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A Versatile pH-Responsive Platform for Intracellular Protein Delivery Using Calcium Phosphate Nanoparticles

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Intracellular protein delivery has great biomedical applications; the safe and efficient delivery vectors are crucial for achieving this goal. Here we report a platform for the efficient protein delivery using calcium phosphate (CaP) nanoparticles. The well-dispersed and highly-stable nanoparticles (~100 nm) are prepared with the protein loading capacity up to 29%. The nanoparticles are stable in the serum condition; however, after being internalized into cells, the particles quickly release protein in the weak acidic endosomes/lysosomes. The decomposition of CaP promotes the endo-lysosomal escape of proteins released from nanoparticles. The protein/CaP conjugates were prepared in a mild condition (aqueous solution, room temperature); hence the protein released from nanoparticles retained its folding and function. In addition, all materials used for the preparation are highly biocompatible. This method has been applied for the loading of three model proteins, BSA, GFP and KillerRed; similar loading properties were observed on these proteins. Therefore, this work offers a general approach for the intracellular protein delivery, which could be applicable for therapeutic proteins.

Introduction

Protein therapeutics have been applied in a wide range of areas, including cancer therapy, regenerative medicine, vaccination, treating loss-of-function genetic diseases and imaging.[1] It provides an alternative method in addition to gene therapy, and is a safer approach since it does not involve random or permanent genetic alterations.[2] Although many proteins have been used successfully in clinic, a number of challenges are faced in the development of protein therapeutics, including protein solubility, stability, immune response as well as endo-lysosomal escape and subcellular trafficking rout.[3] Most proteins are unable to cross the cell membrane to enter cells so that the delivery systems are often needed. Protein delivery systems typically enter cells through endocytosis; hence the delivered proteins should be able to escape from endo-lysosomal pathway to avoid the protein degradation under harsh endo-lysosomal conditions.[4, 5] In addition, the safety of the delivery vector is crucial for the biological applications. Thus, many efforts have been made for effective delivery of proteins through various approaches.

Over the past two decades, the most commonly used protein delivery approach is to link the target protein to cell-penetrating peptides (CPPs), e.g. TAT and polyarginines (R9).[6] Cell-penetrating proteins have demonstrated the capability to deliver functional protein domains into cells.[7] However, CPP-mediated protein delivery mechanism may depend on cell types and cargo molecules.[8] In addition, the fusion proteins may loss bioactivity if the functional sites are located in the protein termini.[9] Recently, nanoparticle-based drug delivery systems (DDS) offer efficient approaches to intracellular protein delivery, such as using lipid-based,[10] polymeric,[11] inorganic[12, 13] and protein-mediated[14] nanoparticles. Those protein delivery systems are fabricated to enhance the physical and chemical stability of proteins, prolong biological half-life, increase cellular uptake, decrease antigenicity and to reduce the uptake by the reticuloendothelial system (RES).[15]

Generally, pharmaceutical nanoparticles are internalized by eukaryotic cells through endocytosis, transported to early endosomes and lysosomes, then trafficked to desired subcellular compartments, such as cytosol, nucleus and mitochondria.[16] In this circumstance, the acidity of endosomes and their fusion with lysosomes can be damaging to proteins[17, 18], causing the loss of original conformational structure and biological functions.[19] Therefore, the endo-lysosomal escape is an important strategy in the delivery of therapeutic proteins. Nanoparticles can utilize different mechanisms to achieve this goal, including fusion in the endosomal membrane, pore formation in the endosomal membrane, photochemical disruption of the endosomal membrane and proton sponge effect.[4] Particularly, pH-sensitive nanomaterials can lead to buffering inside endosomes and induce proton sponge effect, so as to result in endo-lysosomal escape and cytoplasmic drug release.

