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Calcium phosphate-polymer hybrid microparticles having functionalized surfaces prepared by a coaxially electrospray technique

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Keywords: Calcium phosphate, Electrospray, Inorganic microcapsules, Surface modification, Phosphoryl choline polymer

Abstract

Microparticles composed of calcium phosphate (CaP) and chitosan covered with 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer were prepared in a single step by coaxial electrospraying. An aqueous solution containing calcium chloride, chitosan and an MPC polymer ethanol solution were electrosprayed from coaxial double needles into a phosphate solution. CaP/chitosan microparticles were successfully formed and their surfaces were simultaneously covered with MPC polymer. The resulting microparticles had an average diameter of around 400 μm. Investigation using fluorescently labeled MPC polymer revealed surface coverage of the CaP/chitosan microparticles with MPC polymer. The formation of CaP, mainly hydroxyapatite, was confirmed by X-ray diffraction measurement. A protein adsorption study revealed that bovine serum albumin (BSA) adsorption on the microparticles was effectively suppressed by the MPC polymer. Model substances

(dextran and BSA) were successfully encapsulated within the microparticles, with high encapsulation efficiencies (more than 80%), in a single step by coaxial electrospraying. Finally, we succeeded in the selective immobilization of a target protein on the surface of the CaP/chitosan microparticles covered with MPC polymer.

1. Introduction

Calcium phosphate (CaP) is a major component of body tissue that has recently attracted increased attention as a biomaterial because of its appropriate biodegradation and excellent biocompatibility. CaP is widely applied in artificial bone, drug delivery carriers, and cell scaffolds [1-5], and is also exploited in biomimetic mineralization research [6,7]. Although CaP crystals can be synthesized under mild conditions, it is usually difficult to control the crystal growth and the size (also shape) of CaP particles while preventing their aggregation [8]. To overcome these difficulties, there have been many reports on the preparation of CaP microparticles, including mechanochemical processing, chemical precipitation, microemulsion, and hydrothermal treatment [9-12]. An electrospray technique is an effective method to create a fast reaction to produce CaP microparticles in a single step. We reported the preparation of microparticles composed of calcium phosphate and organic polymer additives in a single step by electrospraying, and showed excellent encapsulation properties for various substances [13].

The surface properties of CaP particles play an important role in the application for life sciences, because its surface is in direct contact with biological substances. Indeed, CaP adsorbs a wide variety of molecules (proteins, organic acids, saccharides and nucleic acids) [14-18]. Therefore, the control of the surface properties of CaP particles is of great importance. To our knowledge, however, there has been no attempt to prepare CaP microparticles with low-fouling surfaces. In the present study, we used 2-methacryloyloxyethyl phosphorylcholine (MPC) polymers for surface coating to prevent nonspecific protein adsorption on CaP microparticles. MPC polymers, which are inspired by the surface structure of biomembranes and synthesized by Ishihara et al., are well-known to show excellent suppression of

protein adsorption onto various surfaces [19]. Here, we used a coaxial electrospray technique to prepare CaP-polymer hybrid microparticles covered with MPC polymer in a single step, and demonstrated low-fouling of the microparticles. Moreover, we succeeded in the selective immobilization of a target protein on the low-fouling surfaces of the microparticles using avidin-biotin interaction [20], which proposes high potential of CaP-polymer hybrid microparticles functionalized with biomolecules.

2. Materials and methods

Calcium chloride, chitosan (commercially available chitosan 10, with an aqueous solution viscosity below 20 mPa s when dissolved in 0.5 wt% acetic acid solution at 20 °C), acetic acid, disodium hydrogen-phosphate anhydride and ethanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). MPC polymer (Lipidure®-CM) and amine-modified MPC polymer (Lipidure®-NH01) were purchased from NOF America (White Plains, NY). Albumin−fluorescein isothiocyanate conjugate (FITC-BSA), fluorescein isothiocyanate-dextran (FITC-dextran, MW = 2,000,000 Da), albumin, tetramethylrhodamine isothiocyanate bovine (TRITC-BSA) and anti-mouse IgG (whole molecule)-TRITC antibody produced in goat (TRITC-IgG) were purchased from Sigma (St. Louis, MO). Tetramethylrhodamine isothiocyanate (TRITC) was purchased from Invitrogen (Carlsbad, CA). 9-(Biotinamido)-4,7-dioxanonanoic acid *N*-succinimidyl ester (Biotin-PEG$_2$-NHS) was purchased from Tokyo Chemical Industries (Tokyo, Japan). Avidin D, TRITC conjugate (TRITC-avidin) was purchased from Funakoshi (Tokyo, Japan). Other chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

TRITC-labeled MPC polymer was prepared as follows. Amine-modified MPC polymer (87 mg) was dissolved in a triethylamine/acetate buffer (pH 8, 0.1 M, 9 mL). A dimethylformamide solution (1 mL) containing 30 mg of TRITC was added to the amine-modified MPC polymer solution. After 4 h at room temperature, the reaction solution was dialyzed with an excess amount of a

triethylamine/acetate buffer using a dialysis membrane (Slide-A-Lyzer MWCO 3 kDa, Thermo Fisher Scientific, Waltham, MA) for 14 days, followed by freeze-drying.

