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Development of porous spherical hydroxyapatite granules: application towards protein delivery

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A new method for the preparation of porous spherical hydroxyapatite granules is reported. It may be clinically applied towards orthopaedic or maxillofacial surgery as fillers or packing materials, and as biological chromatography supports. Its application towards delivery of macromolecules or protein drugs is discussed utilizing human serum albumin (HSA) as a model protein.

1. Introduction

Hydroxyapatite (HA) is the major component of the skeletal tissue of vertebrates. Studies have shown that synthetic HA is totally biocompatible, non-toxic and osteoconductive [1]. HA-based bone graft materials have considerable potential as bone substitute in medical and dental treatments. HA is also an attractive material for high performance liquid chromatography (HPLC) [2, 3]. Several forms of HA ceramic have been clinically and experimentally used: solid and porous blocks, and solid and porous particulate. Recently the particulate form has received much attention from the research field as well as from industry. Various particulate products are commercially available and are widely used as fillers or packing materials. They are manufactured and sold primarily for the oral surgery market. The different forms of particulate HA include irregular multifaceted particles and rounded smooth particles, with solid or porous structure. They are osteoconductive and help ingrowth and attachment of bone [4, 5], and are clinically used in oral surgical procedures to augment the alveolar ridge [6] and in periodontal repair [7]. Kent *et al.* [8] reported improved denture stability and retention when dense particulate HA has been used for augmentation of atrophic mandibular and maxillary alveolar ridges. However, irregular morphology caused inflammatory reactions [9], and also bone formation was slower compared to smooth particles.

A number of studies reported the development of granulates by hydrothermal reaction of corals [10], by crushing sintered blocks, by means of vibration and rolling [11], by dripping [12] and drip casting [13] procedures, etc., with irregular or spherical geometry. In this investigation, a new method to produce porous spherical particulate form of HA is developed, utilizing chitosan as a binding agent. This particulate form could be designed for a wide range of biomedical applications such as fillers, delivery of antibiotics [14] and macromolecules or contraceptives, and as a matrix for haemoperfusion [15] or (HPLC), etc. The preparation and characterization of spherical particulate are reported and its application towards the sustained delivery of macromolecular drugs is demonstrated utilizing human serum albumin (HSA) as a model protein.

2. Materials and methods

Hydroxyapatite powder was prepared from analytical grades $Ca(OH)_2$ and H_3PO_4 by precipitation method similar to that reported earlier at a reaction temperature of 100 °C [16, 17]. Chitosan is a biopolymer, a deacetylated product of chitin. Biomedical grade chitosan (degree of deacetylation, 86.98%; viscosity average molecular weight, 6.9×10^5 Da; intrinsic viscosity, 5.77 mPa s) was obtained as a gift from Central Institute of Fisheries Technology, Cochin, India. Glutaraldehyde (25% aqueous solution), sorbitan monooleate (Span 80), and human serum albumin (HSA, Fraction V) was from Sigma Chemical Co., USA. Poly(DL-lactic acid) (PLA, M_w 100 000) was from Polysciences, USA. Liquid paraffin (heavy; viscosity 0.009 Pa s at 30 °C and light; viscosity 0.0018 Pa s at 30 °C) was obtained from SD Fine Chem, Bombay, India. Solvents such as acetic acid, petroleum ether and acetone were of analytical grades.

2.1. Preparation of hydroxyapatite spherical particles

Fine HA powders 20 g, was mixed with 40 g of 2% (w/v) chitosan solution in 2% (v/v) acetic acid. Well-mixed HA/chitosan slurry was dispersed in 500 ml of dispersion medium, which was a mixture of heavy and light liquid paraffin and 0.4 mg of span 80 as stabilizing agent in a 1000 ml reaction flask by stirring at 400 r.p.m. with a halfmoon paddle stirrer. After about 5 min, required amounts of glutaraldehyde were added to harden the spheres obtained. After 30 min spheres were filtered,

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washed with acetone and petroleum ether, and dried at 60 °C for 3 h. These chitosan-bonded, spheres were heated initially at 500 °C for 1 h, for burning off chitosan and finally sintered at 1100 °C for 1 h. These particles were thoroughly washed in distilled water to remove any partially burned-off chitosan and finally heated at 200 °C for 24 h.

