.5.1).<sup>[4]</sup>

## 1. Introduction

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Carbohydrate based cellular recognition plays a critical role under physiological as well as pathological conditions<sup>[5]</sup> such as binding of microbes (viruses, bacteria) to an eukaryotic cell surface, cell-cell communication, and binding of polyvalent molecules, such as antibodies or toxins may rely on specific carbohydrates (Figure 1).



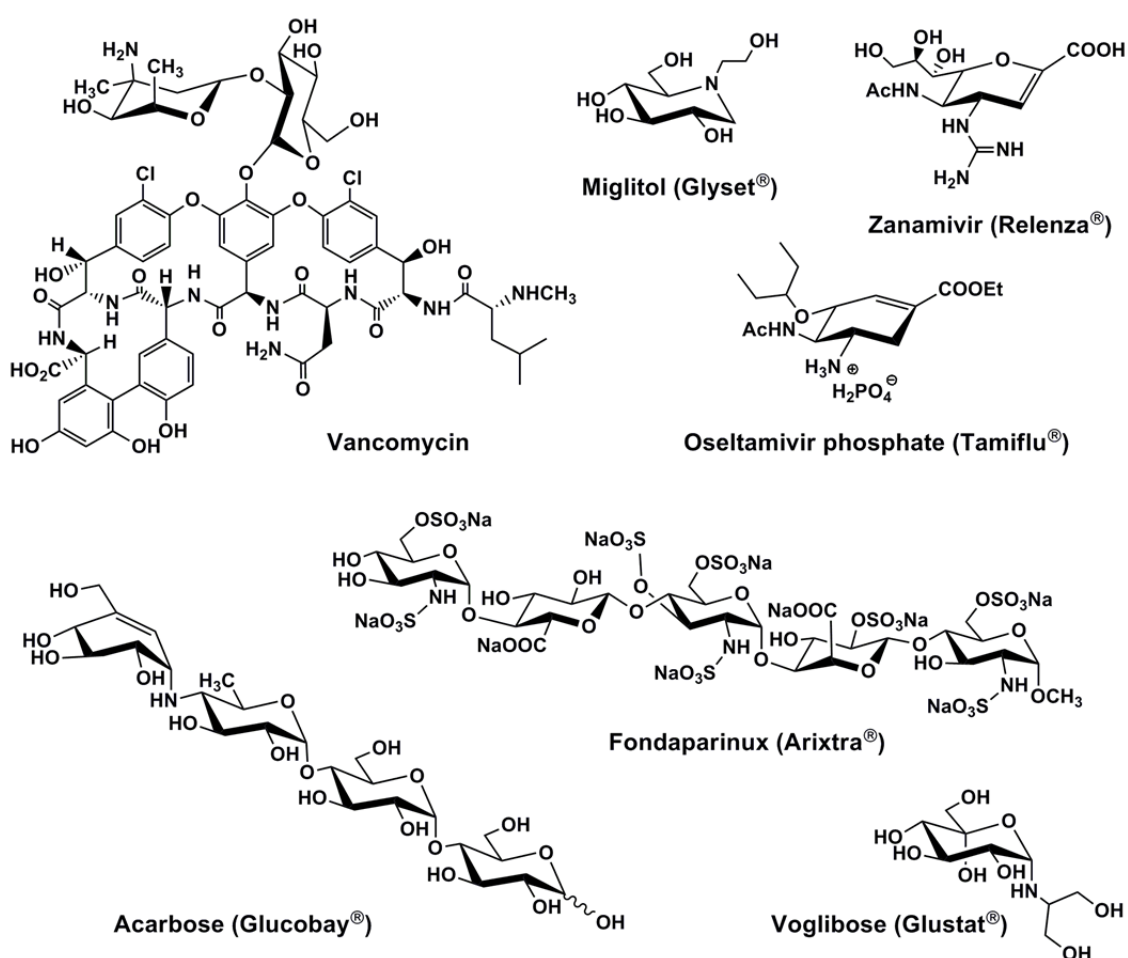
**Figure 1.** The picture in the left panel shows an electron microscopic image of glycocalyx (on the surface of a blood vessel) looking like a lot of hairs sticking up from the cell surface. The depth of the glycocalyx can range up to 500 nm.<sup>[6]</sup> The right panel shows a graphical representation of cell surface protein-carbohydrate interactions. Oligosaccharides bind to lectins, thus providing receptor sites for cell-cell communication, cell-microbe adhesion (bacterial, viral) and cell-antibody/toxin binding. The sugar chain can be linked to proteins (red ribbons) or anchored in the plasma membrane via a lipid.<sup>[7]</sup>

Interactions between a single carbohydrate ligand and its protein receptor are weak,<sup>[8]</sup> dissociation constants of the formed monovalent complexes typically being in the millimolar range ( $K_d = 10^{-3}$ - $10^{-4}$  M).<sup>[5a,9]</sup> To overcome this limitation natural carbohydrates are often organized as multivalent structures, so-called glycoclusters.<sup>[10]</sup> The clustered arrangement of complementary binding partners, a carbohydrate and its specific lectin (a class of carbohydrate-recognition proteins) on biological surfaces enables their interaction with higher affinity and better specificity.<sup>[10-11]</sup> Therefore, this glycoside cluster effect promoted the development of synthetic multivalent glycoconjugates with enhanced lectin binding properties.<sup>[12]</sup> The lectin-carbohydrate binding specificity strongly depends on the lectin type. Several of these are very sensitive to the structure of the carbohydrate (e.g. Man vs. Gal), whereas others are more sensitive to the orientation of the anomeric substituent ( $\alpha$  vs.  $\beta$ ).

## 1.2 Biologically relevant protein-carbohydrate interactions and their characteristics

Many microbes, including viruses, bacteria and their toxins, require binding to the glycocalyx, which is essential for infection to occur. Although carbohydrates are involved in diverse biological processes, the development of carbohydrate-based therapeutics has been

problematic, due to the complex glycan architectures. However, there are some examples of successful carbohydrate based drugs (“sweet medicines”, Figure 2): the low-molecular-weight heparins, derived from animal tissue, and Fondaparinux (Arixtra<sup>®</sup>; GlaxoSmithKline), which are used as anticoagulants; Vancomycin (generic drug) is a glycopeptide antibiotic; inhibitors of viral neuraminidase by Zanamivir (Relenza<sup>®</sup>, GlaxoSmithKline) and Oseltamivir (Tamiflu<sup>®</sup>, Gilead/Roche)<sup>[13]</sup>; or the treatment of diabetes by Voglibose (Basen<sup>®</sup>/Glustat<sup>®</sup>/Volix<sup>®</sup>, Takeda), Miglitol (Glyset<sup>®</sup>, Pfizer), and Acarbose (Glucobay<sup>®</sup>/Prandase<sup>®</sup>/Precose<sup>®</sup>, Bayer).<sup>[14]</sup> A number of vaccines are also based on carbohydrates. An important vaccine is the Hib vaccine against infections, caused by *Haemophilus influenzae* type b (Hib), a gram-negative coccobacillus.<sup>[15]</sup>



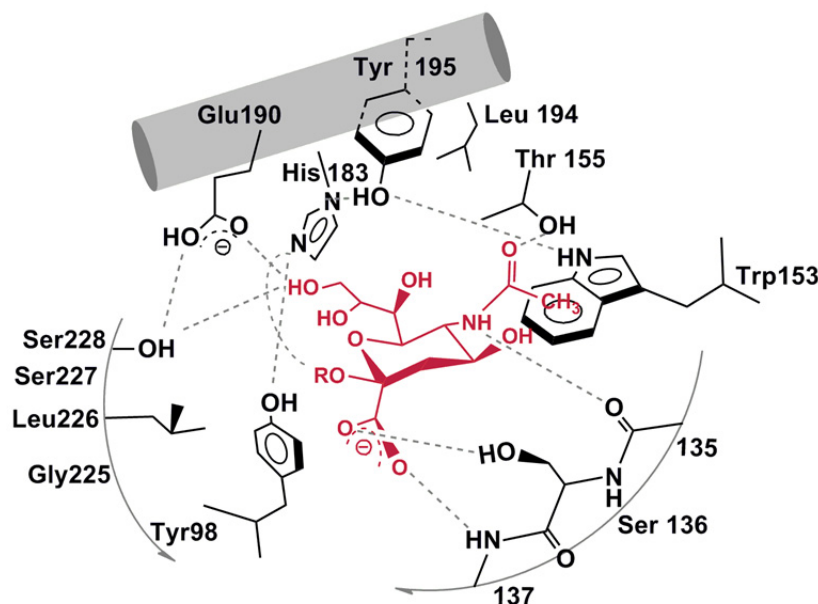
**Figure 2.** Structures of approved carbohydrate and carbohydrate-derived drugs (trade name in brackets).

As mentioned before, protein-carbohydrate interactions are characteristic for their low affinity. These interactions are driven by a favorable enthalpy derived from the multiple contact points between carbohydrate and the protein.<sup>[9a,9b,16]</sup> However, the favorable enthalpy is counteracted by an unfavorable entropy term that might arise from restricted carbohydrate



flexibility.<sup>[16-17]</sup> These enthalpy-entropy compensations are characteristic for weak chemical interactions.<sup>[18]</sup>

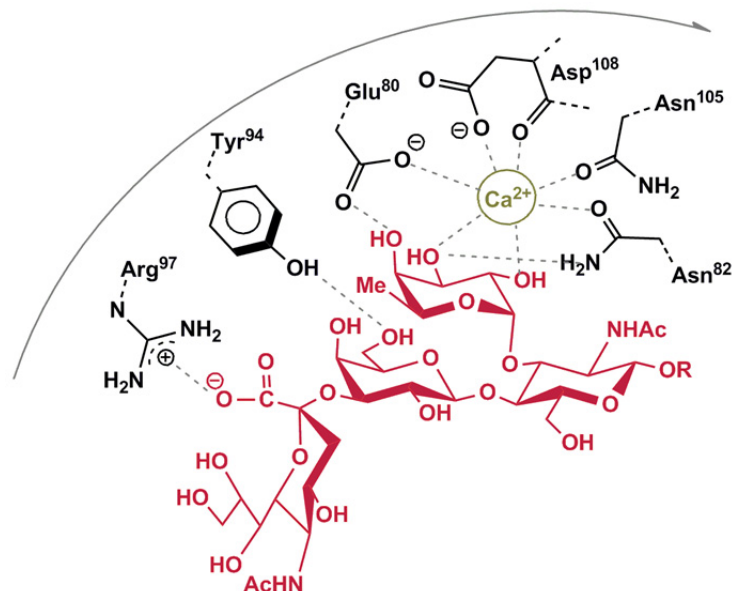
Different types of interactions can be found in protein-carbohydrate complexes, such as hydrogen bonds, hydrophobic effects, coulombic interactions, and interactions with calcium ions and water. Cooperative hydrogen bonding is the most characteristic interaction of lectins with carbohydrates. Carbohydrates display a high density of hydroxyl groups, which can act as an acceptor of two hydrogen bonds and as a donor of a single hydrogen bond, simultaneously. One example of hydrogen-bond mediated interaction can be found in influenza virus binding, where the viral surface protein hemagglutinin (HA) specifically binds sialic acids (SA) on the cell surface. A schematic diagram of SA in the hemagglutinin binding pocket is displayed in Figure 3. The protein uses tyrosine (Tyr), tryptophane (Trp), glutamic acid (Glu), serine (Ser), leucine (Leu), and histidine (His) residues to form direct hydrogen bonds with the sialic acid residue.<sup>[19]</sup>



**Figure 3.** Schematic representation by Wiley *et al.* of residues that form the HA receptor binding site and the location of bound sialic acid (in red). Hydrogen bonds are represented as grey dotted lines.<sup>[20]</sup>

Hydrophobic effects (hydrophobic stacking) are other important interactions between carbohydrate and lectins. Although carbohydrates possess many polar functional groups (-OH), they present several nonpolar surface area that can interact with hydrophobic amino acid residues, i.e. the methyl moiety of the acetamide in SA can interact with aromatic residues in the protein (Figure 3). Carbohydrates bearing aromatic aglycones are regularly bound with significantly greater affinity than the corresponding glycosides lacking such hydrophobic substituent.<sup>[21]</sup> As described by Weis *et al.* the aliphatic protons of the sugar ring bear a small partial positive charge, which leads to weak, favorable interactions with the  $\pi$ -cloud of aromatic residues in protein structures.<sup>[8]</sup>

The role of calcium ions is also important in the orientation of protein functional groups for ligand coordination. For example, during binding of sialyl Lewis x (sLe<sup>x</sup>) to E-selectins it has been shown by Graves *et al.*<sup>[22]</sup> in the crystallographic data that amino acid residues (i.e. asparagine, Asn) simultaneously coordinate a calcium ion and participate in multiple hydrogen-bonding interactions with the bound carbohydrate (Figure 4).<sup>[23]</sup>



**Figure 4.** Binding site for sLe<sup>x</sup> (Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuca1-3)GlcNAc $\beta$ 1-OH) on E-selectin.<sup>[22-24]</sup> The functional groups important for binding to the selectins are involved in many interactions: coordination to Ca<sup>2+</sup> ion (the 2- and 3-OH groups from fucose) as well as H-bonding to glutamic acid, tyrosine, or amino acid side chains (3- and 4-OH groups from fucose, 6-OH group from galactose) and in ion pairing with an arginine side chain (carboxylate of SA). Hydrogen bonds above are represented as grey dotted lines.

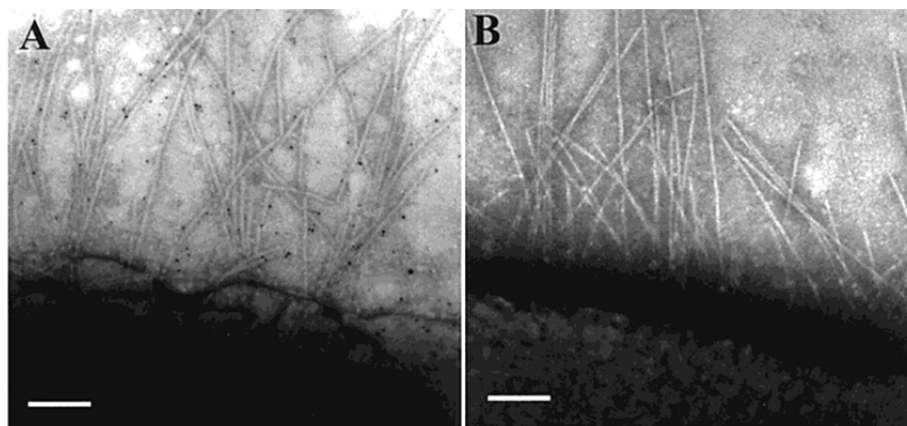
Water molecules are also explicitly involved in the binding of proteins to carbohydrates by mediating ligation of the carbohydrate residue to the binding gap of the protein.<sup>[25]</sup> When a glycan surface matches to the protein-binding site, water can be displaced and binding occurs. Water molecules readily form hydrogen bonds with other water molecules as well as with hydrophilic surfaces of proteins and carbohydrates. During binding, the protein-water and carbohydrate-water interactions are replaced by protein-carbohydrate interactions and the water molecules are released to the bulk. The reorganization of water molecules provides the driving force for protein-carbohydrate complexation.<sup>[25]</sup>

Structure-function studies performed in several systems highlight the importance of anionic carbohydrate substituents in binding their protein targets (coulombic interactions).<sup>[26]</sup> Sialyl Lewis x (sLe<sup>x</sup>) and other sialic acid derivatives, such as sialyl Lewis a (sLe<sup>a</sup>: Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3(Fuca1-4)GlcNAc $\beta$ 1-OH) and heparin (sulfated complex polysaccharide composed of glucuronic acid, iduronic acid, and glucosamine) contain anionic groups that are recognized by their respective receptors (Figure 7).

### 1.2.1 Bacterial adhesins and other lectins

Bacterial infections constitute a major global health problem. The most common serious neonatal infections involve bacteraemia, meningitis, and respiratory tract infections.<sup>[27]</sup> Key pathogens in these diseases are *Escherichia coli* (*E. coli*). To cause infection, bacteria often need to target cells and to colonize the glycosylated surface. For the attachment to cells, most bacteria depend on the expression of specialized adhesive organelles, which are hair-like, 1-2  $\mu\text{m}$  long and  $\sim 7$  nm wide protein structures on the bacterial cell surface. They are referred to as fimbriae (or pili). *E. coli* mainly utilizes two different types of fimbriae, the so-called P-fimbriae (containing the PapG adhesive protein) and type 1 fimbriae (containing the FimH adhesin). The two types of fimbriae are classified according to their sugar specificities; P-fimbriae show specificity for galabiose, whereas type 1 fimbriae bind to  $\alpha$ -D-mannopyranosides (Man) residues.<sup>[28]</sup>

As Man specific adhesion is among the most widely distributed types of carbohydrate specific bacterial adhesion,<sup>[29]</sup> several studies have described the mechanism. The direct binding of FimH to Man, attached to a carrier protein, was demonstrated for the first time by Klemm and his co-workers in 1990 using transmission electron microscopy (TEM) measurements.<sup>[30]</sup> In 2002 Wu *et. al.* confirmed the specific binding of Man functionalized gold nanoparticles (AuNPs) to FimH protein (the Man binding protein) by TEM measurements.<sup>[31]</sup> Two *E. coli* strains, the wild-type (wt) expressing FimH and a mutant, devoid of FimH ( $\Delta$  FimH) were incubated with Man-AuNP separately in buffer solution at different temperatures (4, 25, and 37  $^{\circ}\text{C}$ ). The TEM results showed that Man-AuNP selectively bound to the pili of the *E. coli* (wt) (Figure 5), demonstrating specific binding of Man to FimH.



**Figure 5.** Specific binding of Man-AuNP to FimH. Typical TEM images of sectioned areas of (A) pili of the *E. coli* (wt) decorated with Man-AuNP; (B) pili of *E. coli* ( $\Delta$  FimH) are not labeled. Scale bar 100 nm.<sup>[31]</sup>

The nanoparticles were localized at the lateral ends and distributed at intervals along the shaft of the pili (average of 100-150 nm intervals). The competition assay with free Man suggested that Man-AuNP bound FimH better than free Man did, due to the multivalent presentation of Man on gold surface. This work demonstrated that carbohydrate attached nanoparticles can be used as an efficient affinity label and a multiligand carrier in a biological system.

