Associative polymers for novel nanostructure formation and cell surface engineering

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Associative polymers for novel nanostructure formation and cell surface engineering

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Chapter 1. General introduction

1.1. Associative polymers

In the biomedical field such as drug delivery systems or tissue engineering, various polymers with multiple-interaction side chain have been widely-applied. Such polymer binds to target molecule more strongly than interactive monomer because of multiple interactions, which is called “multivalent effect”. Increment of avidity is attributed to content and density of interaction sites on the single polymer backbone. The multivalent effect has an impact on avidity to target and formation of self-assembly. We focused on two kinds of non-covalent interaction, electrostatic interaction and hydrophobic interaction to design and evaluate polymers having multiple binding sites in this thesis, and summarize the application of self-assembly based on polymer structure. In the design of multiple-interactive polymers, content of interactive moieties should affect dramatically to the polymer self-assembly characteristics. Mode of each interaction will be also important for the design of such polymers. I used here two different interaction modes, electrostatic and hydrophobic interactions.

1.2. Electrostatic interaction for polymer self-assembly

Electrostatic interaction is much weaker than hydrophobic interaction in aqueous milieu [1]. However, the interaction has been widely-used to form self-assembly because various therapeutic agents have ionic charge [2, 3] and microenvironment in vivo has tissue-specific pH [4-6] so that ionic charges can be varied dependent on target tissues.

1.2.1. Increasing content and density of ionic groups

The high ionic strength of physiological conditions attenuates electrostatic interaction. Thus, increasing content of ionic groups is essential to prepare stable polyion complex.
Chapter 1. General introduction

**Polyion complex colloids**

Polyion complex colloids composed of cationic polymer and anionic polymer has been applied to polymeric carriers for carrying low molecular drug and protein carrier [7-14]. For the anionic polymer to form polyion-complex, great variety of natural anionic polymers, such as poly(glutamic acid) [15], poly(aspartic acid) [16], carboxymethyl cellulose [17], glucomannan [18] and dextran sulfate [19] can be available. Polyion complex colloids were also applied to gene delivery carrier by forming cationic polymers – DNA complex. A variety of polycations including both biopolymers [20-29] and synthetic polymers [30-38] have been examined as novel carriers. These polyion complexes form tight aggregation so that the PIC is hindered from degradation by protease. To obtain a colloidal dispersion, high dilution and non-stoichiometric charge ratio conditions are required [39-41].

**Tunable polyion complex structure**

Kishimura et al succeeded in controlling precise structure of PIC on nano scale [42-46]. They synthesized oppositely charged diblock copolymers consisting of water-soluble poly(ethylene glycol) (PEG) segment, and charged poly(amino acid)s segment. When the diblock copolymers are mixed to neutralize their charge, the primary amino groups on the polycations and the carboxylate groups on the polyanions form multivalent ion pairs to give PICs. A large volume of PEGs, or higher PEG weight fraction ($f_{PEG}$), prevents the unlimited growth of PIC and drives micelle formation. In contrast, a small volume of PEGs, or lower weight fraction ($f_{PEG}$), is favorable for vesicle-like structure (PICsome) formation. They succeeded in formation of stable PIC micelle and PICsome in physiological condition, containing 150 mM NaCl. PIC region of both structure forms tight packing layer, thus the size of molecules permeable to PIC layers in PICsome is limited.
(a) PIC colloid (general PIC structure)

(b) Precisely controlled PIC structure

**Figure 1.1.** PIC structure composed of polyionic polymers with higher content density of ionic groups. (a) General PIC structure and (b) precisely controlled PIC structure. Both PIC structure have tight PIC region.
1. 2. 2. Decreasing content of ionic groups in PIC formation

Decreasing the content of ionic groups make the PIC unstable, however increasing the net charge on each ionic group can improve the stability of PIC. The polyelectrolyte with low content but high net charges of ionic groups forms unique PIC structure as follows;

(1) Loose aggregate forms due to the large exclusion volume of main chain
(2) Dissociation of PIC structure may easily occur in response to the alteration of condition that can change the net charges.

Katayama group reported on a graft copolymer composed of a neutral main chain and oligocationic graft chains [47-49]. They employed cationic peptides as the graft chains, which are the substrates of intracellular enzyme such as protein kinases or protease specifically activated in diseased cells. The enzymatic reaction of the grafted cationic peptides reduces their net cationic charge, thus weakening the PIC to afford a gene expression. The binding of the graft copolymers to pDNA is not so strong but has a sticky nature, which has not been shown in previous PIC colloids. This unique binding mode of the graft copolymers to pDNA creates an environment that selects the accessible enzymes. The graft copolymers afforded the nucleases or protein kinase reactions, while it suppressed DNA amplification and gene expression [50].

We focused on the loose PIC environment that allows the access of protein, and effect of ionic groups interacting with pDNA. From this finding, we propose novel PIC structure composed of the polycation and polyanion with low content of oligoionic groups. The PIC is expected to have large space to accommodate various macromolecules, such as proteins or pDNA due to loose PIC environment. When oligo-cationic and ologo-anionic groups have the same net charges, the oligoionic groups are expected to form stable crosslinking points and form PIC structure. In addition, increased ionic strength to attenuate electrostatic interaction
or pH change to disrupt the charge balance between oligocationic ionic groups may induce
dissociation of PIC structure. The novel PIC structure which is expected to have these
property has not been reported.

**Figure 1.2.** Property of the PIC structure composed of the amphiphilic polymer bearing
oligocation and DNA.
(a) Loose aggregate accommodating a variety of molecules

![Diagram of PIC structure with oligocation and oligoanion](image1)

(b) Dissociation of PIC structure responding to charge change

![Diagram of PIC structure with high ionic strength and pH change](image2)

**Figure 1.3.** Expected property of the PIC structure, composed of the amphiphilic polymers bearing oligoionic groups. (a) This PIC structure is expected to have large free space to accommodate a variety of molecules such as proteins and DNA. (b) This PIC structure is expected to dissociate in response to high ionic strength and pH change.
1. 3. Hydrophobic interaction for polymer self-assembly

Hydrophobic interaction is main driving force to fold proteins [1] and accumulate signaling proteins on the cell surface [51] because it is the strongest interaction in physiological condition with high ionic strength. The interaction has been widely-used to form self-assembly.

1. 3. 1. Increasing content of hydrophobic groups

**Block copolymer**

Formation of infinite aggregate in aqueous milieu has been tried in many previous researches, especially in biomedical applications. Until recently, much focus has been made on the self-assembly of block copolymers due to their unique and excellent assembly behavior [52, 53]. Amphiphilic block copolymer composed of one or two hydrophilic blocks and one hydrophobic block was reported to form well size-defined self-assembly in aqueous solvent. The structure of self-assembly was tunable by changing the molecular weight balance between hydrophilic and hydrophobic blocks. Polymer-micelle [54-56] having hydrophobic core and hydrophilic shell, and polymersome [57-59], having hydrophilic surface and hydrophobic layer have been widely used to encapsulate therapeutic drugs stably.

**Random copolymer**

Recently, self-assembly of amphiphilic random copolymer has been developed and mechanism of self-assembly has been elucidated. Such random copolymer is easy to prepare comparing with that in block copolymer [60]. Tuning the preparation condition and content of hydrophobic groups results in formation of size-defined self-assembly like block copolymer. In contrast, the random copolymers with high contents of hydrophobic groups can form unimer micelle because hydrophobic groups can interact with adjacent groups on same polymer chain and the number of the hydrophobic groups on the same polymer backbone is large enough to form micelle [61]. Regardless of solution concentration change, the conformation of the
aggregate is constant.

(a) Amphiphilic block copolymer  
(b) Amphiphilic random copolymer

![Figure 1.4.](image)

**Figure 1.4.** (a) Self-assembly structure of amphiphilic block copolymer [62] and (b) amphiphilic random copolymer [60].

1. 3. 2. Decreasing content of hydrophobic groups

Decreasing the content of hydrophobic groups results in formation of the unique self-assembly such as the following property. Akisyoshi’s group first reported that cholesterol-modified pullulan (CHP) forms nanogels and incorporate proteins due to interaction between cholesteryl group and hydrophobic domain of protein [63]. The nanogels have large space to accommodate proteins thanks to loose aggregate property. Cholesteryl groups are held in equilibrium between association for polymer crosslinking and dissociation for interaction with proteins [64]. Other similar reports are described in chapter 2.

Sunamoto’s groups reported that cholesteryl groups of CHP were anchored to the membrane of liposome [65-67], surface of monolayer [68, 69] and that of O/W emulsion [64], which improve the stability of each assembly. They modified the surface of liposome just by adding
prepared dispersive CHP nanogels, indicating that dissociation of cholesteryl group in nanogel proceeded to change its counterpart to hydrophobic layer in each liposome. This shows that dynamic property of hydrophobic group in such polymers is useful not only for infinite self-assembly but also for modification of hydrophobic domain on target.

Especially, cell surface is attractive target for the polymers modification, because a variety of event such as cellular incorporation, adhesion, recognition and signal transduction are controlled on the cell surface [70]. Previously, Iwata’s group reported that palmitate-modified polyvinyl alcohol (Pal-PVA) was anchored to cell surface by mixing cells with aqueous polymer solutions [71, 72]. Similarly, Katayama’s group reported that palmitate-modified dextran (Pal-Dex) was anchored to cell surface by same methods [73]. They reported that these polymers bound stably on cell surface due to multivalent interaction with plasma membrane. However, relation between design of the polymers and behavior on cell surface has been poorly understood. It has possibility that altering the kind and content of hydrophobic groups improve the stability on cell surface dramatically. In addition, aggregation of the polymers on cell surface is expected to induce cellular assembling, which may be controllable by optimal design of polymer.
Figure 1.5. Dynamic property of the self-assembly composed of the amphiphilic polymer bearing hydrophobic groups.
**Figure 1.6.** Expected property of the amphiphilic polymers bearing hydrophobic side chain. The hydrophobic groups of the polymer are expected to anchor cell membrane thanks to dynamic property of the groups (①). The polymers are expected to interact with cell membrane via multivalent hydrophobic interaction, though high stability on cell surface will be achievable (②). The amphiphilic polymers tend to form self-assembly and are expected to induce cellular assembling (③). These property is thought to be controlled by changing the design of the polymers.
Chapter 1. General introduction

1.4. Composition of the paper

Associative polymers with hydrophobic groups or ionic groups have been mostly applied to formation of tight and stable self-assembly by increasing interacting points. However, as described in this chapter, decrease in interaction points results in unique self-assembly, which is expected to result in novel applications for biomedical fields.

In chapter 2, I tried developing a novel PIC structure, which is categorized to physically-crosslinked nanogels. Characteristics of the PIC structure were analyzed by dynamic light scattering (DLS) and static light scattering (SLS), to confirm that the PIC has large space enough to accommodate payload molecules. In addition, the effect of ionic strength and pH on the PIC were evaluated because this PIC structure is expected to respond to the solution conditions such as ionic strength and pH. Then, I evaluated the effect of partial-crosslinking salt bridge on the stability against increasing ionic strength or lowering pH.

In chapter 3, we proposed different amphiphilic polymers to three applications, such as cellular incorporation via artificial receptor, ligand presentation, and cellular assembling. The desired behavior of the polymers on cell surface is vary among these three applications. In order to elucidate the optimum design for each application, we synthesized 12 kinds of amphiphilic polymers with different molecular weight of the dextran backbone, acyl groups, and their contents. Judging from both solution property of the polymers in test tube and polymer behavior in vitro, I elucidated the relationship of polymer design and behavior on cell.

Finally in chapter 4, I summarized the findings and discussions described in this paper. Then, I described the remaining problems and the perspectives of the research.
1.5. Reference


[40] H. Dautzenberg, Macromoeules 1997, 30, 7810-7815
Chapter 1. General introduction


Chapter 2. Polyion complex nanogel

2.1. Introduction

2.1.1. General properties of nanogels

Nanogels (NGs) are submicron-size hydrogels composed of polymer chains crosslinked by covalent bonds or non-covalent interaction, such as hydrogen bonds, electrostatic and hydrophobic interactions. The former type nanogels are called chemically-crosslinked NGs, which was reported firstly by Kabanov groups [1]. The latter type nanogels are called phisycally-crosslinked NGs, which were reported firstly by Akiyoshi group [2]. NGs have received much attention because of the following three interesting characteristics (Figure 2.1), and have been applied to various fields such as carriers for drug delivery system (DDS), artificial chaperone matrix [3], colloidal dispersant [4].

![Figure 2.1. General three property of NGs.](image-url)
2.1.1.1. Inner free space accommodating various molecules

NGs can accommodate various molecules in their free space more highly than other nanosized self-assembled carrier such as polymeric micelles, liposomes and so on [5]. The main reason for this is that swollen nano-sized network mainly consists of water (90%), therefore provides a large space for the incorporation of various molecules. Furthermore, high loading in NGs can be achieved by self-assembly and under relatively mild conditions compared with other carriers [5]. In fact, various molecules (both hydrophilic and hydrophobic low molecular weight drugs [6-10], DNA [11-14], siRNA [15], proteins [16-22]) have been applied to the target molecules for NGs.

2.1.1.2. Swelling-shrinking behavior response to outer stimuli

Second attractive feature of NGs is that their swelling-shrinking behavior can be regulated by outer stimuli (change in pH, ionic strength and so on) and the response to the environmental change is much quicker than that of macroscopic gels [23-25]. This behavior of NGs is controlled by both NGs structure (chemical structure, degree of crosslinking and charge density in the polyelectrolyte NGs).

