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Zn and Mg Doped Hydroxyapatite Nanoparticles for Controlled Release of Protein

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Abstract

Bovine serum albumin (BSA) protein incorporated with hydroxyapatite (HA) nanoparticles (NPs) were synthesized by *in situ* precipitation process. 2 mol% Zn^{2+} and Mg^{2+} were used as dopants to synthesize $\text{Zn}^{2+}/\text{Mg}^{2+}$ doped HA-BSA NPs by *in situ* synthesis route. In our study we used BSA as a model protein. The amount of BSA uptake by doped and undoped HA NPs and subsequent release of BSA from NPs were investigated. Zn doped HA NPs showed the highest amount of BSA uptake, whereas the amount of BSA loaded in undoped HA NPs was the lowest. A two-stage BSA release profile from doped and undoped HA NPs was observed in phosphate buffer solution (PBS) at pH 7.2 ± 0.2 . Initial burst release was due to the desorption of BSA from the HA surface. The later stage of slow release was controlled by the dissolution of BSA incorporated HA NPs. BSA release rate from Zn doped HA NPs was found to be the highest, whereas undoped HA NPs released BSA at the slowest rate. Our study showed that the protein release rate from HA NPs can be controlled by the addition of suitable dopants and doped HA based NP systems can be used in bone growth factor and drug release study.

Keywords

Nanoparticles; Hydroxyapatite; Dopant; Bovine serum albumin; Protein release

1.0 Introduction

In the recent years there has been increasing interest in inorganic nanoparticles (NPs) as carriers for macromolecules such as proteins, vaccines, and drugs. Numerous studies have shown that NPs can not only improve the resistance of therapeutic agents against enzymatic degradation, but also provide the possibility of transporting biomolecules to specific tissues, cells, and cell compartments in a controlled manner with a minimal invasive procedure.[1,3] Inorganic NPs have some potential advantages over other polymeric nanoparticulate based carrier systems, because of their low susceptibility to immune response as compared to viral vectors, low toxicity as opposed to organic NPs, and resistance to lipases and bile salts unlike liposomes.

Among the inorganic NPs, hydroxyapatite (HA) has attracted much attention as a carrier for biomolecules because of its excellent biocompatibility and bioactivity. For orthopedic applications, porous HA-based implants infiltrated with bioactive agents or drugs have been reported. [4–5] However, limited surface area and unpredictable bioresorbability of HA implant have been the issues that remain to be resolved for the development of a controlled drug carrier system. In the past decade, these problems have been addressed in some research efforts directed towards the synthesis of HA micro or nano-carriers delivering antibiotics and growth factors with controlled release kinetics. Intema *et al.* employed HA microcrystals as microcarriers to load bovine serum albumin (BSA) of 5–10 wt.% and concluded that these can

be used for biomedical applications such as drug delivery, orthopedics, and dentistry.[6] Matsumoto *et al.* investigated the influence of protein concentration and crystallinity of HA particles on protein release from the HA nano-carrier.[7] In all of these cases, the biomolecules were adsorbed on the surface of HA carrier, however, burst release kinetics of protein was observed. For a protein or drug carrier system to be therapeutically effective, it should show prolonged and steady release kinetics to function as a sustained delivery system. Liu *et al.* synthesized *in situ* BSA-loaded calcium deficient hydroxyapatite (CDHA) nanocarriers varying synthesis parameters and observed a continued steady protein release for 4 days from such a system.[8] Boonsongrit *et al.* studied protein release from BSA-loaded HAp microspheres encapsulated with poly(lactic acid-co-glycolic acid) (PLGA) and found that the BSA release could be remarkably prolonged from such a system.[9]

