Selected Paper

Nanogel–Calcium Phosphate Hybrid Nanoparticles with Negative or Positive Charges for Potential Biomedical Applications

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Self-assembled polysaccharide nanogels with negative or positive charges were used as templates for mineralization of calcium phosphate for potential biomedical application. The formation of stable and negatively- or positively-charged amorphous calcium phosphate nanoparticles was confirmed by transmission electron microscopy, energy-dispersive X-ray spectroscopy, and ζ-potential measurements.

Organic–inorganic hybrid materials are of great interest in biotechnology and medicine.[1] Calcium phosphate, a major inorganic constituent of bone and teeth, shows excellent biocompatibility and biodegradability.[2] Recently, organic–inorganic hybrid nanomaterials containing calcium phosphate have been extensively investigated for application in drug-delivery systems (DDS) because of their excellent biocompatibility and biodegradability in addition to their appropriate mechanical stability.[3] It is important to control the structural properties of these hybrid nanomaterials such as size, colloidal stability, phase, and surface properties since these factors greatly affect the functions of the materials.

We previously reported the preparation of organic–inorganic nano-hybrid consisting of hydrogel nanogels of cholesterol-bearing pullulan (CHP) and calcium phosphate.[4] CHP nanogels are self-assembled physical gels in which the association of hydrophobic cholesteryl groups provides physical cross-linking points.[5] The biomedical applications of CHP nanogels have been investigated, especially for use as DDS carriers of proteins, drugs, and semiconductor nanocrystals.[6] However, their stability is not sufficient especially in the blood stream because of their physical cross-linking structure. The mineralized nanogels with calcium phosphate allow more stable carriers with controlled-release properties. To apply them to nanocarriers in DDS, surface properties of the nanoparticles are very important, especially in terms of desired tissue distribution and cellular uptake. For example, cationic self-assembled nanogels enabled effective transport of various proteins into cells.[6a,6c,6d] We report here preparations of both anionic and cationic hybrid calcium phosphate nanoparticles by using templates as electrically charged CHP nanogels consisting of β-alanine-modified CHP anionic nanogels (CHPCOOH) and ethylenediamine-modified CHP cationic nanogels ($CHPNH_2$) (Figure 1).

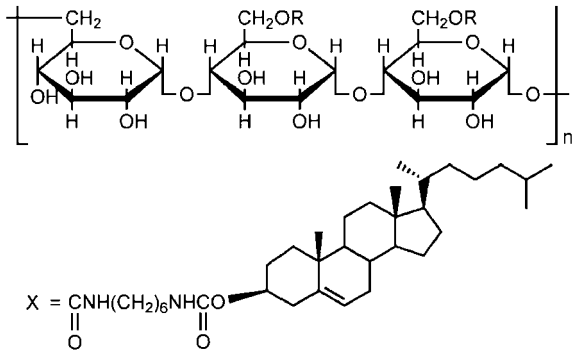

Figure 1. Chemical structures of cholesterol-bearing pullulan (CHP), carboxyl group-bearing CHP (CHPCOOH), and amino group-bearing CHP ($CHPNH_2$).

Nanogel–calcium phosphate hybrid nanoparticles were prepared by pH-gradient method as previously reported.[4] A dilute solution of calcium phosphate was used as a mineralization medium. This enables the formation of calcium phosphate only in nanogel templates, whereas homogeneous nucleation in bulk solution does not take place under the present conditions.[4] First, hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$) ($[Ca^{2+}] = 0.8$ mM) was dissolved in acidic water by introducing CO_2 gas to the HAp-suspended solution. CHPCOOH or $CHPNH_2$ nanogels were then added to this solution (0.5 mg mL^{-1}). The pH of the mixed solution slowly increased from 5.6 to 7.9 for 8 h at 25 °C with the loss of CO_2 gas. Calcium phosphate–nanogel hybrid was obtained because calcium phosphates are more insoluble at basic pH. CHPCOOH and $CHPNH_2$ formed nanogels with diameters of 25.1 and 33.4 nm, respectively, similar to CHP (36.1 nm). Under the present condition for mineralization, the COOH and NH_2 groups of the nanogels are mostly negatively (COO^-) and positively (NH_3^+) charged, respectively. The ζ-potentials of CHP, $CHPNH_2$, and CHPCOOH nanogels in are -0.74,[6d] $+6.6$,[6d] and -1.9 mV (deionized water at pH 7.4), respectively. The morphology and size of hybrid materials were investigated by transmission electron microscope (TEM) images and selected area electron diffraction (SAED) patterns. Since samples were unstained, only inorganic constituents in the hybrids are visible in the images (Figure 2). Well-dispersed spherical inorganic nanoparticles (CHP nanogel: 26 ± 9 nm, CHPCOOH nanogel: 40 ± 13 nm, and $CHPNH_2$ nanogel: 34 ± 11 nm) were formed in the case of any nanogels employed here. The calcium phosphates are thought to be amorphous or low-crystalline materials due to the absence of sharp electron diffraction rings.

