

High pressure synthesis procedure to obtain nanostructured composites for regenerative medicine

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Traditional chemical techniques to produce materials with specific applications, for example in cosmetics, drugs, orthopaedic surgery are incredible time consuming and inefficient. Scientists are now concentrating on analyzing how nature produces a useful structure or attribute, for example structure and properties of human bone [1]. It is well known that human bone is a natural composite material made by 80% hydroxyapatite (HAp) nanocrystals and 20% collagen fibres, forming a unique structure with exceptional properties. The aim of this study is to obtain nanostructured hydroxyapatite-collagen composite by *in situ* hydrothermal synthesis at low temperature and different pressures. Nanostructured composites were characterized by FT-IR, SEM, HRTEM and XRD. Structure and composition of the resulted powders for various HAp:collagen molar ratios will be compared. Best synthesis parameters (composition, pressure, time, etc.) will be selected for applications in regenerative medicine or tissue engineering.

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1. Introduction

Regenerative medicine is an emerging multidisciplinary field involving biology, medicine, and engineering that is likely to revolutionize the ways we improve the health and quality of life for millions of people worldwide by restoring, maintaining or enhancing tissue and organ function [2].

During the last decades, different biomaterials of biological or synthetic origin have been designed, aiming to act as artificial bones in orthopaedic, maxillofacial, craniofacial or reconstructive surgery [3]. The most direct approach for the development of a real bioactive artificial bone is to realize a material with similar composition, nanostructure and biological response to bone [4]. It is well known that human bone is a natural composite material made by 80% hydroxyapatite (HAp) nanocrystals and 20% collagen fibres, forming a unique structure with exceptional properties. Many researchers have prepared hydroxyapatite-collagen composites (HAp/COL) with composition similar to human bone but no one reported a similar nanostructure [4]. The aim of this study is to obtain nanostructured hydroxyapatite-collagen composite by *in situ* hydrothermal synthesis at low temperature and different pressures. Hydrothermal method represents a process that takes place in aqueous solution at low temperatures (25-350°C) and variable pressures $p > 100$ kPa. Hydrothermal reactions in homogenous or heterogeneous phase are followed by hydrothermal crystallization, resulting nanocrystalline materials. Usually, pressure is given by vapour pressure of

components in solution. It is known that vapour pressure depends on temperature, as Clausius-Clapeyron equation shows:

$$\frac{dp}{dT} = \frac{\Delta H_{vap}}{T_f \Delta V} \quad (1)$$

where:

ΔH_{vap} – vaporization enthalpy

T_f – boiling temperature

$\Delta V = V_g - V_l$ (difference between gas volume and liquid volume)

If $V_g \gg V_l$ and $V_g = RT/p$ then equation (1) becomes:

$$d \ln p = \frac{\Delta H}{RT^2} dT \quad (2)$$

Integrated relation transforms in:

$$p = C e^{\frac{-\Delta H_{vap}}{RT}} \quad (3)$$

vapour pressure above liquid component has an exponential increase with temperature.

All above mentioned equations consider vapour pressure as the result of liquid vaporization in vacuum. To increase vapour pressure values and work in high pressure conditions, an external pressure may be applied. In this case, gases above solution will be a mixture of molecules from component in liquid phase and molecules of inert gas introduced in reaction system. If P is the total pressure above liquid (external pressure) and p is vapour pressure,

inert gas will induce increasing of both pressures: external pressure from P to $P+dP$ and vapour pressure from p to $p+dp$. Temperature remains constant [5]. Vapour pressure of components in solution always increases with the increase of inert gas pressure.

2. Experimental

2.1 Synthesis of nanocomposite hybrid powder

Collagen powder used in present study is extracted from young calf skin. Two types of collagen were used: hydrolyzed collagen (HC) and jellified collagen (HG). Hydroxyapatite-collagen (HAp-COL) nanocomposites were prepared *in situ* by hydrothermal method in CORTEST autoclave. External pressure of 2 MPa was added into reaction system. Solid salts of Ca and P as hydroxyapatite precursors were previously mixed in water with hydrolyzed or jellified collagen. Chemical composition of final powder is similar with that of bone tissue (80% HAp and 20% COL). Acid-base titration of precursor solution using a mineralized as a base was performed. Automatic microburette Radelkis was used for this purpose. Optimum precipitation pH for HAp/COL nanopowders was determined from analytical titration curves.

2.2 Characterization of nanocomposite hybrid powders

The Fourier transform infrared spectroscopy (FT-IR) was performed using JASCO FT/IR-620 spectrometer. Nanostructured composite powders were mixed with spectroscopic grade KBr and then pressed as pellets.

Hydroxyapatite-collagen composite powders were observed by scanning electron microscopy (SEM) on HITACHI microscope type S-2600N, equipped with energy dispersive X-ray spectrometer (EDAX). Small amounts of powder were dispersed onto a conductive and gold coated carbon adhesive foil.

Qualitative analysis using X-ray diffraction was performed on a SHIMADZU XRD 6000 diffractometer working in Bragg-Brentano geometry in 2θ . This equipment uses $\text{CuK}\alpha$ radiation and Ni filter.

