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Hollow hydroxyapatite microspheres as a device for controlled delivery of proteins

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Abstract Hollow hydroxyapatite (HA) microspheres were prepared by reacting solid microspheres of Li_2O–CaO–B_2O_3 glass (106–150 μm) in K_2HPO_4 solution, and evaluated as a controlled delivery device for a model protein, bovine serum albumin (BSA). Reaction of the glass microspheres for 2 days in 0.02 M K_2HPO_4 solution (pH = 9) at 37°C resulted in the formation of biocompatible HA microspheres with a hollow core diameter equal to 0.6 the external diameter, high surface area ($\sim$100 m^2/g), and a mesoporous shell wall (pore size $\approx$13 nm). After loading with a solution of BSA in phosphate-buffered saline (PBS) (5 mg BSA/ml), the release kinetics of BSA from the HA microspheres into a PBS medium were measured using a micro bicinchoninic acid (BCA) protein assay. Release of BSA initially increased linearly with time, but almost ceased after 24–48 h. Modification of the BSA release kinetics was achieved by modifying the microstructure of the as-prepared HA microspheres using a controlled heat treatment (1–24 h at 600–900°C). Sustained release of BSA was achieved over 7–14 days from HA microspheres heated for 5 h at 600°C. The amount of BSA released at a given time was dependent on the concentration of BSA initially loaded into the HA microspheres. These hollow HA microspheres could provide a novel inorganic device for controlled local delivery of proteins and drugs.

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1 Introduction

Over the past few decades, there has been considerable interest in the development of devices for controlled local delivery of proteins such as growth factors and drugs [1]. A controlled-release system consists of a biologically active agent (e.g., protein) in a carrier material (commonly a polymer or ceramic). The objective of the controlled-delivery device is to provide a means for local delivery of the protein to the target site at concentrations within the therapeutic limits and for the required duration. Since the delivery device is implanted, injected, or inserted into the body, the biocompatibility and toxicity of the carrier material must also be considered.

Natural and synthetic biodegradable polymers have found wide application as carrier materials for protein delivery [2]. The delivery systems include microspheres, hydrogels, and three-dimensional porous scaffolds [3, 4]. These polymers degrade in vivo, either enzymatically or non-enzymatically, to produce biocompatible or non-toxic by-products along with progressive release of the dispersed or dissolved protein. Natural polymers and their derivatives in the form of gels or sponges have been used extensively as delivery vehicles. In particular, collagen is a readily available extracellular matrix component that allows cell infiltration and remodeling, making it an attractive delivery system for proteins [5, 6]. Biodegradable synthetic polymers, such as poly(lactic acid), PLA, and poly(glycolic acid), PGA, as well as their copolymers, poly(lactic co-glycolic acid), PLGA, are the most widely used delivery

systems. In addition to being widely available, they can be prepared with well-controlled, reproducible chemical and physical properties [2–4, 7]. They are also among the few synthetic biodegradable polymers approved by the Food and Drug Administration (FDA) for in vivo use.

Inorganic materials which have been utilized as carriers for protein delivery consist primarily of calcium phosphate materials such as β-tricalcium phosphate, $Ca_3(PO_4)_2$ and hydroxyapatite, HA, $Ca_{10}(PO_4)_6(OH)_2$ [1], and bioinert metal oxides such as silica, SiO_2 [8, 9]. The calcium phosphate materials, composed of the same elements as bone, are biocompatible and produce no systemic toxicity or immunological reactions. In addition to its chemical and structural stability, SiO_2 can be prepared near room temperature by sol–gel methods and other solution-based methods, so the protein activity can be retained. The delivery systems typically consist of nanoparticles, porous particles, granules, or porous substrates in which the protein is adsorbed or attached to the surfaces of the porous material, or encapsulated within the pores [8–11]. Hollow HA microspheres (diameter = 1,500–2,000 μm), consisting of a hollow core and a mesoporous shell, have been prepared by coating chitin microspheres with a composite layer of chitin and HA, followed by thermal decomposition of the chitin and sintering of the porous HA shell [12].

Day and Conzone [13] invented a process for preparing porous phosphate materials with high surface area by converting borate glasses with special compositions in an aqueous phosphate solution near room temperature [14, 15]. A characteristic feature of the process is that it is pseudomorphic, so the HA product retains the same external shape and dimensions of the starting glass object. Using this process, Wang et al. [16] reacted solid glass microspheres (106–125 μm) with the composition (wt%) $4.7Li_2O$, $13.2CaO$, $82.1B_2O_3$ in 0.25 M K_2HPO_4 solution for 5 days at 37°C and pH = 10.0–12.0. They found that the product consisted of hollow microspheres of a calcium phosphate material which, on heating for 4 h at 600°C, converted to HA. Huang et al. [17] prepared hollow HA microspheres by reacting glass microspheres with the composition $10Li_2O$, $10CaO$, $80B_2O_3$ (wt%) for 5 days (microsphere diameter = 106–125 μm) or 14 days (microsphere diameter =500–800 μm) under similar conditions used by Wang et al. [16]. They measured the surface area of the smaller HA microspheres (135 m^2/g) and the rupture strength of the larger HA microspheres (1.6 MPa), and studied the effect of heat treatment on the surface area and rupture strength. Heating the as-prepared HA microspheres for 8 h at 600°C and at 800°C resulted in a marked decrease in surface area and a sharp increase in strength.

Our previous work showed that the microstructure of hollow HA microspheres prepared by converting Li_2O–CaO–B_2O_3 glass microspheres (106–150 μm) in a K_2HPO_4 solution can be manipulated over a wide range by controlling the process variables [18]. By varying the concentration (0.01–0.25 M) and the temperature (25–60°C) of the K_2HPO_4 solution at pH = 9–12, hollow HA microspheres with a hollow core diameter to microsphere diameter (*d*/*D*) of 0.14–0.62, surface area of 78–145 m^2/g, and pore size of 8–20 nm were produced.

The objective of this work was to evaluate hollow HA microspheres prepared by the glass conversion process as a potential device for controlled delivery of proteins. Bovine serum albumin (BSA) was used as a model protein in this work because it is one of the most widely studied proteins. The ability to fill the hollow HA microspheres with an aqueous solution of BSA, and the release kinetics of BSA from the filled microspheres into an aqueous medium were determined. The ability to manipulate the release kinetics of the BSA was studied by altering the microstructure of the shell wall of the as-prepared HA microspheres using a controlled heat treatment (1–24 h at 600–900°C).

2 Experimental procedure

2.1 Preparation of hollow hydroxyapatite (HA) microspheres

Hollow HA microspheres were prepared by reacting solid glass microspheres in an aqueous phosphate solution as described previously [18]. Briefly, borate glass, with the composition (wt%): $15CaO$, $10.63Li_2O$, $74.37\ B_2O_3$, designated CaLB3-15, was prepared by melting Reagent grade $CaCO_3$, Li_2CO_3 and H_3BO_3 (Alfa Aesar, Haverhill, MA, USA) in a Pt crucible at 1,200°C for 45 min, and quenching between cold stainless steel plates. Particles of size 106–150 μm were obtained by grinding the glass in a hardened steel mortar and pestle, and sieving through 100 and 140 mesh sieves. Microspheres were obtained by dropping the crushed particles down a vertical tube furnace at 1,000°C, as described in detail elsewhere [14].

