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Design of Cell-Surface-Retained Polymers for Artificial Ligand Display

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Cell surface engineering remains an important field in biotechnology and medical science, as these techniques allow the addition of tailored systems into cells and thus enable the modification or regulation of cellular behavior through artificial signals.[1] Several approaches to the engineering of the mammalian cell surface have been explored. For example, metabolic incorporation of oligosaccharide precursors allows for subsequent covalent attachment of desired synthetic epitopes onto the cell surface.[2] Recently, several groups have reported the noncovalent display of synthetic molecules on the cellular surface by passive insertion using lipid-tethered molecules.[3,4] These methods will be applicable for wide range of cell types, and they cause less perturbation of inherent signal transduction. Peterson and co-workers have shown that the non-natural membrane anchor, *N*-alkyl-3β-cholesterylamine, acts as an artificial receptor for efficient drug delivery when linked to the binding motifs for proteins and drugs.[3] Added ligands are rapidly delivered into the cell through receptor-mediated endocytosis. Nagamune and co-workers have also reported the use of PEG-lipids with a single functional group attached on the termini of the PEG chain to anchor proteins.[4]

Our aim is to create molecules that can be retained on the cell surface for a long period and function as an effective scaffold for the display of artificial recognition sites on the cellular surface. In this paper, we report our finding that the addition of secondary amino groups to the polymer backbone affords increased retention time on the cell membrane without rapid internalization (Figure 1). Introduction of a lipid moiety to the end of the secondary amine-containing polymer enhanced binding of the polymer on the cell surface but did not affect localization in the cells. Confocal laser scanning (CLS) microscopy and flow cytometry analysis revealed that the secondary amine-containing polymers were retained on the cell surface for over 12 h. In contrast, primary amine-containing polymers were rapidly internalized. These secondary amino group-containing polymers can act as an effective scaffold for the display of artificial ligands on the cell membrane. Ligands displayed on the surface of HeLa cells can efficiently participate in surface binding, thus affording a new technique for the control of cell adhesion events through the specific recognition of these ligands.

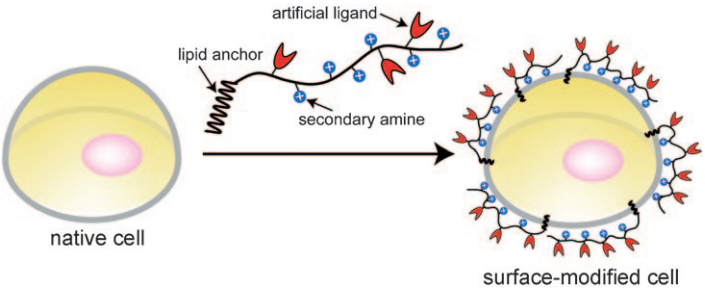

Figure 1. Modification of the cell surface by using cell membrane-retained cationic polymers.

We synthesized the epoxide-bearing, lipid-tethered polymer **3** (M_n: 12520, M_w: 17555, polydispersity index: 1.40) by atom transfer radical polymerization (ATRP) with oleyl initiator **1** (Scheme 1). The lipid moiety was tethered to poly(glycidyl methacrylate) (PGMA); PGMA is of great interest, as its further modification with epoxide groups would offer a simple synthetic route to the introduction of a variety of functional groups into a polymer chain.[5] The oleyl chain was employed as a membrane anchor because it did not affect cell signalling.[4] Oleyl-PGMA **3** was conjugated with FITC and subsequently reacted with ethanolamine, ammonium or sulfuric acid to provide labeled polymers **5**, **6** and **7**, respectively. After coupling with ethanolamine, the polymer has a positive charge (due to secondary amino groups) and is water soluble.[6] The primary amino groups were introduced to the polymer side chain through a reaction in ammonia solution.[7] The acid-catalyzed epoxide-opening reaction provides a water-soluble nonionic polymer.[8] Cationic polymers **5** and **6** and nonionic polymer **7** were incubated separately with HeLa cells for 10 min, and the localization of the polymer was observed by using CLS microscopy. As shown in Figure 2A, strong fluorescence was observed at the periphery but not in the cytoplasm of cells after incubation with polymer **5**. Relative fluorescence intensities of FITC and Hoechst 33342 along the dotted lines drawn on the image clearly demonstrate the retention of polymer **5** on the cell surface (Figure 2B). On the other hand, cationic polymer **6**, which has primary amino groups, was rapidly taken up into the cell (Figure 2A). It is well known that cationic polymers are rapidly internalized into cells, and thus they have traditionally been used as DNA transfection reagents.[9] In fact, highly positively charged polymers, such as polyethyleneimine (PEI, M_w = ~10000) and poly-L-lysine (PLL, M_w: 30000–70000),