Commonly used antibiotics suffer from increasing resistance of pathogens.<sup>[32]</sup> Unlike general antibiotics, prevention of bacterial binding does not stimulate the bacteria to develop resistance because the bacteria survive the treatment. Since bacteria bind to cell surfaces in a multivalent manner, research was performed towards the development of multivalent compounds to overcome the low affinity of monovalent carbohydrates to adhesion proteins. The multivalent binding mode of several Man-glycopolymers was demonstrated by testing them for binding with a highly Man sensitive lectin Concanavalin A (Con A, a model analyte). Con A isolated from jack beans (*Canavalia ensiformis*) is a well known tetrameric protein at neutral pH with four identical carbohydrate binding sites. Its specificity is directed to  $\alpha$ -D-mannopyranosides and to a lesser extent to  $\alpha$ -D-galactopyranosides, and shows no affinity towards  $\beta$ -D-monosaccharides.<sup>[33]</sup>

Over the years several multivalent Con A inhibitors have been synthesized. The different types of polyvalent inhibitors are discussed in Section 1.5.

### **1.2.2 Carbohydrate-protein interactions in inflammation; the role of selectins in inflammation**

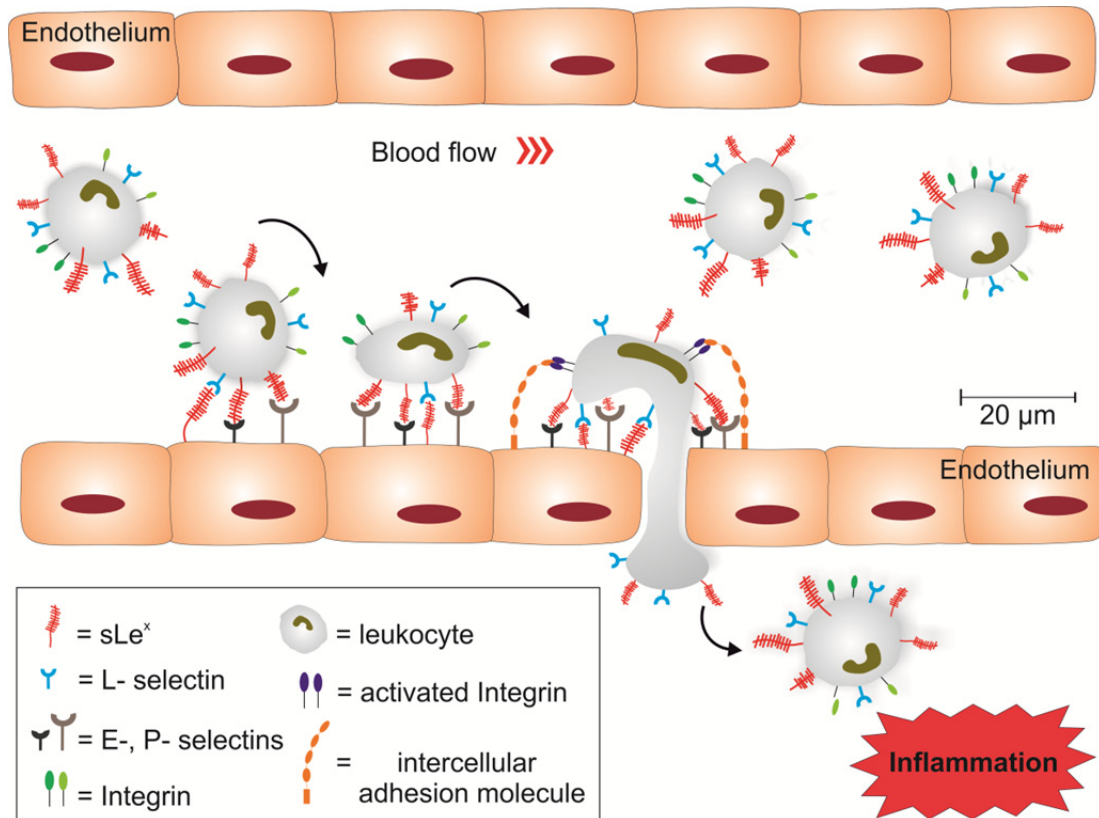
Carbohydrate mediated cell adhesion is an important cell function initiated by tissue injury or infection. Intercellular adhesion events are the origin of the migration of white blood cells to the site of infection and are mediated by a family of sialic acid specific binding proteins known as selectins.<sup>[34]</sup> Expression of specific combination codes of adhesion and signaling molecules on the respective endothelial site determines the leukocyte class that enters the tissues in response to injury or immunologic challenge.

However, in disease (such as arthritis and asthma) a dysregulated expression of adhesion and signaling molecules may result in excessive leukocyte accumulation, which could lead to tissue damage.<sup>[35]</sup>

#### **1.2.2.1 Leukocyte migration and selectins**

Immune responses are critically dependent on the ability of leukocytes to migrate to sites of infection (inflamed tissues). Leukocytes are transported to these sites via blood circulation and extravasate from the blood into the tissues upon specific interactions with activated

endothelial cells which line the blood vessels. The three selectins E-selectin (expressed on activated endothelium), P-selectin (expressed on activated platelets and endothelium) and L-selectin (constitutively presented on leukocytes), responsible for the initial contact to vascular endothelium, are a family of transmembrane glycoproteins (Figure 6).<sup>[36]</sup>



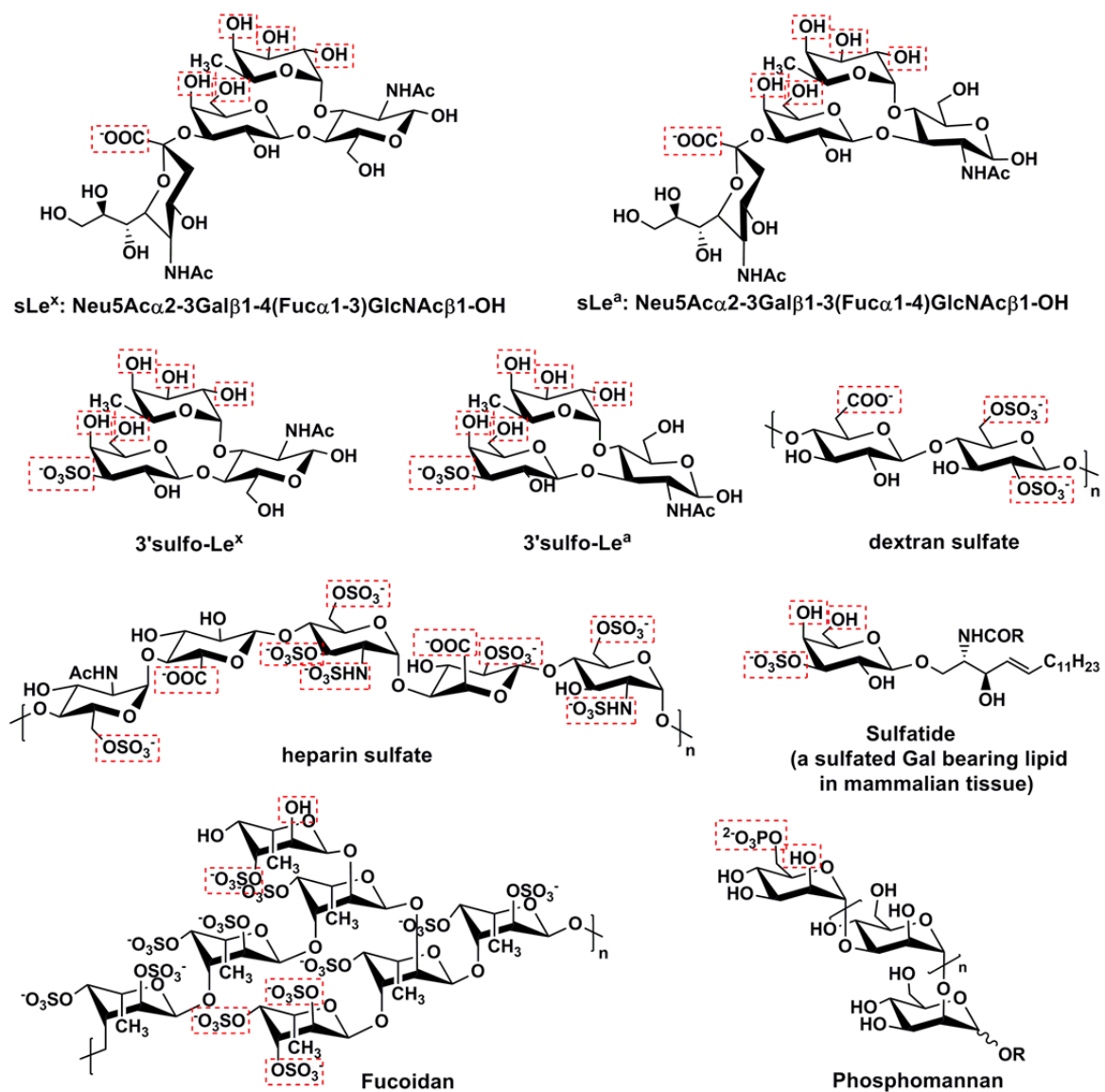
**Figure 6.** The "adhesion cascade": Interactions between leukocytes and endothelium. Leukocyte-endothelial cell contacts are initiated by selectin dependent tethering and rolling of leukocytes on sLe<sup>x</sup> structures, followed by integrin dependent firm adhesion, spreading and, finally, extravasation of leukocytes into the inflamed tissue.

The initial step of the complex adhesion cascade involves activation of vascular endothelial cells; including upregulation of selectins (cytokine induced expression of E-selectin and P-selectin). Transient binding of leukocytes to activated endothelial cells can be observed by tethering and rolling of the white blood cells on activated endothelium. These interactions are mediated by L- and P-selectins, which recognize sLe<sup>x</sup> bearing structures on the opposite cell-surface. Stronger adhesion is subsequently formed by the leukocytes activated integrins, which bind to endothelial proteins of the immunoglobulin superfamily, like the intercellular adhesion molecule-1 (ICAM-1). Transepithelial migration is guided by further adhesive interactions, and finally the leukocytes cleave the paracellular connections and transmigrate through the endothelium into the underlying tissue.<sup>[37]</sup>

## 1. Introduction

The best characterized and studied ligand for the three selectins is the P-selectin glycoprotein ligand-1 (PSGL-1), which is a mucin type glycoprotein expressed on all white blood cells. Crystal structure analysis by Somers *et al.* has shown that binding of P-selectin to PSGL-1 depends on the arrangement of an sLe<sup>x</sup> epitope, presented as an O-glycan in proximity to three sulfated tyrosine residues of PSGL-1 protein backbone.<sup>[24,38]</sup>

A similar arrangement of ligands was observed for high affinity binding of PSGL-1 to L-selectin<sup>[39]</sup>, but not to E-selectin.<sup>[22,40]</sup> In this case sulfated tyrosine residues are not required.<sup>[24]</sup> It was found that 3'-sulfo-Le<sup>a</sup> and 3'-sulfo-Le<sup>x</sup> bound to E-selectin as strong as sLe<sup>x</sup> (Figure 7).<sup>[41]</sup> However, the sulfated analogs were better inhibitors for L- and P-selectins.<sup>[42]</sup>



**Figure 7.** Structure of the selectin ligands sLe<sup>x</sup>, sulfo-Le<sup>x</sup>, sLe<sup>a</sup>, sulfo-Le<sup>a</sup>, dextran sulfate, sulfatide, fucoidan, phosphomannan, heparin sulfate. The functional groups that have been shown to be critical for the binding to the selectins are highlighted.

The hypothesis that sulfation would increase the affinity for L- and P-selectin<sup>[43]</sup> was confirmed by Bertozzi *et al.* with derivatives produced by chemical synthesis<sup>[44]</sup>, e.g. sulfated lactose derivatives.<sup>[45]</sup> According to studies of Yuen *et al.*, the order of ligands affinity for selectins was 3'sulfo-Le<sup>a</sup> > sLe<sup>a</sup> > 3'sulfo-Le<sup>x</sup> > sLe<sup>x</sup> and hexasaccharides > pentasaccharides > tetrasaccharides > trisaccharides, where 3'sulfo-Le<sup>a</sup> binds 15 fold better than sLe<sup>x</sup>.<sup>[46]</sup> Additionally, a number of anionic saccharides such as polyphosphomannan, fucoidan, sulfatide, heparin fragments, keratin and chondroitin sulfates showed low level of binding to L- and P-selectins, but not to E-selectin.<sup>[41,47]</sup>

Recent studies by Dervedde *et al.* showed that sugar moieties are not necessarily required to achieve high L- and P-selectin binding, as long as a high density of sulfate groups are presented in a polyvalent fashion (on AuNPs,<sup>[48]</sup> or on polymeric scaffold).<sup>[49]</sup>

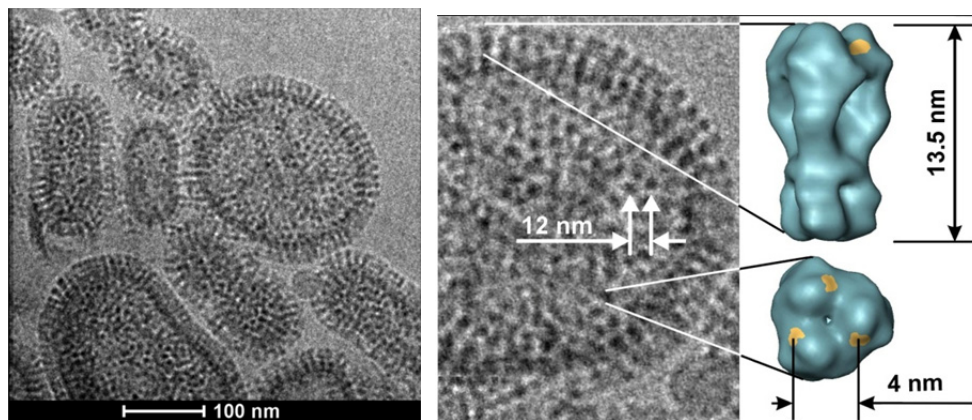
### **1.2.3 The role of carbohydrates in influenza virus inhibition**

The influenza A virus, which causes epidemics and pandemics in human populations, is difficult to eradicate.<sup>[50]</sup> Influenza A virus is a pleiomorphic, enveloped negative-stranded RNA virus, belonging to the Orthomyxovirus family (i.e. their size and shape can vary depending on strain and origin). It consists of eight single-stranded RNA segments of negative polarity, which significantly attribute to the stability of the virus during replication.<sup>[20]</sup>

Viruses propagated in embryonated chicken eggs are mostly spherical<sup>[51]</sup> with a diameter of ca. 100 nm. The surface of influenza virus is decorated with three proteins: an M2 ion channel protein, the lectin hemagglutinin (HA), and the sialidase enzyme neuraminidase (NA).<sup>[19b,50]</sup> HA mediates cell attachment by binding sialic acid (SA) residues on host glycoproteins and glycolipids, thus initiating infection. NA catalyzes the cleavage of terminal SA from glycans on the host surface and is therefore responsible for virus release. The M2 protein is lined in the inner side of the viral envelope and plays an important role in uncoating the virus and exposing its content to the cytoplasm of the host cell.<sup>[52]</sup> Figure 8 shows a cryo-electron micrograph (cryo TEM) of budded influenza virus particles, in which the densely packed HA and NA spikes are clearly visible protruding externally from the viral membrane.

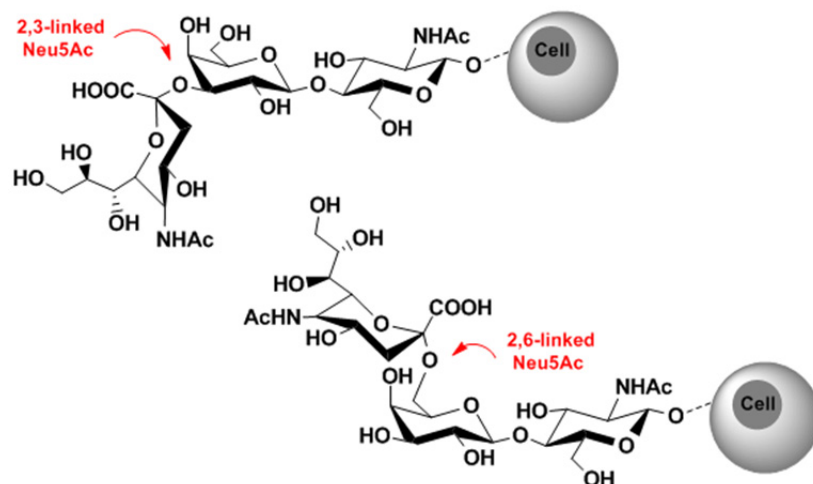
The influenza infection (common flu) is a well-known example of a cell-virus interaction mediated by sialic acids. As opposed to selectin-carbohydrate interactions discussed in the section 1.2.2.1, where sugar moieties are not necessarily required, sialic acid functionalities appeared essential for favorable binding interactions in influenza infections. In general, the influenza virus adheres to the target host cell by using its surface glycoprotein HA to recognize glycoconjugates that present terminal  $\alpha$ -linked N-

acetylneuraminic acid ( $\alpha$ -Neu5Ac) residues, a carboxylated ninecarbon monosaccharide.<sup>[52]</sup>  $\alpha$ -Neu5Ac is the most common form of sialic acid found in humans.



**Figure 8.** Cryo-TEM of pleomorphic influenza A X31 virions. The lipid bilayer is not visible in the intact particles, but the trimeric fusion protein HA and NA (6 nm, tetramer) glycoprotein spikes protrude from it. Most of the spikes are HA molecules (left). The right side shows individual HA sticking out of the viral membrane (2-4 per 100 nm<sup>2</sup>, 600-1200 per virus particle),<sup>[11b]</sup> in side-view projection (enlargement, top, represented by a 3D surface representation) and top view projection (enlargement, bottom, represented by a 3D surface representation) of a HA protein. Individual receptor binding sites, which are 4 nm apart, are depicted in yellow.