A number of pH-sensitive delivery systems have been investigated, such as liposomes, polymers and inorganic nanoparticles.[19] Organic solvents are often needed during the

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encapsulation of proteins, such as polymers nanoparticles; therefore the exposure of proteins to a water/organic interface and the consequent organic solvent removal are required.[20] In addition, the adverse effects of exogenous molecules generated by the degradation of nanoparticles are unpredictable.[21] To address these issues, we present a facile and practical approach for making protein delivery system using calcium phosphate nanoparticles. Calcium phosphate (CaP) is highly biocompatible as it is the primary mineral phases of biological hard tissues, such as teeth and bones. CaP is a well-known pH sensitive inorganic material, which has been used for intracellular delivery.[21, 22] In addition, the proton sponge effect of CaP enhances endosomal escape of cargoes.[23, 24] Three proteins, including a bovine serum albumin (BSA), and two fluorescent proteins, the green fluorescent protein (GFP) and KillerRed (KRed) were used as model proteins in this study. With the simple preparation in mild conditions, a reasonably high loading capacity (~29%) of protein/CaP hybrid was obtained. In addition, the pH-sensitive decomposition of CaP nanoparticles allows the efficient release of the cargo protein in cells.

Experimental

Materials

The analytical pure $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ were purchased from Sinopharm Chemical Reagent Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) was purchased from Sigma, USA. Poly(ethylene glycol) (PEG$_{4000}$, 4 kDa), BSA, Bradford protein assay kit and H_2O_2 quantitative assay kit (water-compatible) were purchased from Sangon Biotech (Shanghai) Co., Ltd. Ultrapure water (18.2 MΩ) was obtained from Millipore Direct-Q purification system containing a 0.22 μm filter and was used in all experiments.

Protein expression and purification

The GFP was overexpressed from *E. coli* BL21 (DE3). The gene encoding GFP was inserted into pET21 vector. The plasmid was transformed into *E. coli* and cells were grown in LB broth at 37°C until OD$_{600}$ reached 0.6-0.8. Then the protein expression was induced with 0.8 mM IPTG at 16°C for 20 h. The cells were harvested, centrifuged at 4000 rpm for 20 min at 4°C. The cell pellet was resuspended in binding buffer (Tris·HCl 20 mM and NaCl 150 mM, pH 8.0) and sonicated in ice bath. Then the lysate was centrifuged at 16,000 rpm for 30 min at 4°C. The supernatant was filtrated by 0.8 μm, 0.45 μm and 0.22 μm cellulose acetate membrane and loaded on Ni^{2+}-nitrilotriacetic acid-Sepharose column. Impure proteins were washed with washing buffer (Tris·HCl 20 mM, NaCl 150 mM and 50 mM, pH 8.0). Then the GFP protein was eluted with elution buffer (Tris·HCl 20 mM, NaCl 150 mM and 250 mM, pH 8.0).

KillerRed protein was overexpressed in *E. coli* BL21 (DE3) by the transformation of pET-15b-KRed plasmid. The protein expression was induced with 0.8 mM IPTG at 37°C for 6 h. The rest procedures were the same as mentioned above.

Preparation of protein loaded CaP particles

29.5 mg $Ca(NO_3)_2 \cdot 4H_2O$ was dissolved in 50 ml ultrapure water and 2 ml protein (5 mg/ml) was added to with stirring at room temperature. The solution was further stirred for 10 min before adding 2 g PEG$_{4000}$. The pH of the solution was adjusted to 10.0 by $NH_3 \cdot H_2O$ and stirred at 250 rpm for 30 min. 16.5 mg $(NH_4)_2HPO_4$ was dissolved in 50 ml water and adjusted to pH 10.0. The $(NH_4)_2HPO_4$ solution was added dropwise to the protein solution (2 ml/min) and stirred for another 20 min. Then the mixture was centrifuged for 20 min in 10,000 rpm. The precipitate was collected and washed with ultrapure water three times, and resuspended in water or DMEM with 10% FBS.

Characterization of protein loaded CaP particles

The size of nanoparticles was determined in water at 25°C using dynamic light scattering (DLS) with a Zetasizer Nano (ZS90, Malvern Instruments, Southborough, MA). Scanning electron microscope (SEM) was performed with a Sirion200 instrument (FEI, USA). The X-Ray diffraction (XRD) was recorded on the high-resolution X ray diffracmeter (TTR-III, Rigaku, Japan).

