Synthesis and characterization of etidronic acid (ETD) grafted nano sheets of hydroxyapatite

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Summary

The surface of prepared nano hydroxyapatite (nHA) has been changed into nano sheets by grafting the etidronic acid (ETD) in aqueous media. The modified nano hydroxyapatite was further subjected to sintering at various temperatures i.e. 60, 400, 600, 800°C to obtain different particle size (10-100nm). Nano-sheets of hydroxyapatite was characterized using FTIR, XRD, TEM, SEM. FTIR results confirmed the presence of etidronate fragment bounded to the hydroxyapatite structure and the change in its morphology from cylindrical/grain to sheet was evidenced by TEM and SEM. XRD analysis shows that HA particles are crystalline in nature and the structure of hydroxyapatite does not change after surface modification.

KEY WORDS: etidronic acid; hydroxyapatite (HA); SEM; TEM; contact angle.

Introduction

Thermally stable hydroxyapatite is being widely used for the synthesis of polymer composites. Hydroxyapatite (HA) is most interesting ceramics due to its excellent mechanical properties, osteoconductivity, biocompatibility and its chemical and structural properties are very similar to the mineral constituents of human bones (1). Because of its colloid stability, HA having several problems in the synthesis of HA based polymeric materials for bone tissue engineering applications. Agglomeration tendency of nano particles in suspension is one of the most common problems that make it difficult to synthesis of polymeric materials with high density of nano particles as filler dispersion (2). Aggregates of nano particles in composite materials are increase the possibility of mechanical and chemical properties failure. Having good dispersion of filler in polymer matrix is increase the interfacial bond between polymer and inorganic reinforcing material (3). Ultrasonication and mechanical stirring are two most techniques have been used to reduce agglomeration during sample preparation of HA. However, both techniques are less effective and temporary (4). Only chemical methods can provide permanent solution to solve dispersion problem by altering of HA surface. It has been found that two functional groups ≡Ca-OH and ≡P=OH presents on active sites of hydroxyapatite surfaces for further modification (5). Surface modifications is an effective technique to further enhance the biocompatibility which significantly improves and accelerate invasion of bone tissues in scaffolds (6). The surface of hydroxyapatite can be modified with various organic substances consisting of bisphosphonate group because of improved interaction between calcium or phosphate ions on the apatitic surface and the anionic groups of the organic molecule (7). Bisphosphonates are used in the treatment of a variety of bone diseases which are associated with high bone resorption, such as metastatic bone disease, paget’s disease, and osteoporosis as the two phosphonate groups in its structure (P-C-P) helps in binding of calcium on hydroxyapatite (8). Several studies on the modification of hydroxyapatite using different compounds containing bisphosphonate groups such alendronate (9), zoledronate (10), risedronate (11) and clodronate (12) have been reported. Among these etidronate [disodium (1-hydroxyethylidene) bisphosphonic acid] is now the most widely used compounds for the treatment of osteoporosis (13). Etidronate display a common backbone structure of P-C-P with the two phosphonate groups such alendronate (9), zoledronate (10), risedronate (11) and clodronate (12) have been reported. Among these etidronate [disodium (1-hydroxyethylidene) bisphosphonic acid] is now the most widely used compounds for the treatment of osteoporosis (13). Etidronate display a common backbone structure of P-C-P with the two phosphonate groups helping in binding of calcium to hydroxyapatite (14, 15). Modified hydroxyapatites possess properties such as excellent surface morphology, particle size and real microstructure. Optimizing these properties is of crucial importance for their applications in tissue engineering.

The aim of this research was to enhance the biocompatibility of HA by its surface modification with etidronic acid using precipitation technique. The modified HA was sintered at different temperatures i.e. 60, 400, 600 and 800°C to obtain mHA with different crystallite sizes and morphologies. The effect of sintering on chemical, structural and morphological properties of the modified HA was studied.

Materials and method

Materials

All chemicals were purchased of AR grade from commercial sources. Calcium nitrate tetra hydrate (Ca(NO3)2•4H2O) (99%) and potassium dihydrogenphosphate (KH2PO4) (99%) were
procured from Merck India Ltd. Liquid Ammonia 25% concentration and Etidronic acid (60% aqueous solution) were purchased from Rankem India and Sigma-Aldrich, respectively.

