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Mineralized cyclodextrin nanoparticles for sustained protein delivery

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ABSTRACT

The extensive therapeutic potential of protein drugs has been severely limited by their instability and short biological half-lives *in vivo*. To prolong their therapeutic effects, a sustained delivery system is required. In this study, cyclodextrin-based polymeric nanoparticles (CD-NPs), mineralized by calcium phosphate as the diffusion barrier, were developed as a carrier for sustained protein delivery. Spherical CD-NPs were readily prepared by a conjugate, composed of β-CD as the protein-binding moiety and carboxymethyl dextran as the substrate for mineralization in a physiological solution. Owing to the presence of carboxylic acids in CD-NPs, they were effectively mineralized by sequential addition of calcium nitrate and ammonium phosphate. The physicochemical characteristics of mineralized CD-NPs were characterized using FT-IR, thermogravimetric analysis, transmission electron microscopy and energy dispersive X-ray photoelectron spectroscopy. Mineralization reduced CD-NP particle size from 310 nm to 121 nm in PBS (pH 7.4) indicating the formation of compact nanoparticles. Carbonic anhydrase B (CAB), chosen as the model protein, was loaded into the mineralized CD-NPs with a high loading efficiency (80%) by a simple dialysis method. *In vitro* release tests showed that CAB was completely released from bare CD-NPs in 3 days. Interestingly, the mineralized CD-NPs released CAB in a sustained manner for 21 days, which was due to the stable calcium phosphate barrier inhibiting CAB release. The enzymatic activity of CAB, which was released from the nanoparticles, did not significantly deteriorate compared to native CAB. Overall, mineralized CD-NPs could be a promising carrier for sustained protein delivery.

1. Introduction

There have been more than 130 therapeutic proteins approved by the Food and Drug Administration (FDA) for clinical applications (Leader, Baca, & Golan, 2008; Mullard, 2011). These proteins are highly specific in their actions and have minimal side effects thereby allowing for their use in a wide range of diseases. However, due to their inherent characteristics such as rapid renal clearance, fragile tertiary structure and susceptibility to proteolytic enzymes, therapeutic proteins must be administered systemically by frequent injections. Therefore, much effort has been devoted to the development of sustained protein delivery systems to prolong their therapeutic effect following administration. Temperature-sensitive Pluronic hydrogels have been investigated as a sustained protein delivery system (Chung, Lee, & Park, 2008; Park, Chung, Lee, & Park, 2008; Wenzel et al., 2002). However, most proteins are released within several days because Pluronic-based hydrogels dissociate rapidly upon dilution with body fluid which is primarily ascribed to the weak physical interactions between polymer chains (Barichello, Morishita, Takayama, & Nagai, 1999). Injectable and biodegradable microspheres derived from poly(lactic-co-glycolic acid) have been also studied (Bartus, Tracy, Emerich, & Zale, 1998; Tamber, Johansen, Merkle, & Gander, 2005; Wei, Pettway, McCauley, & Ma, 2004). Unfortunately, harsh preparation conditions including the use of organic solvents during the formulation often results in loss of biological activity (Fu, Klibanov, & Langer, 2000; Fu, Pack, Klibanov, & Langer, 2000).

Cyclodextrins (CDs) composed of cyclic oligosaccharides with high biocompatibility have been widely used in pharmaceutical applications in order to improve the physicochemical properties of drugs such as their solubility, stability and bioavailability (Davis & Brewster, 2004; Szejtli, 1998). CDs have the unique property of forming inclusion complexes with various lipophilic molecules and hydrophilic polymers through hydrogen bonding and/or hydrophobic interactions (Harada, 1996; Harada & Kamachi, 1990; Kang, Kumar, Yang, Chowdhury, & Hohl, 2002; Liu & Guo, 2002; Liu et al., 2003). Several CD/drug inclusion complexes have been approved by the FDA and are currently available in the markets

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(Davis & Brewster, 2004). In recent years, CDs have been emerging as constituents of protein and drug delivery sytems (Gao, Wang, Fan, & Ma, 2007; Gao, Wang, Fan, & Ma, 2005; Gao, Yang, Fan, & Ma, 2006; Prabakaran & Jayakumar, 2009). Self-assembled hydrogels based on the inclusion complexes between CDs and hydrophobic molecules were found to release proteins in a susatined manner for 11 days (van de Manakker, Braeckmans, et al., 2009).