Biotin-conjugated MPC polymer was prepared as follows. Amine-modified MPC polymer (64 mg) was dissolved in a triethylamine/acetate buffer (pH 8, 0.1 M, 8 mL). A dimethylformamide solution (2 mL) containing 25 mg of biotin-PEG_2-NHS was added to the amine-modified MPC polymer solution. After 24 h at room temperature, the reaction solution was dialyzed with an excess amount of diluted water using a dialysis membrane (Slide-A-Lyzer MWCO 3 kDa, Thermo Fisher Scientific, Waltham, MA) for 2 days, followed by freeze-drying.

2.1. Electrospray

The coaxial electrospray (NF-102, MECC Co., Ogori, Japan) experimental equipment consisted of syringe pumps, a stainless steel outlet comprising an inner needle nested inside an outer needle, and a high-voltage generator. Typically, the inner needle was loaded with an aqueous solution (pH 4.0) containing calcium chloride (5.0 wt%), chitosan (2.0 wt%), and acetic acid (200 mM), and the outer needle was loaded with an ethanol solution containing MPC polymer (0.1, 0.2 and 0.5 wt%) and TRITC-labeled MPC polymer (0.005 wt% for visualization experiments). The solutions were simultaneously sprayed from the inner and outer needles (cathode), into an oppositely charged stainless steel dish (anode) containing an aqueous solution (receiving solution, 7 mL, pH 8.9) of disodium hydrogen phosphate (5.0 wt%) for 3 min to form CaP/chitosan microparticles covered with MPC polymer (CaP/chitosan/MPC microparticles). During electrospraying, the aqueous solution in the dish was gently stirred continuously using a magnetic stir bar (at approximately 100 rpm). The CaP/chitosan microparticles without MPC polymer were prepared by the same procedure but omitting MPC polymer.

The feed rates for both the inner and outer solutions were set at 0.2 mL/h, and the working voltage was 23 kV. The distance from the needle to the collector was 5.0 cm. The inner and outer diameters of the inner needle were 330 and 630 μm, respectively, and those of the outer needles were 1.0 and 2.5

mm, respectively.

After electrospraying, a microparticle suspension (7 mL) was left to allow precipitation. The supernatant was replaced with phosphate buffered saline (PBS, pH 7.4) and was left to allow precipitation. This washing procedure was repeated twice and, finally, the microparticles were dispersed in PBS (pH 7.4).

2.2. Characterization of CaP/chitosan/MPC microparticles

CaP/chitosan/MPC microparticles were observed using an inverted microscope (IX71, Olympus Co., Tokyo, Japan) and a confocal laser scanning microscope (CLSM) (FV1000-D, Olympus Co, Tokyo, Japan). Based on the microscope images, the diameters of 100 microparticles were measured.

The crystalline state of the microparticles was characterized by X-ray diffraction (XRD) (SmartLab, Rigaku, Tokyo, Japan) with Cu-Kα incident radiation.

2.3. Evaluation of protein adsorption onto CaP/chitosan/MPC microparticles

FITC-BSA was dissolved in PBS (pH 7.4) to prepare a 1 mg/mL solution. After mixing 500 μL of a CaP/chitosan/MPC microparticle suspension (or CaP/chitoan microparticle suspension) with 500 μL of FITC-BSA solution, the mixture was incubated for 1 h at 37 °C. The concentration of FITC-BSA in the supernatant was measured using a fluorescence spectrometer (JASCO, Tokyo, Japan) (excitation wavelength (λ_{ex}) = 490 nm, emission wavelength (λ_{em}) = 520 nm) to quantify the amount of FITC-BSA adsorbed on the microparticles.

2.4. Encapsulation of substances in CaP/chitosan/MPC microparticles

To evaluate encapsulation, FITC-dextran and FITC-BSA were used as core substances. The inner needle was loaded with an aqueous solution (pH 4.0) containing calcium chloride (5.0 wt%), chitosan (2.0 wt%), acetic acid (200 mM), and the core substrate (100 μg/mL FITC-dextran or 100 μg/mL FITC-

BSA), and the outer needle was loaded with an ethanol solution containing MPC polymer (0.5 wt%) and TRITC-labeled MPC polymer (0.005 wt%). The solutions were simultaneously sprayed into an aqueous solution containing 5.0 wt% disodium hydrogen-phosphate under typical conditions. The CaP/chitosan microparticles encapsulating FITC-dextran and FITC-BSA were prepared by the same procedure but omitting MPC polymer, for the release study. Microparticles encapsulating fluorescent substances were observed using CLSM.

