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Molecular Recognition of Glycopolymer Interface

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1. Introduction

Saccharides on the cell surfaces play important roles in the living systems. For example, it mediate the cell-cell adhesion, fertilization, protein transportation, infection of pathogens and cancer metastasis etc [1, 2]. The saccharide-protein interactions also involve the various biological events (Table 1). Actually, the saccharides are the model compounds of some of the medicines like oseltamivir [3]. The interaction between galactose and asialoglycoprotein receptor is a possible mechanism for the hepatocyte-specific drug delivery systems [4]. Therefore, it has been pointed out that the saccharide-protein interaction can be utilized for the novel bio-functional materials such as cell cultivation, medicine target, and drug delivery systems.

	Target	Saccharide structure
Lectin	Concanavalin A (ConA)	α -Man/ α -Glc
	Wheat germ agglutinin (WGA)	GlcNAc, Neu5Ac
Cell	Hepatocyte	β -Gal/ β -GalNAc
Pathogen	Shiga toxin (from E. coli O-157etc)	Gb3: Gal1 α -4Gal β 1-4GlcCer
	Cholera toxin	GM1:Gal β 1-3(NeuAca2-3)GalNAc β 1-4Gal β 1-4GlcCer
	Influenza Type A for human	Neu5Aca2-6Gal β 1-4(3)GlcNAc β 1-, Neu5Aca2-6Gal β 1-3GalNAc β 1

Table 1. The saccharide recognition of proteins, cells and pathogens.

The saccharide-protein interactions are also important in terms of protein analyses (proteome), because the interaction is important to clarify the biological function of proteins. [5] The saccharide immobilized substrates are investigated for the saccharide-microarray. In addition, the saccharide-protein interactions is a potential marker of various diseases like infection of pathogens (e.g. viruses, bacteria, Cholera, and Shiga toxin) and cancer. Therefore, the saccharide-protein interactions are also utilized for the biosensor of diseases.

In this chapter, we describe the materials with molecular recognition ability of sugars. Section 2 reviews the multivalent interaction between sugar and proteins. Section 3 presents the physical chemical properties of glycopolymers. Section 4 presents the graft of glycopolymers and the biomaterial fabrication. Section 5 presents the glycopolymer interface with dendrimer.

2. Multivalent interaction

The saccharide-protein interaction plays important roles in the living system, and the novel biomaterial fabrication is expected using the interaction. However, the saccharide-protein interaction is basically weak, and it is difficult to utilize and detect the interactions. It has been reported that the saccharide-protein interaction can be amplified by the multivalency [6, 7, 8]. Actually, saccharides on the cell-surfaces are displayed in a multivalent manner. The glycolipids form densely saccharide structures of lipid-rafts [9], and glycoproteins usually have multivalent saccharide structures, which provides the multivalent saccharide-protein interactions.

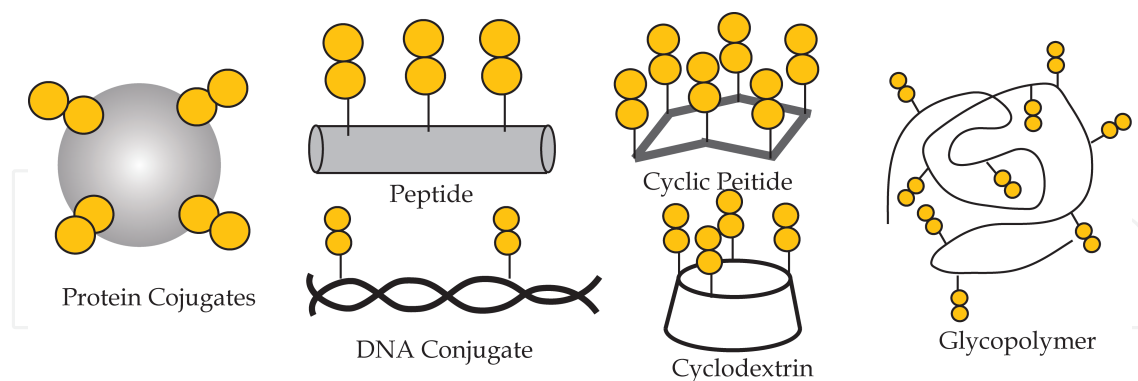


Figure 1. Schematic illustration of multivalent saccharide compounds.