Particle size analysis was carried out by fractionation using standard test sieves.

2.2. X-ray diffraction (XRD) and infrared spectroscopy (IR)

HA spheres were finely powdered and dispersed in KBr powder and the pellets analyzed using an IR spectrophotometer (Nicolet, Impact 400D). The diffraction profiles of this powder were recorded using a powder X-ray diffractometer (PW 1710, Philips, Cu radiation, 15 mA, 40 kV).

2.3. Microscopy

Spheres were observed and photographed using a scanning electron microscope (SEM) (S 2400, Hitachi, Japan) and an optical microscope (Labophot, Nikon, Japan).

2.4. Calcium/Phosphate (Ca/P) ratio

Finely powdered spheres (1 g) were dissolved in minimum quantity of 1 N HCl (approximately 5 ml) and made up to 100 ml using distilled water. Two point five milliliters of this solution was made up to 100 ml using distilled water to make the concentration of calcium and phosphorus, similar to that in blood. The amounts of calcium and phosphorus in the final solution were estimated using reagent kits (Autopak, Baroda, India) by standard procedures employing *o*-cresophthalein complexone [18] and ammonium molybdate [19], respectively. Ca/P ratios were calculated from this.

2.5. Determination of pore volume

Dry spheres were placed in distilled water ($d = 1.0\,\mathrm{g\,cm^{-3}}$) and in ethanol ($d = 0.810\,\mathrm{g\,cm^{-3}}$) at 30 °C. The solvents were allowed to penetrate into the spheres for 24 h under reduced pressure. The spheres were removed from the solvents and the surface of the spheres was lightly wiped with filter paper and reweighed. The solvent accessible volume or the internal pore spaces in the spheres were estimated using the formula

$$V_p = \frac{(w_2 - w_1)}{w_1 d}$$

where w_1 is the weight of empty spheres, w_2 the weight of solvent filled spheres and d the density of the solvent.

2.6. Solubility testing

The dissolution of HA spheres in physiological conditions was investigated after immersion of HA samples in a balanced salt solution. HA spheres (2 g) of uniform size (400–500 μm diameters) were placed in 50 ml of calcium-free Hank's balanced salt solution (HBSS), pH 7.4. These were subjected to ultraviolet light for a period of 16 h in an attempt to subdue any ensuing bacterial proliferation. Calcium content of the medium was estimated at one day intervals for one week. The pH of the medium was maintained at 7.4.

2.7. Protein loading into HA spheres

Empty spheres (200–400 μm) were placed in albumin solution (2 to 5 g%) under the vacuum at 0 °C and allowed to stand overnight. This was evaporated to dryness at subzero temperature under the vacuum in a freezer. To modulate the release rate, albumin loaded HA spheres were coated with poly (DL-lactic acid) (PLA). For this 1 g of albumin-loaded spheres was added to 1 ml of PLA solution (10% w/v in dichloromethane) and mixed using a shaker for 2 min, and the spheres separated by vacuum filtration and dried at 0 °C under reduced pressure. This was stored in vials under reduced pressure at 0 °C.

2.8. *In vitro* protein release studies

The release of albumin from the microspheres was carried out in simulated body fluid (SBF, pH 7.4) at 37 °C. SBF was prepared fresh in the laboratory with the composition of NaCl, 6.51 g; KCl, 0.35 g; $CaCl_2$, 0.28 g; KH_2PO_4, 0.16 g; $MgSO_4$, 0.29 g; $NaHCO_3$, 1.40 g, and Na_2HPO_4, 1.85 g in 1 l distilled water and pH adjusted to 7.4. One hundred milligrams of PLA-coated albumin-loaded HA spheres was introduced into 50 ml of SBF in a screw-capped bottle under sterile conditions. Aliquots of 1 ml were withdrawn at various time intervals and albumin content was estimated by noting the optical density in a UV spectrophotometer (UV 160A, Shimadzu) at 270 nm. An equal amount of SBF was added in order to maintain a constant volume.