After electrospraying, the fluorescence of the supernatant of the receiving solution was measured by a fluorescence spectrometer. An encapsulation efficiency was calculated on the basis of the fluorescence in the supernatant and the amount of a fluorescent substance sprayed.

The release of encapsulated substances (FITC-dextran and FITC-BSA) was evaluated as follows. A microparticle suspension was left to allow precipitation. The supernatant was replaced with a PBS (pH 7.4) and was left to allow precipitation. This washing procedure was repeated twice. A pH adjusted aqueous buffer was added to the precipitate to disperse microparticles. A HEPES buffer (0.1 M, pH 7.4) and an acetate buffer (0.1 M, pH 5.5) were used as a pH-adjusted buffer. The suspension was gently mixed using a test tube rotator at 25 °C for 48 h in the dark. Supernatant samples were periodically drawn from the suspension and subjected to fluorescence measurements.

2.5. Immobilization of avidin on the surface of CaP/chitosan/biotin-conjugated MPC microparticles

Biotin-conjugated MPC polymer was coated onto CaP/chitosan microparticles as follows. The inner needle was loaded with an aqueous solution (pH 4.0) containing calcium chloride (5.0 wt%), chitosan (2.0 wt%) and acetic acid (200 mM), and the outer needle was loaded with an ethanol solution containing MPC polymer (0.5 wt%) and biotin-conjugated MPC polymer (0.005 wt%). The solutions were simultaneously sprayed into an aqueous solution containing 5.0 wt% disodium hydrogen-phosphate under typical conditions.

A microparticle suspension was washed by the same procedure described above. Each protein

solution containing TRITC-avidin, TRITC-BSA or TRITC-IgG (0.01 mg/mL each) in PBS (pH 7.4) was added to the microparticle precipitate. After incubation for 1 h at 37 °C, each of the supernatant was replaced with phosphate buffered saline (PBS, pH 7.4) and was left to allow precipitation. This washing procedure was repeated twice and, finally, the microparticles were dispersed in PBS (pH 7.4). The CaP/chitosan/MPC (non-conjugated with biotin) microparticles were also incubated in TRITC-avidin solution by the same procedure. After washing, the microparticles were observed using CLSM.

3. Results and discussion

3.1. Preparation and characterization of CaP/chitosan/MPC microparticles

An ethanol solution containing MPC polymer and TRITC-labeled MPC polymer, and an acetic acid aqueous solution containing calcium chloride and chitosan were electrosprayed from the outer and inner needles, respectively, of the coaxial electrospray apparatus shown in Fig. 1. An aqueous solution of disodium hydrogen phosphate (5.0 wt%) was used as a receiving solution. The ejected microdroplets (composed of an aqueous solution containing Ca^{2+} and polyelectrolyte (chitosan) and ethanol solution containing MPC polymers) fell into the receiving solution containing PO_4^{3-}. PO_4^{3-} diffused to react with Ca^{2+} to form calcium phosphate (CaP), resulting in CaP/chitosan microparticles. Simultaneously the microparticles were covered with MPC polymer (Fig. 1) because of the poor solubility of MPC polymer in water and strong interaction between CaP and many kinds of organic polymers [13].

Fig. 2a and b show bright-field microscope and CLSM images of CaP/chitosan/MPC microparticles prepared with a 0.5 wt% MPC polymer solution. Fig. 2a shows that pleated microparticles ranging 300–400 μm in diameter were successfully prepared. As shown in Fig. 2b, the surface of CaP/chitosan microparticles exhibited red fluorescence derived from TRITC-labeled MPC polymer, suggesting surface coverage with the MPC polymer, which supports the scheme for formation of CaP/chitosan/MPC microparticles proposed above. As proposed in the scheme (Fig. 1), TRITC-labeled MPC polymer mainly exists in the surface of the particles, however, a little amount of TRITC-labeled

MPC polymer also exists inside the particles, which suggests that a part of MPC polymer diffused inside microdroplets of CaP/chitosan during being sprayed. The mean diameter of the microparticles was 410 μm (C.V. 17%), with a relatively narrow distribution (Fig. 2c). The relatively narrow diameter distribution of microparticles prepared by the electrospray technique agrees with our previous reports [13,21,22].

The XRD spectrum of the prepared microparticles shows peaks (26° and 32°) typical of hydroxyapatite crystals (Fig. 2d), which is also consistent with our previous report [13]. These results support our proposed mechanism for the formation of CaP/chitosan microparticles covered with MPC polymer.

The effect of the inner diameter of the inner needle was also studied (130, 190 and 330 μm). The diameter of microparticles increased (140, 240 and 410 μm in average diameters, respectively) as the inner diameter of the needle increased. Size control was thus easily achieved by varying the needle diameter.

In the present study, we also confirmed a stable electrospraying to continuously produce the microparticles under the present electrospray conditions.