A balance between the osmotic pressure and the polymer elasticity determines the physical behavior of NGs. The osmotic pressure of the polyelectrolyte NGs results from the difference in the concentration of mobile ions between the interior of NGs and exterior solution. The ionized groups attract hydrated counterions and lead swelling of the NGs. Conversely, the entropy elasticity of the polymer chains opposes the expansion. The ionization of the polyelectrolyte NGs depend on the pH value. A reduction in the total charge and the number of counterions as the pH change results in compression of the NGs (Figure 2.2a). Similarly, the swelling of polyelectrolyte NGs depend on the ionic strength because of decrease in effective charge (Figure 2.2b) [26].

The stimuli-responsive behavior is an attractive feature of NGs for various applications, in
particular drug delivery. For example, Na and Bae reported on pH-sensitive NGs, which was composed of a sulfonamide (SA) tagged-pullulan acetate conjugate [27] and hydrophobidized pullulan bearing Na-Boc-Histidine for the tumor specific release of doxorubicin [28]. These NGs were stable at pH 7.4 in the normal tissue, while at pH 6.8 in the tumor tissue [29-31] the SA tagged NGs shrank and aggregated due to the SA deionization (Figure 2.2c), and histidine tagged NGs swelled due to histidine protonation. Both NGs showed fast response to pH change and released drug in the tumor microenvironment.

Figure 2.2. (a) An increase in the pH value results in the collapse of a NG composed of weak base chains, and the swelling of NGs comprised of a weak polyacid. (c) Weak polyacid NGs loaded with protein compress with decrease in pH or increase in calcium ion concentration, and results in release of proteins [26].
2.1.1.3. Effect of elasticity on biodistribution

J. M. DeSimone groups reported that the elasticity of the NGs significantly affects their biodistribution [32, 33]. They synthesized the NGs with various elasticity by changing the number of cross-linker, and evaluated biodistribution of the NGs. The stiffer NGs were cleared quite rapidly, while the most deformable ones were eliminated over 30 times more slowly. In addition, the stiffer NGs were poorly tolerated while the more flexible ones were well tolerated due to the avoidance of filtration in the lung (Figure 2.3). The more flexible NGs were largely sequestered into spleen. These findings are useful for designing novel drug carrier whose elasticity is controlled (Figure 2.3).

**Figure 2.3.** Tissue distribution of chemically-crosslinked NGs. (a) Distribution of NGs into various tissues 2 h postdosing by percent recovered dose normalized for tissue weight. Lung tissue from a mouse dosed with (b) 10% crosslinked NGs or (c) 1% crosslinked NGs. NGs are shown in red, with cell nuclei in purple and cytoskeleton stained green. Compared to 10% crosslinked NGs, few 1% NGs accumulate to this tissue [32].
Chapter 2. Polyion complex nanogel

2.1.2. Preparation of NGs

Most of the NGs reported so far employ covalent crosslinking because of the stable nature comparing with non-covalent crosslinking. That is why various methods how to prepare chemically cross-linked NGs have been reported.

2.1.2.1. Chemically-crosslinked NGs

Typically, chemically-crosslinked NGs have been synthesized under diluted conditions using a crosslinking reactions of water soluble polymers bearing reactive groups, such as vinyl groups or thiol groups. To obtain the NGs with a well-controlled size, various advanced methodologies to prepare NGs have been reported. Such methodologies to synthesize chemically-crosslinked NGs are shown in figure 2.4.

Copolymerization in heterogeneous colloidal environments such as oil-in-water (O/W) [34] and water-in-oil (W/O) [35] emulsions has been widely used. The size of NGs is dependent on that of internal colloidal space.

Cross-linking of block copolymer micelle is also one of the promising methods to control the size of NGs [36]. Amphiphilic block copolymers are known to self-assemble monodispersive polymer micelles in water. Stable nanogels with a narrow size distribution were obtained by covalent chemical-crosslinking of the hydrophilic or hydrophobic main chains.

Nano template method using liposome [37] or inorganic nanoparticles [38] has been developed to control size of NGs precisely. NGs can be prepared using aqueous cores of the nanosize liposomes as a reaction vessel. Upon removal of the lipid molecules, the NGs that closely matched the size of liposomal template can be obtained.

Lastly, top-down methods using lithography is able to control not only size but also shape, composition, and surface functionality of NGs [39]. This lithography technique uses nonwetting elastomeric molds of perfluoropolyether network prepared on patterned silicon templates by photochemical crosslinking of dimethacrylate-functionalized perfluoropolyether oligomers.
Chapter 2. Polyion complex nanogel

The nonwetting molds eliminate the formation of a residual interconnecting film between the molds objects, thereby allowing the production of monodisperse, shape-specific NGs from an extensive liquid precursors.

**Figure 2.4.** Methodologies to prepare chemically-crosslinked NGs. (a) Emulsion polymerization: with o/w emulsion (upper), w/o emulsion (lower). (b) Crosslinking of amphiphilic copolymer at core or shell of the polymer micelles in water. (c) Nanotemplate methods using liposome (upper) or gold nanoparticles (lower). (d) Top-down method using lithography.
2.1.2.2. Physically-crosslinked NGs

Physically-crosslinked NGs can be prepared using non-covalent interactions between polymer chains. In contrast to chemically-crosslinked NGs, this methods have advantages with respect to their biomedical applications, because toxic crosslinkers, catalysts are not necessary in the preparation process. However, this technique has two challenges as follows.

1. To design NGs with non-covalent crosslinking, interparticle crosslinking formation should be avoided because it results in macroscopic aggregation.
2. It is difficult to adjust size of NGs using these association due to their relatively weak interaction.

Akiyoshi’s group firstly succeeded in the production of physically cross-linked NGs with narrow size distribution by modifying the hydrophobic groups in small amount [2]. The small amount of modification reduced the chance to form the interparticle crosslinking. When highly hydrophobic cholesterol groups were employed to modify the hydrophilic polysaccharide main chain, the degree of modification of ~1.5 mol% was enough to form NGs, in which the main chains of the NGs were much contracted when compared with those of the original polysaccharide coils. Since the first report from akiyoshi’s group, phisycally-crosslinked NGs bearing various crosslinking points, such as fatty acid, cholesterol, deoxycolic acid, lactic acid, and combination of lauryl acid-cycrodextrin, have been developed. Physically-crosslinked NGs reported previously are summarized to table 2.1.
Table 2.1. Physically-crosslinked NGs reported previously.

<table>
<thead>
<tr>
<th>Category of crosslinking</th>
<th>Side chain</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophobic interaction</td>
<td>Cholesterol</td>
<td>Protein encapsulation</td>
</tr>
<tr>
<td></td>
<td>[2, 40-50]</td>
<td>Artificial chaperon</td>
</tr>
<tr>
<td></td>
<td>Fatty acid</td>
<td>[43, 47]</td>
</tr>
<tr>
<td></td>
<td>Deoxycholic acid</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>Cholic acid</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>PNIPAM</td>
<td>Temperature responsive-formation/disruption</td>
</tr>
<tr>
<td></td>
<td>L-lactide</td>
<td>Protein encapsulation</td>
</tr>
<tr>
<td></td>
<td>[55]</td>
<td>Biodegradable</td>
</tr>
<tr>
<td>Host-guest interaction</td>
<td>β-CD</td>
<td>Hydrophobic reagent-encapsulation</td>
</tr>
<tr>
<td></td>
<td>/lauryl acid</td>
<td>[57-59]</td>
</tr>
<tr>
<td>Stereocomplexation</td>
<td>L-lactide</td>
<td>Protein encapsulation</td>
</tr>
<tr>
<td></td>
<td>/D-lactide</td>
<td>Slow biodegradation rate</td>
</tr>
<tr>
<td></td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td>Complexation</td>
<td>PIPOZ</td>
<td>Temperature responsive-formation</td>
</tr>
<tr>
<td></td>
<td>[60]</td>
<td></td>
</tr>
<tr>
<td>Electrostatic interaction</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
</tbody>
</table>
2.1.3. Property and applications of physically-crosslinked NGs

Because of the reversible nature of the physical crosslinking, physically-crosslinked NGs show unique properties that can’t be realized in chemically-crosslinked NGs. These properties can be controlled by changing the hydrophobic side chains and determines various applications as follows.

2.1.3.1. Trapping of proteins via dynamic hydrophobic side chains

One of the most notable characteristics of the NGs bearing hydrophobic side chains is their dynamic property, which trap various proteins and accommodate them into the nanoscale hydrophilic network. Akiyoshi’s group reported that cholesteryl group-bearing pullulan (CHP) forms NG with about 30 nm in size by self-assembly in aqueous media and can incorporate various proteins in its network (Figure 2.5). For example, one CHP NG forms complex with approximately one bovine serum albumin (Mw 66,000), two α-chymotrypsin (Mw 25,000), two myoglobin (Mw 17,800), four cytochrome c (Mw 12,500), and five insulin (Mw 5735) [43]. Although the maximum amount of protein accommodated in one CHP NG depends on the molecular weight and hydrophobicity of the protein, it is difficult for chemically-crosslinked NGs to accommodate so many kinds of proteins due to their static network. Thus, the feature allows the CHP NGs useful protein carrier.

![Figure 2.5. Formation of NGs via self-assembly of amphiphilic polymers bearing small amounts of hydrophobs and trapping of proteins via dynamic hydrophobe.](image-url)
2.1.3.2. Control of association and dissociation depending on crosslinking candidates

Physically-crosslinked NGs are held in equilibrium between dissociation and association, which can be controlled by additive and outer stimuli. For example, CHP NGs, described in 2.1.3.1, are dissociated upon complexation with β-CD due to forming CHP-CD complex [45, 49]. The main driving force of NG formation in CHP is the association of hydrophobic cholesteryl groups of CHP in water. β-CD is able to dissolve cholesteryl group in water by incorporation into the hydrophobic cavities. Thus, the dissociation of CHP NGs-protein via β-CD complexation allows the release of proteins from NGs (Figure 2.6a).

Furthermore, preparation of NGs that enable heat-induced association and dissociation have been reported by using hydrophilic main chains partially grafted with short poly(N-isopropylacrylamide) (PNIPAM) chains [53, 54]. These polymers dissolve in water at temperature less than lower critical solution temperatures (LCSTs). Above a LCST, PNIPAN grafted polysaccharides form NGs that are physically-crosslinked by the hydrophobidized PNIPAN domains (Figure 2.6b).

![Figure 2.6](image-url) 

**Figure 2.6.** (a) Protein release from NGs via dissociation of cholesteryl groups upon competitive complexation with cyclodextrin [45, 49]. (b) Heat induced association and dissociation of polysaccharides partially substituted with short PNIPAM chains [53, 54].
2.1.4. Strategy for preparing novel type nanogel, “polyion complex nanogel (PIC-NG)”

The purpose of this study is to design a new class of physically crosslinked NGs referred to as polyion complex NGs (PIC-NGs), which are crosslinked by electrostatic interactions among the polycations and polyanions. Figure 2.7 shows the brief strategy for preparation of PIC-NG. When the polycations and polyanions are mixed together, polyion complexes (PICs) are formed, which usually results in macroscopic coacervation [61]. We tried to avoid this macroscopic coacervation by minimizing the content of ionic groups in the polycations and polyanions. However, the minimized content of ionic groups was expected to destabilize the interaction between the polymer chains. Therefore, to strengthen the salt bridges, we employed oligoionic groups to modify the main chain. We further studied the stabilization of the PIC-NGs by partial chemical crosslinking. The PIC-NGs may be applicable to drug delivery vehicles by electrostatically accommodating payload molecules. The present work will open up new areas of NGs research and will serve as the basis for designing PIC-NGs for specific applications.

![Strategy for preparation of PIC-NG](image)

**Figure 2.7.** Strategy for preparation of PIC-NG stable in aqueous milieu. When the contents of ionic side chains and the net charges on the each side chain are optimized, stable PIC-NG is expected to form.
2.2. Results and discussions

2.2.1. Synthesis of polymers

We selected trivalent cationic and anionic groups to modify on the neutral dextran main chain. \( pK_a \) values of four amino groups of the tetramine are higher than 7.4 and those of four carboxyl groups of the tetracarboxylic acid side chain are lower than 7.4 [62, 63]. Therefore, after modification of these ionic groups on dextran main chain, each group is expected to exist as trivalent ions at pH 7.4. Thus, the cationic and anionic dextrans were synthesized following Scheme 2.1. For the synthesis of cationic dextran (CD), after hydroxyl groups of the dextran were activated with \( p \)-nitrophenyl carbonate, it was treated with tetramine. Crosslinking reaction of dextran with tetramine can be avoided by adding the dextran solution dropwise to tetramine solution with excess concentration. We prepared three cationic polymers with different tetramine content by controlling the activation amount of hydroxyl groups. The contents of tetramine group per glucose unit of the polymers were determined from \(^1\)H NMR (Figure 2.8b) to be 5.5, 10.9 and 15.5 mol\%. These three polymers were abbreviated as CD6, CD11 and CD16, respectively.

