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Research of Preparation Conditions for Regeneration of Hydroxyapatite and Influence on Crystalline Forms

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Abstract

The process of hydroxyapatite (HAp) precipitation in ionic liquid (ChCl-urea) was studied and the influence of various factors on the crystalline forms and yields of HAp were analysed. It was concluded that Na-citrate made the regenerated HAp appear as a long cylindrical crystal (perfect rod-like grain) when chosen to be an additive/surfactant. The optimum conditions for HAp precipitation (regenerated HAp) were applied to extract HAp from chicken bone in ChCl-urea (product HAp). The product HAp was characterized by FTIR, SEM XRD and Particle Size Analyser. The results showed that there is no obvious difference between HAp extracted from chicken bone (product HAp) and regenerated HAp regarding crystalline forms and compositions. The product HAp is relatively pure, and its average particle size is 555.6 nm, meaning that this product has the application value.

Keywords

Hydroxyapatite (HAp), ionic liquid, dissolving process, optimum conditions, particle size

1 Introduction

Hydroxyapatite (HAp), which has good biological activity and compatibility, is an important inorganic component in human and animal bones. It is an ideal bone substitute and can be used as a filling material for bone damage.[1,2] The breakthrough research of tissue engineering and biomaterials cannot be done without nano-HAp. At present, the nanometer HAp has been very important in various fields.[3–5] Researchers have begun to seek a technology for the preparation of natural HAp in response to the advocacy of "green chemistry".[6] Therefore, it has become a hot topic of researchers to choose a "green and efficient" solvent for the dissolution of animal bones in order to obtain Hap.[7,8] Ionic liquids are "green and pollution-free" compared to traditional organic solvents, and are widely used in organic synthesis, catalysis, separation and purification, electrochemistry and nanometer materials.[9–11] HAp with different crystal morphology, shape and size has different effects in the field of medical biology. HAp with different crystal shapes can be gained through exploring various influencing factors, and finding the optimum conditions. These optimum conditions can be applied to the preparation of hydroxyapatite. The aim of this work was to study the recrystallization process of HAp dissolved in a deep eutectic ionic liquid (ChCl-urea), and the influences of various factors on the crystalline forms of regenerated HAp. These conditions were extended to extract product HAp by dissolving chicken bone in ChCl-urea. The product HAp was characterized by FTIR, SEM XRD, and Particle Size Analyser.

2 Experimental

2.1 Materials

Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) of medical grade was provided by China Xi'an Ruiying Biotechnology Co. Ltd.; choline chloride ($ChCl,C_5H_{14}ClNO$) of food grade was provided by China Tianjin Shiyuan Biotechnology Co. Ltd.; urea (CN_2H_4O), sodium citrate (Na-citrate), polyethylene glycol (PEG 600) and sodium dodecyl benzene sulfonate (LAS) of analytical regent (AR) grade were purchased from Tianjin Chemical Reagent wholesale department.

Lab preparation: ionic liquid (ChCl-urea): 1 : 2 (mole) ChCl and urea were added to a 3-mouth flask, and then placed in a thermostatic water bath with a multi-function electric mixer. This reaction was kept at 80 °C for 2 h. Finally, it became a deep eutectic transparent ionic liquid ChCl-urea. It is a colourless viscous liquid at room temperature.[12] The equation of the synthesis of ion liquid (ChCl-urea) is as follows:

$$[HOC_2H_4N(CH_3)_3]^+[Cl]^- + 2NH_2CONH_2 \leftrightarrow \leftrightarrow [HOC_2H_4N(CH_3)_3]^+ + (NH_2CONH_2)_2[Cl]^-$$

Chicken bone powder (40–60 mesh): the meat residue, fat and connective tissue on fresh chicken bones were

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Table 1 – Effects of additives on regenerated HAp

Additives	Na-citrate		Peg-600		LAS	
dosage/%	0.2	0.5	0.2	0.5	0.2	0.5
crystalline form	rod-like	rod-like	tetrahedral	tetrahedral	irregular	irregular
yield/%	30.89	20.08	21.94	23.69	31.35	26.58

removed and washed, then heated until boiling and kept at 70–80 °C for 4 h. Thereafter, bone oil and bone marrow were wiped off, dried in a vacuum vent 60 °C for 12 h, and shattered with a high-speed multi-function crusher.