Though some reports are available on effects of crystallinity, particle size, and surface area of HA micro and nanocarriers on protein loading and release, to the best of our knowledge the role of dopants in modifying protein release behavior from HA nanocarriers has not been studied. Modification of crystallinity of HA nanocarrier by heat treatment is undesirable in many cases, as heating may lead to denaturation and inactivation of many biomolecules loaded on the nanocarriers. Addition of a dopant generates defects and disorders in the HA crystal lattice and thus changes the crystallinity of HA nanocarriers without any application of heat. Biological apatites in enamel, dentin and bone contain trace amounts of Mg and Zn. Zinc is an essential trace element, with stimulatory effects on bone formation *in vitro* and *in vivo*. HA doped with metal ions have shown to stimulate promotes osteoblast function and subsequent bone formation.[10,11] Our research also indicates that trace elements, such as Sr, Mg and Zn, enhance bone-cell materials interaction. [12,13] Therefore, Mg and Zn-substituted HA NPs are expected to have excellent biocompatibility and biological properties. Studies have also indicated that Zn deficiency in elderly subjects may cause osteoporosis [Otsuka '04] [14] Incorporation of Mg and Zn in HA lattice results in a decrease in crystallinity, and hence an increase in dissolution of HA NPs in physiological environment.[15] Here we studied BSA-HA nanocarriers to achieve controlled release kinetics of BSA protein. We also investigated the role of Mg and Zn in controlling the protein release from HA nanocarriers. In our study we used BSA as a model protein. Our hypothesis is that a controlled dissolution and thus controlled protein or drug release from HA NPs can be obtained if the rate of dissolution of HA NPs can be modified by doping them with Zn and Mg.

2. Materials and Methods

2.1 Materials

2 mol % Mg and Zn doped BSA-HA NPs were synthesized by *in situ* precipitation process. The doped and undoped BSA-HA NPs were characterized by X-ray diffraction (XRD), dynamic light scattering (DLS) technique, transmission electron microscopy (TEM), and Fourier transform infra-red (FTIR) spectroscopy. Differential scanning calorimetry (DSC) was used to understand the effect of dopants on the interaction between BSA and HA NPs. The protein release behaviors of the BSA-loaded HA NPs were studied in phosphate buffer solution at pH 7.2 ± 0.2 .

For synthesis of BSA loaded HA NPs, calcium nitrate [$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, J.T. Baker, Phillipsburg, NJ] and ammonium hydrogen phosphate [$(\text{NH}_4)_2\text{HPO}_4$, Alfa Aesar, Ward Hill, MA] were used as source of Ca^{2+} ion and PO_4^{3-} ion, respectively, and BSA (Sigma-Aldrich, St. Louis, MO) was used as a model protein. Mg^{2+} and Zn^{2+} doped BSA-HA nanopowder was synthesized using magnesium nitrate [$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, J. T. Baker, Phillipsburg, NJ] and zinc nitrate [$\text{Zn}(\text{NO}_3)_2 \cdot 10\text{H}_2\text{O}$, J. T. Baker, Phillipsburg, NJ] as a source of Mg^{2+} and Zn^{2+} respectively. Ammonium hydroxide (NH_4OH , J. T. baker, Phillipsburg, NJ) was used to adjust the pH of the reaction mixture.

2.2 Synthesis of HA-BSA NPs

5 M aqueous solution of Ca^{2+} -ion was prepared by dissolving 0.03 moles of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ in 6 ml distilled water. A BSA aqueous solution of 1500 $\mu\text{g}/\text{ml}$ was separately prepared by dissolving BSA powder into distilled water. The solutions were stirred still a clear suspension was obtained without any visible solids. NH_4OH was added dropwise to adjust the pH of Ca^{2+} solution to 9. Five ml of BSA was dropped into Ca^{2+} solution with constant stirring. To maintain Ca to P molar ratio of 1.67:1 in the reaction mixture, 0.018 moles of $(\text{NH}_4)_2\text{HPO}_4$ was added to Ca^{2+} - BSA solution. The pH of the reaction mixture was readjusted to 9 by dropwise addition of NH_4OH . The suspension was aged for 24 h at room temperature and centrifuged to drain supernatant out and the resulting precipitate was then washed with phosphate-buffered solution (PBS at pH 7.0) and DI water for 3 times to remove NO_3^- ions and loosely bound BSA. 2 mol% Mg^{2+} and Zn^{2+} doped HA-BSA NPs were synthesized by the addition of the required amount of $\text{Mg}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ and $\text{Zn}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$, respectively, in the Ca^{2+} aqueous solution. After washing, all the powders were dried at room temperature and stored in a freezer at -10°C .

All the supernatants after every washing were collected and analyzed for concentration of BSA using a BCA protein assay kit. The total amount of BSA incorporated into doped and undoped

HA NPs was calculated by using the equation $\frac{[\text{BSA}]_I - [\text{BSA}]_S}{[\text{BSA}]_I} \times 100\%$ where $[\text{BSA}]_I$ = total amount of BSA added to the reaction mixture, $[\text{BSA}]_S$ = total amount of BSA present in the supernatant.

