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Evaluation of BSA protein release from hollow hydroxyapatite microspheres into PEG hydrogel

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Abstract

Implants that simultaneously function as an osteoconductive matrix and as a device for local drug or growth factor delivery could provide an attractive system for bone regeneration. In our previous work, we prepared hollow hydroxyapatite (abbreviated HA) microspheres with a high surface area, mesoporous shell wall and studied the release of a model protein, bovine serum albumin (BSA), from the microspheres into phosphate-buffered saline (PBS). The present work is an extension of our previous work to study the release of BSA from similar HA microspheres into a biocompatible hydrogel, poly(ethylene glycol) (PEG). BSA-loaded HA microspheres were placed in a PEG solution which was rapidly gelled using ultraviolet radiation. The BSA release rate into the PEG hydrogel, measured using a spectrophotometric method, was slower than into PBS, and it was dependent on the initial BSA loading and on the microstructure of the microsphere shell wall. A total of 35–40% of the BSA initially loaded into the microspheres was released into PEG over ~14 days. The results indicate that these hollow HA microspheres have promising potential as an osteoconductive device for local drug or growth factor delivery in bone regeneration and in the treatment of bone diseases.

Keywords

Hollow hydroxyapatite microspheres; controlled release; bovine serum albumin; poly(ethylene glycol) hydrogel

1. Introduction

The regeneration of bone lost as a result of trauma, bacterial infection of prosthetic implants, and congenital diseases represents a common and challenging clinical problem. Local delivery of protein growth factors is often required for tissue regeneration [1,2], while local delivery of high doses of antibiotics is desirable in treating bone infections [3,4]. An ideal system for bone regeneration should simultaneously function as a controlled local delivery device for growth factors or drugs and as an osteoconductive matrix to support bone formation.

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Poly (methyl methacrylate) (PMMA) cement is widely used clinically as a carrier material for antibiotics in the treatment of bone infections [4], but PMMA is not biodegradable, and it provides a surface upon which secondary bacterial infection can occur. While collagen is the most widely used biodegradable carrier material for growth factors and antibiotics [5,6], the synthetic polymers, such as poly(lactic acid), PLA, and poly(glycolic acid), PGA, and their copolymers, poly(lactic co-glycolic acid), PLGA, have received considerable interest. In addition to being widely available, they can be prepared with well-controlled, reproducible chemical and physical properties [7–10]. They are also among the few synthetic biodegradable polymers approved by the U. S. Food and Drug Administration (FDA) for in vivo use.

Inorganic biomaterials have been investigated as alternatives to polymeric carrier materials in the repair of hard tissues because they can better mimic the physical and chemical properties of bone [11,12]. Calcium sulfate hemihydrate, $\text{CaSO}_4 \cdot 0.5\text{H}_2\text{O}$ (plaster of Paris, POP), has been widely used as a biodegradable carrier, but POP has a limited ability to stimulate bone regeneration, high resorption rate, rapid elution in vitro, and low mechanical strength [13–15]. The calcium phosphates such as hydroxyapatite (abbreviated HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and beta-tricalcium phosphate (β -TCP), $\text{Ca}_3(\text{PO}_4)_2$, are attractive because they can provide an osteoconductive matrix for bone regeneration plus a matrix to fill bone defects [16–18]. The delivery systems typically consist of porous particles, granules, or scaffolds in which the protein is adsorbed or attached to the surfaces of the porous material, or encapsulated within the pores [19–22]. A disadvantage of those systems is that the release rate of growth factors or antibiotics can be rapid. Inorganic organic composites are also receiving interest because they can take advantage of the properties of the different components to better control the release of growth factors and antibiotics [23–25].