Hollow HA microspheres were prepared by reacting the glass microspheres for 2 days in 0.02 M K_2HPO_4 solution at 37°C and pH = 9.0. These conditions were used because our previous work showed that they resulted in the formation of hollow HA microspheres with large *d*/*D* value (ratio of the hollow core diameter, *d*, to the sphere diameter, *D*), high surface area, and mesoporous shell wall [18]. In all the experiments, 1 g of glass microspheres was placed in 200 ml solution, and the system was gently stirred continuously. Upon completion of the conversion process, the HA microspheres were washed three times with distilled water, soaked in anhydrous ethanol to displace residual water, dried for at least 12 h at room

temperature, then for at least 12 h at 90°C, and stored in a desiccator.

These 'as-prepared' HA microspheres were subjected to a controlled thermal treatment in order to modify the microstructure of the shell wall. Microspheres were heated in a Pt crucible for 1, 5, and 24 h at temperatures between 600 and 900°C. These heat treatment temperatures were used because they are below and above the onset temperature for sintering (densification) of porous, fine-grained HA.

2.2 Characterization of hollow HA microspheres

The phase composition of the converted microspheres was checked using X-ray diffraction, XRD (*D*/mas 2550 v; Rigaku; The Woodlands, TX) and Fourier transform infrared (FTIR) spectroscopy (NEXUS 670; Thermo Nicolet; Madison, WI). XRD was performed using Cu K_α radiation ($\lambda = 0.15406$ nm) at a scan rate of 1.8°/min in the 2θ range 20–70°. The HA microspheres were ground to a powder for the XRD and FTIR analyses. FTIR was performed in the wavenumber range of 400–4,000 cm^{-1} (resolution = 8 cm^{-1}). A mass of 2 mg of the powder was mixed with 198 mg KBr, and pressed to form pellets for the FTIR analysis.

The rupture strength of individual HA microspheres, as-prepared or heat treated, was measured in a nano-mechanical testing machine (Nano Bionix; Agilent Technologies; Oak Ridge, TN) using a procedure described in detail elsewhere [17]. Because of the difficulty of manipulating microspheres of size 106–150 μm in the testing machine, larger spheres (diameter 200–250 μm) were tested. At least eight microspheres were measured for each group (as-prepared or heat-treated), and the rupture strength was expressed as a mean value ± standard deviation.

The microstructure of the external surface and the cross section of the as-prepared and heat-treated HA microspheres was examined in a scanning electron microscope, SEM (S-4700; Hitachi, Tokyo, Japan), at an accelerating voltage of 10 kV and working distance of 12 mm. Local composition of the external surface and across the wall of the microspheres was determined using energy dispersive X-ray (EDS) analysis in the SEM, with an electron beam spot size of 1 μm.

The specific surface area and the pore size distribution of the shell wall of the HA microspheres were measured using nitrogen adsorption (Autosorb-1; Quantachrome, Boynton Beach, FL). Prior to the measurement, a known mass of microspheres (in the range 300–500 mg) was weighed, and evacuated for 15 h at 120°C to remove adsorbed moisture. The volume of nitrogen adsorbed and desorbed at different relative gas pressures was measured and used to construct adsorption–desorption isotherms. The first five points of the adsorption isotherm, which initially followed a linear trend implying monolayer formation of adsorbate, were fitted to the Brunauer–Emmett–Teller (BET) equation for the determination of the specific surface area [19]. The pore size distribution was calculated using the Barrett–Joiner–Halenda (BJH) method applied to the desorption isotherms [20].

2.3 Biocompatibility of hollow HA microspheres

The biocompatibility of the hollow HA microspheres was evaluated by examining their ability to support cell proliferation in vitro. For these experiments, thin discs of hollow HA microspheres were formed by pouring the borate glass microspheres into a graphite die, heating the system for 1 h at 550°C to join the glass microspheres, and then reacting the disc in 0.02 M K_2HPO_4 solution to convert the glass microspheres to hollow microspheres. After sterilization by washing three times with water and ethanol, followed by heating for at least 24 h at 120°C, the discs of hollow HA microspheres were seeded with 60,000 MC3T3-E1 cells suspended in 60 μl medium, and incubated for 4 h to permit cell attachment. The cell-seeded constructs were then transferred to a 24-well culture plate containing 2 ml of complete medium per well. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 with the medium changed every 2 days. At selected time intervals, disks with attached cells were removed, washed, stained with Sanderson Bone Stain™, and examined in an optical microscope.

2.4 Loading of BSA into hollow HA microspheres

The ability to load a model protein, bovine serum albumin (BSA) into the converted microspheres was studied using optical microscopy. To permit visual observation of the protein distribution within the microspheres, a fluorescein isothiocyanate-labeled BSA (referred to as FITC-BSA) (Sigma–Aldridge, St. Louis, MO) was used. A mass of 0.1 g microspheres was immersed in 5 ml of a solution consisting FITC-BSA in phosphate-buffered saline (PBS) (FITC–BSA concentration = 5 mg/ml). A small vacuum was applied to the system to remove air trapped within the microspheres, thereby assisting the incorporation of the FITC-BSA into the microspheres. When the removal of air bubbles from the microspheres had ceased (as determined visually), the microspheres loaded with FITC–BSA were dried in air at room temperature, and observed in an optical microscope.

2.5 Release kinetics of BSA from hollow HA microspheres into PBS

The hollow HA microspheres were loaded with a solution of BSA (without FITC labeling) using the method outlined above, removed, and immersed in PBS to determine the release of BSA from the microspheres into the PBS. In the loading step, 200 mg of the microspheres was placed in 2 ml of a PBS solution containing 5 mg/ml BSA (molecular weight = 66 kDa; Sigma–Aldrich). The BSA-loaded microspheres were removed, rinsed three times with PBS, and placed in a beaker containing 20 ml PBS. The system was kept at 37°C, and the solution was stirred continuously. At selected time intervals, 50 µl aliquots were taken from the solution and used for determining the amount of BSA released into the solution.

The concentration of BSA in each aliquot was measured using a micro bicinchoninic acid (BCA) protein assay reagent (Product # 23240ZZ; Thermo Fisher Scientific, Rockford, IL). This assay is very sensitive to dilute concentrations of proteins and has a linear working range of 0.5–20 µg/ml for BSA [21]. The aliquots were mixed with 50 µl PBS and 100 µl of the working reagent, reacted for 2 h at 37°C and cooled to room temperature. The absorbance of each solution was measured at 550 nm using an HP 8452A diode array spectrophotometer (BMG LABTECH Inc., Cary, NC). The concentration of BSA was determined from a standard curve calibrated from measurements of the absorbance of PBS containing known concentrations of BSA. At the completion of the BSA release experiments, the microspheres were washed three times with distilled water and dried in air at room temperature. The dried microspheres were crushed and the residual BSA in the microspheres was measured using the BCA technique described above.

3 Results

3.1 Microstructure of as-prepared and heat-treated HA microspheres

Figure 1 shows an optical image of the starting CaLB3-15 glass microspheres (Fig. 1a), and SEM images showing the external surface and the cross-section of a hollow HA microsphere formed by reacting the glass microspheres for 2 days in 0.02 M K_2HPO_4 solution (pH = 9) at 37°C (Fig. 1b, c). The HA microspheres had a hollow core diameter, relative to the external diameter of the microspheres, $d/D = 0.61 \pm 0.03$, surface area = 101 ± 5 m^2/g, and a pore size of the shell wall = 13 ± 2 nm (Table 1).

