Cell-Specific Delivery of Polymeric Nanoparticles to Carbohydrate-Tagging Cells

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Carbohydrates on cell surfaces contribute a variety of communications between the cell and its environment, and they have been assumed to act as markers for cellular recognition. In this research, 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer nanoparticles, which can react with specific carbohydrates of target cells, were newly prepared to serve as novel drug carriers. A water-soluble MPC polymer bearing hydrazide groups (PMBH) was synthesized by conventional radical polymerization. The MPC polymer showed amphiphilic nature and worked as an emulsifier to form nanoparticles. The nanoparticles covered with PMBH were prepared by the solvent evaporation method and exhibited monodispersity. They were approximately 200 nm in diameter and −2.0 mV in surface potential. According to a surface analysis of the nanoparticles, phosphorylcholine and hydrazide groups were observed, and the surface was fully covered with PMBH. Unnatural carbohydrates having ketone groups on human cervical carcinoma cell (HeLa) surfaces were expressed by treatment with levulinoyl mannosamine (ManLev). When the PMBH nanoparticles were in contact with the ManLev-treated HeLa cells, they accumulated in the cells. In contrast, the nanoparticles were not observed in native HeLa cells (without unnatural carbohydrates). These results indicate that the hydrazide groups of the nanoparticles selectively reacted to the ketone groups of the carbohydrates on the cell surface. The PMBH nanoparticles immobilized with anticancer drugs such as doxorubicin or paclitaxel were in contact with either ManLev-treated or untreated HeLa cells. The viability of the ManLev-treated HeLa cells was effectively reduced, but that of the untreated cells was preserved. This indicated that the anticancer drugs were selectively delivered to the ManLev-treated cells. Nonspecific cellular uptake of the nanoparticles was effectively reduced by MPC polymer coating. Furthermore, the immobilization processes of the drugs differed because of the solubility of the drugs. In conclusion, cellular-specific drug delivery by means of the novel nanoparticles was demonstrated with the selective reaction between unnatural carbohydrates on the cell surface and the hydrazide groups bearing the phosphorylcholine polymer nanoparticles.

Introduction

The use of polymeric nanomaterials to deliver drugs to specific types of tissues has recently been studied in both research and practical applications.[1−5] Almost all of these nanomaterials are formed through the association of some polymers. In particular, polymeric micelle and polymer nanoparticles are made from amphiphilic polymers, and they have hydrophilic shells and hydrophobic cores. The surface of nanomaterials is generally covered with water-soluble polymers such as poly(ethylene glycol)[6,7] and polysaccharides,[8,9] resulting in the stable dispersion of nanoparticles and their stealth properties. Furthermore, the surface of nanoparticles is immobilized with some ligands to deliver drugs to cells or tissues. However, almost all of the ligands demonstrated do not uniquely interact with a specific target and show broad affinity.

In living cells, carbohydrates on the cell surface contribute to most communications between the cell and its environment.[10] Active cell targeting can then be possible with polymers, which can recognize a specific carbohydrate on a cell surface. For recognition of a target cell, the tagging of carbohydrates on the cell membrane as well as the polymer design might be effective.

The incorporation of unnatural carbohydrates into living cells provides an opportunity to study the specific contributions of sialic acid and its *N*-acyl side chains to sialic acid-dependent ligand−receptor interactions at a submolecular level. It has been shown that synthetic *N*-acyl-modified D-mannoseamines can be taken up by cells and efficiently metabolized to the responsive *N*-acyl-modified neuraminic acids.[11−16] Since the initial demonstration of cell-surface engineering with *N*-levulinoylmannosamine (ManLev), this technique has been applied to the development of targeted imaging reagents[17] and methods for gene transfer.[18] The tagging technique has led to great progress in cell biology and biomedical science.[19] Prescher and coworkers alternatively reported that cell-surface modification with monosaccharides could be applied to living animals.[20]

We have synthesized 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers by mimicking their biomembrane structure and clarified that MPC polymers have surface proper-