3. Results and discussion

3.1. Particle morphology and size distribution

The method reported here seems to be suitable for the preparation of porous spherical HA particles ranging from 212 to 1000 μm in diameter. Spheres were prepared by varying the mixing ratio of high and low viscosity paraffin oil. The particle size distribution of HA spheres obtained by varying the oil mixing ratios are given in Fig. 1. High viscosity oil alone gave smaller spheres whereas low viscosity oil alone gave spheres of bigger sizes; about 85% were above 500 μm. Microspheres prepared in heavy oil with stirring speed of 1000 r.p.m. gave particles less than 100 μm which could be suitable for chromatographic column supports. Different commercial grades of HA granules are available, indicated for a wide application of general dental, periodontal, and oral/maxillofacial surgical procedures, including augmentation, fill-in and repair. Some examples are INTERPORE® 200 granules (425–1000 μm diameter), PRO OSTEON® 500 granules (1–9 mm), Osteograf/N® (225–400 μm),

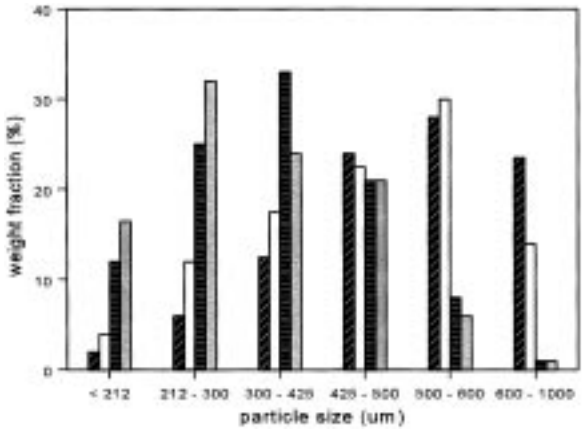


Figure 1 Particle size distribution of HA spheres prepared in paraffin liquid with various oil mixing ratios: ■, heavy : light (1 : 1); □, (3 : 1); ▬, (7 : 1); and ▭, heavy oil only.

OSTEOGEN® (300–1000 μm) and BIO-OSS®. Granules with an average overall diameter of 425 to 600 μm are recommended for periodontal applications and with an overall diameter of 425 to 1000 μm for oral surgical applications [20], and 300–400 μm for alveolar ridge augmentation.

HA spheres prepared had an overall spherical geometry as evidenced by Figs 2 and 3. These spheres have an irregular porosity ranging from 1 to 30 μm. The surface morphology of HA spheres before and after heat treatment is shown in Fig. 4. Before heating each individual HA particle was surrounded by chitosan and

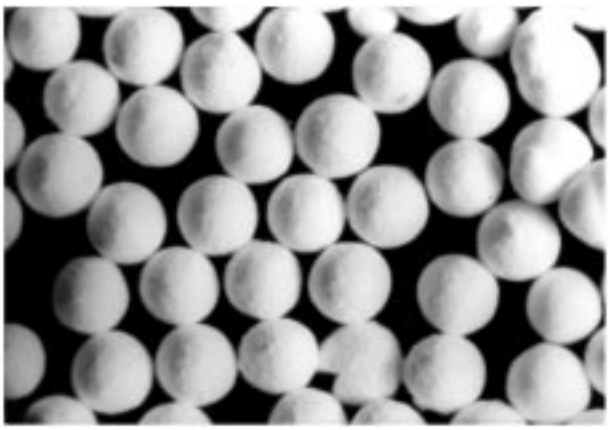


Figure 2 Optical micrograph of HA spheres (300 μm diameter).