XRD, FTIR, and EDS analyses (Fig. 2) confirmed that the hollow microspheres had a structure and composition corresponding to an HA-type material. The starting CaLB3-15 glass had a diffraction pattern with no measurable peaks (Fig. 2a), typical of an amorphous glass. In comparison, the patterns of the converted microspheres (as-prepared and heat treated for 5 h at 600°C) contained peaks that corresponded to those of a reference HA (JCPDS 72-1243). The broad peaks in the XRD pattern of the as-prepared microspheres may indicate that the material was poorly crystallized, or consisted of nanometer-sized crystals, or a combination of both. The intensity of the peaks increased markedly for the heat-treated microspheres, presumably as a result of increased crystallization.

The FTIR spectrum of the as-prepared glass (Fig. 2b) was similar to that of a binary Li_2O–$3B_2O_3$ glass [22, 23], consisting of two broad resonances, at 600–750 cm^{-1} and 1,200–1,600 cm^{-1}, which corresponded to vibrations of the trigonal BO_3 groups, and a broad resonance centered at ~975 cm^{-1} corresponding to the vibration of tetrahedral BO_4 groups. The most dominant resonances for the converted microspheres (as-prepared or heat-treated) were the phosphate v3 resonance, centered at ~1,040 cm^{-1}, and the phosphate v4 resonance, with peaks at ~605 and 560 cm^{-1}, which are associated with crystalline HA [24, 25]. EDS analysis (Fig. 2c) showed that Ca and P were the main metallic elements present in the converted microspheres.

SEM images of the surface and cross section of the hollow HA microspheres, as-prepared and after heat treatment for 5 h at 600 and 900°C, are shown in Fig. 3. As-prepared, the surface of the microspheres consisted of a mesoporous structure of fine, plate-like (or needle-like) HA particles (Fig. 3a). The cross section (Fig. 3b) shows that the shell wall consisted of two distinct layers: a less porous surface layer of thickness ~5 µm and a more porous inner layer. Heating for 5 h at 600°C did not produce a measurable change in the porosity of the hollow HA microspheres, but resulted in a marked change in the surface microstructure (Fig. 3c). The particles in the surface layer have a more rounded morphology, with a size<50 nm. Except for coarsening, little change in the microstructure of the inner layer of the shell wall is observed (Fig. 3d). Heating for 5 h at 900°C resulted in a porous surface layer (Fig. 3e), but the cross section of the shell wall appeared to be almost fully dense (Fig. 3f).

Figure 4 shows the effect of heating time at 600°C on the microstructure of the surface layer of the hollow HA microspheres. Heating for 1 h resulted in a fairly homogeneous microstructure of nearly rounded HA particles (or grains) (Fig. 4a, b). After 5 h, coarsening of the surface microstructure increased (Fig. 4c), but the cross section showed a more heterogeneous microstructure, consisting of

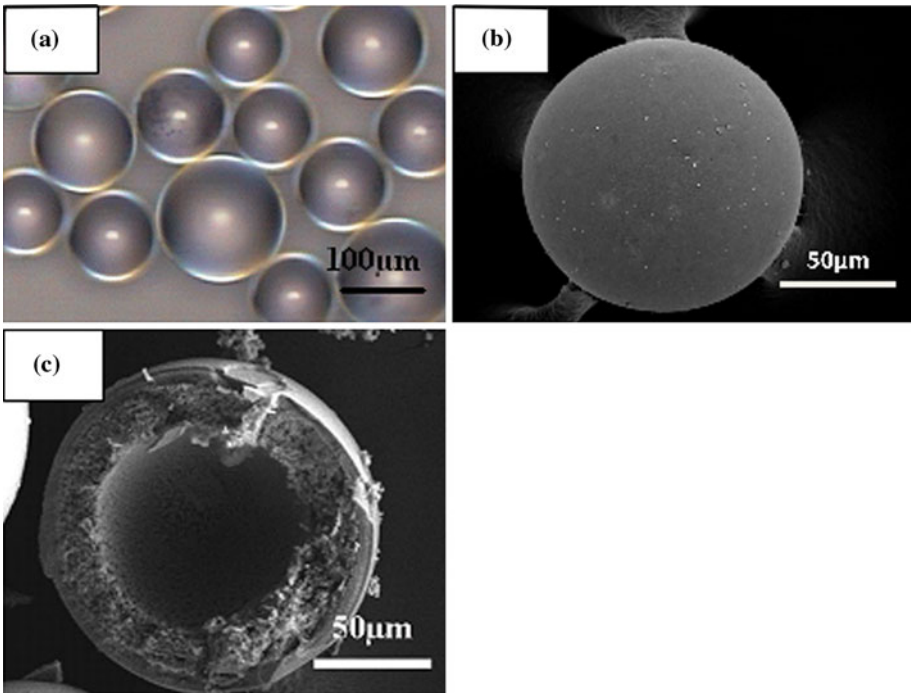

Fig. 1 **a** Optical image of starting glass (CaLB3-15) microspheres, and SEM images of **b** external surface of hollow HA microsphere prepared by converting the glass microspheres for 48 h in 0.02 M K_2HPO_4 solution at 37°C and pH = 9, **c** cross section of hollow HA microsphere

Table 1 Characteristics of as-prepared hollow HA microspheres formed by reacting Li_2O–CaO–B_2O_3 glass microspheres (106–150 μm) in K_2HPO_4 solution, and after heat treatment under the temperature/time conditions shown

Sample	*d*/*D*	Surface area (m^2/g)	Rupture strength* (MPa)
As-prepared	0.61 ± 0.03	102	11 ± 6
600°C/1 h	0.61 ± 0.03	21	17 ± 8
600°C/5 h	0.62 ± 0.03	19	–
600°C/24 h	0.66 ± 0.02	14	23 ± 14
700°C/5 h	0.73 ± 0.02	7	–
900°C/5 h	0.80 ± 0.02	2	30 ± 10

The ratio of hollow core diameter to microsphere diameter, *d*/*D*; specific surface area, and rupture strength are shown

* Measured for HA microspheres of size 200–250 μm prepared under the same conditions

a less porous layer (red arrow) between two more porous layers (Fig. 4d). For the HA microspheres heated for 24 h, the microstructure of the surface layer appeared to be a coarsened version of that for the microspheres heated for 5 h (Fig. 4e, f).

3.2 Strength of hollow HA microspheres

The rupture strength of the as-prepared hollow HA microspheres (200–250 μm) was 11 ± 6 MPa (Table 1). After heating for 1 h at 600°C, the rupture strength increased to 17 ± 8 MPa, while heating for 5 h at 900°C resulted in a further increase in the rupture strength (30 ± 10 MPa).

3.3 Biocompatibility of hollow HA microspheres

Figure 5 shows optical images of MC3T3-E1 cells (stained with Sanderson Bone Stain™) on hollow HA microspheres after incubation times of 2, 4, and 6 days. (The inset shows SEM images of the same constructs.) The cells seen in the micrographs appeared to attach to the HA microspheres by day 2 (Fig. 5a), and increased in density with the culture duration (Fig. 5b, c). After 4 days, the cells tried to colonize the surface of the spheres. The cells were in physical contact with each other and aggregated with the neighboring cells via extensions (Fig. 5b; inset). The optical and SEM images for the 6-day culture show almost complete coverage of the scaffolds with the MC3T3-E1 cells and increased cell density (Fig. 5c; inset). Viewed as a group, the continuous increase in cell density during the 6 days culture period shows the ability of the hollow HA microspheres to support cell proliferation.