Protein loading capacity

The protein content in nanoparticles was measured with Bradford assay. The net mass of nanoparticles was measured by weighing the protein loaded nanoparticles after freeze-drying. 0.1 ml GFP/CaP nanoparticles (5 mg/ml) were centrifuged at 12,000 rpm for 10 min, then discarded the supernatant. The nanoparticles was dissolved in 1 ml MES buffer (0.5 M, pH 5.0) for 1 h in room temperature. The solution was centrifuged at 12,000 rpm for 10 min. The protein in the supernatant was quantified by UV absorption using Bradford protein assay kit. The loading capacity (w/w) was quantified as [(mass of protein in nanoparticles)/(total mass of nanoparticles)]×100%.[25]

Stability of GFP/CaP nanoparticles

Nanoparticles were dispersed in DMEM with or without 10% FBS in 37°C. The size of nanoparticles was measured by DLS after different incubation time.

pH responsive protein release from CaP nanoparticles

The nanoparticles were incubated with 100 mM MES buffer with different pH for 1 h, 4 h and 8 h in room temperature.

Then the amount of released GFP was measured by using Bradford assay.

Toxicity assays

HepG2 cells were seeded at 5.0×10^3 cells per well in a 96-well plate one day before the treatment of nanoparticles. 100 µl different concentrations of GFP/CaP nanoparticles suspended in DMEM were added into the cells. After 48 h incubation, the medium was replaced by fresh medium containing 0.5 mg/mL of MTT. After 4 h incubation, the medium was removed and 150 µl of DMSO was added to dissolve tetrazolium salt. The absorbance at 490 nm were measured by a microplate reader (Biorad, USA). Cell viabilities were normalized to the absorbance of non-treated cells.

Circular dichroism measurement

The circular dichroism measurements were performed with a Jasco-715 spectropolarimeter at 20°C using matched 10 mm path length quartz cells. Spectra were recorded as the average value of three scans in a range of 190 - 250 nm.

SDS-PAGE analysis

Protein samples in loading buffer were heated in boiling water for 8 min. Then the samples were loaded into the wells of SDS-PAGE gel. The electrophoresis lasted for about 1 h at 120 V voltage.

Cellular uptake and intracellular localization of protein

A total of 60,000 or 240,000 HepG2 cells were cultured in a 24-well plate or 3.5 cm dish and maintained at 37°C in a 95% humidified atmosphere with 5% CO_2 for 24 h. After the incubation with GFP/CaP or KRed/CaP nanoparticles (1 mg/ml) for different time, the cells were washed three times with 500 µl PBS buffer. Then the cells were analyzed by flow cytometer, fluorescence microscope or confocal microscope. To study the co-localization with LysoTracker®Red, cells were incubated with the dye for 20 min before the detection.

Quantification of radical oxygen species generated from KRed

100 µl native KRed and KRed released from CaP nanoparticles were placed in 96-well plate in the same concentration. After the irradiation with white LED light (1 W/cm^2) for 30 min, the radical oxygen species (ROS) generated by the irradiation of KRed was quantified by measuring H_2O_2 using Hydroperoxide Quantitative Assay Kit (Sangon).

Results and discussion

### Characterizations of protein-loaded nanoparticles

The protein loaded nanoparticles were prepared by adding $(NH_4)_2HPO_4$ solution into the $Ca(NO_3)_2$ solution containing protein in the presence of PEG. The protein was incorporated into calcium phosphate while the formation of nanoparticles. The particles were collected by centrifugation and re-suspended in water. The GFP and KRed loaded particles clearly showed the green and Red colour, respectively; while the BSA loaded particles demonstrated white colloid phase (Figure 1A). The scanning electron microscope (SEM) micrograph of GFP/CaP nanoparticles showed the spherical and uniform morphology of nanoparticles (Figure 1B). The dynamic light scattering (DLS) measurement indicated the narrow size distribution of GFP/CaP nanoparticles (Figure 1C). Similar results were observed on KillerRed and BSA loaded nanoparticles (Figure S1, S2). The XRD pattern showed that the calcium phosphate were mainly in amorphous phase with very low crystalline order (Figure 1D). The amount of protein loaded in the nanoparticles was analysed using fluorescence spectra by measuring the protein released from particles. The loading capacity increased with the protein concentration in the preparation; it reached 29% at the GFP concentration 0.20 mg/ml (Figure 1E). The average particle sizes did not change obviously with the variation of GFP concentration.