Calcium phosphate (CaP), the principal mineral component of bone and teeth, is biocompatible and biodegradable with high stability in physiologocal fluids (pH 7.4). Owing to these unique properties, it has been extensively investigated for drug delivery application (Epple et al., 2010; Han, Han, et al., 2011; Kim et al., 2010). In order to develop a carrier for sustained delivery of proteins, we prepared a polymeric conjugate which can be self-assembled into nanoparticles consisting of β-CD as the protein-binding core and carboxymethyl dextran (CMD) as the substrate shell for mineralization. The β-CD-bearing nanoparticles (CD-NPs) were mineralized in the presence of calcium nitrate and ammonium phosphate. The physiochemical properties of mineralized CD-NPs (MCD-NPs) were characterized using FT-IR, thermogravimetric analysis (TGA), transmission electron microscope (TEM), dynamic light scattering (DLS) and energy dispersive photoelectron spectroscopy (EDS). Carbonic anhydrase B (CAB), chosen as the model protein, was loaded into the nanoparticles by a simple dialysis method. The *in vitro* release behavior of CAB was monitored as a function of time in PBS (pH 7.4). In addition, the enzyme activity of CAB, released from MCD-NPs, was evaluated using a 4-nitrophenyl acetate (NPA) assay.

2. Materials and methods

2.1. Materials

CMD (MW = 10,000–20,000 Da), β-CD, ethylenediamine (EDA), 1-ethyl-3(3-dimethylaminopropyl) carbodiimide·hydrochloride (EDC·HCl), *N*-hydroxy succinimide (NHS), CAB (MW = 30,000 Da), 2-amino-2-hydroxymethyl-1,3-propanediol·hydrochloride (Tris·HCl), 2-amino-2-hydroxymethyl-1,3-propanediol sulfate (Tris–sulfate), NPA, ammonium phosphate and calcium nitrate were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). The water used in the experiments was prepared using an Aqua Max-Ultra water purification system (Yonglin Co., Anyang, Korea). All other chemicals were analytical grade and used without further purification.

2.2. Synthesis of CMD-β-CD conjugates

CMD-β-CD was prepared by chemical conjugation of amine-functionalized β-CD to the backbone of CMD in the presence of EDC and NHS. The detailed synthetic scheme for amine-functionalized β-CD is shown in Fig. S1. In brief, carboxymethyl β-CD was first synthesized by a previously reported procedure with slight modification (Sivasubramanian, Kim, et al., 2012). A mixture of β-CD (3 g, 2.6 mmol) and sodium hydroxide (2.64 g, 66 mmol) were dissolved in 15 mL of distilled water. The resulting solution was treated with monochloroacetic acid (0.25 g, 2.6 mmol) at 50 °C for 5 h. The solution was subsequently neutralized with 0.1 M HCl, precipitated in acetone and dried for 2 days at 40 °C under vacuum. Next, the carboxymethyl β-CD (1 g, 0.8 mmol) obtained was dissolved in 25 mL of distilled water containing EDC·HCl (0.24 g, 1.2 mmol) and NHS (0.14 g, 1.2 mmol). The mixture was stirred for 12 h at room temperature to prepare the succinimide derivative of β-CD (β-CD-NHS). Subsequently, the mixture was purified by dialysis (MWCO = 1000 Da) against distilled water for 1 day followed by lyophilization. In order to obtain β-CD-NH_2, β-CD-NHS (1 g, 0.76 mmol) in dimethyl formamaide (5 mL) was reacted with EDA (5.13 mL, 76 mmol) for 7 h at room temperature. The solution was then purified by dialysis (MWCO = 1000 Da) against distilled water for 1 day followed by lyophilization.

The synthetic route for CMD-β-CD is shown in Fig. S2. In brief, CMD (100 mg, 0.4 mmol) was dissolved in 25 mL of distilled water containing EDC·HCl (322.3 mg, 1.68 mmol) and NHS (193 mg, 1.68 mmol), and the solution was stirred for 30 min. Subsequently, β-CD-NH_2 (290.5 mg, 0.235 mmol) was added and stirred at room temperature for 24 h. The resulting mixture was purified by dialysis (MWCO = 12–14,000 Da) against distilled water for 2 days followed by lyophilization. The chemical structure of the conjugate was analyzed using ^{1}H NMR spectroscopy (JEOL JNM-AL300, Tokyo, Japan) in which the sample was dissolved at a concentration of 1 mg/mL in D_2O.

2.3. Preparation of mineralized nanoparticles

Mineralization of CD-NPs was carried out by the addition of aqueous solutions of calcium nitrate and ammonium phosphate using a slightly modified method compared to the previous report for mineralization of carboxylic acid-bearing nanoparticles (Han, Han, et al., 2011). In brief, calcium nitrate (0.29 M) and ammonium phosphate (0.24 M) solutions were prepared in Tris·HCl buffer (pH 7.4). CD-NPs were prepared by sonication of the CMD-β-CD conjugate solution in Tris·HCl buffer (pH 7.4) for 2 min using a probe-type sonifier (VCX-750, Sonics & Materials, CT, USA) at 90 W. For the mineralization of CD-NPs, the calcium nitrate solution (60 μL) was slowly added to 5 mL of the CD-NP solution and stirred for 5 min. Subsequently, the ammonium phosphate solution (60 μL) was slowly added and stirred for 5 min. This procedure of sequential addition of the stock solutions was repeated 15 times in order to achieve effective mineralization. The molar ratio of calcium to the carboxyl group was fixed at 1:2 as CaP was deposited effectively onto the CD-NPs without the homogeneous growth of CaP.

