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Preparation, characterization and in-vitro release of gentamicin from coralline hydroxyapatite–chitosan composite microspheres

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Abstract

Composite microspheres have been prepared from bioactive ceramic such as coralline hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] granules, a biodegradable polymer, chitosan and an antibiotic, gentamicin. In our earlier work, we have shown a simple method of converting the calcium carbonate skeleton of the Indian corals into hydroxyapatite granules. The composite microspheres containing coralline hydroxyapatite and chitosan were prepared by dispersion polymerization technique and the gentamicin was incorporated by absorption method. The crystal structure of the composite microspheres was analyzed using X-ray powder diffractometer. The Fourier transformed infrared spectra clearly indicated the presence of amide and hydroxyl groups in the composite microspheres. Scanning electron micrographs and optical micrographs shows that the composite microspheres are spherical in shape and porous in nature. The particle size of composite microspheres was analyzed and the average size was found to be 18 microns. The thermal behavior of composite microspheres was studied using thermogravimetric analysis and differential scanning calorimetric analysis. The cumulative in-vitro release profile of gentamicin from composite microspheres showed near zero order patterns.

Keywords: Coralline hydroxyapatite; Chitosan; Composite microspheres; Gentamicin; In-vitro release

1. Introduction

Bone repair is a subject of intensive investigation in human health care. Drug delivery technology presents an interesting interdisciplinary challenge for the pharmaceutical, chemical engineering, biomaterials and medical communities. Owing to their physiochemical and biological properties, calcium phosphates have recently been considered as a potential material for a bone drug delivery system (Bajapi & Benghuzzi, 1988; Guicheux, Grimandi, Trecant, Faivre, Takahashi & Daculsi, 1997). This type of drug delivery system, using a bioactive matrix, can release a therapeutic agent in-situ to produce an action associating the osteoconductivity of the material (Ragel & Vallet-Regi, 2000). Bone derived apatites have a considerable potential as remodeling implants, prosthetic bone replacements and drug delivery devices (Gautier, Caillon, Le Ray, Daculsi & Merle, 2000; Krajwski, Ravaglioi, Roncari & Pinasco, 2000). Many composite materials have been developed for biomedical applications. They may include ceramic or polymer matrix composites (Doyel, 1990; Greish & Brown, 2000). Various biocomposites exist in nature where an organic matrix is associated with an inorganic fraction (Calvert & Mann, 1988). These composites fulfill the mechanical properties required for their function as skeleton, teeth or cells of organisms. Many efforts have been made towards the development of new bone substitute materials. Among these, hydroxyapatite/polymer composites have attracted much attention since such composites may have osteoconductivity due to the presence of hydroxyapatite (HA) (Liu, de Wijn & van Blitterswijk, 1998; Tanner, Doyle & Bonfield, 1990; Labella, Braden & Deb, 1994).

Hydroxyapatite granules have been used clinically as substitutes for autografts in filling bone defects. HA is biocompatible, nontoxic, resorbable, has excellent osteoconductive ability, possesses a structure similar to bone mineral and can form a direct bond with bone. Using HA is advantageous since it is non-inflammatory and causes no immunological, foreign body or irritating response. However, the migrations of individual particles from the implant side before the tissue ingrowth starts cause in

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convenience and also it is difficult to fill the surface of irregular bone defects and to reconstruct the shape of bones completely with either powder or block form of HA implant. In recent years, several kinds of bioactive bone cements have been developed to overcome these problems. This shortcomings of HA granules was overcome by employing biodegradable HA particulate composites (Dandurand, Delpech, Lebugle, Lamure, & Lacabanne, 1990). Several particle composites based on degradable biopolymers such as collagen (Hsu, Chueh & Wang, 1999), fibrin glue (Wittkarmpf, 1988), starch based material (Mano, Vaz, Mendes, Reis & Cunha, 1999), gelatin (Katsumura, Koshino & Saito, 1998), chitosan (Ito, Hidaka, Nakajima, Yagasaki & Kafrawy, 1999) and alginate (Paul & Sharma, 1997) with inorganic powders, as bone filler were developed (Bigi, Panzavolta & Roveri, 1998; Wan, Eugene Khor & Hastings, 1998).

