JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **13** (2002) 623–632

Microstructured microspheres of hydroxyapatite bioceramic

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Hydroxyapatite (HAP) particles having spherical geometry and 125–1000 μm in size range were prepared using a solid-in-water-in-oil (S/W/O) emulsion, cross-linking technique. An aqueous solution of chitosan containing different loading of HAP was dispersed as droplet in liquid paraffin using a stabilizing agent. Cross-linking of chitosan was induced by adding appropriate amount of glutaraldehyde saturated toluene. Chitosan microspheres containing HAP were sintered at 1150 °C to obtain pure HAP microspheres. The spheres thus produced were examined by scanning electron microscopy. The percentage yield and size distributions of the spheres were also determined.

Introduction

In recent years, synthetic hydroxyapatite (HAP) has emerged as one of the most promising bioceramic material for many applications such as orthopedic, dental, drug release, biomolecules separation etc. [1–4]. Having the chemical formula $Ca_{10}(PO_4)_6(OH)_2$, HAP is considered as the most biocompatible implant material for orthopedic applications because of its similarity in chemical structure to that of the inorganic matrix of the living bone and teeth [5, 6]. HAP does not show any foreign body reaction and has got excellent bonding ability to the host bone [7, 8]. The particulate form of ceramic materials finds various applications in medical and non-medical fields. Particles in the form of microspheres have special application in treating tumors located within organs supplied by a single afferent arterial blood supply. Microspheres have traditionally been used for targeted delivery of chemotherapeutic and radio-therapeutic agents. Kunieda *et al.* [9] have used HAP spheres with the diameter 1000 μm for sustained release of doxorubicin by direct implantation into hepatic tumors in mice and rabbits. HAP particles have also been utilized for the purification of nucleic acid [10]. Dense and porous hydroxyapatite and bioactive glass particles of different sizes have been used for the reconstruction of various bone and dental defects such as cystic defects, periodontal pockets, surgical bone defects and resorption of the alveolar ridge. The reconstruction of these defects is attempted with ceramic particles, to achieve an enhanced repair and growth of the defected part. Oonishi *et al.* used porous and dense HAP granules to pack into the massive bone defect cavity for studying the long-term bone growth up to 12 months [11]. Extensive bone growth was observed around these particulates near the wall of the osseous defects. Schepers *et al.* indicated that particle size of ceramic material is also important in its application [12]. They have used a narrow size range, i.e. 300–360 μm, for dental osseous lesions, and observed that the particles with a size exceeding the above mentioned range do not corrode throughout and therefore are not resorbed in their centers; the protective pouches will not be formed. Particles with a smaller diameter are fully resorbed and cannot act as a substrate for upregulation of mesenchymal cells.

Ceramic parts fabrication is a demanding task, requiring number of carefully controlled processing steps. In early days the simplest way to obtain small particles for chromatography and enzyme immobilization was to crush the bulk materials. For example, large pieces of porous silica or dried dextran gels were crushed in ball mills and then fractionated according to size. Although easy to prepare, these particles pose some acute problems because of their irregular shape. Particles of irregular shape packed poorly in columns and created large voids that robbed the systems of their efficiency, and sharp edges of the particles fractured easily, clogging the systems. Spherical beads do not suffer from the above mentioned problems.

Various studies have been conducted using different polymers like poly(methyl methacrylate), poly(ethyl methacrylate), bisphenol-A-glycidyl methacrylate, polyethylene etc. as matrices for HAP [7, 13–15]. Ethylene vinyl acetate co-polymer (EVA) has been used as a binder for the preparation of HAP in intricate shapes

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[16, 17]. Here we report the preparation of smooth perfectly spherical microspheres of HAP in different size, ranging from 125–1000 μm, using chitosan, a deacetylated derivative of chitin, which is chemically (1,4)-2-acetamido-2-deoxy-β-D-glucan, is obtained from crab shell, as a binder for HAP particles.

Materials and methods

Materials

Chitosan (viscosity grade 50) obtained from The Central Institute of Fisheries and Technology, Kochi, India, was used without further purification. Glutaraldehyde (25% aqueous solution), hexane L.R. (fraction from petroleum) were from S.D. Fine Chemicals Ltd., India; liquid paraffin I.P. grade (heavy) was from Teecee Pharma Thiruvananthapuram, India; toluene (for synthesis) E. Merck (India) Ltd., Mumbai, India; dioctyl sulphosuccinate purchased from Sigma Chemical Company, USA. All other chemicals used in the present study were of analytical grade and were used as received.

Methods

Preparation of HAP

The HAP powder was prepared by a precipitation route, which has been described elsewhere [18] Briefly, ammonium di-hydrogen phosphate solution was added dropwise to the calcium nitrate solution to get a precipitate. The precipitate was aged in the mother liquor for 24 h. Then it was filtered and washed to get a filter cake. The filter cake was adequately washed several times with distilled water to get rid of the surface impurities such as nitrates and ammonium ions. The filter cake was then dispersed in distilled water and spray dried using BUCHI B191 Spray Dryer (Switzerland).