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Table 1. Synthetic Results of MPC Polymers

	molar fraction of monomer unit (MPC/BMA/MH)		conversion[b]		
	in feed	in copolymer[a]	%	Mw[c] ($\times 10^{-4}$)	Mw/Mn[c]
PMB50	0.50/0.50/−	0.42/0.58/−	50.8	2.0	1.8
PMBH50	0.50/0.45/0.05	0.44/0.55/0.01	36.8	1.6	1.9

[a] Determined by phosphorus and elemental analysis. [b] [Monomer] = 1.0 M; [AIBN] = 5mM; polymerization temperature = 60 °C. [c] Determined by GPC.

ties that reduce nonspecific protein adsorption[21] and cell adhesion.[22] Polymeric amphiphiles with MPC can be easily obtained with appropriately composed hydrophobic comonomers.[23] The polymeric nanoparticles were prepared[24] by using the amphiphilic MPC polymers as emulsifiers, and they were not distinguishable from immunocytes. That is, the nanoparticles could be applied as excellent drug carriers in the bloodstream.[25] It can be assumed that the stealth surface produced by MPC polymers is important for cell-specific drug delivery.

Although cell-surface engineering is a robust procedure for making unique markers on cell surfaces and has been applied to enhance the immunotargeting of cancer cells[26] and gene delivery with biotin−avidin interaction,[18] the study of drug delivery using carriers with the loading capacity of anticancer drugs is still limited. In addition, the reduction of nonspecific interaction of drug carriers to cells must be important for reliable drug delivery. In the current research, new MPC polymer nanoparticles that can react with unnatural carbohydrates on a target cell are prepared, and the cell-specific delivery of drugs is demonstrated.

Materials and Methods

Materials. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was synthesized by a previously reported method.[27] Methacryloyl hydrazide (MH) was synthesized from the reaction of methyl and hydrazine monohydrate.[28] *N*-Levulinoylmannosamine (ManLev) was synthesized by a previously described method.[14] *n*-Butyl methacrylate (BMA) was purified by conventional distillation. Other reagents and solvents were commercially available in extra-pure grade and were used without further purification.

Poly(MPC-co-BMA) (PMB) and poly(MPC-co-BMA-co-MH) (PMBH) were synthesized by radical polymerization using 2,2′-azobisisobutyronitrile (AIBN) as an initiator. The mole fraction of monomer units in the copolymers was determined by phosphorus analysis and elemental analysis. The apparent molecular weights of the MPC polymers was determined by GPC with a Jasco GPC system with a refractive index detector and size-exclusion columns, Shodex, SB-804 HQ and SB-806 M with a poly(ethylene glycol) (PEG, Tosoh standard sample) standard in distilled water containing 10 mM LiBr. The results of polymerization are summarized in Table 1.

Preparation of Nanoparticles. Polymer nanoparticles were prepared by the method previously described.[24] Briefly, a 40 mL aqueous solution containing 1 wt % MPC polymer was placed in a sample tube and stirred at 400 rpm with cooling in an ice bath. Poly(L-lactic acid) (PLLA, 20 mg) was dissolved in 2.0 mL methylene dichloride. The PLLA solution was then added dropwise into the MPC polymer aqueous solution. The mixture was sonicated using a probe-type generator (Sonifier 250, Branson, CT, USA) for 30 min and kept under reduced pressure for 2 h to evaporate the methylene dichloride. The formed nanoparticles were fractionated by centrifugation at 10300*g* at 4 °C for 30 min. The nanoparticles were resuspended as a precipitate in distilled water and centrifuged again under the same conditions. This procedure was repeated three times to completely remove any free MPC polymer. The nanoparticles were finally lyophilized.

Characterization of Nanoparticles. The particle size, size distribution, and ζ-potential of the nanoparticles were determined using Malvern Instruments Zetasizer. Scattering was performed with a vertically polarized incident beam at a wavelength of 633 nm supplied by a He−Ne ion laser. The scattering angle was 90°. The measurements were performed in phosphate-buffered saline (Dulbecco's PBS, pH 7.4; ionic strength, 0.14 M) at 25 °C. Elemental analysis of the surface of the nanoparticles was performed with an X-ray photoelectron spectroscope (XPS, ESCA-200, Scienta, Uppsala, Sweden).