2.3 Characterization of HA-BSA NPs

2.3.1. Powder x-ray diffraction (XRD) of the NPs—The powder x-ray diffraction (XRD) method was used for the determination of the crystal phase, lattice constants, and crystallinity. The XRD analyses of synthesized BSA-HA NPs were performed using a Philips fully automated x-ray diffractometer with CuK_α radiation (1.54018 Å) and a Ni- filter. The diffractometer was operated at 35 kV and 30 mA. The XRD data was collected at room temperature over the 2θ range of $20^\circ - 60^\circ$ at a step size of 0.02° and a count time of 0.5 s/step.

The crystallinity of the apatite crystallites was evaluated by measuring the broadening of the XRD peaks in two regions: peak (002) between 25.0 and 27.0° and peak (310) between 37.5 and 41.0° . [16] Broadening of the diffraction peaks was measured at half maximum intensity of the peaks using the Scherrer formula, [17]

$$D = k\lambda / \beta_{1/2} \cos \theta \quad (1)$$

where D is the crystallite size (Å) estimated using the reflection (002) and (310), k is a shape factor equal to 0.9, λ is the X-ray wavelength (1.5405 Å), θ is the diffraction angle related to the reflection (002) and (310), and $\beta_{1/2}$, expressed in radians, is defined as

$$\beta_{1/2} = (B^2 - b^2)^{1/2}, \quad (2)$$

B being the diffraction peak width at half height and b the natural width of the instrument.

2.3.2. Dynamic light scattering (DLS)—Particle size of synthesized doped and undoped HA-BSA NPs was measured by DLS technique using NICOMP™ 380 (Santa Barbara, CA, USA) particle size analyzer. Approximately, 0.002 gm HA-BSA nanopowder was added to 50

ml of water at pH-10 and ultrasonicated for 15 min to minimize the degree of agglomeration. The aqueous suspension of HA-BSA NPs at pH-10 was stable against flocculation for a long time under the influence of strong electric double layer repulsion around the negatively charged particle surface. The NP suspension was filled in a 6 × 50 mm size borosilicate glass tube and inserted in NICOMP™ 380 chamber for particle size analysis. The particle sizes were automatically determined from the autocorrelation function using the Stokes–Einstein equation: $r = kT/6D\pi\eta$, where r is the particle radius, k is the Boltzmann constant, T is the absolute temperature, D is the diffusion coefficient, and η is the viscosity of the liquid in which the NPs were suspended. The values for the above parameters at room temperature were, [18]

$$k=1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}, T=293\text{K}, \eta=0.891 \text{ cP:}$$

2.3.3. Transmission electron microscopy—A dilute aqueous suspension of doped and undoped HA-BSA NPs in water was prepared following the aforementioned method. One drop, approximately 5 μl , of particle suspension was deposited onto a formvar coated Cu grid (Ted Pella, Inc.) and allowed to equilibrate for 3 min. The grids were then allowed to air dry. Images were taken using a JEOL, JEM 120 (MA, USA) transmission electron microscope (TEM) set to an accelerating voltage of 100 KV.

2.3.4 Elemental analysis of HA-BSA NPs—The elemental composition of both undoped and doped HA-BSA NPs were determined with inductively coupled plasma-optical emission spectroscopy (ICP-OES) using Optima 3200 RL (Perkin Elmer, CA, USA) instrument. For this analysis, ~ 0.1 g of both doped and undoped HA-BSA NPs were calcined in a muffle furnace at 600 °C for 2 h to burn out BSA in it. The calcined powders were then dissolved in 10 % HNO_3 . All of the standards and samples were diluted with 4 % HNO_3 for final measurements.

2.3.5 Differential Scanning Calorimetric (DSC) analysis—To understand the interaction between HA and BSA, both doped and undoped NPs were tested on a DSC thermal analyzer (NETZSCH, Burlington, MA, USA). The measurements were performed with a differential scanning calorimeter model STA 409 PC at a heating rate of 10 K/min in argon atmosphere with a flow rate of 40 ml /min. Samples were kept in an alumina crucible for analysis with an empty crucible as reference. Pure HA nanopowder was used for baseline correction.