Stability of GFP/CaP nanoparticles

The stability of GFP/CaP nanoparticles was evaluated by time dependent size change in DMEM media at 37°C for 6 days. The DMEM media containing 10% fetal bovine serum (FBS) is a mimic of the physiological environment and is widely used for cell culture. The DSL measurements indicated that the size of GFP/CaP nanoparticles in DMEM without FBS was stable only for a few hours; the hydrodynamic radius increased from 200 nm to 6 µm in 6 days. Nevertheless, the presence of 10% FBS remarkably stabilized the nanoparticles and the size remained unchanged in 6 days (Figure 2). This observation indicated that FBS improved the stability of CaP nanoparticles. Previous study showed that FBS exhibited a great negative effect on the aggregation of calcium phosphates so as to inhibit the process of mineralization of calcium phosphates[26]; this effect can be utilized to stabilize the CaP nanoparticles for making protein delivery vectors. FBS contains various of proteins, including lipoproteins, glycoproteins and globulins; these biomolecules could contribute to the stabilization of CaP nanoparticles.[27] It has been reported that the cell culture media with FBS caused irreversible conformational transitions of amorphous silica nanoparticles and polyisobutylcyanoacrylate nanoparticles, and led to aggregation of the particles.[28, 29] Nevertheless, results in this work indicated that the calcium phosphate nanoparticles were well-dispersed and stable the DMEM/FBS media, suggesting that this protein/CaP hybrid was suitable for the delivery in the physiological condition.
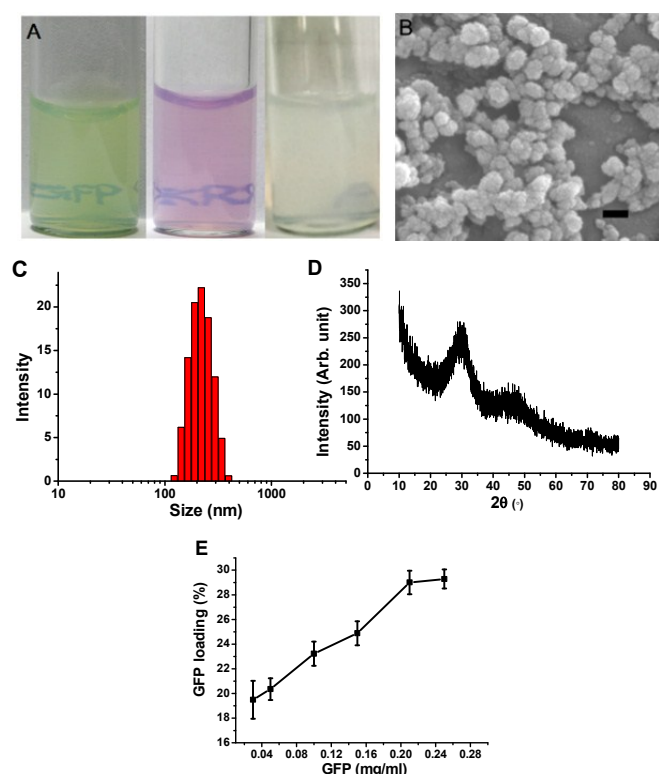
Figure 1. Characterizations of GFP/CaP nanoparticles: (A) image of GFP, KRed and BSA loaded CaP nanoparticles suspended in water; (B) SEM micrograph of GFP/CaP nanoparticles (bar = 100 nm); (C) DLS result of GFP/CaP nanoparticles suspended in water; (D) XRD pattern of GFP/CaP nanoparticles; (E) GFP loading capacity of the CaP nanoparticles loaded from 0.03 to 0.25 mg/ml GFP initial loading solution. Data reported as mean ± SE, n = 3.
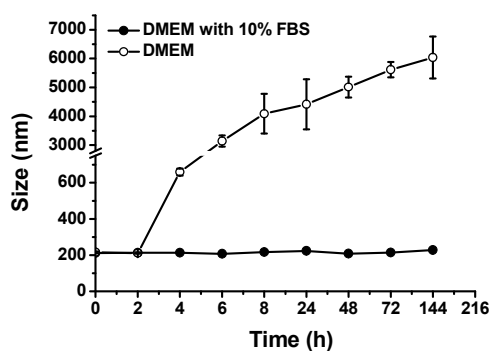
Figure 2. Size variation of GFP/Cap nanoparticles in DMEM with 10% FBS (solid circles) and DMEM free of FBS (empty circles) against time in a period of 6 days.