As for the anionic polymer, dextran was first modified with ethylenediamine, then the resulting polymer was reacted with excess amount of tetracarboxylic acid by using amide-selective condensing reagent, DM-TMM. The quantitative reaction of tetracarboxylic acid with dextran’s amine group was confirmed by Keiser test, where residual amine group was not detected. Figure 2.8d shows \(^1\)H NMR spectra of the anionic polymer. The contents of tetracarboxylic acid group in two kinds of anionic polymers were determined to be 4.9 and 16 mol\%. These two polymers were abbreviated as AD5 and AD16, respectively.
Scheme 2.1. Synthesis of (a) CD and (b) AD.
Chapter 2. Polyion complex nanogel

(a) 

(b)
Figure 2.8. $^1$H NMR spectra of (a) Dex-PNC (DMSO-d6), (b) CD (10 mM NaOD in D₂O),
(c) Dex-EDA (10 mM NaOD in D₂O) and (d) AD (10 mM NaOD in D₂O).
2.2.2. Preparation of PIC-NG

Obtained cationic and anionic polymers with the similar charged group contents were mixed at various cation/anion charge (C/A) ratios in 10 mM phosphate buffer (pH 7.4) at 2 mg/mL of total concentration. Soon after mixing, the solution became turbid, which is typical appearance of coacervate of PIC. Then, the solution was shaken for 12 hours for equilibration of the PIC formation. Most of the resulting solutions were still turbid then the solutions were centrifuged to separate clear supernatant and precipitate. The yield of PIC remained in the supernatant was quantified by phenol-sulfuric acid colorimetric method [64] and the result was summarized in Figure 2.9. The yield was constantly high (80 to 90%) for the pair of CD6 and AD5 irrespective of the C/A ratios, although the yield of PIC with the pairs of CD11 and AD5, and CD16 and AD16 was significant low especially at high C/A ratios. The PIC existing in the supernatant was analyzed by dynamic light scattering (DLS) (Figure 2.10). The mean hydrodynamic radius of the PIC of CD6 and AD5 was less than 15 nm and it had relatively narrow size distribution irrespective of the mixing ratio. The nanoparticle formation of CD6 and AD5 pair in high yields indicates that the relatively large fraction of hydrophilic dextran main chain prevent the interparticle crosslinking which result in macroscopic coacervate formation. In contrast, low yields in the pairs of CD11 and AD5, and CD16 and AD16 showed that there is critical content of ionic groups in the polymers for closed association.

Table 2.2-2.4 summarized the results of DLS and ζ-potential measurements of each PIC-NG formed from CD6/AD5, CD11/AD16, and CD16/AD16. $R_h$ decreased with increasing C/A ratios slightly in CD6/AD5 and significantly in CD11/AD16 and CD16/AD16 with almost constant polydispersity indexes (PDI). The ζ-potentials of the PIC-NGs are negative for all the examined C/A ratios. The residual small negative charge of the PIC-NG even above the C/A ratio of unity indicates that triamine groups of the cationic polymer may not be fully protonated at pH 7.4.
Chapter 2. Polyion complex nanogel

Figure 2.9. Effect of C/A ratio on the yields of PIC-NG

Figure 2.10. Size distribution of PIC-NGs with various C/A ratios. Concentration = 2.0 mg/mL.
### Table 2.2. Parameters of PIC-NGs prepared from CD6/AD5 at various C/A ratio

<table>
<thead>
<tr>
<th>C/A</th>
<th>( R_h ) (nm)</th>
<th>( \zeta )-potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>14.8</td>
<td>-5.0</td>
<td>0.18</td>
</tr>
<tr>
<td>0.8</td>
<td>14.1</td>
<td>-4.5</td>
<td>0.17</td>
</tr>
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<td>0.13</td>
</tr>
<tr>
<td>1.2</td>
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<td>0.14</td>
</tr>
<tr>
<td>2.0</td>
<td>11.5</td>
<td>-0.2</td>
<td>0.13</td>
</tr>
</tbody>
</table>

### Table 2.3. Parameters of PIC-NGs prepared from CD11/AD6 at various C/A ratio

<table>
<thead>
<tr>
<th>C/A</th>
<th>( R_h ) (nm)</th>
<th>( \zeta )-potential (mV)</th>
<th>PDI</th>
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</thead>
<tbody>
<tr>
<td>0.5</td>
<td>12.7</td>
<td>-4.7</td>
<td>0.19</td>
</tr>
<tr>
<td>0.8</td>
<td>11.5</td>
<td>-6.0</td>
<td>0.19</td>
</tr>
<tr>
<td>1.0</td>
<td>10.7</td>
<td>-6.0</td>
<td>0.17</td>
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<td>7.8</td>
<td>-4.7</td>
<td>0.16</td>
</tr>
<tr>
<td>2.0</td>
<td>6.5</td>
<td>-2.1</td>
<td>0.19</td>
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### Table 2.4. Parameters of PIC-NGs prepared from CD16/AD16 at various C/A ratio

<table>
<thead>
<tr>
<th>C/A</th>
<th>( R_h ) (nm)</th>
<th>( \zeta )-potential (mV)</th>
<th>PDI</th>
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</thead>
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<td>0.24</td>
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<td>-3.4</td>
<td>0.15</td>
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<td>0.15</td>
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<tr>
<td>2.0</td>
<td>5.8</td>
<td>-0.3</td>
<td>0.44</td>
</tr>
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</table>
2.2.3. Partial chemical crosslinking of PIC-NG

The salt bridges are essentially weak especially when the ionic strength of the solution is high. Thus, we also examined the effect of chemical crosslinking of PIC-NG comprising from CD6/AD5 for the further stabilization of NG. We partially crosslinked the salt bridges by the condensing reagent, DM-TMM, to form amide bond. The amount of added condensing reagent was kept low (5 or 10 mol% of total amount of salt bridges) because we found that high concentration of the condensing reagent (20 mol% of total amount of salt bridges) resulted in interparticle crosslinking. The obtained crosslinked PIC-NG was purified by membrane filtration to remove the condensing reagent. The crosslinked PIC-NGs with 5 and 10 mol% were abbreviated PIC-NG(5) and PIC-NG(10), respectively. The size distribution of the crosslinked PIC-NG is shown in Figure 2.11. The diameter of the PIC-NG slightly shrank after crosslinking.

Figure 2.11. Size distribution of PIC-NG(CD6/AD5) before and after chemical crosslinking estimated by DLS study. Concentration = 2.0 mg/mL
To obtain the molecular information of crosslinked PIC-NG in detail, we conducted static light scattering (SLS) analysis (Figure 2.12) and the results were compiled in Table 2.5. $M_w$ of the PIC-NG slightly decreased with crosslinking ratio. The association number of polymer chains in one PIC-NG was calculated to be ca. 4. Thus the number of salt bridges in one PIC-NG was about 40 by assuming that all the cationic and anionic groups form one-to-one salt bridges. Therefore, the crosslinking of 5 and 10 mol% of the salt bridges means decrease of 2 and 3.5 crosslinking points in one PIC-NG, respectively.

The ratios of radius of gyration ($R_g$) and hydrodynamic radius ($R_h$) of PIC-NG ($R_g/R_h$) approached to the theoretical value for a homogeneous sphere (0.776) with increasing crosslinking ratio, indicating that the salt bridges in dynamic equilibrium became more static with converting just a small amount of salt bridges to chemical crosslinkings. The static nature of the salt bridge of the crosslinked PIC-NG was also reflected by the density ($\rho$). The density slightly increased with the crosslinking ratio. It is notable that few number of crosslinking points in one PIC-NG resulted in these clear changes from the non-crosslinked PIC-NG. Thus, these crosslinking points seem to enhance the strength of the other non-crosslinked salt bridges interaction. The water contents of PIC-NG were kept more than 90% even after crosslinking. This enough free space may contribute to accommodate payload when PIC-NG is applied to drug carrier.
Figure 2.12. Result of static light scattering analysis of each PIC-NG (CD6/AD5).

Concentration = 1.0 mg/mL.

Table 2.5. Parameters of chemically crosslinked PIC-NGs (CD6/AD5).

<table>
<thead>
<tr>
<th>Crosslinking (mol%)</th>
<th>$R_n$ (nm)</th>
<th>$\xi$-potential (mV)</th>
<th>$R_g$ (nm)</th>
<th>$R_g/R_h$</th>
<th>$M_w \times 10^5$</th>
<th>Association number</th>
<th>$\rho$ (g/cm$^3$)$^a$</th>
<th>Salt bridges</th>
<th>Chemical crosslinks</th>
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</thead>
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<tr>
<td>0</td>
<td>13.4</td>
<td>-3.3</td>
<td>12.5</td>
<td>0.94</td>
<td>3.4</td>
<td>4.6</td>
<td>0.069</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>12.4</td>
<td>-0.0</td>
<td>11.6</td>
<td>0.93</td>
<td>3.0</td>
<td>4.0</td>
<td>0.076</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>12.3</td>
<td>-1.7</td>
<td>10.6</td>
<td>0.86</td>
<td>3.5</td>
<td>3.5</td>
<td>0.088</td>
<td>35</td>
<td>3.5</td>
</tr>
</tbody>
</table>

$^a$Calculated from $M_w/(4\pi R_h^3/3)$
2.2.4. Improved stability of PIC-NG by chemical crosslinking

2.2.4.1. Effect of ionic strength

To check the improved stability of the PIC-NG by crosslinking, we examined the effect of the increasing ionic strength of the solution which will reduce the electrostatic interaction of the salt bridges. In the case of non-crosslinked PIC-NG, the diameter and scattered intensity steeply decreased with increasing concentration of MgCl₂, indicating the reorganization of polymer chains in PIC-NG due to the weakening of the salt bridges (Figure 2.13). In contrast, the decrease in diameter of crosslinked PIC-NG is not so significant because of the minimized reorganization of polymer chains in the PIC-NG.

The crosslinked PIC-NG gave a minor peak resulting from interparticle aggregation at high MgCl₂ concentration (Figure 2.14). The interparticle aggregation should be brought by the salting out which is typically observed in charged particles. The critical MgCl₂ concentration where interparticle aggregation was observed was 4 and 10 mM for 5 and 10 mol% crosslinked PIC-NG, respectively. Thus, the chemical crosslinking seems to raise the colloidal stability of the PIC-NG against the high ionic strength.

![Figure 2.13](image-url)

**Figure 2.13.** Effect of chemical crosslinking of PIC-NGs on the stability against increasing ionic strength evaluated from (a) diameter and (b) relative scattered intensity. Concentration = 2.0 mg/mL.
2.2.4.2. Effect of pH

Next we studied the effect of acidic pH on the stability of the PIC-NG (from CD6/Ad5). The pH decrease will reduce the anionic charge of the tricarboxyl group and increase the cationic charge of the triamine group. As for the non-crosslinked PIC-NG, macroscopic precipitate was observed at both pH 6 and 5 (Figure 2.15). The precipitation will be a result from the enhancement of salt bridges due to the changes in protonation degree of the ionic groups. This property of the non-crosslinked PIC-NG may be interesting as a pH-responsive material. As for the crosslinked PIC-NGs, such macroscopic precipitate was not observed at pH 6 for both crosslinking ratios although some fractions of interparticle aggregates were observed. At pH 5, only PIC-NG(10) avoided macroscopic precipitation, indicating that the tolerance to acidic pH increased with increasing crosslinking ratio. Therefore, the suppression of dynamic exchange of salt bridges by partial crosslinking stabilized the PIC-NG against the pH lowering as well as increasing ionic strength.

**Figure 2.14.** Interparticle aggregation of each PIC-NG observed at a critical MgCl₂ concentration. (a) PIC-NG(5) at 2 mM and 4 mM MgCl₂, (b) PIC-NG(10) at 4 mM and 10 mM MgCl₂. Red arrow indicates the peak derived from interparticle aggregate. Polymer concentration = 2.0 mg/mL.
Figure 2.15. Effect of chemical crosslinking of PIC-NG on the stability against acidic pH. Concentration = 2.0 mg/mL
2.3. Conclusion

In this chapter, we successfully obtained a new class of nanogel, PIC-NG, comprising from dextran grafted with oligo-cationic and oligo-anionic moieties, by simply mixing the solutions of the cationic and anionic polymers. To avoid the interparticle salt bridge formation, it was found that the content of ionic groups should be kept relatively low, ~ 5 mol%. This critical content of the ionic groups is much larger than that in the NGs crosslinked by a quite hydrophobic cholesterol [2] or by a less hydrophobic palmitoyl [47]. When more longer ionic groups are employed for PIC-NG preparation, the critical content will become smaller due to the enhancement of the electrostatic interaction.

The salt bridges of the PIC-NG could be crosslinked covalently through amide bonds to stabilize against increasing ionic strength or lowering pH. In spite of the improved stability after chemical crosslinking, the inner free space of the PIC-NGs was still more than 90% of the original value in Ng without any chemical cresslinks. The free space is much larger than those of the NGs crosslinked by hydrophobic interaction between cholesterol groups (50–87%) [47], indicating weaker interaction among the oligoionic groups of the PIC-NGs.