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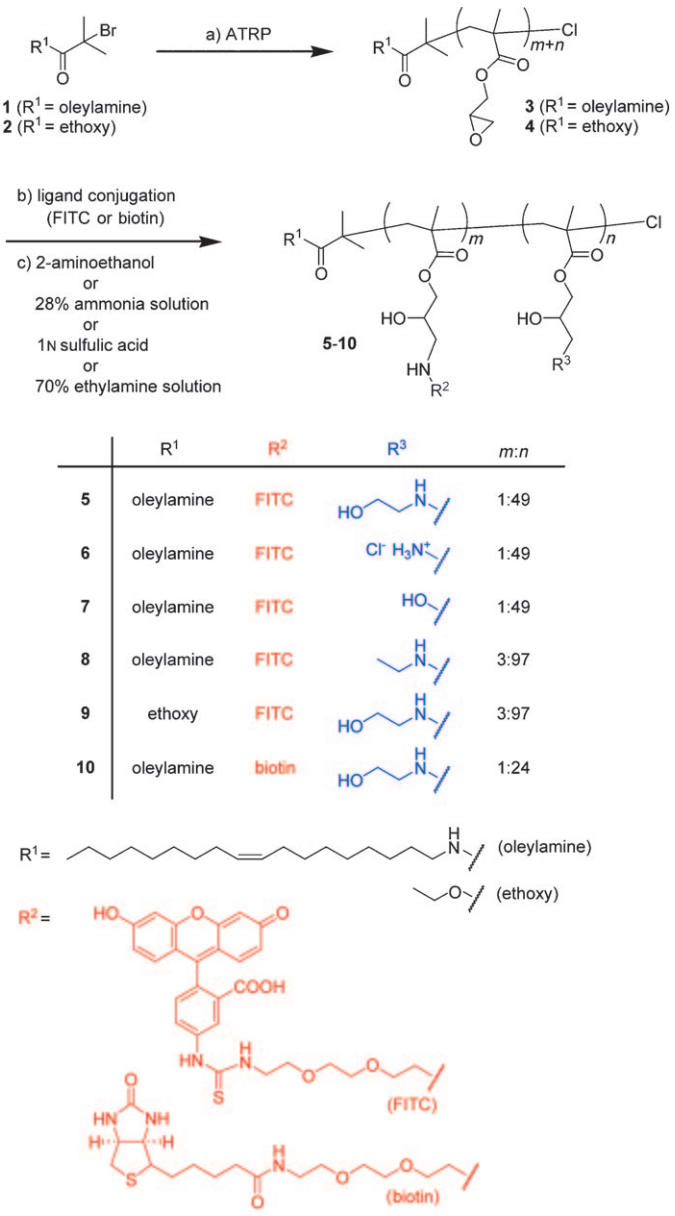

	R^1	R^2	R^3	m:n
5	oleylamine	FITC	HO	1:49
6	oleylamine	FITC	$Cl^- H_3N^+$	1:49
7	oleylamine	FITC	HO	1:49
8	oleylamine	FITC		3:97
9	ethoxy	FITC	HO	3:97
10	oleylamine	biotin	HO	1:24

Scheme 1. Synthetic scheme and chemical structures of cell membrane retained-cationic polymers.

were immediately internalized into the cells (see the Supporting Information). Therefore, we speculated that unlike the primary amine-displaying cationic polymers, secondary amine-displaying polymer **5** does not induce rapid endocytosis due to its weak charge interaction with the cell-surface membrane. No fluorescence was observed after the addition of nonionic polymer **7** (Figure 2A). In order to investigate the effect of the terminal hydroxyl group on the side chain of polymer **5**, we synthesized polymer **8**, which has a number of the ethyl groups on its side chain. After its addition to HeLa cells, polymer **8** was localized on the surface of cells in a similar manner to that of polymer **5** (Figure 2A). These results indicate that the binding of the polymer is caused by charge-mediated interactions between the cationic charge of the polymer and the negatively charged cell membrane. Interestingly, polymer **9**, which has a short anchoring moiety constructed by the substitution of an oleylamine to an ethoxy group, also bound to the cell membrane. These data indicate that the presence of secondary amines, rather than the anchoring effect of the lipid moiety, is essential for polymer retention on the membrane. Quantitative flow cytometry revealed that the binding of polymer **5** was four-fold higher than that of polymer **9** (Figure 2C). This shows that the lipid moiety of these polymer acts as an anchor to the membrane. However, we believe that the lipid moiety is effective in increasing the initial rate of plasma membrane modification due to its hydrophobic character, but has little effect on the retention of the polymer on the cell surface.