The polysaccharide chitin is found in nature as a major component of the organic fraction for several biocomposites (Maruyama & Ito, 1996). Chitosan is the deacetylated derivative of chitin, which is one of the most abundant natural polysaccharides containing amino and hydroxyl groups. The primary unit of chitin is 2-acetamido-2-deoxy-D-glucose while that of chitosan is 2-amino-2-deoxy-D-glucose linked by β, 1-4 glucosidic linkage (Muzzarelli, 1985). Chitosan is insoluble in water, alkali and many organic solvents but is soluble in many dilute aqueous solutions of organic acids, of which the most commonly used are formic and acetic acid. Chitosan's major attractions include its biocompatibility and its acceptable biodegradation properties by virtue of its biopolymer origin.

A composite biomaterial of HA and chitosan therefore is expected to show increase osteoconductivity and biodegradation together with sufficient mechanical strength for orthopeadic use (Yamaguchi, Tokuchi, Fukuzaki, Koyama, Takakuda, Monma et al., 2001). Irregularly or densely packed granules often cause inflammatory reaction or the bone formation may be slower. Uniformly packed spherical particles with uniform pore distribution help in faster bone in-growth. Polysaccharides are known to activate macrophages and induce their proliferation (Chang, Lee, Hong, Youn, Ryu, Chung et al., 2000). Many researchers have been using the active drugs in hard composites for orthopedic surgery (Bajpai & Billotte, 1995; De Real, Padilla & Vallet-Regi, 2000). The utilization of antibiotic delivery systems for treatment and prevention of bone infection is a very important subject because the poor circulation of blood in the osseoous tissue makes necessary the supply of great amount of antibiotics to reach the adequate therapeutic level in the affected region. In this work, an attempt was made to prepare implantable materials that are able to lead to bone growth and also can, at the most critical inflammation-infection step, release an antibiotic. For this purpose, composite materials were prepared with bioactive ceramic granules such as coralline hydroxyapatite, a biodegradable polymer, chitosan and an antibiotic, gentamicin, for its release in the implant area. The in-vitro release profile of gentamicin was carried out in phosphate buffer of pH 7.4 at 37°C.

2. Materials and methods

2.1. Materials

Chitosan (80% deacetylation, soluble in 5% acetic acid) was obtained as a gift from the Central Institute of Fisheries Technology, Cochin, India, Gultaraldehyde 25% solution (Fluka, Germany) and Gentamicin (Hindustan antibiotics, India). The coralline hydroxyapatite granules and PMMA powder used in this study were prepared in our laboratory. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Preparation of coralline hydroxyapatite–chitosan (CHA–C) composite microspheres

The composite microspheres were prepared by dispersion polymerization technique using the prepared ceramic and polymeric powders. The preparation method of coralline hydroxyapatite granules (Sivakumar, Sampath Kumar, Shantha & Panduranga Rao, 1996) and PMMA powder (Sivakumar & Panduranga Rao, 2000) used in this study was reported earlier. 1 gm of coralline hydroxyapatite granules were mixed with 40 ml of chitosan solution via sonication for 15 min. This mixture was added drop by drop into a 5% PMMA dispersion solution (PMMA containing chloroform/toluene 1:1) with constant stirring (stirring speed 400 rpm) until the microspheres were obtained. About 2 ml of glutaraldehyde-saturated toluene was added to the medium as a cross-linking agent. After half an hour of cross-linking reaction, the PMMA was removed by washing several installments of solvents with toluene, acetone and finally with water. These CHA–C composite microspheres were obtained by centrifugation, filtered and dried at room temperature for 24 h. These composite spheres were in the form of free flowing powder.