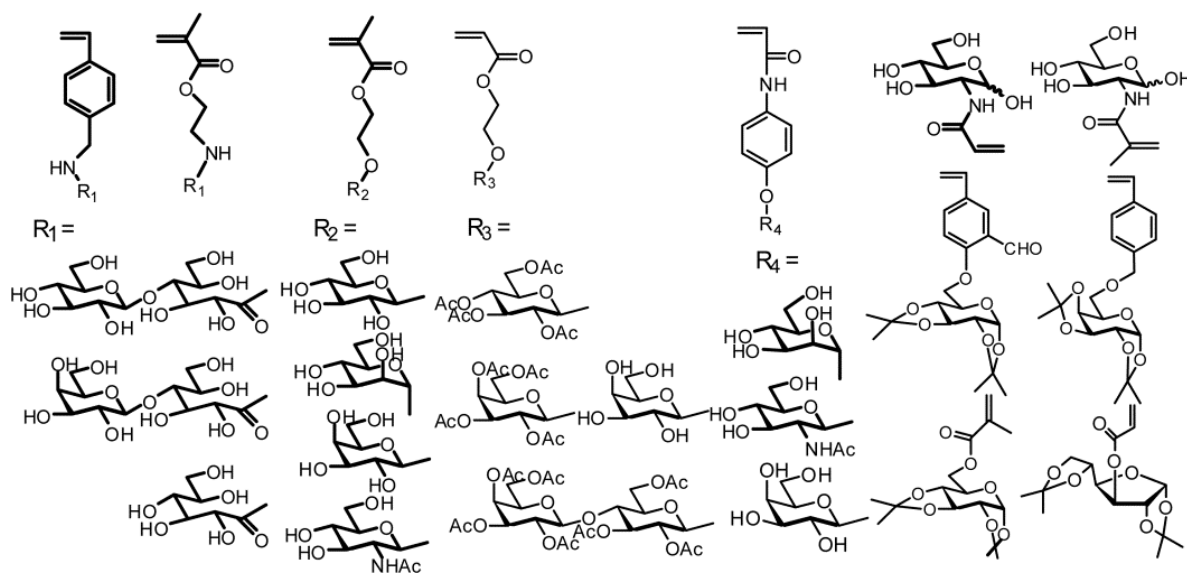
The artificial multivalent saccharide displays also enables the multivalent interaction between saccharide and protein. Various artificial compounds with multivalent saccharides have been reported (Figure 1). Proteins are commonly used as carriers for the multivalent presentation of antigens, and bovine serum albumin (BSA) is the representative [10]. Peptides are used as a scaffold of saccharide display [11]. Saccharide conjugates with DNA [12], cyclodextrin [13] and polymers have been also reported to exhibit multivalent interactions.

Saccharide conjugates with peptides and proteins are appropriate structure for pharmaceutical substances because of the biocompatibility and the fine structures. The glycopeptides toward shiga toxins (toxins from *E.coli* O-157 and enterohemorrhagic *E.coli*), influenza virus [14] and lectins [15] were reported.

2.1. Glycopolymer

There have been various multivalent saccharide derivatives as we described in the above section. Glycopolymers have been reported to exhibit larger multivalent effect comparing to other multivalent saccharides, because glycopolymers form large multivalent cluster [16]. The glycopolymers are the interesting compounds with large molecular weights and diverse structures. The glycopolymers are prepared by saccharide addition to polymer via polymer reaction, or by polymerization of saccharide monomers. The technique of synthetic polymer enables the preparation of versatile biomaterials. Especially, living radical polymerization is applicable to various saccharide monomers and provides the facile strategy for functional material preparation [17].

(a) Living radical polymerization



(b) Ring-opening metathesis polymerization (c) Polymerization and saccharide addition

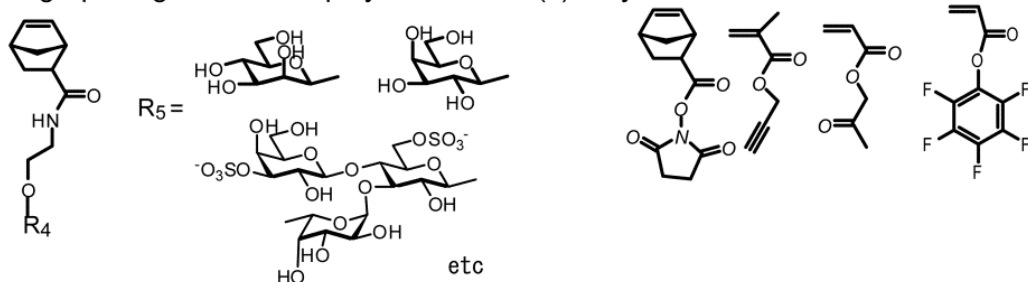


Figure 2. Chemical structure of monomers for glycopolymer preparation for (a) living radical polymerization, (b) ring-opening metathesis polymerization and (c) polymerization with saccharide addition.