Another slightly modified form of sialic acid is  $\alpha$ -Neu5Gc ( $\alpha$ -linked *N*-glycolyneuraminic acid), which bears a hydroxyl group at the *N*-acyl position, and can be found mostly in animals. In addition to Neu5Ac and Neu5Gc there are at least 50 forms of sialic acid found in nature, which are in detail described in a review by Varki *et al.*<sup>[53]</sup> The carbohydrate binding specificity of the virus is dependent on the species of origin. Human viruses (i.e. H3N2) bind to glycans with terminal Neu5Ac $\alpha$ 2-6Gal glycan structures,<sup>[54]</sup> whereas, viruses of avian origin (i.e. H5N1) preferentially bind to glycans with terminal Neu5Ac $\alpha$ 2-3Gal structures<sup>[55]</sup> (Figure 9).



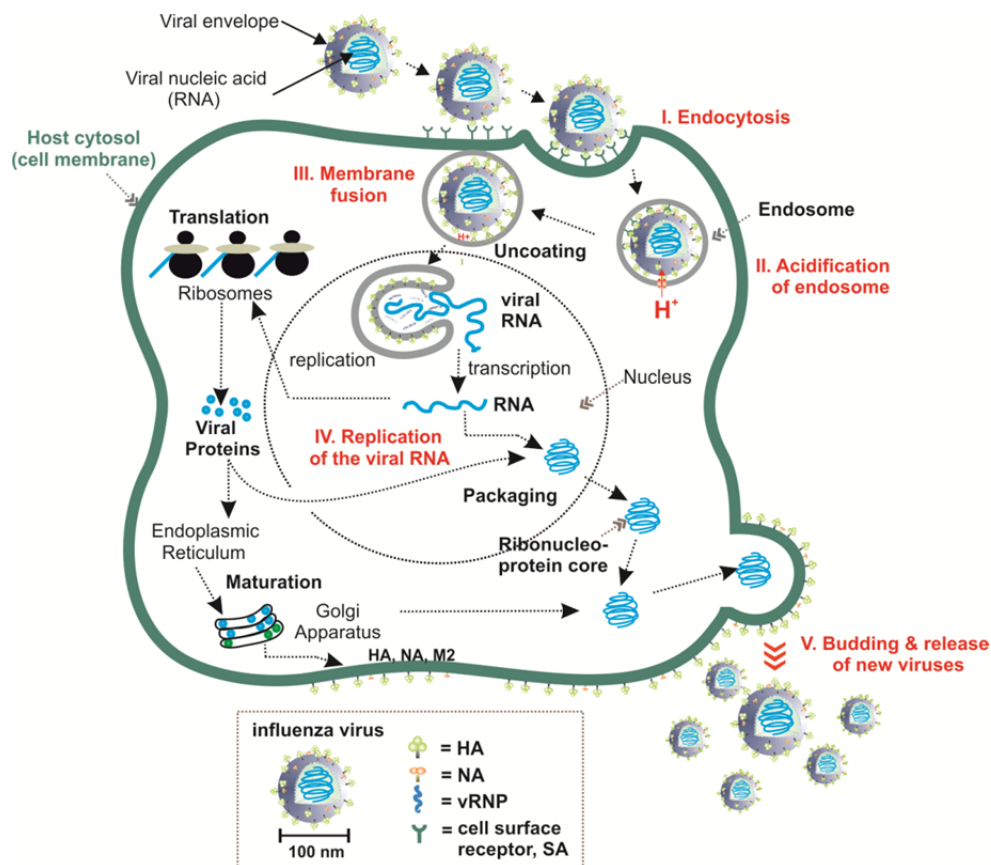
**Figure 9.** Structures of sialic acids that are attached to the 3 (top) or 6 (bottom) position of the penultimate galactose residue.



Neu5Ac $\alpha$ 2-3Gal structures can only be found in the lower respiratory tract of human tissue and not in the nasal and throat tissue (which would be more readily exposed to inhaled virus). Consequently, humans are not highly disposed to avian influenza and human-to-human transmission of avian influenza is inhibited. Although a mutation of a single amino acid in the HA protein of the virus may be sufficient to change the binding specificity of avian strains from  $\alpha$ 2-3- to  $\alpha$ 2-6-glycosidic linkage.<sup>[56]</sup>

### 1.2.3.1 Influenza virus attachment, replication, transcription, and translation

The hemagglutinin binds with the sialic acids present on glycoprotein receptors of the host cell. After adsorption, it is internalized as an endosome due to the acidic environment (pH ~5.5) of the host cell. This acidic medium activates the M2 protein ion channel in the viral membrane, allowing the internal capsid to be acidified. The cell then begins digesting the contents of the endosome, by acidifying its interior and transforming it into a lysosome (Figure 10).



**Figure 10.** Viral replication cycle: I. endocytosis; II. acidification of endosome, HA conformation change; III. membrane fusion, the HA fusion protein inserts in endosomal membrane; IV. RNA enters nucleus, RNA replication; V. release of newly assembled viruses.

When the pH drops, the original folded structure of the HA molecule becomes unstable, partially unfolds, and exposes a very hydrophobic peptide sequence, that was previously buried in the protein. This so-called fusion peptide is responsible for the fusion of viral and endosomal cell membrane and results in the release of viral RNA and RNA-dependent RNA-Polymerase. After transfer to the nucleus mRNA (the positive strand) is generated and copied. By usage of the host translation machinery viral proteins are produced and assemble at the plasma membrane, where the virions finally are released. The release of virions from the host cell surface is completed by the viral enzyme sialidase (NA), which cleaves the terminal  $\alpha$ -Neu5Ac residues from both the newly synthesized virion glycoproteins as well as from the host-cell surface. The action of NA thus enables the host-cell-surface aggregated progeny virions to leave the infected cell and seek for new host cells to infect.<sup>[51]</sup>

Currently, two distinct strategies, vaccines and small molecule therapeutics, are used to control the spread of the virus. Vaccination offers limited protection but is hindered by the production of sufficient quantities of vaccines for large populations in a short period of time.<sup>[57]</sup> With respect to small molecule therapeutics both HA and NA have been proposed as potential anti-influenza drug discovery targets.<sup>[58]</sup> There are currently two antiviral drugs for the treatment and/or prevention of influenza infection: Zanamivir (Relenza<sup>®</sup>) and Oseltamivir (Tamiflu<sup>®</sup>). They both efficiently block the sialidase, and therefore significantly inhibit the release mechanism.<sup>[13]</sup> Although these drugs may reduce the severity and duration of influenza infections, they have to be administered within 24-48 hours after the development of symptoms in order to be effective.<sup>[59]</sup> The former two drugs Amantadine and Rimantadine inhibit the M2 ion channel protein. They block the influx of H<sup>+</sup> ions through the M2-proton channel so that uncoating and the release of free ribonucleoproteins into the cytoplasm is inhibited. However, these drugs have serious side effects.<sup>[60]</sup>

An alternative to interfere with influenza replication is to target HA<sup>[61]</sup> and thereby preventing viral adhesion but these compounds have failed to become drugs so far.<sup>[19b]</sup> This was mainly due to weak HA binding properties shown by monomeric sialic acid derivatives. The viral adhesion uses a multivalent effect because monovalent binding between HA and SA is weak with an affinity constant of  $10^{-3}$  M only, as mentioned previously.<sup>[11b]</sup> This is why the role of multivalency in the design of potent multivalent HA inhibitors has been investigated by numerous researchers. Several synthetic compounds have been tested and the most important ones are described in Section 1.5.

### 1.3 The importance of multivalency in biological processes

The “small area, weak binding” characteristic of protein-carbohydrate interactions provides the basis for a fast exchange rate that seems essential under physiological conditions. The

monosaccharide-lectin interactions are relatively weak, yet the strength and specificity required for recognition in physiological settings is high.<sup>[11b]</sup> In nature this problem is solved by the use of multivalency, the simultaneous and specific association of two or more ligands to one biological entity, which can result in strong binding (up to 1000-fold) between proteins and carbohydrates.<sup>[11b,62]</sup> In the case of glycoligands it is commonly referred as a “cluster effect”, which greatly increases the overall avidity of carbohydrate ligands towards their receptors.<sup>[10,11b,62]</sup>

Multivalent interactions usually result in much higher specificity and thermodynamic and kinetic stability than those arising from simple monovalent interactions. In particular, avidity is higher since the dissociation-rate of a multivalent species is much slower than that of a monomer, because rebinding events easily can occur.<sup>[63]</sup>

Multivalent ligands can exhibit enhanced binding to biological surfaces as a consequence of the lesser entropic cost of organizing them at the binding sites. The higher binding affinities of multivalent complexes originate from two distinct mechanisms that is steric stabilization and entropically enhanced binding. Steric stabilization is found in inhibitors with a relatively large backbone structure, which can sterically hinder the receptor from reaching other ligands, as well as shield a large part of the existent receptors without actually binding to all of them.<sup>[64]</sup>

Entropically enhanced binding arises from the fact that multivalent protein-carbohydrate interactions are associated with both a favorable enthalpy and entropy, as opposed to the general mechanism in protein-carbohydrate interactions. This is in contrast to monovalent interactions, where the binding between the ligand and its receptor induces a conformational retention on the carbohydrate ligand that leads to an entropy cost. In the multivalent interaction this restriction of carbohydrate flexibility has already been induced, at least partly, by the backbone carrier so that the entropy cost is smaller than in a monovalent situation. The net result is a more negative free energy ( $\Delta G^\circ$ ) and a higher affinity: [Eq. (1)].<sup>[7,11a-b,65]</sup>

$$\Delta G^{poly} = \Delta H^{poly} - T\Delta S^{poly} \quad (\text{Equation 1})$$

Due to the better binding properties multivalent interactions control many important biological processes such as cell-cell adhesion or cellular recognition of foreign antigens (viruses, bacteria) by macrophages.<sup>[5b, 11b]</sup> Prevention of influenza virus hemagglutinin binding to host cells was among the first application of developing multivalent inhibitors.<sup>[11b]</sup>

## 1.4 Mechanism of multivalent ligand binding

In the design of synthetic multivalent molecules with desired properties an important fact is the understanding of the structure and thermodynamics of the system. There are several different mechanisms by which a ligand can interact with a receptor contributing to high activities often observed for multivalent ligands. The possible binding mechanisms according to Kiessling *et al.*<sup>[68]</sup> are presented in Figure 11.

The monovalent ligand can commonly bind only to a single site on the receptor or it can heterodimerize a receptor via two receptor binding faces. In contrast, multivalent interactions are more diverse. Relevant mechanisms include (Figure 11):

a) Chelate effect (chelation) occurs with proteins having closely separated carbohydrate binding sites. The multimeric ligand must be capable of bridging the distance between the receptor sites. Chelate effects is primarily used for small molecules (mainly metals and ions) binding to multivalent hosts. A typical example of chelate effect is the ability of bidentate ligands (ethylene diamine, 2,2-bipyridine) to form a more stable complex with transition metals than corresponding monodentate ligands (ammonia, pyridine).

b) In subsite binding the primary binding to the receptor promotes secondary binding interaction to other receptors, which is in close distance to the first one.

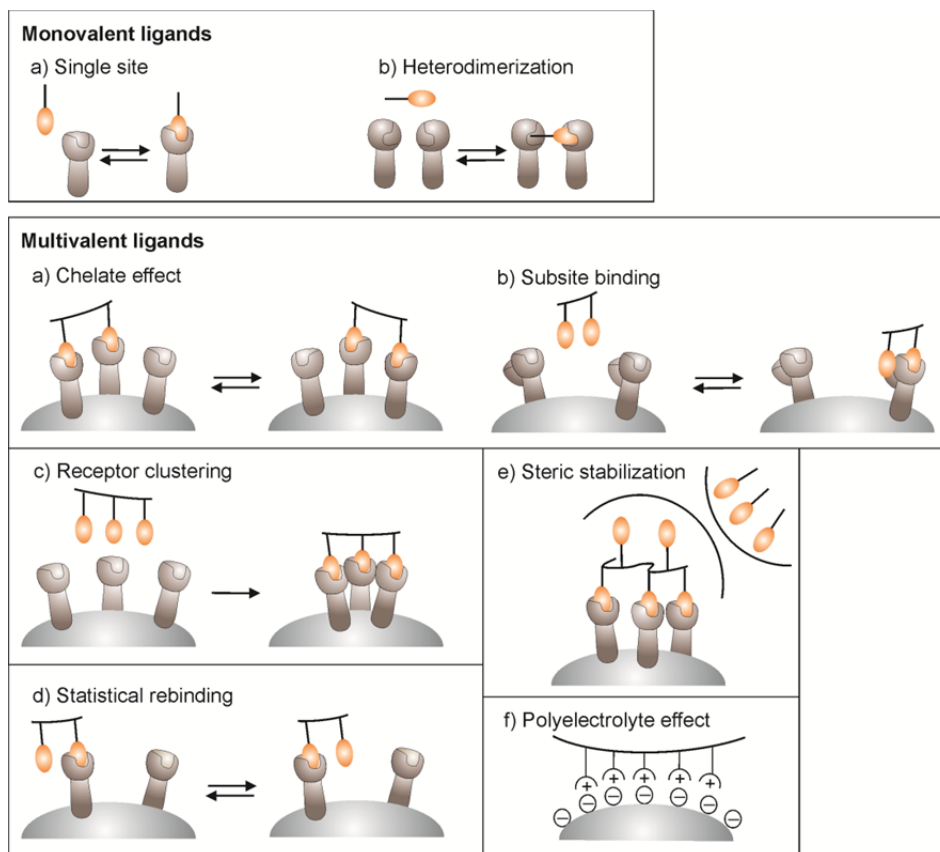
c) Multivalent binding collects receptors (receptor clustering), thereby altering the signalling properties of the receptors.

d) Statistical effect. Multivalent ligands display higher local concentrations of binding species, which promotes rebinding.

e) Steric stabilization. Binding of a large multivalent ligand inhibits further ligands from binding by sterical blocking the surface.

f) Polyelectrolyte effect. The entropically favorable release of counterions into solution, as the protein binds, provides a driving force for association of the two macromolecules, which plays an important role in e.g. protein-DNA association.<sup>[66]</sup>

Cooperativity, a conceptually related term, differs from multivalency but is frequently used in literature as a substitute for multivalency. It usually describes systems which do not involve multivalency. The best known example of such a cooperative system in biology is the cooperative binding of oxygen to hemoglobin subunits, where the binding strength of the second oxygen molecule is increased by the first one and the sum of both binding energies is higher than two times the binding energy of the first oxygen.<sup>[67]</sup>



**Figure 11.** Potential binding modes of mono and multivalent ligands with cell-surface receptors according to Kiessling et al.<sup>[68]</sup> Monovalent ligands: a) Single site binding, b) Receptor heterodimerization. Multivalent ligands: a) Chelate effect, b) Subsite binding, c) Receptor clustering, d) Statistical effect, e) Steric stabilization, f) Polyelectrolyte effect.

## 1.5 Synthetic multivalent neoglycoconjugates as modulators of biological processes

### 1.5.1 Carbohydrates as terminal substituents

A variety of multivalent structures have been synthesized that display carbohydrates on their surfaces, including peptides, proteins, lipids, polymers, nanoparticles, and dendrimers. The biological properties of these compounds can be investigated by well-established methods like Hemagglutination Inhibition Assay (HIA), Enzyme-Linked Lectin Assay (ELLA), Surface Plasmon Resonance Spectroscopy (SPR), and Isothermal Titration Calorimetry (ITC).<sup>[10]</sup> Usually the affinities are given as  $K_d$  (dissociation constant) or  $IC_{50}$  value ( $IC_{50}$  represents the concentration of an inhibitor that causes 50 % reduction in the binding of the labeled reference ligand *in vitro*). It was found that the observed multivalent effects depend very much on the assay used. The assays operate in greatly different concentrations and

measure different physical properties, therefore the values are only comparable within one distinct binding assay.<sup>[10]</sup>

Carbohydrates as terminal substituents are ideally suited to participate in a variety of recognition processes between cells and molecules. Sialic acids that are typically found attached to the outermost ends of glycoproteins can mediate a wide variety of physiological and pathological processes. In a recent review by Varki<sup>[69]</sup> lectins and their respective sialoside ligands are described which can contribute to disease development. Some examples are listed in Table 1.

Carbohydrate binding protein	Cell surface sialoside ligands*
Influenza A virus hemagglutinin (human)	Neu5Ac $\alpha$ 2-6Gal $\beta$ 1-4GlcNAc $\beta$ 1-R
Influenza A virus hemagglutinin (avian)	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-R
Sendai virus hemagglutinin	Neu5Ac $\alpha$ 2-8Neu5Ac $\alpha$ 2-3Gal $\beta$ -R
<i>E. coli</i> adhesin	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4Glc $\beta$ -R
<i>Vibrio Cholerae</i> toxin	Gal $\beta$ 1-3GalNAc $\beta$ 1-4(Neu5Ac $\alpha$ 2-3)Lac $\beta$ -R
E-, L- and P-selectin	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuca1-3)GlcNAc $\beta$ -R Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3(Fuca1-4)GlcNAc $\beta$ -R
Bone marrow macrophage lectin	Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3GalNAc $\beta$ -R

**Table 1.** Examples of lectins from different origin that bind to sialic acid on human cell surfaces \*(R indicates the remaining carbohydrate core structure to which the sialoside sequence is attached).