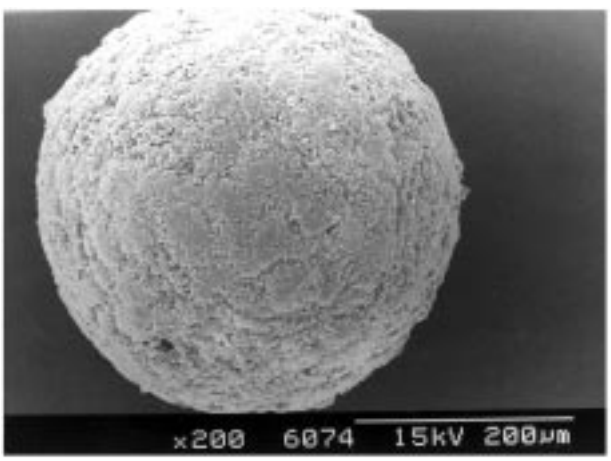
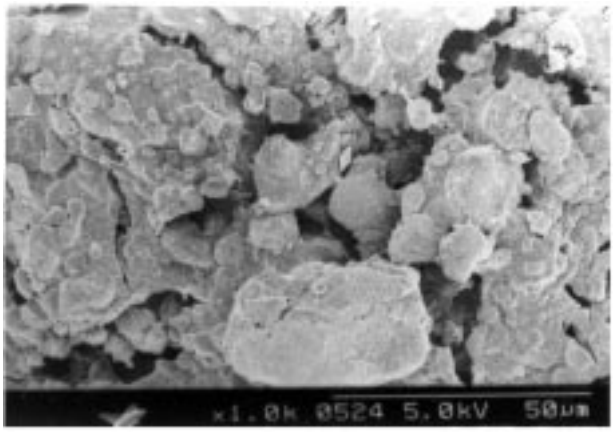
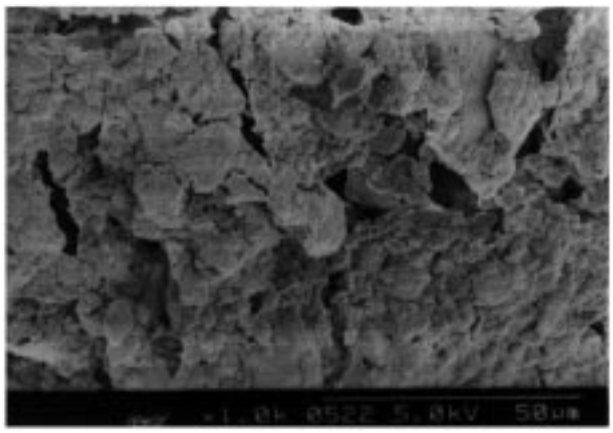


Figure 3 SEM micrograph of HA sphere.



(a)



(b)

Figure 4 SEM micrograph of HA spherical particle surface: (a) before heat treatment, (b) after heat treatment.

this is evident by its smooth texture (Fig. 4a). After heat treatment chitosan was burned off and HA crystals were visible (Fig. 4b). It is known that surface texture [21], and implant shape [9, 21] affect tissue response. Smooth and round particles induce a less favorable reaction than rough and angular materials. It has been reported that significantly faster ingrowth of bone was observed with rounded granules than polygonal-shaped granular material [22]. Rounded particles seem to be clinically more suitable for implantation in the oral cavity [9], especially in stress-bearing areas such as the mandibular alveolus. Spherical particles were preferred because of their unique packing characteristics. Spheres, when packed together, form a matrix with uniform pores between particles and this configuration promotes efficient conduction of bone from particle to particle [23].

3.2. XRD and IR studies

X-ray diffraction analysis indicated that the particles did not contain any distinguishable crystalline impurity, and the diffractogram shown in Fig. 5 matched the standard pattern for hydroxyapatite (JCPDS file number 9-432).

IR spectra of HA sphere obtained from KBr pellets containing fine powder is shown in Fig. 6 (b). The PO_4 bands were at 476, 570, 603, 961, 1049 and 1090 cm^{-1}. The sharp bands at 3579 and 633 cm^{-1} were the vibrations from OH. There was a sharp peak at 1648