2.3.6 ATR-FTIR analysis of HA-BSA NPs—Fourier transform infrared spectroscopy (FTIR) was conducted on BSA loaded doped and undoped HA NPs. For this, 1mg of each of the synthesized powders was put on an ATR (attenuated total reflection) diamond crystal and pressed with an indenter so that powder could remain in contact with the ATR diamond crystal. The FTIR spectra of these samples were then obtained using a FTIR spectrophotometer Nicolet 6700 FTIR (Madison, WI, USA). Before every measurement a background FTIR spectra was taken and deducted from the sample spectra. All of the spectra were collected in the 400–4000 cm^{-1} wave number range.

2.3.7 Secondary structure analysis by FTIR spectroscopy—Background-corrected spectra were analyzed in the amide I band regions for their component compositions and peak frequencies using Microcal origin 7.0 software. Gaussian curve-fitting, using GRAMS/386, was performed on the original (non-smoothed) amide I band region. The number of components and their peak positions were used as starting parameters. In all cases, linear baseline was fitted. The secondary structure content was calculated from the areas of the individual assigned bands

and their fraction of the total area in the amide I region [19] The determined areas were averaged, and standard deviations were calculated.

2.4 Study of release kinetics of BSA from HA-BSA NPs

20 ml of phosphate buffer solution at pH 7.2 was added to 5 mg of BSA-loaded doped and undoped HA NPs. The NPs were dispersed with a stirring rate of 100 rpm at 37 °C. The sample vials were centrifuged and 0.5 ml of supernatants were removed at each time point, kept in cryovials and frozen at -10 °C. The BSA release test was carried out by taking 0.1 ml of the supernatant and mixing it with 0.1 ml of bicinchoninic acid solution (Sigma, bicinchoninic acid protein assay kit, BCA-1 and B9643). UV-visible spectroscopy (Agilent 8453) was used for the characterization of absorbance peaks at 562 nm to determine the BSA concentration through the use of a pre-determined standard concentration-intensity calibration curve.

2.5 Statistical Analysis

Statistical analysis of data was performed using the software package SAS 9.1 (Cary, NC, USA).[20] All quantitative tests were carried out in triplicate and then mean values with standard deviations were calculated. After verifying normal distribution and homogeneity of variance, one way analyses of variance (ANOVA) was done for comparison between groups at a significance level of $p < 0.05$. Finally, Tukey's test for pairwise multiple comparison was performed to detect significant differences between groups.

3.0 Results

Figure 1 shows the x-ray diffraction (XRD) patterns of BSA-loaded doped and undoped HA nanopowders. All the powders showed pure HA according to JCPDS No. 09-0432.[21] As evident from Table 1, the peaks for (002) and (310) reflections were broader for doped HA-BSA NPs. Zn doped HA-BSA NPs showed the highest broadening of the above peaks among the three powders. The relative broadening of (002) and (310) diffraction lines indicated that crystallinity was the lowest for Zn doped NPs, whereas the undoped HA-BSA NPs showed the highest crystallinity.

Figure 2 shows FT-IR spectra of BSA loaded doped and undoped HA NPs. All the NPs showed characteristic peaks for PO_4^{3-} and BSA. TEM micrographs of NPs in Figure 3 reveals that aspect ratios of doped NPs were higher compared to undoped NPs. Zn doped HA-BSA NPs showed the highest aspect ratio of 6.5 among the three powders. Mg doped HA-BSA NP showed an aspect ratio of 4.5, which was higher than the aspect ratio of 3.4 in undoped HA NP. The average length of NPs was found to be 37 ± 13 nm, 85 ± 10 nm and 104 ± 12 nm for undoped, Mg doped and Zn doped NPs, respectively. Similarly, the average width was 11 ± 3 nm, 19 ± 3.5 nm and 16 ± 3 nm for undoped, Mg doped and Zn doped NPs, respectively. From the number average particle size (NICOMP) distribution data in Figure 4, it was evident that doped HA NPs showed broader particle size distribution as compared to undoped HA nanopowder. The number average particle size of these NPs varied between 30 – 110 nm.