The PIC-NGs may offer a unique environment to accommodate payload molecules through electrostatic interaction. The PIC-NGs also sensitively responded to the solution conditions to reorganize. This property may be useful as stimuli responsive characteristics in drug delivery application.
2.4. Experiment

2.4.1. Materials

Dextran (Mw 60,000-90,000) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Lithium chloride was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). These reagents were dried over diphosphorus pentoxide at 90°C. N, N’-Bis(3-aminopropyl)-1,3-propanediamine and 1,2,3,4-butanetetracarboxylic acid were purchased from Sigma (St. Louis, MO). p-Nitrophenyl chloroformate (PNC) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Pyridine, dimethylsulfoxide (DMSO), and dimethylformamide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.4.2. Synthesis of cationic dextran (CD)

The hydroxyl groups of dextran were activated with various concentrations of PNC following the literature method [65]. The content of p-nitrophenyl groups was determined by 1H-NMR to be 9.5 and 41.2 mol% of glucose units. Then, the PNC-activated dextran (200 mg; containing 0.11 and 0.36 mmol nitrophenyl groups) was dissolved in DMSO (20 mL). This solution was added dropwise to a DMSO solution of N,N’-bis(3-aminopropyl)-1,3-propanediamine (230, 330 and 730 μL; 1.1, 1.6 and 3.6 mmol). The reaction mixture was stirred for 24 h at room temperature, and dialyzed with a Spectra/Pore 7 dialysis bag (MWCO 10,000) against diluted sodium hydroxide solution (pH = 11) for 2 days and distilled water for a day, and then lyophilized. The degree of substitution of the cationic dextran (CD) was determined by 1H-NMR.

^1H-NMR (300 MHz, D2O): δ 1.60 (6H, (NHCH2CH2CH2)3NH2), 2.55 (10H, NHCH2CH2CH2(NHCH2CH2CH2)2NH2), 3.13 (2H, NHCH2), 3.4-3.95 (6H, glucosidic protons without anomeric proton), 4.93 (1H, anomeric proton).
2.4.3. Synthesis of anionic dextran (AD)

PNC-activated dextran (200 mg; containing 0.11 and 0.36 mmol nitrophenyl groups) was dissolved in DMSO (20 mL). This solution was added dropwise to a DMSO solution of ethylenediamine (75, 110 and 240 µL; 1.1, 1.6 and 3.6 mmol). The reaction mixture was stirred for 24 h at room temperature, and dialyzed with a Spectra/Pore 7 dialysis bag (MWCO 10.000) against diluted sodium hydroxide solution (pH = 11) for 2 days and distilled water for a day, and then lyophilized. The degree of substitution of the ethylenediamine-modified dextran was determined by 1H-NMR.

$^1$H-NMR (300 MHz, D$_2$O): $\delta$ 2.66 (2H, CH$_2$NH$_2$), 3.12 (2H, NHCH$_2$) 3.42-3.95 (6H, glucosidic protons without anomeric proton), 4.93 (1H, anomeric proton).

The ethylenediamine-modified dextran (200 mg; containing 0.06 and 0.18 mmol amine groups) was dissolved in distilled water (20 mL). Then, 1,2,3,4-butanetetracarboxylic acid (140 and 430 mg; 0.6 and 1.8 mmol amine groups) was added to this solution, and pH of the solution was adjusted to 9 with sodium hydroxide solution. To this solution was added DMT-MM (49 and 150 mg; 0.18 and 0.55 mmol). The reaction mixture was stirred for 24 h at room temperature, and dialyzed with a Spectra/Pore 7 dialysis bag (MWCO 10.000), using diluted hydrochloric acid solution (pH = 4) for 2 days and distilled water for a day, and then lyophilized.

$^1$H-NMR (300 MHz, D$_2$O): $\delta$ 2.18-2.63 (6H, protons of tetracarboxylic acid), 3.25 (4H, NHCH$_2$CH$_2$NHCO), 3.48-3.95 (6H, glucosidic protons without anomeric proton), 4.92 (1H, anomeric proton).

2.4.4. Preparation of PIC-NG

CD and AD were dissolved in 10 mM phosphate buffer (pH 7.4) at 2.0 mg/mL. After these polymer solutions were mixed at various cation/anion (C/A) charge ratios, the mixture was sonicated for 30 min with an SU-9TH ultrasonic water bath to equilibrate the PIC formation.
Macroscopic aggregates were removed by centrifugation (13,000g) for 30 min at 25oC, and the supernatant was collected to obtain a PIC-NG solution. The yield of PIC-NG was determined using the phenol-sulfuric and colorimetric method [64].

2.4.5. Crosslinking PIC-NG

An aqueous solution of DMT-MM (0.4 mg/mL) was added to 1 mL of 1.0 mg/mL PIC-NG in 10 mM phosphate buffer (pH 7.4). The reaction mixture was shaken for 24 hr at room temperature and purified using a cellulose ultrafiltration filter (MWCO 3000).

2.4.6. Dynamic Light Scattering (DLS) and $\zeta$-potential measurement

The diameters of the PIC-NGs were measured using Zetasizer Nanoseries (Malvern Instruments, Worcestershire, UK) at a detection angle of 173° and a temperature of 25°C. The $\zeta$-potential of the PIC-NGs was measured using a Zetasizer Nanoseries at a scatter angle of 17° and a temperature of 25°C.

2.4.7. Static Light Scattering (SLS)

SLS analysis of PIC-NG (10 mM phosphate buffer (pH 7.4)) was conducted on DLS-7000 (Otsuka Electronics Co., Ltd., Osaka, Japan) at 25oC. Measurements were carried out over the angular range from 30°C to 130°C. The concentration of PIC-NG was 1.0 mg/mL.
2.5. Reference

Chapter 2. Polyion complex nanogel

Chapter 3. Optimum design of amphiphilic polymers for cell surface engineering

3.1. Introduction

3.1.1. Cell surface engineering

Cell surface is filled with many kinds of proteins such as receptors, ligand proteins, and adhesive proteins to recognize a variety of molecules and neighboring cells for cellular response to these outer stimulations [1]. This cellular response can be modified by the artificial presentation of bioactive molecules on the cell surface. Such strategy is called as [2-4]. Key applications of cell surface engineering are encocytotic uptake of desired molecules through receptor modification [5, 6] and to the development of cell delivery system capable of targeting specific tissue through ligand modification [4, 7, 8]. In addition, this technique can be applied to tissue engineering by controlling cell-cell adhesion and inducing 3D-cell structure formation [9].

3.1.2. Cell surface modification

Artificial cell surface modification can be classified into two main categories, genetic transformation and chemical transformation.

3.1.2.1. Genetic transformation

Genetic engineering is well established as a robust and highly versatile method for introducing specific proteins on a cell surface to modulate cell functions. Introducing of targeting proteins results in success of targeted immunotherapies using chimeric antigen receptors (CARs) in B cell malignancies [10, 11], and of improvement of hematopoietic stem cells (HSCs) engraftment [12]. Expression of neuronal cell adhesion molecule (N-CAM) was reported to enhance cell-cell adhesion [13]. However, technical challenge of efficient gene transfection and the safety concerns of activating oncogenes through random transgene insertion hinder the technique from clinical applications [14]. In addition, the materials which can be presented on cell surface using
this methodology, are limited to biological molecules, such as proteins.

3.1.2.2. Chemical transformation

The chemical modification enables presentation of the materials, which can not be presented in genetic transformation technique, by various interactions including covalent and non-covalent interaction (Figure 3.1). The property of each method is shown in Table 3.1.

Covalent interaction

Functional groups naturally present on the cell surface as a part of proteins and carbohydrates are regarded as docking sites for the covalent conjugation with other molecules to be displayed. The most straightforward approach is direct chemical reaction of amino (-NH\textsubscript{2}) or thiol (-SH) groups. The main concern of this approach is to fluctuate the function of membrane proteins. To dissolve the problem, the concept of metabolically introducing chemical functional groups that are orthogonal to native plasma membrane, which was reported by Reutter groups [15] and Bertozzi groups [16-18]. The main advantage of this method is to present the materials of interest on cell surface longer than that based on non-covalent interaction due to the retarding the release from cell surface. However, chemical modification processes are unfavorable to cells and modification efficiency may vary in cell-type dependent manner.

Non-covalent interaction: electrostatic interaction

The net surface charges of mammalian cells negative due to the existence of carboxylate groups of membrane proteins and sialic acids terminating glycoproteins sugar chains. Chaikof groups functionalized PEG polymers with cationic poly-L-lysin through electrostatic adsorption to alter the surface of pancreatic islets [20, 21]. However, the polymers with a number of charges are reported to damage cell surface.
**Non-covalent interaction: hydrophobic interaction**

Among chemical transformation methods, the hydrophobic interaction for the cell surface modification is reported to be less cytotoxic and widely applicable to variety of cell lines. It has been reported that bioactive molecules such as proteins [22, 23], peptides [24], carbohydrate [25] and DNA [26-31] were successfully presented via hydrophobic interaction. However, the method has main drawback, which is rapid disappearance of presented materials from cell surface via (1) release from cell surface and (2) incorporation into cells.

**Figure 3.1.** Methods for cell surface engineering based on chemical transformation. (a), (b) Covalent conjugation of functional groups on cell surface. (c), (d) Exogenous insertion of amphiphilic materials on cell surface via hydrophobic interaction. (h) Nonspecific electrostatic adsorption of cationic materials to the negatively charged cell membrane. (e), (f), (g) are explained in ref. [32].
### Table 3.1. Property of methods for cell surface engineering.

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<tr>
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<th>Chemical transformation</th>
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<td>~ hours</td>
</tr>
<tr>
<td></td>
<td></td>
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</tbody>
</table>

*1 Amount of target proteins is constant because the proteins are produced constantly.

*2 Toxicity is highly dependent on technical procedure.
Chapter 3. Optimum design of amphiphilic polymers for cell surface engineering

3.1.3. Application of amphiphilic materials for cell surface engineering

Amphiphilic materials have been applied to ligand/receptor presentation for control of endosomal uptake and cell homing capability, and crosslinker to form 3D cell structure rapidly. However, the function of amphiphilic materials is expected to be improved based on logical manners. Therefore, in following sections, the strategy to develop optimum design of amphiphilic materials is discussed for the efficient ligand/receptor presentation and cellular crosslinking.

3.1.3.1. Ligand/receptor presentation

To apply the materials to ligand/receptor presentation, suppression of the release of the presented molecules from cell surface is needed. In addition, enhancing cellular uptake is necessary to endosomal uptake application, while retarding cellular uptake is necessary to stable ligand presentation.

Strategy of amphiphilic polymer design for ligand/receptor presentation based on natural molecules

To be applicable to a wide variety of target molecules, synthetic ligand/receptors should be designed by learning from endogenous cellular receptors. The endogenous receptors are categorized into following two classes; transmembrane receptors and lipid-associated receptors such as glycosylphosphatidylinositol (GPI)-anchored receptors [33, 34], glycosphingolipids [35] and lipid-modified proteins [36] (Figure 3.2). Because of their simple structures, lipid-based receptors are good models for designing synthetic receptors.

The number of lipids modified on proteins determines the stability on cell membrane. For example, peptide and proteins modified with a single farnesyl chain dissociate from membranes within 1-2 minites. In contrast, signal proteins such as H-Ras and N-Ras contains farnesyl and palmitate chains to achieve membrane binding [37, 38]. From this finding, increasing the
content of hydrophobic anchor modified on the materials is expected to enhance the stability on cell surface, due to retardation of release.

**Figure 3.2.** Natural receptors involved in receptor mediated endocytosis and recognition of target molecules. Transmembrane structure is shown in left and lipid-based structure is shown in right. Lipid based structures are categorized into GPI anchor protein, ganglioside GM1 and lipid-modified protein. Stability of lipid-modified protein on cell surface is enhanced with increase in the number of modified lipid.

**Synthetic materials**

Peterson et al. firstly designed synthetic receptors, such as cholesterol modified-low molecule [39] or -short peptide [40-42]. Silvius et al. also reported another synthetic receptor that was a lipid modified with biotin via a long hydrophilic polyethyleneglycol spacer [43]. However, a single lipid may not stably anchor the receptor to the cell surface because the modified molecules are easy to disappear from the cell surface due to the dissociation from the cell surface and endocytotic uptake. To address the issue, two different approaches have been
(1) Increasing contents of the hydrophobic anchoring groups

(2) Extending acyl chain length of the anchoring groups

The strategy using approach (1) was already described above. Nagamune et al. reported that lipid-based divalent acyl groups were more stable than monovalent acyl anchors [44]. Similarly, we reported peptide based anchors bearing two acyl groups were more stable than that bearing one acyl group [45] to keep on the cell surface. Our groups [46] and Iwata groups [47, 48] have previously reported polymer-type anchor bearing multiple hydrophobic moieties along the main hydrophilic polymer, which prolonged the longevity of the anchor on the cell surface.

With regard to the approach (2), Iwata et al. clearly showed that the use of phospholipid-based anchors with longer acyl chains led to an increase in the retention time of the anchor on the cell surface [47, 48]. This effect has been confirmed by other groups based on their extensive studies [31].

To achieve long time presentation of the molecules on the cell surface, however, the disappearance via endocytosis should be avoided in the case of application to ligand presentation. However suppression of endocytosis has not been achieved in the amphiphilic materials.

Optimum design of artificial receptor/ligand for cell surface modification

It is known that the larger particle is difficult to be endocytosed because of the size limitation of endosome. Irvin’s group reported that liposome with a diameter of more than 100 nm which is covalently modified on to the cell surface stayed long time with efficient suppression of the endocytotic uptake [49, 50].

We showed the effort to achieve the long time presentation of the polymeric anchors on the cell surface by using high molecular weight polymers. Our polymeric anchors are amphiphilic molecules, which will cause self-aggregation (Figure 3.3). The self-aggregation of the
polymeric anchor on the cell surface will further attenuate the cellular uptake due to the larger size of the aggregate than a single polymer chain.