Preparation of chitosan-HAP microspheres

A 2.5% solution of chitosan was prepared in 5% aqueous acetic acid. This solution (5 mL), mixed with appropriate amounts of HAP, was dispersed in 50 ml of 1 : 1 solution of liquid paraffin (heavy) and hexane containing 0.1 g dioctyl sulphosuccinate in 100 ml beaker. The dispersion was stirred using a stainless steel half-moon paddle stirrer at 350 rev min^{-1} (± 15%) for 5 min, and 5 ml of glutaraldehyde saturated toluene was added. The reaction was allowed to continue for another 2 h with stirring at room temperature (27 ± 2 °C). After the reaction, the beads were filtered off, washed several times with hexane, acetone and finally with distilled water. The beads were then dried in an air oven at 60 °C. Microspheres were fractionated by sieving them through standard test sieves (Retsch, Haan, Germany) and particle size distribution of the microspheres were determined.

Thermogravimetric analysis

TGA analysis of chitosan–HAP microspheres were conducted using SDT 2960 simultaneous TGA–DTA analyzer, TA Instruments Inc. USA., to study the degradation behavior of chitosan in the microspheres. Here the weight loss of the beads having 40% HAP content were measured continuously by heating the sample from room temperature to 1200 °C at a constant rate of 20 °C/min.

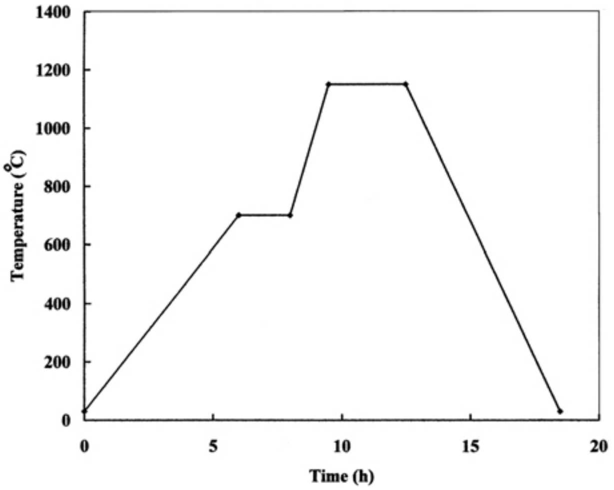


Figure 1 Temperature – Time profile of thermal de-binding and sintering of HAP–Chitosan microspheres.

Thermal de-binding and sintering process of microspheres

The HAP/chitosan microspheres of various HAP contents were allowed to decompose at a very high temperature, so that the polymer decomposes completely to leave HAP alone in a microsphere form. The temperature–time profile of the thermal de-binding and sintering process is shown in Fig. 1. The thermal de-binding and sintering of HAP/chitosan microspheres were carried out in a high temperature furnace (OKAY Raising Hearth High Temperature Furnace, Calcutta, India). The heating of the green microspheres was programmed in such a manner that the rate of heating was kept at 2 °C per minute up to a temperature of 700 °C and held at this temperature for 2 h. The microspheres were then heated steadily to 1150 °C and sintering was continued at this temperature for 3 h. Then it was allowed to cool down to room temperature.

Scanning electron microscopy (SEM)

Particle size, shape and microstructure of the microspheres were determined by SEM analysis. The samples for SEM observations were prepared by mounting the microspheres on aluminum stubs with double-sided tape. The samples were then coated with a layer of gold in a sputter coater and the SEM photographs were taken using the Hitachi Model S-2400, SEM.

Results and discussion

The HAP powder produced by precipitation-followed-spray-drying method yielded fine particles of HAP in the size range of 2–15 μm. The spray-drying technique made the HAP particles to be in the spherical form. To produce HAP microspheres having higher size range, a new

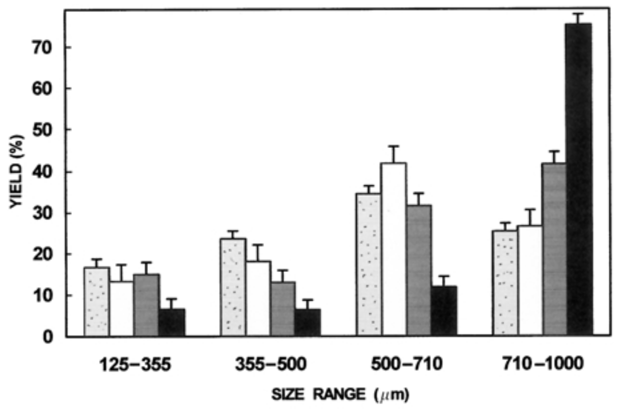


Figure 2 Particle size distribution of HAP–Chitosan microspheres. ░ 40% HAP, ☐ 60% HAP, ▓ 70% HAP, ■ 80% HAP

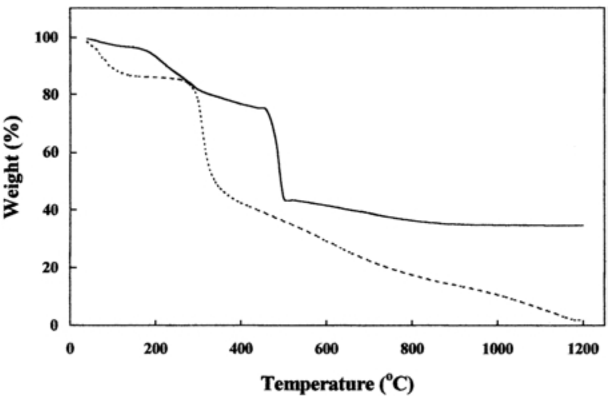


Figure 3 Thermogram of Chitosan (- - -) and HAP–Chitosan microspheres loaded with 40 wt % of HAP (—).