The various saccharide monomers have been reported, which were shown in Figure 2. There are various saccharide vinyl compounds. Styrene [18, 19], methacrylate [20], acrylate [21], acrylamide [22] and methacryl amide [23] with saccharide were reported. Living radical polymerizations were reported with them. Norbornene saccharide derivatives were also reported, which provides the fine-tuned polymers via ring opening metathesis polymerization (ROMP) [24]. Reactive functional monomers were also utilized for glycopolymer synthesis. Monomers with acetylene [25] and active ester [26] were reported, where glycopolymers were obtained by polymerization and successive sugar addition.

Saccharide recognition proteins are called lectin, which basically have multiple domain structures [27]. The multivalent saccharides gain in enthalpy due to multiple binding to sugar recognition sites, and gain in entropy due to the various binding modes. The glycopolymers are large sugar cluster to gain the Gibbs free energy in both enthalpy and entropy, and lectins have multiple and valuable structure, which is advantage for binding. The distance of sugar binding sites is different with each lectin, which is easily tuned by copolymerization. The density, distance, and the size of multivalent compounds can be easily adjusted by copolymerization, which can be applied to variable lectins.

The glycopolymers are water soluble polymers, which can be utilized as artificial polymeric ligands or polymer drugs. Choi et al reported polyacrylic acid with sialic acid, and the polymer efficiently inhibited the sialidase of Influenza virus [6]. Kobayashi et al reported the various glycopolymers. The lactose substituted polystyrene (poly(*N*-vinylbenzyl-*O*- β -D-galactopyranosyl-(1-4)-D-gluconamide (PVLA))) strongly interacted with lectin, and it was applied for hepatocyte culture [18]. Polystyrene with sialyl lactose showed the strong binding to influenza virus A [28]. Gestwicki et al prepared various glycopolymers via metathesis reaction, and reported the glycopolymers to bind lectin and *E. coli* [29]. Nishimura reported the glycopolymers interacting with glycosyl transferases to synthesize oligosaccharides, and they developed the oligosaccharide synthesizer with glycopolymers [30].

2.2. Amphiphilic property of glycopolymer

We defined glycopolymers as polymers with pendant saccharides. As we described above, the glycopolymers showed the strong multivalent effect based on the multivalency, with lectins, cells, viruses and bacteria. Another interesting property of glycopolymer is amphiphilicity. Glycopolymers via addition polymerization have hydrophobic backbones with C-C bond, and are amphiphilic due to the hydrophilic side chain. The side chain of glycopolymer is bulky structure, and so the glycopolymer easily form the self-assembling structure. The structure of PVLA in aqueous solution was analyzed by small angle X-ray scattering [31], and it was found that PVLA formed rod-like structure, where the rod had the structure with long axis 10 nm and short axis 5 nm. The rod-structure was similar to some polysaccharides of amylose and sizofiran.

It has been known that amylose is a host of hydrophobic compound as it is known starch-amylose complex. PVLA also became a host compound of hydrophobic substances. We investigated the supramolecular polymer complex of PVLA. PVLA formed su-

pramolecular complex with various hydrophobic fluorophore and π -conjugate polymer of polythiophen [32].

