Synthesis, characterization and *in vitro* cytotoxicity assessment of hydroxyapatite from different bioresources for tissue engineering application

SUDIP MONDAL, RAJASHREE BARDHAN, BISWANATH MONDAL*, APURBA DEY[†],
SUDIT S MUKHOPADHYAY[†], SYAMAL ROY[‡], RAJAN GUHA[‡] and KOUSHIK ROY[‡]

Centre for Advanced Materials Processing, CSIR-Central Mechanical Engineering Research Institute,
Durgapur 713 209, India

[†]Department of Biotechnology, National Institute of Technology, Durgapur 713 209, India

[‡]CSIR-Indian Institute of Chemical Biology, Kolkata 700 032, India

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Abstract. In the present study, hydroxyapatite (HAp) is synthesized from different biosources like eggshell, fish scale and bovine bone in a cost effective and ecofriendly way. HAp materials were synthesized from eggshell by wet precipitation method whereas thermal decomposition method was applied in case of fish scale and bovine bone. The phase purity and crystallinity of different calcined HAp powder were determined by XRD and FTIR analyses. The thermogravimetric analysis was carried out to show thermal stability of HAp powder. Average grain sizes of sintered samples were in submicron range. The morphology of the powders were observed under scanning electron microscopy (SEM). The dried powders were wet ball milled for several hours and surfactants like Triton-X small fillers (2 / 4 mm long rod-shaped) were made for *in vitro* testing. In order to verify the biocompatibility of HAp powders, cytotoxicity evaluation was carried out in RAW macrophage like cell line media for an incubation period of 72 h. The cell attachment studies on HAp compacts show an excellent affinity between cells and compact surface. These results proved high biocompatibility of HAp powders obtained from different biosources for tissue engineering applications.

Keywords. Hydroxyapatite; biomaterials; sintering; tissue engineering; cytotoxicity.

1. Introduction

Hydroxyapatite and calcium phosphate based materials have attracted considerable interest in the field of tissue engineering because of similarities with the mineral fraction of natural bone and high biocompatibility with living tissues. It has good bio-affinity, stimulates osteo-conduction and is slowly replaced by the host bone after implantation. Application of hydroxyapatite biomaterial as a porous or granulated material is useful in bone surgery or coating on metallic bio-implants. A number of methods have been used for HAp powder synthesis such as solid state reaction, hydrothermal reaction, co-precipitation reaction, sol-gel synthesis, mechano chemical synthesis, etc (Kim *et al* 2000; Balamurugan *et al* 2002; Tadic and Epple 2003; Mobasherpour *et al* 2007; Parthiban *et al* 2009).

The present study illustrates synthesis and processing parameter of HAp powder from egg shell, fish scale and bovine bone. Natural structural HAp material from these biosources (Mondal *et al* 2010; Bardhan *et al* 2011) not only provides an abundant source for novel bone and cartilage replacement surgery but it also inspires investigations

to develop biomimetic composites. In this study HAp powder was synthesized at low temperature from egg shell by wet precipitation method using nitric acid and di-ammonium hydrogen phosphate. Eggshell is made up by a three-layered structure, viz. the cuticle, the spongy layer and the lamellar layer. Chemical composition of egg shell crust is calcium carbonate (94%), magnesium carbonate (1%), calcium phosphate (1%) and organic matter (4%) (Prabakaran *et al* 2005). Eggshell is a good source of calcium carbonate and this source can be utilized for synthesis of hydroxyapatite. Wet precipitation route is quite favourable because of its ease in experimental operations, low working temperature and high percentages of pure products. This study also illustrates the development of hydroxyapatite biomaterial from fish scales. Fish scales are mainly composed of collagen, connective tissue proteins, water and the remaining 41–84% (Sankar *et al* 2008) of other proteins. This study shows that the biomimetic property may lead to the development of new biomaterials. There are several types of calcium phosphate salts, which are present in fish scales due to their extreme biological response in physiological environment. HAp powder was extracted by calcining the chemically treated fish scales at different temperatures. Extraction of hydroxyapatite from bovine bone is biologically safe and economic, since it is easy to obtain. Bone is a unique composite, containing