**Synthesis of nano Hydroxyapatite (HA) ceramics**

Hydroxyapatite was synthesized according to a well established sol gel method using calcium nitrate and potassium dihydrogenphosphate used as calcium and phosphorus precursors, respectively. Solution of calcium nitrate was added drop wise to a solution of potassium dihydrogenphosphate under stirring. The molar ratio of the Calcium nitrate and potassium dihydrogenphosphate was 1.67 and the pH was maintained at 10 using liquid ammonia. Gel obtained after precipitation was aged for 24 hrs and then centrifuged 3-4 times at 3000 RCF with double distilled water which was then dried overnight at 60°C in a vacuum oven.

**Grafting of etidronic acid and sintering of Hydroxyapatite**

The surface of the above prepared hydroxyapatite was modified using a bisphosphonate compound i.e. etidronic acid. An etidronate solution of 0.1M concentration in double distilled water was used for modification. 0.5 gm of prepared hydroxyapatite and 100 ml of 0.1M etidronate solution was mixed using mechanical stirring for 48hrs at 25±2°C temperature and pH was maintained using ammonia solution. Mixture so obtained was ultra centrifuged 3-4 times at 6000 RCF in double distilled water for 10 minutes to remove the unreacted etidronic acid and ammonia. The obtained composite material was dried in vacuum oven at 60°C for 24 hrs. Finally the surface modified hydroxyapatite was sintered at different temperatures 400, 600 and 800°C in electrical furnace in air with heating rate of 10°C/min to obtain different particle size. Based on sintering temperature, samples were designated as SF60, SF400, SF600 and SF800. Scheme for surface modification of hydroxyapatite by grafting of etidronic acid is shown in Figure 1.

**Characterization of Hydroxyapatite nano sheets**

**Fourier Transform Infrared Spectroscopy (FTIR)**

The chemical structures of unmodified, modified and modified sintered hydroxyapatite were characterized by Fourier transform infrared spectroscopy (FT-IR) in Perkin Elmer RZX spectrometer using KBr technology. FTIR spectra were recorded in a spectral range of 4000-450 cm⁻¹ with a resolution of 2 cm⁻¹ with two scans for each sample.

**X-ray diffraction**

Phase analysis was performed by X-ray diffractometer (XRD) using a Philips X’Pert Pro X-ray diffractometer system. The radiation was Cu Ka (λ = 0.15406 nm) with 40 kV voltage and 40 mA intensity. The mean crystallite size (D) of the particles was calculated from the XRD line broaden measurement using the Scherrer equation, equation 1)

\[ D = \frac{0.9λ}{β \cos θ} \]

Where λ is the wavelength (Cu-Ka), β is the full width at the half maximum of the HA (211) line and θ is the diffraction angle.

The fraction of crystalline phase (Xc) of the HA powders was evaluated by equation 2)

\[ Xc = \frac{1 - \nu_{112/300}}{300} \]

Where I300 is the intensity of (3 0 0) diffraction peak and ν112/300 is the intensity of the hollow between (1 1 2) and (3 0 0) diffraction peaks.

**Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)**

The morphology of unmodified, modified and sintered modified hydroxyapatite was examined using transmission electron microscope (TEM) and Scanning Electron Microscope (SEM). TEM was also used to determine particle size. Model Hitachi-2100 was used for TEM and Images were taken at 80 kV accelerating voltage. A drop of a dilute sample in suspension was deposited on the carbon-coated grids and allowed to dry at room temperature. Particle size distribution of unmodified, modified and modified sintered hydroxyapatite in TEM images was undertaken using a UTHSCSA Image Tool image analyzer program (IT version 3). The images were loaded into the software and particle size measured using a two point measuring analysis. The scale of the soft-
ware was calibrated using the scale bars on each TEM image (given below each TEM image). Approximately, 300 measurements were taken to obtain the distribution. Powder of unmodified, modified and modified sintered hydroxyapatite was gold coated in order to make the surface conductive.