3.2. Protein adsorption onto CaP/chitosan/MPC microparticles

To evaluate the effect of MPC polymer coating of CaP/chitosan microparticles, protein adsorption experiments were conducted. Fig. 3 shows the amount of BSA adsorbed on the microparticles with various concentrations of MPC polymer (concentration in the feed ethanol solution). Several research groups reported that CaP exhibits high protein adsorption properties [14-16]. Indeed, a large amount of BSA (approx. 250 μg/mg-microparticles) was adsorbed on CaP/chitosan microparticles without the MPC polymer coating. The amount of BSA adsorbed decreased with increasing MPC polymer concentration. The amount of BSA adsorbed on the microparticles with 0.5 wt% MPC polymer was about 18% of that without the MPC polymer. These results indicate that protein adsorption on CaP can

be suppressed by an MPC polymer coating and that CaP/chitosan microparticles covered with MPC polymer can be easily prepared by the electrospray technique.

3.3. Encapsulation of substances in CaP/chitosan/MPC microparticles

One of the advantages of microcapsule preparation using an electrospray technique is to readily encapsulate various substances within microparticles, with high encapsulation efficiency. CaP/chitosan/MPC microparticles can be expected to encapsulate substances efficiently because CaP has a mesoporous structure that provides enough room to accommodate a large amount of substances [13]. Here, we investigated the encapsulation of FITC-dextran and FITC-BSA within CaP/chitosan/MPC microparticles. CLSM observation revealed that these fluorescent substances (green fluorescence) were evident over the entire CaP/chitosan/MPC microparticles, indicating that they were successfully encapsulated within the microparticles (Fig. 4). The encapsulation efficiencies of FITC-dextran and FITC-BSA were 84% and 99%, respectively. The encapsulation efficiency of FITC-BSA was higher than that of FITC-dextran, probably because of a strong interaction between FITC-BSA and CaP. These results show that the electrospray technique can encapsulate substances within the CaP/chitosan/MPC microparticles, with high encapsulation efficiency.

We investigated the release of encapsulated substances from the CaP/chitosan/MPC and CaP/chitosan microparticles under different pH conditions. Fig. 5 shows the release profiles of dextran and BSA. The release of dextran was slower than that of BSA at both pH 5.5 and pH 7.4. Because of the large molecular size of dextran, it could be difficult to permeate through the microparticle surface. The release of dextran was accelerated under an acidic condition (at pH 5.5) due to the partial dissolution of calcium phosphate. On the other hand, the release of BSA was much more accelerated under an acidic condition than that of dextran, because calcium phosphate has a relatively strong interaction with BSA under neutral condition (at pH 7.4). The release of encapsulated substances from the microparticles coated with MPC polymer was slower than the uncoated microparticles at pH 7.4.

These results indicate that the MPC polymer shell worked as a barrier for the encapsulated substances, which is consistent with the suppression of the foulants adsorption onto the CaP/chitosan/MPC microparticles discussed above.

3.4. Immobilization of avidin on the surface of CaP/chitosan/biotin-conjugated MPC microparticles

The present study reports the biocompatible microparticles with low-fouling surfaces. The functionalization of the surfaces of microparticles is of large importance in biomaterial. We investigated the immobilization of a target protein on the CaP/chitosan/MPC microparticles (Fig. 6a). The CaP/chitosan/MPC microparticles was prepared using biotin-conjugated MPC polymer and the selective binding of fluorescently-labeled avidin was observed using CLSM. Fig. 6b-e shows the adsorption of each protein to the microparticles with and without biotin-conjugated MPC polymer. While the microparticles without biotin-conjugated MPC polymer did not adsorb avidin (Fig. 6b), the CaP/chitosan/biotin-conjugated MPC microparticles obviously adsorbed avidin on their surfaces (Fig. 6c). Other kinds of proteins (BSA and IgG) were not adsorbed on the CaP/chitosan/biotin-conjugated MPC microparticles due to the low-fouling property of MPC polymer (Fig. 6d and e). These images also show that any protein tested did not penetrate in the microparticles, suggesting that the CaP/chitosan microparticles were fully covered by MPC polymer. These results mean the selective immobilization of a target protein on the microparticle surfaces and demonstrate that CaP/chitosan/MPC microparticles can be functionalized with biomolecules using MPC polymer as a chemically designable platform, while keeping the low-fouling property of their surfaces.

4. Conclusions

We prepared microparticles composed of CaP and chitosan covered with MPC polymer in a single step by coaxial electrospraying. The microparticles were around 400 μm in an average diameter, and effectively suppressed protein adsorption by their surface coverage with MPC polymer. This technique

successfully encapsulated substances (dextran and BSA) with high encapsulation efficiencies and their release from the microparticles could be delayed by the coverage of MPC polymer. Finally, the highly selective immobilization of a target protein (avidin) on the microparticles was realized using biotin-conjugated MPC polymer. The findings in the present study propose that the coaxial electrospray technique can provide great potential for the production of functional microparticles with the controlled surface properties and release property.