The confirmation of the reactivity of the hydrazide groups on nanoparticles in an aqueous medium was determined by the reaction with 2,4,6-trinitrobenzene sulfonic acid (TNBS).[29] The PMBH− and PMB nanoparticles were suspended in aqueous solution. TNBS (1 mg/mL) was introduced in the suspensions and stored for 60 min. The suspensions were then centrifuged and the supernatant removed. This process was repeated for thee times to rinse nanoparticles. Finally, the suspension was centrifuged, and the color of the precipitate was observed.

Cellular Uptake of Nanoparticles. Human uterine cervical cancer cells (HeLa) were purchased from Riken Cell Bank and maintained in RPMI 1640 with 10% fetal bovine serum at 37 °C in a humidified atmosphere of air containing 5% CO_2. The medium was changed to one containing 10 mM ManLev, and the cells were stored for 3 days. The cells were then washed with fresh medium and centrifuged to remove any free ManLev in the medium. The expression of ketones on the cell membrane was confirmed by fluorescence staining with 5 mM Alexa Fluor 350 hydrazide (Molecular Probes, OR, USA).

The PMBH or PMB nanoparticles containing a hydrophobic fluorescence probe were prepared by desorbing 0.1 mg of Nile Red with PLLA in 2.0 mL of methylene dichloride and placed in contact with native or ManLev-treated HeLa cells for 2 h. The cells were rinsed with fresh medium to remove any free nanoparticles and observed by fluorescence microscope (IX-70, OLYMPUS Co., Tokyo, Japan). The density of the nanoparticles incorporated in the cells was analyzed by flow cytometry (FACSCalibur, Becton Dickinson, NJ, USA). To identify the ketone groups on the cellular uptake of the nanoparticles, the nanoparticles were also placed in contact with cells treated with sialidase. Sialidase buffer (20 mM Hepes, 140 mM NaCl, pH 6.8) was prepared, and the concentration of sialdase was adjusted at 1 U/mL. The sialidase buffer (100 μL) was added to each medium and stored at 37 °C for 30 min.

Delivery of Anticancer Drugs. PMBH and PMB nanoparticles immobilized with doxorubicin (DOX) or paclitaxel (PTX) were prepared. DOX was adsorbed on the surface of the nanoparticles from an aqueous solution. The DOX (final conc. 0.4 mg/mL) was introduced in a nanoparticle suspension (1 mg/mL) and stored for 48 h. The suspension was then centrifuged to precipitate the nanoparticles and decanted. This process was repeated several times to remove any free DOX in the suspension. For the PTX-immobilized nanoparticles, PTX (1 mg) was desorbed in methylene chloride with PLLA (20 mg) and the nanoparticles prepared as mentioned previously. The amount of DOX and PTX incorporated in/on the nanoparticles was determined by solubilizing the nanoparticles in methylene chloride. DOX (0.02 mg) or PTX (0.03 mg) was independently conjugated in 1 mg of nanoparticles.

The release of DOX from the nanoparticles was determined by a spectroscopic method. The nanoparticles suspended in PBS and the

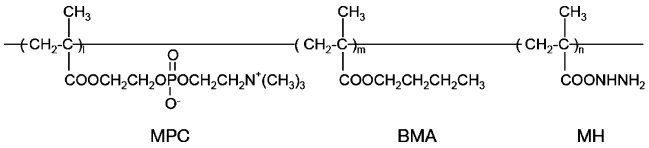

Figure 1. Chemical structure of PMBH.