## Protein release from CaP Nanoparticles

Protein release is a crucial step for the controlled delivery. It is well-known that the nanoparticles in the size of 100-200 nm size are typically internalized into cells via endocytosis.[30] Therefore, the weak acidity in endosomes/lysosomes (pH is 4-6) provides a unique environment for the responsive release of nano-delivery systems. The protein release assay was conducted at 37°C at different pH (4.5 to 7.4). The protein released into solution was measured using Bradford assay after removing nanoparticles with centrifugation. The result on GFP/CaP showed that nanoparticles were stable at neutral condition; nearly no protein was detected in solution at pH 7 and 7.4. (Figure 3) However, lowing pH to 6.5 led to protein release, and complete protein release was detected at pH < 5.5. No obvious difference was observed with different incubation time (1 h, 4 h, 8 h), suggesting that the nanoparticles decomposed rather quickly at relevant pH values. Similar result was observed on the KillerRed or BSA loaded nanoparticles. This result indicated that the protein/CaP hybrid nanoparticles were remained stable in physiological conditions (pH = 7.4) and readily release the protein in the weak acidic environments of endosomes and lysosomes.
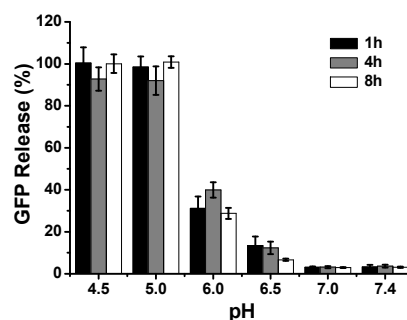
Figure 3. pH-sensitive protein release from GFP/CaP nanoparticles in 100 mM MES buffer of different pH and different reaction time by protein concentration measurement.

Toxicity assays

The cytotoxicity of protein loaded CaP nanoparticles was analyzed based on the cell viability measurement using MTT assays on HepG2 cells. The result on GFP/CaP showed that cells exhibited more than 85% viability with the treatment of nanoparticles in the range of 0.25 to 2.50 mg/mL for 48 h (Figure 4). The concentration of nanoparticles in this range is able to deliver sufficient amount of protein into cells. (see next section) Similar results were observed on KRed/CaP and BSA/CaP (Figure S4). These data clearly indicate the low cytotoxicity of CaP nanoparticles, and further highlight the advantage of CaP nanoparticles as desirable protein carriers.
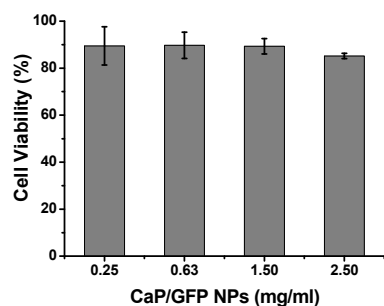
Figure 4 Toxicity of GFP/CaP nanoparticles to HepG2 cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to determine the toxicity of GFP/CaP nanoparticles. The nanoparticles were incubated with HepG2 cells for 48 h. Data points represented the average of three experiments; error bars represented the standard deviation.