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Figures

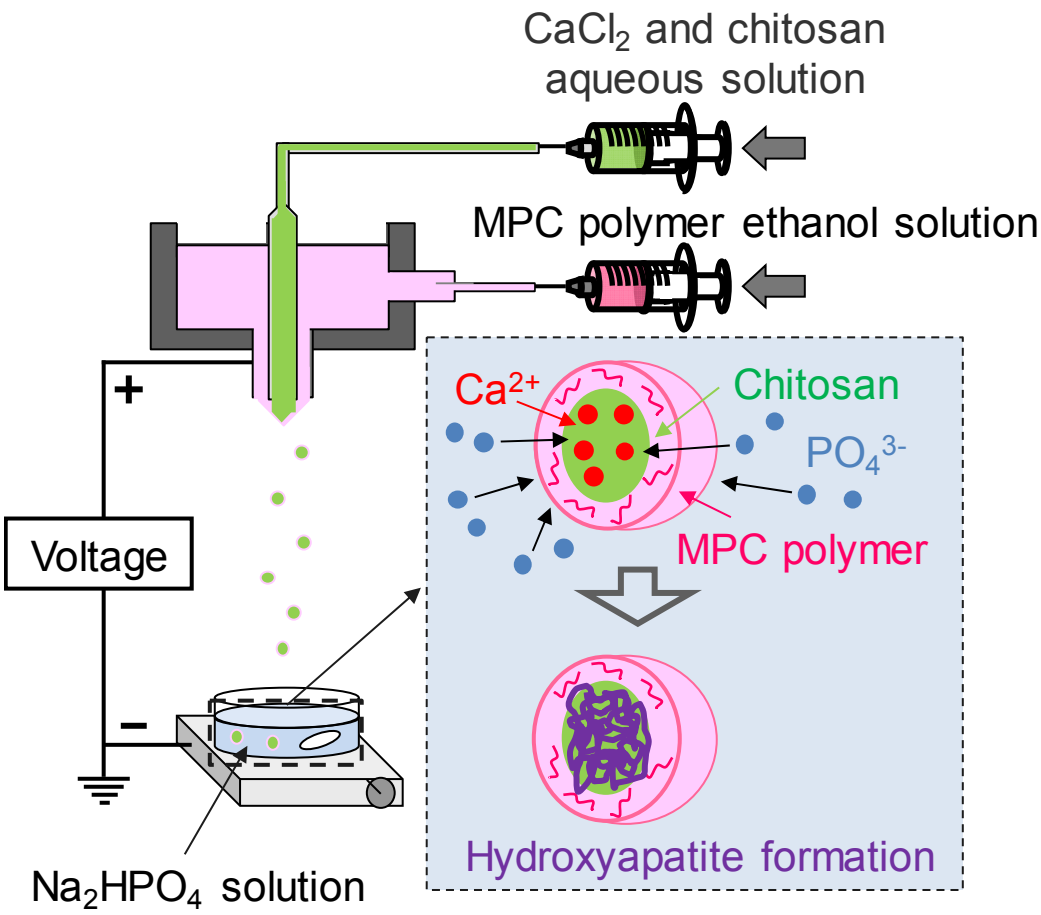

Fig. 1 Schematic of the method of preparation of CaP/chitosan/MPC microparticles.