**Figure 3.3.** Synthetic amphiphilic materials applied to receptors or ligands presentation and behavior on cell surface. As shown in left and center, release from cell surface decreases with increase in the number of hydrophobic anchor, while cellular uptake is not affected (lipid-capped molecules and peptide-type anchor). Our expectation is in the right panel. The amphiphilic polymer forms self-aggregate on cell, and is expected to retard the incorporation into cells because of limitation in the material size for cellular uptake via endocytosis.

### 3.1.3.2. Cellular assembling

**Strategy of design in artificial crosslinker for cell assembly based on natural molecules**

For the wide applicability to many kinds of cell lines, synthetic cellular crosslinker should be designed by learning from natural cellular crosslinking molecules. Previously, immunoglobulin [51, 52] and lectin molecules [53] have been applied to red blood cell aggregation (Figure 3.4). In addition, immunoglobulin G (IgG), containing two interaction points, can not induce cellular assembling because distance between interaction points is too short to connect two red blood cells. In contrast, distance between interaction points of immunoglobulin M (IgM), containing ten interaction points, is large enough to induce cellular assembly. These findings indicate that modification of cellular crosslinker with multiple hydrophobic groups is necessary to prepare highly-assembled cell structure.
Figure 3.4. Natural cellular crosslinker involved in formation of cellular assembly. Although IgG molecule has two interaction points, the molecule can not induce cellular aggregation due to short distance between the interaction points (left). IgM has ten points interaction with target antigen molecules and distance between interaction points is long enough to induce highly cellular aggregation (right).
Synthetic materials for cell aggregation

Nagamune et al. reported PEG derivatives capped with hydrophobic groups such as cholesterol or oleyl group to prepare β-cell [54] and liver cell spheroid [55, 56] as intercellular cross-linker. When one hydrophobic groups anchor to cell membrane, the other one is expected to interact with other cells and cross-link both cells. However, the efficiency of the cellular interaction using linear polymeric linkers will be low because the linear polymer has the tendency to fold back to the same cell surface thereby forming a loop around the cell instead of bridging between two different cells [57].

To address this issue, amphiphilic polymers, such as dendrimeric derivatives [58] bearing hydrophobic groups, were applied to cellular crosslinking. The materials possess hydrophobic groups incorporated randomly and are capable of cellular crosslinking.

Optimum design of the artificial crosslinker

In the case of cellular crosslinking, linker length of polymers are expected to be longer than that of glycocalyx on cell surface (> 50 μm) [59], while single amphiphilic polymer is difficult to exceed the distance between two cells. The polymers bearing acyl groups cause self-aggregation, which is thought to be dependent on the design of the polymer structures, indicating that the potential possibility in the use of these polymerss for cellular crosslinking (Figure 3.5).
Figure 3.5. Synthetic cellular crosslinker involved in the formation of cellular assembly. Lipid-capped PEG has two interaction points and was reported to be successful to induce cellular assembly, while the linear polymer has the tendency to fold back to the same cell surface thereby forming a loop around the cell instead of bridging between two different cells (left). Dendrimer-type materials has multiple interacting points and was reported to be succeeded in formation stiff cellular assembling (middle). Our strategy is to form self-assembly with the polymers bearing hydrophobic chain, and induce cellular assembly (right). Cellular assembling independent of cell-type having different glycocalyx is expected because of longer distance between interaction points than that of unimer.
3.1.5. Strategy of this study

Amphiphilic polymers bearing hydrophobic side chains are promising materials for cell surface modification via hydrophobic interaction due to retardation of release from cell surface. Here we tried to elucidate the optimal design of amphiphilic polymers bearing hydrophobic side chains for two applications, ligand/receptor presentation and cellular assembling. We synthesized twelve polymers with different molecular weight of the dextran main chain (M.W. 10k, 60k, 500k, 1500k), acyl groups (palmitoyl, behenyl), and their contents (low, high). The polymers were labeled with rhodamine to evaluate behavior and stability on cell surface. In addition, the polymers were biotinylated to present materials of interest on cell surface via biotin-avidin interaction. Judging from both solution properties of polymers in test tube and polymer behavior on cell surface in vitro, we elucidated the relationship of polymer design and behavior on cells and concludes the optimum design for two applications in a logical manner.
3.2. Results and discussions

3.2.1. Synthesis and characterization of the amphiphilic polymers

The structure of the acyl-modified polymers used in this study is shown in Figure 3.6. The polymers are composed of hydrophilic dextran main chain, hydrophobic acyl anchor, and biotin. In order to establish the optimum design for two applications such as ligand presentation and cellular crosslinker, we synthesized 12 kinds of polymers with different molecular weight of the dextran main chain (M.W. 10k, 60k, 500k, 1500k), acyl groups (palmitoyl, behenyl), and their contents (low, high). The polymers were synthesized by converting hydroxyl groups of dextran main chain into amino groups with ethylenediamine, and then modifying the other groups (acyl, biotin and rhodamine) via amide bond formation. The content of each group was determined by $^1$H-NMR and absorbance at 550 nm. Characteristics of polymers are summarized in Table 3.2 and 3.3. The acyl contents was adjusted to be low (~ 5 mol%) or high (~10 mol%), while the biotin content was kept nearly constant (1.3-4.1 mol%) in all the polymers.

Table 3.2. Characteristics of polymers with low molecular weight.

<table>
<thead>
<tr>
<th>L.D.</th>
<th>M.W. (g/mol × 10^3)</th>
<th>Hydrophobic group</th>
<th>Substitution degree (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hydrophobic group</td>
</tr>
<tr>
<td>D(10)-PL</td>
<td>10</td>
<td>Palmitoyl (C16)</td>
<td>6.5</td>
</tr>
<tr>
<td>D(10)-BL</td>
<td></td>
<td>Behenyl (C22)</td>
<td>5.3</td>
</tr>
<tr>
<td>D(60)-PL</td>
<td>60</td>
<td>Palmitoyl (C16)</td>
<td>6.0</td>
</tr>
<tr>
<td>D(60)-BL</td>
<td></td>
<td>Behenyl (C22)</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Table 3.3. Characteristics of polymers with high molecular weight.

<table>
<thead>
<tr>
<th>L.D.</th>
<th>M.W. (g/mol × 10^3)</th>
<th>Hydrophobic group</th>
<th>Hydrophobic group</th>
<th>Biotin</th>
<th>Amine group</th>
<th>Rhodamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>D(500)-PL</td>
<td>500</td>
<td>Palmitoyl (C16)</td>
<td>4.8</td>
<td>2.2</td>
<td>9.6</td>
<td>0.7</td>
</tr>
<tr>
<td>D(500)-PH</td>
<td></td>
<td></td>
<td>10.5</td>
<td>1.3</td>
<td>4.7</td>
<td>0.8</td>
</tr>
<tr>
<td>D(500)-BL</td>
<td></td>
<td>Behenyl (C22)</td>
<td>4.5</td>
<td>2.2</td>
<td>9.9</td>
<td>0.7</td>
</tr>
<tr>
<td>D(500)-BH</td>
<td></td>
<td></td>
<td>9.6</td>
<td>1.3</td>
<td>6.9</td>
<td>0.8</td>
</tr>
<tr>
<td>D(1500)-PL</td>
<td>1500</td>
<td>Palmitoyl (C16)</td>
<td>4.7</td>
<td>1.5</td>
<td>10.9</td>
<td>0.9</td>
</tr>
<tr>
<td>D(1500)-PH</td>
<td></td>
<td></td>
<td>10.3</td>
<td>2.4</td>
<td>4.5</td>
<td>0.8</td>
</tr>
<tr>
<td>D(1500)-BL</td>
<td></td>
<td>Behenyl (C22)</td>
<td>4.6</td>
<td>1.8</td>
<td>10.8</td>
<td>0.8</td>
</tr>
<tr>
<td>D(1500)-BH</td>
<td></td>
<td></td>
<td>8.8</td>
<td>1.5</td>
<td>7.4</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Figure 3.6.** Structure of synthetic polymers for cell surface modification.
3.2.2. Aggregation properties of the polymers

Because the polymers prepared here have amphiphilic characteristics, the polymers will aggregate by hydrophobic interaction between the acyl groups in aqueous milieu. Thus, we first evaluated the aggregating property of the polymers in two kinds of isotonic buffers; A (10 mM HEPES containing 150 mM NaCl, pH 7.2) and B (10 mM HEPES containing 300 mM mannitol, pH 7.2). After dispersing each polymer in the buffer, the solution was filtered with 0.45 μm-filter to remove large size aggregate, and then the amount of remaining polymer in the filtrate was quantitated (Figure 3.7) and the hydrodynamic diameter of the remaining aggregate was measured (Figure 3.8). We categorized the polymers into low Mw group (Mw 10k, 60k) and high Mw group (Mw 500k, 1500k), obtained results are explained based on the following four points; effect of buffer content, molecular weight of the dextran, length of acyl chain and content of acyl chain.

3.2.2.1. Effect of buffer

Low Mw group

As shown in Figure 3.7, in the low Mw group, the amount of polymers in the filtrate was higher in the buffer B than A in these polymers. This indicates larger aggregate formation of the polymer in buffer A containing NaCl due to the high ionic strength. This result agrees with the general theory that aggregation of hydrophobic low molecule is enhanced with increase in ionic strength.

High Mw group

On the other hand, in the high Mw group, larger amount of the remaining polymers was contained in the filtrate of buffer A than buffer B in most of the polymers, indicating the smaller aggregate formation of the polymers in buffer A which includes 150 mM NaCl. This will probably due to the enhancement of the hydrophobic interaction to fold the polymer chain into tighter conformation in the solution with a high ionic strength.
3.2.2.2. Effect of molecular weight

**Low M\textsubscript{w} group**

In the case of low Mw group bearing parmitoyl chains, amount of the polymer remained in the filtrate was larger for D(10) polymer (containing 10k dextran) than that in D(60) polymer (60k dextran). This result indicates the smaller size of the aggregate in the D(60)-PL. In contrast, the polymers with behenate show no difference in the amount of remaining polymer.

**High M\textsubscript{w} group**

Comparing between the polymers with the same acyl groups and their contents, D(500) polymer (500k dextran) remained more in the filtrate than the D(1500) polymer (1500k dextran). This result indicates the smaller size of the aggregate in the 500k-polymers.

3.2.2.3. Effect of acyl groups

**Low M\textsubscript{w} group**

In case of the polymers with smaller molecular weight (D(10)), the polymers with behenate remain more in the filtrate than those with palmitate, indicating larger aggregate formation in the polymer with behenate-polymers. In contrast, the polymers with higher molecular weight (D(60)) shows no difference between D(60)-PL and D(60)-BL.

**High M\textsubscript{w} group**

Comparing between the polymers with the same M.W. and the acyl contents, the polymers with palmitate remains less in the filtrate than behenate. This indicates that the polymers with palmitate formed the larger aggregate. Akiyoshi’s group reported similar results [60]. They examined the effect of length of alkyl groups (C12, C16, C20) on the size of aggregate, and found that the size of the polysaccharides with shorter alkyl groups form larger aggregates including larger number of the polysaccharide chains. This similar tendency observed here and Akiyoshi’s group will be explained as follows. The polymers with longer alkyl groups form tighter aggregate with less number of the polymer chains due to the stronger hydrophobic
interaction to fold the polymer chain. However, the polymers with the shorter alkyl groups can’t tightly fold the polymer chains, resulting in the larger size aggregate with larger number of polymer chains.

3.2.2.4. Effect of acyl group content

*High M<sub>w</sub> group*

With regard to the polymers with higher molexular weight (500k, 1500k), comparing between polymers with the same M.W. and acyl groups, amount of the polymers with high acyl content was higher in the filtrate than low acyl-content polymers. As shown in Figure 3.8, the polymers with the high acyl content form the aggregates with narrower size distribution. These results are probably due to the formation of the tighter aggregate in the high acyl chain content polymers.
Figure 3.7. (a) Ratio of remaining polymers in the filtrate after filtration of each polymer solution dissolved in either buffer A or B. (b) Diameter and (c) polydispersity index of the polymers in buffer A or B after filtration. Results are expressed as mean ± SD for n = 3.
Figure 3.8. Size distribution of the polymers in high $M_w$ group in buffer B after filtration.
3.2.3. Optimization of conditions for polymer modification on cell surface

We compared the efficacy of cell surface modification with our polymers in the buffer A with or without solubilizing agents and in buffer B. We applied some kinds of solubilizer, α-cyclodextrin, methyl-β-CD, and pluronic F-68, because these solubilizers are expected to inhibit polymer aggregation and enhance the efficacy of cell surface modification (Figure 3.9a). The cell surface was modified with the polymers by suspending the K562 cells in the polymer solution for 30 min at low temperature (4°C) to inhibit the endocytotic uptake of the polymers. After removing the unbound polymers by washing, the cells were incubated at 37 °C. Figure 3.9b shows microscopic images of the cells modified with four kinds of polymers soon after the modification. Red fluorescence resulting from the rhodamine labeled on to the polymers was observed on the cell surface, indicating the successful modification with the polymers on the cell surface. Then, we quantified the amount of the polymer modified on cells by image based cytometry (Figure 3.9c).