In addition to the proven osteoconductivity of HA [26], carriers composed hollow HA microspheres with a mesoporous shell wall can provide advantages as a device for loading and release of proteins such as growth factors. (Mesopores are defined as pores of size 2–50 nm). The hollow core provides a reservoir for loading proteins, while the shell provides a structure to control the release of proteins by diffusion through the mesopores. Hollow HA microspheres typically have a higher strength and stiffness than hydrogels and some polymers. Consequently, the loaded protein cannot be easily squeezed out of the HA microspheres, which could result in a more controlled protein release profile in vivo. The preparation of hollow HA microspheres with a mesoporous shell wall by a glass conversion process near room temperature and the characterization of the microspheres have been described elsewhere [27–31].

Previously, we studied the effect of the process variables on the microstructure of hollow HA microspheres prepared by the glass conversion process [32], the ability to load a protein, bovine serum albumin (BSA) into the microspheres, and the release profile of the BSA into phosphate buffered saline (PBS) [33]. BSA was used a model protein because it is one of the most widely studied proteins and it is inexpensive when compared to growth factors.

This study is an extension of our previous work to evaluate the release of BSA from hollow HA microspheres into a biocompatible hydrogel. When compared to PBS, advantages of the hydrogel medium includes its structural similarity to the extracellular matrix (ECM) [34–36], and the potential for developing injectable implants composed of hollow HA microspheres dispersed in the hydrogel. Although it is not biodegradable, poly(ethylene glycol) (PEG) is used as model photopolymerizable hydrogel. Because of their nearly stoichiometric composition, the hollow HA microspheres did not show a measurable degradation rate in an aqueous medium (PBS). A key objective of the present study was to

measure the release profile of BSA from the HA microspheres into the PEG hydrogel and to compare the data with the BSA release from similar HA microspheres into PBS. HA microspheres loaded with BSA were placed in a PEG solution and following photopolymerization with UV radiation, the release of BSA into the PEG was determined as a function of time.

2. Experimental Procedure

2.1. Preparation and characteristics of hollow HA microspheres

The preparation of hollow HA microspheres by a glass conversion process is described in detail elsewhere [32]. Two groups of microspheres were used in this work: (1) as-prepared microspheres, formed by reacting borate glass microspheres (106–150 μm) with the composition 15CaO, 10.6 Li₂O, 74.4 B₂O₃ (wt%) (designated CaLB3-15) for 2 days in 0.02 M K₂HPO₄ solution at 37°C, followed by drying for at least 24 h at 90°C, and (2) heat-treated microspheres formed by heating the as-prepared HA microspheres for 5 h at 600°C. The glass microspheres (in the range 106–150 μm) used for preparing the hollow HA microspheres were obtained by sieving. While the size distribution of the microspheres was not measured, it may approximate to that of a log-normal distribution found for many milled and sieved particles. The characteristics of the two groups of microspheres are summarized in Table I. These two groups of microspheres were used because they showed a large difference in BSA release kinetics into PBS in our previous work [33].

For ease of measuring the BSA release in the present work, the hollow HA microspheres were lightly bonded at their contact points to form thin discs (4.5 mm in diameter $\times$ 1.5 mm) with a mass of 10 mg each. The discs were prepared by pouring the borate glass microspheres into a graphite die, heating the system for 1 h at 560°C to lightly bond the glass microspheres at their contact points, and reacting the disc under the conditions described above to convert the glass into hollow HA microspheres.

The macroporosity of discs composed of the borate glass microspheres was measured by the Archimedes method, using the procedure described in ASTM C 830. Mineral spirits (density = 0.752 g/ml; Sigma-Aldrich; USA) was used as the liquid instead of water to avoid surface degradation of the borate glass. Since there is no shrinkage of the borate glass microspheres during the conversion to HA, the macroporosity of the discs composed of hollow HA microspheres was the same as the measured macroporosity.

2.2. Loading of BSA into hollow HA microspheres

Fluorescein isothiocyanate-labeled bovine serum albumin, referred to as FITC-BSA (molecular weight = 66 kDa; Sigma-Aldrich, St. Louis, MO) was used in order to permit visual observation and spectrophotometric measurement of the BSA released from the disc of microspheres. Ten disks of hollow HA microspheres (mass = 10 mg each) were immersed in 2 ml of a solution consisting of 5 mg of FITC-BSA per ml of PBS. 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 discs loaded with FITC-BSA were washed with deionized water twice and dried in air at room temperature in the dark.