Protein stability analyses

Maintaining stability and biological functions of proteins is crucial for delivery systems. The integrity of GFP released from the CaP nanoparticles was verified by SDS-PAGE analysis. (Figure 5A) The protein release from GFP/CaP nanoparticles displayed the same mobility as native GFP on SDS-PAGE, indicating that no protein degradation occurred during the preparation of CaP nanoparticles and release of protein in acidic condition. Circular dichroism (CD) spectroscopy is a sensitive method to analyze the secondary structure of proteins. The native GFP demonstrated a negative band at 213 nm and a positive band between 195 - 200 nm, which indicates the well-folded protein with large amount of β-strand (Figure 5B). The GFP released from CaP nanoparticles exhibited a CD spectrum nearly identical to the native GFP. This result indicates that the released GFP retained its original secondary structure due to the mild protein loading conditions. The same result was observed on the KRed and BSA proteins.

To further verify whether the proteins released from CaP nanoparticles can retain their functions, the activity of KRed was analyzed. KRed is a genetically encoded photosensitizer, which generates O^{2-} with the irradiation at 585 nm.[31] O^{2-} is a reactive oxygen species (ROS) that can oxidize water to generate hydrogen peroxide. Thus, the activity of KRed can be analysed by the quantification of H_2O_2 after irradiation using H_2O_2 quantitative assay kit (Sangon, Shanghai). Figure 5C indicated that native KRed and released KRed generated nearly the same amount of H_2O_2, which confirmed that protein loaded on CaP nanoparticles retains its function.
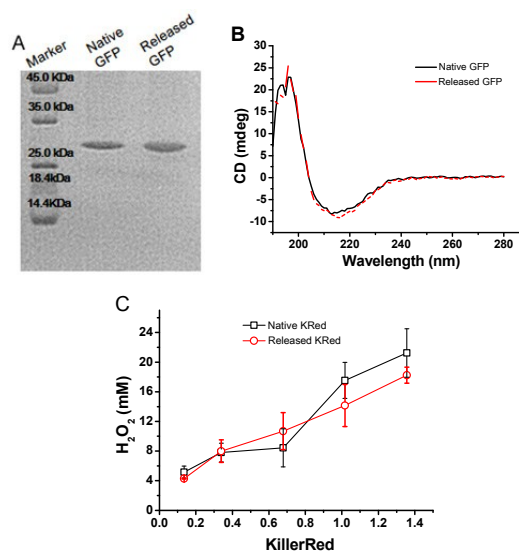
Figure 5. (A) SDS-PAGE analysis of native and released GFP in pH 5.0 MES buffer; (B) circular dichroism spectra of native and released GFP for confirmation of secondary structure of protein; (C) Quantification of H_2O_2 generated from native KRed and KRed released from KRed/CaP nanoparticles after irradiation with LED white light for 30 min.