D(500)-BH were anchored to the cell surface only in buffer B with weak ionic strength, whereas the polymer was not anchored to the cell surface in the other solutions. The high ionic strength of the buffer A is expected to enhance the hydrophobic interaction among the acyl groups. Thus, the D(500)-BH have less chance to expose the acyl group which leads to the anchoring of the polymers to the cell membrane through the hydrophobic interaction. Furthermore, the solubilizers are expected to form complex with polymer and inhibit the acyl groups from exposing, although such agent increased the solubility and dispersion of the polymers. Hereafter, we applied isotonic buffer B to in vitro experiment due to the capability of the efficient modification on the cell surface.
Figure 3.9. Effect of solvents on the surface modification of acyl-modified polymers. Chemical structures of solubilizers are shown in (a). K562 cells were modified with D(500)-BH in isotonic buffer A with or without solubilized, or B. The modification efficiency was evaluated by (b) fluorescence microscopic observation and (c) image-based cytometry.
3.2.4. Selection of isotonic buffer for polymer modification on cell surface

We compared the efficacy of cell surface modification in the different isotonic buffer A and B. Figure 3.10a - 3.13a shows microscopic images of the cells modified with 12 kinds of polymers soon after the modification. Red fluorescence resulting from the rhodamine labeled on to the polymers was observed again on the cell surface, indicating the successful modification of the polymers on the cell surface. Then, we quantified the amount of the polymer modified on cells by image based cytometry (Figure 3.10b - 3.13b). To compare the modification efficacy among the different polymers, we obtained relative fluorescence intensity (RFI) derived from polymer per single cell and calculated the ratio of RFI in buffer A to in buffer B. Here we explain these results based on the following three points.

3.2.4.1. Homogeneity of cell surface modification with the amphiphilic polymers

As shown in figure 3.10a – 3.13a, almost all the polymers bound on cell surface homogeneously in buffer B with low ionic strength. In contrast, most polymers were not modified homogeneously in buffer A containing NaCl with high ionic strength. These results show that high ionic strength enhance association of polymers in aqueous milieu and on cell surface. Hereafter, we applied isotonic buffer B to in vitro experiment due to the capability of the efficient modification on the cell surface.

3.2.4.2. Effect of molecular weight

Comparing between the polymers with the low content of acyl groups, most of the palmitate-modified polymers were anchored to the cell surface in both of the isotonic buffers, whereas the behenate-modified polymers with high molecular weight were anchored to the cell surface slightly in isotonic buffer A containing 150 mM NaCl (Figure 3.10 – 3.13). The high ionic strength of the buffer A is expected to enhance the hydrophobic interaction among the acyl groups. Thus, the behenate-modified polymers have less chance to expose the acyl group which
leads to the anchoring of the polymers to the cell membrane by the hydrophobic interaction. This effect will be enhanced more in the behenate-modified polymers than the palmitate-modified ones due to its higher hydrophobicity.

Comparing between the polymers with low content of behenate, the ratio of RFI in buffer A to in buffer B decreased with increase in molecular weight of the polymers (Figure 3.14). This indicates that the effect of ionic strength on behenate association and dissociation gets higher with increase in molecular weight.

3.2.4.3. Effect of acyl content

Among the polymers with the same molecular weight (500k, 1500k) and content of acyl groups, the palmitate-modified polymers were anchored to the cell surface in both of the isotonic buffers, whereas the behenate-modified polymers were anchored to the cell surface just slightly in isotonic buffer A containing 150 mM NaCl (Figure 3.12, 3.13).

Comparing between the polymers with low behenate, the ratio of RFI in buffer A to in buffer B decreased with increase in the behenate content (Figure 3.14). This indicates that the effect of ionic strength on behenate association and dissociation gets higher with increase in content of acyl groups.
Figure 3.10. Effect of solvents on the surface modification of acyl-modified polymers with molecular weight of 10k. K562 cells were modified with two kinds of polymers either in isotonic buffer A or B. The modification efficiency was evaluated by (a) fluorescence microscopic observation and (b) image-based cytometry.

Figure 3.11. Effect of solvents on the surface modification of acyl-modified polymers with molecular weight of 60k. K562 cells were modified with two kinds of polymers either in isotonic buffer A or B. The modification efficiency was evaluated by (a) fluorescence microscopic observation and (b) image-based cytometry.
**Figure 3.12.** Effect of solvents on the surface modification of acyl-modified polymers with molecular weight of 500k. K562 cells were modified with four kinds of polymers either in isotonic buffer A or B. The modification efficiency was evaluated by (a) fluorescence microscopic observation and (b) image-based cytometry.
Figure 3.13. Effect of solvents on the surface modification of acyl-modified polymers with molecular weight of 1500k. K562 cells were modified with four kinds of polymers either in isotonic buffer A or B. The modification efficiency was evaluated by (a) fluorescence microscopic observation and (b) image-based cytometry.
Figure 3.14. Effect of solvents on the surface modification of twelve kinds of acyl-modified polymers. K562 cells were modified with four kinds of polymers either in isotonic buffer A or B. The modification efficiency was evaluated by image-based cytometry and the ratio of RFU in buffer A to that in buffer B was calculated.
3.2.5. Modification efficacy of each polymer onto cell surface and its cytotoxicity

We quantitated the amount of polymers modified on the cell surface at various polymer concentrations (Figure 3.15). Here we compared the amount of the bound polymers with PEG-lipid. The polymer concentration in Figure 3.15 - 3.17 is actual polymer concentration after sterilization filtration of 100 \( \mu \text{g/mL} \) polymer solution. These concentrations are marked with red in color in Figure 3.15 - 3.17. The amount of the modified polymers proportionally increased with the polymer concentration in both the acyl-modified polymers and PEG-lipid. It is noteworthy that the slope of the curves for the acyl-modified polymers was steeper than PEG-lipid, showing that higher modification efficacy of the acyl-modified polymers. In fact, the calculated modification ratio of most of the acyl-modified polymers is higher than that of PEG-lipid (~ 40%) (Figure 3.16). One of the reasons for the lower modification ratio of the PEG-lipid is its micelle formation with hydrophobic acyl core surrounded by PEG chains \([\ldots]\). This will result in the less chance of acyl groups to anchor on the cell surface. Another reason for the lower modification ratio of the PEG-lipid may be in its single layer coverage of the cell surface. As mentioned above, the acyl-modified polymers will anchor on the cell surface as the aggregate of the polymer chains. This speculation seems to be valid according to the following results. When we assumed random coil conformation of each polymer chain on the surface of the K562 cell, the hydrodynamic diameter of 10k, 60k, 500k and 1500k should be ca. 4, 13, 30 and 50 nm, respectively \([23]\). Therefore, coverage of the polymer chain on the K562 cell surface with diameter of 14 \( \mu \text{m} \) exceeds 100%.

Next we examined the cytotoxicity of the polymer modification after 24-hours incubation at 37 °C by using a membrane-impermeable dye (YOYO-1). Figure 3.17 shows that higher cytotoxicity was observed in the polymers with molecular weight of 60k. As shown in Figure 3.17, weaker cytotoxicity was observed in D(500)-PH at high concentration (≥ 60 \( \mu \text{g/mL} \)). This point is discussed later.
Figure 3.15. Polymer concentration dependence on the amount of polymer bound to the K562 cell surface. The symbols colored in red indicates the polymer concentration applied to in vitro experiments.
Figure 3.16. Polymer concentration dependence on the ratio of polymer bound to the K562 cell surface. The symbols colored in red indicate the polymer concentration applied to in vitro experiments.
Figure 3.17. Viability of cells treated with acyl-modified polymers. Cell viability was measured at 24 hours after cell surface modification by staining the dead cell with YOYO-1. The symbols colored in red indicate the polymer concentration applied to in vitro experiments.
3.2.6. Cellular assembly induced by polymer modification on cell surface

Next, we examined the effect of the polymer modification on the assembling behavior of the cells. After the modification of the polymers on the cells in buffer B, the assembling behavior of the cells was monitored by microscopy. We applied a non-adherent cell line (K562 cells) and two adherent cell line (RAW 264.7, HepG2) to cellular assembling.

3.2.6.1. Non-adherent cell line

We evaluated the effect of the polymers on cell assembling in detail by using K562 cell line, because the cell is non-adherent and we can exclude the effect of cell-plate adherence. Figure 3.18 shows microscopic images of the cells modified with polymers after 6 and 24 hours incubation at 37 °C. At 6 hours, the assembly of the cells were clearly observed in the palmitate-modified polymers of D(500)-PH and D(1500)-PH. However, the cellular assembly was observed in all the polymers at 24 hours. The more efficient assembling was observed in the palmitate-modified polymers than in the behenate-modified ones. It seems to reflect the more aggregating property of the palmitate-modified polymers in the aqueous milieu observed in Figure 3.7a. Figure 3.19 shows the magnification image of the cells. The significant accumulation of the polymers to cell-to-cell contact points was observed in the palmitate-modified polymers. These results clearly indicate that the polymer functions as the crosslinker between the cells. The rate of the cellular assembling was found to be dependent on the cellular concentration (Figure 3.20). The higher cellular concentration facilitated the cellular assembling.

As observed in Figure 3.17, the most efficient crosslinking polymer, D(500)-PH, shows weak cytotoxicity at the high concentration (≥ 60 μg/mL), where almost all of the cells were assembled to be a single assembly (Figure 3.21). In this case, the nutrient and oxygen may difficult to diffuse homogenously throughout this large cellular assembly.
Figure 3.18. Intercellular assembling of K562 cells modified with polymers modified on the cell surface. After modification of the cells with the filtrate of each polymer solution (100 \( \mu \text{g/mL} \)) with 0.45 \( \mu \text{m} \) filter, cells were incubated in RPMI medium containing FBS at 37 °C for 6 and 24 hours. Cell concentration were 1\( \times 10^5 \) cells/mL.
Figure 3.19. Fluorescent microscopic image of K562 cells treated with four different acyl-modified polymers labeled with rhodamine. After modification of cells with filtrate of 100 μg/mL of polymers with 0.45 μm filter, cells were incubated in RPMI medium containing FBS in 5% CO₂ at 37°C for 3 hr. Cell concentration were 5×10⁵ cells/mL both during incubation and for observation after incubation. Arrow indicates the contact point between two adherent cells where the polymers accumulated.
Figure 3.20. Cellular aggregation of K562 cells caused by D(500)-PH modified on cell surface. After modification of cells with filtrated of 100 μg/mL of D(500)-PH with 0.45 μm filter, cells were incubated in RPMI medium containing 10% FBS at 1×10^5 or 5×10^5 cells/mL in 5% CO₂ at 37°C for 6, 24 hr.
Figure 3.21. Cellular aggregation of K562 cells caused by four different acyl-modified polymers modified on cell surface. After modification of cells with filtrate of 100 μg/mL of polymers with 0.45 μm filter, cells were incubated in RPMI medium containing 10% FBS in 5% CO₂ at 37°C for 24 hr. Cell concentration were $5 \times 10^5$ cells/mL both during incubation and for observation after incubation.
3.2.6.2. Adherent cell line

We applied cell assembling evaluation to other cell lines, RAW 264.7 cells (murine macrophage-like cell line) and HepG2 cells (human hepatocarcinoma cell line).

Figure 3.22 shows microscopic images of RAW 264.7 cells modified with D(500)-PH or BH after 0 and 1 hour incubation at 37 °C. Soon after the modification, weak assembly of the cells were clearly observed in D(500)-PH. Then, the cellular assembly was observed in both D(500)-PH and D(500)-BH at 1 hour. The more efficient assembling was observed in the palmitate-modified polymers than in the behenate-modified ones. It seems to reflect the higher aggregating property of the palmitate-modified polymers in the aqueous milieu observed in Figure 3.22. Figure 3.23 shows the magnification image of the cells. Soon after polymer modification, the significant accumulation of the polymers to cell-to-cell contact points was observed in the palmitate-modified polymers. These results clearly indicate that the polymer functions as the cellular crosslinker. The assembling rate of RAW 264.7 cells was higher than K562 cells.

Figure 3.24 shows microscopic images of HepG2 cells modified with polymers D(50)-PL or B10 after 0 and 2 days incubation at 37 °C. When the culture medium contains 10% FBS, the cell assembling was observed independent on the polymer existence. Thus, we tried proceeding same experiment with the culture medium without FBS and succeeded in suppressing the spontaneous cellular aggregation independent on the polymers. However, assembling of HepG2 cells was much weaker than that of the other cells with D(500)-PH and D(500)-DH.

In conclusion, our cellular assembling technique showed the dependence on cell line. Hereafter we need to elucidate the reason of cell line dependence and optimize the condition of cellular assembling.
**Figure 3.22.** Intercellular assembling of RAW 264.7 cells modified with polymers modified on the cell surface. After modification of the cells with the filtrate of each polymer solution (100 μg/mL) with 0.45 μm filter, cells were incubated in DMEM medium containing FBS at 37 °C for 1 hour. Cell concentration were 5×10^5 cells/mL.