Quantitative estimates of weight and atomic percents of different element present in doped and undoped HA NPs are shown in Table 2. The variation in measured atomic percentages of dopants suggests that not all of the dopants added precipitated out as a part of the HA. Zn^{2+} appeared to be more effective in substituting Ca^{2+} in HA NPs as compared to Mg^{2+} . For all the powders Ca/P ratio was found to be higher than ideal Ca/P ratio of 1.67 for HA.

The schematic representation of interaction between HA crystal and BSA molecule has been presented in Figure 5. The amount of BSA uptake by *in situ* synthesized doped and undoped HA-BSA nanopowders changed with addition of dopants. Zn doped HA NPs showed the

maximum of 24 wt % of BSA uptake, followed by 21 wt % by Mg doped and 18 wt % by undoped HA NPs. Table 3 shows the compositional analysis of secondary structure in pure solid BSA powder, doped HA-BSA NPs, and undoped HA-BSA NPs. The α -helix content as determined from FTIR spectra in amide –I region was found to be 37%, 33% and 28% for Zn doped, Mg doped, and undoped HA-BSA NPs respectively. The result showed a relative decrease in α -helix content in HA-BSA NPs as compared to α -helix content of 41% in pure solid BSA powder

The interaction between BSA and HA was explored by DSC analysis as shown in Figure 6. The endothermic peak at 70 °C was attributed to thermal denaturation of BSA. The removal of water molecule from BSA was evident from the exothermic peak at 160°C. Thermal decomposition of BSA molecule was detected by the exothermic peak at 350 °C.[22] The most noticeable difference in DSC plot of undoped HA-BSA NPs from that of doped HA-BSA NPs was the presence of a sharp and intense exothermic peak in the former which was absent for doped HA-BSA NPs at around 470 °C. The concerned exothermic peak was probably attributed to the thermal decomposition of the HA-BSA complex.[22]

The BSA release profiles of the BSA-loaded doped and undoped HA nanocrystals are shown in Figure 7. It was found that all samples showed a burst release within the first 4 h initial time period when almost 40% of BSA was released. This was followed by a slower BSA release, which continued for 4 days. BSA release rate from Zn doped HA nanocrystal was found to be significantly higher compared to Mg doped HA-BSA ($p=0.0044$) and undoped HA-BSA ($p<0.0001$) NPs. Again Mg doped HA-BSA NP exhibited a significantly higher ($p<0.0001$) BSA release rate compared to undoped HA-BSA NP.

4.0 Discussion

In atomic structure, HA builds up with a central $\text{Ca}(\text{OH})_2$ and three surrounding $\text{Ca}_3(\text{PO}_4)_2$ groups. When $\text{Zn}^{2+}/\text{Mg}^{2+}$ are incorporated into the lattice structure of HA, the central Ca atom is substituted by Mg or Zn. The ionic radii of Mg^{2+} and Zn^{2+} are 0.72 and 0.74 Å respectively, which are significantly smaller than .99 Å of Ca^{2+} . Substitution of Ca^{2+} by Mg^{2+} and Zn^{2+} distorts the HA lattice and the structure more resembles β -TCP which has an increased c-axis bond length as compared to HA. Thus doping with $\text{Mg}^{2+}/\text{Zn}^{2+}$ caused bond strain and decreased crystallinity in HA-BSA nanocrystals as shown in Figure 1 and Table 1. FTIR spectra in Figure 2 reveal typical ν_3 asymmetric PO_4^{3-} stretching mode at 1093 cm^{-1} and 1024 cm^{-1} , ν_1 symmetric PO_4^{3-} stretching mode at 974 cm^{-1} , labile PO_4^{3-} at 634 cm^{-1} , triply degenerate ν_4 PO_4^{3-} bending mode at 601 and 561 cm^{-1} , and doubly degenerate ν_2 bending mode at 478 cm^{-1} in all the NPs.[23,24] The decrease in relative peak intensities at 561, 601, and 1024 cm^{-1} for Mg and Zn doped HA-BSA NPs as compared to undoped HA-BSA NPs also reflected a relative decrease in crystallinity with addition of dopants into HA-BSA nanocrystal.[22] The doubly degenerate ν_2 CO_3^{2-} bending mode at 871 cm^{-1} indicated the formation of a β -type carbonate in these HA NPs.[11] The presence of BSA in all the powders were confirmed from the band at 1655 cm^{-1} assigned to amide I C=O stretching mode, 1542 cm^{-1} assigned to amide II N–H bending mode, and 1475 cm^{-1} assigned to amide III C–N stretching mode.[25] As synthesis of BSA loaded HA NP was carried out in ambient atmosphere, the presence of carbonate in HA-BSA NP is expected due to the presence of carbon dioxide (CO_2) in the reaction mixture during the synthesis process.

