Preparation and evaluation of highly dispersible and Ca^{2+} level responsive PEGylated calcium phosphate nanoparticle with PEG-*block*-poly(4-vinylbenzylphosphonate) for the application of drug carrier

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PEGylated calcium phosphate nanoparticles (CaPNPs) were prepared through the simple mixing of calcium and phosphate solutions in the presence of a PEG-*block*-poly(4-vinylbenzylphosphonate) (PEG-*b*-PVBP). The PEG-*b*-PVBP possesses the phosphonate side chains and the hydrophobic polystyrene segment which enhance the stability of the PEG brush layer on the CaPNP surface. The size of CaPNPs was controlled within the range of several tens of nanometer depending on the [PVBP unit]/[phosphate ion] feed-molar ratio upon the CaP coprecipitation reaction. The obtained CaPNPs showed excellent colloidal stability and it was sustained even after the lyophilization. In vitro cell cytotoxicity assay suggested that the CaPNPs do not induce serious cytotoxicity in the murine colon adenocarcinoma 26 (C-26) cells. Light scattering study revealed that the CaPNPs can be dissolved into the low Ca^{2+} condition ($[\text{Ca}^{2+}] = 0.1 \text{ mM}$). Since intracellular Ca^{2+} concentration is four orders of magnitude lower than the extracellular one, the Ca^{2+} concentration responsive dissolution behavior of CaPNPs is promising for the intracellular delivery of therapeutic materials.

Key words: PEG-based block copolymer, surface modification, calcium phosphate nanoparticle, calcium responsive

1. INTRODUCTION

A number of nano-sized drug carriers have been developed for drug delivery system and several of them showed long circulation in blood. However, the delivery efficiency of drugs into the target cells is still insufficient due to unintended release of the drugs during the circulation. In order to improve circulation tendency in vivo, high colloidal stability and non-fouling character are very important factors. We have previously found that size-controlled nanoparticles with high dispersion character could be prepared by co-precipitation method in the presence of poly(ethylene glycol) (PEG) -based block copolymers. For example, CdS quantum dot was prepared by the reaction between CdCl_2 and Na_2S in the presence of PEG-*b*-poly(2-(*N,N*-dimethylamino)ethyl methacrylate) (PEG-*b*-PAMA) [1]. The size and dispersion stability was controllable in this method. This method could be applied to other nanoparticles such as gold [2] and iron oxide [3].

Because intracellular calcium ion concentration is known to be 10^4 times lower compared to the extracellular surroundings [4], calcium phosphate (CaP) is erodible selectively in intracellular environment. Recently, Kakizawa and co-workers reported CaP nanoparticles by the co-precipitating method of calcium chloride and disodium hydrogen phosphate in the

presence of PEG-*b*-poly(aspartic acid) (PEG-*b*-PAA) and applied for nucleic acid delivery system [5], [6]. This system worked very well because of the erosion of CaP in intracellular environment. Since carboxyl groups as a side chain in the PAA segment of PEG-*b*-PAA are not best choice for coordination of the CaP surface, however, they modified the side chain of PAA to improve the stability on the CaP surface [7]. Using these materials, they investigated the intracellular kinetics. In order to use this kind of materials in vivo, we have to pay infinite attention to improve its colloidal stability and non-fouling character. We have designed several types of PEG-based block copolymers for versatile nanoparticles. PEG-*b*-poly(4-vinylbenzylphosphonate) (PEG-*b*-PVBP) is one of the promising materials for surface modification of nanoparticle system. For example, erbium doped yttrium oxide nanoparticles modified by PEG-*b*-PVBP showed extremely high stability under the physiological conditions and could be applied for in vivo near-infrared imaging [8].

PEG-*b*-PVBP possesses phosphonate groups as a side chain of PVBP segment which improves adsorption stability via multi-point coordination of phosphonate moieties. Ginger and co-workers previously reported PEGylated CaPs using PEG-functionalized bisphosphonate [9]. From our experiences, however,

Figure 1. Schematic illustration of PEGylated CaP nanoparticle (CaPNP).

mono-phosphonate-ended PEG was rapidly exchanged to low molecular weight phosphate in PBS buffer [8] and thus cannot be applied even in vitro conditions.