2.4. Characterization of nanoparticles

Morphology of the nanoparticles was observed using a TEM (JEOL-2100F, Tokyo, Japan), at an accelerating voltage of 200 kV. A drop of the sample solution (1.0 mg/ml) was placed onto a copper grid coated with carbon. After 2 min, the grid was tapped with filter paper to remove the surface water and was subsequently air-dried. Negative staining was performed for non-mineralized nanoparticles using a drop of a 2 wt% uranyl acetate solution while mineralized nanoparticles were observed without the staining process. FT-IR spectra of the nanoparticles were obtained by a Bruker IFS-66v FT-IR spectrometer (Germany) using KBr pellets. TGA was performed for samples with a heating rate of 10 °C min^{-1} using a SDT Q600 (TA Instruments, DE, USA) under nitrogen atmosphere up to 700 °C. EDS measurements were carried out using a JEOLJEM-2100F equipped with an EDX genesis series γ-TEM at 200 kV. EDS was used to determine the atomic components of the selected nanoparticle area. DLS measurements were conducted using a FPAR-1000 fiber optics particle analyzer (Otsuka Electronics, Osaka, Japan). The ζ-potential of the nanoparticles was measured using a 90 PLUS (Brookhaven Instruments Co., NY, USA).

The inclusion complexation behavior of CMD-β-CD was investigated using pyrene as the hydrophobic fluorescent probe by fluorescence spectroscopy. In brief, a pyrene solution (12×10^{-7} M) was prepared in distilled water and was mixed with the conjugate solution to obtain a polymer concentration ranging from 1.0×10^{-4} to 1 mg/mL. The final concentration of pyrene in each sample was fixed at 6.0×10^{-7} M. The fluorescence spectra were recorded using an ISS K2 multifrequency phase and modulation fluorometer (ISS,

Champaign, IL, USA). The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 336 and 390 nm, respectively.

2.5. In vitro protein release test

In order to prepare the CAB-loaded nanoparticles (CD-CAB-NPs), CMD-β-CD (10 mg) and CAB (1 mg) was dispersed in Tris·HCl buffer (pH 7.4) and stirred for 10 min. Calcium nitrate and ammonium phosphate was further added sequentially as described in the previous section in order to obtain mineralized CAB-loaded CD-NPs (MCD-CAB-NPs). Free CAB was removed by dialysis (MWCO = 100,000 Da) against distilled water at 4 °C. The samples were obtained as white powder after lyophilization and stored at −50 °C prior to use. The amount of CAB loaded was estimated by a bicinchoninic acid (BCA) assay using the microplate reader (Model BIO-TEK, Synergy HT).

The *in vitro* release profile of CAB from nanoparticles was evaluated in a PBS solution (pH 7.4). CAB-loaded nanoparticles (1.0 mg/ml) were dispersed in PBS (pH 7.4) and carefully transferred to a cellulose membrane tube (MWCO = 100,000 Da). The dialysis tube was then placed in a release medium and gently shaken in a 37 °C water bath at 100 rpm. At various time intervals, the medium (10 mL) was withdrawn and replaced with an equal volume of fresh medium. The amount of CAB released was estimated by the BCA assay. All values are expressed as a mean ± SD ($n = 5$).

2.6. Biological activity of released CAB

The biological activity of released CAB was determined using the NPA esterase assay (Sivasubramanian, Kang, et al., 2012). Briefly, 10 μL of NPA (100 mM) in dry acetonitrile was added to 1 mL of the native CAB solution (0.03 mg/mL in 50 mM Tris–sulfate). After mixing for 10 s, the hydrolysis product (p-nitrophenolate) was quantified by measuring the absorbance at 400 nm with a microplate reader. A similar procedure was followed in order to determine the biological activity of released CAB using the samples obtained on day 3 from CD-CAB-NPs and on day 12 from MCD-CAB-NPs. The enzymatic activity of released CAB was determined by comparing the activity to the rate of NPA hydrolysis by the native enzyme at the same concentrations.