Addition of dopants, Zn^{2+} and Mg^{2+} into HA crystal lattice caused a reduction in a-axis and an increase in c-axis and thereby increased the aspect ratio of HA-BSA NPs as reflected in TEM micrographs in Figure 3.[26] The broader particle size distribution for doped HA-BSA NPs rather than undoped HA-BSA NPs, as reflected in Figure 4, is primarily attributed to their greater degree of anisotropy in translational motion in water suspension during DLS study

because of higher aspect ratio of the former as compared to the latter. HA exhibited higher selectivity for Zn^{2+} compared to Mg^{2+} as shown in Table 2, which explains the higher amount of Zn^{2+} incorporation into HA lattice.[27] The higher Ca/P ratio in all of the HA NPs was resulted from PO_4^{3-} substitution by CO_3^{2-} in HA lattice, which was also reflected in FTIR analysis in Figure 2.

As BSA was added to the reaction mixture an intermediate HA-BSA complex was formed through the electrostatic interaction between Ca^{2+} on the c-sites of HA lattice and COO^- of BSA molecule.[28] The c-sites were developed as a result of Ca^{2+} ions on the HA surface located on the crystal planes that were perpendicular to a-axis and b-axis of apatite crystal as depicted in Figure 5.[29] Preferential inhibition effect on the growth of HA nanocrystals along the a and b axis, resulted in higher aspect ratio doped HA-BSA NP and exposed a higher spacing distance between the two planes of ions, as shown in Figure 5, to allow for higher electrostatic interaction between Ca^{2+} of HA lattice and COO^- ions of BSA molecules. Thus aspect ratio of Zn doped HA-BSA NPs being the highest, exhibited the maximum amount of BSA uptake, followed by Mg doped HA-BSA and undoped HA-BSA NPs[22] BSA molecules were incorporated into HA nanocrystals by two types of interaction; one is physical adsorption on the surface of HA NPs, which is weak in nature, and the other one is strong electrostatic interaction between Ca^{2+} of HA lattice and COO^- ions of BSA molecules. The adsorption of BSA onto HA nano-crystals can be considered as pseudo-Langmuir type, where a number of the BSA layers were adsorbed onto HA surface that were not tightly bound with HA nanocrystals.[26] Therefore, for all the HA NPs an exothermic peak was observed at 350 °C due to thermal decomposition of adsorbed BSA molecule, which is close to that of pristine BSA as shown in Figure 6. The exothermic peak at 470 °C is attributed to the thermal decomposition of tightly bound BSA molecules into HA crystals, which were bonded through strong electrostatic force. A sharper and more intense exothermic peak at around 470 °C in undoped HA-BSA nanocrystals indicates a stronger HA-BSA interaction in undoped HA-BSA NP compared to doped HA-BSA NP.[22] Undoped HA-BSA NP being more crystalline than doped HA-BSA NPs, exhibited stronger Ca^{2+} - COO^- electrostatic interaction as compared to doped HA-BSA NP.

In the present study we have used BSA as a model protein, as it is abundantly available in the physiological environment. The decrease in α -helix content in doped and undoped HA-BSA NP indicates that BSA's secondary structural integrity was distorted due to its interaction with HA crystal lattice. The undoped HA NP interacted most strongly with BSA, which resulted in a maximum decrease in α -helix content in loaded BSA molecules as depicted in Table 3. Retention of α -helix structural integrity was found to be the greatest for BSA molecules in Zn doped HA-BSA NP primarily because of less intense BSA-HA interaction in Zn doped NP. In our previous study using NP of tricalcium phosphates and calcium silicates, we have shown that no significant distortion of BSA structure happened when they were used as BSA nanocarrier.[30,31]