**Figure 3.23.** Fluorescent microscopic image of RAW 264.7 cells treated with two different acyl-modified polymers labeled with rhodamine. After modification of cells with filtrate of 100 μg/mL of polymers with 0.45 μm filter, cells were incubated in DMEM medium containing FBS in 5% CO2 at 37°C for 1 hr. Cell concentration were 5×10^5 cells/mL both during incubation and for observation after incubation. Arrow indicates the contact point between two adherent cells where the polymers accumulated.
Figure 3.24. Microscopic image of HepG2 cells treated with two different acyl-modified polymers. After modification of cells with filtrate of 100 μg/mL of polymers with 0.45 μm filter, cells were incubated in DMEM medium (a) with or (b) without 10% FBS in 5% CO₂ at 37°C for 2 days. Cell concentration were 5×10⁵ cells/mL both during incubation and for observation after incubation.
3.2.7. Stability of ligand presented on cell surface

We then checked the stability of the polymer on the cell surface, which is crucial characteristic for the application to present ligand or receptor on the cell surface. Here we examined by using both adhesive and non-adhesive cell lines; RAW264.7 and K562 cells, respectively. After the polymer modification at 100 \( \mu \text{g/mL} \) of the polymer concentration, the cells were incubated at the lower cell concentration \((1.0 \times 10^5 \text{ cells/mL})\) to retard the intercellular assembly. As shown in Figure 3.10 – 3.14, the fluorescence on the cell surface resulting from the modified polymers became weaker with time in K562 cell, especially in the case of the polymers with palmitate. Especially, D(10)-PL disappeared from cell surface after 24 hours incubation compared with the polymers with higher molecular weight (500k, 1500k). In the both cell lines, the fluorescence was observed inside of the cells, showing the cellular uptake. Adhesion of RAW264.7 cells on the dish surface was observed at 6 hours, indicating that the polymer modification does not inhibit the cellular adhesion to the dish surface.

The polymer’s fluorescence remaining on the cell surface was quantified from the microscopic images and is plotted against incubation time in Figure 3.29. D(500)-BH remained longer duration on the cell surface than D(500)-PH in the both cell lines. The longer duration of the behenate-modified polymer was also observed in the other polymers (Figure 3.29). As a comparison, the duration of PEG-lipid is also plotted in Figure 3.29. The acyl modified polymers were found to be much more stable than PEG-lipid, indicating that the multiple anchoring is advantageous for the longer duration of the polymers on cell surface.

Figure 3.30a, b shows percentage of remaining polymer on cell surface after 6 hours incubation. Compared between the polymers with same acyl groups and low content of acyl groups, the stability of polymer on cell surface increased with increasing in the molecular weight. In addition, compared the acyl group content among the polymers with same acyl groups and same molecular weight, the stability of the polymer with high contents of acyl groups on cell surface was higher than that with low contents of acyl groups.
Figure 3.25. Time-lapse image of localization of rhodamine-labeled polymers with molecular weight of 10k and fluorescein-labeled PEG-lipid on the surface of K562.

Figure 3.26. Time-lapse image of localization of rhodamine-labeled polymers with molecular weight of 500k on the surface of K562.
Figure 3.27. Time-lapse image of localization of rhodamine-labeled polymers with molecular weight of 1500k on the surface of K562.

Figure 3.28. Time-lapse image of localization of rhodamine-labeled polymers with molecular weight of 500k on the surface of RAW264.7 cells.
Figure 3.29. Time-dependent change of polymers’ RFI on the surface of (a) K562 and (b) RAW 264.7 cells. Results are expressed as mean ±SD for 30 cells, *, **, *** indicate a statistically significant difference respectively; 0.01 < p < 0.05, 0.001 < p < 0.01, p < 0.001.
Figure 3.30. (a), (b) Polymers’ RFI on the surface of K562 after 6 hours incubation. Results are expressed as mean ±SD for 30 cells. (c) Polymer’s RFI on single K562 cell after 6 and 24 hours incubation. Results are expressed as mean ±SD for three experiment. (d) Cellular uptake index is calculated by subtracting Polymer’s RFI on single cell to that on the surface of cell.
In conclusion, to improve the stability of acyl-modified polymer on cell surface, we need to design the polymers based on following three points based on polymer stability on cell surface (Figure 3.30a, b).

(1) Hydrophobic side chain (stability: behenate > palmitate)

(2) Content of hydrophobic side chain (stability: high > low)

(3) Molecular weight of polymer chain (stability: high > low)

First, approach 1) is expected to increase the stiffness of cell membrane and retard the invagination of plasma membrane. This proof is described later. Second, approach 2) is also expected to increase the stiffness of cell membrane. In addition, increment of cationic charge is considered to be one reason affecting the incorporation, because polycationic materials are highly incorporated into cells that polyanionic and neutral ones. Lastly, approach 3) is expected to form large polymer self-aggregate on cell surface. As figure 3.25 – 3.27 shows, the polymers with high molecular weight (500k, 1500k) form self-aggregate on cell surface more rapidly than that with low molecular weight (10k). This finding agrees with our suggestion.

Thus, for the polymer used to cell-surface presentation of artificial ligand or receptor, above three factors should be higher. In contrast, if the polymer is applied to the incorporation of target molecules into cell, above all three factors should be smaller. Lastly, the polymer for cell assembly should have high molecular weight (500k or 1500k) main chain.
3.2.8. Recognition of streptavidin by biotin presented on cell surface

We examined SA recognition with biotin presented on the cell surface via the polymers. Biotin-presenting K562 and RAW264.7 cells were mixed with fluorescein-labeled SA at 25 °C. After removing the unbound SA, the cells were incubated at 37 °C and the fluorescences resulting both from SA and the polymer were monitored (Figure 3.31). The yellowish color resulting from the colocalization of the green fluorescence of SA with the red fluorescence of the polymer was observed on the cell surface just after mixing with SA (0 hr). With time passed, yellowish color resulting from the SA/polymer complex on the cell surface became weaker, while the complex more accumulated in the cell-to-cell contact points comparing with the case of the polymer alone. Compared with the polymer with low molecular weight (10k), SA/polymer complex was not incorporated into cells in the high molecular weight polymer (500 k). Thus the lower uptake of the SA/polymer complex observed here may result from the enlargement of the polymer aggregates on the cell surface through the crosslinking via SA of the polymer aggregates.

Figure 3.32 shows fluorescence of SA from the polymer-modified K562 cells which is measured by image-based cytometry. The remaining SA presented with D(500)-BH was higher than that with D(500)-PH and lower molecular weight polymers especially at 6 hours. This will be probably due to the stronger anchoring of the behenate-modified polymer to the cell membrane. Thus, for the presentation of SA, the behenate-modified polymer is found to be more suitable. In spite of rapid disappearance of SA from cell surface, the remaining total SA existing with the polymers with low molecular weight (10k) was higher than that with high molecular weight (500k) at 24 hours. Thus, for the incorporation of SA, the polymers with low molecular weight are found to be suitable.
Figure 3.31. Time-lapse image of localization of rhodamine-labeled polymers and SA on the surface of (a) K562 and (b) RAW264.7 cells.
Figure 3.32 Relative fluorescent intensity (RFI) of the polymer (a) and fluorescein-labeled SA (b) per single K562 cell after 6 and 24 hours incubation. Error bar shows SD of independent three experiments. *** indicate a statistically significant difference respectively; $p < 0.001$. 
3.2.9. Intracellular distribution of the polymers

In order to apply the acyl-modified polymers to display an artificial receptor for intracellular delivery, we evaluated the intracellular localization of the polymers with fluorescent microscope (Figure 3.33). After 24 hours incubation of polymer modification, incorporation of the polymers in K562 cells was observed in all polymers. However, the size of some organelles where the D(500)-BH and D(1500)-BH localized seems to be larger than normal one. To investigate this phenomenon, we evaluate the intracellular localization in detail. Figure 3.34 shows that the acyl-modified polymers was localized to specifically in the plasma membrane, endosome and lysosome. Especially, D(500) and D(1500) were localized to the membrane of organelles larger than normal ones, which was proved to be endosome and/or lysosome in Figure 3.34. Interestingly this indicates that D(500) and D(1500) increased the size of endosome/lysosome without damaging the cells (Figure 3.17).

We suspect the mechanism of this phenomenon as follow. The polymers are incorporated into cell via invagination of plasma membrane, and covered with vesicle. The vesicle are delivered to endosome through microtubule and fused with endosome membrane. Normally, the polymers are delivered to other organelle, such as lysosome and recycling endosome, via budding from the membrane of endosome. We suspect that D(500)-BH and D(1500)-BH retard the budding from cell membrane, while fusion of vesicles happens constantly. Thus, the size of endosome are thought to be increased.
Figure 3.33. Microscopic images after 24 hours incubation of polymer modified K562 cells. Red fluorescence is derived from rhodamine labeled on to the polymers. Arrow indicates the organelles larger than normal endosome/lysosome.
Figure 3.34 Cellular uptake of acyl-modified polymers by K562 cells modified with the polymer after 24 hours incubation. These are merged images of red fluorescence of rhodamine labeled on to the polymers, green fluorescence of endosome/lysosome, and blue fluorescence of nucleus. Yellow color indicates the colocalization of the polymers and endosome/lysosome. The polymers with behenate localized to the membrane of cell surface and endosome/lysosome. Arrow indicates the organelle larger than normal endosome/lysosome.
3.3. Conclusion

Here we pursued for the first time the suitable design of the acyl-modified polymers for three applications, the biotin presentation, SA incorporation and the cellular assembling. Our findings are illustrated in Figure 3.35.

The behenate (C22)-modified polymers were found to be more suited for the presentation of biotin due to the longer stability on the cell surface leading to the efficient recognition of SA. On the other hand, the palmitate (C16)-modified polymers with high molecular weight (500k, 1500k) was the most suitable as a crosslinker to induce cellular assembling. This result seems to reflect superior aggregating ability of the palmitate-modified polymers in the aqueous milieu. In the same way, the acyl-modified polymers with low molecular weight (10k) were found to be more suited for artificial receptor of SA due to the high efficiency of SA incorporation, compared with that with high molecular weight (500k). Thus, we obtained findings helpful for designing acyl-grafted type polymers for cell surface engineering.

![Diagram illustrating the interaction of acyl-modified polymers with living cell and behavior on the cell surface.](image)

**Figure 3.35.** Illustration of the interaction of acyl-modified polymers with living cell and behavior on the cell surface. Behavior of the polymers is mainly dependent on length of acyl groups (palmitate, C16; behenate, C22) and molecular weight of polymers.
3.4. Experiment

3.4.1. Materials

Dextran (Dex, $M_w$ 500 000 and 1 500 000) was purchased from Sigma Aldrich. Palmitic acid, behenic acid, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), and Triethylamine (TEA) were purchased from Tokyo Kasei Industry (Tokyo, Japan). Ethylenediamine (EDA), biotin, $N,N'$-carbonyldiimidazole (CDI), Dimethylsulfoxide (DMSO), ethanol were purchased from Wako Pure Chemicals (Osaka, Japan). Lithium chloride were purchased from Kanto Chemical (Tokyo, Japan). Hoechst 33342, YOYO-1 and Rhodamine-NHS were purchased from Invitrogen (Carlsblad, USA). NHS-Fluorescein and FITC-labelled streptavidin (SA) were purchased from Thermo Scientific (Massachusetts, USA). N-(aminopropyl polyethyleneglycol)carbamyl-distearoylphosphatidyl-ethanolamine (DSPE-PEG(5000)-NH2, PEG chain $M_w$ 5 000) was purchased from NOF Corporation (Tokyo, Japan).

3.4.2. Synthesis of Dex-EDA-Pal and Dex-EDA-Beh

Dex-EDA, which is the dextran derivatives substituted with EDA, was synthesized as described previously [17]. After Dex-EDA (260 mg, 1.47 mmol of glucose units) was dissolved in DMSO/DMF (26 mL, v/v = 1/1) at 80 °C for 30 minutes, the solution was cooled to 50 °C. Then, to introduce 5 or 10 mol% hydrophobic moieties onto glucose units of Dex-EDA, palmitic acid (4.7 and 9.4 mg, 18.4 and 36.8 μmol) or behenic acid (6.3 and 12.5 mg, 18.4 and 36.8 μmol), and DMT-MM (6.1 and 12.2 mg, 22.1 and 44.2 μmol) were added and then the mixture was placed at 50 °C for 12 h. After the reaction, the solution was reprecipitated using ethylacetate. The obtained polymer (Dex-EDA-Pal and Dex-EDA-Beh) was dissolved in DMSO containing 1 w/w% LiCl and dialyzed with Spectra/Pore6 Dialysis bag (MWCO 3 500) against diluted sodium hydroxide solution (pH = 11) for 2 days and distilled water for a day, and then lyophilized. The degree of substitution was determined by $^1$H-NMR.

$^1$H-NMR (300 MHz, DMSO): $\delta$ 0.85 (3H, $CH_3(CH_2)_{14or20}CONH(CH_2)_{2}NHCO$), 1.23 (24H or
36H, CH3(CH2)_{12or18}(CH2)2CONH(CH2)2NHCO), 1.46 (2H, CH3(CH2)_{12or18}CH2CH2CONH(CH2)2NHCO), 2.29 (2H, CH3(CH2)_{12or18}CH2CH2CONH(CH2)2NHCO), 3.51-3.99 (6H, protons without anomeric proton), 5.01 (1H, anomeric proton).

3.4.3. Synthesis of Dex-EDA-Pal-Bio and Dex-EDA-Beh-Bio

Dex-EDA-Pal and Dex-EDA-Beh (30 mg) were dissolved in DMSO (30 mL). Then, biotin (2 mol equivalents to 100 glucose units of Dex-EDA-Pal and Dex-EDA-Beh) and DMT-MM (2.4 mol equivalents to 100 glucose units of Dex-EDA-Pal and Dex-EDA-Beh) were added and resulting solution was stirred at room temperature for overnight. After the reaction, the solution was dialyzed with Spectra/Pore6 Dialysis bag (MWCO 3 500) against diluted sodium hydroxide solution (pH = 11) for 2 days and distilled water for a day, and then lyophilized. The degree of substitution of the obtained polymer (Dex-EDA-Pal-Bio and Dex-EDA-Beh-Bio) was determined by 1H-NMR.