2.7. Calcium dissolution behavior of mineralized nanoparticles

Mineralized nanoparticles (1 mg/mL), dispersed in PBS (pH 7.4) or acetate buffer (pH 5.0), were transferred to a dialysis cellulose membrane tube (MWCO = 1000 Da). The dialysis tube was then placed in the release medium (PBS (pH 7.4) or acetate buffer (pH 5.0) and gently shaken in a 37 °C water bath at 100 rpm. At various time intervals, the medium (1 mL) was withdrawn and replaced with an equal volume of fresh medium. The release medium (100 μL) was mixed with Arsenazo III solution (2 mL) in HEPES-buffered saline (pH 7.4). The concentration of calcium ions was determined using UV–vis spectrophotometer (Optizen 3320, Mecasys Inc., Korea) at a wavelength of 656 nm.

3. Results and discussion

The formulation and delivery challenges associated with protein drugs are long standing issues. Factors limiting the clinical applications of proteins are their short *in vivo* half-lives, chemical or physiological degradation and unstable tertiary structures (Frokjaer & Otzen, 2005; Putney & Burke, 1998). In recent years, CDs have drawn attention as promising controlled protein delivery systems. CD-based biomaterials such as hydrogels and polypseudorotaxanes have shown potential for sustained delivery

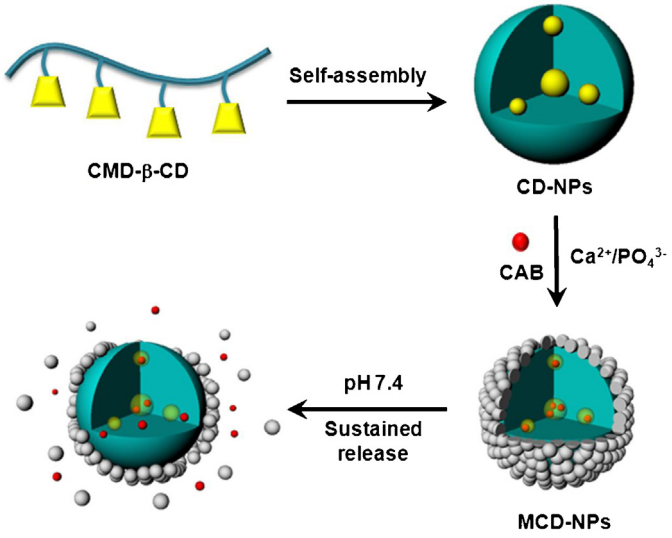

Fig. 1. Schematic illustration of mineralization of CD-NPs and sustained release.

of proteins (Higashi, Hirayama, Misumi, Arima, & Uekama, 2008; van de Manakker, Braeckmans, et al., 2009; van de Manakker, Vermonden, van Nostrum, & Hennink, 2009). Our research group has previously demonstrated that the polymeric conjugate bearing α-CD forms an inclusion complex with PEGylated human growth hormone thereby allowing its sustained release without denaturation (Sivasubramanian, Kim, et al., 2012). In this study, we attempted to develop mineralized CD-NPs as a novel carrier for sustained delivery of proteins in which calcium phosphate was introduced into the CD-NPs as the diffusion barrier (Fig. 1).

3.1. Synthesis and characterization of CMD-β-CD

In order to prepare the CMD-β-CD conjugate, we synthesized an amine-functionalized β-CD, followed by its coupling reaction with CMD in the presence of EDC and NHS (see Supporting information Figs. S1 and S2 for the detailed synthetic routes). The chemical structure of the conjugate was confirmed using ^{1}H NMR as shown in Fig. 2. The degree of substitution (DS), defined as the number of β-CD molecules per 100 sugar residues of CMD, was calculated by

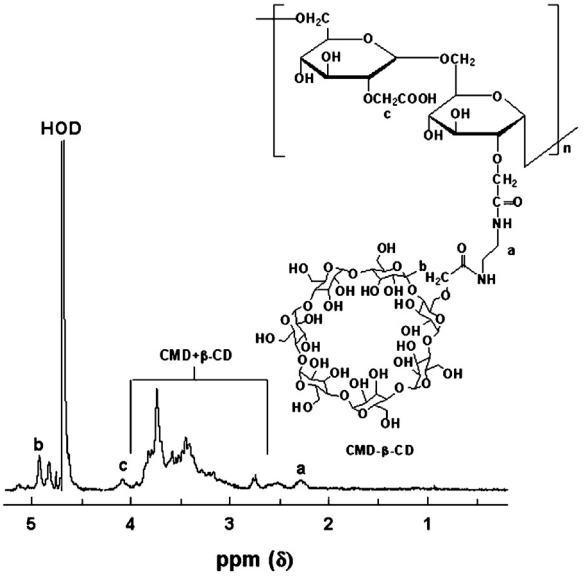

Fig. 2. ^{1}H NMR of CMD-β-CD conjugate.

Table 1
Physicochemical characteristics of CD-NPs.