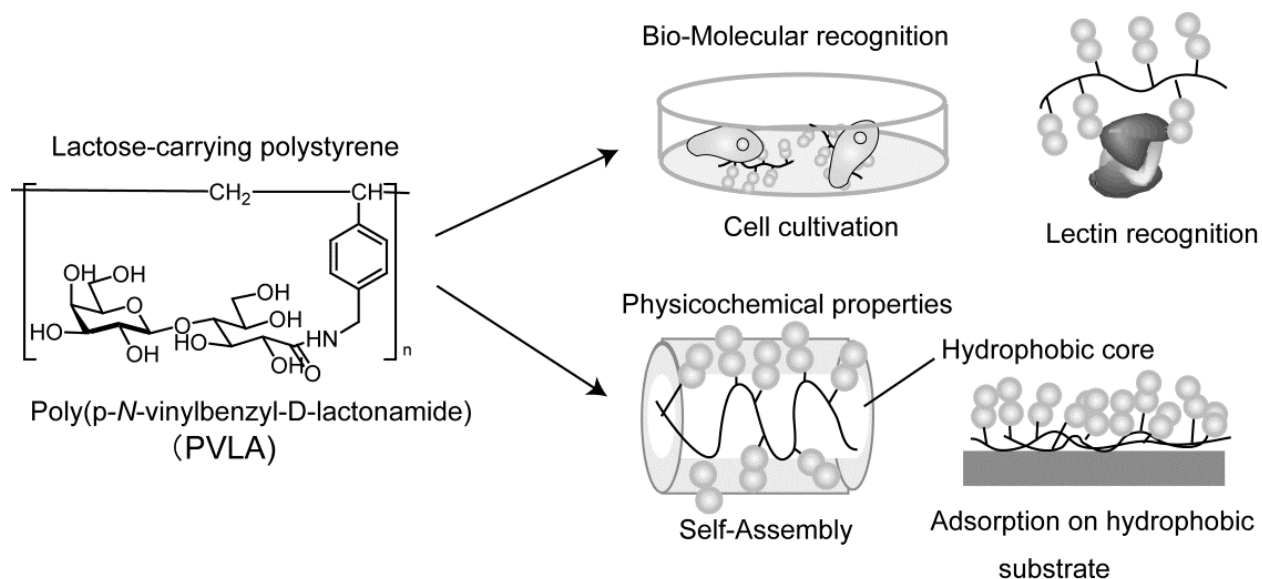


Figure 3. Chemical structure and properties of lactose-carrying polystyrene.

Self-assembling properties of amphiphilic polymers were used in order to organize the glycopolymer interface. The glycopolymer, PVLA, had amphiphilic structure and adsorbed to the hydrophobic interface [33]. PVLA adsorbed the hydrophobic polystyrene culture dish, and the culture dish was used as hepatocyte culture [34]. The adsorption of PVLA was investigated with hydrophobic self-assembled monolayer (SAM) of octadecyltrimethoxysilane [35]. PVLA selectively adsorbed onto the hydrophobic substrate, exhibiting the lectin and hepatocyte affinity. We utilized the adsorption process to fabricate the micropatterned cell cultivation system and protein display.

On the other hand, the self-assembling properties were expanded to the complex and micropatterned cell cultivation systems. We fabricated the micropatterned substrate with hydrophobic and cationic SAM. The micropatterned substrates were fabricated by the formation of SAM and micropatterning with photolithography. The orthogonal self-assembly was performed with PVLA and anionic polysaccharide of heparin. PVLA and heparin bound to hydrophobic and cationic part, respectively. PVLA showed affinity to hepatocyte, and heparin binds to bFGF that has affinity to fibroblast cell. The multiple cell cultivation was accomplished with PVLA/hepatocyte and heparin/bFGF/fibroblast in a self-assembling manner [36].

3. Grafted glycopolymers

Glycopolymer-coated substrates were facilely prepared by self-assembly of hydrophobic interaction. However, it is difficult to control the density of glycopolymer by self-assembling

process. In addition, the physical adsorbed polymers were fragile in a specific solvent condition. The coatings with spin-coat and Langmuir-Blodgett (LB) technique also provide the well-defined coating, but they are also fragile.

On the other hand, the surface-attached polymers are robust and practical to various purposes. In order to attach the polymer to the substrate, the covalent bond formation between polymers and substrates was necessary. The polymers with functional groups on the side chain and the polymer terminal were subjected to covalent bond formation with substrate. Those method is called "grafting-to" process. The grafting of the polymer was also reported via surface-initiated polymerization, which is called "grafting-from" method. The polymerization was possible to start from the substrate by surface activation with γ -beam, VUV and plasma irradiation, and by the radical initiator immobilization. The properties of the substrates depend on the polymer density, thickness and flatness. The grafting polymers are categorized as "pancake", "mushroom", and "brush". Generally, the grafting to method provides the non-dense grafting substrate like "pancake" or "mushroom" and the grafting from method enables "polymer brush" structure [37].

3.1. Glycopolymer-grafted nanoparticle via RAFT polymerization

In order to prepare the polymer-grafted materials, the living radical polymerizations are actively utilized by many groups. Living radical polymerization provides the uniform polymer, and polymer terminals can be modified. Atom-transfer-radical-polymerization (ATRP) enabled the dense-polymer brush. Since living radical polymer has active terminal end, the polymer terminal is possible to be modified. Specially, the polymers via RAFT process have the active terminal end with dithio- or trithioester. The polymer terminal with reversible-addition-fragmentation chain-transfer polymerization (RAFT) is converted to thiol by reduction or hydrolysis. Thiol is highly reactive and relates to various reaction like thiol-ene reaction, thiol-maleimide coupling, and Au-S interaction [38].