**Contact angle measurement**
Contact angles of unmodified, modified and sintered modified hydroxyapatite were measured on DSA 10 Mk 2 (Kruess) equipped with a video-imaging system. Sessile deionized-water drops were placed on the surface in the ambient environment, with a drop volume of 5μL. Images were recorded after every 3 minutes and 5 images every second were taken with a video system. Contact angle values were calculated using the drop shape analysis system (DSA 1) and selected at 10 s.

**Wavelength Dispersive X-ray Fluorescence (WD-XRF)**
The elementary composition and Ca/P ratio of unmodified, modified and sintered hydroxyapatite was determined with Wavelength Dispersive X-ray Fluorescence-S8 Tiger from Bruker (WD-XRF), Germany.

**Results and discussion**

**FTIR analysis**
FTIR spectra of unmodified, surface modified sintered hydroxyapatite at various temperatures, i.e. 60, 400, 600 and 800°C, designated as HA, SF60, SF400, SF600 and SF800, respectively are shown in Figure 2. The spectrum of unmodified HA consists characteristic peaks of \((PO_4)^{3-}\) group near wave number 1023-1047 cm\(^{-1}\), 961 cm\(^{-1}\), 560-599 cm\(^{-1}\) and 474 cm\(^{-1}\) (16). The two weak peaks near wave number 1419 cm\(^{-1}\) and 1455 cm\(^{-1}\) also confirms the presence of traces of carbonate in pure HA during the synthesis of via sol gel, calcium monophosphate and calcium dehydrates are formed which disappear during sintering. The peak near wave number 875 cm\(^{-1}\) belongs to \((HPO_4)^{2-}\) groups due to protonation of phosphate groups present in pure HA (18). A new absorption band at 810-820 cm\(^{-1}\) corresponding to v (P- C- P) group is seen in all the spectra of modified HA. A peak attributing to v (P- O) group at 920-996 cm\(^{-1}\) is observed in modified HA samples while absorption band of v (P -O) group confirming the surface modification is seen at 1150-1160 cm\(^{-1}\). The formation of these bisphosphonate groups in the modified samples confirms the reaction of etidronic acid with hydroxyapatite. The weak bands near 3000 and 3200 cm\(^{-1}\) are attributed to the C- H and the O- H stretching vibration mode of etidronates (19). The formation of these band in the modified samples further indicates grafting of etidronic groups i.e. etidronates on the HA surface. The FTIR spectra further illustrates the effect of sintering of HA at higher temperatures. The new bands appeared in the modified HA at 810-820, 920-996 and 1150-1160 cm\(^{-1}\) increased in intensity with increase in temperature from 60 to 800°C. However, the intensity of band near wave number 875 cm\(^{-1}\) belonging to \((HPO_4)^{2-}\) group, remained conserved at higher sintering temperatures (20). It indicates that the organic moieties have
not interacted by \((HPO_4)^{2-}\) groups, particularly the P-OH active sites, but etidronates are superficially attached with hydroxyapatite via covalent bonding (21).

**XRD analysis**

The X-ray diffraction profiles of unmodified and modified hydroxyapatite (HA) sintered at different temperatures i.e. 400, 600 and 800°C are shown in Figure 3. The XRD spectra of modified hydroxyapatite sintered at different temperatures shows that all the samples are in structural resemblance with that of pure HA material. There is no significant phase change in HA after modification with etidronic acid. From Figure 3 it is revealed that as the sintering temperature increased from 60 to 800°C, several of the HA peaks become more distinct and narrower signifying an increase in the crystallite size and crystallinity confirming the important role of sintering. Table 1 shows value of crystallite size (CS) and crystalline index (CI) calculated for different samples. The crystallite size and crystalline index increased tremendously for SF400, SF600 and SF800 samples. For pure HA crystallinity index is about 39% and crystallite size is about 10nm, while for the modified HA sample SF800, crystallinity index is about 90% and crystallite size about 91nm as depicted in Table 1 and also confirmed by the TEM results.

**TEM and SEM analysis**

TEM images of unmodified and modified hydroxyapatite sintered at various temperatures are shown in Figure 4. From these images it is clear that all samples possess crystalline rod like morphology. From Figure 4a, it is depicted that pure HA are individual particles with irregular rod like shapes. When the HA particles are modified with etidronic acid, the particle morphology steadily changes from rod like to plate type with increase in particle size. This continues till sintering temperature of 600°C but beyond this temperature the plates start diffusing to small particle with tendency of agglomeration that can be clearly seen in Figure 4e.