2.2 Regenerated process of HAp

1 g HAp and 40 g ChCl-urea were added into a 3-mouth flask, which was fixed in a thermostatic oil bath with hot magnetic stirring. The dissolution process was kept at 100 °C for 4 h. After cooling for certain time at room temperature, the mixture was separated by a centrifuge. (1) The clear liquid was the solution of dissolved HAp and ChCl-urea. It was heated continuously in the oil bath until 100 °C and kept for a while, then removed from the oil bath and cooled slowly. It was divided averagely into several portions, and precipitant water (1 : 1 $g g^{-1}$) was added (for subsequent experiments). The precipitated HAp was set aside for 24 h and filtered using a biofilm of 0.22 microns diameter, then washed three times with deionized water and dried in vacuum oven at 60 °C for 24 h. The HAp precipitated accordingly was designated as regenerated HAp. (2) The sediment from centrifugal precipitation was washed and filtered using filter paper of medium pore size and dried under the same conditions. This was undissolved HAp.

2.3 HAp prepared by dissolving chicken bone in ionic liquid

Product HAp was extracted by dissolving chicken bone powder in ChCl-urea under the optimum technique conditions of regenerated HAp, which was obtained from 2.2.

2.4 FTIR, SEM, XRD and Particle Size Analysis

Infrared spectrum of product HAp was obtained using KBr pellets, and recorded on NICOLET6700 Fourier infrared spectrometer. The crystalline forms of HAp were observed by SEM (JSM-6380LV), as well as a Universal Research Microscope (OLYMPUS, U-CMAD3). Numbers of complete clear grain shapes were used as the evaluation index. XRD surveys crystalline form changes of product HAp. The particle size distribution of HAp was determined by Zeta particle size analyser (Delsa Nano C).

3 Results and discussion

3.1 Experimental analysis of regenerated HAp

3.1.1 Effects of additives on crystalline forms and yields of regenerated HAp

Additives, which are divided into non-ionic, cationic, and anionic type, can have different effects on the structure, dispersion, and morphology of crystal. Effects of additives on crystalline forms and yields of regenerated HAp are shown in Table 1. It can be seen that the crystalline form is rod-like when the additive is Na-citrate; it presents tetrahedral shape for PEG 600, and it is irregular for LAS. Na-citrate (cationic) presents alkalescence, and its function is to prevent HAp from being reunited, *i.e.*, the crystal is dispersed. Therefore, the right amount of surfactant Na-citrate can allow the regenerated HAp to present a long cylindrical crystal or a perfect rod-like crystal. PEG 600 (non-ionic) can interact with calcium ions when they coexist simultaneously. It will distract crystals as it is added in nucleation stage, and probably promote the growth of HAp, which improves crystal regularity. LAS (anionic) lowers the crystalline degree of HAp, thus crystal growth is slow, and crystalline form incomplete.[13,14]

Fig. 1 shows the crystalline forms of regenerated HAp under a Universal Research Microscope, magnified 500 times. Additives as surfactants can promote the crystallization of HAp. Different additives make the crystal direction of particles different, thus the crystalline forms of HAp are diverse. Obviously, the additive Na-citrate improves the rod-like shape of HAp crystalline forms.