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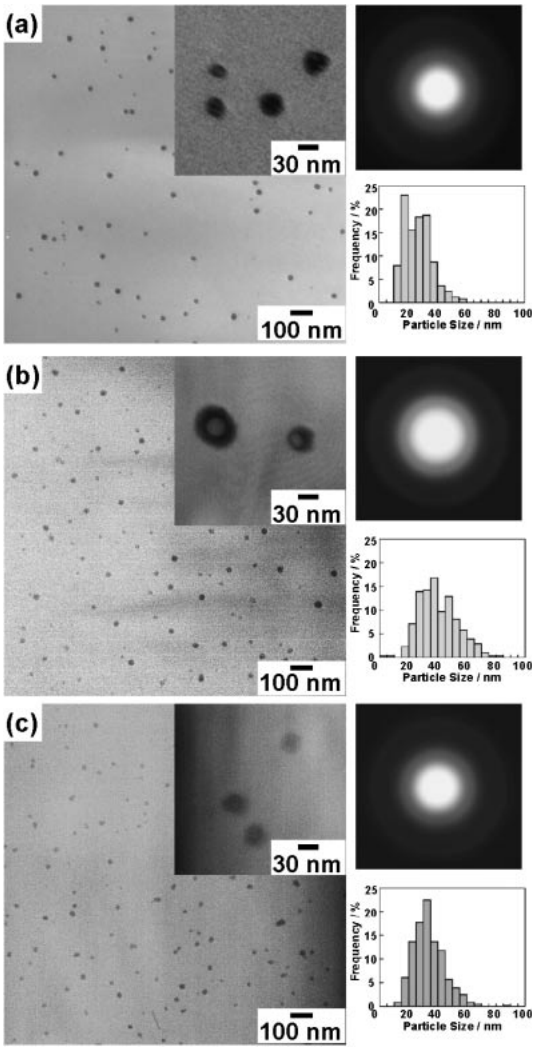

Figure 2. TEM images, SAED patterns, and particle-size distributions determined from TEM images of calcium phosphate nanoparticles formed in the presence of a) CHP, b) CHPCOOH, and c) $CHPNH_2$. Insets show magnified images of the corresponding samples.

Nonionic CHP nanogels gave well-dispersed hybrid amorphous calcium phosphate (ACP) nanoparticles as previously reported.[4] CHPCOOH and $CHPNH_2$ nanogels also acted as templates for the formation of dispersed ACP nanoparticles without an induction of crystallization. Calcium phosphate could nucleate in nanogels due to the increase in the local concentration of calcium ions and phosphate ions by the interaction with COO^- groups/NH_3^+ groups or the dense polyhydroxy groups of the nanogels.[7] The mineralized nanoparticles obtained were further analyzed by energy-dispersive X-ray spectroscopy (EDS) to confirm the particles contain Ca and P (Figure 3). EDS shows that the Ca/P ratio in the hybrid nanoparticles was different depending on the template nanogels. The Ca/P ratio of ACP nanoparticles formed in the presence of nonionic CHP nanogels is 1.6.[4] This value is identical to the composition of Ca^{2+} and PO_4^{3-} ions in starting solution of HAp. In contrast, the Ca/P ratios of CHPCOOH–ACP (2.9) increased and that of $CHPNH_2$–ACP (1.3) decreased

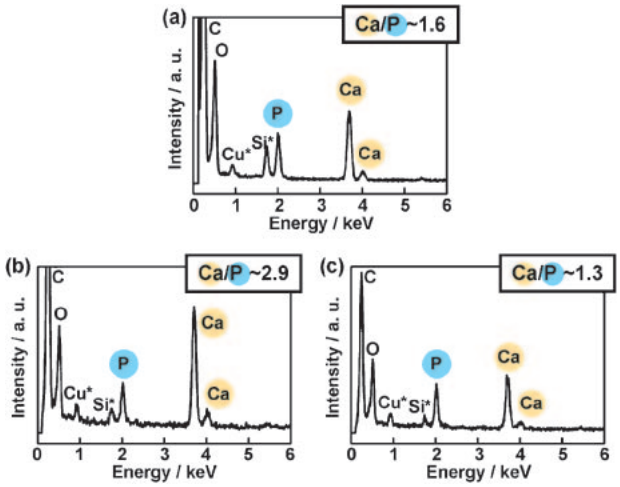

Figure 3. EDS spectra of a) CHP–ACP, b) CHPCOOH–ACP, and c) $CHPNH_2$–ACP. *The Cu and Si signals were attributed to the substrate.