2.2.2. Encapsulation of gentamicin in the CHA–C composite microspheres

Encapsulation of gentamicin into CHA–C composite microspheres was carried out in phosphate buffer saline (PBS) pH 7.4 at room temperature for 24 h. 100 mg of CHA–C composite microspheres was immersed in 10 ml of PBS containing 100 mg of gentamicin (10 mg ml^{-1}). After 24 h, the microspheres were separated by centrifugation and dried at room temperature for 48 h. The estimation of gentamicin uptake by the composite microspheres was carried out through an indirect method, by finding the difference in gentamicin concentration in the loading buffer, before and after loading. Percentage of drug loading was

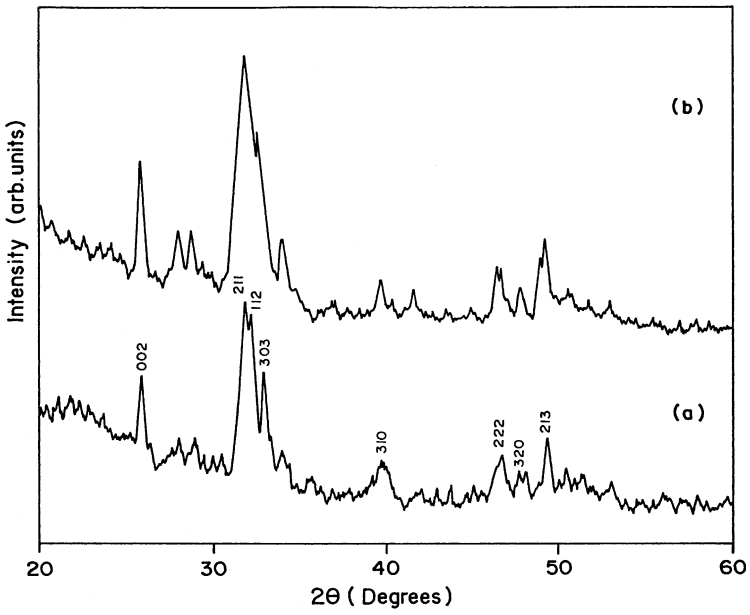

Fig. 1. XRD patterns of (a) CHA and (b) CHA–C composite microspheres.

calculated using the formula:

$$\text{Percentage drug loading} = \frac{X - Y}{X} 100$$

where X and Y represent the initial and final drug concentrations, respectively. The experiments were performed in triplicate.

2.2.3. In-vitro release of gentamicin from CHA–C composite microspheres

In-vitro release of gentamicin from CHA–C composite microspheres (100 mg) was carried out at 37°C in PBS (10 ml) pH 7.4. The release medium was collected at predetermined time intervals, and replaced with a fresh buffer of PBS (3 ml) each time. The collected samples were filtered through a 0.45 μm millipore filter. The amount of gentamicin released was then measured at 257 nm using a shimadzu UV-2100S spectrophotometer. These experiments were carried out in triplicate.

2.3. Characterization of CHA–C composite microspheres

The powder samples of the coralline hydroxyapatite and CHA–C composite microspheres were examined with a high resolution X-ray powder diffractometer in Guinier geometry using monochromatic CuKα radiation ($\lambda = 1.54059$ Å, XRD 3000, Seifert, Germany). The X-ray diffraction (XRD) patterns were recorded in steps of 0.01° intervals with 1 s counting time at each step. FT-IR spectra of composite microspheres were recorded using a FT-IR spectrometer (Nicolet 20DXB model spectrophotometer, Madison, USA). The particle size distributions of CHA–C composite microspheres were determined using a Malvern Master Sizer/E particle size analyzer, UK. The optical microscope (Reichart 2 polyvar mat) was used for determination of shape of CHA–C composite microspheres and the morphological characteristic features of CHA–C composite microspheres were studied using a scanning electron microscope (SEM, Leika Stereo Scan, UK). Thermogravimetric analysis (TGA) and Differential scanning calorimetric analysis (DSC) of the CHA and CHA–C composite microspheres was performed with a heating rate of 5°C min^{-1} under a flowing high purity nitrogen atmosphere (Dupont 2000, USA).