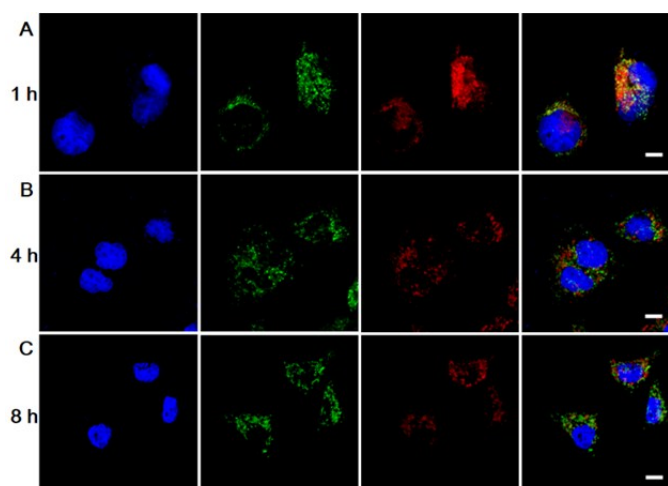## Cellular uptake of CaP nanoparticles

The cytosolic delivery of CaP nanoparticles was investigated using flow cytometry and fluorescent microscopy imaging on HepG2 cells. 1.0 mg/ml of GFP/CaP or KRed/CaP nanoparticles were incubated with HepG2 cells for different time. The flow cytometry analysis showed that 66.3% cells exhibited green fluorescence after 2 h incubation with GFP/CaP nanoparticles, and this percentage increased to 78.9% and 94.6% in 4 h and 8 h, respectively (Figure 6A,B). These data indicate that the protein loaded nanoparticles were internalized into cells rather efficiently. In contrast, the control experiments showed that the incubation with pure GFP protein did not lead the fluorescence increase in cells, indicating that the GFP protein alone was unable to enter cells. In addition to the number of cells, the amount of protein in cells also increased with the incubation time. The fluorescent microscopy clearly showed the time dependent increase of green and red fluorescence with the incubation of GFP/CaP and KRed/CaP, respectively (Figure 6C). Only weak and scattering fluorescent spots of GFP and KRed were observed in cells after 2 h treatment with nanoparticles. The fluorescence intensity increased with the incubation time and strong fluorescence was observed throughout the cytosol in 8 h. The cellular uptake of GFP/CaP was also observed on A549 cells (Figure S5). These data confirm that CaP nanoparticles can efficiently deliver proteins into cells.
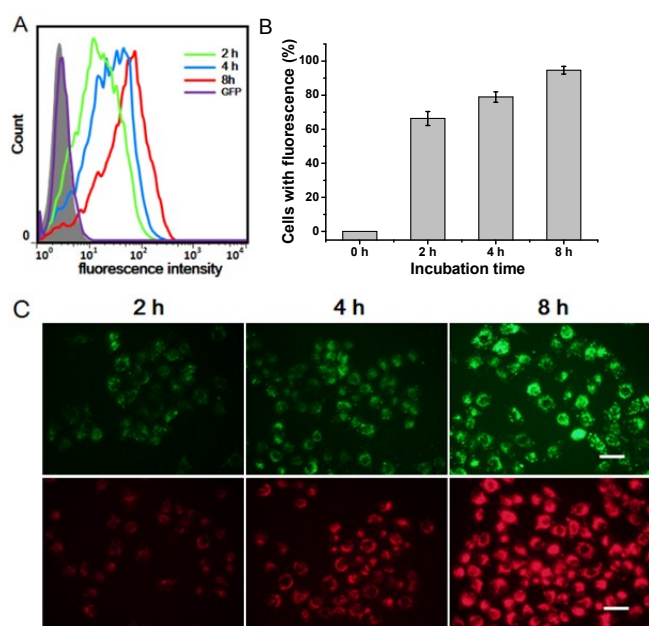
Figure 6. (A) flow cytometry results of intracellular delivery of GFP/CaP nanoparticles. HepG2 cells were incubated with GFP/CaP nanoparticles (1 mg/ml, containing 250 µg/mL GFP) for 2 h, 4 h, 8h and GFP (250 µg/mL) for 2 h. Grey: non-treated, purple: GFP for 2h; green, blue and red: treated with GFP/CaP nanoparticles for 2 h, 4 h and 8h respectively; (B) the delivery efficiency of GFP/CaP nanoparticles analyzed by flow cytometry. Data shown the average of three experiments; (C) Fluorescent images of HepG2 cells treating with GFP/CaP (green) and KRed/CaP (red) nanoparticles for 2 h, 4 h and 8 h at 37 °C (bar = 40 µm).

Intracellular distribution of proteins released from CaP nanoparticles

The encapsulation of particles in endosomes/lysosomes is an obstruction for drug delivery through endocytosis. In addition, the protein degradation in the acidic environment is a major concern for protein delivery.[16] To verify whether the protein delivered through CaP system can escape from endosomes/lysosomes, the protein distribution in cells was measured using confocal laser scanning microscope (CLSM). HepG2 cells were analyzed after the incubation with GFP/CaP nanoparticles for different time (1, 4, 8 h). The lysosomes and endosomes were stained with a red fluorescent dye LysoTracker®Red and nuclei were stained with a blue fluorescent dye DAPI. The CLSM result showed that, after 1 h incubation with GFP/CaP nanoparticles, the majority of the green fluorescence (GFP) overlaid with the red fluorescence (lysosomes and endosomes), which confirmed the endocytosis of the protein/nanoparticles hybrid. (Figure 8A) Less GFP protein was localized in endosomes/lysosomes after 4 h incubation, and the protein was more dispersed in cytosol after 8 h. This observation highly suggested broader cytoplasmic release and distribution of the protein after endo-lysosomal escape.