2.3. Measurement of BSA release profile from hollow HA microspheres into PEG hydrogel

Each disc of hollow HA microspheres, loaded with FITC-BSA as described above, was immersed in a solution (1 or 2 ml) of poly(ethylene glycol diacrylate), (molecular weight = 3.4 kDa; Laysan Bio Inc., Arab, AL) in a 12- or 24-well plate. The system was placed for 5 minutes under an ultraviolet lamp (365 nm; Glo-Mark Systems, NY) to crosslink the

polymer to form a gel (referred to as PEG hydrogel), sealed with a plate sealer, and stored at room temperature in the dark. At selected times, the samples were removed from the dark, and optical images of the fluorescent FITC-BSA were taken. The amount of FITC-BSA released into the PEG hydrogel, at specific distances from the circumference of the HA disc, was also measured in a BMG FLUORstar Optima plate reader using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Each well of a 12-well plate contained an HA disc in 2 ml PEG hydrogel; at each selected time, the amount of BSA at different positions in each well was measured in the BMG FLUORstar Optima plate reader using 96-well plate reading program. The concentration of FITC-BSA was determined from a calibration curve determined from measurements of the fluorescent intensity of PEG containing known concentrations of FITC-BSA.

2.4 Statistical analysis

All of the BSA release experiments were performed in triplicate, and the amount of BSA released at each time point was determined as a mean $\pm$ standard deviation (SD).

3. Results

3.1. Microstructure of HA microspheres

Figure 1a shows an optical image of a disc (4.5 mm in diameter $\times$ 1.5 mm) composed of hollow HA microspheres; an SEM image (Fig. 1b) shows that the HA microspheres were bonded at their contact points. The discs had a macroporosity of $34 \pm 3\%$, as determined by the Archimedes method. SEM images of the surface and cross section of the two groups of hollow HA microspheres used in this work, as-prepared and heat treated for 5 h at 600°C, are shown in Fig. 2. As-prepared, the surface of the microspheres consisted of a mesoporous structure of fine, plate-like (or needle-like) HA particles (Fig. 2a-inset), while the cross section (Fig. 2a) shows that the shell wall consisted of two distinct layers differing in porosity. The heat treatment for 5 h at 600°C did not produce a measurable change in the total porosity of the hollow HA microspheres, but resulted in coarsening of the particles and pores in the shell wall (Fig. 2b), presumably by a surface diffusion process [33]. The particles at the surface of the shell developed a more rounded morphology, with a size <50 nm (Fig. 2b; inset). Except for coarsening, little change in the microstructure of the inner layer of the shell wall was observed. As a result of the coarsening process, the surface area of the heat-treated microspheres was significantly smaller than that of the as-prepared microspheres. The characteristics of the as-prepared and heat-treated HA microspheres used in this work are presented in Table 1.

3.2. Release of BSA from hollow HA microspheres into PEG hydrogel

Figure 3 shows the BSA release profile from the HA microspheres loaded with FITC-BSA into a surrounding medium of PEG hydrogel, for the as-prepared and heat-treated HA microspheres. The figure also shows a schematic diagram of the HA microspheres (disc) and the position (center of the hydrogel) at which the BSA concentration was determined. Initially, for the first 4–5 days, there was no difference in the amount of BSA released from both groups of HA microspheres. After this initial period, the BSA released from the as-prepared microspheres slowed considerably, and almost ceased after 5–7 days. The total amount of BSA released was $\sim 25 \mu\text{g}$ per ml of PEG. In comparison, release of BSA from the heat-treated microspheres continued up to ~ 14 days, after which time it slowed markedly. The total amount of BSA released after 14 days was $\sim 37 \mu\text{g}/\text{ml}$.