The initial burst release of BSA as shown in Figure 7 from HA NPs could be attributed to desorption of BSA molecules, which were not tightly bound with HA nanocrystals. The slower BSA release profile in the later stage can be assigned to gradual release of BSA molecules which were incorporated into HA crystals forming HA-BSA complex. The later stage of BSA release was governed by crystal dissolution along the c- axis to release incorporated BSA from HA-BSA complex.[32] It is generally accepted that with decrease in the crystallinity of HA NPs, their rate of dissolution in phosphate buffer solution also increases, which can lead to faster BSA release of doped HA NPs. BSA release rate from Zn doped HA-BSA was the fastest, because Zn doped HA-BSA NPs being the least crystalline dissolved at the highest rate in phosphate buffer solution at $\text{pH-}7.2 \pm 0.2$. Undoped HA-BSA NPs showed the slowest BSA release rate as they showed the highest crystallinity and dissolved at the slowest speed in

phosphate buffer solution at pH-7.2±0.2. Based on our study described in this manuscript we are currently studying the calcium phosphate /HA nanocarrier system for delivering bone growth factors and osteoporosis drugs.

5.0 Conclusions

The amount of BSA uptake was found to be the highest for Zn doped HA-BSA NP, while undoped HA-BSA NP exhibited the lowest amount of BSA uptake. BSA was found to interact with undoped HA nanocrystal more strongly compared to doped HA-BSA nanocrystals to form HA-BSA complex. The BSA release profile was first dominated by its desorption from NP surface, followed by a slow release controlled by crystal dissolution. Moreover, addition of dopants significantly altered the protein release behaviors of BSA-loaded HA NPs. Zn doped HA-BSA NP being the least crystalline, released BSA at the fastest rate followed by Mg doped HA-BSA NP and undoped HA-BSA NP. It is feasible to employ HA nanocarriers to load protein, drug and other biomolecules and control their release by incorporation of suitable dopants into the crystals with predesigned parameters for particular therapeutic effect.

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Figure 1.
X-ray diffraction pattern of doped and undoped HA- BSA nanopowders

Figure 2.
FTIR spectra of doped and undoped HA-BSA nanopowders.

Figure 3. TEM micrographs of *in situ* synthesized HA-BSA nanopowders. (a) undoped (b) Mg doped and (c) Zn doped.

Figure 4.
Particle size distribution of synthesized HA-BSA nanopowders

Figure 5.
Schematic representation of hydroxyapatite- BSA interaction (not shown to scale)

Figure 6.
Amount of BSA uptake by *in situ* synthesized doped and undoped HA-BSA nanopowders

Figure 7.
DSC curves of BSA loaded undoped and doped HA nanopowders

Figure.8.
BSA release profiles of the BSA-loaded undoped and doped HA nano-carriers

Table 1

Peak width measurements of the (002) and (310) reflections of BSA loaded doped and undoped HA nanopowders

Sample	Peak width (2 θ)	
	(002)	(310)
HA-BSA	0.456	0.608
MgHA-BSA	0.476	0.656
ZnHA-BSA	0.512	0.752

Table 2

Concentration of Ca, P, Mg and Zn in HA- BSA, Mg HA- BSA and Zn HA-BSA nanopowders, as determined by ICP-OES

	Undoped HA-BSA	Mg doped HA-BSA	Zn doped HA-BSA
Ca (wt %)	38.23±1.54	37.48 ±1.61	37.27± 1.54
P (wt %)	16.41±0.89	16.61±0.34	16.78 ±0.45
Mg (at %)	-----	0.143 ±0.08	-----
Zn (at %)	-----	-----	0.178 ±0.12
Ca/P (atomic)	1.81 ±0.02	1.75 ±0.39	1.72 ± 0.024

Table 3

Infrared band positions of BSA and band assignments

Band position (cm ⁻¹)	Secondary structure assignment	Area			
		Pure BSA	HA - BSA	MgHA-BSA	ZnHA-BSA
1616±3	Self association	6±1	8±2	6±1	4±1
1628±3	β-sheet	9±2	12±1	14±2	11±2
1638±3	β-sheet	15±2	19±2	16±1	16±2
1648±3	unordered	18±1	20±1	17±1	16±1
1658±3	α-helix	26±1	17±2	18±2	21±1
1665±3	α-helix	15±1	11±2	15±1	16±1
1671±3	β-turns	9±3	9±2	9±1	10±2
1685±2	β-turns	4±2	3±2	6±1	6±1