From SEM micrographs shown in Figure 5 it was evident that modification of HA followed by sintering results in small particles that have tendency to agglomerate. This is the most important morphological characteristic which is appropriate for its use as a bioactive element in tissue engineering. The structure of human bone and teeth represents similar morphologies and hence such a characteristic provide a better biocompatibility with human bone. The modification of nano HA with etidronic acid further provide enhanced mechanical strength due to its high specific surface area, superior defect free chemical structure and improved resemblance with the human bone due to its modification at micro level (22, 23). The morphology of modified HA samples shows that the crystallite size of the nanoparticles increased from 10 to 100 nm with increasing sintering temperature from 60 to 800°C. This is because etidronic acid increased the surface reactivity of the individual HA nanoparticles thus promoting the formation of layer particles which is also the reason for agglomeration (19). The change in morphology with sintering temperature may also be attributed to the relative specific surface energies of modified HA. Best results were obtained for sample SF-600 as it shows plate morphology of HA resembling the structure of bone. These results are in coherence with results of TEM.

**Contact angle analysis**

The contact angle characterizes the wettability of the surface of a solid by a liquid i.e. the interaction between a solid and a liquid surface at the interface. It is a highly desirable parameter for loading of drug to the ceramics and delivering to the desired target. The contact angles of unmodified and modified hydroxyapatite (HA) sintered at different temperatures i.e. 400, 600 and 800°C are shown in Figure 6. No significant differences were demonstrated in contact angles and were below 40˚ revealing the hydrophilic behavior thus allowing the loading of drug to the ceramics and delivery of drug by aqueous solution incubation (14).

**WD-XRF analysis**

The chemical composition, Ca/P ratio and percent carbon of unmodified and modified HA sintered at different temperatures determined using WD-XRF (Wavelength Dispersive X-ray Fluorescence) are given in Table 1. From the data in the Table 2 it is clear that etidronic acid did not significantly affect the stoichiometry of hydroxyapatite and the Ca/P molar ratio for modified HA is close to the stoichiometry value of...
1.67±0.02 (24). The incorporation of etidronic acid in HA structure is also confirmed by the total carbon analysis, showing almost constant amount of the total carbon ~0.90% in the powder while the total amount of carbon in unmodified HA is less than 0.30.

Conclusions

The overall purpose of the study was to modification of HA nanoparticles using etidronic acid. Modification of nano-hydroxyapatite was successfully done by grafting of etidronic acid.
acid on the surface of HA as observed in XRD and FTIR results. The chemical compositions and structural study showed undisturbed stoichiometry and phase stability of surface modified HA which is a good indication. Further, morphology and particle size studied using SEM and TEM technique confirmed improvement in agglomeration tendency, crystallite size and shape of HA nanoparticles. The study revealed that SF600 sample modified at 600°C showed plate type shape with high agglomeration tendency and can be best used in tissue engineering. Introduction of bisphosphonate groups in HA nanoparticles resulted in its enhanced surface area along with high reactivity. The surface modifying agent used in this study can act as an interface providing a strong bond between HA and polymer matrices that can possibly improve bioactivity of scaffolds in bone tissue engineering.

Figure 5 - SEM micrographs of unmodified and modified hydroxyapatite sintered at various temperatures (a) HA (b) SF60 (c) SF400 (d) SF600 (e) SF800.
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References


Table 1 - Crystallite size, crystallinity, Ca/P ratio and total % carbon of unmodified and modified hydroxyapatite sintered at various temperatures.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sintering Temperature (°C)</th>
<th>Crystallite size (nm)</th>
<th>Crystallinity (%)</th>
<th>Ca/P ratio</th>
<th>Total %C</th>
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<td>80</td>
<td>1.71</td>
<td>0.86</td>
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<tr>
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<td>~91</td>
<td>90</td>
<td>1.72</td>
<td>0.85</td>
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</tbody>
</table>

Figure 6 - Contact angle images of unmodified and modified hydroxyapatite sintered at various temperatures (a) HA (b) SF60 (c) SF400 (d) SF600 (e) SF800.