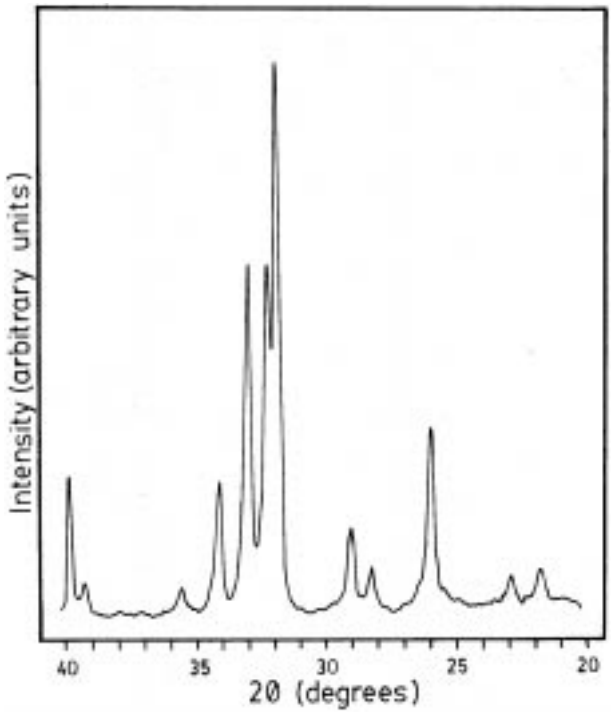


Figure 5 X-ray diffraction pattern of HA sphere.

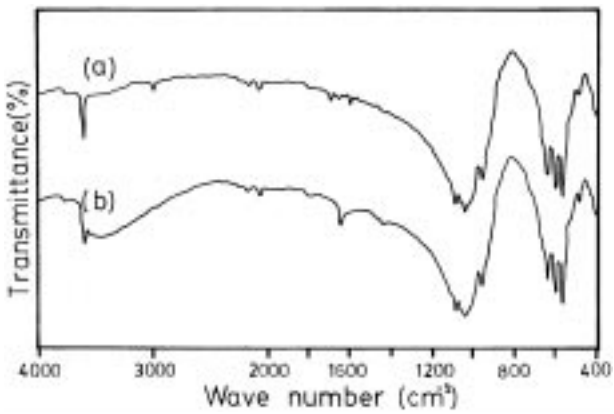


Figure 6 IR transmission spectra of (a) calcined starting powder (1100 °C, 1 h), (b) powdered HA sphere.

and a broad band near 3400 cm^{-1} which are an indication of adsorbed water. The adsorbed water gave bands near 3400 cm^{-1} [24, 25]. High purity of HA spheres are confirmed from X-ray diffraction and IR studies.

3.3. Ca/P ratios and pore volume

The most thermally stable HA was prepared with nearly stoichiometric mixing ratios from 1.68 to 1.70 [16]. A similar mixing ratio was followed for preparing the HA powder used for preparing the spheres. Ca/P ratios estimated and pore volumes of spheres are given in Table I. Ca/P ratios were almost equal to that of the theoretical value of 1.67. To estimate the solvent accessible pore volume in HA spheres the beads were placed in distilled water and ethanol. Both showed similar results. Pore volume decreased with increasing calcination temperature and duration of treatment. From SEM studies it was evident that the internal pores were not uniformly distributed. Also there was no significant variation in pore volume of spheres having different particle size, as evidenced in Table II.

3.4. Solubility of HA spheres

The dissolution of calcium phosphate ceramics is governed by its crystallinity, chemistry, density, secondary phases and environment (pH) [26]. The results of the analysis for calcium release performed on the Ca-free HBSS after HA samples had been in solution for 7 days, are plotted graphically in Fig. 7. This indicated that dissolution of HA microspheres were in fact occurring but comparatively much less than HA powder. An equilibrium of Ca^{+} ion concentration was achieved with all samples within 7 days. Similar results were reported for various commercially available HA particulate [27] and HA coatings [26]. The dissolved Ca^{2+} may combine with carbonate ions in solution and precipitate onto the spheres, and this carbonated HA will significantly increase the stability of HA. It has been reported that stability of carbonated HA is significantly high, and also HA is stable in water vapor atmosphere upto a temperature of 1200 °C [28].