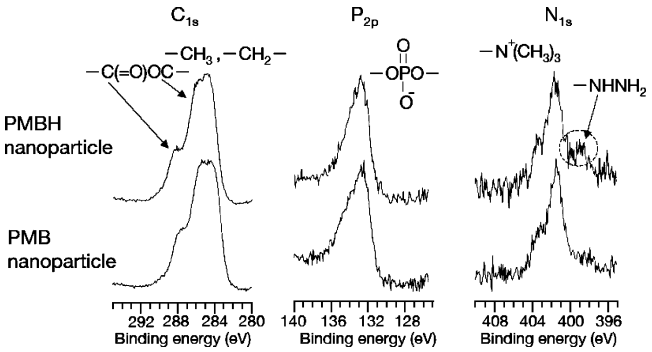

Figure 2. XPS spectra of PMBH and PMB nanoparticles.

suspension were centrifuged for varied periods. The concentration of drugs in the supernatant was determined by use of a UV−vis spectrometer. After 4 days, the pH was decreased to 5.2 by changing the buffer solution. The effect of the pH change on the release profile was also monitored.

The nanoparticle suspension (0.033, 0.1, and 0.33 mg/ mL) was placed in contact with ManLev-treated or untreated HeLa cells and stored for 2 h. After rinsing with fresh medium three times, the cell culture was then continued for various durations. Cell viability was determined by trypan blue staining.

Statistic Analysis. The data are represented as the mean ± standard deviation (SD). Statistical comparisons ($n = 3$) were performed with Student's *t*-test.

Results and Discussion

Synthesis of Amphiphilic Phosphorylcholine Polymers. To achieve selective ligation with ketone groups on a cell surface, an amphiphilic phosphorylcholine polymer with hydrazide groups was synthesized. Poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-methacryloyl hydrazide (MH)] (PMBH, Figure 1) was synthesized by conventional radical polymerization. Poly(MPC-co-BMA) (PMB) was also synthesized as a control polymer. The mole fractions of PMBH and PMB were 0.42/0.57/0.01 (MPC/BMA/MH) and 0.42/0.58 (MPC/BMA), respectively. Both polymers were soluble in water and showed surface-active behavior. The apparent critical micelle concentration (cmc) was 1.0×10^{-2} g/dL, which was determined from the middle point of the transition state of the surface tension.

Preparation of Polymeric Nanoparticles. The polymeric nanoparticles were prepared as described in the cited literature with some modifications.[23] To identify the distribution of nanoparticles in the following study, Nile red was mixed with PLLA, forming the nanoparticle core. Figure 2 shows the XPS spectra of the nanoparticles. In the case of the nanoparticles covered with MPC polymers, nitrogen and phosphorus peaks were observed at 402.5 and 133.0 eV, respectively. These are attributed to the phosphorylcholine group in the MPC units. Moreover, nitrogen peaks at 399.0 and 400.0 eV due to the hydrazide groups were also observed on the surface of PMBH. The surface XPS spectra of the nanoparticles were completely different from those of PLLA, and it was clear that the nanoparticles were completely covered with MPC polymers.

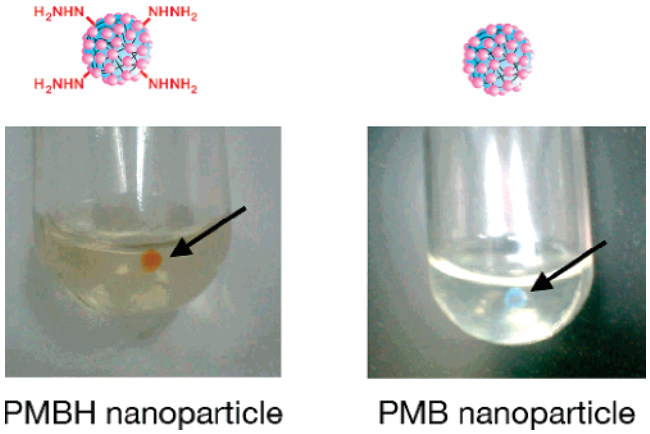

Figure 3. Centrifuged nanoparticles after contact with TNBS for 1 h.