Optical fluorescent images, taken at different times, of the PEG medium surrounding a disc of heat treated HA microspheres loaded with FITC-BSA are shown in Fig. 4. The intensity of green color is an indication of the amount of the FITC-BSA released. As shown, little

fluorescence can be observed in the first 1–2 h, but the intensity of the green color became very noticeable after 3–4 h, and increased at longer times. The images provide visual evidence for the release of FITC-BSA from the HA microspheres into the PEG hydrogel.

The amount of BSA released into the PEG at three different distances from the edge of the HA disc is shown in Fig. 5 as a function of time. The disc was composed of the heat-treated HA microspheres, and the BSA concentration in the PEG hydrogel was measured near the edge of the disc (position A); 4.5 mm from the edge (B), and 13.5 mm from the edge (C). The BSA concentration at these 3 positions showed a markedly different dependence on time. Near the edge of the disc (A), the BSA concentration in the PEG increased rapidly in the first 2 days, remained nearly constant ($\sim 38 \mu\text{g/ml}$) during days 2–5, then decreased gradually in the next 3 days to $\sim 35 \mu\text{g/ml}$, and remained nearly stable at this concentration for up to 2 weeks. At position B, the BSA concentration increased continuously, with a decreasing slope, and reached a value of $\sim 32 \mu\text{g/ml}$ by day 14. The BSA concentration at position C initially increased far more slowly than that at B in the first 2–3 days, but then showed an almost linear increase with time in the next 8–10 days, after which the concentration increased slowly to $\sim 33 \mu\text{g/ml}$ by day 14.

4. Discussion

The present system, consisting of BSA-loaded hollow HA microspheres dispersed in a hydrogel, can provide an approach for controlled local delivery of proteins and an osteoconductive matrix in a single device. The *in vitro* results obtained in the present study for BSA release from the hollow HA microspheres in a PEG hydrogel show promise for the potential use of these hollow HA microspheres in bone repair applications.

When compared to the results from our previous work for the release of BSA from similar HA microspheres into PBS [33], the present results for BSA release into the PEG hydrogel showed similarities and differences (Fig. 3). For the same group of HA microspheres, the BSA release into the PEG was far slower initially. However, later in the process when the release essentially ceased, the total amount of BSA released into the PEG was approximately the same as that into PBS. For example, the BSA release from the as-prepared HA microspheres into PBS increased rapidly and ceased within 1–2 days. In comparison, the release into the PEG increased more slowly and ceased after ~ 7 days. The total amount of BSA released into the PEG ($\sim 25 \mu\text{g/ml}$) was approximately the same as that released into PBS. For the heat-treated HA microspheres ($600^\circ\text{C}/5\text{h}$), the BSA release into the PEG was initially slower than that into the PBS; however, after ~ 7 days the release into the PEG continued to increase at approximately the same rate as that into the PBS. The total amount of BSA released from the heat-treated microspheres into the PEG or the PBS was $\sim 37 \mu\text{g/ml}$.

Because of the ease of diffusion in a liquid, the release of the BSA into the PBS is expected to be controlled by diffusion through the mesoporous shell wall of the HA microspheres and presumably, by desorption from the pore surface of the HA microspheres. In comparison, the BSA release into the PEG is expected to be controlled by the diffusion/desorption process present in the PBS system as well as by diffusion through the PEG hydrogel. The relationship between the diffusion coefficient of a solute in a gel (D_g) and the diffusion coefficient of the solute in a liquid (D_l) has been described by the equation [37]:

$$D_g/D_l = \left(1 - \frac{r_s}{s}\right) \exp \left[-y \left(\frac{\varphi}{1-\varphi} \right) \right] \quad (1)$$

where r_s is the radius of the solute, ζ is the scaling correlation length between crosslinks, $y > 0$ is a parameter related to the critical volume required for a successful translational movement of the solute molecule and the average free volume per molecule of the liquid, and ϕ ($0 < \phi < 1$) is the volume fraction of polymer in the hydrogel. Since the solute transport within a hydrogel occurs primarily within the water-filled regions delineated by the polymer chains, ζ must be larger than r_s to provide enough space for the movement of the solute, that is $r_s/\zeta < 1$, and $0 < (1 - r_s/\zeta) < 1$. The term $\exp[y \phi/(1 - \phi)]$ is always less than 1 since $y > 0$ and $0 < \phi < 1$. Therefore, Equation (1) predicts that $D_g/D_1 < 1$, and the diffusion rate in the PEG hydrogel is always smaller than the diffusion rate in the PBS solution.