3. Results and discussion

The CHA–C composite microspheres was prepared by dispersion polymerization technique using coralline hydroxyapatite granules and chitosan. Chitosan is a biodegradable cationic polysaccharide composed of *N*-acetylglucosamine residues which is known to accelerate wound healing and bone formation. The antibacterial drug such as gentamicin was loaded in the composite microspheres and the release studies were discussed.

XRD patterns of CHA and CHA–C composite microspheres are shown in Fig. 1a and b, respectively. Both the diffractograms show identical patterns and the XRD pattern of composite microspheres shows that the peaks are shifted when compared to CHA peaks. The merging of (211) and (112) peaks in the CHA–C composite microspheres clearly indicates the interaction of the polymer backbone with the CHA lattice. The XRD pattern of the CHA and CHA–C composite microspheres has been indexed based on hexagonal structure. The lattice parameters were obtained by refining the diffraction lines using standard least-square method and the cell parameters were $a = 9.425 \pm 0.016$;

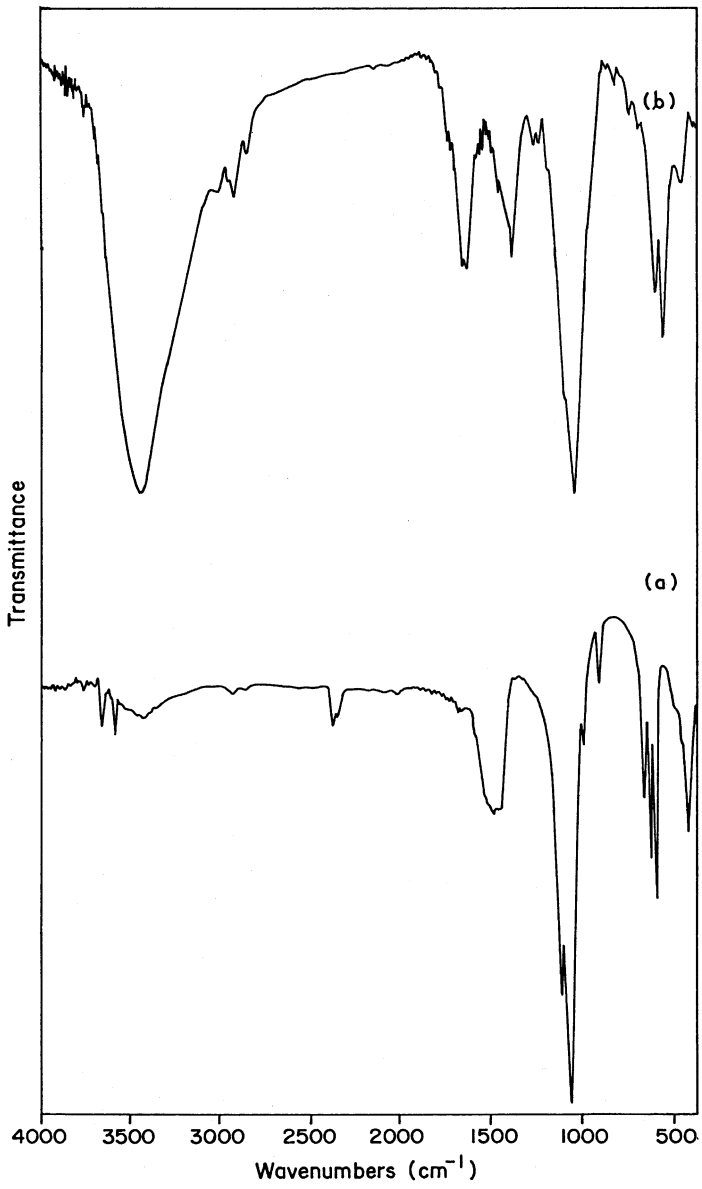

Fig. 2. FT-IR spectra of (a) CHA and (b) CHA–C composite microspheres.