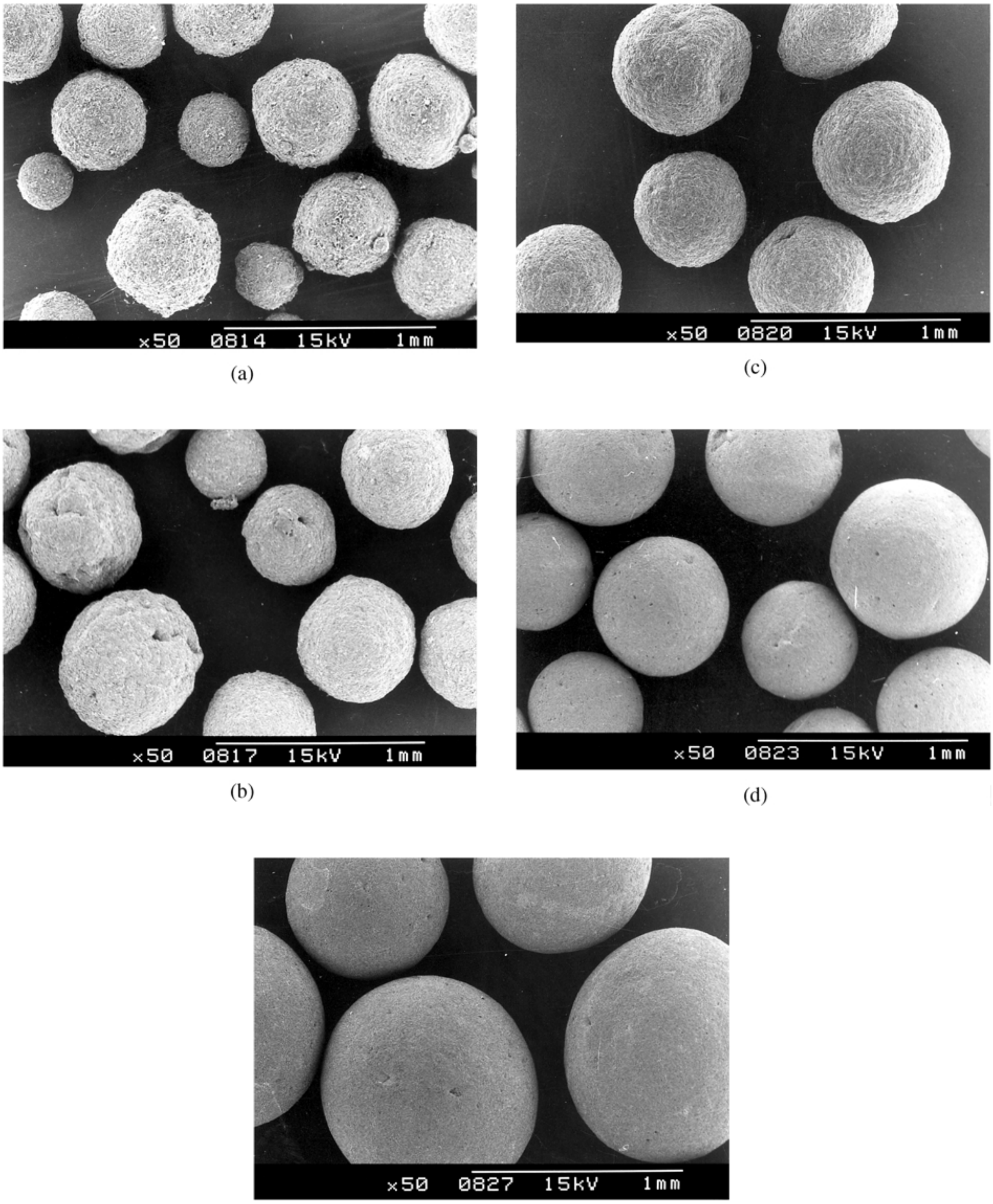
technique using s/w/o emulsion solidification has been developed. Chitosan, a polysaccharide was used as a binder for small HAP particle to form microspheres of desired size. The water insoluble chitosan can be solubilized in dilute acetic acid due to the formation of the acetate salt. Viscosity of the chitosan solution is important in the incorporation of HAP particles. Good dispersibility of HAP–chitosan solution in liquid paraffin was obtained with 2.5% solution of chitosan, even with high ($> 90\%$) loading of HAP. Below 2.5%, the size of the micospheres produced was very small. The inorganic nature of HAP may also be one of the reasons for high loading of HAP in an aqueous chitosan medium. The solidification of the individual HAP–chitosan droplets was accomplished by cross-linking of chitosan by glutaraldehyde and this reaction is an instantaneous one. When aqueous glutaraldehyde was added to the dispersion of chitosan in paraffin oil, instantaneous cross-linking occurred and the product obtained did not show good spherical geometry. To induce a slow and uniform cross-linking of the dispersed HAP–chitosan droplets for the generation of spheres of good sphericity, glutaraldehyde saturated toluene was chosen. Toluene saturated with glutaraldehyde (GST), because of its solubility in oil medium would be uniformly available for cross-linking the surface of the droplets. Microspheres having different HAP contents (30%, 40%, 50%, 60%, 70%, 80% and 90%) were prepared. In all cases the percentage yield is ranging from 90–95%. This data clearly indicate that there is not much loss in HAP while preparing the microspheres.