In addition, polystyrene moiety of the PVBP segment would enhance the anchoring efficiency because of its hydrophobic nature, which could prevent the detachment of the surface-bound PEG-*b*-PVBP under hydrophilic conditions (Figure 1). Considering this additional hydrophobic enhancement of the anchoring, this block copolymer is expected to enable the robust PEG brush layer on the CaP surface and high colloidal stability of the nanoparticles, which can be applicable to in vivo conditions.

In this study, we report the formation of highly dispersible CaP nanoparticles (CaPNPs) by mixing calcium and phosphate solutions in the presence of PEG-*b*-PVBP. The colloidal stability was examined using dynamic light scattering (DLS). The Ca^{2+} concentration-dependent dissolution of CaPNP was investigated by the measurement of scattered light intensity (SLI).

2. EXPERIMENTAL SECTION

2.1 Materials

Poly(ethylene glycol)-*block*-poly(4-vinylbenzylphosphonate) (PEG-*b*-PVBP) was synthesized as previously described [8]. The molecular weight of PEG segment was 5000 g mol^{-1} and the degree of polymerization of the PVBP segment was 14. Hydrochloride, sodium hydride, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), tris(hydroxymethyl)aminomethane (Tris), disodium hydrogenphosphate 12-water, calcium chloride were purchased from Kanto Chemicals, Japan. Sodium chloride was purchased from Wako Pure Chemical Ind., Japan. Murine colon adenocarcinoma 26 (C-26) cells were obtained from RIKEN BioResource Center.

2.2 Preparation of calcium phosphate nanoparticles covered with PEG-*b*-PVBP

A solution of 2.5 M CaCl_2 was added to a buffer (1 mM Tris-HCl , pH 7.6) to a final volume (calcium ions 250 mM). PEG-*b*-PVBP was dissolved in the phosphate solution ($1.5 \text{ mM Na}_2\text{HPO}_4$, 50 mM HEPES , 140 mM NaCl , pH 7.1). One volume of this phosphate solution was immediately added to an equal volume of the calcium solution (final concentrations; calcium ions 125 mM , phosphate ions 0.75 mM). The mixed solutions were stirred by a vortex mixer for a few seconds and incubated at room temperature for 1 d.

2.3 Evaluation of colloidal stability upon freeze drying

The dispersant of as-prepared CaPNP solution

([PEG-*b*-PVBP] = $420 \mu\text{g/mL}$) was exchanged into another calcium containing buffer (10 mM HEPES , 1.5 mM CaCl_2 , pH 7.4) by centrifugal ultrafiltration with Amicon Ultra (MWCO 10,000 Millipore, US) at $2,580 \times g$, followed by the measurement of hydrodynamic diameter by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments, Ltd., U.K.). The CaPNP solution was frozen in a liquid N_2 , and then lyophilized. The resulting powder was rehydrated with deionized water to reach the initial volume and suspended by ultrasonication. The resulting solution was analyzed by DLS.

2.4 Cell culture and cell cytotoxicity assay

C-26 cells were maintained as an adherent culture and grown as a monolayer in a humidified incubator (95% air, 5% CO_2) at 37°C in a Petri dish containing Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS, Gibco). 5×10^3 cells were seeded in each 96-well culture plate and incubated for 24 h. The CaPNP solution was prepared using PEG-*b*-PVBP of $420 \mu\text{g/mL}$ followed by condensation by centrifugal ultrafiltration. The medium of each well was then replaced with $90 \mu\text{L}$ of fresh DMEM containing 10% FBS and $10 \mu\text{L}$ of CaPNP dispersion in HEPES buffer (10 mM HEPES , 1.5 mM CaCl_2 , pH 7.4) at selected concentrations. Control experiments were carried out using the same HEPES buffer instead of the CaPNP dispersion. The culture plate was returned to the incubator and incubated for 48 h. After this incubation period, the cell viability was evaluated by means of the colorimetric WST assay using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

2.5 Evaluation of Ca^{2+} concentration-dependent solubility

The dispersants of as-prepared CaPNP solution ([PVBP unit]/[phosphate ion] = 1) were exchanged into aqueous buffer solutions containing variety of calcium ion concentration from 100 nM to 1.5 mM (pH 7.4) by means of centrifugal ultrafiltration with Amicon Ultra (MWCO 10,000). The solutions were incubated at room temperature for 1 d and the scattered light intensity (SLI) was measured by DLS. In order to investigate the solubility of CaPNP in the condition like inside and outside the cells, as-prepared CaPNP solutions were dialyzed (MWCO 3,500) for one day against the aqueous buffer solutions containing calcium ions similar to intracellular (100 nM) or extracellular (2 mM) fluids, followed by measurement of SLI of these solutions.