Sample name	Size (nm)[a]	Loading efficiency (%)[b]	Loading contents (%)[b]
CD-NPs	310.2 ± 1.98	–	–
MCD-NPs	120.5 ± 3.37	–	–
CD-CAB-NPs	330.3 ± 2.35	28.5 ± 3.2	2.9
MCD-CAB-NPs	161.7 ± 1.98	80.3 ± 5.4	8.1

[a] Mean diameter measured using the particle size analyzer.
[b] Determined using the microplate reader.

using the peak integration ratio of carboxymethyl groups in CMD (δ = 4.09 ppm) to the anomeric protons of β-CD (δ = 4.93 ppm). The DS value of the conjugate prepared in this study was 52.3.

CDs, consisting of a hydrophilic outer surface with an inner hydrophobic cavity, are able to form self-assembled nanoparticles at higher concentrations (Bonini et al., 2006; Messner, Kurkov, Jansook, & Loftsson, 2010). Hydrogen bonding among the hydroxyl groups of CDs plays a significant role in the self-assembling process (Häusler & Müller-Goymann, 1993; Szente, Szejtli, & Kis, 1998). Owing to this self-aggregation behavior of CDs, the CMD-β-CD conjugate also forms nano-sized particles (310 nm in diameter) in an aqueous solution (Table 1).

In order to understand the self-assembly and inclusion complex formation behavior of CMD-β-CD with hydrophobic molecules in the aqueous condition, fluorescence spectroscopy experiments were performed using pyrene as the fluorescent guest molecule. Due to its high hydrophobicity, pyrene is located inside or closer to the hydrophobic environment rather than in the aqueous phase. Upon exposure to CMD-β-CD solutions, pyrene molecules may either fit inside the hydrophobic cavities of β-CD or could be accommodated by the local hydrophobic domain created by the association of β-CD-pyrene inclusion complex units. Fig. 3A shows the fluorescence excitation spectra of CMD-β-CD at various concentrations in the presence of pyrene (6.0×10^{-7} M). At higher concentrations, a considerable increase in the fluorescence intensity of pyrene was observed with a red shift and broadening of the excitation spectra implying formation of self-assembled nanoparticles (Zhang, Ellsworth, & Ma, 2010). However, no significant change in fluorescence intensity was observed at lower concentrations. In addition, the pyrene intensity ratios (I_{339}/I_{334}) of the excitation spectra increased with increasing concentration (Fig. 3B). These results suggest that hydrophobic pyrenes lie not only in the hydrophobic cavity of β-CD but are also encapsulated by local hydrophobic domains in the nanoparticles.

3.2. Mineralization of the CD-NPs

CaP was deposited onto CD-NPs by the sequential addition of calcium nitrate and ammonium phosphate. Owing to electrostatic interactions, calcium ions were attracted toward the anionic carboxyl groups of CMD. Subsequent addition of ammonium phosphate as the phosphate ion source induced the growth of CaP. As shown in Table 1, mineralization of CD-NPs significantly reduced the mean diameter (121 nm), which was determined using the DLS. This may be due to the electrostatic localization of calcium ions on carboxyl groups in the CMD backbone thereby resulting in the formation of compact nanoparticles. Fig. S3 shows the TEM images of nanoparticles. In general, the nanoparticles possessed a spherical morphology. For TEM measurements, the mineralized nanoparticles were visualized directly without any staining agents while bare CD-NPs could be observed only after being negatively stained. The particle sizes of all the nanoparticles, measured using the DLS, were larger than those observed from TEM images. This is due to the difference in the sample preparation condition; *i.e.*, while measurements using the DLS were carried out for hydrated nanoparticles, TEM images were obtained from dried samples.

The elemental components of MCD-NPs were determined using the EDS (Fig. 4A). The results demonstrated that MCD-NPs predominantly contained Ca and P, suggesting the formation of CaP. Based on the selected area diffraction pattern of MCD-NPs in Fig. 4B, no crystalline electron diffraction rings were observed thereby implying formation of amorphous CaP. The existence of CaP on MCD-NPs was also supported by FT-IR spectra (Fig. 4C) in which the peak corresponding to the asymmetric P—O stretching appeared at 1000 cm^{-1}. The TGA spectra of nanoparticles showed that the weight loss behavior of CD-NPs was different from that of MCD-NPs (Fig. 4D). In particular, at high temperatures above 600 °C, the MCD-NPs produced more residues than CD-NPs which was due to the presence of inorganic CaP. The calcium content of MCD-NPs, calculated from the TGA spectrum, was found to be 9.2 wt%. The ζ potential of CD-NPs increased from −24.91 to −3.09 mV following mineralization thereby indicating that CaP shielded the negatively charged carboxyl groups.