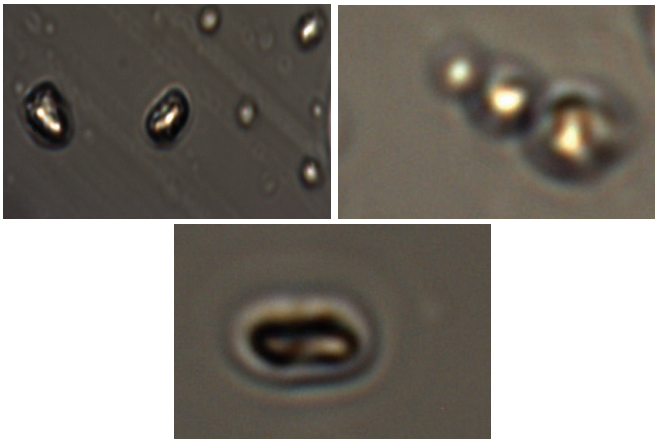

Fig. 1 – Crystalline forms of Na-citrate (left), Peg-600 (middle), and LAS (right) as additives

Table 2 – Basic factors of orthogonal experiment conditions

Factors	Dosage/%	Acoustic wave/dB	pH
1	0.2	80	7
2	0.4	100	8
3	0.6	120	9

Table 3 – Orthogonal experimental data

Factors	Additive	Acoustic wave	pH	No. of rod-like grain	Yield/%
1	1	1	1	8	20.98
2	1	2	2	12	23.50
3	1	3	3	7	17.82
4	2	1	2	4	29.42
5	2	2	3	6	25.58
6	2	3	1	5	19.20
7	3	1	3	4	21.10
8	3	2	1	6	18.19
9	3	3	2	10	18.84
K1	9	5.33	4.67		
K2	5	8	8.67		
K3	6.67	7.33	5.67		
range R	4	2.67	3.92		
results	1	3	2		

3.1.2 Orthogonal experiments

The orthogonal conditions are presented in Table 2. Table 3 gives the orthogonal experimental data on different dosages of additive Na-citrate.

The extreme difference (range R) indicates that the additive dosage has the greatest influence on crystallization. Therefore, the optimum preparation conditions of HAp are Na-citrate (w = 0.2 %), acoustic intensity 120 dB, pH = 8.[15,16]

3.1.3 Validation experiments

Verification experiments were conducted three times in parallel under optimum conditions. The results show that the number of rod-like grain is relatively stable, and its average reaches 9. This suggests improved reproducibility of the optimum preparation technique conditions for HAp.

3.2 Characterization and performance analysis of product HAp

Product HAp was extracted by dissolving chicken bone powder in ChCl-urea under the optimum conditions mentioned previously.

3.2.1 HSPOM Analysis

The dissolving situation was observed by means of HSPOM (thermal stage polarized optical microscope). HSPOM pictures indicate that ChCl-urea had a certain solubility, and chicken bone power dissolved gradually in ChCl-urea with time. After the dissolution experiment, the yield of HAp was determined at 38.36 %. It can be preliminarily concluded that this dissolving process was a direct physical dissolution.