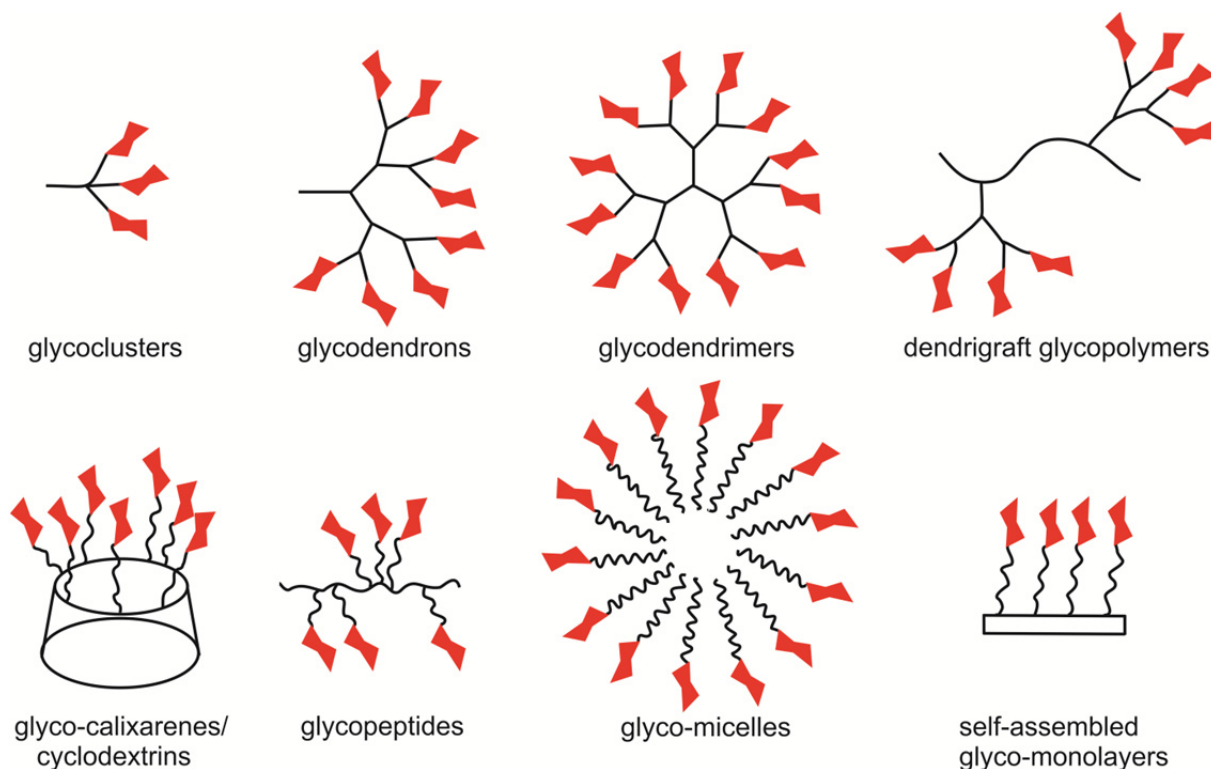
Three families of carbohydrate-binding receptors that have been thoroughly investigated are Gal/GalNAc receptors, Man binding proteins, and SA-binding receptors. A review by Roy describes in details the synthetic efforts that have been applied towards the synthesis of multivalent neoglycoconjugates which contains ligands for these protein receptors.<sup>[11c]</sup>

### 1.5.2 Different classes of multivalent model systems. Effect of scaffold structure on function

A multivalent ligand consists of a main core called the scaffold/carrier, which bears several covalent connections, linkers or spacers, to the peripheral ligating (binding) units. Binding can be modulated by varying the structure of the carrier or the binding unit (saccharide) or by altering their spacing (introducing flexibility between the scaffold and the carbohydrate). The relative sizes of the scaffolds used for multivalent interactions can be determined by electron microscopy or by dynamic light scattering (DLS) measurements.

A number of different molecules, e.g. linear polymers such as polyacrylamides,<sup>[11b,70]</sup> peptides,<sup>[71]</sup> bovine serum albumins, dendrimers,<sup>[72]</sup> cyclodextrins,<sup>[73]</sup> calixarenes,<sup>[73a]</sup> and gold nanoparticles,<sup>[31,74]</sup> have been used as scaffolds for multiple presentations of mono- and

oligosaccharides in the attempt to create multivalent binding of the corresponding receptors. An alternative approach involves non-covalent association of the carbohydrates in liposomes,<sup>[75]</sup> membranes or other surfaces (Figure 12).<sup>[11b]</sup>



**Figure 12.** Diverse scaffolds used for multivalent interactions: low-molecular weight displays (e.g. dimers, trimers), dendrons, dendrimers, 3-D cavity containing scaffolds (glyco-cyclodextrins/ calixarenes), peptides, liposomes (are not shown), 2-D self-assembled monolayers (SAM) on Au/quartz.

Adhesion of *E. coli* was extensively studied by Lindhorst *et al.* They created several Man glycoclusters,<sup>[76]</sup> carbohydrate centered cluster mannosides (octopus glycosides),<sup>[77]</sup> oligo- and multivalent systems<sup>[78]</sup> and glyco-SAMs, which were targeted as inhibitors of Man-specific bacterial adhesion. In the preparation of SAMs they applied the click-chemistry approach.<sup>[79]</sup> By altering the spacer lengths of mannosyl clusters, the ligand preferences to the type-1 fimbriae lectin of *E. coli* could be elucidated.<sup>[76,80]</sup>

Carbohydrate ligands can naturally assemble to multivalent structures (clusters). This has been observed for glycolipids, which are part of the lipid bilayer of the cell surface. For example, randomly distributed glycolipids may respond to binding interactions by assembling themselves into patches of high density.

Based on these findings Bruehl *et al.* prepared postpolymerized bifunctional liposome assemblies with embedded sLe<sup>x</sup> or sulfo-Le<sup>x</sup> analogs (5 %) and distinct anionic (-SO<sub>3</sub><sup>-</sup>, -CO<sub>2</sub><sup>-</sup>) or cationic (-NH<sub>3</sub><sup>+</sup>) or neutral (-OH) head groups in the matrix of liposomes.<sup>[81]</sup> As it was mentioned in Section 1.2.2, sLe<sup>x</sup> itself had only moderate affinity to selectins with K<sub>d</sub> values reported in the range of ~ 0.1 mM (for comparison the K<sub>d</sub> of physiological PSGL-1 is 778 nM). They demonstrated that the presentation of additional anionic functional groups in the form of

sulfate esters on a polymerized liposome surface containing a multimeric array of sLe<sup>x</sup>-like oligosaccharides, generated a highly potent, bifunctional macromolecular assembly. This assembly inhibited L-, P-, and E-selectin binding to GlyCAM-1 (a physiological ligand) better than sLe<sup>x</sup>-like liposomes without additional anionic charges. The IC<sub>50</sub> values (217-425 nM) were four orders of magnitude higher than the IC<sub>50</sub> values of monovalent carbohydrates (Figure 13).

In influenza virus inhibition studies, numerous divalent linear as well as branched multivalent clusters were prepared using a synthetic and enzymatic approach. The synthesized HA inhibitors reported to date have been low molecular weight scaffolds forming clusters of mono-,<sup>[19b,82]</sup> bi-,<sup>[83]</sup> tetra-,<sup>[84]</sup> and octavalent sialosides. In the last case, the scaffold used was a polyazido-calix[4]arene.<sup>[85]</sup> Two types of carriers were adopted for obtaining glycoconjugates bearing multiple sialic acid residues: natural backbones such as self-assembling sialo-glycopeptides,<sup>[86]</sup> proteins<sup>[87]</sup> or polysaccharides (chitosan backbone)<sup>[88]</sup> and synthetic backbones like spherical (liposomes<sup>[89]</sup> and dendrimers), and linear polymers, and nanostructures.

Unverzagt *et al.* prepared two types of the target ligand made of Neu5Acα2-6Galβ1-4GlcNAcβ1-Asn, which were scaffolded enzymatically on preformed βGlcNAc-Asn linked residues interspaced by various oligoglycine or oligo-L-proline spacers. The spacers were prepared using solid-phase synthesis on PAM-resin by applying the Boc strategy.<sup>[90]</sup> The synthesized influenza virus ligands had different geometries and distances between the sialic acids. Their studies showed that the inhibitory properties of these divalent sialosides were critically dependent on the inter sialoside distances and the geometry imposed by the peptide backbones. The compound with more flexible oligoglycine spacers [AcGly-Gly-Asn(Neu5Acα2-6Galβ1-4GlcNAcβ1-NH-CO-)-Gly<sub>15</sub>-Asn(Neu5Acα2-6Galβ1-4GlcNAcβ1-NH-CO-)-Gly-Gly-OH] was eight-fold more potent than the monosialidase. On the other hand, the compound with more rigid proline rich spacers ([AcAsn(Neu5Acα2-6Galβ1-4GlcNAcβ1-NHCO-)-Gly-Pro<sub>3</sub>]<sub>2</sub>-Lys-Gly-OH) showed only a moderate increase of binding.

An interesting way to accomplish multivalent interactions with influenza viruses is to synthesize liposomes with sialic acid residues exposed on their surface. Studies by the Whitesides group highlighted the ability of carbohydrate functionalized liposomes to function as efficient inhibitors of protein-carbohydrate recognition events. Kingery-Wood *et al.* prepared liposomes with O-linked sialosides, and demonstrated their biochemical activity by HIA assay. They found that SA presented on liposome had an inhibitory concentration of 20 nM. It was 10<sup>4</sup> more potent than bare SA, missing the lipid tail.<sup>[89b]</sup>

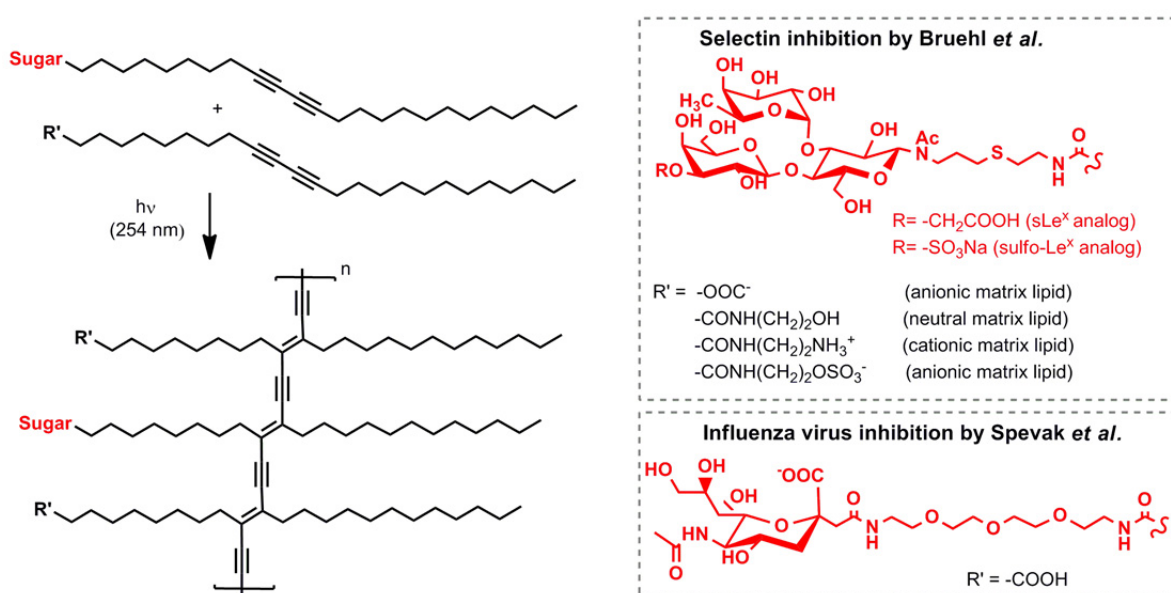
The major drawback of these liposomes was that they initiated lysis at concentrations above 10 μM. A more metabolically and physically stable class of sialylated liposomes was



## 1. Introduction

synthesized by Spevak *et al.* by partially polymerizing liposomes containing  $\alpha$ -C-linked sialylosides.<sup>[89c,89d]</sup>

Spevak *et al.* prepared liposomes carrying SA residues and crosslinked them with varying amounts of unsubstituted lipid to give covalently crosslinked liposomes that displayed different concentrations of sialic acid residues (0-60 %). They discovered that liposomes with 5 % and 10 % SA content resulted  $IC_{50}$  values  $3 \times 10^4$  times lower than the SA alone, whereas liposomes with a higher level of sialosides (30 % and 60 %) did not inhibit hemagglutination (Figure 13).

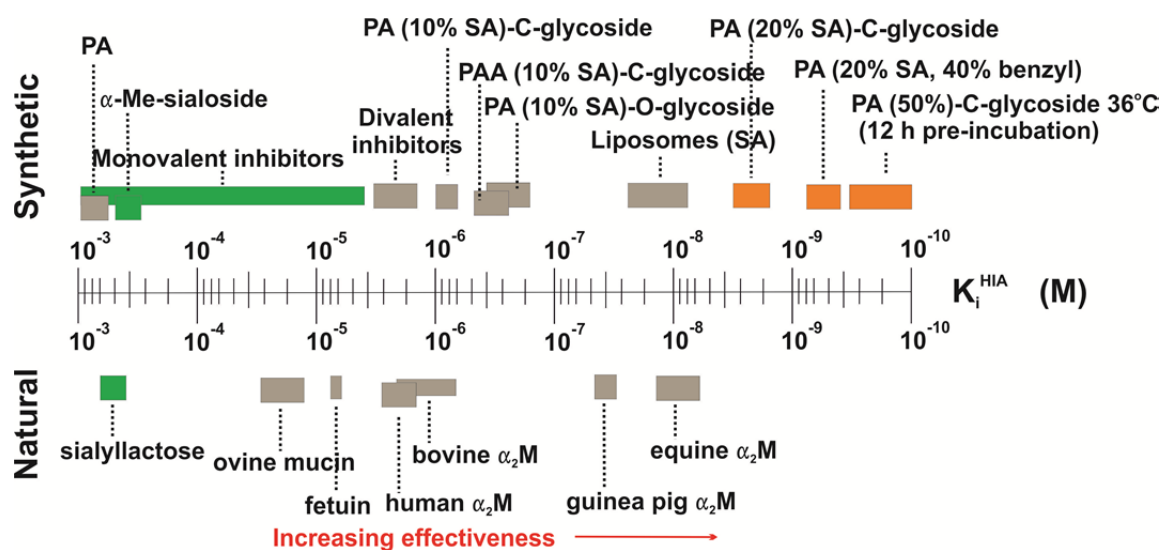


**Figure 13.** Synthesis of sugar liposomes used by Spevak<sup>[89d]</sup> and Bruehl *et al.*<sup>[81]</sup>

These findings show that a higher density presentation of carbohydrates does not necessarily exhibit higher potency. Liposomes present a polyvalent surface array that is quite large; consequently some of the inhibitory potency may derive from steric stabilization (see Section 1.3). Most examples of multivalent ligands focused on increasing the specific interactions between the carbohydrates and lectins were based on polymeric scaffolds. The vast majority of amorphous linear polymers, used in influenza virus inhibition studies, were either copolymers of acrylamide esters (polyacrylamide, PA) used by Roy,<sup>[70b,91]</sup> Whitesides,<sup>[11b,64,70c,92]</sup> Bovin,<sup>[70a,93]</sup> Matrosovich,<sup>[61,94]</sup> or copolymers of acrylic acid esters (poly(acrylic acid), PAA),<sup>[92b,95]</sup> polystyrene,<sup>[96]</sup> and others.<sup>[60]</sup> The first example of polyacrylamide polymer was reported by Roy *et al.* in 1987.<sup>[91]</sup> Since then, an increase in research activity has been noticeable in the literature in this area.<sup>[60]</sup> Polymers incorporating varying mole fractions of SA on a PA or PAA backbone have been synthesized by two general strategies. In the first method, both sialic acid bearing acrylamide and acrylamide itself were copolymerized in aqueous solutions using radical initiation chemistry. In this

manner,  $O$ -<sup>[91,92c,97]</sup>,  $N$ -<sup>[87d]</sup>,  $S$ -<sup>[70b]</sup>, and  $C$ -<sup>[92d]</sup> linked sialopolymers have been prepared. The  $N$ -,  $S$ -, and  $C$ -sialosides are resistant to the action of viral neuraminidase (NA), which catalyzes the cleavage of terminal SA. In the second approach, suitable functionalized sialosides were grafted onto preformed polymers bearing reactive functionalities.<sup>[94,98]</sup>

Whitesides and co-workers incorporated a range of SA groups into the PA and PAA polymeric scaffold and tested the ability of the resulting compounds to inhibit virus induced agglutination of red blood cells. The inhibitory concentrations were determined based on the amount of SA. In Figure 14 the  $K_i^{HIA}$  values are summarized for natural as well as some of the synthetic inhibitors of hemagglutination based on sialic acid.