3.4 Loading and distribution of BSA in hollow HA microspheres

Optical images of the as-prepared hollow HA microspheres prior to loading with FITC-labeled BSA showed no fluorescence (Fig. 6a). The brightness of the image was enhanced, and the microspheres were circled to reveal their presence. In comparison, the surfaces of the microspheres filled with the FITC-labeled BSA solution showed a high degree of fluorescence, indicating the presence of BSA (Fig. 6b). In order to show the distribution of the BSA,

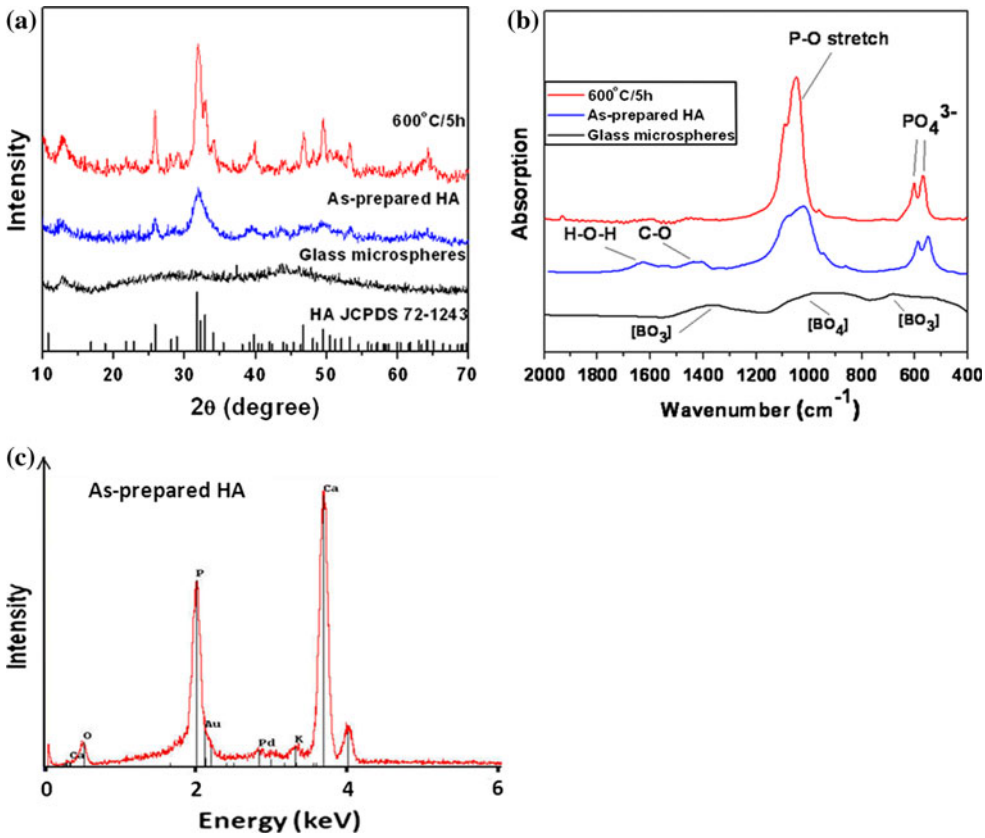

Fig. 2 **a** XRD patterns and **b** FTIR spectra of the starting glass microspheres, the as-prepared hollow HA microspheres, and the hollow HA microspheres heated for 5 h at 600°C; **c** EDS spectrum of the as-prepared hollow HA microspheres

after filling with the BSA solution and drying, the microspheres were sectioned and observed in the microscope. The fluorescence in Fig. 6b (inset) shows that the BSA was incorporated within the hollow core of the microspheres as well as within the mesoporous shell wall.

3.5 Release kinetics of BSA from hollow HA microspheres

Figure 7 shows the BSA release kinetics from the BSA-loaded HA microspheres into a PBS solution, for the as-prepared microspheres and for microspheres heat treated for 5 h at 600 or 900°C. For the as-prepared HA microspheres, the release of BSA was initially rapid (~2.0 μg/ml/h) during the first 10 h, then slowed considerably, with 95% of final amount released within 24 h, and almost ceased after 24–48 h. The total amount of BSA released into the PBS was ~22 μg/ml. Heating the as-prepared microspheres for 5 h at 600°C resulted in a marked increase in the amount and duration of the BSA released into the PBS. The amount of BSA released was comparable to that for the as-prepared microspheres during the first 48–72 h, then continued to increase, with 95% of final amount released in 7 days, and almost ceased after 14 days. The total amount of BSA released (after 14 days) was ~35 μg/ml.

For the HA microspheres heated for 5 h at 900°C, release of the BSA from the microspheres into the PBS was limited. The cumulative amount of BSA released after 3–5 h was ~2 μg/ml, and it remained at this value thereafter. The release of BSA from hollow HA microspheres heated for 5 h at 700°C was also studied. The trend in the release kinetics was approximately similar to that for the HA microspheres heated for 5 h at 900°C, and the results are omitted for the sake of brevity.

The effect of heating the as-prepared HA microspheres for varying times (1–24 h) at 600°C on the BSA release kinetics is shown in Fig. 8. For a heating time of 1 h, the release kinetics showed a trend similar to that for the as-prepared microspheres, but the total amount of BSA released was far higher (~35 vs. ~22 μg/ml). As discussed above, an increase in the heating time to 5 h at 600°C had a marked effect on the release kinetics. When compared to the microspheres heated for 1 h, the duration of the release increased (7–14 days) but the total amount of BSA released (~35 μg/ml) was approximately the same. For a longer heating time (24 h) at 600°C, the duration of the release did not increase further. Instead the duration of the release became shorter, with 95% of the final amount