Table 1 shows the physiochemical characteristics of CAB-loaded nanoparticles. As expected, the encapsulation of CAB slightly increased the mean diameters of CD-NPs and MCD-NPs. Interestingly, the loading efficiency of CAB for MCD-NPs (80.3%) was significantly higher than that for CD-NPs (28.5%). This is likely due to the formation of inclusion complexes between β-CD in nanoparticles and CAB followed by mineralization, generating CaP as the diffusion barrier. The cavity size of β-CD is favorable for binding the aromatic amino acids and large alkyl groups of CAB. Therefore, β-CD molecules can form inclusion complexes with hydrophobic residues of CAB. Furthermore, CAB is a negatively charged protein at pH 7.4 since its isoelectric point is 5.7. During the mineralization process, CD-CAB-NPs were sequentially exposed to Ca^{2+} ions and PO_4^{3-} ions. Consequently, negatively charged CD-NPs and CAB would be attracted toward positively charged Ca^{2+} ions via electrostatic interactions, followed by growth of the CaP mineral as the diffusion barrier of CAB. In the case of bare CD-NPs, while CAB could form inclusion complexes with CD-NPs, the electrostatic repulsion between negatively charged CMD and CAB would result in the poor loading efficiency of CAB.

3.3. In vitro protein release behavior

In order to observe the *in vitro* release pattern of CAB from nanoparticles, the amount of CAB released from CAB-loaded nanoparticles in PBS (pH 7.4) was determined as a function of time (Fig. 5A). Both nanoparticles showed burst release of CAB for an hour followed by the significant differences in the release rates. Bare CD-NPs showed a high initial burst: 33.51 ± 1.14% of CAB was released in 1 h. CAB was completely released from CD-NPs in 3 days. Interestingly, MCD-NPs exhibited a minimal initial burst: 4.06 ± 1.38% of CAB followed by sustained release for up to 21 days. The prolonged release of CAB with a low initial burst from MCD-NPs suggests that mineralization effectively inhibited the release of CAB. Owing to their high stability in biological fluids (pH 7.4), the CaP layer in MCD-NPs acted as the diffusion barrier resulting in the reduced release rate. For a successful protein delivery system, the released proteins must be biologically active. Therefore, evaluation of the biological activity of the released CAB was very

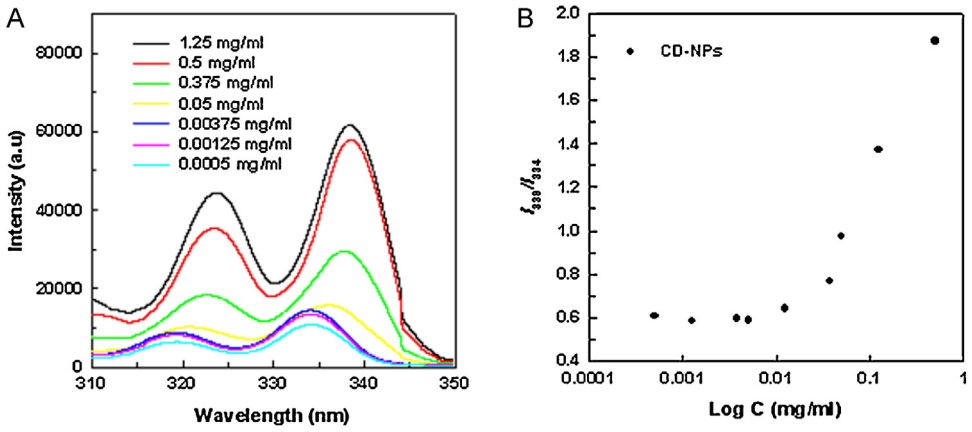

Fig. 3. (A) Excitation spectra of pyrene as a function of CD-NPs in water. (B) Plot of pyrene intensity vs. CD-NPs concentration.