3. RESULTS AND DISCUSSION

3.1 Preparation of CaPNP

Simple mixing of calcium and phosphate solution without polymer results in large CaP precipitation. In order to control the crystal growth through the absorption of PVBP segment of PEG-*b*-PVBP on the crystal surface, the polymer was added at the same time as the mixing calcium and phosphate solution. The sizes and polydispersity indices (PDI) of the nanoparticles formed with varying [PVBP unit]/[phosphate ion] feed-molar ratio upon the CaP coprecipitation reaction

were determined by DLS measurement and are shown in Figure 2. The diameters of the particles ranged from 60 nm to 180 nm depending on the [PVBP unit]/[phosphate ion] with the minimum value at [PVBP unit]/[phosphate ion] = 1. Though the reason of this phenomenon is not clear, it might be balance between coordination rate and crystal growth of the particles. At the [PVBP unit]/[phosphate ion] = 0.5–1.5, the diameters and polydispersity indices were 60–97 nm and 0.09–0.17 respectively. This result indicates that appropriate size and defined structure were obtained from the perspective of application as a drug carrier, which is probably due to the multi-point and robust anchoring of PEG-*b*-PVBP.

3.2 Colloidal stability of CaPNP upon freeze drying

In order to investigate the integrity of CaPNP upon freeze drying, particle solution ([PVBP unit]/[phosphate ion] = 1) was frozen and lyophilized. The resulting powder was rehydrated with water and suspended by ultrasonication. The size distributions of the particle solutions before and after freeze drying were measured and shown in Figure 3. Upon re-dissolution, the CaPNP recovered the size and colloidal stability. The mean sizes before and after freeze drying were 60.5 ± 0.2 nm and 62.2 ± 0.1 nm respectively. These results suggest that CaPNP can be re-dispersed and recover the colloidal stability after freeze drying even in the absence of cryoprotectants. This colloidal stability upon freeze drying is an important point for a pharmaceutically viable formulation because drug formulation in powder form is easier to handle, store and transfer than in solution.

3.3 Cytotoxicity of CaPNP

The development of non-cytotoxic drug carrier is one of the main challenges to the effective drug delivery. The *in vitro* cytotoxicity of CaPNP was evaluated in the C-26 cell line by WST assay over a dosage range of 4.2–420 $\mu\text{g/mL}$ of PEG-*b*-PVBP concentration ([PVBP unit]/[phosphate ion] = 1). The WST assay showed no significant cytotoxic response (the cell viability >80%) at the tested range after 48 h of exposure (Figure 4). This result shows the *in vitro* biocompatibility of CaPNP, which is advantageous for a delivery system.

3.4 Calcium concentration-dependent dissolution of CaPNP

Once the drug carriers transferred into the cells, they need to fulfill their functions. As the switching stimulus to release the loaded drugs, we focused on the difference between the intra- and extracellular calcium concentration, which is four orders of magnitude lower inside than outside of the cells, and it influences the dissolution behavior of the CaP. The extracellular calcium ion concentration is about 1–2 mM, while that in the intracellular is order of 100 nM [4]. To investigate the Ca^{2+} concentration responsive property of CaPNP, the dispersants of as-prepared CaPNP solution were exchanged by means of centrifugal ultrafiltration method into aqueous buffer solutions containing variety of calcium ions from 100 nM to 1.5 mM (pH7.4). After the exchange of the dispersant solution, the SLI of each solution was measured and normalized by that of as-prepared CaPNP solution. Figure 5 (a) shows the

Figure 2. Size(○) and PDI(◆) of CaPNP depended on [PVBP unit]/[phosphate ion] molar ratio determined by DLS measurement.

Figure 3. Size distribution of CaPNP determined by DLS measurement (×) before and (●) after freeze drying.