1H-NMR (300 MHz, DMSO): δ 0.85 (3H, CH3(CH2)_{14or20}CONH(CH2)2NHCO), 1.23 (24H or 36H, CH3(CH2)_{12or18}(CH2)2CONH(CH2)2NHCO), 1.46 (2H, CH3(CH2)_{12or18}CH2CH2CONH(CH2)2NHCO), 2H, (CHCH2CH2(CH2)2CONH(CH2)2NHCO), 2.29 (2H, CH3(CH2)_{12or18}CH2CH2CONH(CH2)2NHCO; 2H, (CHCH2CH2(CH2)2CONH(CH2)2NHCO), 2.79 (1H, CH2CHSCH(CH2)4CONH(CH2)2NHCO), 3.05 (1H, CHCH(CH2)4CONH(CH2)2NHCO), 3.51-3.99 (6H, protons without anomeric proton), 4.46 (1H, CHCH(CH2)4CONH(CH2)2NHCO), 4.63 (1H, CHCH2SCH(CH2)4CONH(CH2)2NHCO), 5.01 (1H, anomeric proton).
3.4.4. Rhodamine labeling of Dex-Pal-Bio and Dex-Beh-Bio

Dex-EDA-Pal-Bio and Dex-EDA-Beh-Bio were dissolved in DMSO. Then, Rhodamine-NHS (2 mol equivalents to 100 glucose units of each polymers) and TEA (2 mol equivalents to amine group of dextran of each polymers) were added and stirred at room temperature for 24 hours. After the reaction, the solution was dialyzed with Spectra/Pore6 Dialysis bag (MWCO 3 500) against saturated sodium bicarbonate solution for a day and distilled water for 2 days. Thereafter, the solution was lyophilized. The degree of substitution was determined by UV-2550 spectrophotometer (Shimadzu). The resulting polymer was dissolved in DMSO to be 10 mg/mL.

3.4.5. Fluorescein labeling of DSPE-PEG(5000)-NH2

DSPE-PEG(5000)-NH2 was labeled with NHS-Fluorescein. DSPE-PEG(5000)-NH2 was dissolved in acetone. Then, NHS-Fluorescein and TEA (3 equivalents to DSPE-PEG(5000)-NH2) were added and stirred at 25°C for 12 hours. After the reaction, the solution was dialyzed with Spectra/Pore6 Dialysis bag (MWCO 3 500) against ethanol for 1 day, ethanol/distilled water (v/v = 1/1) for 2 days and distilled water for a day. Thereafter, the solution was lyophilized. The degree of substitution was determined to be 39 mol% by UV-2550 spectrophotometer.

3.4.6. Characterization of solution property of polymers

After polymers were dissolved in isotonic buffer A (10 mM HEPES buffer containing 150 mM NaCl, pH 7.2) or B (10 mM HEPES buffer containing 300 mM mannitol, pH 7.2). Polymer suspension was centrifuged to remove macroscopic aggregation and the supernatant was sterilized with syringe filter (0.45 μm pore size). The remaining polymer in the filtrate was determined by the fluorescent intensity of rhodamine labeled on to polymer. Light scattering intensity and hydrodynamic diameter of polymers in the filtrate were evaluated by using the Zetasizer Nano instrument (Malvern Instruments Limited, Malvern, UK).
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3.4.7. Cell culture

K562 (human myelogenous leukemia) cells were cultured in RPMI-1640 medium (WAKO). RAW 264.7 (mouse peritoneal macrophage) and HepG2 cells (human hepatocarcinoma) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (WAKO). Both medium contain 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all from Gibco Invitrogen Co., Grand Island, NY, USA). Cells were harvested in a humidified atmosphere containing 5% CO2 and 95% air at 37°C.

3.4.8. Modification of cells with polymers

K562 cells, RAW 264.7 cells and HepG2 (1×10⁶ cells) were collected by centrifugation. After removal of the supernatant, cells were washed with isotonic buffers. The polymer dissolved in DMSO (10 mg/ml) was diluted by isotonic buffers and sterilized by filtration using 0.45 µm pore size filter. Then, 50 µL of polymer dissolved in the isotonic buffer was added to the cell suspension and incubated for 30 min with gentle agitation at 4°C. After cell surface modification, the isotonic buffer was added to the mixture and the cells were collected by centrifugation. These procedures were repeated twice to remove unbound polymer. The modification ratio of the polymer was determined from the fluorescent intensity in the supernatants derived from the unbound. After treatment with the polymer, cells were incubated in the medium containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B at 37°C under 5% CO2. Fluorescent intensity on the cell surface and total fluorescence per one cell were estimated by BZ-8000 fluorescent microscopy (Keyence) and Tali™ Image-Based Cytometry (Life Technologies), respectively. Analysis of the cell surface fluorescence from the microscopic images are as follows: (1) Analyze fluorescence intensity along the line drawn on the microscopy image. (2) Read the fluorescence intensity on the cell surface. (3) Quantify the fluorescence on the cell surface similarly for 30 cells for each condition.
3.4.9. Cellular assembling with polymers

The polymers dissolved in isotonic buffers B (100 µg/mL) were sterilized by filtration using 0.45 μm pore size filter. Then, 50 μL of polymer solution to the cell suspension (2.0 × 10^7 cells/mL) and incubated for 30 min with gentle agitation at 4 °C. K562 cells, RAW 264.7 cells and HepG2 cells were modified with acyl-modified polymers according to the same procedures described above. Then, the concentration of the cell suspension was adjusted to 5×10^5 cells/mL or 1×10^5 cells/mL and the cells were incubated in RPMI medium containing 10% FBS at 37°C under 5% CO₂. Localization of the acyl-modified polymers on the cell surface and the cellular assembly caused by the polymers were examined with a fluorescent microscope.

3.4.10. Ligand-receptor presentation on the cell surface with polymers

The polymers dissolved in isotonic buffers B (100 µg/mL) were sterilized by filtration using 0.45 μm pore size filter. Then, 50 μL of polymer solution to the cell suspension (2.0 × 10^7 cells/mL) and incubated for 30 min with gentle agitation at 4 °C. Then, SA (0.19 nmol) was added to the polymer-modified K562 cells and RAW 264.7 cells (1×10^6 cells) suspended with isotonic buffer B and the mixture was gently agitated for 30 min at 25°C. After SA presentation on the cell surface, isotonic buffer B (2 mL) was added to the mixture and the cells were collected by centrifugation. These procedures were repeated twice to remove unbounded SA. Then the concentration of the cell suspension was adjusted to 1×10^5 cells/mL and the cells were incubated in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B at 37°C under 5% CO₂. Fluorescent intensity on the cell surface and total fluorescence per one cell were estimated by using a fluorescent microscope and image-based cytometer, respectively.
3.4.11. Cytotoxicity evaluation of polymers

At 24 h after incubation of polymer modified K562, $5 \times 10^6$/mL cell suspension (100 μL) was transferred to 96 well plate and treated with 0.5 mM YOYO-1 staining solution in PBS (50 μL). The resulting mixture was incubated for 30 min. Then, cytotoxicity of polymers was evaluated by calculating percentage of the dead cells based on measurement with image-based cytometer.
3.5. Reference


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Chapter 4. Conclusion Remarks

In chapter 1, I summarized the previous report on the associative polymers bearing ionic groups or hydrophobic groups. In case of electrostatic interaction, most previous approach was to make the PICs tightly by increasing ionic groups. However, decreasing content of ionic groups results in unique property such as loose PIC structure and high avidity of ionic groups. In case of hydrophobic interaction, most previous report was to prepare closed-associate by increasing the content of hydrophobic groups and selecting highly hydrophobic groups. However, decreasing the hydrophobic groups results in dynamic property of the groups, while maintain self-associating property.

In chapter 2, I successfully obtained a new class of nanogel, PIC-NG, comprising from dextran grafted with oligo-cationic and oligo-anionic moieties, by simply mixing the solutions of the cationic and anionic polymers. The salt bridges of the PIC-NG could be crosslinked covalently through amide bonds to stabilize against increasing ionic strength or lowering pH. In spite of the improved stability after chemical crosslinking, the inner free space of the PIC-NGs was still more than 90% of the original value in NGs without any chemical crosslinks. The PIC-NGs also sensitively responded to the solution conditions to reorganize. This property may be useful as stimuli responsive characteristics in drug delivery application.

In this research, I established the basis of designing PIC-NG, categorized into novel physical-crosslinking NGs. However, I did not evaluate the effect of net charge of ionic groups, molecular weight and structure of main chain on the property of PIC. For example, increasing the net charge of ionic groups may improve the stability of PIC-NG against increasing ionic strength without chemical crosslinking.
In chapter 3, I pursued the suitable design of the acyl-modified polymers for three applications, ligand presentation (biotin presentation), intracellular delivery via artificial receptor (cellular incorporation of SA) and the cellular assembling. The behenate (C22)-modified polymers were found to be more suited for the presentation of biotin due to the longer duration on the cell surface leading to the efficient recognition of SA. On the other hand, the palmitate (C16)-modified polymers with high molecular weight (500k, 1500k) was the most suitable as a crosslinker to induce cellular assembling. This result seems to reflect superior aggregating ability of the palmitate-modified polymers in the aqueous milieu. In the same way, the acyl-modified polymers with low molecular weight (10k) were found to be more suited for intracellular delivery due to the high efficiency of SA incorporation thanks to inferior aggregating ability. Thus, I obtained findings helpful for designing acyl-grafted type polymers for cell surface engineering.

In this research, two issues to be confirmed are remained. One issue is that cellular assembling with the acyl-modified polymers is dependent on cell-type. K562 cells and RAW 264.7 cells were aggregated in the medium containing 10% FBS with the polymer, while HepG2 cells was not aggregated with the polymers in the medium not containing 10% FBS. It is necessary to elucidate the reason of this cell type dependency and optimize the condition of cellular assembling. The other issue is that D(500)-BH and D(1500)-BH increase the size of endosome/lysosome without damaging the cells. I elucidated the localization of the polymer with Lysotracker Green DND-26, which stain the acidic organelles such as endosome and lysosome. However, this reagent can not differentiate whether the organelle with large size was endosome or lysosome. It is important to elucidate the phenomenon, because the technology to retain incorporated molecule in early endosome is reported to be important in the field of immunotherapy.
Overall conclusion and perspective

In the field of not only biomedical but also energy and electronic engineering, the technique controlling self-assembly of associative polymers has been advanced in a rapid pace. In the long history of chemistry, novel concepts often push up the level of technology. For example, application of polymer micelle to encapsulation of anti-tumor agent lead to drastic development of polymer micelle type carrier for DDS. In this research, I developed novel type PIC structure, “PIC-NG”, and established the basis to design optimum acyl-modified polymers for cell surface engineering. I hope development of the field based on the findings described in this paper.
Achievement

Poster award in Japanese

「細胞表面修飾を基盤としたがん転移抑制デバイスの開発」

第 50 回化学関連支部合同九州大会、2013 年 7 月
List of publications


List of supplementary publications


List of international oral presentations

“Development of circulating tumor cell-specific macrophage based on cell surface modification for suppression of cancer metastasis”, The 4th Asian Biomaterials Congress, June 2013

List of international poster presentations

“Polymer modification on cell surface for modulation of cellular function”, The 9th SPSJ International Polymer Conference, Hyogo, December 2012
List of oral presentations in Japanese

1. 「タンパク質キャリアとしてのポリイオンコンプレックス型ナノゲル」、第 61 回高分子学会年次大会、2012 年 5 月
2. 「マクロファージと細胞膜アンカーポリマーの融合によるがん転移抑制デバイスの構築」、第 62 回高分子学会年次大会、2013 年 5 月
3. 「がん転移抑制を指向したリポソーム型オプソニンの開発」、第 63 回高分子学会年次大会、2014 年 5 月
4. 「がん転移抑制を指向したリポソーム型オプソニン材料の開発」、第 24 回バイオ・高分子シンポジウム、2014 年 7 月

List of poster presentations in Japanese

1. 「細胞内タンパク質デリバリーの高効率化を目指した新規ポリイオンコンプレックス型ナノゲルの創製」、第 33 回バイオマテリアル学会大会、2011 年 11 月
2. 「細胞内タンパク質デリバリーの高効率化を目指した新規ポリイオンコンプレックス型ナノゲルの構築」、2011 年度九州地区高分子若手研究会・冬の講演会
3. 「細胞表面修飾に基づく共エンドサイトーシス法の開発」、バイオマテリアル学会第二回九州地区講演会、2012 年 9 月
4. 「マクロファージと人工分子の融合によるがん転移抑制デバイスの構築」、日本バイオマテリアル学会大会シンポジウム 2012、2012 年 11 月
5. 「マクロファージと人工分子の融合によるがん転移抑制デバイス」、2011 年度九州地区高分子若手研究会・冬の講演会
6. 「細胞表面修飾を基盤としたがん転移抑制デバイスの開発」、第 50 回化学関連支部合同九州大会、2013 年 7 月
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