**Figure 14.** Values of  $K_i^{HIA}$  for natural and synthetic inhibitors of hemagglutination based on SA. Monomeric inhibitors (green boxes), previously studied polymeric inhibitors (grey boxes), and the best PA polymeric inhibitors (orange boxes) are shown. The synthetic inhibitors are based on liposomes, poly(acrylic acid) (PAA) or polyacrylamide (PA).<sup>[92b]</sup>

The incorporation of SA groups into the side chain of polyacrylamide strongly enhanced its ability to inhibit hemagglutination mediated by influenza A X31. The inhibitory potency was found to depend on the density of the SA groups displayed on the polymer backbone (10-20 % SA). However, serious concerns have been raised about the toxicity of polymeric sialosides with polyacrylamide backbones.<sup>[28a]</sup> Food and drug administration (FDA) has not approved the use of polyacrylamide and similar polymer based compounds as therapeutics because of their pharmacokinetical problems.<sup>[19]</sup>

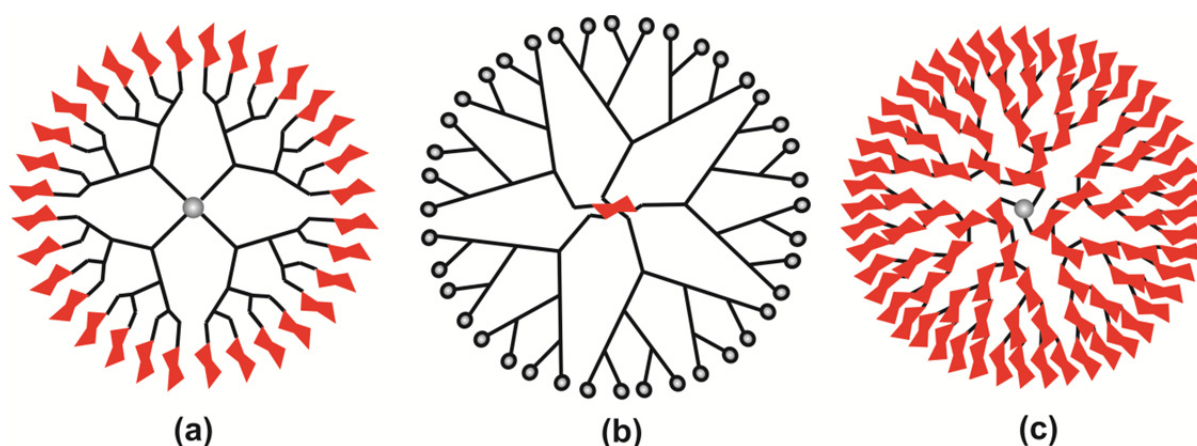
### 1.5.3 Glycodendrimers and hyperbranched polymers

In order to allow the synthesis of strictly monodisperse, nontoxic, multivalent glycoconjugates, dendrimers have been selected as molecular scaffolds. Dendritic molecules constitute one of the most exciting areas of modern nanochemistry, largely as a

consequence of the unique properties, associated with their branched architectures. Dendrimers were originally described independently by Newkome<sup>[99]</sup> and Tomalia<sup>[100]</sup> in 1985. They are oligo- to polymeric structures but offer some of the advantages of both polymers and small molecules. Thus dendrimers are comprised of repeating branched building blocks (dendrons) displaying a large number of functional groups, yet are monodisperse. Dendrons can be considered as individual dendritic branches, while dendrimers are structures in which a number of dendrons are attached to a single core unit.<sup>[101]</sup> Dendrimers can be prepared in a series of iterative steps by either a convergent or a divergent approach, initiating synthesis at the periphery or the core of the macromolecule.<sup>[102]</sup> The dense display of functional groups in dendrimers has driven to their derivatization with diverse, biologically relevant molecules. An application of dendrimers as therapeutics was recently developed by Starpharma. The polylysine dendrimer based drug (VivaGel<sup>®</sup>) has a multivalent sulfonated surface, which prevent infections with HIV (Human immunodeficiency virus) and HSV-2 (Herpes simplex virus 2). It also has potential application for other sexually transmitted infections and as a contraceptive.<sup>[103]</sup>

The potential of dendrimers to act as scaffolds for the multivalent presentation of ligands has awoken the interest of several research groups studying protein-carbohydrate interactions.<sup>[11c,72i,104]</sup> Carbohydrate functionalized dendrimers can mimic cell surface glycan arrays and are used as optical and electrochemical probes to sense lectins when they are complexed onto a tris(bipyridine)ruthenium(II) ( $[\text{Ru}(\text{bipy})_3]^{2+}$ ) derivative.<sup>[72b,105]</sup>

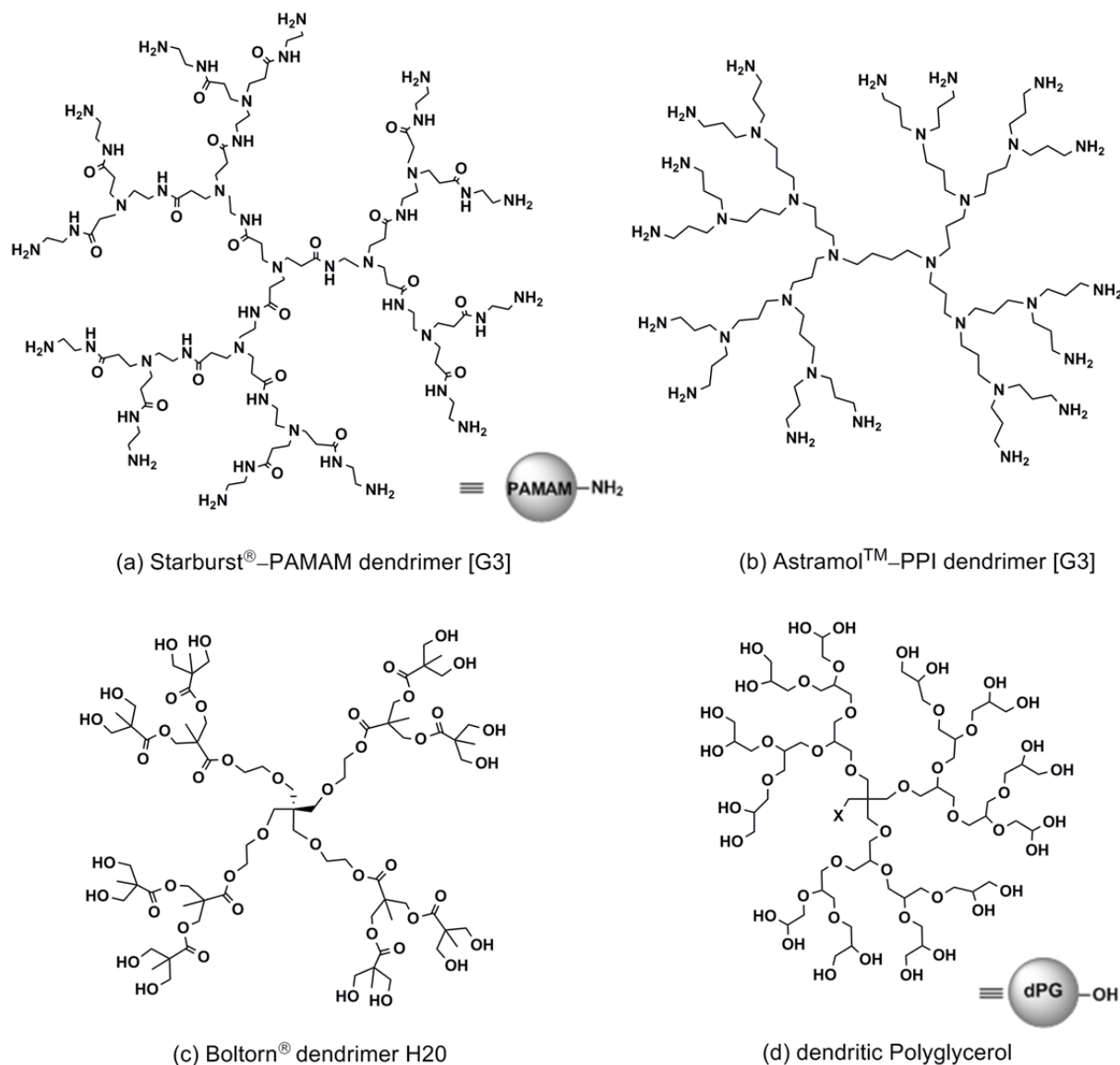
For classification of glycodendrimers, three groups have been distinguished: (a) carbohydrate coated non-carbohydrate dendrimers,<sup>[12a]</sup> (b) dendrimers containing a carbohydrate core molecule,<sup>[104]</sup> and (c) glycodendrimers grown from carbohydrate-building blocks<sup>[106]</sup> (Figure 15). From the two synthetic approaches the convergent one is better suited for the preparation of monodisperse, homogeneous materials.



**Figure 15.** Schematic representation of glycodendrimers varying by their synthetic constructions: (a) carbohydrate coated, (b) carbohydrate centered, and (c) carbohydrate based glycodendrimers.

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Most of the research groups are using sugar coated non-carbohydrate dendrimers with different dendrimer scaffolds. In Figure 16 the commercially available dendrimers which are mostly used as scaffolds are shown.



**Figure 16.** Structures of commercially available dendritic scaffolds bearing amine or alcohol functionalities (a-c), excluding dPG (d), which is not commercially available.<sup>[12a,106]</sup>

Both the PAMAM (poly(amido amine)) and PPI (poly(propylene imine)) dendrimers were frequently applied in glycodendrimer synthesis as scaffolds. They contain tertiary amine at the generation defining branching points and display primary amine groups on their periphery, to which other functional groups can be attached. Rare exceptions are the Boltorn<sup>®</sup> dendrimers with ester linkages in their structure and dendritic polyglycerol, which are not commercially available. Many reviews have been published analyzing the

chemistry,<sup>[11c,12a,28a,106-107]</sup> biophysical evaluation,<sup>[10]</sup> and the biological applications<sup>[11c,12,108]</sup> of glycodendrimers and glycoclusters.<sup>[109]</sup>

The groups of Roy and Stoddart developed the use of multivalent dendritic saccharides to enhance weak sugar binding processes with biological systems. Roy *et al.* used solid phase synthesis to generate sialic acid displaying dendrimers, based on poly-L-lysine core structures as ligands for influenza virus hemagglutinin. These dendrimers contained 2, 4, 8, or 16 terminal  $\alpha$ -thio-SA residues<sup>[110]</sup> and inhibited HA-mediated agglutination of erythrocytes in micromolar concentration.

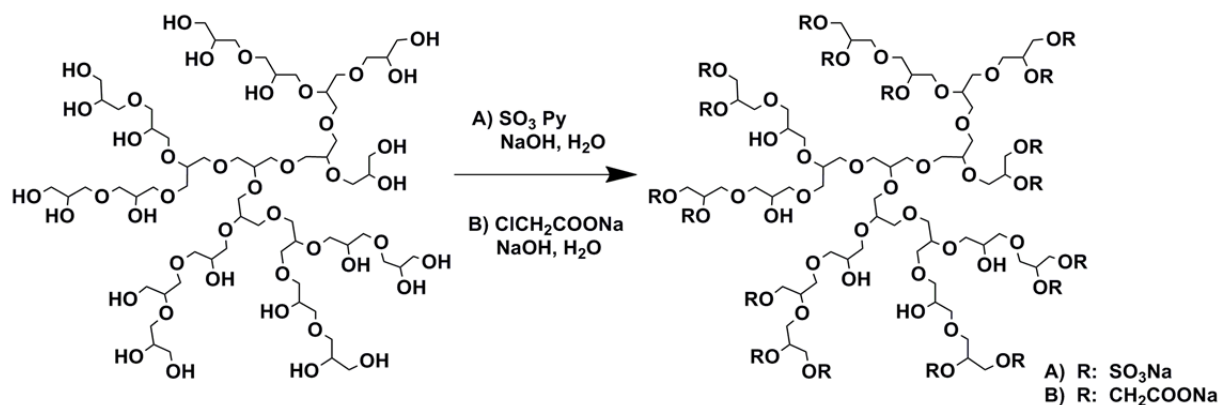
The majority of globular shaped glycodendrimers are constructed on PAMAM or related scaffolds, frequently applied in Con A studies by Cloninger,<sup>[72d,72f-k]</sup> Roy,<sup>[12a,72c,72e]</sup> Okada,<sup>[111]</sup> and others. PAMAM dendrimers have strong generation dependent binding affinities. Schlick and Cloninger have recently prepared large PAMAM dendrimers (G2 up to G6) conjugated with up to 95 Man residues which were tested using SPR binding assays for binding to Con A lectin. The glycodendrimers were efficient inhibitors of protein-carbohydrate interactions. IC<sub>50</sub> values ranged from 260 nM to 13 nM (4.2  $\mu$ M up to 1.2  $\mu$ M per mannoside).<sup>[72f]</sup> In general, observed affinity enhancement for dendrimer-lectin binding appears to be a result of protein-dendrimer aggregation, which is dependent on the dendrimeric architecture, valency and protein concentration. However, it is still relatively difficult to synthesize bulk quantities of higher generation dendritic molecules at a low price and with high purity.

In comparison to perfect dendrimers the hyperbranched polymers are imperfectly branched structures that can be prepared in bulk quantities for several applications.<sup>[26a]</sup> Even though hyperbranched polymers are not monodisperse systems, they have defined properties derived from their three-dimensional structure and a controlled number of functional groups.<sup>[112]</sup>

A biocompatible hyperbranched polymer is the hyperbranched polyglycerol (hPG), a polyether polyol<sup>[113]</sup> which has already been used in several biological applications.<sup>[114]</sup> hPGs are highly branched polydisperse macromolecules with a large number of polar end groups (OH) on the surface of the polymer. It can be easily synthesized by anionic ring-opening polymerization of glycidol.<sup>[115]</sup> Analytical studies by Brooks *et al.* reported similar biocompatibility profiles for hPG and polyethylene glycol (PEG).<sup>[116]</sup> Furthermore, branched polyglycerols exhibit a relatively higher thermal and oxidative stability compared to PEG.<sup>[113,116]</sup> The versatile and tunable properties of hyperbranched polymers together with the ease of synthesis, excellent water solubility, and biocompatibility make them promising materials in different biomedical applications.

An application of hPG was reported in 2004 by Türk *et al.* as a fully synthetic, non-carbohydrate based heparin analog (antithrombotic drug).<sup>[117]</sup> Due to several limitations of

heparin there is a large interest in developing alternatives. They synthesized several anionic hPG derivatives: sulfates with different molecular weights as well as hPG carboxylate analogs and evaluated them for their anticoagulant and anticomplement activities (Figure 17).



**Figure 17.** Synthesis of polyanionic polyglycerol derivatives, sulfates (A) and carboxylates (B).

In contrast to the nonderivatized and the carboxylated polyglycerols, which were inactive, the polyglycerol sulfates revealed low anti-coagulant activity (a max. activity of only 30 % compared to heparin). But in an additional complement-induced hemolysis assay, the hPG sulfate revealed 25 times better activity than heparin, which was a clear indication for the anti-inflammatory potential of hPG sulfates.<sup>[117]</sup>

Hyperbranched polyglycerol sulfates as multivalent inhibitors in inflammation was further studied by Dervede *et al.*<sup>[49]</sup> The biocompatible and well-tolerated hPG sulfate acted as a multivalent selectin ligand mimetic and efficiently blocked leukocyte migration. Analyses by SPR spectroscopy revealed that L- and P-selectin binding to a synthetic ligand (20 mol% sLe<sup>x</sup> and 5 mol% sulfotyrosine conjugated to PA and immobilized on the sensor chip) was drastically reduced by the addition of hPG sulfates and gave IC<sub>50</sub> values in the low nanomolar range. The inhibition was strongly dependent on the core size and the degree of sulfation for different derivatives. Further administration of hPG sulfates *in vivo* in mice models suppressed leukocyte extravasation and complement activation. Thus, sulfated hPG represent an innovative class of a fully synthetic polymer therapeutic that may be used for the treatment of inflammatory diseases.<sup>[49]</sup>

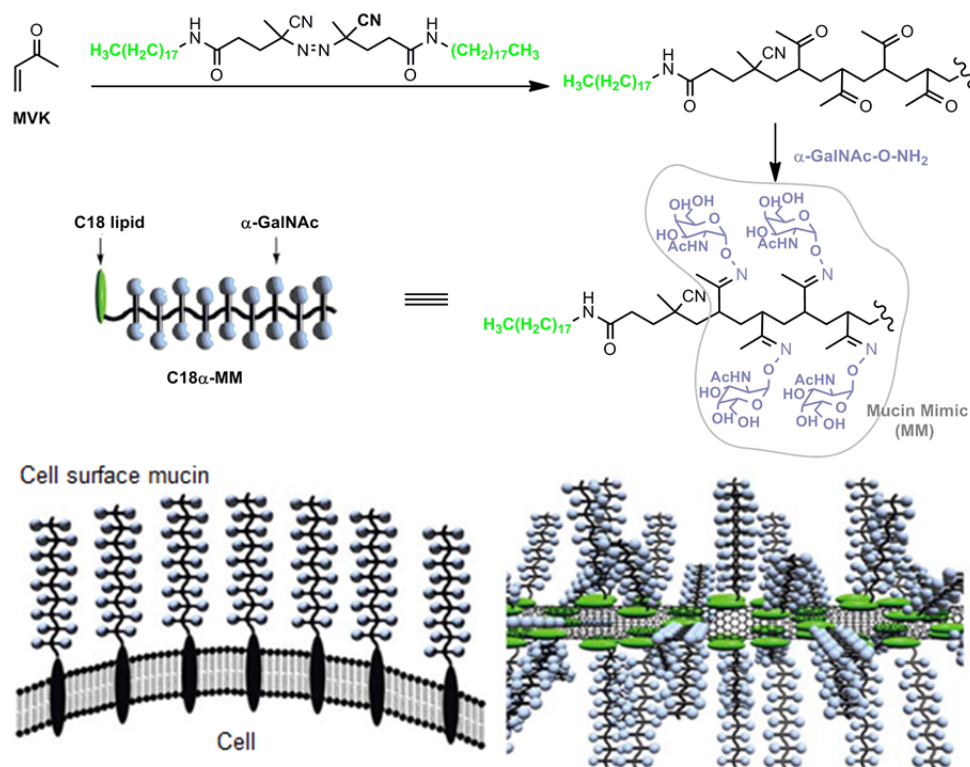
## 1.6 Dimension and scaling in multivalency

At present, there is increasing excitement in the field of chemistry, biology, and physics, which reveal new, unique properties of nanometer-sized objects.<sup>[118]</sup> Working on the

## 1. Introduction

nanoscale (from a few nm to less than 100 nm), specifically designed molecular architectures can be created. These nanomolecules provide greater, performance-enhancing properties such as higher, more flexible, and less expensive surface coating.<sup>[118]</sup>

Among the most interesting nanoscale molecular architectures developed over the past 20 years are dendrimers, micelles, several polymer based nanoparticles, hydrogels, nanogels, colloids, nanorods, and carbon nanomaterials (carbon nanotubes, fullerenes, mesoporous carbon, and more recently graphene). Some of these materials have found applications in electronics, sensors, catalysis, drug delivery, composites, etc.<sup>[119]</sup> Creating of a bridge between nanomaterials and biological systems would provide a strategy for biomimetic surface engineering. Bertozzi *et al.* described a biomimetic surface modification of single-walled carbon nanotubes (SWNTs) using glycosylated polymers (a lipid-terminated poly(methyl vinyl ketone) = polyMVK) to mimic cell-surface mucins (Figure 18).<sup>[120]</sup>



**Figure 18.** (top) Synthesis of C18 $\alpha$ -MM. The C18 lipid was conjugated to 4,4'-azobis(4-cyanopentanoic acid) (ACPA) and the amide-linked product was used to initiate radical polymerization of MVK to produce C18-poly(MVK). C18- $\alpha$ -MM was obtained by chemoselective ligation of C18-poly(MVK) with aminoxy- $\alpha$ -GalNAc. (bottom) A model for the self-assembly of C18 $\alpha$ -MMs on the surface of carbon nanotubes (right), which is similar to the proposed arrangement of cell-surface mucins (left).<sup>[120b]</sup>