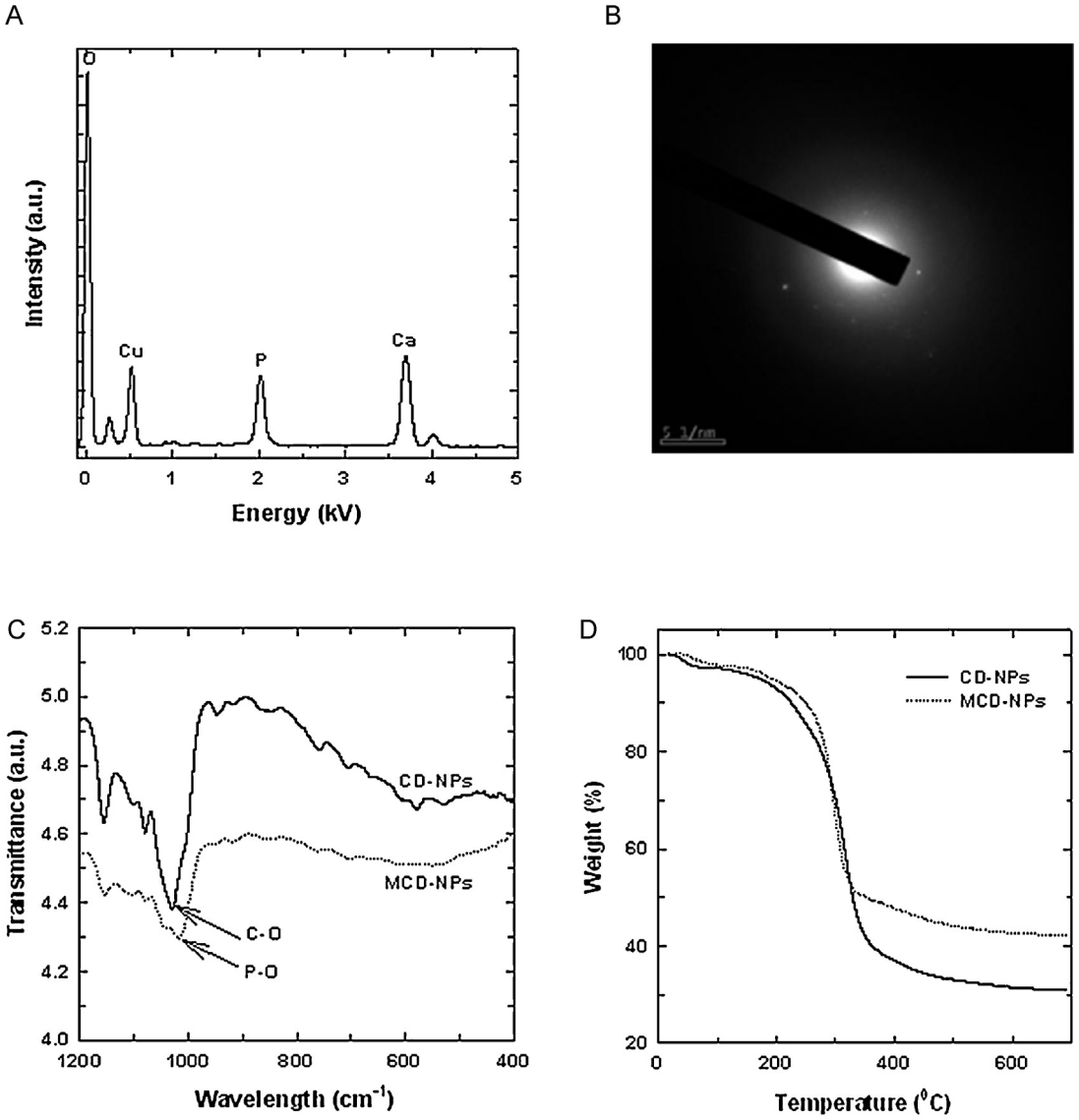

Fig. 4. Mineralization of CD-NPs confirmed by various methods; (A) TEM-EDS analysis of MCD-NPs, (B) selected area diffraction pattern of MCD-NPs, (C) FT-IR spectra and (D) TGA curves of CD-NPs and MCD-NPs.

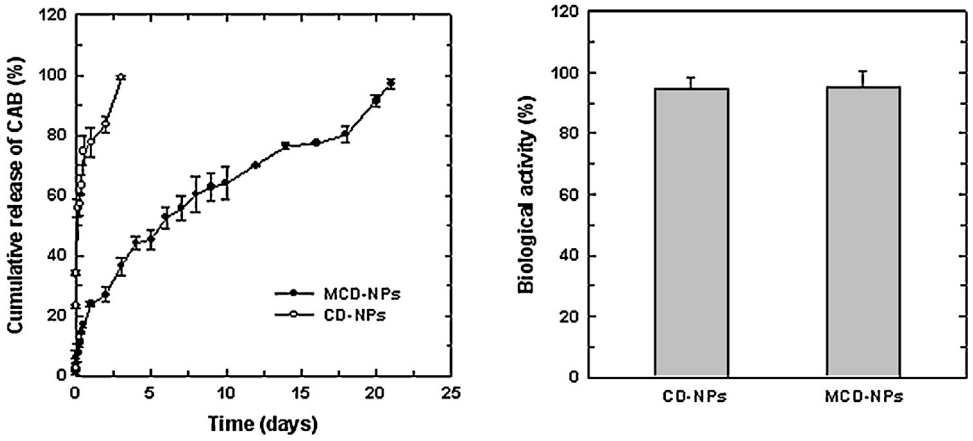

Fig. 5. (A) *In vitro* release profiles of CAB from the nanoparticles (B) biological activity of the released CAB. Error bars represent standard deviations ($n = 5$).

important. Fig. 5B shows the enzymatic activity of CAB released from nanoparticles. Regardless of the nanoparticles, the activity of the released CAB was greater than 90% implying that both CD-NPs and MCD-NPs released biologically active CAB. These results suggest that biological activity of CAB was not significantly deteriorated inside CD-NPs, primarily owing to the formation of inclusion complexes with β-CD, which might effectively prevent aggregation of CAB. Similarly, evidence suggests that β-CD could form inclusion complexes with proteins and improve their stability by preventing irreversible aggregation (Serno, Geidobler, & Winter, 2011).

