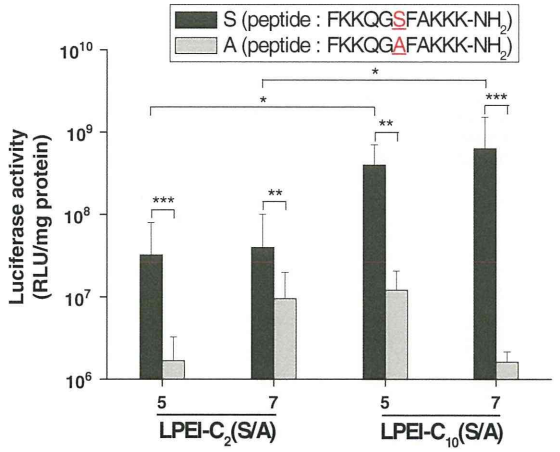

Fig. 8. Transfection of polyplexes of LPEI-peptide conjugates with luciferase encoding pDNA into A549 cells (n = 3). Data were expressed as the RLU/mg protein and the mean ± standard deviation from three different measurements (*p < 0.05, **p < 0.01, and ***p < 0.005).

LPEI-C_2(A). This would be caused by the stabilization of the polyplex via the enhanced hydrophobic interactions in LPEI-C_{10}(A). As a result of both the higher gene expression in LPEI-C_{10}(S) and the suppressed gene expression in LPEI-C_{10}(A), the LPEI(S)/LPEI(A) ratio [defined as the ratio of gene expression from LPEI(S) and that from LPEI(A)] was improved in LPEI-C_{10} from LPEI-C_2. Notably, LPEI-C_{10}(S) showed a 390 times higher gene expression than LPEI-C_{10}(A) at the *N/P* ratio 7.

4. Conclusions

Here, we demonstrated the ability of a hydrophobically modified LPEI-peptide conjugate as a cancer-specific gene carrier. A simple modification of a long alkyl chain as a spacer between the LPEI main chain and a substrate peptide enhanced the cellular uptake of the resultant polyplex because of the effective stabilization of the polyplex via hydrophobic interaction. It is important to note that the alkyl chain spacer did not affect the reactivity of the substrate peptide toward PKCα and the endosomal escaping ability. Because of these advantageous characteristics of the hydrophobically modified polyplex, the polyplex showed excellent performance in the cancer-signal responsive gene expression. Thus, the hydrophobically modified LPEI-peptide conjugate represents a promising carrier for cancer-specific gene therapy especially when cytotoxic suicide genes are employed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.06.011.

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Rapid and serum-insensitive endocytotic delivery of proteins using biotinylated polymers attached *via* multivalent hydrophobic anchors

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ABSTRACT

We have designed biotinylated polymers as synthetic receptors that have multiple alkyl groups for endocytotic delivery of target proteins. The polymers were stably attached to a cell surface via multivalent anchoring. The presented biotin was bound to streptavidin (SA) on the cell surface, and, via an endocytotic pathway, the cell rapidly internalized the biotinylated polymer/SA complex. The cell's uptake of the complex was not inhibited by the presence of 10% fetal bovine serum, and its efficacy for the uptake of SA was the highest when compared with commercial reagents and single-anchored-type synthetic receptors. The synthetic receptor-mediated endocytosis can be used generally for other kind of protein by using SA as an adaptor molecule between a target protein and the cell-surface presented biotin.

1. Introduction

Intracellular delivery of DNA, RNA, and proteins is a fundamental process in biochemistry and biology. Proteins are regarded to be the most difficult to deliver because of their heterogeneous nature [1]. Intracellular protein delivery is needed in cancer immunotherapy [2] and for preparation of induced-pluripotent stem cells [3]. Many protein delivery reagents that form complexes with the protein have been reported, and can be categorized as lipid [4,5] peptide [6], or synthetic polymers [7], possessing cationic charges to interact with both the protein and the cell membrane. However, these cationic reagents are difficult to apply to weak anionic or cationic proteins.

To be applicable to a wide variety of proteins, synthetic receptors should be designed by learning from endogenous cellular receptors. The endogenous receptors are categorized into those with transmembrane structures and those that are lipid-based such as glycosylphosphatidylinositol (GPI)-anchored receptors [8,9] and glycosphingolipids [10]. Because of their simple structures, lipid-based receptors are good models for designing synthetic receptors.

Peterson et al. were the first to design synthetic receptors, such as cholesterol-modified biotin [11] or a short peptide [12–14], that successfully delivered target proteins into cells by endocytosis. However, because the receptors are hydrophobic, its modification on the cell surface will not be efficient because of its low solubility in water. Silvius et al. reported a synthetic receptor that was a lipid modified with biotin via a long hydrophilic polyethyleneglycol spacer [15,16]. Receptor modification thus improved because of its relatively hydrophilic character. However, a single lipid may not stably anchor the receptor to the cell surface.

Here, we report on polymeric synthetic receptors that have multiple alkyl groups and biotin, as shown in Fig. 1. The alkyl groups allow the receptors to be anchored stably on the cell surface, while the biotin binds with SA. Not being endogenous, the synthetic receptors cannot actively induce receptor-mediated endocytosis. However, because endocytosis is known to take place frequently [17], the receptor/SA complex that diffuses into endocytosis-initiating membrane domains will "sneak" into an endosome. We applied this synthetic receptor-mediated endocytosis for intracellular delivery of a target protein by using SA as a crosslinker between the receptor and the protein.