The particle size and ζ-potential of the nanoparticles were determined by Malvern Instruments Zetasizer. The diameters of the PMB and PMBH nanoparticles were 242 ± 10 and 218 ± 9 nm, respectively. The ζ-potentials of the PMB and PMBH nanoparticles were −2.0 ± 0.5 and −2.0 ± 0.6 mV, respectively. There was no evidence of any influence of the hydrazide groups on these physicochemical properties.

Figure 3 shows photographs of the precipitated nanoparticles after contact with TNBS for 60 min. The color of the PMBH nanoparticles became yellow, while the PMB nanoparticles remained colorless. This result indicated that the hydrazide groups were located on the surface of the PMBH nanoparticles and that they were reactive.

Cellar Uptake of Nanoparticles. Metabolic oligosaccharide engineering is of interest for the surface modification of mammalian cells.[30] Various types of unnatural carbohydrates are expressed using a sialic acid biosynthesis pathway. Yarema, et al., have recently succeeded in expressing thiol groups on a cell surface.[31] The surface modification of living cells is powerful technology for use in tagging cells.

Figure 4 shows phase-contrast and fluorescence micrographs of cells after contact with nanoparticles (0.05 mg/mL) for 2 h. When the PMBH nanoparticles were placed in contact with ManLev-treated HeLa cells, the cells could be observed by fluorescence microscopy due to emission of Nile red incorporated into the nanoparticles. In contrast, the fluorescence images of adherent cells were not observed when the PMBH nanoparticles were placed in contact with native HeLa cells or when the PMB nanoparticles were placed in contact with ManLev-treated cells. The intensity of cell fluorescence after being in contact with the nanoparticles (0.33 mg/mL) is shown in Figure 5. Similar to the microscopic observations, the fluorescence intensity of ManLev-treated HeLa cells was significantly higher than that under the other conditions. The fluorescence intensity of ManLev-treated HeLa cells in contact with PMB nanoparticles and untreated HeLa cells in contact with PMBH nanoparticles was almost same as that of cells without nanoparticle treatment. When the ManLev-treated cells were incubated with sialidase before contact with the PMBH nanoparticles, the fluorescence intensity was reduced by approximately 90%. These results presented in Figures 4 and 5 indicate that cellular uptake by the nanoparticles specifically occurred through the reaction of the hydrazide of the nanoparticles and the ketone groups expressed on the cell surface.

The PMBH nanoparticles were distributed homogeneously into the ManLev-treated cells (Figure 4). The mechanism of cellular uptake through the unnatural carbohydrates has been