Natural mucins are characterized by dense clusters of O-linked glycans bound to Ser/Thr residues of a polypeptide. The use of SWNTs in living systems requires several strategies in order to diminish the high toxicity of these nanomaterials.<sup>[121]</sup> They proved that the surface

modification of SWNTs with glycopolymers lowered their toxicity and increased their water solubility. However, the coating of carbon nanotubes (CNTs) had an irregular surface and a non-uniform thickness, which was due to the high polydispersities of the used polymer.<sup>[120b]</sup>

The surface modification was further improved by the same group. By applying glycodendrimers (modified with Man, Gal, or Lac units), a homogeneous bioactive coating of CNTs was achieved. In addition these nanomaterials were applied for various biological binding studies, such as binding to the lectins: Con A, *Arachis hypogaea* agglutinin (PNA), and *Psophocarpus tetragonolobus* agglutinin (PTA), which recognize Man, Lac, and Gal carbohydrates, respectively. Thus it was proven that glycodendrimers can be used for homogeneous bioactive coatings that reduces cytotoxicity for SWNTs.<sup>[122]</sup>

Two recent reviews by Jayakumar *et al.* describe chitin and chitosan biopolymers, which could be chemically modified to generate novel properties and applications in biomedical area. These materials are biocompatible, biodegradable, and possess antimicrobial activity and blood anticoagulant ability.<sup>[123]</sup> They can be easily processed into hydrogels, sponges, membranes, beads, and scaffold forms. These nanomaterials were used in a variety of nanostructured forms such as nanofibers, nanoparticles, and nanocomposite scaffolds in several biomedical applications, such as tissue engineering, drug delivery, and cancer diagnosis.<sup>[123a]</sup>

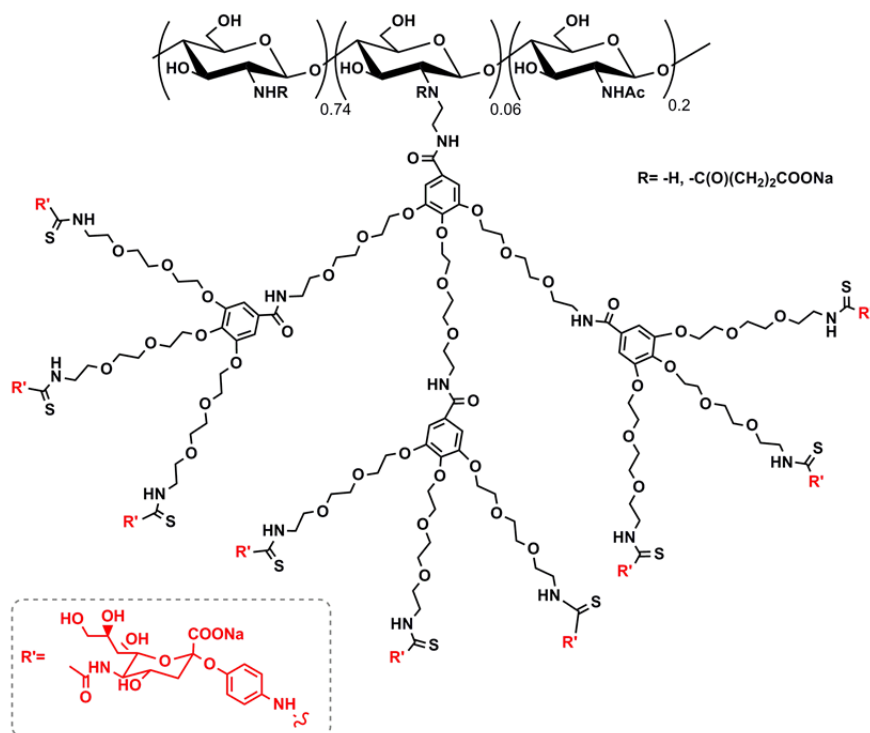
Sugar modification of chitosan was applied by numerous researchers, first reported in 1980 by Hall and Yalpani.<sup>[124]</sup> They synthesized lactose-bound chitosan by reductive *N*-alkylation, using sodium cyanoborohydride (NaCNBH<sub>3</sub>) with an unmodified-Lac or an aldehyde-Lac derivative. At the beginning the chitosan based sugars were mainly used in rheological studies. Afterwards, as the specific recognition of cells, viruses, and bacteria by sugars was discovered, several cell-specific sugars (Fuc,<sup>[125]</sup> Lac,<sup>[126]</sup> GlcNAc,<sup>[125b]</sup> Gal,<sup>[127]</sup> Man<sup>[126a,127a]</sup>) were introduced into chitosan backbones and applied in cell recognition studies.

Sialic acid (SA) modified chitosans are of relevant importance due to the previously described characteristics of SA. A variety of chitosan based dendronized polysaccharides were synthesized by well established methods. As dendrimers the commercially available amino dendrimers such as poly(amidoamine) (PAMAM) or poly(ethylenimine) (PPI) were used.<sup>[128]</sup> A different type of sialylated chitosan-dendrimer hybrid can be seen in Figure 19.

The dendron with a focal aldehyde group was synthesized by iterative amide bond strategy. Trivalent and nonavalent dendrons with gallic acid as the branching unit and triethylenglycol as the spacer arm were prepared and attached to *p*-phenylisothiocyanate-SA derivatives. The focal aldehyde group was then convergently attached to the chitosan backbone.<sup>[129]</sup>

Biological evaluation of the sialylated chitosan dendrimers as well as chitosan sialoglycopeptides<sup>[88]</sup> showed much higher inhibitory activity for influenza virus hemagglutination as the monomeric SA.





**Figure 19.** Hybridization of chitosan with sialodendrimer, composed of gallic acid as junction point.<sup>[129]</sup>

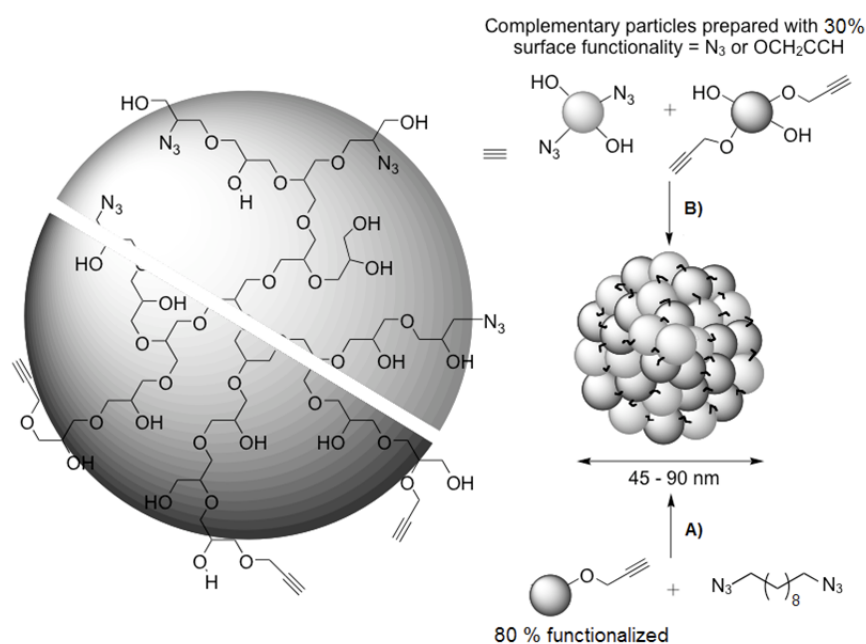
### 1.6.1 Polyglycerol based nanoparticles, nanogels, macrogels

Among the most potent molecular architectures for several biological applications are the dendritic polyglycerols (hPG). They are biocompatible and have favorably low toxicity and similar properties to the widely used poly(ethyleneglycol)s.<sup>[130]</sup> They are globular, multivalent, facilely prepared dendrimer analogs. However, their synthesis (anionic polymerization of glycidol)<sup>[115a]</sup> is limited, and affords just small nanoparticles in the range of 1–5 nm. By this method hPGs can be prepared with a controllable  $M_n$  in the range of a few thousand Daltons. Recently Brooks *et al.* developed a synthesis of hPGs with up to 1 MDa molecular weight, by conducting the ring-opening multibranching polymerization of glycidol in dioxane as an emulsifying agent.<sup>[131]</sup> The synthesized polymers are densely packed, have spherical conformations in water with no indication of aggregate formation, and have a diameter in the order of ~10 nm with narrow polydispersities (PDI = 1.1–1.4).<sup>[131]</sup>

The first polyglycerol based nanogels (20–200 nm) were recently developed by our group. Initially, we covalently assembled lower molecular weight polyglycerol fragments to larger hPG analogs, by using miniemulsion polymerization.<sup>[132]</sup> Miniemulsions are heterophase dispersions of relatively stable nanodroplets in a continuous nonsolvent phase.<sup>[133]</sup> A high shear energy input to this biphasic system in the presence of a surfactant results in droplets with a narrow size-distribution tunable within the 20–200 nm range. This size is considered to be ideal for drug delivery purposes which may accumulate in tumor

tissues by the enhanced permeation and retention effect (EPR).<sup>[134]</sup> A recent review by Landfester gives an overview about the mechanism of polymerizations in miniemulsions and about the current state in this field.<sup>[133]</sup>

As shown in Figure 20 Sisson *et al.* described an approach, where they crosslinked previously functionalized hPGs dendritic macromonomers (2 nm,  $M_n = 5$  kDa) to higher homologs using Huisgen azide/alkyne cycloaddition in miniemulsion polymerization.<sup>[132]</sup> The particle size diameter varied between 20–90 nm. However, larger particle could not be obtained in a stable homogeneous dispersion.

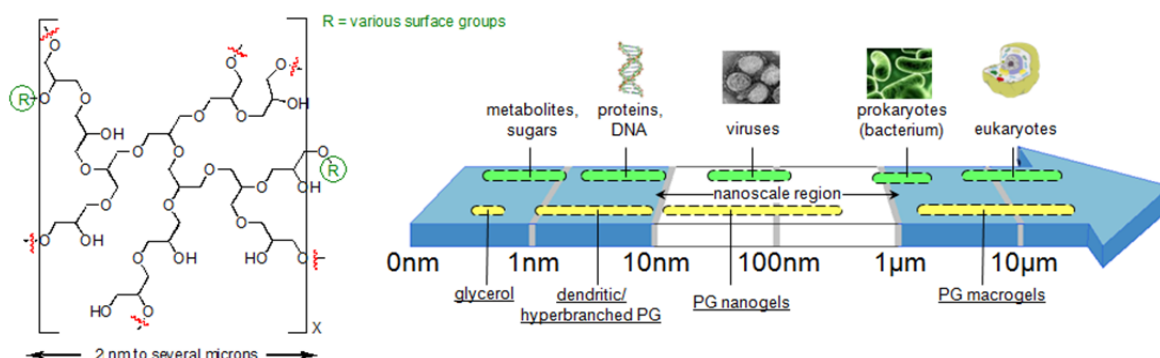


**Figure 20.** Procedure employed to form nanoparticles. Miniemulsion templated click coupling ( $CuSO_4$ /sodium ascorbate). A) Formation of hydrophobic nanoparticles, highly functionalized particles and B) Formation of hydrophilic nanoparticles, minimally functionalized particles.

This nanoparticle synthesis was further optimized by Sisson *et al.* They prepared well-defined polyglycerol nanogels (nPG) from 25 nm up to 350 nm in diameter through inverse miniemulsion polymerization.<sup>[119c]</sup> These nanoparticles were purely polyglycerol based and synthesized from the cheap commercially available starting materials: triol glycerol and trisepoxide, glycidyl glycerolether. A poly(ethylene-co-butylene)-block-poly(ethylenoxide) surfactant was used as stabilizer of the glycerol/trisepoxide nanodroplets.<sup>[119c]</sup> Nanoparticles could be formed bearing unreacted epoxide units for further functionalization by controlling the reaction time (number of remaining epoxides per glycerol unit can be determined by NMR spectroscopy). By addition of sodium azide ( $NaN_3$ ), azido nPGs are created, which can be readily functionalized with a wide range of groups by click chemistry. The biocompatible

## 1. Introduction

nature of these nanogels is very promising for future applications as drug/dye delivery vehicles.



**Figure 21.** (left) General structure of a typical branched polyglycerol repeat unit; (right) Depiction of relative sizes of nanoparticles (scaffold structures of multivalent ligands) in relation to other biological objects. Viruses are smaller than the smallest bacteria and larger than macromolecules. They can be examined by electron microscope (Illustration demonstrates relative sizes and is not drawn to scale).<sup>[119d]</sup>

Biodegradable polyglycerol based nanogels have also been synthesized by the same group, incorporating redox active disulfide branching units within the nanogel structure. They used inverse miniemulsion via an acid catalyzed ring-opening polyaddition of disulfide containing polyols and polyepoxides.<sup>[135]</sup> The particle size and disulfide content could be tuned by varying reaction conditions and the obtained particle size was in a range from 30–300 nm. The particle degradation was analyzed by size exclusion chromatography, which indicated that the degraded product had low molecular weights (< 5 kDa). Additionally performed cell culture studies proved their high biocompatibility. Furthermore, dye labeled nanogels readily internalize into cells by endocytotic mechanisms, as it was shown by optical microscopy techniques.<sup>[135]</sup>

These studies show that polyglycerol based nanogels are excellent materials for different biological applications, due to their size and biocompatible, as well as biodegradable properties.

### 1.6.2 Gold nanoparticles (AuNPs)

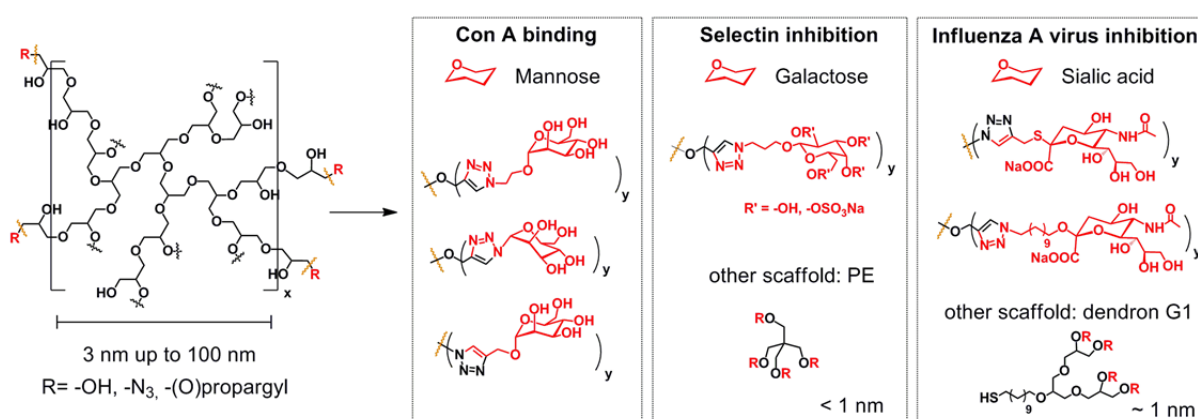
AuNPs<sup>[136]</sup> are biocompatible nanomaterials and have attracted a remarkable interest in the area of nanobiotechnology, electronic, optical, magnetic and biomedical applications.<sup>[137]</sup> They are colloidally stable, but their stability is highly dependent on parameters such as concentration of the colloid, pH, and total ionic strength of the solution.<sup>[138]</sup> As most of the biological interactions are multivalent in nature, Bowman and his co-workers have stopped HIV from infecting human white blood cells by attaching multiple copies of a low-acting HIV drug onto AuNPs.<sup>[139]</sup> Weak single molecule interactions could be disproportionately

enhanced by presenting them on a AuNPs scaffold in a multivalent manner. Some recent publications described that functionalized AuNPs with suitable targeting molecules act as effective inhibitors of various viruses, such as HIV<sup>[140]</sup> and Herpes simplex virus<sup>[141]</sup> or as inhibitors in selectin binding, as shown by Dervede *et al.*<sup>[48]</sup> In the latest case AuNPs with terminally functionalized sulfated thiol shells and branched acyclic epitopes were used, which were found to bind to P- and L-selectins with IC<sub>50</sub> values in the picomolar range. The branched acyclic epitopes showed the highest affinity, whereas a sulfated carbohydrate mimetics (aminopyran derivatives) provided the best selectivity.<sup>[48]</sup> These compounds, as free soluble material, gave no or only moderate IC<sub>50</sub> values in the micromolar range. Their immobilization on the surface of gold particles greatly enhanced selectin binding (IC<sub>50</sub>= 30 pM).

While metallic nanoparticles have been functionalized with peptides, proteins, and DNA for the last 20 years, carbohydrates as functional groups were not used until 2001. Afterwards the number of published articles significantly increased in this area. Glyconanoparticles (GNP) are biofunctional nanomaterials that combine the unique physico-chemical and optical properties of the metallic nucleus with the characteristics of the carbohydrates. They constitute a good biomimetic model of carbohydrate presentation at the cell surface. Penadés *et al.* prepared a small library of multivalent water soluble GNPs presenting bridged (oligo)mannosides of the high mannose undecasaccharide Man<sub>9</sub>GlcNAc<sub>2</sub> and tested them as inhibitors for HIV. The (oligo)manno-GNPs had different spacers and variable density of Man. They mimicked the cluster presentation of oligomannosides on the virus surface. The tested compounds completely inhibited the binding of HIV from micro- to the nanomolar range, determined by SPR spectroscopy measurements. Furthermore, it was found that increasing the density of mannosides on the gold surface from 50 % to 100 % the level of inhibition did not improve.<sup>[140a]</sup> AuNPs offer a rigid scaffold with an adjustable size. By synthesis of GNP further flexibility could be introduced by varying the length and flexibility of the linkers as well as the sugar density, thereby allowing the preparation of multifunctional structures as potential carbohydrate based therapeutics.