2. Experimental methods

2.1. Materials

Dextran (Dex, M_w 40,000), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), and triethylamine (TEA) were purchased from Tokyo Kasei Industry (Japan). Ethylenediamine (EDA), biotin, fluorescein isothiocyanate (FITC), *N,N′*-carbonyldiimidazole (CDI), *N,N*-dimethyl-4-aminopyridine (DMAP), dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF), ethanol, 2-propanol and cytochalasin D were purchased from Wako Pure Chemicals (Osaka, Japan). Palmitoyl chloride and lithium chloride were purchased from Kanto

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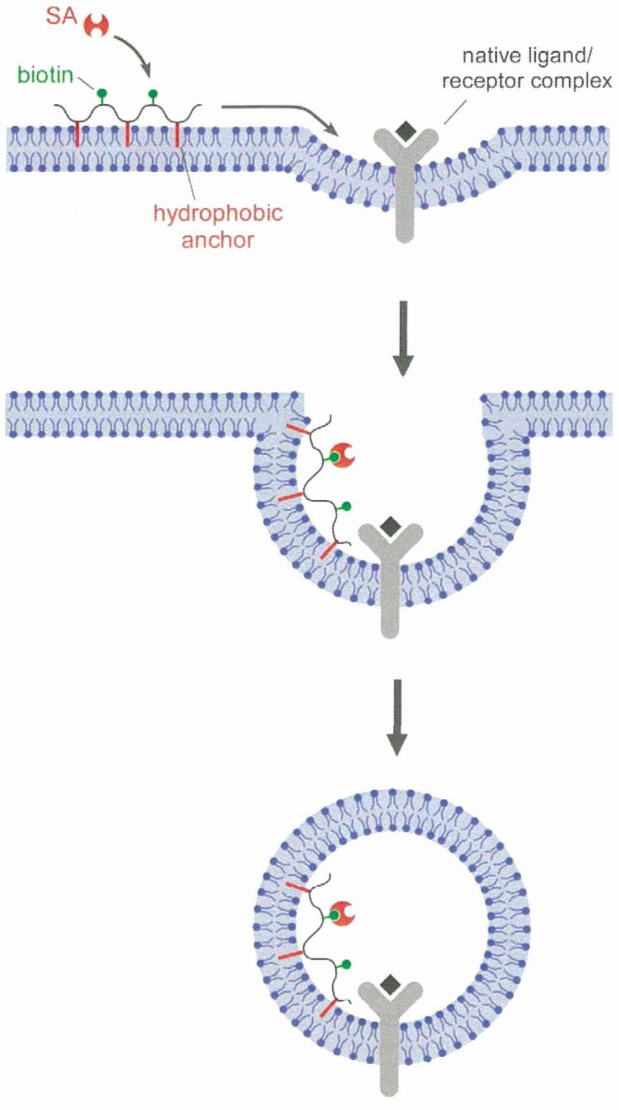

Fig. 1. A biotin-modified polymer anchored via multivalent hydrophobic anchors is used as synthetic receptor for streptavidin (SA). Polymer/SA complex which is formed on the cell surface diffuses into endocytosis-initiating membrane domain and then taken up via endocytosis.

Chemical (Tokyo, Japan). FluoroLink™Cy™3-labeled streptavidin (Cy3-SA) was purchased from Amersham Biosciences (UK). FITC-Streptavidin (FITC-SA) was purchased from Wako Pure Chemicals (Osaka, Japan). Hoechst 33342 and Lysotracker®Red DND-99 were purchased from Invitrogen (USA). NHS-Rhodamine was purchased from Thermo Scientific (Japan). CarryMax-R, CarryMax-H and biotin-PE were kindly provided by Dojindo Laboratories (Japan).

2.2. Synthesis of Dex-EDA

After dissolving dextran (200 mg) into 20 mL of DMSO, CDI (1.24 mmol) was added and the solution stirred at room temperature for 4 h. EDA (15 mmol) was added dropwise to avoid dextran crosslinking, and the solution was stirred at room temperature for 24 h. The resulting polymer was reprecipitated, and the aqueous polymer solution was dialyzed with a Spectra/Por6 dialysis membrane (MWCO 10,000) against water for 3 days. After lyophilization, the polymer was obtained as white powder (yield = 129 mg). The degree of substitution was determined by ^{1}H NMR (nuclear magnetic resonance, Bruker BioSpin).

^{1}H NMR (300 MHz, D_2O): δ 2.95 (2H, $NH_2CH_2CH_2NHCO$), 3.37 (2H, $NH_2CH_2CH_2NHCO$), 3.51–3.99 (6H, protons without anomeric proton), 5.00 (1H, anomeric proton).

2.3. Synthesis of Dex-EDA-biotin

Dex-EDA (80 mg: containing 0.1 mmol EDA) was dissolved in DMSO (8 mL). Biotin (25 μmol) and DMT-MM (37 μmol) were added and the resulting solution was stirred at room temperature overnight. After the reaction, the solution was dialyzed with a Spectra/Por6 Dialysis bag (MWCO 10,000) against water for 3 days and then lyophilized. The degree of substitution was determined by ^{1}H-NMR.