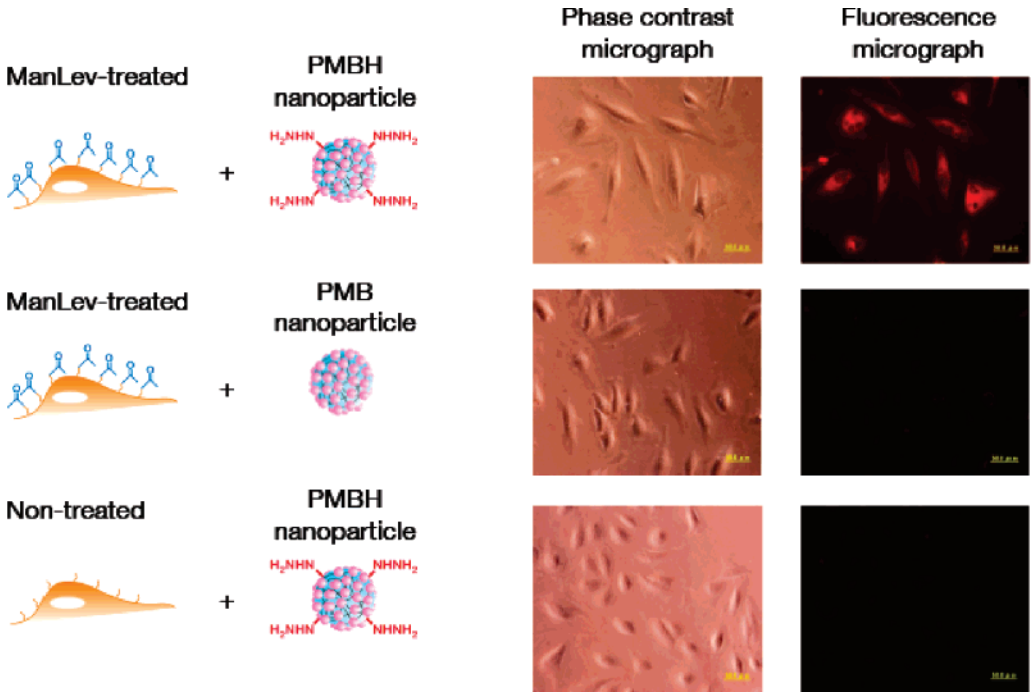

Figure 4. Phase-contrast and fluorescence micrographs of HeLa cells after contact with Nile red-loaded nanoparticles (0.05 mg/mL) for 2 h.

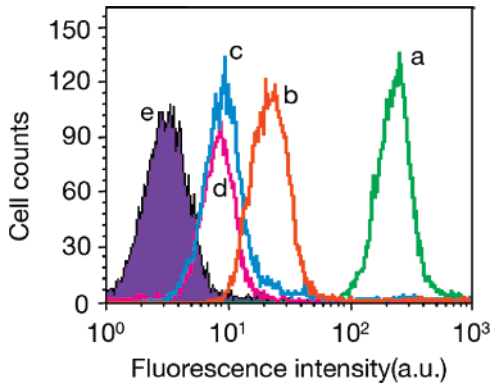

Figure 5. Fluorescence intensity of HeLa cells after contact with Nile red-loaded nanoparticles (0.33 mg/mL). (a) PMBH nanoparticles/ManLev-treated cells; (b) PMBH nanoparticle/sialidase-treated cells; (c) PMBH nanoparticles/untreated cells; (d) PMB nanoparticles/ManLev-treated cells; (e) untreated cells.

proposed as endocytosis.[32] The size of nanoparticles strongly influences the endocytosis process.[33] Alternately, Chan and co-workers reported the effect of the size and shape of nanoparticles on the uptake into mammalian cells; nanoparticles approximately 50 nm in diameter and a 1:1 aspect ratio were the most effective.[34] Although the nanoparticles prepared in this study were relatively larger than the optimal size, cellular uptake of nanoparticles effectively occurred. Nanoparticle size can be controlled by changing the ratio of MPC polymer and PLLA in the feed.[24] The efficiency of cellular uptake will is improved by controlling the size of the PMBH nanoparticles.

Selective Delivery of Anticancer Drugs. Nanoparticles were immobilized with anticancer drugs, and the process was varied because of drug solubility (Figure 6). A water-soluble drug, DOX, was adsorbed on the surface of the nanoparticles. Although DOX is water soluble (10 mg/mL in H_2O), hydrophobic aromatic carbon is present in the molecules. Thus, small amphiphilic molecules such as DOX are preferably adsorbed on the nanoparticles. In contrast, PTX is a highly hydrophobic drug and is barely soluble in water (0.3 μg/mL in H_2O). Because of its poor solubility in water and the availability of many acceptable pharmaceutical solvents other than water, specific emulsifiers, such as Cremophor EL, are used to formulate PTX in commercial injection solutions. However, serious hypersensitive reactions have been reported in some individuals because the amount of Cremophor EL, which is used in the PTX formulation, is significantly higher than in any other solubilizers.[35,36] Therefore, alternative forms of PTX dosage administration are needed to reduce the undesirable side effects induced by Cremophor EL. Applications of liposomes,[37] mixed micelles,[38,39] parenteral emulsions,[40−42] cyclodextrin complexes,[43] and hydrotropic dendrimers[44] have been reported. In this study, PTX is dissolved in methylene chloride and incorporated into the hydrophobic core of nanoparticles.