$c = 6.902 \pm 0.013$ Å for CHA and $a = 9.412 \pm 0.018$; $c = 6.896 \pm 0.014$ Å for CHA–C composite microspheres, respectively. These lattice constants are comparable with hydroxyapatite reported ($a = 9.418$ and $c = 6.884$ Å, respectively, JCPDS file no. 9-432). The *a*-axis dimension of CHA lattice of composite microspheres decreases with the increased amount of chitosan.

Fig. 2 shows the FT-IR spectra of CHA (Fig. 2a) and CHA–C composite microspheres (Fig. 2b). The CHA spectrum shows absorption bands at 571, 601, 962, 1046 cm^{-1} corresponding to the PO_4^{3-} ions of the apatite whereas in the CHA–C composite microsphere, the PO_4^{3-} ions appeared at 564, 603, 964 and 1385 cm^{-1}. The broad band appeared at 3445 and 665 cm^{-1} is due to the OH group of CHA–C. The spectrum of composite microspheres is similar to those of the original chitosan, while a new peak appeared for phosphate and carbonate groups (1423 and 865 cm^{-1}). The broad peaks from 1260–1000 cm^{-1} can be explained owing to the C–O stretching vibrations. The sharp intense peak at around 1385, 1635 and 1720 cm^{-1} is due to the amide bond present in the composite microspheres, where as amide bonds are not appeared in the case of CHA. The two sharp peaks at 2923 and 2824 cm^{-1} are assigned for amide groups present in the composite microspheres. The FT-IR study indicated the presence of chitosan and coralline hydroxyapatite in the composite microspheres.

The particle size distribution curve of CHA–C composite microspheres are shown in Fig. 3. It is clearly evident that the microspheres have a narrow size distribution. These microspheres fall in the size range of 5–30 μm with a volume average diameter of about 18 μm.

Fig. 4 shows the representative optical micrographs of

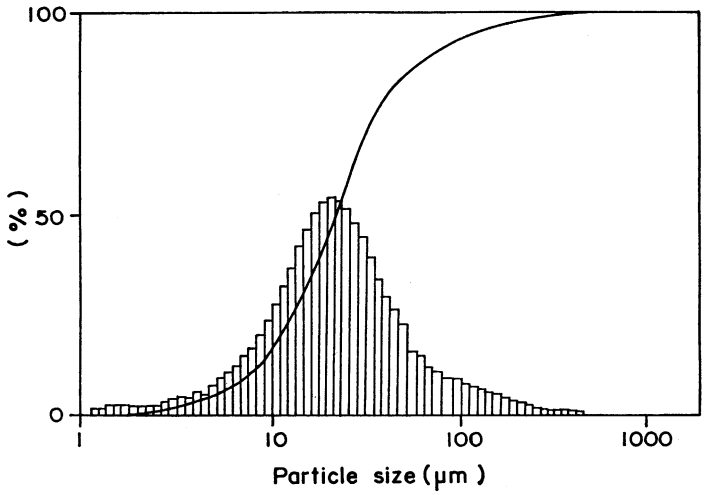

Fig. 3. Particle size distribution of CHA–C composite microspheres.

CHA–C composite microspheres. The photograph indicated that the microspheres are spherical in shape and uniform in size. Representative scanning electron micrographs of the CHA–C composite microspheres are shown in Fig. 5. These composites were found to be spherical in shape and porous in nature. The structural property of porous HA is more resorbable and more osteoconductive than dense HA (Chang et al., 2000). Taking advantage of the porosity of the composite microspheres, it is possible to incorporate a greater amount of antibiotic to the composite microspheres.