^{1}H NMR (300 MHz, D_2O): δ 1.46 (2H, $(CH)_2CH_2CH_2(CH_2)_2CONH(CH_2)_2NHCO$), 1.65 (4H, $(CH)_2CH_2CH_2CH_2CH_2CONH(CH_2)_2NHCO$), 2.29 (2H, $(CH)_2$ $(CH_2)_3CH_2CONH(CH_2)_2NHCO$), 2.79 (1H, $CH_2CHSCH(CH_2)_4CONH(CH_2)_2NHCO$), 3.05 (1H, $CHCH(CH_2)_4CONH(CH_2)_2NHCO$), 3.51–3.99 (6H, protons without anomeric proton), 4.46 (1H, $CHCH(CH_2)_4CONH(CH_2)_2NHCO$), 4.63 (1H, $CHCH_2SCH(CH_2)_4CONH(CH_2)_2NHCO$), 5.01 (1H, anomeric proton).

2.4. Synthesis of Dex-EDA-biotin-palmitoyl

Dex-EDA-biotin (20 mg) was dissolved in LiCl/DMF (20 mg/2 mL) at 80 °C for 30 min. Then, palmitoyl chloride (10.4 μmol) and DMAP (20.8 μmol) were added and reacted at 80 °C for 30 min, followed by stirring at room temperature for 15 h. After the reaction, the solution was reprecipitated using 2-propanol and filtered. The resulting polymer was dialyzed with a Spectra/Por6 Dialysis bag (MWCO 10,000) against water for 3 days and then lyophilized. The degree of substitution was determined by ^{1}H-NMR.

^{1}H NMR (300 MHz, D_2O): δ 0.88 (3H, $CH_3(CH_2)_{14}CONH(CH_2)_2NHCO$), 1.27 (24H, $CH_3(CH_2)_{14}CONH(CH_2)_2NHCO$), 1.62 (2H, $CH_3(CH_2)_{12}CH_2CH_2CONH(CH_2)_2NHCO$), 2.28 (2H, $CH_3(CH_2)_{12}CH_2CH_2CONH(CH_2)_2NHCO$), 3.51–3.95 (6H, protons without anomeric proton), 5.01 (1H, anomeric proton).

2.5. FITC or rhodamine labeling of Dex-EDA-biotin-palmitoyl

Dex-EDA-biotin-palmitoyl (10 mg) was dissolved in DMSO (1 mL). Then, FITC (0.93 μmol) and TEA (5.6 μmol) were added and stirred at room temperature for 24 h. After the reaction, the solution was dialyzed with a Spectra/Por6 Dialysis bag (MWCO 10,000) against a methanol/water (= 1/1) solvent for 1 day and water for 2 days. The solution was then lyophilized. The degree of substitution was determined by a UV-2550 ultraviolet/visible spectrophotometer (Shimadzu). Rhodamine labeling of Dex-EDA-biotin-palmitoyl was conducted similarly. The resulting polymer was dissolved in water or DMSO and the solution was sterilized by filtration (0.22 μm pore size). The polymer solutions were stored at 4 °C.

2.6. Preparation of rhodamine- and biotin-modified OVA

One mg/ml NHS-rhodamine in anhydrous DMSO was added dropwise into 10 mg/ml OVA in a 50-mM sodium bicarbonate solution (pH 8.5) at a 1:2 molar ratio, and the resulting mixture was stirred for 5 h to form rhodamine-modified OVA. The unreacted rhodamine was removed with a VIVASPIN 20 tube (MWCO 30,000). Biotin modification to the resulting rhodamine-modified OVA was conducted similarly with NHS-biotin. The content of rhodamine in one OVA was calculated to be 0.6.

2.7. Cell culture

K562 cells were cultured in a RPMI-1640 medium (Wako) containing 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% CO_2 at 37 °C.

2.8. Modification of cells with polymer

K562 cells (1×10^6) were collected by centrifugation. After removal of the supernatant, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS). Then, 50 μL of polymer (0.1 mg/mL, containing 1.8 nmol of biotin) dissolved in DPBS was added to the cell suspension and incubated for 30 min with gentle agitation at 4 °C. After cell surface modification, DPBS (2 mL) was added to the mixture and the cells were collected by centrifugation. These procedures were repeated twice to remove unbound polymer. The cells were then incubated in 1 mL of 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% CO_2. Fluorescence intensities of the polymer on the cell surface were estimated with a BZ-8000 fluorescence microscope (Keyence) and a Tali™ Image-Based Cytometer (Life Technologies).

2.9. Cellular uptake of SA and SA/OVA complex

K562 cells were modified with polymer 2 or 3 according to the same procedures described above. Then, Cy3-SA (0.07 nmol) was added to the polymer 2-modified K562 cell (1×10^5) dispersion in RPMI-1640 medium (100 μL) and was gently agitated. FITC-SA was mixed with rhodamine- and biotin-modified OVA at a 1:1 ratio to prepare the SA/OVA complex. The resulting complex (0.07 nmol) was added to the polymer 3-modified K562 cell (1×10^5) dispersion in RPMI-1640 medium (100 μL) and was gently agitated.

As for DSPE-PEG-biotin and PE-biotin, they were modified on K562 cells according to the same procedures with polymer 2. They were first dissolved in DMSO and diluted with DPBS to prepare solutions for the cellular modification. The biotin concentration of DSPE-PEG-biotin and PE-biotin in the solutions for the cellular modification was adjusted to be the same with the solution of polymer 2 (1.8 nmol).

2.10. Cytotoxicity evaluation of polymer

Cytotoxicity of the polymer was evaluated using a WST-8 kit (Dojindo, Kumamoto, Japan). K562 cells were modified with different polymer concentrations (0.01, 0.1 mg/mL) and placed in a 96-well plate at an initial density of 7500 cells/well and incubated for 4 h at 37 °C. To measure cell viability, 10 μL of WST-8 was added to each well and the cells were incubated for another 4 h at 37 °C. The WST-8 formazan absorbance at 450 nm was measured by a microplate spectrum reader AD200 (Beckman Coulter) and expressed as a percentage relative to non-modified cells.