Figure 4. Cytotoxicity of CaPNP determined by WST assay ($n = 6$, mean $\pm$ S.D.).

relative SLI of the solutions plotted versus calcium concentrations of substitute solutions. The SLI showed an abrupt decrease in scattering intensity with the decreasing in calcium concentration. This result suggests the dissolution of the particles in response to the decreasing of Ca^{2+} concentration in the dispersant. In order to investigate the solubility of CaPNP in the condition similar to inside and outside the cells,

Figure 5. (a) Relative scattered light intensity as a function of calcium ion concentration of exchanged solution. (b) Relative scattered light intensity of dialyzed solutions.

as-prepared CaPNP solution was dialyzed against the aqueous buffer solutions containing Ca^{2+} similar to intra- (100 nM) or extracellular (2 mM) fluids, followed by measurement of SLI of these solutions. An excess amount of dialysate was used to avoid the concentration change accompanied by the dissolution of the CaP. Figure 5 (b) shows the changes of SLI between before and after dialysis. Obviously, the SLI decreased with dialysis under the condition containing 100 nM of calcium mainly due to the particle dissolution, while there was slight decrease under 2 mM of calcium. The diameter could not be measured after the dialysis under 100 nM of calcium, which suggested the particle dissolution, while there was slight change of the diameter and PDI under 2 mM of calcium (95% and 108%, respectively). These results suggest the possibility of the selective dissolution of CaPNP inside the cells.

4. CONCLUSIONS

CaPNPs, the diameter of which was controlled <100 nm, were synthesized by the mixing of calcium and phosphate solution in the presence of PEG-*b*-PVBP which has multi-point and robust anchoring property. The CaPNP showed no serious cytotoxicity and the colloidal stability, and it was sustained even after the lyophilization, which suggests the facility in handle from the point of view of pharmaceuticals. The Ca^{2+} concentration responsive behavior of CaPNP was verified by the light scattering study, which suggests the intracellular selective dissolution of CaPNP. Since several nanoparticles robustly coated by the PEG-*b*-PVBP have showed excellent colloidal stability and long blood circulation, we expect the high performance of the CaPNP as a drug carrier.

REFERENCES

- [1] Y. Nagasaki, T. Ishii, Y. Sunaga, Y. Watanabe, H. Otsuka and K. Kataoka, *Langmuir*, 20, 6396-6400, (2004).
- [1] Y. Bae, S. Fukushima, A. Hradek and K. Kataoka, *Angew. Chem., Int. Ed.*, 42, 4640-4643, (2003).

- [2] T. Ishii, H. Otsuka, K. Kataoka and Y. Nagasaki, *Langmuir*, 20, 561-564, (2004).
- [3] K. Ujiie, N. Kanayama, K. Asai, M. Kishimoto, Y. Ohara, Y. Akashi, K. Yamada, S. Hashimoto, T. Oda, N. Ohkohchi, H. Yanagihara, E. Kita, M. Yamaguchi, H. Fujii and Y. Nagasaki *Colloids Surf. B.*, 88, 771-778, (2011).
- [2] S. Zalipsky, M. Qazen, J. A. Walker, II, N. Mullah, Y. P. Quinn and S. K. Huang, *Bioconjugate Chem.*, 10, 703-707, (1999).
- [3] S.-Q. Gao, Z.-R. Lu, B. Petri, P. Kopečková and J. Kopeček, *J. Control. Release*, 110, 323-331, (2006).
- [4] D.E. Clapham, *Cell*, 80, 259-268, (1995).
- [5] Y. Kakizawa and K. Kataoka, *Langmuir*, 18, 4539-4543, (2002).
- [6] Y. Kakizawa, S. Furukawa and K. Kataoka, *J. Control. Release*, 97, 345-356, (2004).
- [7] F. Pittella, M. Zang, Y. Lee, H.J. Kim, T. Tockary, K. Osada, T. Ishii, K. Miyata, N. Nishiyama and K. Kataoka, *Biomaterials*, 32, 3106-3114, (2011).
- [8] M. Kamimura, N. Kanayama, K. Tokuzen, K. Soga and Y. Nagasaki, *Nanoscale*, 3, 3705-3713, (2011).
- [9] E.V. Ginger, J. Puigmartí-Luis, B. Castagner, P.S. Dittrich and J.-C. Leroux, *J. Control. Release*, 150, 87-9, (2011).
- [10] M. Kamimura, D. Miyamoto, Y. Saito, K. Soga and Y. Nagasaki, *Langmuir*, 24, 8864-8870, (2008).

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