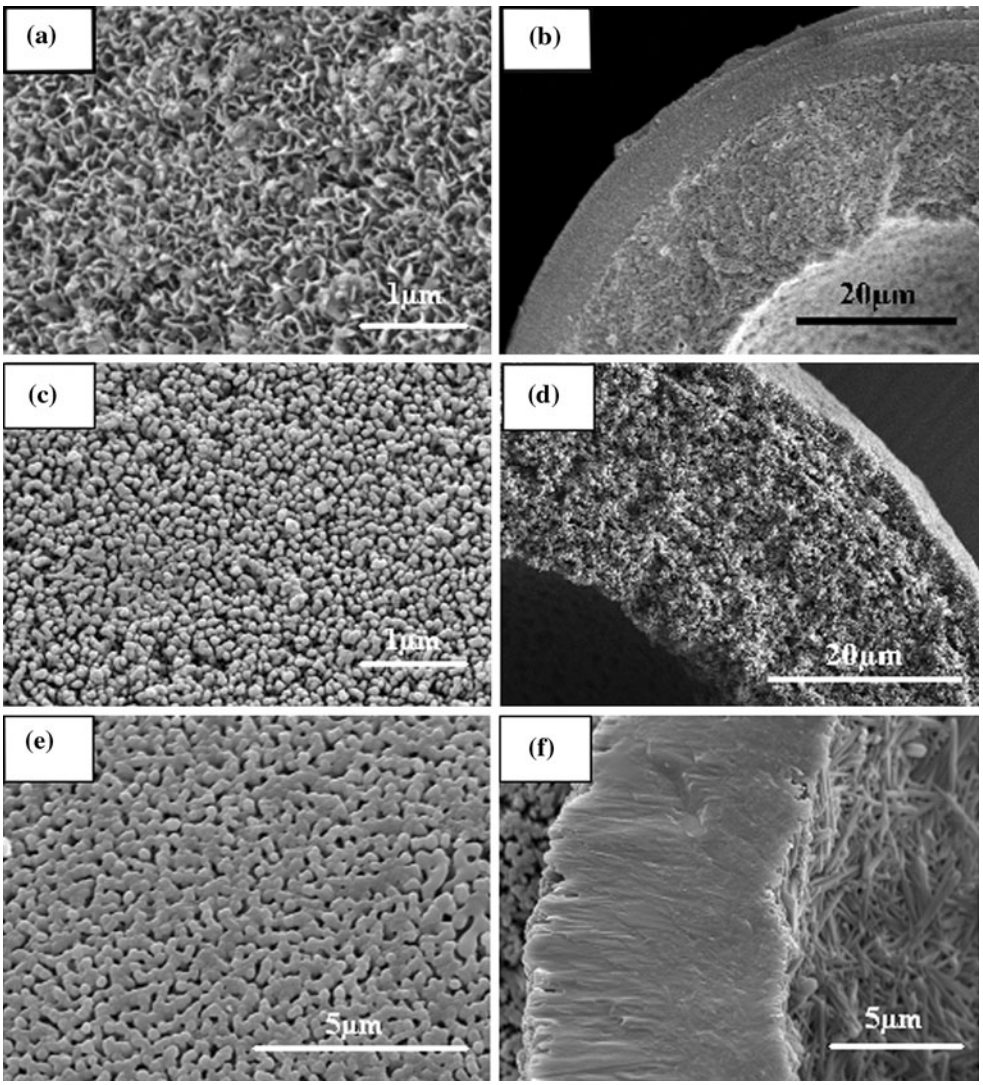

Fig. 3 SEM images of the surface (*left*) and cross section (*right*) of the shell wall of hollow HA microspheres: **a**, **b** as-prepared; **c**, **d** heated for 5 h at 600°C; **e**, **f** heated for 5 h at 900°C

released after 3 days, and BSA release almost ceased after 4–5 days. However, the total amount of BSA released (∼35 μg/ml) was approximately the same as that for microspheres heated for 1 or 5 h.

Figure 9 shows the effect of varying the amount of BSA loaded into the hollow HA microspheres (1–10 mg BSA per ml of PBS) on the release kinetics. The microspheres used in these experiments were heated for 5 h at 600°C. For a BSA concentration of 1 mg/ml, the total amount of BSA released was limited (∼5 μg/ml). Higher BSA loading markedly enhanced the amount of BSA released, as well as the duration of the release. The release kinetics from microspheres loaded with 10 mg/ml BSA followed the same trend as those for microspheres loaded with 5 mg/ml BSA, but the total amount of BSA released was higher (45 μg/ml compared to 35 μg/ml) after 14 days.

4 Discussion

The results of the present investigation show that hollow HA microspheres, prepared by a low-temperature glass conversion route, have promising potential for use as a controlled delivery device for proteins. By modifying the microstructure of the as-prepared hollow HA microspheres using a controlled heat treatment, and varying the concentration of BSA initially loaded into the microspheres, both the amount of protein (BSA) released from the

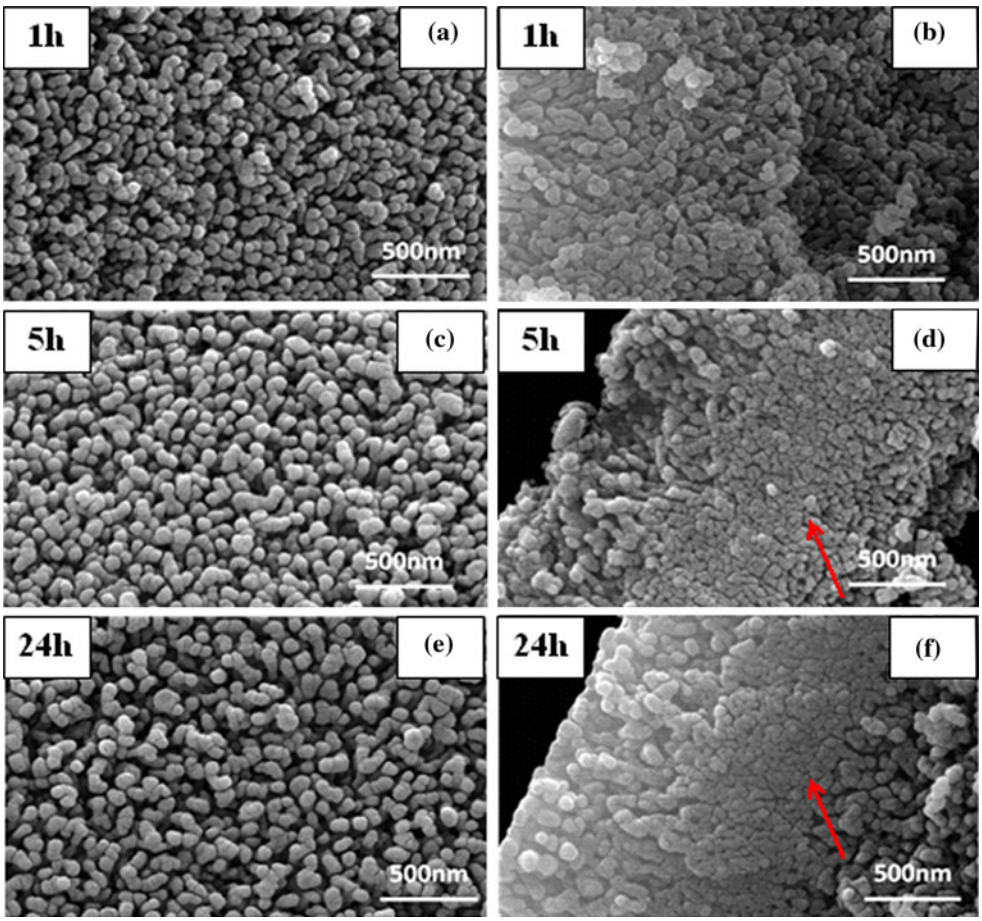

Fig. 4 SEM images of the surface (*left*) and cross section (*right*) of the surface layer of the hollow HA microspheres after heat treatment at 600°C for **a**, **b** 1 h; **c**, **d** 5 h; **e**, **f** 24 h, showing coarsening of the pores and particles, and the formation of less porous layer (*arrow*) within the surface layer

microspheres into a PBS medium and the duration of the release (1–14 days) were controlled. These HA microspheres, consisting of the same ions as human bone, could provide a novel inorganic system for controlled delivery of proteins, such as growth factors and drugs.

Our previous work showed that the microstructure of hollow HA microspheres prepared by converting Li_2O–CaO–B_2O_3 glass microspheres in a K_2HPO_4 solution can be modified over a wide range by controlling the process variables, such as the conversion temperature, K_2HPO_4 concentration and pH of the solution [18]. In this study, a group of as-prepared HA microspheres with a mesoporous shell wall (average pore size = 13 nm), high surface area (~ 100 m^2/g), and a hollow core diameter, d, equal to 0.6 the external diameter, D, of the microspheres, was evaluated as a delivery device for a model protein, BSA. Based on their ability to support the proliferation of MC3T3-E1 cells, the as-prepared HA microspheres prepared by this glass conversion route were shown to be biocompatible (Fig. 5).

The present study showed that the microstructure of the as-prepared HA microspheres can be further modified by a controlled heat treatment at temperatures between approximately 600 and 900°C. Modification of the microstructure by heat treatment resulted in the ability to markedly influence the release of BSA from the microspheres into a PBS. Both the amount of BSA released as well as the duration of the release was influenced by the heat treatment. The microstructural changes produced by the heat treatment and, hence, the ability to control the BSA release kinetics, were dependent on the time–temperature schedule used in the heat treatment.