Our preliminary studies with different types of binders showed that chitosan is a better matrix to get higher incorporation of HAP [19]. The amount of the HAP initially incorporated was found to influence the particle size distribution of the microspheres. The particle size distribution of the microspheres having four different loadings of HAP, is as shown in the Fig. 2. It has been observed that at lower concentration of HAP content, the maximum spheres come in the middle region and as the HAP content increased, most of the spheres fall in the higher size range. So HAP loading can be adjusted to get microspheres having required size range.

Fig. 3 shows the TG curves of chitosan and of 40% HAP loaded HAP–chitosan microspheres. It has been found that, in the case of pure chitosan, the sample weight rapidly decreases with the increase of temperature, especially in the ranges 50–100 °C and 260–340 °C. The initial weight loss observed at 50–100 °C can be assigned to the loss of water while the second weight loss starting at 260 °C may be due to the thermal decomposition of chitosan [20, 21]. The thermal decomposition of chitosan is comparatively slow and a steady decrease in weight is observed. This high resistance of chitosan upon heating may be due to the intermolecular hydrogen bonds formed between hydroxyl and amino groups [22, 23]. But in the case of HAP loaded microspheres, the commencement of decomposition has been observed at elevated temperature. This shows that the composite possessed a high heat-resistance temperature up to 500 °C. There is not much weight loss after this temperature. After sintering, it has been seen that about 36% of HAP remained in the microspheres.

Scanning electron microscopy analysis revealed that the HAP–chitosan microspheres obtained were in good spherical geometry as evidenced by Fig. 4(a)–(e). These micrographs show that the spheres become more and more spherical as the HAP content is increased in the spheres. It is clear that at 80% and 90% HAP loading perfect microspheres were obtained. At higher magnification (× 4 K) these figures reveal that each tiny HAP particle is embedded in the chitosan matrix (Figs. 5(a)–(e)). In 80% HAP loaded microspheres it is seen that the HAP particles are very close to each other and very closely packed, leaving no space between the particles. As expected the gap between the HAP particles become wider and wider as the HAP loading is reduced. In 30% HAP loaded microspheres, Fig. 5(a), we can see that the gap between the particles is more and most of the particles are not in contact with each other. The higher magnification (× 400) of the Fig. 4(a)–(e), i.e. Fig. 6(a)–(e) show that surface of the spheres are covered with chitosan and as HAP content increases each individual HAP particle become clearer at the surface.

During sintering, binder burn out and particle fusion, are taking place. This process is very important in retaining the shape of the microspheres. The heating