2.11. Effect of endocytosis inhibitor

Cytochalasin D is known to specifically inhibit actin polymerization, and can therefore inhibit endocytosis. K562 cells (2×10^6 cells/mL) were collected by centrifugation. After removal of the supernatant, 10 μL of cytochalasin D (10 μM) was added to the cell suspension and incubated for 30 min with gentle agitation at room temperature and washed with DPBS. Then, 50 μL of polymer (0.1 mg/mL) was added to the cytochalasin D-treated cells and incubated for 30 min with gentle agitation at 4 °C. The cells were washed, incubated for 24 h at 37 °C under 5% CO_2, and examined with a fluorescence microscope.

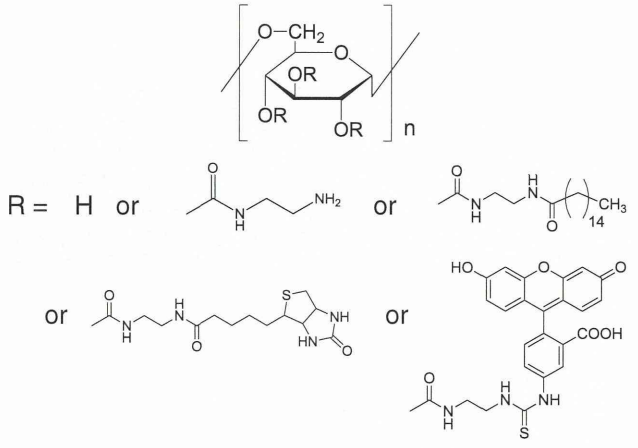

Chart 1. Structure of biotinylated polymers.

3. Results and discussion

3.1. Synthesis of polymers

Chart 1 depicts the structure of the biotinylated polymer that was synthesized by adding ethylenediamine to dextran using carbonyldiimidazole. Then other groups (palmitoyl, biotin, and FITC), were added to the dextran by amide or thiourethane bond formation with the amine group of a modified ethylenediamine. Three polymers (1–3) were obtained, and the content of each group per glucose unit is summarized in Table 1.

3.2. Polymer modification on cell surface

Polymer 1 was attached to K562 cells by mixing the polymer solution with a cell dispersion in PBS for 30 min at a low temperature (4 °C) to avoid endocytosis. Fig. 2A shows the fluorescence image of the cells right after modification with the polymer. The fluorescence from FITC labeling of polymer 1 was observed exclusively from the cell surface, and was not observed when the cells were mixed with palmitoyl-free dextran (data not shown). These results indicate that polymer 1 was successfully incorporated onto the cell surface via hydrophobic anchoring by palmitoyl groups in the cell membrane. The number of polymer chains attached to the cell surface was determined from the fluorescence intensity. Fig. 2B plots the number of the polymer chains present per single cell as a function of the polymer concentration used for the modification. The number of polymer chains increased with polymer concentration until saturation at 15×10^6 chains/cell at concentrations above 0.1 mg/mL. Because the average size of the cells was 8 μm, the polymer density on the cell surface was estimated to be 54 nm^2/chain. Assuming random coil conformations of the polymer chains with a hydrodynamic radius of 5 nm [18] on the cell surface, the surface area occupied by one polymer chain is 79 nm^2/chain. Thus there is good agreement between these densities, indicating that the

Table 1
Contents of each component in the biotinylated polymers.

Polymer	Content (mol%)				Number of palmitoyl[a]
	Ethylenediamine	Palmitoyl	Biotin	FITC	
1	16	5.4	–	0.6	13
2	5.7	10	5.0	0.3	25
3	18	6.0	8.1	–	15

[a] Number of palmitoyl groups per one polymer chain.

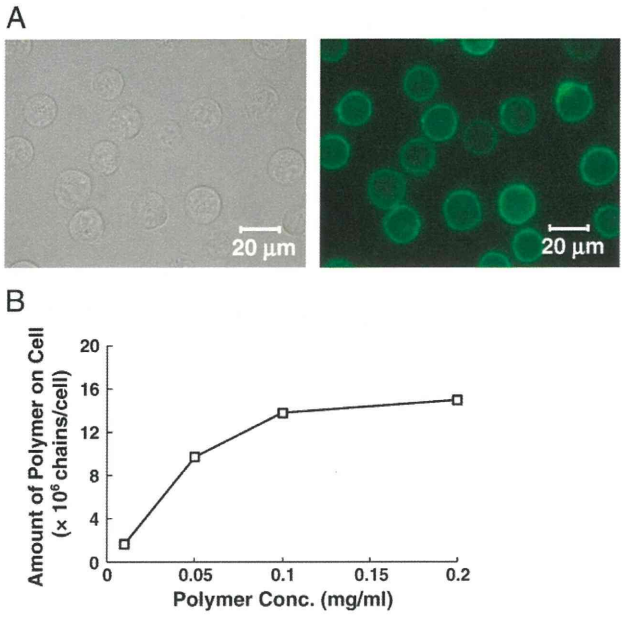

Fig. 2. (A) Bright field (left) and fluorescence images (right) of K562 cells modified with polymer 1. (B) Polymer concentration dependence of the number of polymer chains bound to the cell surface.

cell surface is almost filled with the polymer chains at the 0.1 mg/mL concentration.