Upon heating the as-prepared hollow HA microspheres, surface diffusion presumably dominated at lower temperatures (e.g., approximately 600°C or below), resulting in rounding of the high surface area plate-like (or needle-like) particles present in the microspheres, as well as coarsening of the pores and particles (Fig. 3a–d). These processes are driven by the need of the system to reduce its surface free energy [26]. At these lower temperatures, there is rearrangement of the porosity but little or no reduction in the total porosity of the shell wall. At higher temperatures, other types of matter transport processes become more

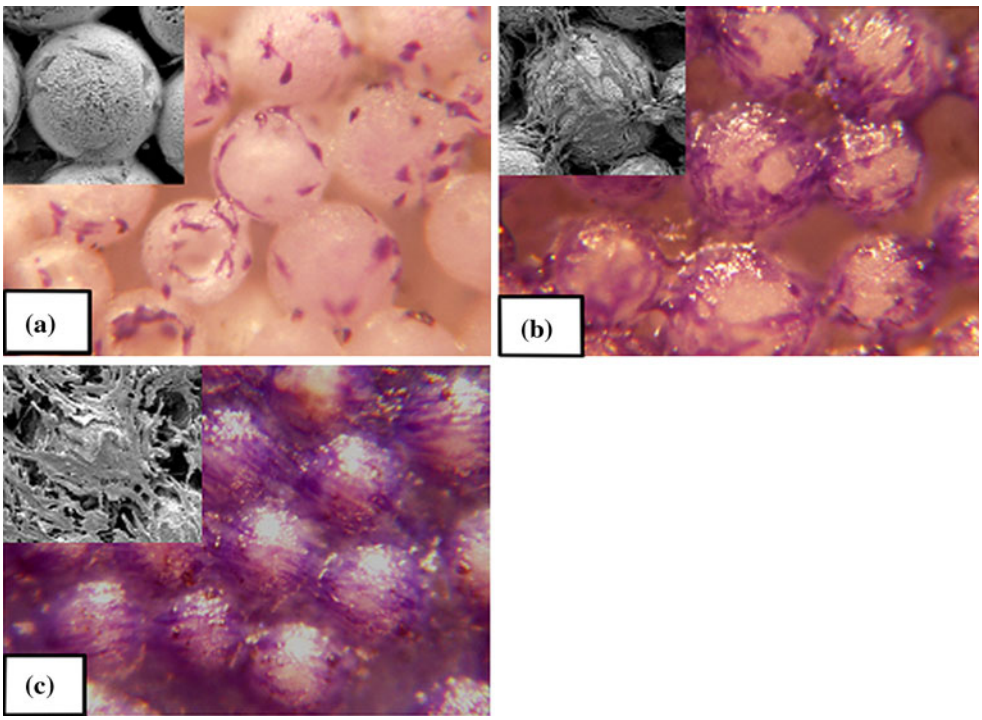

Fig. 5 Optical images and SEM images (*inset*) of MC3T3-E1 cell morphology on hollow HA microspheres (106–150 μm) after culturing for **a** 2, **b** 4, and **c** 6 days

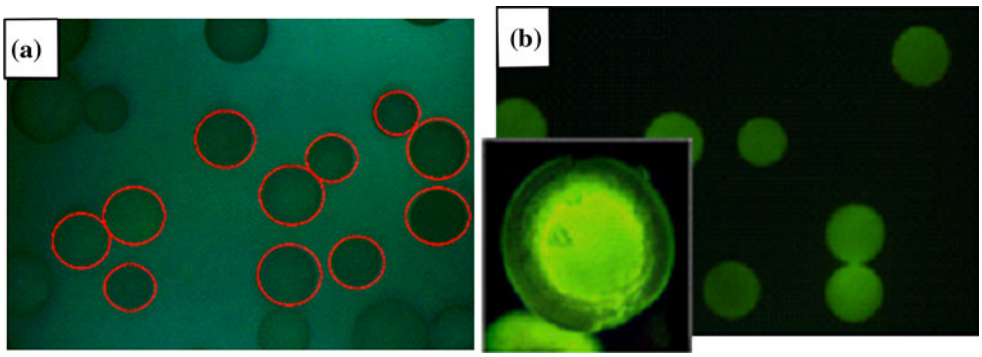

Fig. 6 Optical images of the surface of **a** as-prepared HA microspheres (brightness enhanced to show microspheres (*circles*)), and **b** HA microspheres loaded with fluorescent FITC-labeled BSA. (*Inset*: cross section of microspheres loaded with FITC-labeled BSA.) (Diameter of microspheres = 106–150 μm)

dominant, resulting in actual densification of the shell wall (reduction in the porosity). At 900°C, densification dominated to the extent that the shell wall became almost fully dense, although some porosity remained on the microsphere surface (Fig. 3e, f).

Using an FITC-labeled BSA, it was clearly shown that the hollow HA microspheres can be loaded with BSA by immersing the microspheres in a solution of BSA in PBS and applying a small vacuum to replace the entrapped air with the solution (Fig. 6). Furthermore, it was shown that the BSA loaded into the microspheres was distributed both in the pores of the shell wall and in the hollow core.

The release of BSA from the as-prepared HA microspheres into a PBS medium was rapid, and essentially ceased after 24–48 h (Fig. 7). The cumulative amount of BSA in the medium when the release of BSA ceased was ~ 22 μg/ml. Little release of BSA was found for the HA microspheres heated for 5 h at 900°C. As described earlier, SEM observation showed that the shell wall for this group of HA microspheres was dense (Fig. 3f), so presumably no BSA was loaded into the microspheres. The little BSA released was presumably due to BSA adsorbed on the surface of the microspheres.

Heating the as-prepared HA microspheres for varying times (1–24 h) at 600°C provided the most favorable conditions for manipulating the amount and duration of the BSA released from the microspheres (Fig. 8). Presumably, the transition from coarsening of the microstructure to

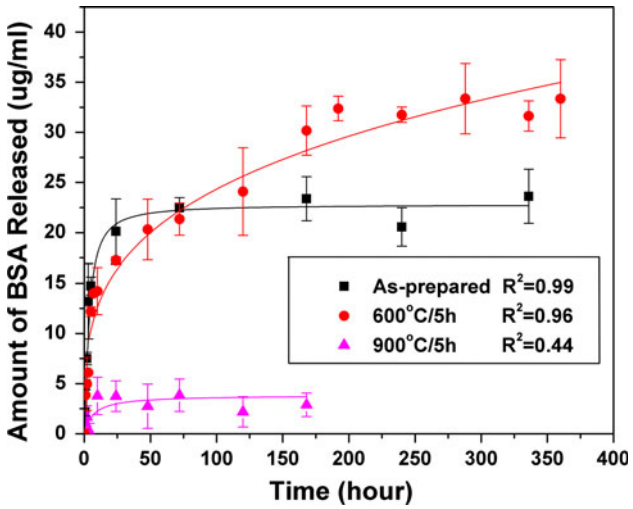

Fig. 7 Amount of BSA released from hollow HA microspheres into PBS, for the as-prepared HA microspheres, and after heat treatment under the conditions shown. (The R^2 value for each fitted curve is also shown.)