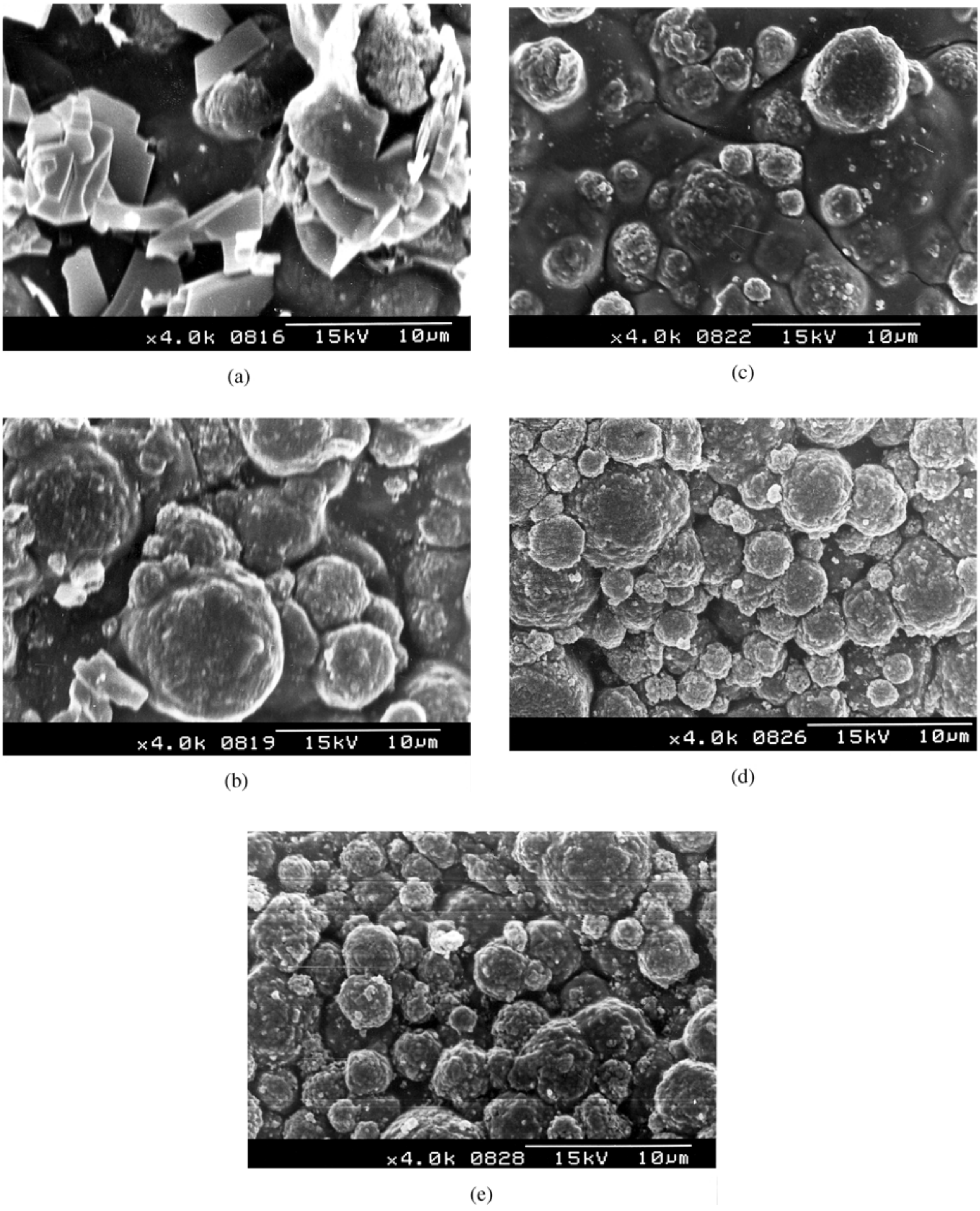


(e)

Figure 4 Scanning electron micrograph of HAP–Chitosan microspheres having different HAP loading (a) 30%, (b) 40%, (c) 60%, (d) 80% and (e) 90%.

process progresses with a steady increase in temperature up to 700 °C. Slow heating process helps to retain the shape of microspheres intact. At 1150 °C each HAP particles fuse together without changing the shape of the spheres. At this temperature almost all the binder material burns out. The size of the microspheres before and after sintering is given in Table I. The shrinkage is due to both the binder burn out and fusion of the HAP particles.

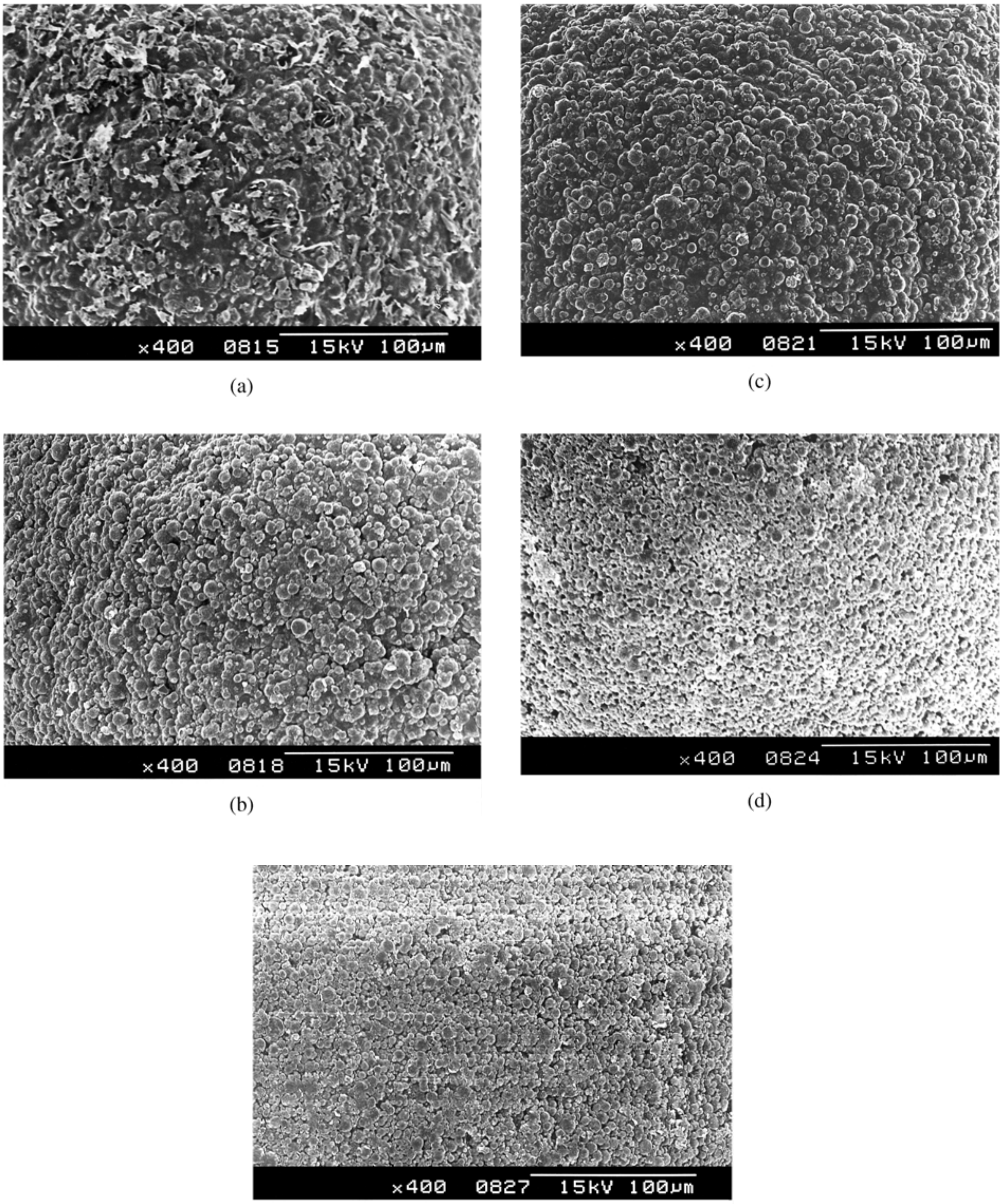
SEM micrographs in Fig. 7(a)–(e) show the sintered HAP–chitosan microspheres, containing 30–90% HAP content. In all cases, except 30% loading, the shape of the