Since the release of the BSA into the PEG hydrogel is controlled by diffusion through the mesoporous shell wall of the HA microspheres and by diffusion through the PEG, the amount of BSA released into the PEG should depend on the distance from the HA disc as well as the time. Figure 5 shows that release of BSA close to the edge of the HA disc was initially rapid, reaching a value of $\sim 35 \mu\text{g/ml}$ after day 1; however, the total amount of BSA released at this position changed by less than $\pm 5\%$ between day 1 and day 7, and remained nearly constant at longer times. This indicated that after day 1, the amount of BSA released near the edge of the HA disc is approximately constant. Therefore, after day 1, the diffusion away from the edge of the HA disc is approximately balanced by the diffusion of BSA out of the HA microspheres. In comparison, the amount of BSA at positions B and C increased with time. This is because of the time taken for the BSA to diffuse from A to B and C down the concentration gradient. At position B (4.5 mm from the edge of the HA disc) the amount of BSA increased approximately as $t^{1/2}$, where t is the time, while the amount of BSA at position C (13.5 mm from the edge of the HA disc) increased approximately linearly with t .

As described above, after day 1 the BSA concentration near the edge of the disc of hollow microspheres (position A) remained approximately constant as a function of time (Fig. 5), while the concentration at position B (4.5 mm from the edge of the disc) and position C (13.5 mm from the edge of the disc) increased with time as the BSA molecules diffused down the concentration gradient (from A to B and C). The diffusion coefficient of the BSA molecules in the PEG hydrogel can be estimated from the measured time-dependent concentrations at B and C. By approximating the system to a semi-infinite source with a constant concentration C_0 for $x < 0$ in contact with a semi-infinite system, such that $C = 0$ for $x > 0$ at $t = 0$, the solution to Fick's second law is [38]:

$$C = C_0 \operatorname{erfc} \left[\frac{x}{2(Dt)^{1/2}} \right] \quad (2)$$

where D is the diffusion coefficient and erfc is the error-function complement. By plotting the argument of the complementary error function, $\operatorname{erfc}^{-1}(C/C_0)$, versus x , D is found from the slope $= 1/[2(Dt)^{1/2}]$. Figure 6 shows the plots for $t = 48 \text{ h}$ and $t = 96 \text{ h}$, from which D was found to be $1.7 \text{ mm}^2/\text{h}$ (48 h) and $2.3 \text{ mm}^2/\text{h}$ (96 h).

In the present study, the hollow HA microspheres were bonded at their contact points into small discs (Fig. 1) for ease of manipulation and for ease of measuring the BSA release from the microspheres into the PEG hydrogel by a spectrophotometric method. However, it is expected that the loose microspheres will also provide an attractive system for biomedical applications. For example, loose HA microspheres loaded with a growth factor can be packed into a bone defect to regenerate bone by osteoconduction and osteoinduction. In addition, the HA microspheres can fill the defect and integrate with new bone. Growth factor-loaded HA microspheres can also be dispersed in a biodegradable hydrogel, injected into a bone defect, and hardened in situ by ultraviolet radiation or by thermal methods.

The present in vitro study showed that these hollow HA microspheres can serve as a device for controlled delivery of BSA into a PEG hydrogel. However, it is not clear whether the duration of the release achieved for BSA (~7 days for the as-prepared HA microspheres and ~14 days for the heat-treated microspheres) is applicable for bone regeneration. The release profile will depend on the molecular weight and composition of the growth factor to be used. However, additional methods, such as coating the protein-containing HA microspheres with a biodegradable polymer, could be used to increase the duration of release and to control the release profile.