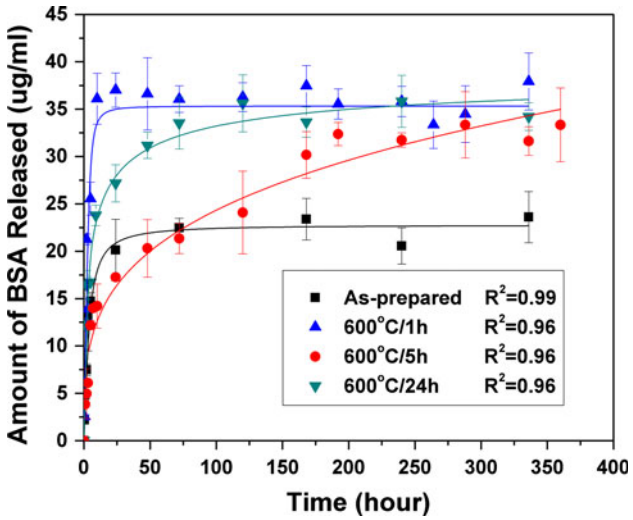

Fig. 8 Amount of BSA released from hollow HA microspheres heat treated at 600°C for the times shown (1–24 h). The data for BSA released from the as-prepared HA microspheres are also shown for comparison. (The R^2 value for each fitted curve is also shown.)

densification of the shell wall occurred above this temperature, and the heating time provided an additional variable to modify the microstructure. With the smallest heating time (1 h), the amount of BSA released at any time was far higher than that for the as-prepared HA microspheres, but the duration of release was approximately the same (24–48 h). Coarsening apparently resulted in a fairly homogeneous surface layer (Fig. 4a, b). The larger pores presumably allowed more BSA to be loaded into the microspheres, as well as faster release through the microsphere wall. When heated for a longer time (5 h), rearrangement of the porosity during the coarsening process

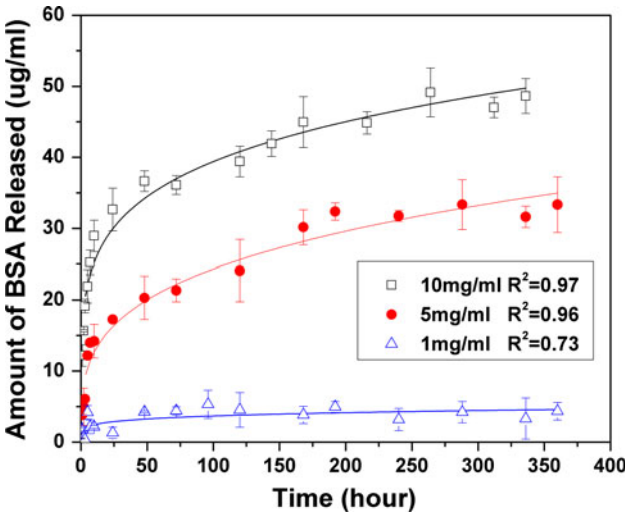

Fig. 9 Amount of BSA released from hollow HA microspheres heat treated at 600°C for 5 h, for different concentrations of BSA loaded into the microspheres. (The R^2 value for each fitted curve is also shown.)

presumably resulted in the formation of a less porous layer (Fig. 4d; arrow) within the coarsened surface layer. This resulted in a more sustained release of BSA, over a period of 7–14 days. With a longer heat treatment (24 h), the release of BSA from the microspheres was initially faster that for the microspheres heated for 5 h, but the duration of the release was shorter (4–5 days). For the 24 h heating, the microstructure of the surface layer of the microspheres (Fig. 4f) appeared to be a coarsened version of that for the microspheres heated for 5 h (Fig. 4d); this resulted in faster release kinetics because of the easier migration of the BSA molecules through the larger pores. Although the duration of the BSA release was different, Fig. 8 showed that the cumulative amount of BSA released (32–35 μg/ml) was approximately independent of the heating time at 600°C. The coarsening of the microstructure at nearly constant pore volume coupled with the small difference in surface area (Table 1) resulted in approximately the same amount of BSA loaded into the microspheres and released into the PBS.

The amount of BSA released from the hollow HA microspheres was also be varied by changing the concentration of BSA initially loaded into the microspheres (Fig. 9). At any time, the cumulative amount of BSA released into the PBS increased with the amount of BSA initially loaded into the microspheres. Furthermore, for BSA concentrations of 5 and 10 μg/ml loaded into the microspheres, the release kinetics showed the same trend, indicating that the mechanism of BSA release was the same for these two concentrations (Table 2).

As described earlier, release of BSA from the as-prepared or heat-treated HA microspheres effectively ceased within 1–14 days. Taking the amount released after

Table 2 Data for release of BSA from hollow HA microspheres into a medium of PBS

Sample	BSA loading (mg BSA/g HA)	BSA released (mg BSA/g HA)	BSA released/BSA loaded (%)	Duration (days)
As-prepared	50	22	44	1–2
600°C/1 h	126	35	30	1–2
600°C/5 h	119	35	30	7–14
600°C/24 h	115	32	30	4–5
700°C/5 h	19	2	–	<1
900°C/5 h	<5	2	–	<1

14 days as the final value, the data for the fractional amount of BSA released (α) as a function of time (t) were fitted by a least-squares regression analysis using software (Fig. 10). For the microspheres heated for 5 h at 600°C (Fig. 10a), the fractional release data could be well fitted by a power-law equation:

$$\alpha = kt^n \quad (1)$$

where k and n are constants, with the values $k = 0.26$ and $n = 0.25$ for this group (Table 3). The coefficient of determination, R^2, which represents the goodness of the fit to the data, was equal to 0.94. The fractional release data for different concentrations of BSA loaded into the microspheres (5 and 10 mg/ml) lie on the same curve, indicating that the release mechanism was independent of the concentration.

Figure 10b shows that the BSA release from the as-prepared HA microspheres and the microspheres heated for 1 h at 600°C could be well fitted by a different power-law equation:

$$\alpha = \frac{kt^n}{c + t^n} \quad (2)$$

where the constants $k = 0.96$; $c = 4.08$, and $n = 1.43$ ($R^2 = 0.95$). The fractional release data for these two groups lie on the same curve, indicating the same BSA release mechanism. Table 3 summarizes the constants in Eqs. 1 and 2 for the different groups of HA microspheres.

Equation 1 was introduced to describe the general release of drugs and other solutes by diffusion from non-swellable polymer devices in the shape of slabs, spheres, cylinders, and discs [27]. The exponent n is characteristic of the shape of the device, with $n = 0.43$ for spheres of the same size. In the present work, using hollow HA microspheres with an external diameter in the range 106–150 μm, which were heated for 5 h at 600°C, the release data can be well fitted by Eq. 1 but the value of the exponent ($n = 0.25$) was smaller than the predicted value of 0.43. In comparison, the BSA release from the as-prepared HA microspheres and the microspheres heated for 1 h at 600°C cannot be well fitted by Equation 1; this may indicate that the release is not controlled by a diffusion process, and that the release mechanism for these two groups of microspheres is different from that for the microspheres heated for 5 h at 600°C.