(a) (c) (b) (d) (e)

Figure 5 Scanning electron micrograph of HAP–Chitosan microspheres higher magnification (× 4 K) of Fig. 4, having different HAP loading (a) 30%, (b) 40%, (c) 60%, (d) 80% and (e) 90%.

spheres remained intact. In 30% HAP loaded microspheres, most of them lost their shape after sintering. Since the gap between the adjacent HAP particles is more in the case of 30% HAP loaded spheres, during sintering, the chances of adjacent HAP particles getting fused is less and therefore the spheres could not retain the spherical geometry. It can be seen that, as the amount of HAP increased, the shape of the microspheres becomes more and more spherical and reaches perfectly spherical at the loading of 60%. The shape of the microspheres having 60% and above HAP loading is perfectly spherical. The increase in sintering density with increase in HAP loading is also evident from the SEM micrographs.



(a) (c)

(b) (d)

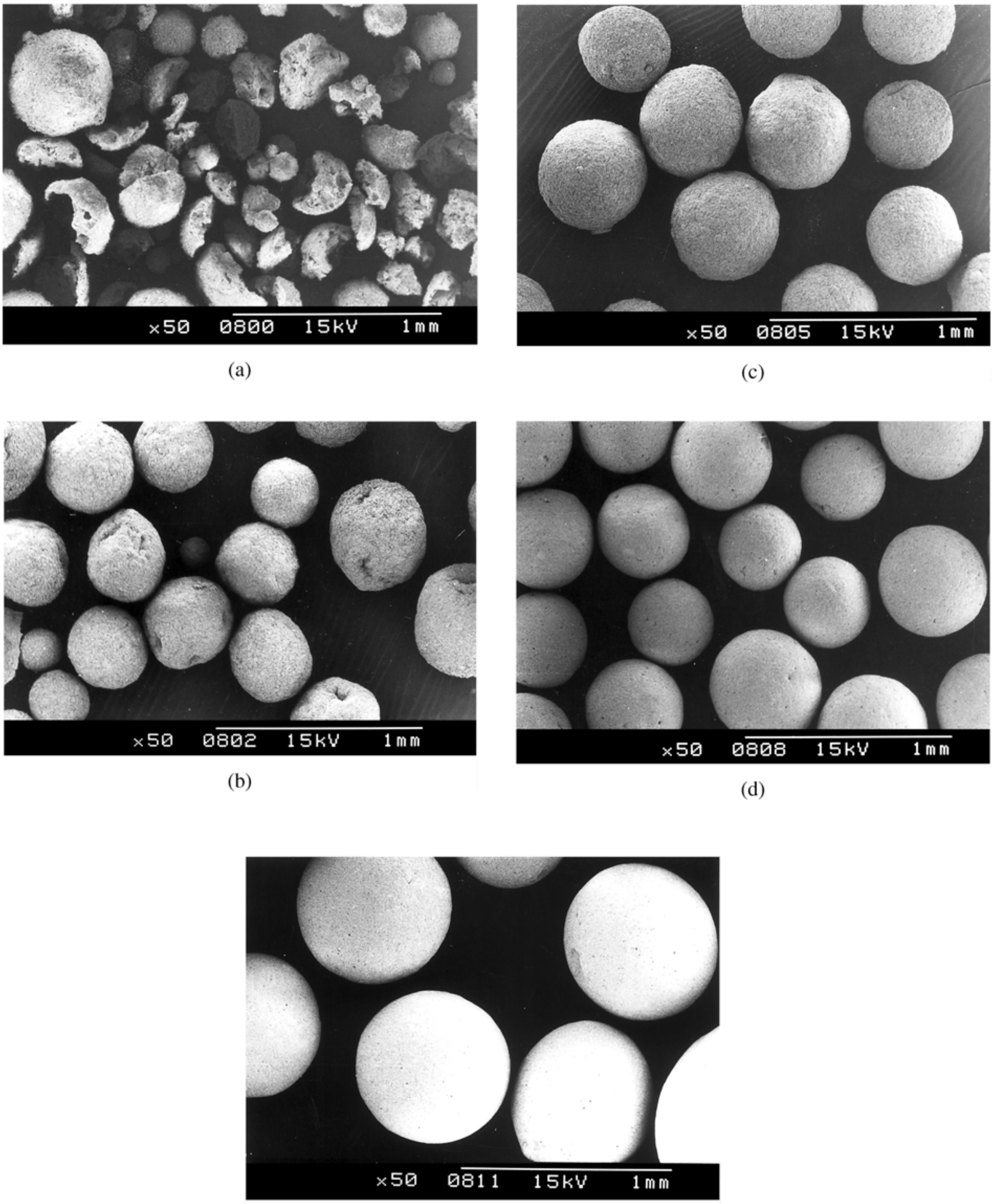
(e)

Figure 6 Scanning electron micrograph of HAP–Chitosan microspheres higher magnification (× 400) of Fig. 4, having different HAP loading (a) 30%, (b) 40%, (c) 60%, (d) 80% and (e) 90%.