* Author for correspondence (bnmondal@rediffmail.com)

a collagenous hydrogel matrix consisting of about 33–43% apatite minerals, 32–44% organics and 15–25% (Kalita *et al* 2007) water on a volumetric basis. HAp material manufactured from bovine bones has the advantage of inheriting some properties of the raw materials viz. its chemical composition and structure. The synthesized powder from these three different biosources is ball milled for several hours and compacted. The synthesized powder and compacted samples were characterized through thermogravimetric analysis, X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). The morphology of the derived hydroxyapatite powder and their sintering behaviour has also been studied by FESEM. Many processing technologies have been employed to obtain porous ceramics for bone tissue engineering. In tissue repair application the macro pores and highly interconnected networks are required for growth of surrounding of host tissues. The milled powder was mixed with surfactant and finally press injection method was applied to make small diameter fillers. As a first parameter of biocompatibility (Sandeep *et al* 2006), the ability of the cells to attach to the material surface during early period of cell/material interaction was used. Then the powders were screened *in vitro* for cytotoxic (Roy *et al* 2011) effects on cultured RAW macrophage cells. Cell attachment (Van der Stok *et al* 2011) study was observed by scanning electron microscopy. Cytotoxicity effects were analysed by Trypan blue staining method.

2. Experimental

2.1 Synthesis of hydroxyapatite powder from egg shell

Raw egg shells were initially washed with water and then treated with 0.5N HCl (Merck 35%) to separate the membrane. The egg shell powder was dissolved in dilute nitric acid, HNO₃ (Merck, 70%) by warming the solution. The chemical reaction is given below:

Then 0.6 M di-ammonium hydrogen phosphate solution was added drop-wise while stirring for 1 h producing white coloured HAp precipitate. pH of the reaction was adjusted to 9 by addition of dilute ammonium hydroxide solution. The chemical reaction is given below:

The precipitate was thoroughly washed and filtered. The residue was dried in an hot air oven at 100 °C for 12 h and then sintered at 1000 °C.

2.2 Synthesis of hydroxyapatite powder from fish scale

Fish scales were collected from fresh water fish (*Labeo rohita*) and washed thoroughly in running tap water. The

collected scales were initially deproteinized through external washing with 1(N) HCl (Merck, 35%) solution (2:1, v/w, water HCl /fish scale) for 24 h at room temperature (28° ± 2 °C). Next the deproteinized fish scales were washed thoroughly several times with distilled water. Remaining proteins of fish scales were treated with 1(N) NaOH solution. The filtered fish scales were washed thoroughly with distilled water and dried at 60 °C in hot air oven for several hours. Treated fish scales were calcined at different temperatures to synthesize hydroxyapatite ceramics.

2.3 Synthesis of hydroxyapatite powder from bovine bone

Bovine bone were initially deproteinized externally with 1 (N) HCl (Merck, 35%) solution and finally 1(N) NaOH solution was added for removal of remaining proteins. The samples were then thoroughly washed with double distilled water and dried at 50–60 °C in an hot air oven. The dried samples were then calcined in air atmosphere for 1 h at different temperatures viz. 900 °C, 1000 °C, 1100 °C and 1200 °C to obtain HAp phase.

2.4 Preparation of small fillers

The synthesized powder was mixed with starch and milled for 48 h in ball mill. Triton-X surfactant was added drop-wise with the powder to make a paste. Injection press method was employed to make small 2 mm diameter and 4 mm long fillers with this paste. The small rod shaped fillers were then dried at 80 °C for several hours. Finally the dried samples were sintered at 1100 °C for 2 h.