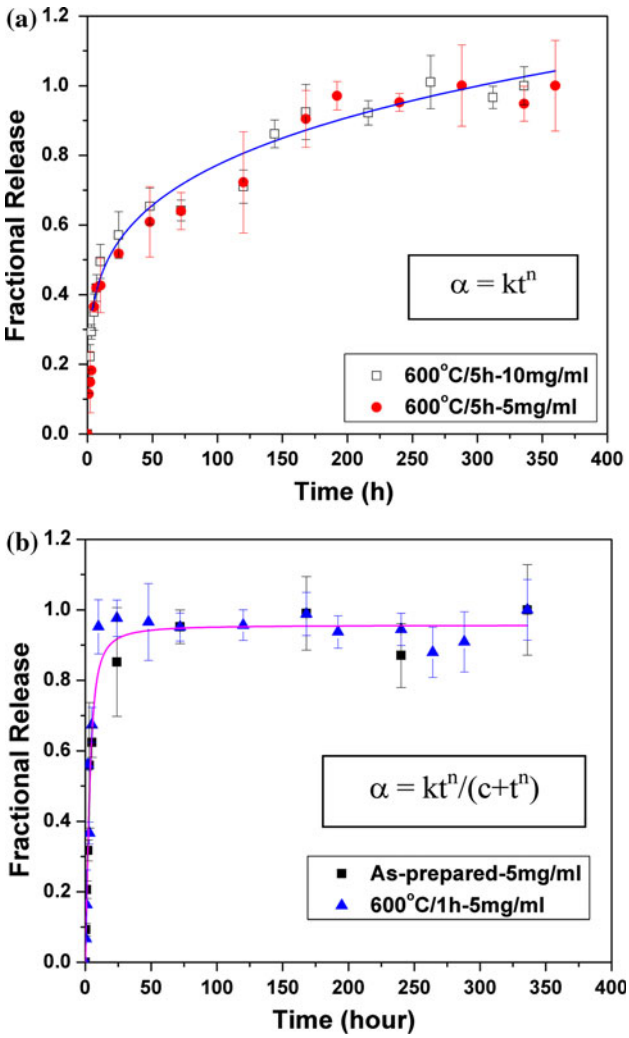

Fig. 10 Least-squares regression fit to the data for the BSA release (as a fraction of the final amount released) versus time: **a** Release from microspheres heated for 5 h at 600°C for two different BSA loading (5 and 10 mg/ml); **b** Release from the as-prepared HA spheres and the microspheres heated for 1 h at 600°C (BSA loading = 5 mg/ml)

Table 3 Parameters in equations used to fit the BSA release data from the as-prepared HA microspheres and the HA microspheres heat-treated HA at 600°C for the times shown

HA microspheres	Loading concentration of BSA (mg/ml)	Equation	Parameters
As-prepared	5	$\alpha = kt^n/(c + t^n)$	$k = 0.96$; $n = 1.43$; $c = 4.08$; ($R^2 = 0.95$)
600°C/1 h	5		
600°C/5 h	5	$\alpha = kt^n$	$k = 0.26$; $n = 0.25$ ($R^2 = 0.94$)
600°C/5 h	10		
600°C/24 h	5	$\alpha = kt^n/(c + t^n)$	$k = 1.20$; $n = 0.55$; $c = 2.40$; ($R^2 = 0.96$)

The amount of BSA loaded into the HA microspheres was determined from the final cumulative amount released into the PBS, plus the amount remaining in the microspheres. For the as-prepared HA microspheres, the total BSA loading was ~50 mg per gram of HA microspheres. The cumulative amount released was ~22 mg per gram of HA microspheres, indicating that ~40% of the total amount of BSA initially loaded into the HA microspheres was released into the PBS. In the case of the HA microspheres heated at 600°C, the total amount of BSA loaded into the HA microspheres was 115–126 mg per gram HA, of which ~35 mg (~30%) was released into the PBS.

The results described above showed that only 30–40% of the BSA initially loaded into the HA microspheres was released into the PBS. The factors that limit the release of larger amounts of BSA into the PBS are not clear. However, it should be recalled that loading of the BSA solution into the hollow HA microspheres was assisted by a small pressure gradient, resulting from the application of a small vacuum to the system to remove the air from within the hollow HA microspheres. On the other hand, the release of the BSA into the PBS was driven by the concentration gradient of BSA between the HA microspheres and the PBS. As the concentration gradient decreased with time, the release of BSA became slower, and eventually ceased.

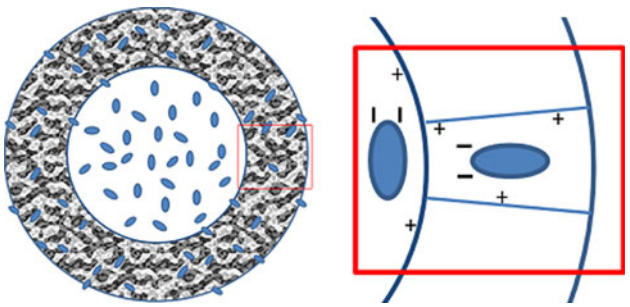

Fig. 11 Qualitative model illustrating the release of BSA from hollow HA microspheres into PBS medium. (*Left*): Cross section of HA microsphere showing BSA molecules (*ellipsoids*) located in the hollow core and mesoporous shell wall; (*Right*): Magnified view of idealized pore in the shell wall and BSA molecules (ellipsoids). At the pH value of the PBS (7.2), the HA surface should have a small positive (+) charge (isoelectric point = 7.4–8.0), while the BSA molecules should have a negative (−) charge (isoelectric point ≈4.7)

The structure (conformation) of the BSA molecule is the subject of some controversy, but based on hydrodynamic experiments, BSA is reported to take up the shape of an oblate ellipsoid with dimensions of 14 and 4 nm along the long and short axis, respectively [28]. The average pore size of the shell wall of the as-prepared HA microspheres was 13 ± 2 nm, approximately equal to the long dimension of the BSA molecules. As indicated above, during the loading step, the application of a small vacuum to the system produced a pressure difference between the surface and the interior of the hollow microspheres as the air was removed from the microspheres. This pressure difference, coupled with some alignment of the ellipsoidal BSA molecules during liquid flow, presumably facilitated the migration of the BSA molecules into the pores of the shell wall and into the hollow core.

After the microspheres are filled, release of the BSA into a surrounding PBS medium (Fig. 11) is controlled by their ability to diffuse through the pores of the microsphere wall down the BSA concentration gradient between the interior of the microspheres and the surrounding PBS medium. Interaction between the functional groups of the BSA and the pore surfaces of the hollow HA microspheres could also influence the BSA release. The pH value of the PBS was 7.2, while the isoelectric point of BSA is reported as 4.7, with a net surface potential of –18 mV at pH = 7.0 [28]. The isoelectric point of hydroxyapatite depends on a few factors, such as preparation method and composition, but it has been reported in the range of 7.4–8.0 [29]. Therefore, at pH = 7.2 (the pH of the PBS), the HA surface should be almost neutral or should carry only a small positive surface charge. Electrostatic interaction between the oppositely charged HA surface and the BSA molecules could enhance adsorption, limiting the amount of BSA released.

5 Conclusion

Hollow hydroxyapatite (HA) microspheres (106–150 μm) with a hollow core diameter equal to 0.6 the external diameter and a mesoporous shell wall were prepared by a

low temperature glass conversion route and evaluated as a device for controlled delivery of a model protein, bovine serum albumin (BSA). Both the hollow core and the mesopores of the shell wall were loaded with a solution of BSA. Release of the BSA from the as-prepared HA microspheres increased linearly time, and ceased after 24–48 h. The amount of BSA released from the microspheres and the duration of the release was varied by heat treating the as-prepared HA microspheres to modify their microstructure. For HA microspheres heated for 5 h at 600°C, 30–40% of the BSA initially loaded into the microspheres was released over 7–14 days. In general, the present results show promising potential for the application of these hollow HA microspheres as a device for controlled local delivery of proteins such as growth factors and drugs.

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