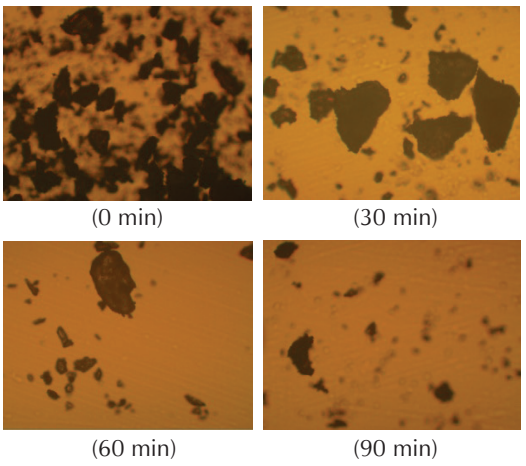

Fig. 2 – HSPOM images of HAp dissolving process

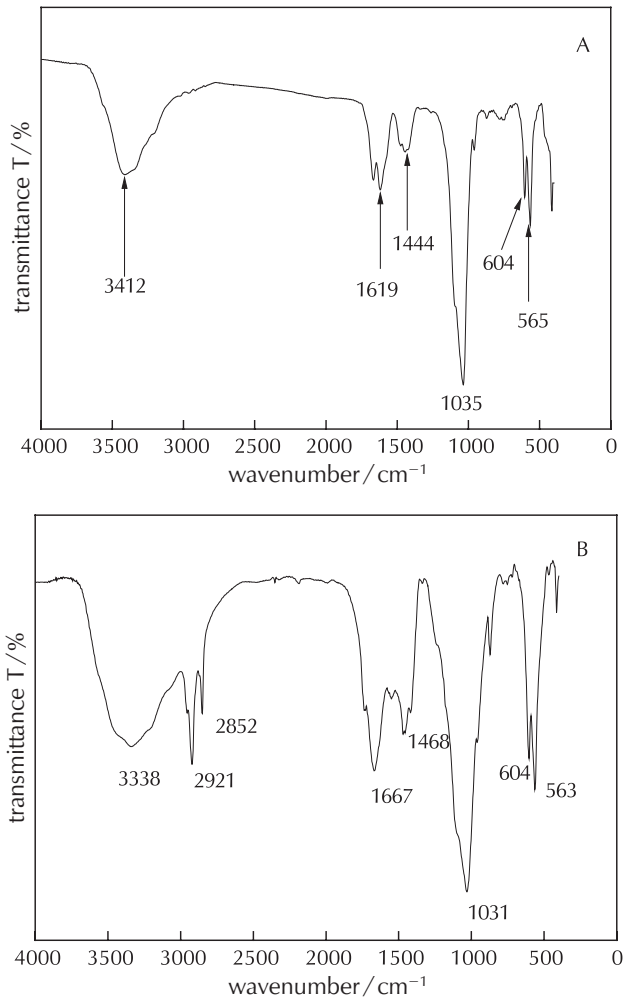

Fig. 3 – FTIR spectra of product HAp (A) and regenerated HAp (B)

3.2.2 FTIR analysis

Fig. 3 presents the IR spectra of product HAp and regenerated HAp. There is a wide absorption peak between 3600 and 3200 cm^{-1}, caused by −OH stretching vibration peak of hydrogen bond association, and absorption peak of adsorption water on the surface of extracted HAp. The weak absorption peak at 1619 or 1667 cm^{-1} suggests that the surface of HAp adsorbed water molecules. The absorption peak at 1444 or 1468 cm^{-1} implies that CO_3^{2-} enters the HAp structure and replaces some PO_4^{3-}. The absorption peaks of carbonate or free radical CO_3^{2-} are single peaks at 1400–1500 cm^{-1}. When the CO_3^{2-} enters into HAp lattice and replaces the PO_4^{3-} group in the lattice, the absorption peaks appeared to split. The strong absorption band at 1035 or 1031 cm^{-1} are stretching vibration peaks of the group PO_4^{3-}. The absorption peaks of 604 and 563 cm^{-1} are related to the bending vibrational absorption peaks of the group PO_4^{3-}. HAp structure of the two samples are basically the same. The only difference is the absorption peak disappearing at 2921 cm^{-1}. This may indicate that the hydrogen bond structure of the product HAp was damaged in the bone dissolving process. IR characterization indicates that the product HAp has application value, *i.e.*, the process of dissolving animal bone in ChCl-urea can be applied for preparation of HAp.

3.2.3 SEM analysis

Fig. 4 shows scanning electron micrographs of the product HAp magnified 5000 and 2000 times, respectively. The two images show that diameters of crystal particles are 5 μm and 10 μm, and the different forms of particles are visible. Most of the particles are micron-sized or even tinier, but their crystal structures are relatively complete. It can be seen that product HAp mostly presents rod-like shape, and crystal structures are relatively complete. However, some of the particles have an irregular shape. That may be due to particle agglomeration or some impurities in the product, such as oil, not removed in the preparation process.