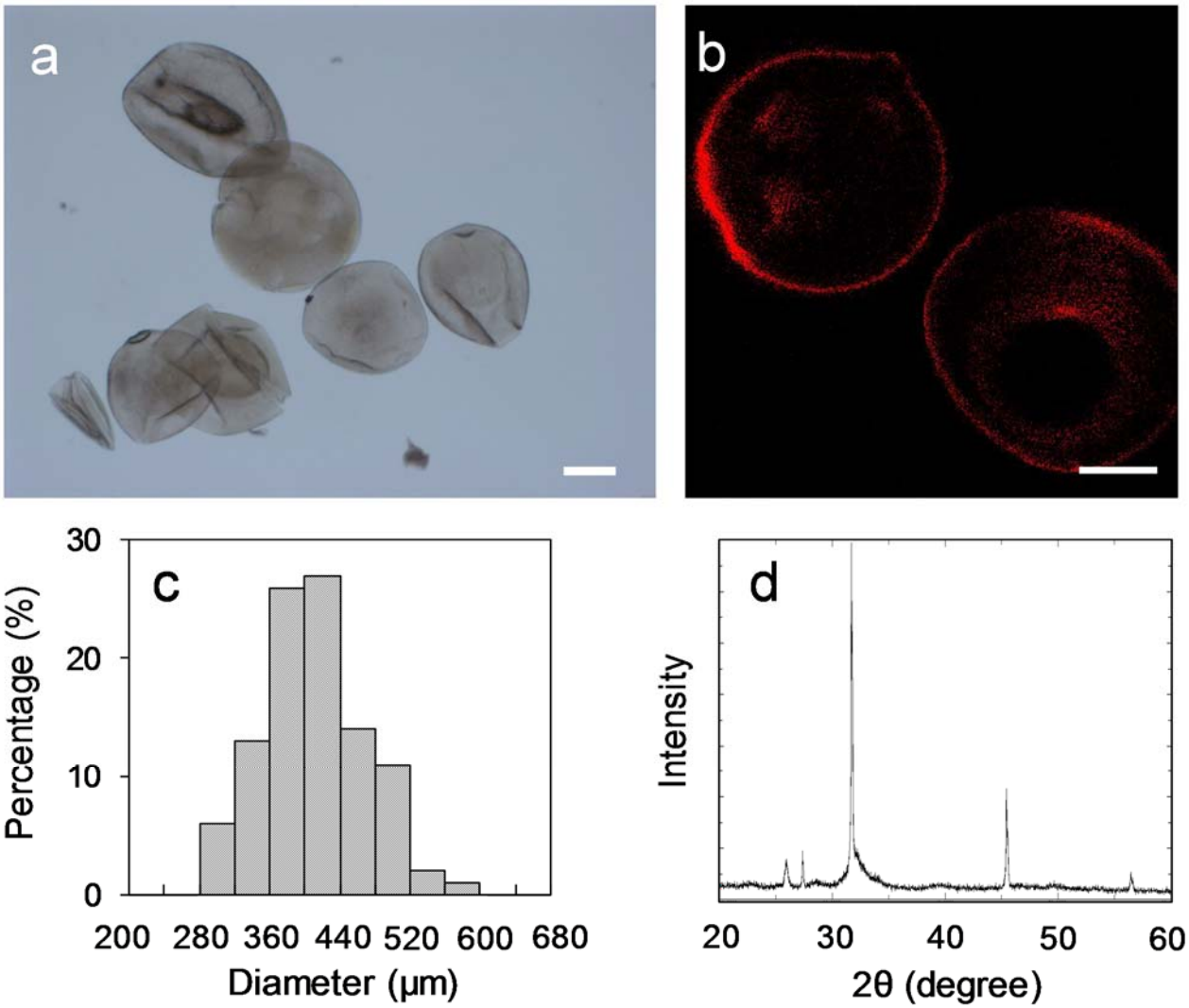

Fig. 2 Characteristics of CaP/chitosan/MPC microparticles. (a) Bright-field microscope image and (b) CLSM image of CaP/chitosan/MPC microparticles. The scale bars represent 100 μm. (c) Size distribution of CaP/chitosan/MPC microparticles. (d) XRD spectrum of CaP/chitosan/MPC microparticles.