The glycopolymer conjugates have been synthesized via RAFT polymerization. Mancini et al reported a protein with glycopolymer via RAFT polymerization and disulfide bond formation [39]. Narain et al reported the preparation of particle by RAFT polymerization and subsequent Au-S bond formation [40].

We prepared the glycopolymer with *p*-amidophenyl glycosides (α -Man, β -Gal and β -GlcNAc) and acrylamide via RAFT process with (thiobenzoyl)thioglycolic acid [41]. The polydispersities were below 1.5 in spite of random copolymer. The obtained glycopolymers had dithioester terminal, which was reduced thiol by addition of NaBH_4 . The thiol-terminated glycopolymers were mixed with gold nanoparticle solution. The gold nanoparticle (40 nm) was successfully modified by glycopolymer, which was confirmed by TEM observation and zeta-potential measurement. The glycopolymer modified gold nanoparticle was water soluble and stably dispersed for more than half a year.

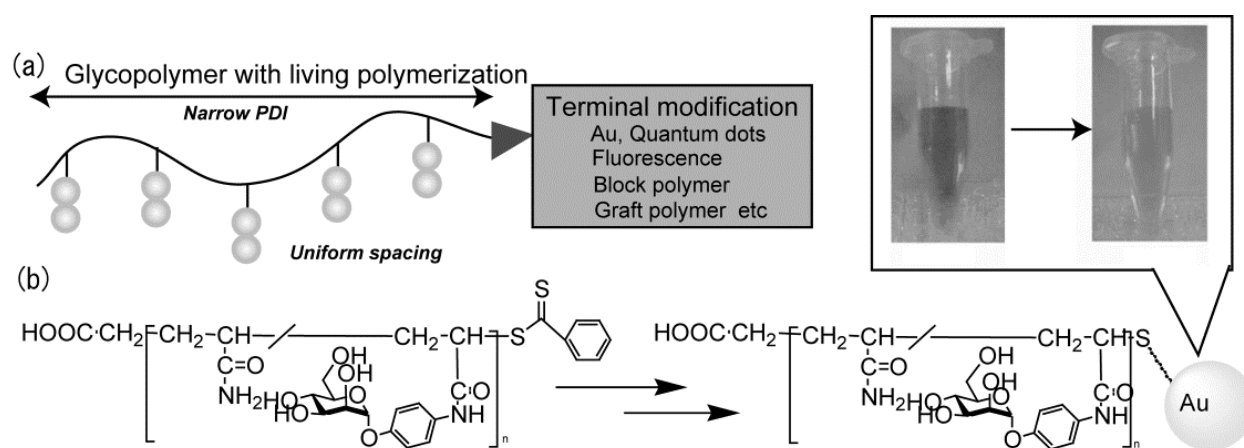


Figure 4. Preparation of a glycopolymer modified nanoparticle via RAFT living radical polymerization. (a) Properties and (b) a synthetic scheme of particle with a color change image of nanoparticle.

A glycopolymer modified gold nanoparticle stained pink color with peak top at 520 nm. ConA (α -Man recognition protein) was added to the α -Man-modified nanoparticle solution, and the glycopolymer-gold nanoparticle showed the lectin recognition property. The color of the particle solution changed to blue, and the spectra showed the red-shift (Figure 4). The color change occurred based on the aggregation of nanoparticle by α -Man-lectin binding. The nanoparticle showed the affinity to a sugar recognition protein, and bacterium. The sugar recognition *E.coli* (ORN 178) was also added to the solution with glycopolymer-modified gold nanoparticle. The nanoparticle was adsorbed onto the periphery of *E.coli*, which was observed by TEM observation. On the other hand, *E.coli* without sugar recognition property (ORN 258) didn't show the change. The color change slowly occurred in 8 hours, while the color change with protein occurred quickly for 1-3 min. The color change occurred specifically with the corresponding lectin and glycopolymer.

Sugar modified gold nanoparticles were reported by other groups. Otsuka et al reported lactose substituted gold nanoparticles with PEG linker [42]. The gold nanoparticle also showed red-shift by addition of lactose-recognition lectin. Narain et al reported nanoparticle of glycopolymer having biocompatibility [43].