Fig. 6a and b shows the TGA trace of CHA and CHA–C composite microspheres, respectively. The trace of composite microspheres showed continuous weight loss from 90°C onwards until 800°C. The total weight loss is significant between 200 and 400°C and the loss is minimal for 420°C onwards. The total weight loss observed for composite microspheres was 45% where as in the case of CHA the total weight loss is very minimal. The weight loss was relatively higher for composite microspheres when compared to CHA sample alone and is attributed to the presence of natural polymer such as chitosan. Fig. 7 shows the DSC trace of CHA–C composite microspheres, the decomposition temperature starts at 40°C and the glass transition temperature was observed at 382°C where as in the case of CHA, it is stable upto 900°C, as confirmed from TGA studies.

CHA–C composite microspheres were loaded with 68.58 mg of gentamicin, by immersing the microspheres in 10 ml of gentamicin solution at 10 mg ml^{-1} concentration for 24 h. The percentage loading of gentamicin in the composite microspheres was calculated using the formula given in Section 2.2.2. The percentage loading of gentamicin was found to be 47.5 ± 1.98%. Fig. 8 shows the in-vitro release of gentamicin from CHA–C composite microspheres carried out at 37°C in PBS pH 7.4. 84.9% of the loaded drug was released within 4 days from CHA–C composite microspheres. The composite microspheres started releasing encapsulated gentamicin with the absorption of surrounding fluid into the microspheres pores,

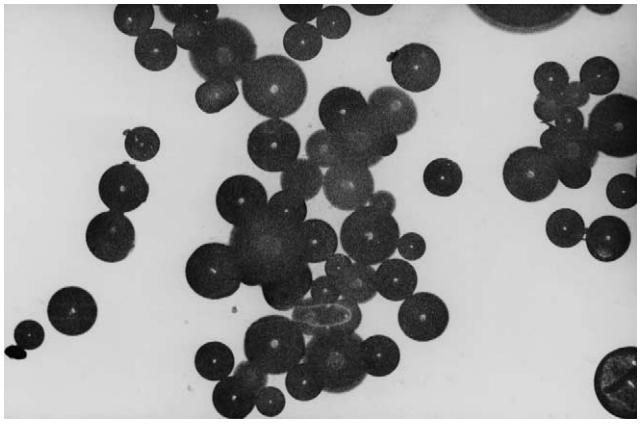

Fig. 4. Representative optical micrographs of CHA–C composite microspheres.

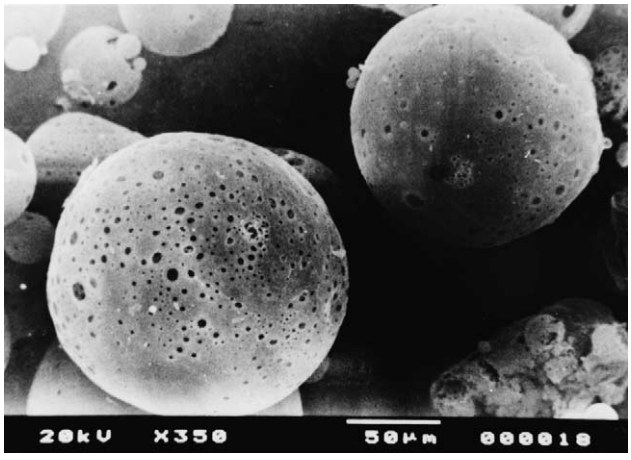

Fig. 5. Representative scanning electron micrographs of CHA–C composite microspheres.