3. Powder characterization

Thermogravimetric analysis of HAp from egg shell, treated fish scale and bovine bone was carried out using a Thermal Analyzer (Netzsch, STA 409) over a temperature range of 30–1250 °C and a heating rate of 10 °C/min under air atmosphere. The crystalline phases of synthesized powders were identified by X-ray diffraction analysis (Shimadzu, XRD-6000, CuKα radiation). FTIR (Perkin Elmer) analysis of calcined powder at different temperatures was carried out to confirm the structural composition. The average particle sizes of powder samples were observed through SEM analysis. The optimized calcined hydroxyapatite powder derived from these three different biosources was wet ball milled for several hours and compacted into cylindrical/square shapes. Transmission electron microscopy (TEM) studies were carried out using JEOL-JEM classic modes such as bright field (BF) to image the texture of the materials (shape, dimension). The samples were prepared using ultra-microtomy to obtain thin samples with a thickness inferior to 80 nm. The preparations were deposited on a copper grid covered by a thin film of amorphous carbon. This protocol was used in order to minimize sample artifacts such as amorphization.

Cytotoxicity and cell attachment studies of synthesized HAP materials were also carried out in RAW macrophage-like cell line media.

4. Results and discussion

4.1 TG DTA analysis

Thermogravimetric plot for synthesized HAP powder from egg shell is shown in figure 1. HAP materials prepared via low temperature wet-chemical processes are known to accommodate various ionic species like H_3O^+ , HPO_4^{2-} and they decompose in a variety of ways. There is around 7.56% weight loss upon heating up to 300 °C due to the elimination of weakly entrapped adsorbed water in HAP powder. Weight loss around 3.41% between 300 °C and 700 °C corresponds to HPO_4^{2-} decomposition which may be present in the chemically synthesized powder (Fung 1993). In the range 700–800 °C, weight loss was due to the removal of interstitial water and decomposition of $\text{P}_2\text{O}_7^{4-}$. However, there was only 0.36% weight loss on further heating up to 1250 °C. Total mass loss of about 11.33% was obtained for HAP powder leaving 88.77% of residual mass. DTA plot shows a broad dip in the range of 300–600 °C corresponding to the decomposition of HPO_4^{2-} . The exothermic peak at a range of 680–810 °C is associated with complete crystallization of synthesized hydroxyapatite powder.

The thermogravimetric analysis of chemically treated fish scales were carried out between 35 °C and 1250 °C in air at a heating rate of 4 °C/min as shown in figure 2. In this analysis actual weight losses (Mortier *et al* 1989; Ozawa and Suzuki 2002) are observed in three steps and the related

temperature range is 30–800 °C. The first loss is due to the weakly entrapped water in the sample material, second and third losses resulted from the decomposition and burning of organic components in the raw scales. As the washing treatment could not completely remove the organic compounds of the inner parts of scale structure, the large weight loss resulted mainly from those organics. However, there was minor weight loss on heating up to 1250 °C which indicates thermal stability of the sample in this range. DTA plot shows a sharp exothermic peak in the range from 480–800 °C, which is due to the crystallization of synthesized powder.

The thermogravimetric analysis (figure 3) of chemically treated bovine bone was carried out from room temperature to 1250 °C in air atmosphere at a heating rate of 4 °C/min. In this analysis actual weight losses were observed with rising temperature. The first loss is due to the removal of trapped water in the sample material, second and third losses resulted from the decomposition and burning of organic components in the bovine bone constituents. However, there was minor weight loss which occurred on heating up to 1250 °C. DTA plot shows a broad exothermic peak in the range from 500–1000 °C, which is due to the crystallization of synthesized powder similar to fish scale derived hydroxyapatite. The endothermic peak around 150 °C is due to loss of incorporated water and decomposition of organic matter of the bovine bone.

Table 1 shows comparative mass loss study of HAP preparation from three different sources. The first two major weight losses in the ranges of 35–200 °C and 200–500 °C were observed in fish scale due to presence of trapped water molecules, organic particles such as connective tissue proteins, collagen etc. In case of egg shell derived HAP powder no such weight loss occurs due to organic materials as it

Figure 1. TG DTA of egg shell derived HAP powder.

is a wet chemical precipitation method where major organic materials are previously removed by chemical treatment. In case of bovine bones the major part of bone is made up of hydroxyapatite materials. For this reason moderate weight loss occurs in respect of the fish scale.

4.2 XRD analysis

The crystalline phase analysis of HAp powder from three different biosources was carried out by X-ray diffraction studies. Figure 4 shows XRD pattern of synthesized HAp

Figure 2. TG DTA of fish scale.