We checked the cytotoxicity of the polymer with the WST assay. No cytotoxicity was observed at the polymer concentration of 0.1 mg/mL (Fig. S1), indicating that the hydrophobic anchors did not affect the membrane structure or any membrane protein functions. Hereafter the polymer modification was conducted at 0.1 mg/mL of the polymer concentration.

Fig. 3A shows the effect of incubation time at 37 °C in the presence of 10% FBS. With elapsed time, the polymer chains on the cell surface decreased and bright spots appeared inside of the cell, indicating cellular uptake of the polymer chains. These bright spots colocalized well with LysoTracker Red (Fig. 3B), revealing that the polymer was endocytosed and was contained within endosomes or lysosomes. The endosomes or lysosomes were adjacent to the nucleus (Fig. S2), which is an organizing center for the termini of microtubules [19–21]. Thus, endosomes containing polymer 1 were most likely delivered along the microtubules. It is important to note that the cellular uptake of polymer did not occur in the presence of the endocytosis inhibitor cytochalasin D, which prevents actin polymerization [22,23] (Fig. S3). This result supports the notion that polymer was taken up via endocytosis.

Fig. 4 shows the time-dependent decrease in fluorescence intensity from the cell surface in the presence and absence of 10% FBS. Both curves are virtually superimposable (no significant difference). After 24 h, the fluorescence intensity drops to 20 and 40% in the absence and presence of FBS, respectively. Thus, the residual 60 and 80% of polymer 1, respectively could either be taken up via endocytosis or dissociate from the cell membrane to be released into the surrounding medium. However, because the amount of polymer 1 released into the medium was found to be negligible, the residual 60 and 80% of polymer 1 will be mainly taken up via endocytosis.

3.3. Cellular uptake of streptavidin via endocytosis

Then, we checked the receptor-ligand recognition on the cell surface. Fig. 5A shows a fluorescence image after adding Cy3-labeled SA to cells modified with polymer 2 at 4 °C. The yellow color is exclusively

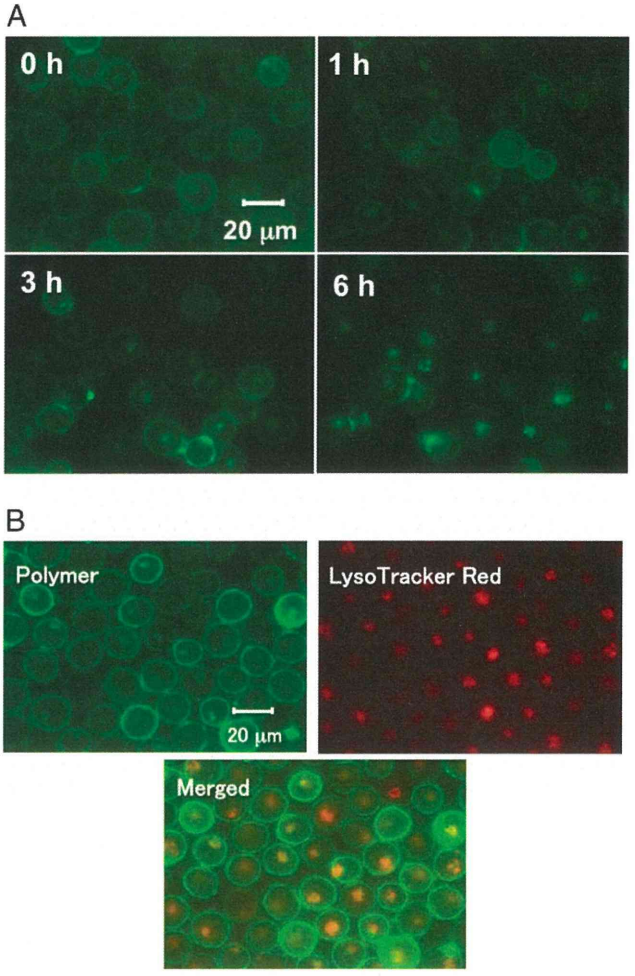

Fig. 3. (A) Time lapse images of cellular uptake of polymer 1. Cells were incubated at 37 °C after polymer modification. (B) Colocalization of polymer 1 with LysoTracker Red.

on the cell surface and is from colocalization of SA and the polymer, indicating that SA recognized the biotin attached to the polymer.

To internalize the cell surface SA/polymer complex, the cells were incubated at 37 °C. As shown in Fig. 5B, after only 1 h of incubation,

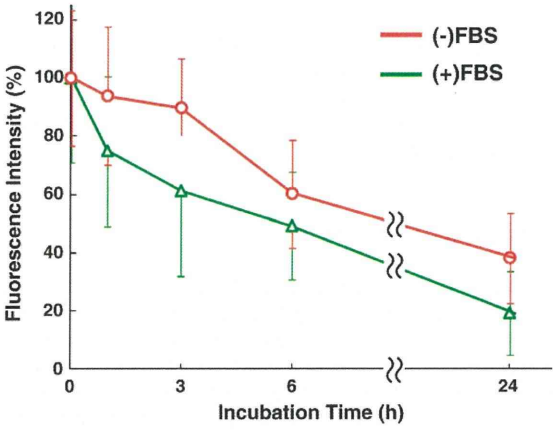

Fig. 4. Time-dependent change of polymer 1s fluorescence intensity on the cell surface. The fluorescence intensity of the cell surface was determined from the brightness of the cell surface of fluorescence images at each time point.