We analyzed the effects of polymer **5** on the endocytosis of HeLa cells. Baba et al. have reported that endocytosis of transferrin through the transferrin receptor was inhibited in poly(ethylene glycol)-linked cholesterol-treated cells.[10] Using the same assay system, we tested the endocytosis of AlexaFluor 546-labelled transferrin after the addition of polymer **5**. In polymer **5**-treated cells, internalization of transferrin was almost totally blocked (Figure 3A), whereas, without the addition of polymer **5**, transferrin immediately accumulated inside the cells (Figure 3B). These results indicate that the endocytic activity of cells was impaired because the cationic polymer **5** fully covered the cell surface.

The molecular weight of a polymer is also an important factor in determining cellular uptake. Small molecules such as fluorescein-labeled 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanol amine (FITC-DOPE, Avanti Polor Lipids, Inc.) are rapidly taken into the cell by passive diffusion (Supporting Information). Thus, we investigated the effect of the molecular weight of the polymer on its retention time on the cellular plasma membrane. Three biotin-labeled polymers **10a–c**, with different molecular weights (51900, 19400 and 8640, respectively) were prepared. Since FITC-labeled streptavidin is only accessible to biotin displayed on the cellular surface (Supporting Information), the amount of membrane-retained polymer can be estimated. After treatment of HeLa cells with polymer **10a–c**, incubation for 0–12 h, and the subsequent addition of FITC-avidin, flow cytometry analyses were carried out as a function of time (Figure 4). In the case of polymer **10a** (M_w: 51900), one-fourth of the polymer was retained on the membrane after 2 h, and more than 10% of the polymer was retained even after incubation for a period of more than 12 h. Approximately 5% of polymer **10b** (M_w: 19400) remained after 12 h, whereas polymer **10c** (M_w: 8640) was removed from the cell surface within 6 h. These results indicate that increasing the molecular weight of the polymer increases its retention time on the plasma membrane. As a control, we performed the same experiment using biotin-labeled DOPE (M_w: 992, Avanti Polor Lipids, Inc.). Due to its rapid internalization, we could not accurately follow the time-course as we did in Figure 4. However, after incubation for 30 min with biotin-labeled DOPE, no binding of the FITC-avidin was observed. These results indicate that small molecules such as DOPE are not retained on the cell plasma membrane, but rapidly migrate into the cells within a few minutes. This result corresponds with that observed in the CLS images taken the addition of FITC-DOPE.