Advantage of the glycopolymer-modified materials is the specific recognition and bioinert property. The detailed protein affinity was investigated with surface plasmon resonance of glyco-polymer-modified gold substrate. The glycopolymer-modified gold substrate had affinity constants of 10^7 (M^{-1}) order, which was much stronger than the monovalent sugar of 10^3 (M^{-1}) order. At the same time, the glycopolymer-modified substrate showed the highly specificity to proteins. The amount of specific protein bounds (α -Man-ConA) was more than 15 times larger than that of non-specific binding (BSA, fibrinogen, and lysozyme) [44]. Interestingly, the glycopolymer-interface showed much better protein specificity than the artificial glycolipid monolayers of self-assembled monolayer (SAM) and LB membrane. The hydrophilicity of the glycopolymer-modified gold substrate contributed the bioinert property.

3.2. Biosensing with glycopolymer-modified nanoparticles

We investigated the biosensing of the glycopolymer-modified gold nanoparticles.

First, the gold nanoparticles have been applied for biotechnology as a marker. We applied the glycopolymer-modified gold nanoparticle for lateral flow assay (immune-chromatography), where we tested the properties of particle with target analyte of lectin (ConA) [45]. Anti-ConA antigen was immobilized on the nitrocellulose strip, and the detection of target ConA was investigated with glycopolymer-modified gold nanoparticle.

Target protein of ConA was detected by the pink color of gold nanoparticle. We tested the glycopolymer with varying sugar ratio of 0, 6, 12 and 50 %. In terms of red-shift, the glycopolymer with higher sugar ratio (50 %) exhibited more red-shift. However, the nanoparticle with higher sugar ratio (50 %) was not appropriate for lateral flow assay. The gold nanoparticle with higher sugar ratio aggregated at the bottom line with addition of ConA. The glycopolymer with modest sugar content (6 %) exhibited the best indicator of ConA. The glycopolymer with modest sugar content was more flexible than that with higher sugar content, which improves the sensitivity in lateral flow assay. What is interesting about lateral flow assay is the biosensing with naked eye, using a simple device. The detection of ConA was possible from 1 nM level with naked eye.

Electrochemical biosensing was also conducted with glycopolymer-modified gold nanoparticle [46]. The gold nanoparticle was assembled on anti-ConA antigen immobilized electrode. The amount of protein bound was estimated by the electrochemical signal of gold nanoparticle, where the gold nanoparticle was electrochemically reduced in differential pulse voltammetry. The amount of ConA bounds were more sensitively monitored than that with lateral flow assay. The detection limit was around 0.1 nM.

These experiments were conducted using the model target of ConA. Since the protein-saccharide interactions are involved in various infection diseases, the detection of serious disease like influenza and cancer will be realizable with the corresponding saccharide modified particle.

3.3. Protein separation with glycopolymer materials

The glycopolymer-modified interface showed specific affinity biomolecules, which can be applied not only for biosensing but also for protein purification devices. We modified the porous filter membrane with glycopolymer grafting, and prepared protein purification device. Basically, the purification and removal of specific biomacromolecules are mainly conducted by the size-exclusion process. For example, bacteria are able to be removed by filtration, which are called "sterile filtration". The size of bacteria was μm order, and so the porous materials with μm order pore are applied for sterilization. However, the size of proteins and viruses are nm level, which is difficult to apply the size-exclusion way. In addition, the filtration speed is strongly dependent on the radius of porous materials, and the flux speed of nano-level porous materials were too slow to use it practically. Therefore, it is almost impossible to attain the protein purification by nm porous membrane, and the affinity purification is appropriate to the purification and removal of protein and viruses [47].