Our recent work has been investigating the release of actual growth factors such as transforming growth factor-beta1 (TGF- β 1) and bone morphogenetic protein-2 (BMP-2) from the HA microspheres in vitro, and the capacity of the growth factor-loaded HA microspheres for repairing bone defects in rat calvarial defects. The results showed that 6 or 12 weeks postimplantation, the hollow HA microspheres loaded with TGF- β 1 enhanced bone regeneration when compared to the hollow HA microspheres without TGF- β 1 [39]. More recent results have shown that the hollow HA microspheres had a greater capacity to enhance bone regeneration when loaded with BMP-2 instead of TGF- β 1. These results for the BMP-loaded HA microspheres will be reported in a future publication.

5. Conclusion

In vitro evaluation of BSA release from hollow hydroxyapatite (HA) microspheres into a PEG hydrogel provided experimental support for the potential use of the HA microspheres as an osteoconductive device for controlled local delivery of proteins such as growth factors and drugs. The BSA release into the PEG hydrogel was slower than the release from similar HA microspheres into PBS. However, the final amount of BSA released into the PEG was approximately the same as that released into PBS. The BSA release profile into the PEG was dependent on the microstructure of the shell wall of the HA microspheres. Approximately 40% of the BSA initially loaded into the HA microspheres was released from the as-prepared HA microspheres after ~7 days when release of the BSA ceased. In comparison, BSA release from heat-treated HA microspheres (600°C/5h) increased continuously, and reached a total value of 37% after 14 days.

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Highlights

- Novel multifunctional system, hollow hydroxyapatite (HA) microspheres, was evaluated
- System can simultaneously provide osteoconductivity and local protein delivery
- HA microspheres showed sustained release of a protein into an ECM-like medium
- HA microspheres have promising potential for use in bone regeneration

Fig. 1. Microstructure of thin disk composed of hollow HA microspheres bonded at their contact points. (a) optical image of thin disk with hollow HA microspheres; (b) higher magnification SEM image showing the hollow HA microspheres bonded at their contact points.

Fig.2. SEM images of the cross section and surface (*insert*) of the shell wall of hollow HA microspheres: (a) as-prepared; (b) heated for 5 h at 600°C.

Fig. 3.

Release of BSA from a disc of hollow HA microspheres into PEG hydrogel as a function to time measured at the position indicated, approximately 4.5 mm from the edge of the disc. For comparison, the BSA release from similar HA microspheres into phosphate-buffered saline (PBS), taken from Ref. 33, is also shown (dashed lines).

Fig. 4. Optical images of BSA released from hollow HA microspheres (600°C/5 h) into PEG hydrogel at different times. The intensity of the green fluorescence is an indication of the concentration of BSA released.

Fig. 5.

Amount of BSA released from disc of hollow HA microspheres (600°C/5 h) into PEG hydrogel at the different positions shown (A: near the edge of the HA disc; B: 4.5 mm from the A position; C: 13.5 mm from the A position).

Fig. 6.

Plot of the argument of the complementary error function, $argerfc(C/C_0)$, vs. distance (x) from the edge of the HA disc, for the BSA release data from the disc of HA microspheres ($600^\circ\text{C}/5\text{ h}$) into PEG hydrogel after $t = 48\text{ h}$ and $t = 96\text{ h}$. The diffusion coefficient for the BSA molecules in the hydrogel was determined from the slope of each plot. (The coefficient of determination, R^2 , is shown for each plot.)

Table 1

Characteristics of as-prepared hollow HA microspheres formed by reacting $\text{Li}_2\text{O}-\text{CaO}-\text{B}_2\text{O}_3$ glass microspheres (106–150 μm) in K_2HPO_4 solution, and after heat treatment under the temperature/time conditions shown. The ratio of hollow core diameter to microsphere diameter, d/D , specific surface area, and rupture strength are shown.

Sample	d/D	Surface area (m^2/g)	Rupture strength* (MPa)
As-prepared	0.61 ± 0.03	102 ± 2	11 ± 6
600°C/5 h	0.62 ± 0.03	19.0 ± 0.4	19 ± 11

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