Figure 7. Intracellular delivery of GFP into HepG2 cells with CaP nanoparticles observed by CLSM. HepG2 cells were incubated with GFP/CaP nanoparticles containing 250 µg/mL GFP for different time (1, 4, 8 h). After the delivery and removal of the nanoparticles, the cells were fixed with formaldehyde. Endosomes and lysosomes were stained with LysoTracker®Red and nuclei were stained with DAPI. Blue: DAPI, green: GFP, and Red: LysoTracker®Red. Bar indicates 10 µm.

For the CaP mediated protein delivery system, the decomposition of nanoparticles in endocytosis generates Ca^{2+} and PO_4^{3-} (in addition to hydroxide ions). Both ions are presented in high concentration (mM level) in body fluids; therefore, the degradation of CaP nanoparticles will not perturb the regular biological processes.[32] On the other hand, the decomposition of nanoparticles consumes large amount of protons, causing additional pumping of protons into the endosomes/lysosomes. This process leads to osmotic swelling and physical rupture of the endosome.[4] As a result, proteins are released from the degradative endo-lysosomal trafficking pathway.

Calcium phosphate nanoparticles, as a well biocompatible material, have already shown the biological applications, such as gene transfection, gene silencing, drug delivery and imaging,[21] yet there are only a few works on CaP nanoparticles as potential protein carriers. CaP nanoparticles were prepared as oral delivery systems for insulin[33, 34] and utilized to deliver protein antigen[35] to target and activate of antigen-specific B-cells. The encapsulation of drug-loaded protein demonstrated efficient cellular uptake and programmed drug release.[36] The loading of hydrophobic drug ibuprofen to BSA/CaP particles in organic solvent results in the sustained release of the drug.[37, 38] A recent work showed that using yolk-shell porous microspheres of CaP can be applied for encapsulating proteins and drugs.[34] However, the complex preparation process limits the general application of these methods. Here we present a general protocol for the facile preparation of CaP nanoparticles with the protein loading capacity up to 29%. The moderate preparation process allows the protein maintaining their structure and functions after release from particles.

Particularly, the nanoparticles prepared in this method show high stability in serum conditions, and the pH-responsive release allows the protein release in endosome. Therefore, this method offers a high potentiality in biological applications, such as the delivery of therapeutic proteins.

Conclusions

In summary, this work offers a facile method for the preparation of pH-responsive protein delivery system using calcium phosphate (CaP) nanoparticles. This method has been used for different proteins, including BSA, GFP and killerRed proteins. The well-dispersed nanoparticles are obtained with the protein loading capacity up to 29%. These conjugates can be efficiently internalized into cells. The protein/CaP particles are highly stable in serum condition and able to quickly release proteins in weak acidic environments in endosomes/lysosomes. The decomposition of CaP nanoparticles promotes the protein escape from endosomes/lysosomes. In vitro assay shows that proteins released from nanoparticles retain their structure and functions, suggesting that the moderate preparation is applicable for the encapsulating functional proteins. In addition, all materials used in the synthesis of protein/CaP nanoparticles are highly bio-compatible. Therefore, this work presents a method of intracellular protein delivery, which could be useful for the application of therapeutic proteins.

## Acknowledgements

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Graphic Abstract

A Versatile pH-Responsive Platform for Intracellular Protein Delivery Using Calcium Phosphate Nanoparticles

Protein/CaP Conjugates   **Dispersion in Media**   **Cellular uptake**
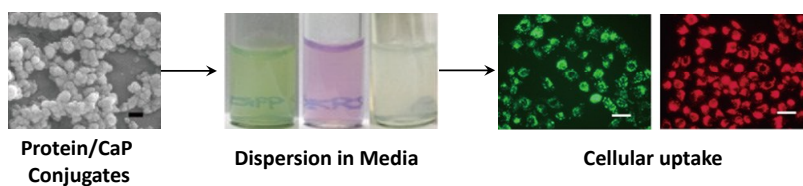
5

A highly biocompatible nanoplatform for intracellular delivery of different proteins, exhibiting pH-responsive release and efficient endosomal escape.

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