TABLE I Size of the microspheres before and after sintering

Before sintering (μm)	After sintering (μm)
250–355	< 180
500–710	300–400
710–1000	500–710

Higher magnification (× 400) of these spheres (Fig. 8(a)–(e)) indicate that each microsphere is composed of a large number of tiny HAP particles. Therefore, these microspheres could be described as microstructured microspheres of hydroxyapatite consisting of tiny HAP microspheres in a microsphere geometry. SEM pictures, (Fig. 9(a)–(e)) are the higher magnification of these



(a) (c)

(b) (d)

(e)

Figure 7 Scanning electron micrograph of sintered HAP microspheres having different HAP loading (a) 30%, (b) 40%, (c) 60%, (d) 80% and (e) 90%.

microspheres, show the porous nature of the spheres. It is clearly seen that 40% HAP loaded microspheres are more porous and the porosity is getting reduced as the HAP content is increased.

Conclusions

HAP particles in the size range 3–15 microns were prepared by spray-drying method. HAP particles were incorporated in a 2.5% chitosan solution in different

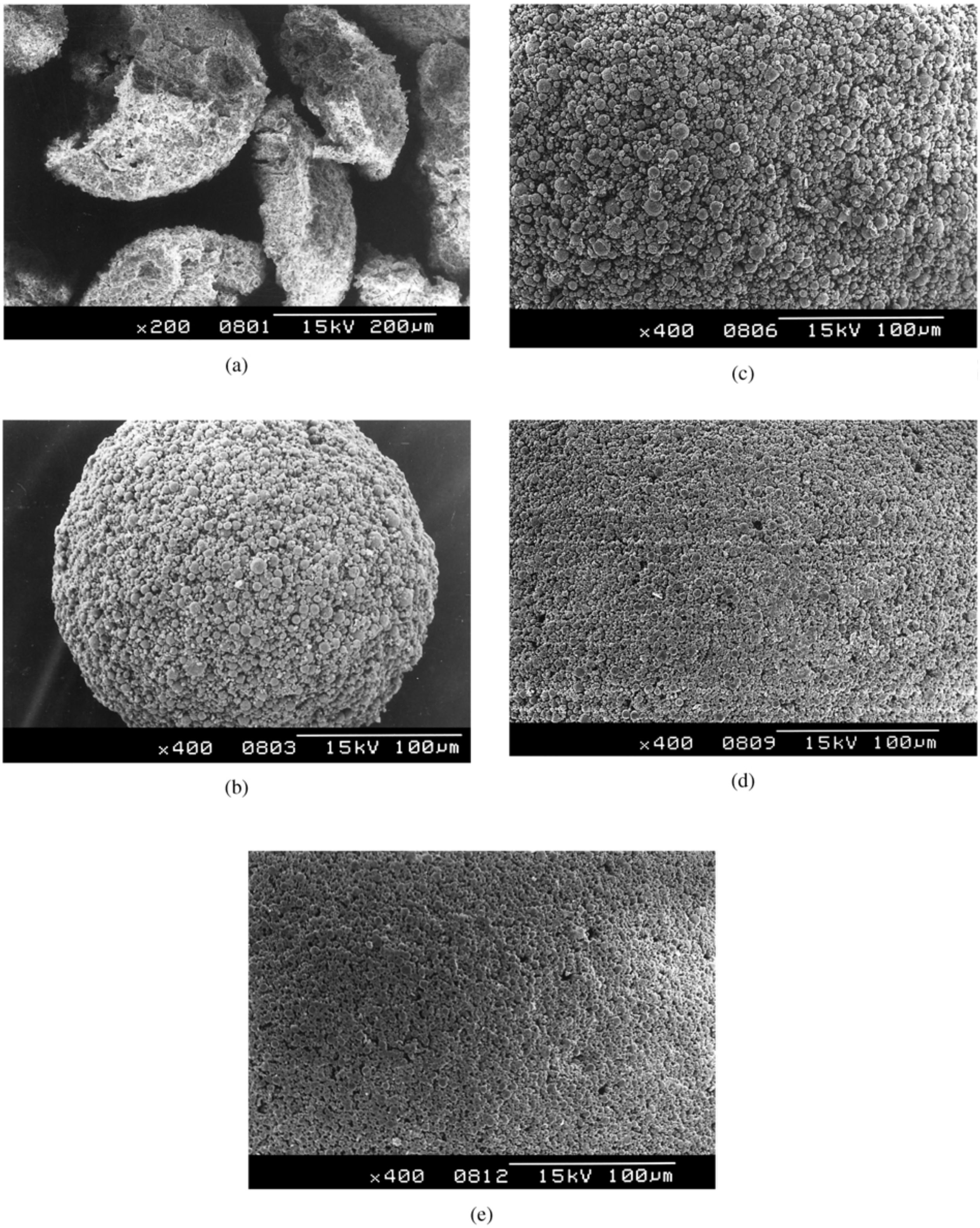
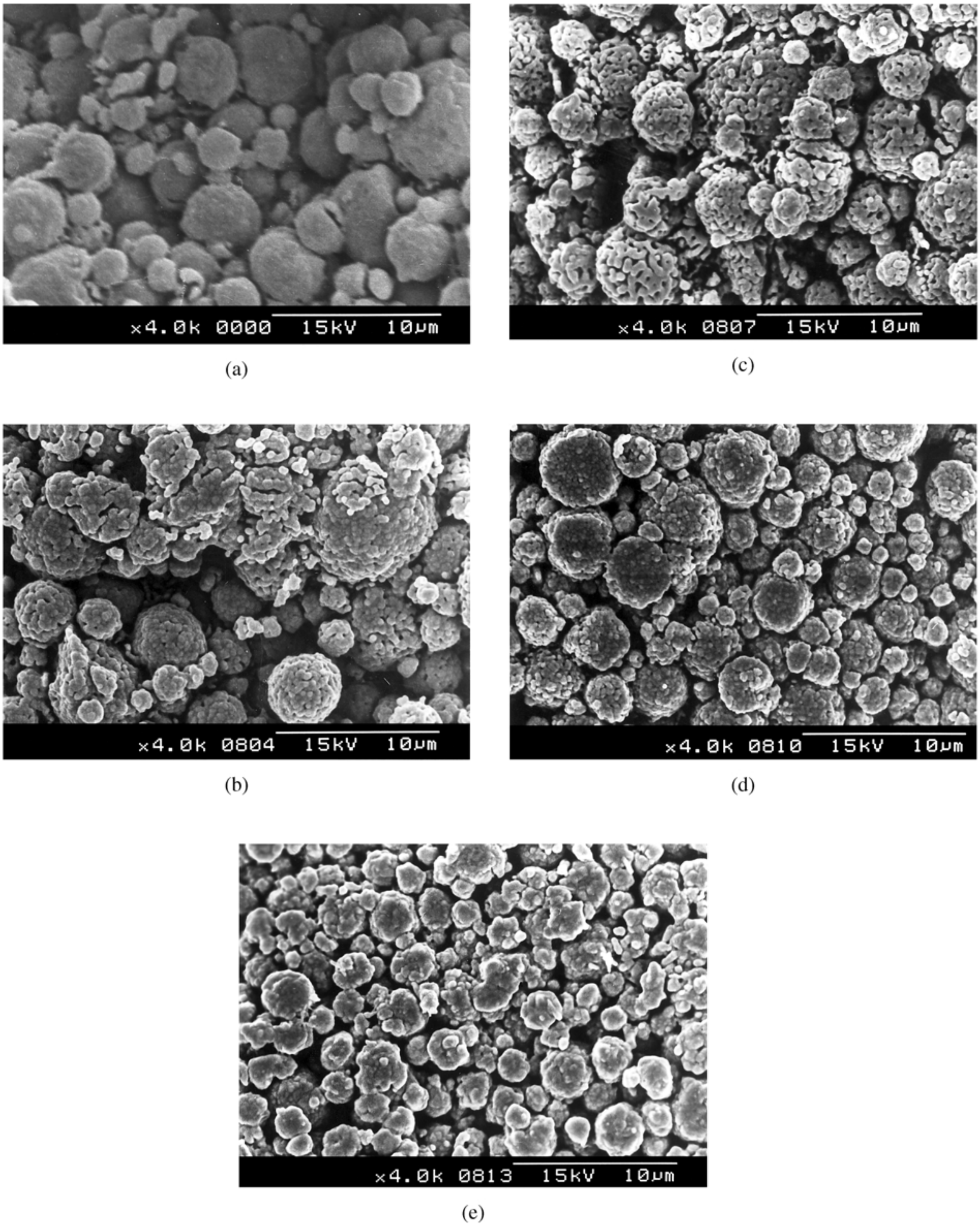


Figure 8 Scanning electron micrograph of sintered HAP microspheres higher magnification (× 400) of Fig. 7, having different HAP loading (a) 30%, (b) 40%, (c) 60%, (d) 80% and (e) 90%.

ratios and resultant mixtures were dispersed in paraffin liquid to obtain a S/W/O emulsion. The cross-linking of chitosan in the emulsion with glutaraldehyde resulted in HAP filled chitosan microspheres. Heating of these spheres at elevated temperature resulted in the binder burn out and the fusion of the HAP particles and perfectly spherical microstructured microspheres of HAP could be obtained. The required size and porosity of the spheres can be adjusted by varying the HAP content. Hydroxyapatite/Chitosan composite as well as the heat



(a) (c)

(b) (d)

(e)

Figure 9 Scanning electron micrograph of sintered HAP microspheres higher magnification (× 4 K) of Fig. 7, having different HAP loading (a) 30%, (b) 40%, (c) 60%, (d) 80% and (e) 90%.

treated or sintered microstructured microspheres will be a candidate material for the further studies on drug delivery, separation of bio-molecules, bone filling and augmentation applications.

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Received May 3
and accepted October 23 2001