3.5. *In vitro* HSA release

Porous HA granules can be utilized towards the delivery of macromolecules, protein drugs or polypeptides. Protein drugs, when encapsulated, may denature within the polymer matrix [29] causing a loss of biological activity and possible changes in immunogenicity. This has been caused mainly by the interaction between the drug and the polymer or the solvent used and also the

TABLE I Calcium/phosphate (Ca/P) ratio and pore volume of HA spheres (300–500 μm diameter) prepared in liquid paraffin having different oil ratio

Oil ratio (heavy : light)	Calcination temperature (°C) & duration (h)	Ca/P ratio	Pore volume (ml g^{-1})	
			Ethanol	Distilled water
1 : 0	1100, 1	1.685	0.3896	0.3912
0 : 1	1100, 1	1.697	0.3914	0.3817
1 : 1	1100, 1	1.671	0.3886	0.3847
3 : 1	1100, 1	1.681	0.3992	0.3916
7 : 1	900, 1	1.699	0.5678	0.5003
7 : 1	1100, 1	1.669	0.3923	0.3811
7 : 1	1100, 4	1.678	0.2732	0.2619
Starting powder	1100, 1	1.692	—	—

TABLE II Pore volume of HA spheres of varying particle size (using distilled water as solvent)

Particle size (μm)	Pore volume (ml g^{-1})
< 212	0.3724
212–300	0.3689
300–425	0.3823
425–500	0.3794
500–600	0.3866
600–710	0.3935
710–1000	0.4103

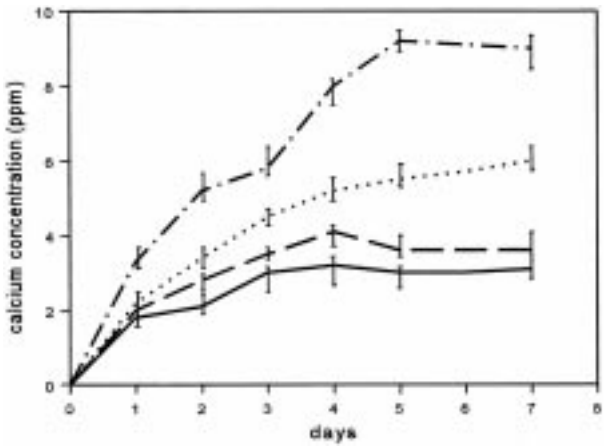


Figure 7 Dissolution of HA sphere in HBSS; –·–·– green powder, ... calcined powder, – – – – HA sphere (1100 °C, 1 h), ——— HA sphere (1100 °C, 4 h).

temperature involved. Also, the degraded by-products may cause side effects reducing the intended effect of the drug. However, hydroxyapatite is a biocompatible material, is used as a matrix for the purification of proteins itself, which do not seem to cause any side-effects, shown to be non-toxic [17, 30] and non-carcinogenic [30]. Thus, HA system could be effectively used as sustained delivery devices in humans. As a preliminary study we have investigated the release of HSA (model protein) from loaded HA spheres coated with PLA.

HSA release profiles from HA spheres coated with PLA with varying amounts of loaded HSA, in SBF at pH 7.4 and 37 °C are shown in Fig. 8. The release rate was dependent on the amount of albumin loaded. The release of albumin within the ceramic starts with the absorption of surrounding fluid into the ceramic reservoir (pores), the dissolution of loaded albumin and diffusion through the PLA film (coating). No significant burst release was observed. The release profile appears to exhibit near-zero-order kinetics. The surface morphology of albumin-loaded PLA-coated HA spheres are shown in Fig. 9. PLA has been investigated as a possible material for degradable scaffoldings required in tissue replantation [31]. PLA is a suitable material for surgical implants because it undergoes hydrolytic de-esterification to lactic acid, a normal product of muscle metabolisms [32]. It is non-toxic, non-tissue-reactive and biodegradable. The same system was studied for the delivery of antibiotics (ampicillin) [14]. Ampicillin was released for a period of 15 days. Hence, sustained