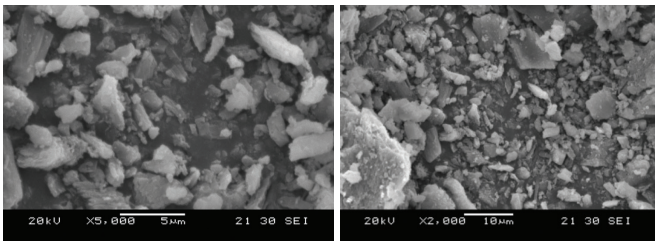

Fig. 4 – SEM image of product HAp

3.2.4 XRD analysis

Fig. 5 shows the XRD diffraction patterns of product HAp (A) and regenerated HAp (B). It explains that their crystalline forms and compositions are the same. There is no other impurity peak, meaning that purity of the product HAp is high. This suggests that crystallinity of product HAp was better in this experiment,[17,18] having the macroscopic composition and microscopic structure of natural bone. Thus, the process of directly dissolving biological bones in ChCl-urea can be used for preparation of HAp.

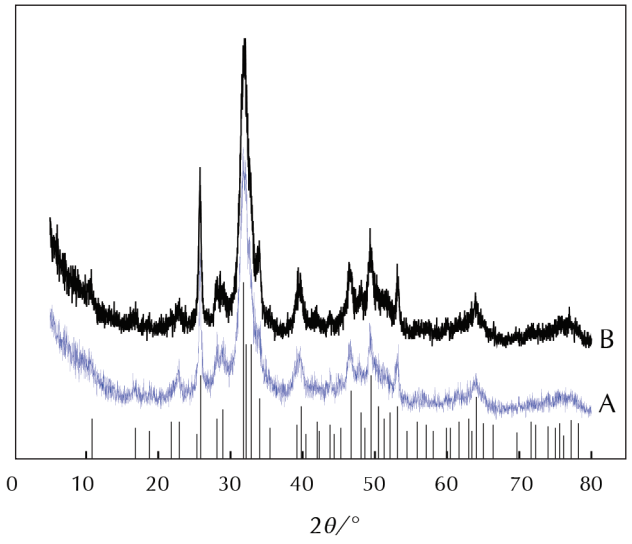

Fig. 5 – XRD pattern of product HAp (A) and regenerated HAp (B)

3.2.5 Particle Size Analysis

According to the detection principle of Zeta potential particle size analyser, particle size distribution is related to particle strength. The bigger the solid particle size, the wider the corresponding peak shape, and the larger the peak area. Therefore, the particle size distribution of product HAp can be indirectly obtained by analysing the intensity distribution of particles, as shown in Fig. 6. The average particle size of the product HAp is 555.6 nm.