Figure 7 shows DOX release from nanoparticles. During the 48-h incubation, 40% of DOX was quickly released, and then the release rate slowed greatly. The adsorption behavior of DOX on nanoparticles is not uniform; some DOX may penetrate into the shell composed of MPC polymer chains. The release rate was influence by the pH of the soaking medium: the rate was accelerated under a lower pH condition (pH 5.2). DOX is partially ionized under physiological conditions by protonation of the amino group, the p*K*a being 7.2−7.6.[45] The increment of the release rate under low pH conditions might be influenced by the protonation of DOX.

Generally, cellular uptake of nanoparticles occurs through endocytosis. The nanoparticles are taken up into endosomes, where the pH is 1.4−2.4 units lower than the physiological pH of 7.4.[46−48] The pH-responsive release profile is of interest because it relates to the controlled release of drugs in the endsomal environment.

The time dependence of cell viability after contact with nanoparticles containing DOX for 2 h is shown in Figure 8. When the PMBH nanoparticles were in contact with the ManLev-treated nanoparticles, cell viability gradually decreased, and approximately 60% of the cells died during the first 3 days of cultivation. In contrast, 80% of the cells in contact with the

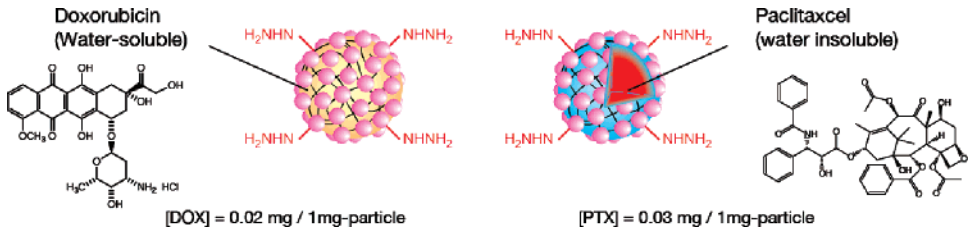

Figure 6. Schematic representation of PMBH nanoparticle immobilized with anticancer drugs.

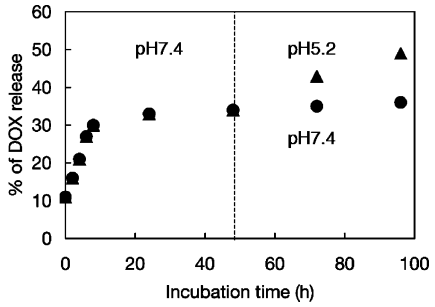

Figure 7. Release profile of doxorubicin from PMBH nanoparticles. (●) pH 7.4; (▲) pH 7.4 → pH 5.2.

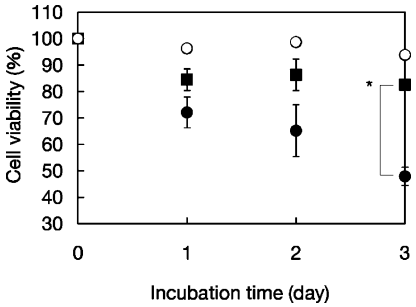

Figure 8. Viability change in ManLev-treated HeLa cells after contact with doxorubicin (DOX)-adsorbed nanoparticles (0.1 mg/mL) for 2 h. (○) PMBH nanoparticles (non-DOX); (■) PMB nanoparticles; (●) PMBH nanoparticles. *$p < 0.01$.

PMB nanoparticles were alive after contact with the PMB nanoparticles for 3 days. DOX-adsorbed PMBH nanoparticles preferably penetrated into the ManLev-treated cells for 2 h, and the DOX was released from the surface of the nanoparticles. In contrast, the amount of PMB nanoparticles that penetrated into the ManLev-treated cells was significantly lower than that of the PMBH nanoparticles, as shown in Figures 4 and 5. The viability of the ManLev-treated cells placed in contact with the DOX-adsorbed PMB nanoparticles was thus preserved.