## 2. SCIENTIFIC GOALS

This thesis contributes to the research on fundamental properties of multivalent model systems for a deeper understanding of multivalency in biological systems, especially regarding surface interactions and the scaling in multivalency. The main goal of this work has been to develop biocompatible multivalent glycoarchitectures using glycerol dendrons and polyglycerol nanoparticles with different dimensions as scaffolds. To study the functions of glycoconjugates in biological systems, reliable and efficient protocols for glycoconjugate synthesis are needed. To reach this goal an easy method is required for their synthesis. The first step would be to focus on the modular functionalization of glycerol dendrons (1 nm) with carbohydrates and their attachment to hybrid systems such as gold nanoparticles (AuNPs) of different sizes (2 nm and 14 nm). Furthermore, dendritic polyglycerol nanoparticles with different length scales (1 nm to 100 nm) should be used as scaffolds and be functionalized with various biologically active carbohydrates. More specifically, the intended polymeric scaffolds could be the hyperbranched polyglycerols (hPG, >10 nm) and recently developed PG-nanogels (nPG) (25 nm to 100 nm). In the latter case size will play an important role, because of their similar dimensions with typical viruses. The hPG and nPG could be functionalized after polymerization to provide a versatile scaffold for the rapid attachment of a variety of different carbohydrates. The grafting of carbohydrate moieties on a polymer backbone could be achieved by copper(I)-catalyzed Huisgen [2+3] cycloaddition. As click chemistry provides highly reproducible formulations, it is easily scalable for large scale production. The versatile nature of this reaction leads to quantitative yields and therefore to carrying out the synthesis in either organic solvents or water.

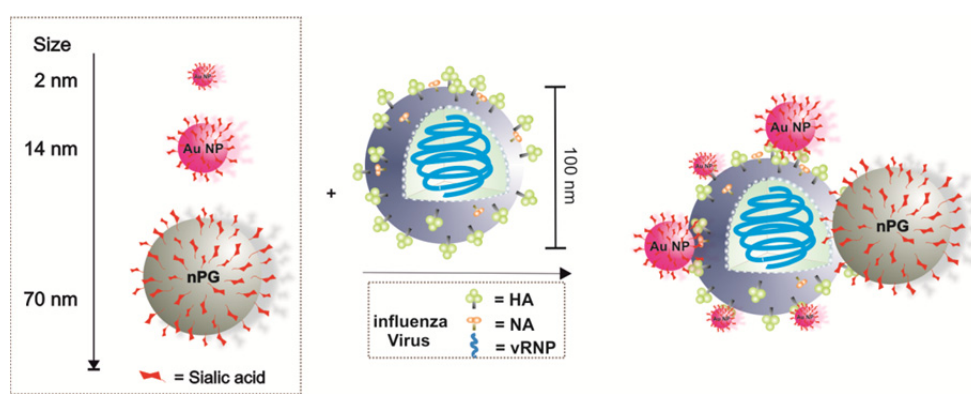


**Scheme 1.** Polyglycerol based glycoarchitectures;  $x$  = shows a small fragment of the actual PG nanoparticles (hPG and nPG);  $y$  = degree of functionalization of the polymeric scaffold.

The simplest carbohydrates, i.e., those which are easy to prepare and handle, are the most desirable candidates for study, e.g., the monosaccharides (Man, Gal) in Concanavalin A and

## 2. Scientific Goals

selectin binding studies; as well as the sialic acid (SA) in inhibition of influenza A virus. Coupling reactions with carbohydrate moieties generally require protection and deprotection steps of the hydroxyl functions. Thus click chemistry should be performed with peracetylated carbohydrates for simplicity using  $\text{CuSO}_4$  and sodium ascorbate in a THF/water mixture, followed by deprotection. Glycopolymers would be rendered water soluble by removal of the protecting acetyl groups. In parallel derivatives based on smaller size scaffold should be synthesized as well, for low valency control, based on the same synthesis. Since the properties of the hyperbranched polymers strongly depend on their functional groups, correlations between the polyether properties and the functionality of the corresponding carbohydrate-coated formulations will be investigated.

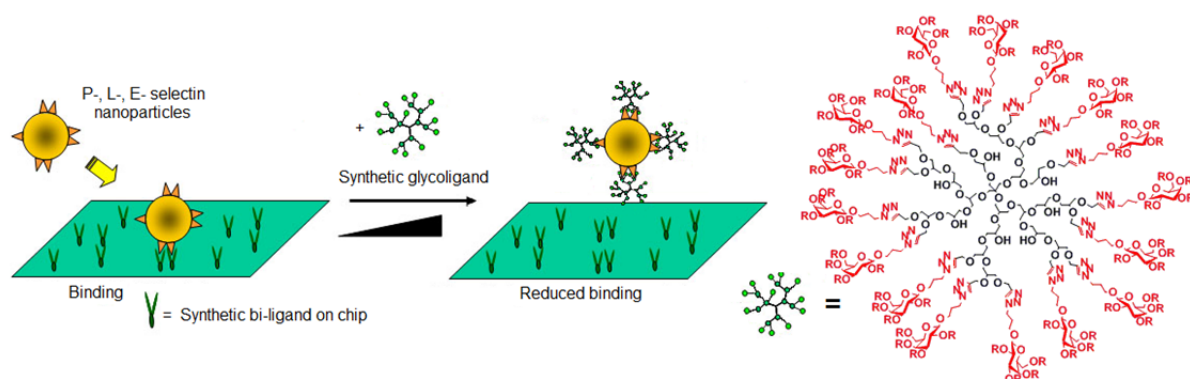


**Scheme 2.** Different sizes of glycoarchitectures based on polymers and on AuNPs for influenza virus inhibition studies. Illustration demonstrates relative sizes and is not drawn to scale.

The second step would be to evaluate the biological interactions of these compounds in cooperation with biologists. The synthesized compounds should be examined by SPR spectroscopy binding assay, hemagglutination inhibition assay (HIA), turbidimetry assay, and several other physico-chemical measurements. Through systematic characterization and on the basis of crossover projects for *in vitro* studies, the multivalent behavior of these spherical architectures with different dimensions can be evaluated. In addition to indirect biochemical techniques, a direct visualization of these multivalent glycoarchitectures by transmission electron microscopy (TEM) should be possible to validate their multivalent binding behavior to biological surfaces. Multiple carbohydrate representation on different scaffolds (hybrid or polymeric) should lead to a dramatic increase in effectiveness compared to the monovalent carbohydrate. The influence of the functionalities and the variety of sugar end groups on biological processes is to be investigated systematically. The impact should be dependent on the size of the multivalent inhibitor and on the degree of functionalization (DF). Particles of similar dimension to a typical virus (50 nm to 100 nm) are expected to be particularly effective.

### 3. PUBLICATIONS

#### 3.1 Modular synthesis of multivalent glycoarchitectures and their unique selectin binding behavior



Click chemistry allows the simple preparation of novel, multivalent galactose modified polyglycerols in high yields independent of their surface functionality ( $R = -OH$  and  $-SO_3^-Na^+$ ). These glycoligands are remarkably strong selectin inhibitors ( $IC_{50} = 1$  nM) as revealed by a surface plasmon resonance spectroscopy (SPR) based competitive binding assay.

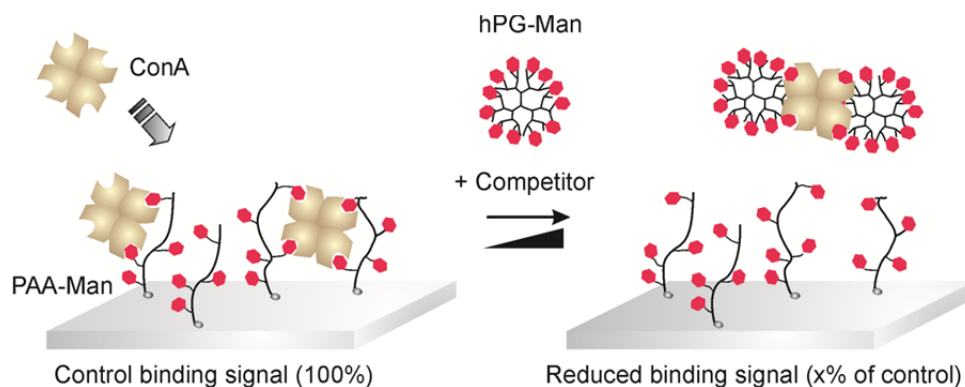
This chapter was published in the following journal:

I. Papp, J. Dervedde, S. Enders, R. Haag, *Chem. Commun.* **2008**, 5851-5853 (DOI: 10.1039/b813414f).

The original article is available at:

<http://pubs.rsc.org/en/content/articlelanding/2008/cc/b813414f>

### 3.2 Multivalent presentation of mannose on hyperbranched polyglycerol and their interaction with Concanavalin A lectin



**Architecture-dependent binding:** Multivalent dendritic glycoconjugates with high binding affinities for Con A were efficiently prepared, and a detailed structure-activity relationship study revealed the best linker and the degree of functionalization for the Man–polymer conjugates.

This chapter was published in the following journal:

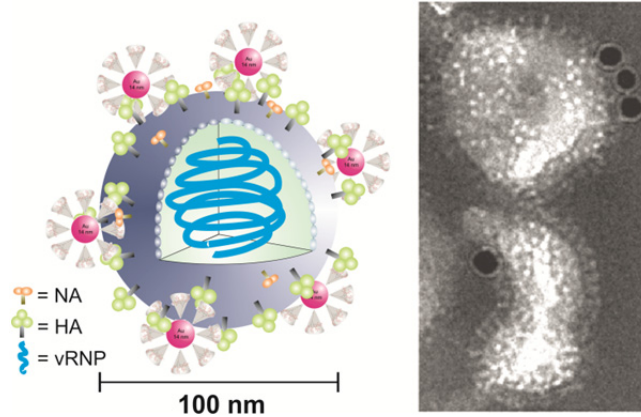
I. Papp, J. Dervedde, S. Enders, S. B. Riese, T. C. Shiao, R. Roy, R. Haag, *ChemBioChem* **2011**, 12, 1075-1083 (DOI: 10.1002/cbic.201000718).

The original article is available at:

<http://onlinelibrary.wiley.com/doi/10.1002/cbic.201000718/abstract>



### 3.3 Inhibition of influenza virus infection by multivalent sialic acid functionalized gold nanoparticles



**Densely sugar-coated gold nanoparticles** are prepared by covalent attachment of sialic acid-modified dendrons to the nanoparticle surface. Such multivalent constructs are designed to bind hemagglutinin envelope-protein arrays on the influenza surface, thus inhibiting viral invasion of host cells. Various chemical and biological assays, as well as electron microscopy, are presented to validate this approach and visualize for the first time the multiple binding of AuNP inhibitors to the virus surface.

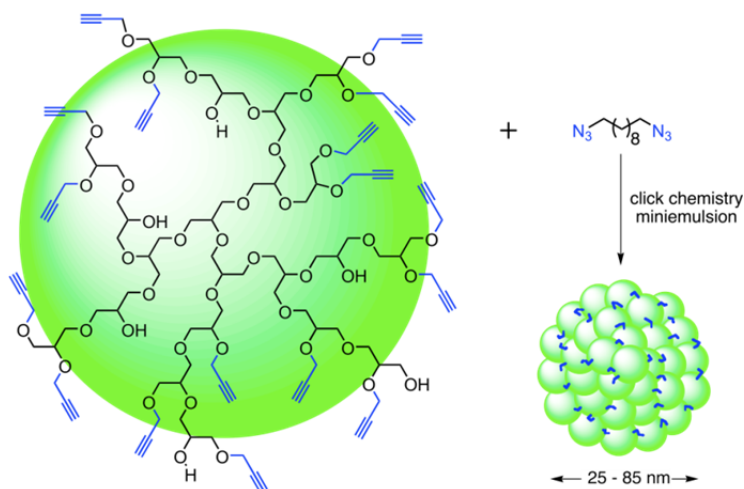
This chapter was published in the following journal:

I. Papp, C. Sieben, K. Ludwig, M. Roskamp, C. Böttcher, S. Schlecht, A. Herrmann, R. Haag, *Small* **2010**, *6*, 2900-2906 (DOI: 10.1002/smll.201001349).

The original article is available at:

<http://onlinelibrary.wiley.com/doi/10.1002/smll.201001349/abstract>

### 3.4 Functional nanoparticles from dendritic precursors: hierarchical assembly in miniemulsion



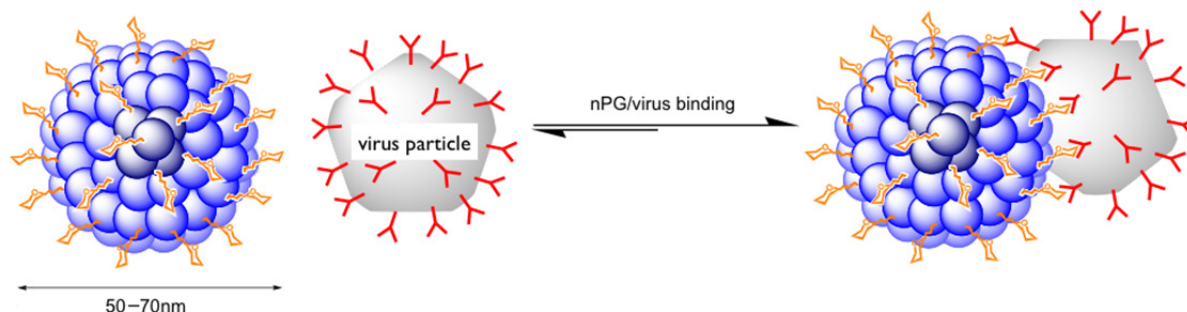
Click chemistry concepts have been utilized in order to polymerize highly branched polyvalent macromonomers in miniemulsion, resulting in dendritic nanoparticle covalent aggregates. Dynamic light scattering and transmission electron microscopy characterize the formation of hyperbranched polyglycerol based particles with narrow size distribution tunable between 25 nm and 90 nm diameter. Two related approaches are discussed allowing the synthesis of either hydrophobic or hydrophilic particles.

This chapter was published in the following journal:

A. L. Sisson, I. Papp, K. Landfester, R. Haag, *Macromolecules* **2009**, *42*, 556-559 (DOI: 10.1021/ma802238e).

The original article is available at: <http://pubs.acs.org/doi/abs/10.1021/ma802238e>

### 3.5 Inhibition of influenza virus activity by multivalent glycoarchitectures with matched sizes



Polyglycerol nanoparticles of diameter 50-70 nm were coated with sialic acid residues to afford excellent inhibitors of influenza virus binding and fusion, and hence infectivity of erythrocytes. This approach highlights the versatility and potential of a growing class of biocompatible, branched, polyether nanogels that benefit from a highly functionalizable, hydrophilic surface.

This chapter was published in the following journal:

I. Papp, C. Sieben, A. L. Sisson, J. Kostka, C. Böttcher, K. Ludwig, A. Herrmann, R. Haag, *ChemBioChem* **2011**, *12*, 887-895 (DOI: 10.1002/cbic.201000776).

The original article is available at:

<http://onlinelibrary.wiley.com/doi/10.1002/cbic.201000776/abstract>

### 4. SUMMARY (ZUSAMMENFASSUNG)

#### 4.1 Summary

Within this thesis several novel glycoarchitectures were synthesized and their biological activities evaluated in Concanavalin A binding, selectin binding, and in the inhibition of influenza A virus infection. Mainly three relevant carbohydrates mannose (Man), galactose (Gal), and sialic acid (SA) were involved by designing these glycoarchitectures presented on diverse scaffolds such as hyperbranched polyglycerols (hPG, 3 and 5 kDa), polyglycerol nanoparticles (nPG, 1000 kDa), glycerol dendrons, and gold nanoparticles (AuNPs). The size of these scaffolds varied from 2 up to 70 nm.

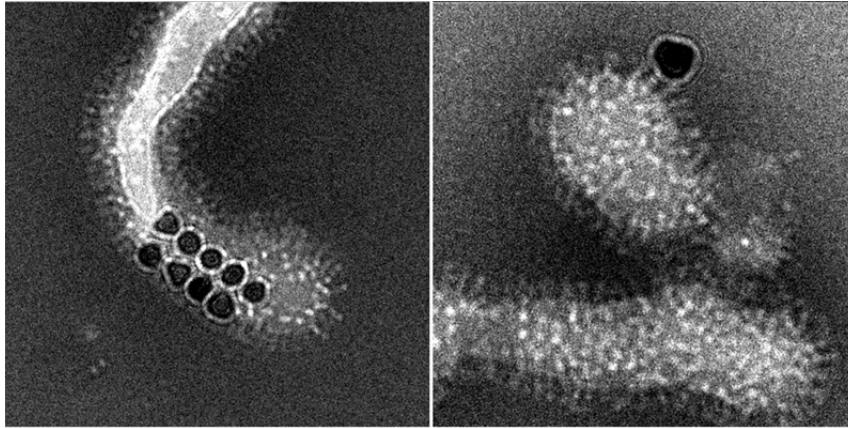
The surface availability and bioactivity of Man and Gal modified polymers were evaluated using a competitive surface plasmon resonance spectroscopy (SPR)-based binding assay by interactions of the Man-glycopolymers with Concanavalin A (Con A, a Man binding lectin), and the Gal-glycopolymers with selectins, respectively. The results of these studies indicated that the designed glycopolymers acted multivalent. Higher sugar loadings onto the polymers promoted higher binding activity. The multivalent presentation of galactose (35 terminal Gal per polymer) significantly lowered the  $IC_{50}$  values of L-, P-, and E-selectin binding in a well-established competitive SPR based binding assay. Furthermore, the binding affinity to L- and especially to P- selectin was dramatically enhanced when sulfated Gal derivatives were used as ligands, resulting in  $IC_{50}$  values in the low nanomolar range. SPR results from Con A studies indicated that the novel glycoarchitectures were able to efficiently recognize Con A with  $IC_{50}$  values from the micro to the nanomolar range, while the corresponding monovalent methyl mannoside (methyl-Man) required millimolar concentrations. Precipitation assays were performed, to gain an insight into the stoichiometry of Con A binding per functionalized hPG. Furthermore, the flexibility of Man to hPG was tested by varying the spacer between the ligand and the scaffold. As carbohydrate flexibility has already been induced (at least partly) by the PG scaffold, smaller rigid spacers were chosen and the best one was determined by several measurements. These studies suggest that for further improvement of the binding affinity, not a higher functionalization, but an increase in size of the hPG core should be appropriate to provide more space for protein binding.