Development of the formulation for prolonging the biological activity of proteins is a critical challenge. The chemical attachment of poly (ethylene glycol) (PEG) to proteins is considered the most successful approach to improving the bio-availability and *in vivo* stability of protein drugs (Gauthier & Klok, 2010). Such PEGylated proteins have shown prolonged activity in the body since PEG can effectively prevent degradation of proteins from proteolytic enzymes (Harris & Chess, 2003). However, recent studies have demonstrated that PEGylation of proteins can reduce their biological activity and generate antibodies against PEG after repeated doses thereby warranting an alternative strategy (Ganson, Kelly, Scarlett, Sundy, & Hershfield, 2006). In this regard, MCD-NPs might be useful for the sustained release of biologically active proteins suggesting their potential as a protein delivery system for prolonged therapeutic effects.

3.4. Ca^{2+} ion release behavior

MCD-NPs were exposed to solutions with different pH (5.0 and 7.4) in order to observe the stability of the CaP layer. The release of calcium ions from MCD-NPs was evaluated (Fig. 6). In general, CaP is stable at physiological solutions (pH 7.4) and is known to be soluble at mildly acidic solutions (Han, Han, et al., 2011; Kim et al., 2010). As expected, the calcium ions were completely released within 2 h at a pH 5.0, indicating the rapid dissolution of CaP in MCD-NPs. In contrast, slow release of calcium ions was observed at pH 7.4; *i.e.*, MCD-NPs released only 40% of calcium ions in 10 days, indicating that the CaP layer in nanoparticles is highly stable at the physiological pH. These results are in agreement with the protein release behavior. The high stability of CaP-coated polymeric nanoparticles has been also reported both *in vitro* and *in vivo* (Han, Han, et al., 2011; Han, Lee, et al., 2013).

In recent years, inorganic nanoparticles composed of CaP have received much interest as a constituent of drug delivery systems. CaP-based nanoparticles have demonstrated success in delivering therapeutics to specific environments, especially intracellular or pathological sites such as tumors with mildly acidic conditions

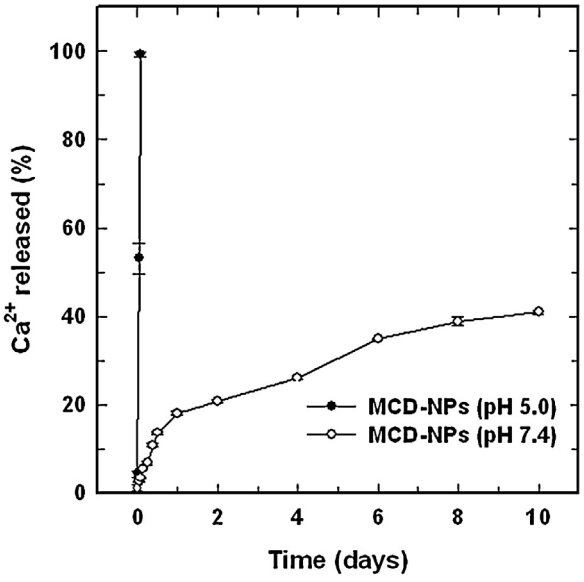

Fig. 6. Release pattern of calcium ions from mineralized nanoparticles in different pH buffers. The error bars in the graph represent standard deviation ($n = 5$).

(Han, Han, et al., 2011; Kim et al., 2010). However, applications of CaP for protein delivery remain unexplored. Due to their high stability in physiological fluids (pH 7.4), CaP could behave as an effective diffusion barrier, thereby reducing the release rate of proteins. As demonstrated in this study, CD-NPs could effectively prevent the aggregation of CAB, chosen as the model protein, by forming inclusion complexes. In addition, the mineralization of CD-NPs allowed for the sustained release of CAB without affecting its biological activity.

4. Conclusions

In this study, we developed mineralized nanoparticles (MCD-NPs) as the potential carrier for sustained protein delivery. Mineralization of CD-NPs reduced particle size from 310 nm to 210 nm in PBS (pH 7.4), indicating the formation of compact nanoparticles. Due to the high stability of CaP as the diffusion barrier, MCD-NPs effectively reduced the release rate of CAB at pH 7.4. In addition, MCD-NPs preserved the biological activity of CAB as demonstrated by the NPA assay. Overall, the results described here suggest that MCD-NPs are useful for the sustained delivery of proteins.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.05.018.

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