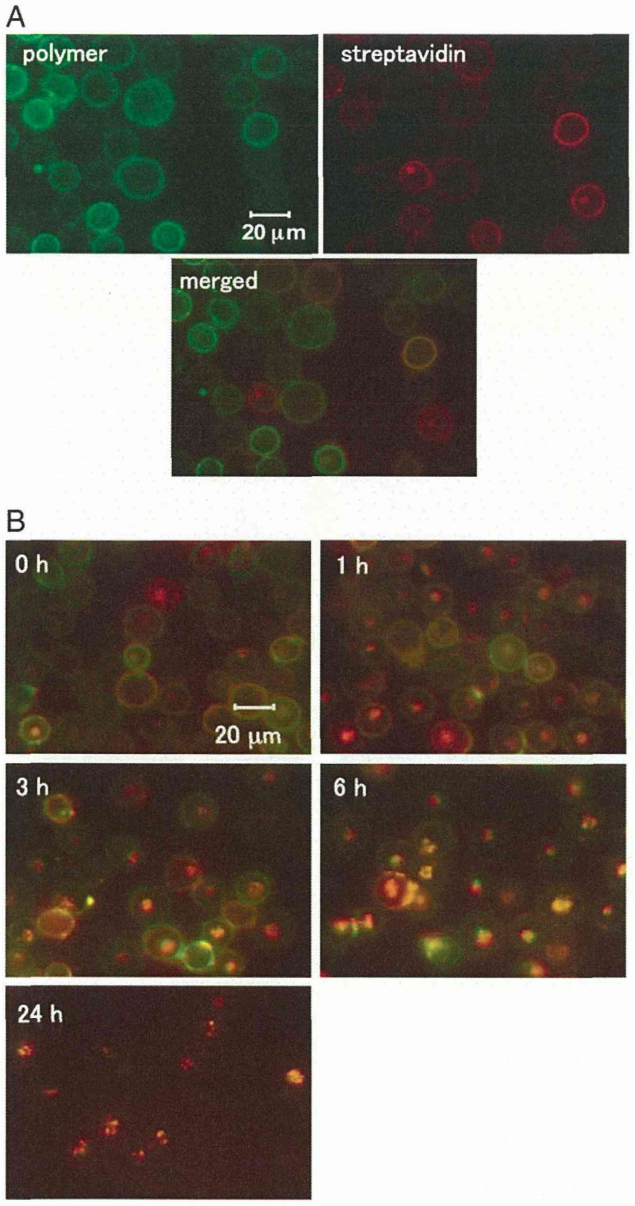

Fig. 5. Binding and cellular uptake of SA by K562 cells modified with polymer 2. (A) Microscopic images of cells modified with polymer 2 after treated with Cy3-labeled SA. (B) Cellular uptake of polymer 2/SA complex monitored by fluorescence microscopy at 37 °C. These are merged images of green and red fluorescence. Yellow color indicates the colocalization of polymer 2 and SA.

yellow fluorescence was clearly observed inside the cell. At longer times, the internal fluorescence became brighter, while the membrane fluorescence weakened and almost disappeared after 24 h. The cellular uptake of SA was not observed without polymer modification on the cell surface (Fig. S4). These results clearly indicate that the SA was taken up via endocytosis which is mediated by the biotinylated polymer.

According to the fluorescence images (Fig. 5B), the amount of SA transferred to the cytoplasm does not seem to be large even after 24 h. However, in order to apply to antigen protein delivery to dendritic cells, cytoplasmic delivery is not a prerequisite and our technique will be applicable to antigen delivery.

Fig. 6 shows the results of fluorescence cytometry in the presence or absence of 10% FBS. In both cases, the cellular uptake of SA increased with incubation time and, importantly, the amounts of SA taken up in

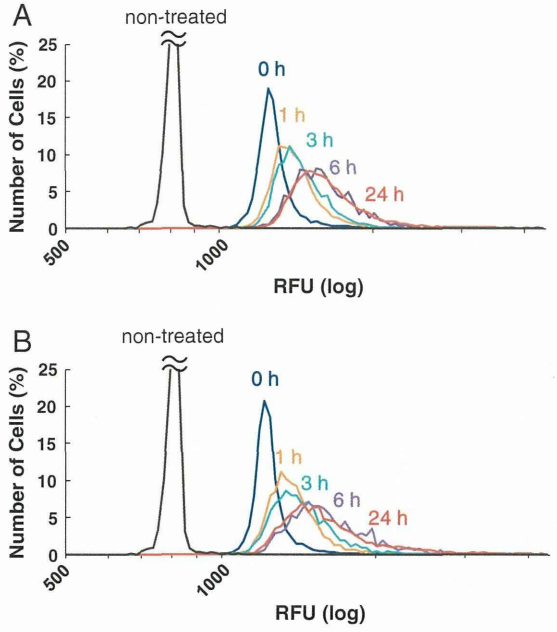

Fig. 6. Fluorescence cytometry analysis of polymer 2-modified K562 cells after mixing with Cy3-labeled SA in the presence (A) and absence (B) of 10% FBS in the medium.

both cases were almost identical for each time interval. Most of the protein-introducing reagents require serum free conditions for cellular uptake [5–7] because serum proteins disrupt the protein/reagent complex. Thus, no FBS interference in the biotinylated polymer-mediated endocytotic is advantageous. The increasing cellular uptake of SA over time may indicate recycling of the biotinylated polymer via a recycling endosome; this is observed for natural receptors such as transferrin and G protein-coupled receptors [24–28].