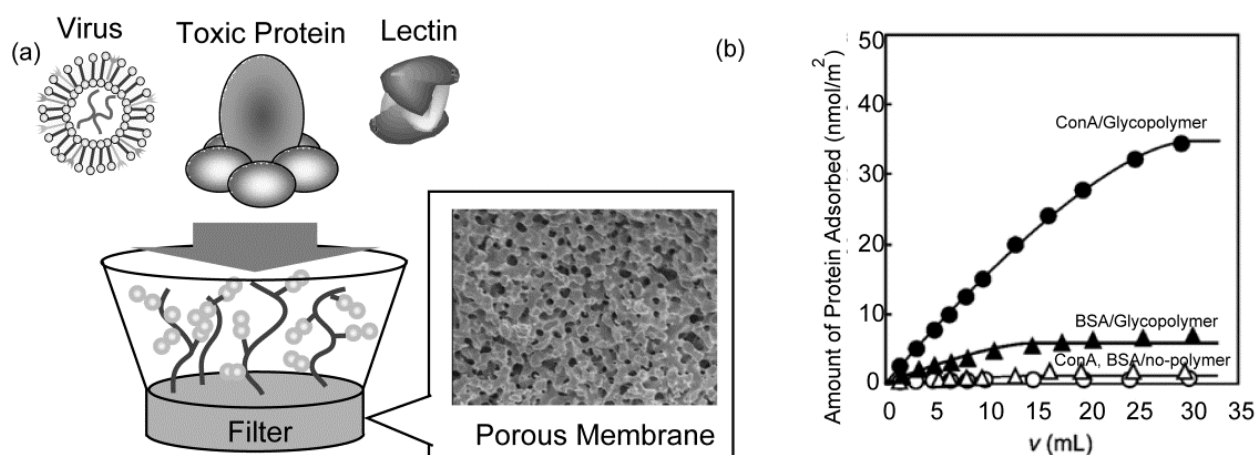


Figure 5. (a) Schematic Illustration of glycopolymer brush for protein and pathogen removal. (b) The amount of protein adsorbed on the glycopolymer brush.

We synthesized the glycopolymer with α -Man and trimethoxysilane units, and the glycopolymer was immobilized onto the porous siliceous materials via Si-O-Si bond [48] (Figure 5). The radius of the porous materials was 2 μm , which was much larger than the size of proteins and viruses. The porous membrane was connected to flow channel, and the protein solutions (ConA and BSA) was injected to the flow. ConA was selectively adsorbed onto the porous membrane, but BSA passed through the membrane due to α -Man-ConA interaction. On the other hand, the porous membrane adsorbed proteins by non-specific interaction. The amount of ConA bound was 34 nmol/m^2 , and that of BSA was 4.2 nmol/m^2 . The modification of glycopolymer provides the affinity to specific protein and the bioinert properties to other proteins.

Li et al reported the filter preparation with sialyl-lactose modified chitosan [49]. The modified chitosan took up the influenza virus. The solution containing influenza virus A was passed through the filter, and the amount of virus was reduced about 1/200. The chitosan filter without sialyl-lactose didn't remove influenza virus. The influenza virus showed the affinity to sialyl-lactose via hemagglutinin. Muschin et al also reported the virus removal by sulfated curdlan modified filter.

Bio-separation with nanomaterials was investigated. Nagatsuka et al reported the protein separation with glycopolymer-modified magnetite. The glycopolymer with lactose modified magnetite was prepared by biotin-streptavidin reaction. The toxic protein of ricin solution was mixed with lactose-substituted nanoparticle [50]. The ricin was separated with magnetic. El-Boubbou et al separated sugar recognition *E.coli* with a similar manner [51].

4. Glyco-interface with precise structure

The affinity between saccharide and protein was strongly affected by multivalency. Therefore, the precise multivalent compound is useful to fabricate the efficient ligand and to clari-

fy the protein function. For example, the precise multivalent sugars were reported with a starfish like compound carrying globotriose to exhibit the strong neutralizer of Shiga-toxin [52]. Matsuura et al reported the multivalent sugar with DNA template [12]. The multivalent sugar with precise sugar distance clarified the multivalent interaction based on the sugar distance.

Glycopolymer shows the strong multivalent effect, but generally the structure was not uniform. Dendrimer is regularly branched polymer with precise structure. Glycopolymer with dendrimer is useful to display saccharide in a precise manner [53]. For example, Roy et al reported various glycodendrimers with sialic acid [54]. The efficient ligand fabrication is expected with glycodendrimers.

The precise structure of glycol-dendrimers is applicable to the saccharide microarray, where the multivalent saccharide structure provides the various information. Those saccharide array can reveals the properties of proteins like multivalency, distance of saccharide, and the saccharide binding site.