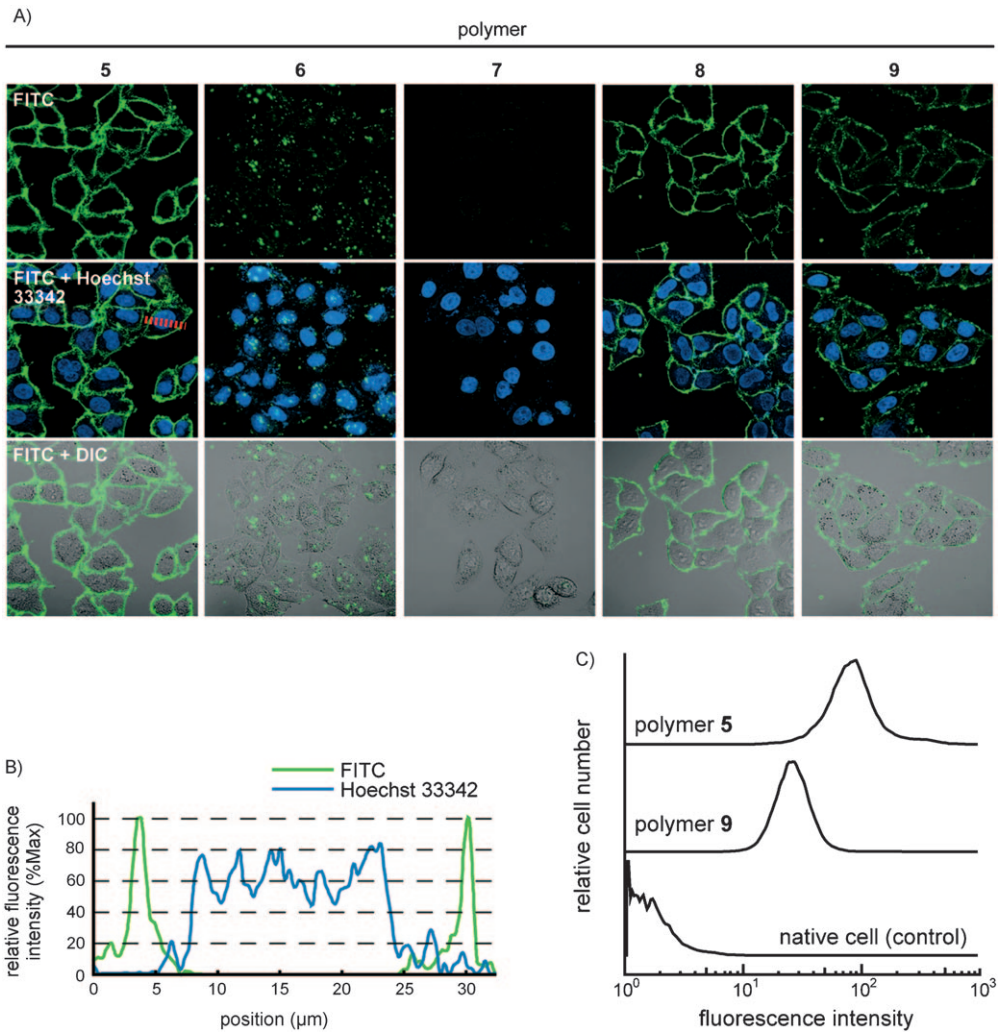

Figure 2. A) CLS and differential interference contrast (DIC) microscopy images of live HeLa cells treated with secondary amine-displaying cationic polymer **5**, primary amine-displaying cationic polymer **6**, nonionic polymer **7**, and polymers **8** and **9** for 10 min. The nuclei of the cells were stained with Hoechst 33342 before imaging. B) The relative fluorescence intensities of FITC and Hoechst 33342. Position (x-axis) refers to the location on the dotted red line shown in A) for polymer **5**. C) Flow cytometry analysis of the HeLa cells treated with polymers **5** and **9**.

The number of biotin molecules displayed on the cell surface after addition of polymer **10a** was calculated to be $7.34 \pm 0.20 \times 10^8$ biotin moieties per cell by using a 2-[4′-hydroxyazobenzene]benzoic acid (HABA) displacement assay.[11] Assuming that HeLa cells are spherical bodies with a diameter of 22 μm, the cell surfaces were estimated to be completely covered with biotin molecules. For the further application of these polymers to live cell studies, we assessed their toxicity using a lactate dehydrogenase (LDH) release assay (Supporting Information). According to these studies, the addition of polymer **5** and **10a** up to a concentration of 100 $\mu g\,mL^{-1}$ and for as long as 24 h had little effect on cell viability.

By using the molecular recognition of the ligands displayed on the cell surface, we tested the potential application of this method for the control of cell adhesion events. The controlled immobilization of cells onto a defined surface is an important step toward the development of array-based cellular analytical systems, drug screening platforms and tissue engineering.[12] In many cases, cell-micropatterns have been made by employing naturally adhering ligands, such as RGDS peptides and fibronectin.[13] It has been, therefore, difficult to extend this approach to a wide range of cell types that do not have such specific receptor ligands. We demonstrate the fabrication of a cell-micropattern

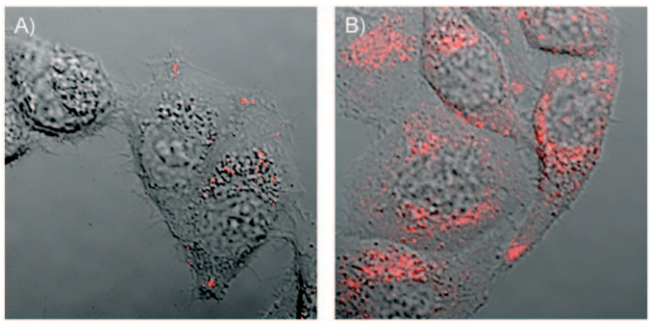

Figure 3. Effect of a cell surface-retained polymer on the endocytosis of HeLa cells. CLS and DIC microscopy images of A) polymer **5**-treated HeLa cells and B) native HeLa cells (control) after incubation with AlexaFluor 546-labeled transferrin for 10 min.