The cytotoxicity of PMBH and PMB nanoparticles without treatment with anticancer drugs was not observed. The slight decrease in cell number due to contact with the DOX-adsorbed PMB nanoparticles was due to the diffusion of DOX in the medium for 2 h. Approximately 20% DOX adsorbed on nanoparticles was released for 2 h under physiological condition as shown in Figure 7. The concentration of DOX in a medium was 40 ng/mL. When the DOX (40 ng/mL) was directly in contact with cells for 2 h, the cell viability after 3 days of cultivation was 82.5%. This amount was coincident with the viability (83.3%) of cells in contact with the DOX-adsorbed PMB nanoparticles.

Figure 9 shows the effect of the concentration of PTX-loaded nanoparticles in the medium on the viability of ManLev-treated HeLa cells. This result also indicates the time dependence of the viability of the cells placed in contact with the PTX-loaded PMBH nanoparticles for 2 h. Cell viability decreased

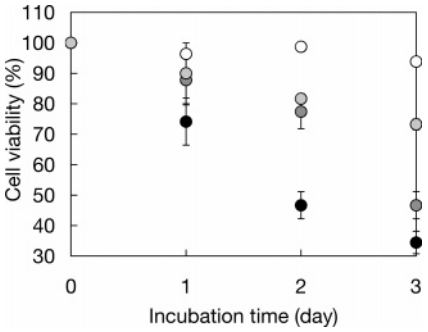

Figure 9. Viability change in HeLa cells after contact with varied composition of Paclitaxel (PTX)-loaded PMBH nanoparticles for 2 h. (Open circle) 0.1 mg/mL (non-PTX); (light gray circle) 0.03 mg/mL; (dark gray circle) 0.1 mg/mL; (black circle) 0.3 mg/mL.

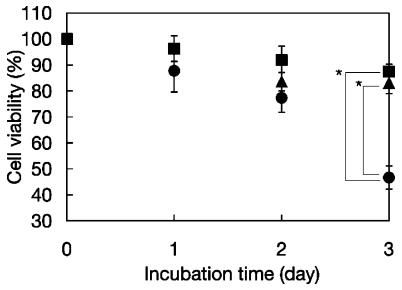

Figure 10. Viability change in HeLa cells after contact with Paclitaxel (PTX)-loaded nanoparticles (0.1 mg/mL) for 2 h. (■) PMB nanoparticles/ManLev-treated HeLa cells; (▲) PMBH nanoparticles/untreated HeLa cells; (●) PMBH nanoparticles/ManLev-treated HeLa cells. *$p < 0.01$.

with an increase in the concentration of PTX-loaded nanoparticles. The dose dependence of the cytotoxicity was thus observed.

The PTX-loaded PMB and PMBH nanoparticles (0.1 mg/mL) were placed in contact with ManLev-treated or untreated cells (Figure 10). Similar to the DOX nanoparticles, cell death was significant when PMBH nanoparticles were placed in contact with the ManLev-treated cells. When the PTX-loaded PMBH nanoparticles were placed in contact with the ManLev-treated HeLa cells for 2 h, 50% of the cells died during the first 3 days of cultivation. In contrast, approximately 90% of the cells were alive when the PTX-loaded PMBH nanoparticles were placed in contact with the native cells or when the PTX-loaded PMB nanoparticles were placed in contact with the ManLev-treated cells.

Cell-specific delivery of anticancer drugs was demonstrated through the ligation of carbohydrates on the cell surfaces and functionalized biocompatible nanoparticles.

Conclusions

Amphiphilic PMBHs are able to form nanoparticles bearing hydrazide groups on their surfaces. The nanoparticles were able to recognize ManLev-treated HeLa cells because the cells had

ketone-functionalized unnatural carbohydrates on their surfaces. Nonspecific delivery of PMBH nanoparticles to cells was effectively reduced because of the stealth the MPC units. It was demonstrated that MPC polymer-coated nanoparticles could specifically interact with carbohydrates on cell surfaces. Because of the immobilization of a molecule that can react with carbohydrates on a natural specific cell, cell-specific delivery to a variety of cells can be enabled.

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