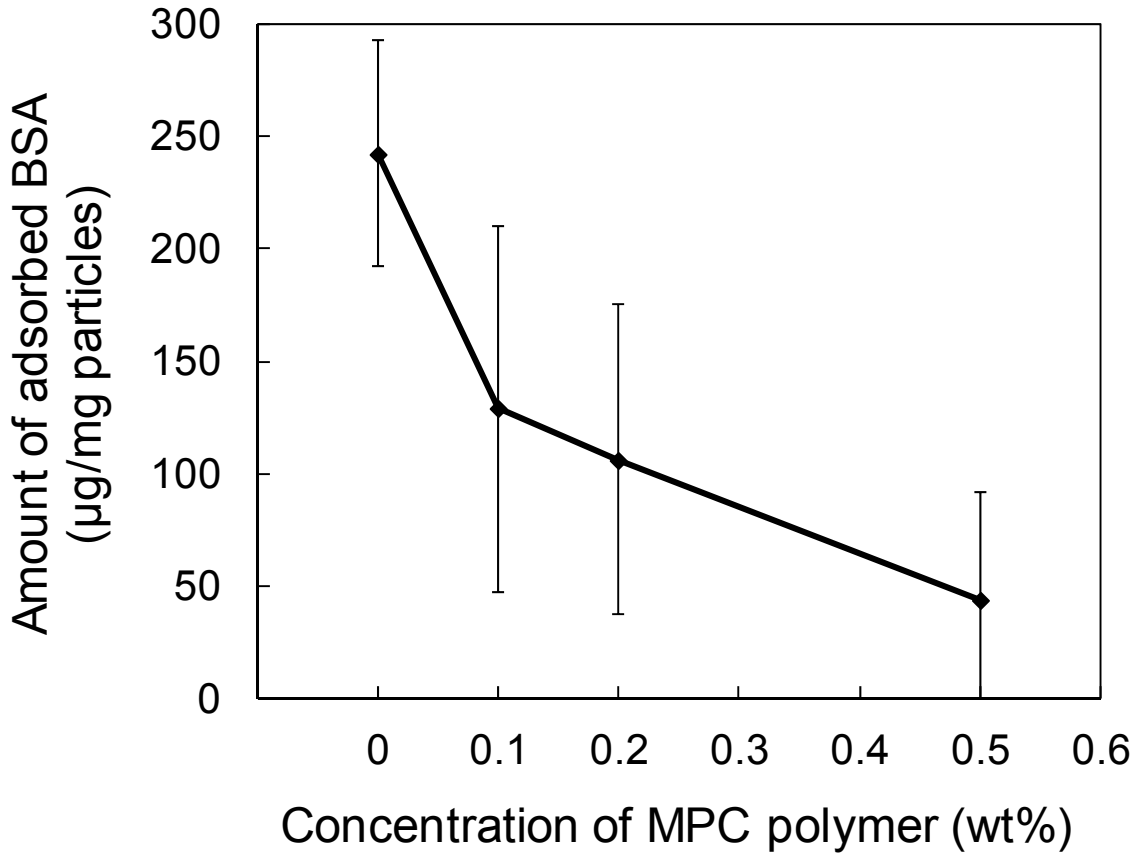

Fig. 3 Protein (FITC-BSA) adsorption onto CaP/chitosan/MPC microparticles with various concentrations of MPC polymer.

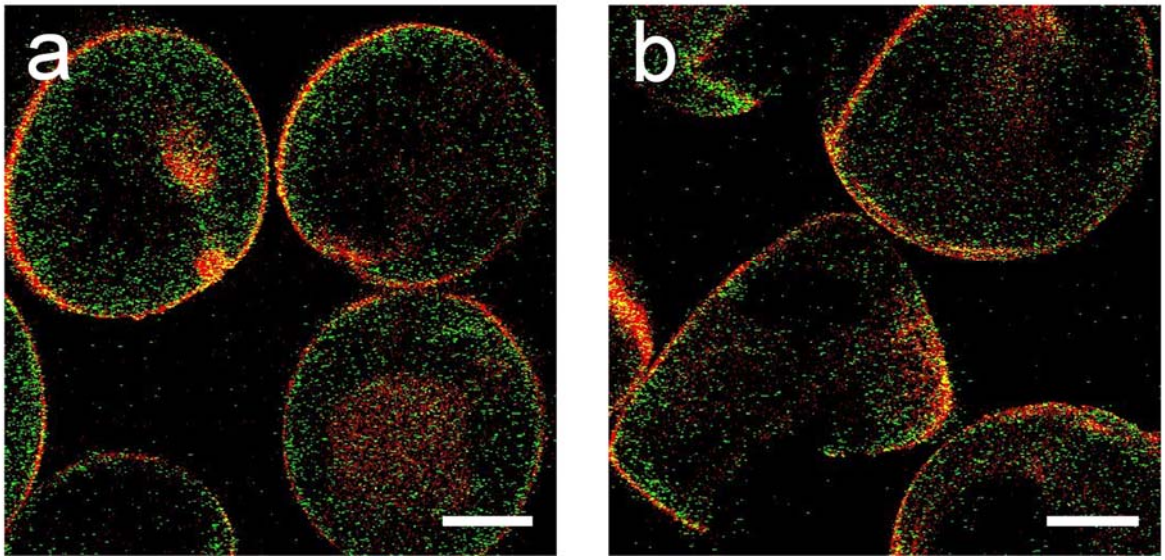

Fig. 4 CLSM images of CaP/chitosan/MPC microparticles containing FITC-dextran (a) and FITC-BSA (b). The scale bars represent 100 μm.