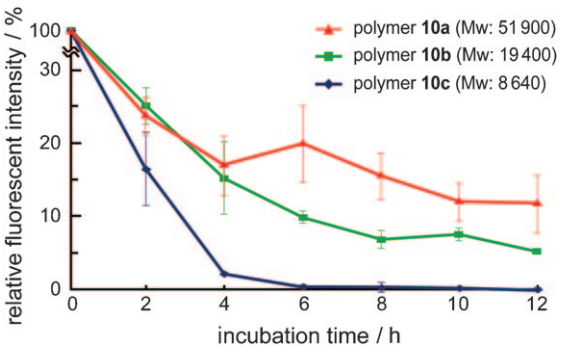

Figure 4. Effect of polymer molecular weight on cellular retention time. HeLa cells were treated with polymers **10a**–**c**, washed, incubated for the time shown on the x-axis, and then FITC-avidin was added. The values were normalized to the mean fluorescence intensity at $t = 0$. Results are expressed as mean ±SD ($n = 3$).

based on the biotin-streptavidin interaction between biotin displaying-cells and a streptavidin-patterned substrate. Biotin-grafted polymer **10a**-treated HeLa cells were seeded on an avidin-patterned surface (Figure 5A). Figure 5B clearly shows

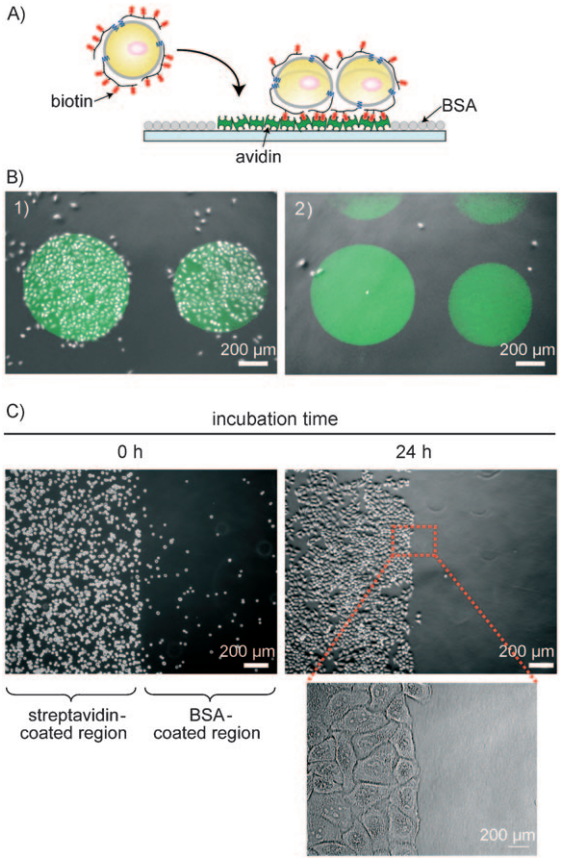

Figure 5. A) Schematic illustration of site-specific immobilization of Hela cells through interaction with biotin-avidin. B) Phase contrast images of: 1) polymer **10a**-treated cells; 2) non-treated HeLa cells (control experiment). Fluorescent images of FITC-avidin were merged. C) Phase contrast images of polymer **10a**-treated HeLa cells cultured on the patterned surface. The left region of the substrate was coated with streptavidin, and the right region was treated with BSA.

that the HeLa cells only adhered to the avidin-immobilized regions. No patterned immobilization was observed using native HeLa control cells. In Figure 5C, polymer **10a**-treated cells immediately adhered to the avidin-immobilized region but not to the bovine serum albumin (BSA)-immobilized region. After incubation for 24 h, the cells were only spread over the avidin-immobilized region. These data demonstrate that ligands displayed on the cell surface can efficiently participate in surface binding.

In summary, we propose a cell surface modification method based on the use of the synthetic polymers that can be retained on the cell surface without rapid internalization. The systematic synthesis of polymers revealed that the introduction of secondary amines into the polymer significantly elongates their retention time on the cellular membrane. Our data support that the localization of polymers on the cellular membrane is due to electrostatic interaction between the secondary amine and the negative-charged cell surface rather than through the effect of the terminal hydroxyl group. Attachment of a lipid moiety to the polymer enhanced binding to the cell membrane due to its hydrophobicity. Furthermore, our secondary amine-containing polymers showed almost no cytotoxicity. Thus, these molecules can act as an effective scaffold for the display of artificial ligands; this provides a basic methodology through which to control cellular behavior, including specific cell attachment and cell–cell contact.

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