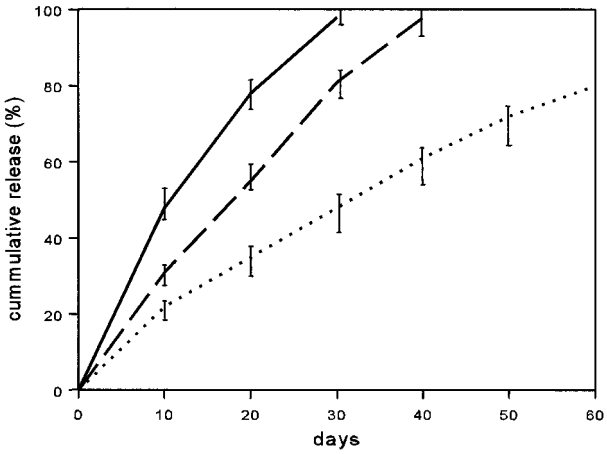
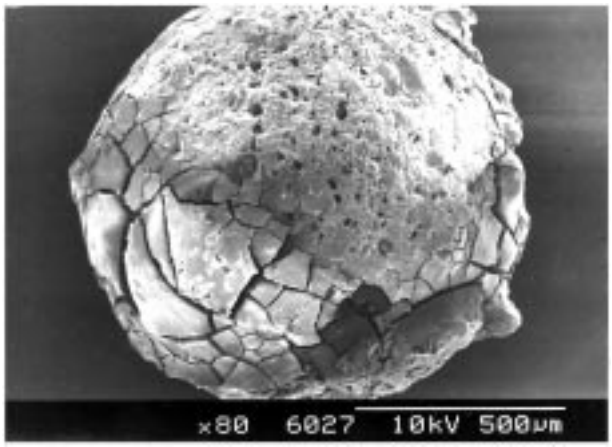
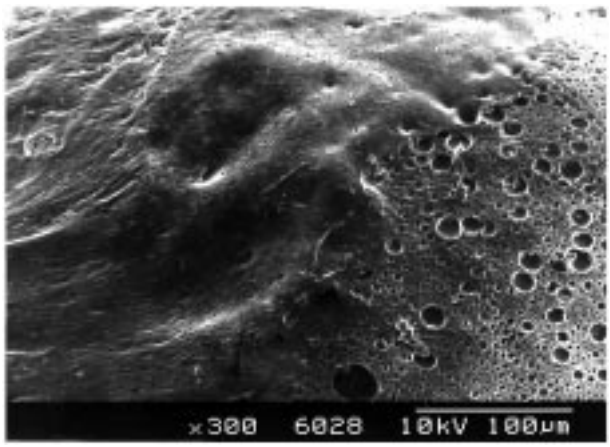


Figure 8 Cumulative release of albumin from poly(DL-lactic acid)-coated albumin-loaded HA sphere: loading (albumin content) ———, 127 mg g^{-1}, – – –, 251 mg g^{-1}; ....., 391 mg g^{-1}.



(a)



(b)

Figure 9 SEM micrograph of (a) albumin-loaded HA sphere, (b) surface morphology after PLA coating.

delivery of HSA, which is a macromolecule, for a period of more than 2 months may be expected.

4. Conclusions

A new method for the preparation of spherical HA particles has been developed. Since smooth particles cause less inflammatory response and facilitate faster bone ingrowth, compared to irregular particles, the

porous spherical particles prepared appear to be a suitable implant material in many orthopaedic and maxillofacial applications as fillers or packing material. Although it has been shown that the granules are pure HA, their biological performance needs to be evaluated in a suitable animal model.

The major limiting factor in utilizing protein drugs for sustained delivery is the lack of suitable delivery systems. Present investigation show that porous HA ceramic beads effectively release HSA *in vitro*. Since HA is biocompatible and preserves the biological activity of proteins and PLA is a suitable material for implantation, the HA–PLA system seems to be a suitable reservoir matrix clinically for the sustained delivery of protein drugs, polypeptides, vaccines, etc. In polymeric delivery systems the release rate of drugs is controlled by the crosslinking density of the structure. This is not possible in HA system. Therefore, further studies are in progress in an attempt to increase the loading as well as to modulate the release rate by increasing the porosity of the HA particles and also the polymer coating.

Acknowledgments

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