Figure 3. TG DTA of bovine bone.

from egg shell, fish scales and cow bone, respectively. Identification of the phases was realized by comparing the experimental XRD pattern to standards compiled by the International Centre for Diffraction Data (ICDD) using the cards 00-009-0432 for hexagonal HAp structure. Each pattern showed HAp as the only phase. Well resolved characteristic peak of highest intensity for HAp was obtained at a 2θ value of 31.77° corresponding to 211 plane in all the cases. The phase formed is pure and matches well with standard pattern. Sharp peak intensity and well resolved peaks in XRD patterns of different synthesized powders proves complete crystallization.

The crystallite sizes of HAp particles were calculated using Scherrer's equation

$$D = 0.9 \lambda / B \cos \theta ,$$

where D represents mean grain size, B stands for full width at half maximum of the peak, λ the diffraction wavelength (0.154059 nm) and θ the diffraction angle.

The Bragg reflection at (002) planes of HAp was considered to calculate the crystallite size. Table 2 shows crystallite size for HAp synthesized from egg shell, fish scales and bovine bone, respectively. It has been observed in all cases that HAp crystallite structure is predominant. From XRD characterization, the crystallite sizes of HAp synthesized from three different biosources minutely differed. However,

some detailed investigations are required for finding out differences of preferred method.

4.3 FTIR analysis

Fourier transform infrared (FTIR) spectroscopy was employed to characterize different functional groups of hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (HAp) powder obtained from three biosources. Figure 5 shows FTIR spectrum of synthesized HAp from egg shell, fish scales and cow bone, respectively. The spectrum was recorded in the range of $4000\text{--}400\text{ cm}^{-1}$. The representative FTIR spectrum shows all characteristic absorption peaks of hydroxyapatite. The first indication for formation of hydroxyapatite is in the form of a strong complex broad FTIR band centred at about $1000\text{--}1100\text{ cm}^{-1}$ due to asymmetric stretching mode of vibration for PO_4 group (Rocha *et al* 2007). The band at 576.30 cm^{-1} corresponds to n_4 symmetric P–O stretching vibration of PO_4 group (Varma and Babu 2005). As a major peak of phosphate group, the n_3 vibration peak could be identified in the region between 1100 and 960 cm^{-1} for all three powders which are due to P–O asymmetric stretching of PO_4^{3-} . The band at $2005\text{--}2079\text{ cm}^{-1}$ is due to overtone of 1040 cm^{-1} band. The presence of peak in the region $1400\text{--}1450\text{ cm}^{-1}$ was due to absorbed carbon dioxide. The crystalline powder

Table 1. Comparative mass loss study of HAp preparation from three different sources.

Temperature range	Egg shell derived HAp	Fish scale	Bovine bone
35–200 °C	–4.70%	–14.54%	–7.55%
200–500 °C	–3.05%	–50.79%	–25%
500–800 °C	–1.67%	–5.20%	–4.61%
800–1100 °C	–0.69%	–4.72%	–5.67%

Figure 4. XRD pattern of HAp synthesized from egg shell, fish scale and bovine bone.

Table 2. Crystallite size of hydroxyapatite powder synthesized from egg shell, fish scale and bovine calcined at different temperatures.

Serial no.	Natural source for HAp synthesis	Crystallite size
1.	Eggshell	78.31 nm
2.	Fish scale	75.36 nm
3.	Bovine bone	77.32 nm

Figure 5. FTIR spectrum of hydroxyapatite powder synthesized from egg shell, fish scale and bovine bone.

Figure 6. FE-SEM of HAp compact synthesized from eggshell.

Figure 7. FE-SEM of HAp powder synthesized from fish scale.

Figure 8. FE-SEM of HAp powder synthesized from bovine bone.

Figure 9. TEM of HAp synthesized from eggshell.

Figure 10. TEM of HAp compact synthesized from fish scale.

Figure 11. TEM of HAp synthesized from bovine bone.

Figure 12. Bio fillers and SEM photomicrograph.

Figure 13. Control RAW cell media.

Hydroxyapatite particles in RAW cell media

Figure 14. Eggshell derived hydroxyapatite particle in RAW cell media.