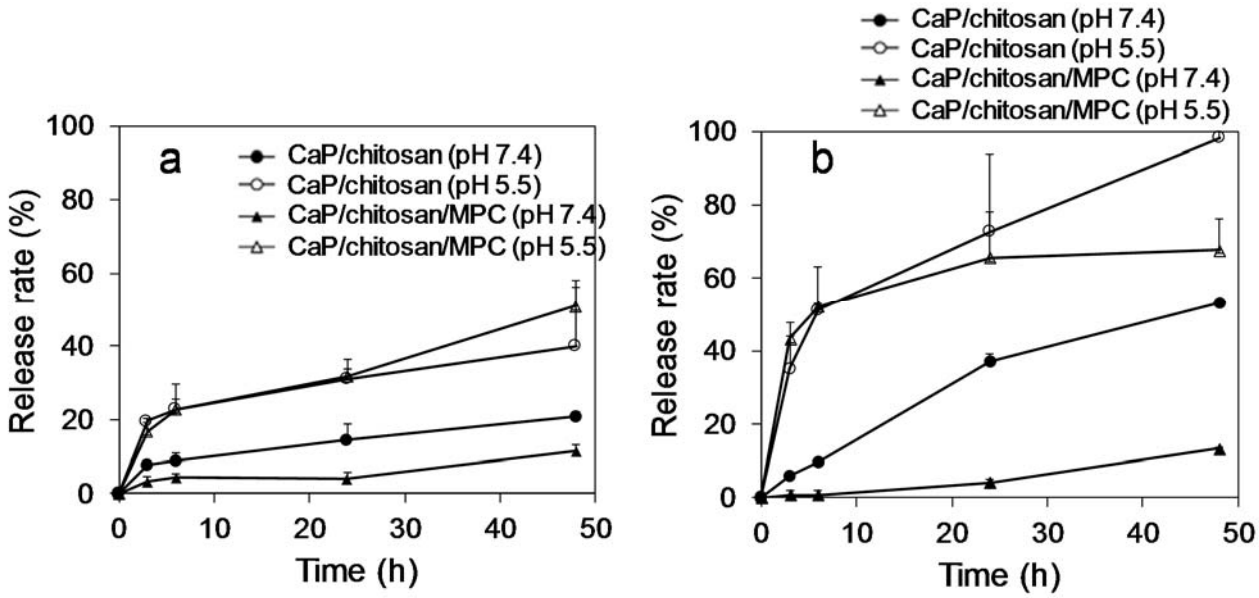

Fig. 5 Release of the encapsulated substances (FITC-dextran (a) and FITC-BSA (b)) from CaP/chitosan and CaP/chitosan/MPC microparticles under different pH conditions (pH 5.5 and 7.4).

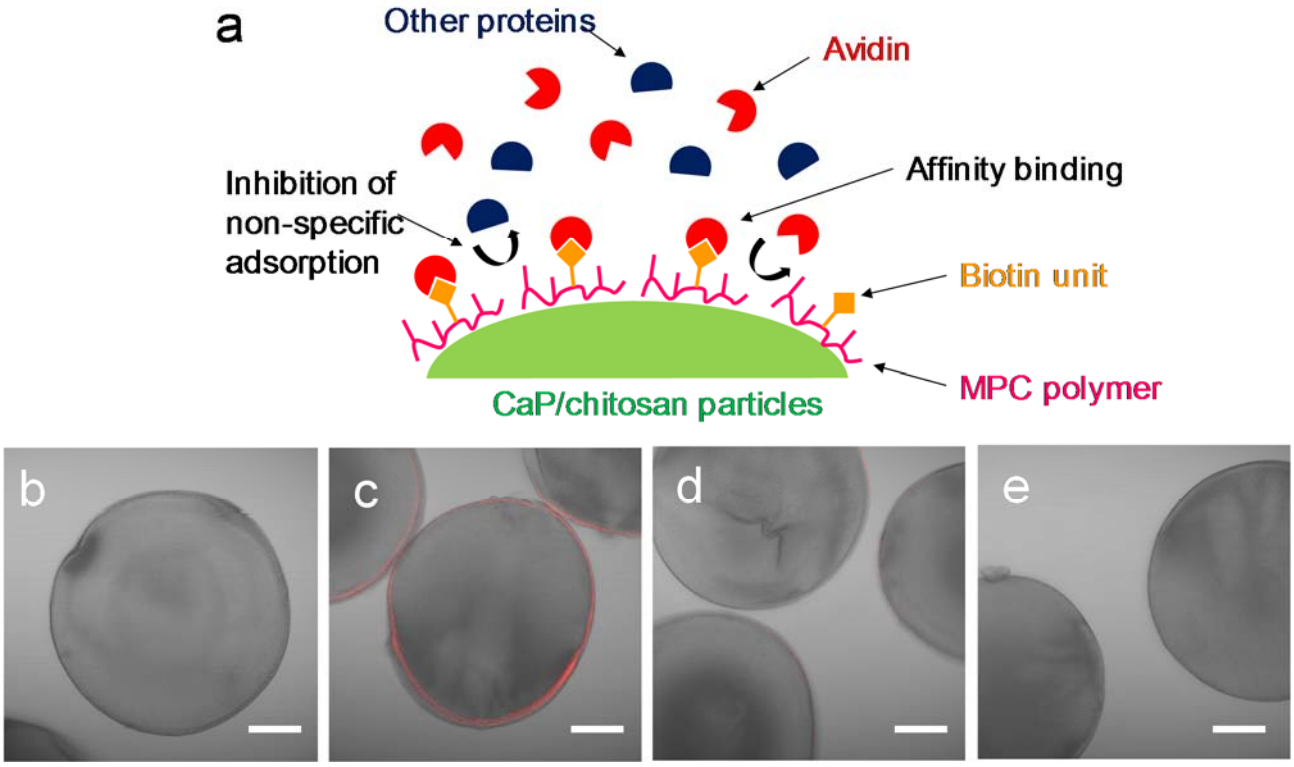

Fig. 6 (a) Schematic of selective immobilization of avidin on CaP/chitosan/biotin-conjugated MPC microparticles**.** (b-e) Merged images of CLSM and differential interference images of the microparticles with fluorescently-labeled proteins. CaP/chitosan/MPC microparticles mixed with TRITC-avidin (b) and CaP/chitosan/biotin-modified MPC microparticles mixed with TRITC-avidin (c), TRITC-BSA (d) and TRITC-IgG (e). The scale bars represent 100 μm