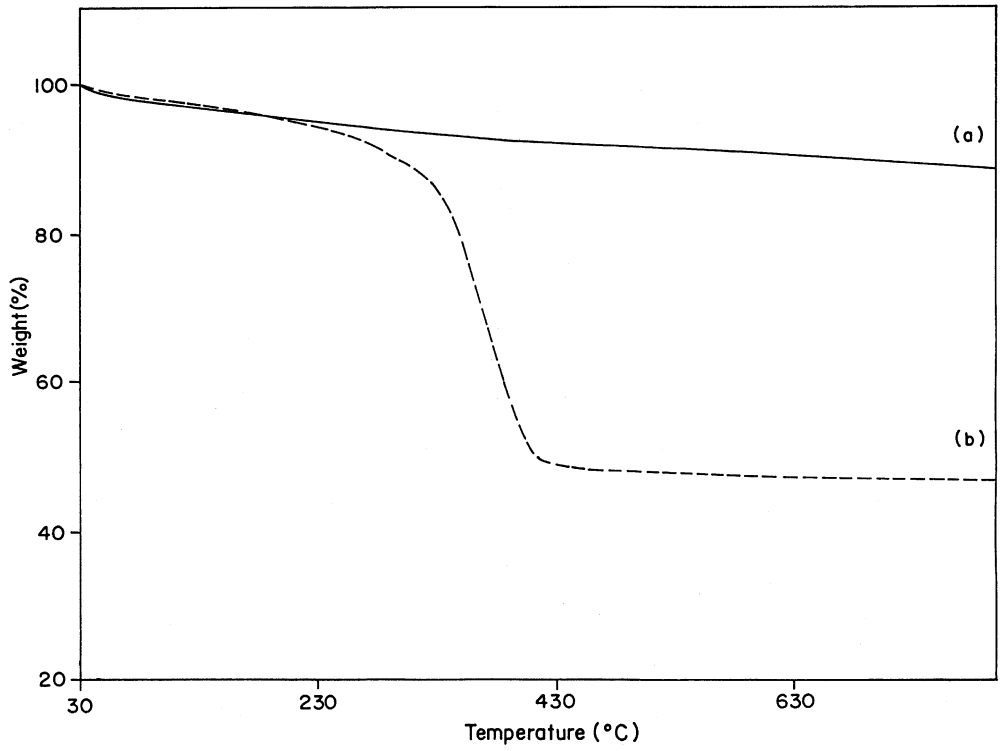

Fig. 6. TGA traces of (a) CHA and (b) CHA–C composite microspheres.

dissolution of loaded drug and exclusion. The composite microspheres releases the drug in a near zero order fashion for prolonged period of time. The cross-linking of functional groups played a vital role in the prolonged release of drug.

4. Conclusions

Composite microspheric systems for bone and dental application based on coralline hydroxyapatite/chitosan