Hydroxyapatite particles in RAW cell media

Figure 15. Fish scale derived hydroxyapatite particle in RAW cell media.

generates two characteristic stretching modes of O–H bands at about 3497 cm^{-1} and 456 cm^{-1} which are noticed in all FTIR spectra of HAP.

4.4 SEM analysis

Figures 6–8 show SEM micrographs of hydroxyapatite samples. Images showed that biocompatible (Liao *et al* 1999) composites consisted of a highly porous network structure in the range between $15\text{ }\mu\text{m}$ and $20\text{ }\mu\text{m}$. SEM micrographs of synthesized HAP powder as depicted in figures 7 and 8 from egg shell, fish scale and bovine bone sources, respectively were soft agglomerated ultrafine HAP particles which break up easily during compaction due to high uniform pressure. All the samples show noticeable porosity.

Ca/P ratio in all samples was closer to stoichiometric ratio of Ca and P (1.67) in the pure hydroxyapatite powder (Mostafa 2005) which again was confirmed through chemical determination.

4.5 TEM analysis

TEM images show characteristics of synthesized HAp powder (figures 9–11) from egg shell, fish scale and bovine bone sources which also showed HAp were granular in shape with dimension of approximately 80–90 nm for these synthetic hydroxyapatite particle.

4.6 Bio fillers made up of HAp powder

The synthesized powder was wet ball milled for several hours and by using surfactants like Triton-X small fillers were made for bio-implants. Figure 12 shows filler structure and

their SEM micrograph which reveals the porous structure. These small fillers were then heated up to 1200 °C temperature and prepared for *in vivo* bio-implantation and subsequent toxicity testing.

4.7 In vitro analysis

Hydroxyapatite powder samples as well as fillers and plates were utilized for further *in vitro* testing. First of all the samples are sterilized by autoclaving at 16 lb/inch² pressure for 20 min. Then the samples were placed into an RPMI culture media. Here RAW cell lines were utilized for experimental purpose. Cell loads are given at 10⁵ cells/200 micro litre. After incubation in cell culture media samples are stained and observed under inverted microscopy. Figure 13 shows control cells attached in one of the 6 well plates culture vessels. Figures 14–16 show HAp particles incubated for 72 h along with macrophage like RAW cell lines. The cultured cells are stained with Trypan Blue and the result shows that there are no dead cells in the culture vessels. It confirms that there is no toxic effect of the synthesized HAp materials.

4.8 Cell attachment

Bio fillers are selected for *in vitro* cell attachment studies. Bio fillers are sterilized and then RAW cell lines are used in RPMI cell culture media for this study. After 72 h of incubation, samples cell culture media is decanted and washed thoroughly with PBS. After washing, 2% paraformaldehyde solution were utilized for cellular fixation. Figure 17 shows SEM image of attached cells on the filler surface. This study reveals that cells can be attached on the hydroxyapatite surface which would be utilized as scaffold material for tissue engineering (Ikada 2006) application.

Hydroxyapatite particles in RAW cell media

Figure 16. Bovine bone derived hydroxyapatite particle in RAW cell media.

Figure 17. SEM image of attached cells at HAp surface.

5. Conclusions

The present study reveals that hydroxyapatite powder can be synthesized by wet-chemical route and thermal decomposition using egg shell, fish scale and bovine bone in a cost effective way. FTIR and X-ray diffraction analyses indicated the phase purity and crystallinity of HAp powder. TGA analysis results showed thermal stability of HAp derived from three different biosources. The microstructural study of compacted powders show the presence of uniformly distributed very fine sub-micron particles. The SEM image of HAp ceramics prepared from these routes are found to exhibit 9–12% porosity, which could be useful for biomedical applications as fillers or scaffold. TEM analysis showed HAp were granular in shape. The small biocompatible osteoconductive fillers appear to have great potential for bone tissue engineering as they showed no toxic effect on cell culture studies and also have good affinity of cellular attachment on the developed material surface. More systematic studies are required for *in vivo*, assessments of bio-ceramic composite scaffolds for bone tissue engineering.

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