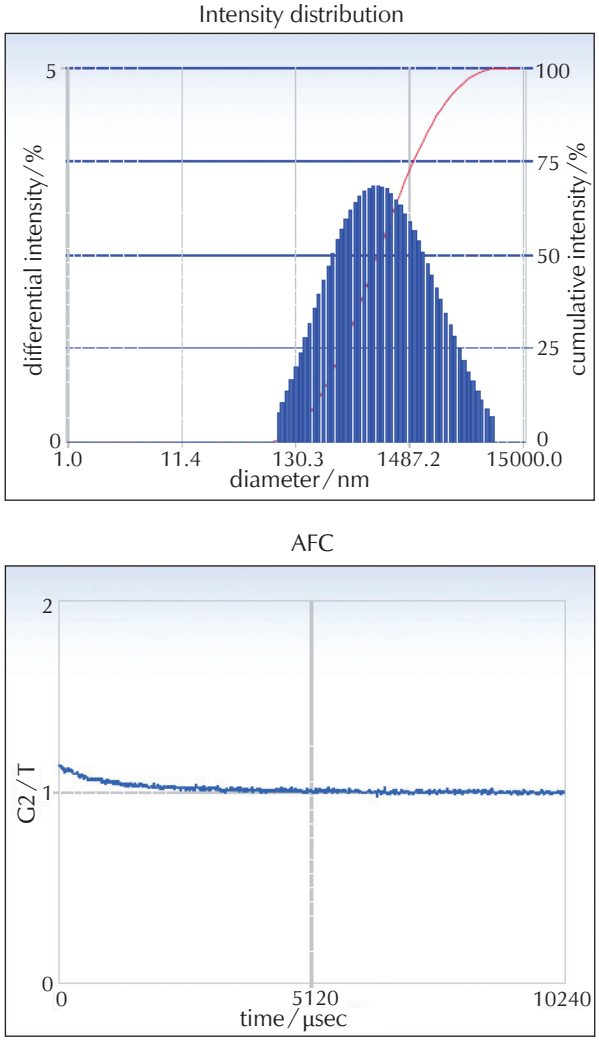

Fig. 6 – Particle size strength distribution

4. Conclusions

The crystallization conditions of HAp are affected and controlled by many factors. Influence of various factors on the crystalline forms of regenerated HAp were analysed. It was concluded that Na-citrate makes the regenerated HAp appear as a long cylindrical crystal or a perfect rod-like grain when chosen to be an additive. The optimum conditions of regenerated HAp in ionic liquid (ChCl-urea) were determined, as follows: Na-citrate as an additive at a dosage of 0.2 % (*w*/%), acoustic intensity 120 dB, and pH = 8.

Under these conditions, the product HAp was extracted by dissolving chicken bone in ionic liquid (ChCl-urea). It can be preliminarily concluded that this dissolving process was a direct physical dissolution based on dissolving situation observed by HSPOM. The product HAp was characterized by FTIR, SEM, XRD and Particle Size analyser. The results showed that there was no obvious difference between product HAp and regenerated HAp for the crystalline form and compositions, and product HAp was relatively pure and its average particle size was 555.6 nm. Thus, the product has application value, and the technique conditions can be applied to the preparation process of HAp by dissolving animal bones in ionic liquid.

ACKNOWLEDGMENT

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List of abbreviations and symbols

AR	– analytical regent
ChCl-urea	– choline chloride-urea
HAp	– hydroxyapatite
HSPOM	– thermal stage polarized optical microscope
FTIR	– Fourier transform infrared spectroscopy
LAS	– sodium dodecyl benzene sulfonat
XRD	– X-ray diffraction
SEM	– scanning electron microscope

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SAŽETAK

Istraživanje uvjeta pripreme hidroksiapatita i utjecaja na oblik kristala hidroksiapatita

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Proučen je proces precipitacije hidroksiapatita (HAp) iz ionske kapljevine (ChCl-urea) i analiziran je utjecaj raznih faktora na pojavu različitih kristalnih oblika i prinos precipitiranog HAp-a. Zaključeno je da u prisutnosti Na-citrata kao aditiva/površinski aktivne tvari, HAp nastaje kao dugi cilindrični kristal. Dobiveni optimalni uvjeti za precipitaciju HAp-a primijenjeni su za ekstrakciju HAp-a iz pileće kosti u ionskoj kapljevini ChCl-urea. HAp dobiven iz pileće kosti karakteriziran je FTIR i SEM XRD analizom, a određene su i veličine čestica. Rezultati pokazuju da između prvotno precipitiranog HAp-a i HAp-a dobivenog iz pileće kosti nema značajne razlike u kristalnom obliku i sastavu. HAp dobiven iz kosti je relativno čist i ima prosječnu veličinu čestica 555,6 nm što znači da je primjenjiv u praksi.

Ključne riječi
Hidroksiapatit (HAp), ionske kapljevine, proces otapanja, optimalni uvjeti, veličina čestica

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