3.4. Comparison of cellular uptake with commercial reagents and other synthetic receptors

We compared the performance of intracellular SA delivery via endocytosis with that of commercially available cationic lipid-based reagents for protein introduction (CarryMax-R and CarryMax-H) and with that of other synthetic receptors that possess a single lipid anchor [PE-biotin, DSPE-PEG-biotin (MW of PEG = 2 k)]. The commercial reagents were used as recommended by the provider. The same procedures as discussed above were used for the synthetic receptors, with the same biotin concentration. As shown in Fig. 7B, the biotinylated polymer 2 had the best performance. The smaller cellular uptake via the other two synthetic receptors probably results from losses of these receptors from the cell membrane due to dissociation of the hydrophobic anchor for DSPE-PEG biotin and membrane permeation for PE-biotin. The relative low solubility of the two synthetic receptors may also be the reason for the smaller cellular uptake of SA due to the inefficient cell surface modification of these two receptors. Thus, polymer 2, which has more stable multivalent alkyl anchors, is an effective synthetic receptor for SA binding and delivery into cells.

3.5. Endocytotic delivery of target protein

We demonstrated the delivery of a target protein based on the biotinylated polymer-mediated endocytosis. As shown in Fig. 8A, a target protein, ovalbumin (OVA), was modified with biotin by using biotin-succinimide and was mixed with an equimolar quantity of SA to prepare an SA/OVA complex. The complex was then transported via

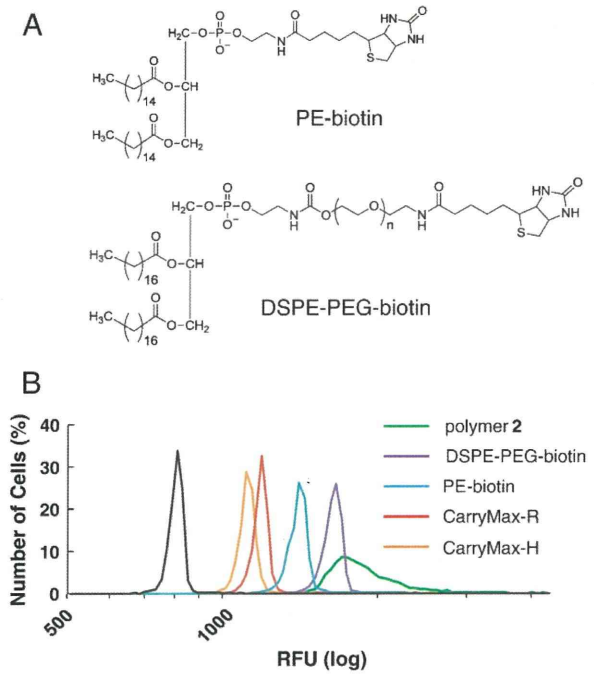

Fig. 7. (A) Chemical structures of synthetic receptors. (B) Comparison of cellular uptake by polymer 2 with commercial reagents and other synthetic receptors.

endocytosis into cells with membranes modified with polymer 3. Fig. 8B shows the fluorescence images after mixing the SA/OVA complex with the cells. The SA (FITC) and OVA (rhodamine) fluorescence images indicated colocalization on the cell surface, verifying that the SA/OVA complex recognized the biotin-modified on the cell surface. After 1 h of incubation at 37 °C, strong fluorescence of SA/OVA complex was observed inside the cells, demonstrating the successful uptake of the SA/OVA complex via the biotinylated polymer-mediated endocytosis. As a negative control, we examined the cellular uptake of biotin-non-modified OVA based on the same procedures as the above-mentioned biotin-modified OVA (Fig. S5). Rhodamine's fluorescence resulting from OVA detected neither on the cell surface nor inside of the cell. These results clearly show that the cellular uptake of OVA was mediated by SA which functions as a crosslinker between the cell surface biotin and OVA.

We further examined the effect of the amount of the cell surface-modified polymer 3 on the cellular uptake of OVA. Polymer 3 was modified on the K562 cell surface with varying concentrations. To the modified cell was added SA/OVA complex and incubated for 1 h at 37 °C for endocytosis. The fluorescence intensity inside of the cells which is originated from the taken up OVA was plotted against the polymer concentration in Fig. 9. The cellular uptake of OVA proportionally increased with the polymer concentration. Thus to raise the amount of cellular uptake of OVA, the modification of polymer 3 on the cell surface should be higher.

4. Conclusions

We presented a method for the intracellular delivery of proteins based on the biotinylated polymer-mediated endocytosis. The biotinylated polymers were modified with multiple palmitoyl groups and stably bound to the cell surface via multivalent anchoring in the cell membrane. Biotin molecules presented on the cell surface by the polymer recognized SA and the resulting SA/biotinylated polymer complex was taken up rapidly by the cells via the endocytotic pathway. The endocytosis was not affected by the presence of 10% FBS. The biotinylated polymers enabled the uptake of an OVA/SA