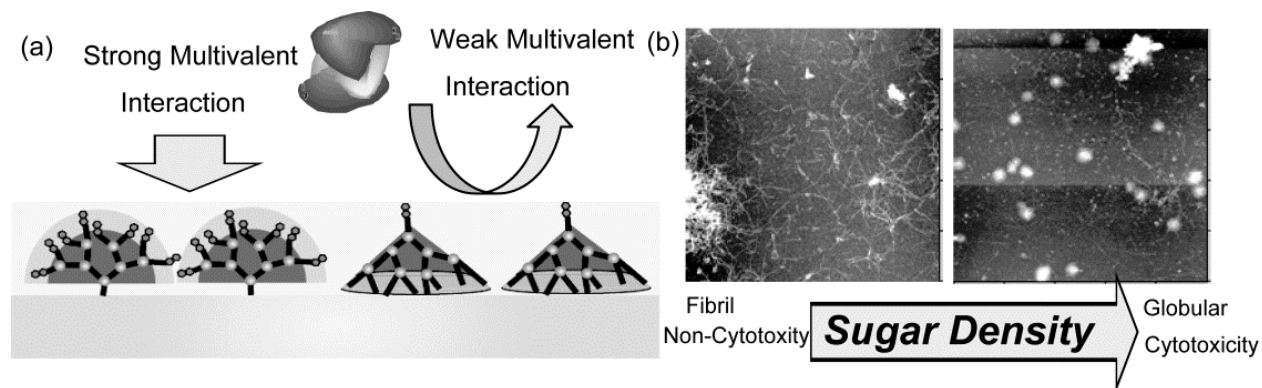


Figure 6. (a) Saccharide microarray with dendrimers. (b) The morphology control of Amyloid beta peptide with sulfonated glyco-dendrimer-interfaces.

The fan-type dendrimers with saccharide terminal was prepared by click chemistry of Huisgen reaction. The saccharide dendrimer with azide-core was immobilized via click reaction onto the acetylene-immobilized SAM. The protein-saccharide interaction of α -Man, β -Gal and β -GlcNAc was measured with surface plasmon resonance. The corresponding lectin showed the remarkable multivalency with higher generation of dendrimer array. Especially, the combination of α -Man and ConA was much affected by dendrimer generation increase [55].

Then, we prepared the glycol-dendrimer interface with 6-sulfo-GlcNAc, which is representative structure in glycosaminoglycans (GAGs) [56] (Figure 6). GAGs have been reported to relate the various biological events. We prepared mono-, di- and tri-valent 6-sulfo-GlcNAc, and the interaction with Alzheimer amyloid $\beta(1-42)$ ($A\beta(1-42)$). First, the interaction was measured with SPR, where the divalent and trivalent 6-sulfo-GlcNAc showed the stronger interaction than monovalent one due to the multivalent effect. Interestingly, the morphology of $A\beta$ was strongly affected by the multivalent array. In case of monovalent array, $A\beta$

formed nanofiber with 8-12 nm width and 1-2 mm long. On the other hand, A β on the divalent and trivalent induced spherical objects. In the case of trivalent array, A β formed spherical objects with 500-600 nm diameter. The cytotoxicity of A β was depend on the microarray used, and A β showed the strong cytotoxicity on the trivalent 6-sulfo-GlcNAc array, where the cytotoxicity of A β was related to the morphology of peptide.

Suda et al reported the dendrimer sugar chip with sulfonated trisaccharide of heparin (Suda et al., 2006). They synthesized mono-, tri- and tetravalent sugar chip with as SAM

They investigated the saccharide-protein interaction with hemostatic proteins. They analyzed the affinity of the protein quantitatively. They found the multivalent sugar chip with dendrimer was a useful tool to investigate the protein-saccharide interaction.

5. Conclusion

The molecular recognizable materials were prepared with glycopolymer immobilized substrates. Since the saccharides interact with sugar recognition proteins, cells and viruses, the glycopolymer immobilized substrates exhibited the biomolecules recognition. The substrate were applicable for the biomaterials.

Generally, the saccharide-protein interaction was weak. Therefore, the multivalent saccharide ligands of glycopolymer showed the strong affinity to proteins. Glycopolymers were amphiphilic polymers, and formed self-assembling structure in aqueous solution based on the hydrophobic interaction. The glycopolymer coating by hydrophobic interaction was also possible in a self-assembling manner, and was used as hepatocyte culture.

The glycopolymers were also prepared via polymer grafting. The glycopolymer grafting was accomplished via both of "grafting to" and "grafting from" methods. The living radical polymerization of glycopolymers was important in both grafting methods. The glycopolymers were immobilized by "grafting to method" via RAFT living radical polymerization. The RAFT polymer terminal was converted to thiol, which modified gold nanoparticle with Au-S bond formation. The modified gold nanoparticle had both properties of nanoparticles and glycopolymers. The color of the modified gold nanoparticle was basically pink, and the color showed red-shift by addition of the corresponding lectin, and bacterium. The modified gold nanoparticle was applied for the biosensing with lateral flow assay and electrochemistry as a marker. The glycopolymer grafted porous materials were prepared, and the porous materials were selectively filtered the saccharide recognition protein. The glycopolymer-modified materials showed the specific binding properties to the corresponding lectin based on the molecular recognition ability and the bio-inert surface property.

The glycopolymer substrates with glycol-dendrimers were also investigated. The glycol-dendrimers were applied to quantitatively measure the saccharide-protein and the multivalent interaction. These interfaces were useful to measure the detailed interaction and mechanism with pathogens or signal proteins.

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