in comparison to that of CHP–ACP. Ca^{2+} and PO_4^{3-} ions are attracted and accumulated by anionic CHPCOOH nanogels and cationic $CHPNH_2$ nanogels, respectively. Modifications of functional moieties that facilitate the mineralization of calcium phosphate by attracting and accumulating Ca^{2+} or PO_4^{3-} ions to organic templates would be a convenient strategy to obtain desired calcium phosphate–organic hybrid materials.

Finally, ζ-potentials of the nanoparticles were examined. The ζ-potentials of CHP–ACP, CHPCOOH–ACP, and $CHPNH_2$–ACP hybrid nanoparticles are -1.1, -4.5, and $+6.7$ mV (hydroxyapatite solution, $[Ca^{2+}] = 0.8$ mM at pH 8.0), respectively. The nanogel–ACP hybrid nanoparticles maintained their electric charge properties derived from functional groups of nanogels. The results show that the polysaccharides were located on the surfaces of the ACP nanoparticles. Calcium phosphate nucleated somewhere in nanogels and then grew, and the mineralization finally halted inside the nanogel. The surface coating explains the colloidal stability of these nanoparticles.

Nano-sized calcium phosphate particles are used as carriers for DNA and siRNA.[3c,8] The activity of transfection increases as a result of dissolution of calcium phosphate nanoparticles in the acidic environment of the endosomal vesicle. We reported that $CHPNH_2$ nanogels enabled effective transport of various proteins into cells.[6a] Calcium phosphate mineralization is also possible by using $CHPNH_2$ nanogel–protein complex. The hybrid nanoparticles are probably useful as a new intracellular protein carrier responsive to environmental pH. The hybrid ACP nanoparticles with various surface charges should offer new opportunities for nanocarriers in DDS. Such applications are under investigation in our laboratory.

Experimental

Materials. Cholesterol-bearing pullulan (CHP) was synthesized as reported previously.[5] CHP was substituted with 1.4 cholesteryl groups per 100 glucose units of the parent pullulan

($M_w = 1.0 \times 10^5$). CHP derivatives containing 22.6 β-alanine groups (CHPCOOH)[9] or 18.2 ethylenediamine groups ($CHPNH_2$)[6a] per 100 glucose units were used in this study.

Preparation of Nanogels. CHP was suspended in water under stirring. The suspended solution was then sonicated using a BRANSON SONIFIER 250 probe-type sonicator (Branson Ultrasonic Co.) for 15 min at 40 W and at an interval of 1 s. The resulting suspension was filtered through membrane filters (pore size 0.22 μm) to remove impurities.

Synthesis of Nanogel–Calcium Phosphate Hybrid Nanoparticles (pH-Gradient Method). HAp was dissolved in acidified water by bubbling with carbon dioxide gas for 3 h. The remaining solid HAp was then removed by filtration (pore size 0.22 μm). The concentration of calcium ions in the solution was determined by back-titration using EDTA. HAp solution was mixed with the CHPCOOH or $CHPNH_2$ nanogel suspension in a pear-shaped flask, and [Ca^{2+}] was then adjusted to 0.8 mM (nanogels 0.5 mg mL^{-1}). The mixture was then stirred for 8 h at 25 °C. Calcium phosphate was precipitated with a slow increase in the pH of the solution from 5.6 to 7.9 with the loss of CO_2 gas from the solution. The resulting solution was aged for 2 days at 25 °C.

Characterization. DLS and ζ-potential measurements were carried out at 25 °C with a Zetasizer Nano ZS (Malvern Instruments Ltd.). The measured autocorrelation function was analyzed by a cumulant method. The ζ-potential measurements were performed using a capillary ζ-potential cell in automatic mode. TEM images of the unstained samples were taken with a Hitachi H-600 (Hitachi High-Technologies Co.) at 100 kV. EDS measurements were obtained using a JEOL JEM-3010 equipped with an EDAX Genesis Series γ-TEM at 300 kV. Sample solutions were applied to a carbon-coated 100-mesh copper grid and then excess samples were removed using filter paper.

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