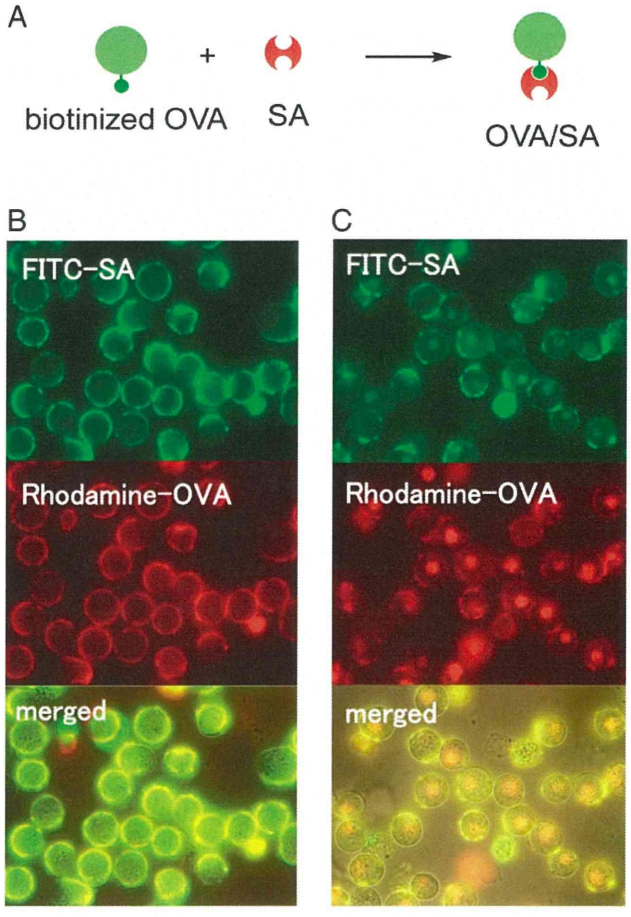

Fig. 8. Complex formation between biotin-modified OVA and SA for cellular uptake (A). Cellular uptake of SA/OVA complex by K562 modified with polymer 3 in serum containing medium right after (B) and 1 hour after (C) the complex addition.

complex. Thus, the biotinylated polymer-mediated endocytosis was found to be potentially applicable to any kinds of proteins.

Acknowledgment

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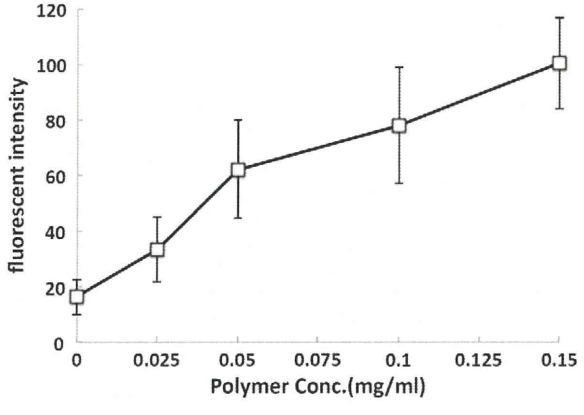

Fig. 9. Effect of polymer 3 concentration used for the K562 surface modification on the fluorescence intensity inside of the cell resulting from the taken up OVA.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.12.024.

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Short communication

Liver cell-specific peptides derived from the preS1 domain of human hepatitis B virus

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A B S T R A C T

The envelope of human hepatitis B virus (HBV) consists of the large (L), middle (M), and small (S) surface proteins. The preS1 domain at the N terminus of the L-protein is essential for recognizing a target cell and for viral infectivity. In the present study, peptides derived from the preS1 domain (amino acid residues 2–19) were synthesized, and their binding affinities for human hepatocellular carcinoma (HCC) cells were determined. Non-myristoylated peptides showed much lower affinity for HepG2 cells than myristoylated peptides. Although all peptides showed significantly higher affinities for two human HCC cell lines (HepG2 and HuH-7) compared with other cell lines (HeLa, B16, NMuLi, and NIH 3T3), a modified peptide exhibited the highest affinity for HCC cell lines. These results suggest that the modified peptide can target liver cells.

The human hepatitis B virus (HBV) is a small enveloped DNA virus that causes acute and chronic infections of the liver. The HBV envelope consists of the large (L), middle (M), and small (S) surface proteins. L- and M-proteins contain the S-protein [226 amino acid (aa) residues] and 55 hydrophilic aa residues at the N terminus of the S-protein (preS2), and the L-protein has an extension of hydrophilic residues (108 or 119 depending on genotype) (called preS1) at the N terminus of preS2 (Glebe and Urban, 2007; Meier et al., 2013; Seeger and Mason, 2000).

preS1 is essential for virus infection, but not preS2. preS1 is myristoylated at glycine residue in position 2 and myristoylation increases viral infectivity (Glebe et al., 2005; Glebe and Urban, 2007; Meier et al., 2013; Seeger and Mason, 2000). Further, the preS1 domain has a receptor binding site containing essential aa residues 9–18 and recognizes the asialoglycoprotein receptor on the surface of human hepatocytes or hepatocellular carcinoma (HCC) cells (Engelke et al., 2006; Glebe et al., 2005; Zhang et al., 2011).

In the present study, preS1-derived peptides were synthesized, and their binding specificity for human HCC cells was examined using two human HCC cell lines (HepG2 and HuH-7), the human epithelial carcinoma HeLa cell line, the mouse melanoma B16 cell line, the normal mouse liver epithelial NMuLi cell line, and the mouse embryo fibroblast NIH 3T3 cell line.

Fluorescent peptides labeled with FITC with or without myristic acid were synthesized by Scrum Inc. (Tokyo, Japan). The purities of the synthetic peptides were determined using high-performance liquid chromatography and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Peptides with purities >95% were used (Table S1 and Fig. S1).

All cell lines were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen Co., Grand Island, NY, USA). Media were supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (all from Gibco). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

To determine the affinity of peptides for each cell line, cells (5×10^5) were grown in 6-well plates at 37 °C for 24 h. After 24 h peptides (5 μM) were added to each plate and were incubated for 24 h. Micrographs of the cells were obtained using fluorescence microscopy, and images were analyzed using AQUACOSMOS 2.6

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