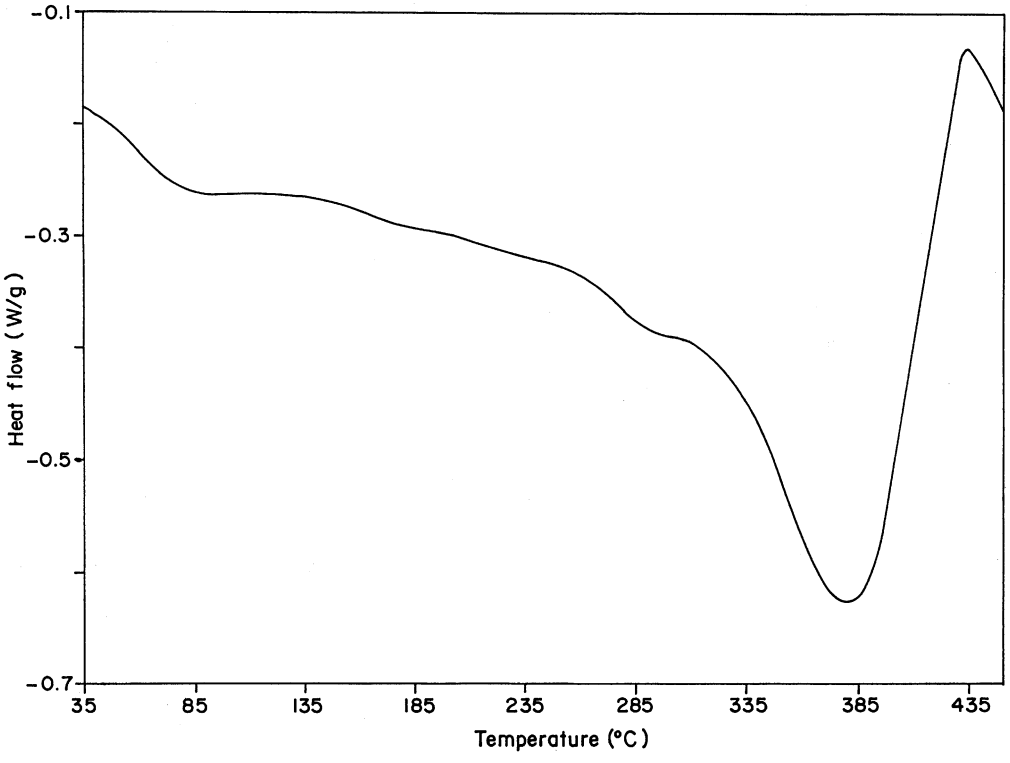

Fig. 7. DSC trace of CHA–C composite microspheres.

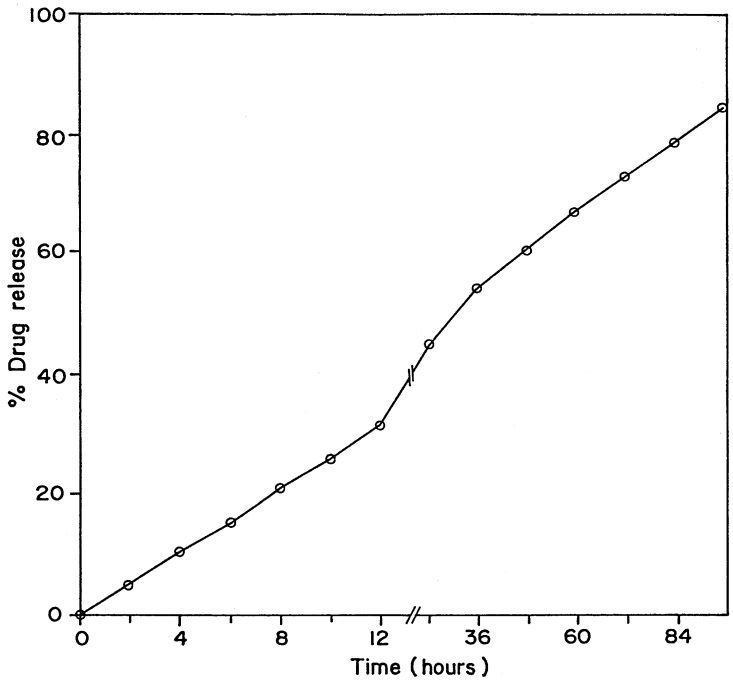

Fig. 8. In-vitro release of gentamicin from CHA–C composite microspheres in phosphate buffer pH 7.4 at 37°C.

may be designed as effective devices for the controlled release of antibiotic agents such as gentamicin. CHA–C composite microspheres were prepared by dispersion polymerization technique. It is evident from the XRD and FT-IR data that the crystal structure and characteristic groups present in the CHA–C composite microspheres. Optical and scanning electron micrographs indicated that the composite microspheres are spherical and porous in nature. The particle size of composite microspheres was determined and it is found to be 18 microns. Thermal studies clearly indicated the presence of natural polymer such as chitosan. Gentamicin, an antibacterial agent which is generally used the infected bone healing, was incorporated in the microspheres and its prolonged release was evaluated. From these data it can be inferred that these composite microspheres could find potential uses in the bone repair and regeneration.

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