In the case of influenza virus inhibition the objective was how particle size and valency affect the inhibition process on scaffolds of different dimensions. Following the concept of multivalency, weak single molecule interactions of sialylated dendrons were disproportionately enhanced by presenting them on a scaffold (AuNPs with different dimensions: 2 nm and 14 nm) in a multivalent manner. Sialylated particles of 14 nm size were found to be effective for influenza virus inhibition, whereas 2 nm analogs did not show significant impact. AuNPs of 2 nm dimension span three binding sites of an individual

#### 4. Summary (Zusammenfassung)

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hemagglutinin (HA) homotrimer, whereas the 14 nm ones could bind several HA trimers. For the first time, the multiple binding of these modified AuNPs to influenza viruses could be directly visualized by electron microscopic imaging (Figure 1).



**Figure 1.** The multiple attachment of modified AuNPs (14 nm) to influenza A virions is clearly visible. Closer inspection suggests that several HA trimers are involved in the particle binding; preparation by negative staining technique with 1 % phosphotungstic acid (Scale bars were omitted for clarity since the well defined size of the AuNPs serve as scale).

Furthermore, the polymer particle sizes were varied along with the degree of functionalization (SA density) to match the corresponding virus size and receptor multiplicity in order to improve affinity. The inhibitory activity of the tested polymeric nanoparticles on influenza virus drastically increased with the nanoparticle size, as observed by virus binding, fusion, and infectivity studies. Larger particles, of dimensions similar to that of a virus (50 nm), showed very efficient virus inhibition (up to 80 %). Additionally it was found that presentation of carbohydrates on polymeric nanoparticles at higher density does not necessarily exhibit higher inhibitory potential. A saturation point in the degree of surface functionalization was observed, whereby inhibition was not significantly improved. This can be explained by the given geometry of both interacting surfaces.

This study emphasizes the importance of matching particle size and ligand density for biological surface interactions.

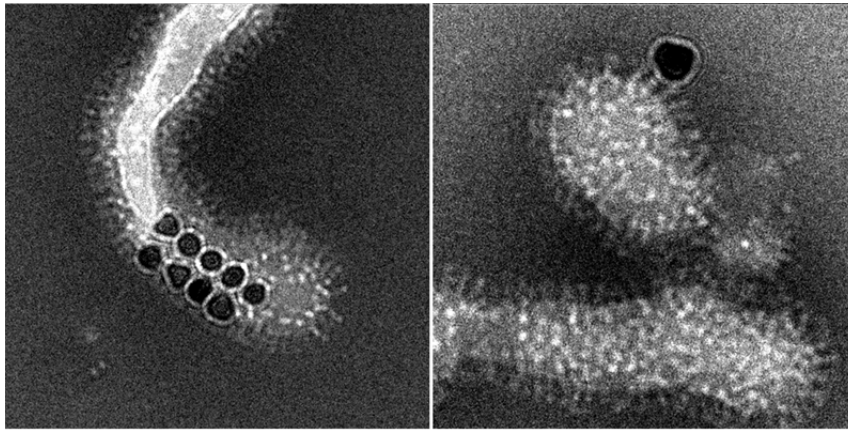
### 4.2 Zusammenfassung

Im Rahmen dieser Arbeit wurden mehrere neuartige dendritische Glykokonjugate synthetisiert, ihre Bindungsfähigkeit an die biologisch wichtigen Lektine Concanavalin A und Selektin, sowie die Inhibitionswirkung auf die Zelladhäsion von Influenza A Viren untersucht. Hauptsächlich wurden drei relevante Kohlenhydrate - Mannose (Man), Galaktose (Gal) und Sialinsäure (SA) - für die terminale Funktionalisierung dieser neuen Glykoarchitekturen verwendet. Als Gerüstmoleküle dienten unterschiedlich strukturierte Polyglycerine: hyperverzweigtes Polyglycerin (hPG, 3 und 5 kDa), *nano*-Polyglycerinpartikel (nPG, 1000 kDa), Glycerol Dendrone und Goldnanopartikel (AuNP), wobei die Größe dieser Gerüste von 2 bis 70 nm variierte. Die Oberflächenverfügbarkeit und Bioaktivität von Man und Gal modifizierten Polymeren wurden durch Oberflächenplasmonenresonanzspektroskopie (SPR) basierten Bindungstests ermittelt. Dabei wurden die Wechselwirkungen der Man-Glykopolymeren mit Concanavalin A (Con A, ein Man bindendes Lektin) und der Gal-Glykopolymeren mit Selektinen getestet. Die Ergebnisse dieser Studien zeigten, dass die verwendeten Glykopolymeren in einer multivalenten Art und Weise binden. Eine erhöhte Funktionalisierung der Polymere mit Kohlenhydraten führte zu einer Erhöhung der Bindungsaktivität. Die multivalente Präsentation von Gal (35 terminale Gal-Einheiten pro Polymer) senkte deutlich die  $IC_{50}$ -Werte bei der L-, P- und E-Selektin Bindung in einem SPR-Bindungstest. Die Bindungsaffinität an L- und vor allem P-Selektin wurde drastisch erhöht, wenn sulfatierte Gal als Liganden verwendet wurden, was zu  $IC_{50}$ -Werten im nanomolaren Bereich führte. SPR Ergebnisse von Con A Studien wiesen darauf hin, dass die neuartigen Glykoarchitekturen in der Lage sind effizient Con A zu binden. Die  $IC_{50}$ -Werte lagen dabei im mikro- bzw. nanomolaren Bereich, während die entsprechenden monovalenten Methyl-Mannoside (Methyl-Man) millimolare Konzentrationen erforderten. Die Stöchiometrie der Bindung zwischen Con A und Kohlenhydrat-funktionalisierten Polymer wurde durch Fällungstests genauer untersucht. Des Weiteren wurde die Linkerflexibilität durch Variation der Bindungseinheit zwischen dem Man-Liganden zum hPG-Polymer systematisch geändert. Da eine grundsätzliche Flexibilität am Kohlenhydrat bereits teilweise durch die recht flexible Polymerstruktur gewährleistet ist, wurde eine Serie von kurzen, starren Linkern untersucht und die optimalen Bindungsparameter in mehreren Messreihen ermittelt. Diese Studien legen nahe, dass für eine weitere Verbesserung der Bindungsaffinität nicht etwa eine höhere Funktionalisierung, sondern eine Vergrößerung der hPG-Gerüststrukturen notwendig ist, um eine größere Anzahl von effektiven Bindungsstellen für die Proteinbindung zu erhalten. Die Untersuchung von Glykokonjugaten hinsichtlich relevanter Parameter wie Partikelgröße und den Einfluß von Valenz auf die Inhibition der Influenzavirus-Zelladhäsion war ein weiteres Forschungsziel dieser Arbeit. Nach dem Konzept der Multivalenz wurden schwache Wechselwirkungen einzelner Moleküle sialylierter Dendrone überproportional verstärkt,

#### 4. Summary (Zusammenfassung)

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indem sie auf einem Gerüstmolekül (AuNPs mit unterschiedlichen Abmessungen: 2 nm und 14 nm) in multivalenter Weise präsentiert wurden. Sialylierte Partikel von 14 nm Größe erwiesen sich als wirksam bei der Influenza-Virus-Hemmung, während die 2 nm Analoga kaum Hemmung zeigten. 2 nm große AuNPs konnten drei Bindungsstellen eines einzelnen Hämagglutinin (HA) Homotrimer besetzen, während die 14 nm großen AuNPs mehrere HA Trimere binden konnten. Zum ersten Mal konnte die mehrfache Bindung dieser modifizierten AuNPs an Influenza-Viren direkt mit elektronenmikroskopischen Aufnahmen dargestellt werden (Abbildung 1).



**Abbildung 1.** Die mehrfache Bindung von modifizierten AuNPs (14 nm) auf Influenza-A-Virionen ist deutlich sichtbar. Bei näherer Betrachtung zeigt sich, dass mehrere HA Trimere an der Teilchenbindung beteiligt sind; Vorbereitung durch negative Maltechnik mit 1% Phosphorwolframsäure (Die definierte Größe der AuNPs dient als Maßstab).

Die Partikelgrößen und der Grad der Funktionalisierung (SA-Dichte) polymerer Gerüste wurde ebenfalls variiert, um diese der entsprechenden Virusgröße und Rezeptormultiplizität anzupassen und somit die Affinität zu verbessern. Die hemmende Wirkung der getesteten Glykokonjugate auf Influenzaviren stieg drastisch mit zunehmender Partikelgröße, wie Bindungs-, Fusions- und Infektionstests zeigten. Größere Partikel wie *nano*-Polyglycerinen, mit Virus-ähnlichen Abmessungen von ca. 50 nm, zeigten eine sehr effiziente Vireninhibition. Es wurde festgestellt, dass eine höhere Funktionalisierung dieser polymeren Nanopartikel mit Kohlenhydraten nicht automatisch zur weiteren Erhöhung der inhibitorischen Wirkung führt. Es wurde vielmehr ein Sättigungspunkt im Oberflächenfunktionalisierungsgrad beobachtet, ab dem die Hemmung nicht mehr signifikant verbessert wurde, was durch die vorgegebene Geometrie der beiden wechselwirkenden Oberflächen gedeutet werden kann. Diese Studie unterstreicht daher die Bedeutung passender Partikelgrößen, Partikelformen, sowie der Ligandendichte für effiziente Wechselwirkungen biologisch relevanter Oberflächen.

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## 6. APPENDICES

### 6.1 Abbreviations

Ac	acetyl	Gal	D-galactose
AuNP	gold nanoparticle	GalNAc	<i>N</i> -Acetyl-D-galactoseamine
br	broad	Glc	D-glucose
Con A	Concanavalin A	GlcNAc	<i>N</i> -Acetyl-D-glucoseamine
conc	concentrated	GlcA	D-Glucuronic acid
d	doublet	h	hour
D	dendritic	HA	hemagglutinin
Da	Dalton	Hex	hexane
DB	Degree of Branching	HIA	Hemagglutination Inhibition Assay
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide	HIV	Human immunodeficiency virus
DF	Degree of Functionalization	HOBt	1-hydroxy benzotriazol
DIPEA	<i>N,N'</i> -diisopropylethylamine	hPG	Hyperbranched Polyglycerol
DLS	Dynamic Light Scattering	HPLC	High Performance Liquid Chromatography
DMAP	4-dimethylaminopyrrodine	Hz	hertz
DMF	<i>N,N'</i> -dimethyl formamide	IC <sub>50</sub>	Inhibiting concentration (50 %)
DMSO	dimethyl sulfoxide	IdoA	L-idunoric acid
<i>E. coli</i>	<i>Escherichia coli</i>	IR	Infrared Spectroscopy
EDC	<i>N</i> -(3-dimethylaminopropyl)- <i>N</i> -ethylcarbodiimide	J	coupling constant
EI-MS	Electron Ionization-Mass Spectrometry	K <sub>a</sub>	association constant
equiv	equivalent	K <sub>d</sub>	dissociation constant
ESI-MS	Electrospray Ionization-Mass Spectrometry	kDa	kilodalton
EtOAc	ethyl acetate	L-	laevus
FAB-MS	Fast Atom Bombardment-Mass Spectrometry	Lac	lactose, Galβ1-4Glcβ
FDA	Food and Drug Administration	LacNAc	<i>N</i> -Acetyl-D-lactoseamin, (Galβ1-4GlcNAcβ)
FTIR	Fourier Transform Infra Red Spectroscopy		
Fuc	fucose		

## 6. Appendices

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Le <sup>x</sup>	Lewis x, Gal $\beta$ 1-4(Fuca1-3)GlcNAc $\beta$ 1-OH
Le <sup>a</sup>	Lewis a, Gal $\beta$ 1-3(Fuca1-4)GlcNAc $\beta$ 1-OH
m	multiplet (NMR)
m	medium (IR)
MALDI	Matrix Assisted Laser Desorption Ionization
Man	mannose
min(s)	minute(s)
$M_w$	molecular weight
$M_n$	number average molecular weight
NA	Neuraminidase
Neu5Ac	<i>N</i> -Acetyl-D-Neuraminic Acid
nm	nanometer
NMR	Nuclear Magnetic Resonance
O.D.	Optical Density
PBS	Phosphate-buffered saline
PDI	polydispersity index
PEG	poly(ethylene glycol)
ppm	parts per million
quant	quantitative
RP-HPLC	Reversed Phase-High Performance Liquid Chromatography
rpm	rotations per minute
r.t.	room temperature
s	singlet (NMR)
s	strong (IR)
SA	sialic acid
SEC	Size Exclusion chromatography
sLe <sup>x</sup>	sialyl Lewis x, Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-4(Fuca1-3)GlcNAc $\beta$ 1-OH
sLe <sup>a</sup>	sialyl Lewis a, Neu5Ac $\alpha$ 2-3Gal $\beta$ 1-3(Fuca1-4)GlcNAc $\beta$ 1-OH
SPR	Surface Plasmon Resonance Spectroscopy
t	triplet
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
UV-Vis	Ultra Violet-Visible Spectroscopy
w	weak (IR)

## 6.2. Publications and conference contributions

### 6.2.1 Publications

1. Ilona Papp, Jens Dervedde, Sven Enders, Rainer Haag; “Modular Synthesis of Multivalent Glycoarchitectures and Their Unique Selectin Binding Behavior”, *Chem. Commun.* **2008**, 44, 5851–5853.
2. Adam L. Sisson, Ilona Papp, Katharina Landfester, Rainer Haag; “Functional Nanoparticles from Dendritic Precursors: Hierarchical Assembly in Miniemulsion”, *Macromolecules* **2009**, 42(2), 556–559.
3. Michaela Mühlberg, Da’san M. M. Jaradat, Rolf Kleineweischede, Ilona Papp, Decha Dechtrirat, Silvia Muth, Malgorzata Broncel, Christian P. R. Hackenberger; “Acidic and Basic Deprotection Strategies of Borane–Protected Phosphinothioesters for the Traceless Staudinger Ligation”, *Bioorg. Med. Chem.*, **2010**, 18(11), 3679–3686.
4. Ilona Papp, Adam L. Sisson, Christian Sieben, Andreas Herrmann, Rainer Haag; “Size-dependent Influenza Virus Inhibition by Multivalent Polyglycerol Glycoarchitectures”, 240<sup>th</sup> ACS National Meeting, Boston, MA, United States, PMSE-36, *Polymer Preprints*, **2010**.
5. Ilona Papp, Christian Sieben, Kai Ludwig, Meike Roskamp, Christoph Böttcher, Sabine Schlecht, Andreas Herrmann, Rainer Haag, “Inhibition of Influenza Virus Infection by Multivalent Sialic Acid Functionalized Gold Nanoparticles”, *Small* **2010**, 6(24), 2900–2906.
6. Ilona Papp, Christian Sieben, Adam L. Sisson, Johanna Kostka, Christoph Böttcher, Kai Ludwig, Andreas Herrmann, Rainer Haag, “Inhibition of Influenza Virus Activity by Multivalent Glycoarchitectures with Matched Sizes”, *ChemBioChem* **2011**, 12(6), 887–895.
7. Ilona Papp, Jens Dervedde, Sven Enders, Sebastian Riese, Tze Chieh Shiao, René Roy, Rainer Haag, “Multivalent Presentation of Mannose on Hyperbranched Polyglycerol and Their Interaction with Concanavalin A Lectin”, *ChemBioChem* **2011**, 12(7), 1075–1083.



### 6.2.2 Conference contributions

1. **International Dendrimer Symposium 5 (IDS5)**, Toulouse (France), August 2007, poster presentation.  
Ilona Papp, Rainer Haag “*Modular Synthesis of Dendritic Glyco-Architectures as Multivalent Ligands.*”
2. **The 4<sup>th</sup> Glycan Forum**, Berlin (Germany), May 2008, poster presentation.  
Ilona Papp, Jens Dornedde, Sven Enders, Rainer Haag “*Modular Synthesis of Biocompatible Dendritic Glyco-Architectures as Selectin Inhibitors.*”
3. **BIO Dendrimer–Symposium on Biomedical Properties and Applications of Dendrimers**, Łódź (Polen), Juli 2008, poster presentation.  
Ilona Papp, Jens Dornedde, Sven Enders, Rainer Haag “*Modular Synthesis of Multivalent Glycoarchitectures and their Unique Selectin Binding Behavior.*”
4. **Bio & Polymers, New Polymer Technologies with Water, Eurogress**, Aachen (Germany), *Biannual Meeting of the GDCh-Division of “Macromolecular Chemistry”*, September 2008, poster presentation.  
Ilona Papp, Adam L. Sisson, Rainer Haag “*Functional Nanoparticles from Dendritic Precursors.*”
5. **Europolymer Conference on “Click“ Methods in Polymer and Materials Science (EUPOC)**, Gargnano (Italy), June 2009, poster presentation.  
Ilona Papp, Jens Dornedde, Sven Enders, Rainer Haag “*Modular Synthesis of Multivalent Glycoarchitectures.*”
6. **11<sup>th</sup> European Symposium on Controlled Drug Delivery (ESCDD)**, Egmond aan Zee (Holland), April 2010, Poster Presentation  
Ilona Papp, Christian Sieben, Adam L. Sisson, Andreas Herrmann, Rainer Haag “*Inhibition of Influenza Virus Activity by Newly Designed Multivalent Glycoarchitectures.*”
7. **8<sup>th</sup> International Symposium on Polymer Therapeutics (ISPT 8): From Laboratory to Clinical Practice**, Valencia (Spain), May 2010, oral and poster presentation.  
Ilona Papp, Christian Sieben, Adam L. Sisson, Andreas Herrmann, Rainer Haag “*Size-Dependent Influenza Virus Inhibition by Multivalent Polyglycerol Glycoarchitectures.*”
8. **Division of Polymeric Materials: Science & Engineering (PMSE)**, Boston (USA), ACS Meeting, August 2010, poster presentation.  
Ilona Papp, Christian Sieben, Adam L. Sisson, Andreas Herrmann, Rainer Haag “*Size-Dependent Influenza Virus Inhibition by Multivalent Polyglycerol Glycoarchitectures.*”

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## **6.4 Curriculum vitae**

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.