Microstructural characterization of nanostructured powders was realized by High Resolution Transmission Electron Microscopy using Philips CM 12 microscope with resolution 2 Å. Small amounts of each sample were immersed in ethanol and homogenized through ultrasonication. Samples were examined in bright field (TEMBF), electron diffraction (SAED) and high resolution transmission electron microscopy (HRTEM).

3. Results

All nanocomposite powders were characterized as described above. Some representative samples are discussed as follows.

FT-IR spectra for composites with 20%, 30% and 40% hydrolyzed collagen (samples named HC) are given in Figs. 1 and 2. Main infrared vibration modes observed in nanocomposite powders are presented in table 1. SEM image of HC1 sample is presented in Fig. 3.

Microstructure of hydroxyapatite-collagen nanopowders was studied using transmission electron microscope (TEM). Some representative images obtained for HC1 sample are given in Figs. 4 and 5.

XRD spectrum presented in Fig. 6 underlines hydroxyapatite peaks as major phase. Morphology of HG1 sample containing 20% jellified collagen and 80% hydroxyapatite is depicted in secondary electron images (SEI) from Fig. 7.

Table 1. Main infrared vibration modes observed in HAp/COL nanocomposites.

| HAp/COL wave number (cm^{-1}) | FT-IR vibration modes |
|--|-------------------------------|
| 3567 | νOH stretching |
| 3432 | $\nu\text{OH} + \nu\text{NH}$ |
| 1639 | amide I |
| 1483 | CONH |
| 1422 | δOH |
| 1092+1030 | PO_4 stretching |

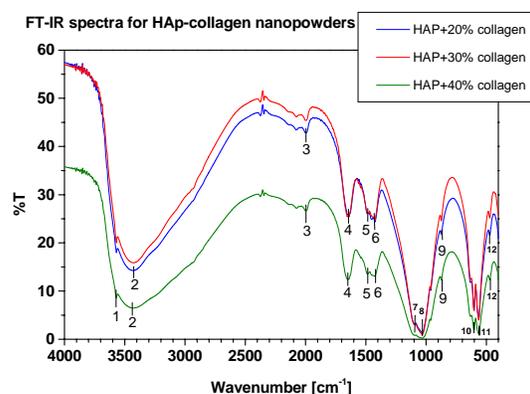


Fig. 1. FT-IR spectra for various compositions of nanocomposites.

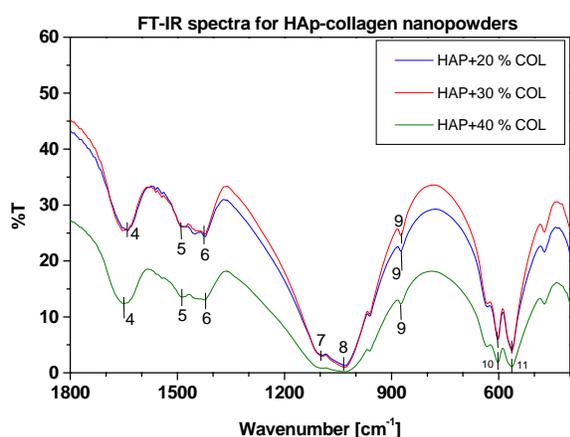


Fig. 2. Detailed area of FT-IR spectrum from figure 1.

4. Discussion

4.1 HAp/COL nanocomposites using hydrolyzed collagen

Hydroxyapatite-collagen nanostructured composites were characterized by FT-IR spectroscopy in order to demonstrate chemical interactions between organic and inorganic phase.

FT-IR analysis of HAp/COL nanocomposites shows the specific bands of hydroxyapatite such as hydroxyl stretching (-OH) at 3567 cm^{-1} , phosphate (PO_4^{3-}) stretching at 1092 cm^{-1} , 1030 cm^{-1} , 874 cm^{-1} and P-O bending bands at 602 cm^{-1} and 563 cm^{-1} . The bands in $1700\text{-}1400\text{ cm}^{-1}$ region suggest the presence of collagen with its characteristic functional groups, as table 1 shows. The bonding between organic and inorganic phases may be argued by the slightly band broadening observed at 3567 cm^{-1} and $1092\text{-}1030\text{ cm}^{-1}$ for nanocomposite sample with 40% collagen. Some shoulders flattened or disappeared with increasing collagen content probably due to the bonding between organic-inorganic phases by participation of PO_4^{3-} . The organic region of FT-IR spectrum (Fig. 2) also shows peaks broadening for HAp with 40% collagen as compared to samples that contain lower amounts of organic (20% COL and 30% COL).

Morphological aspect of hybrid powder was studied by scanning electron microscopy (Fig. 3). Powder seems agglomerated and porous in SEM micrograph but this could be generated by physical attraction between nanosized particles. Higher resolution is needed to examine particle's geometry at nanometre level (Fig. 4). Hydroxyapatite-collagen hybrid powder consists of particles with spherical or polyhedral shape, grouped in small chains and measuring nearly 4-7 nm in diameter. Electron diffraction depicted in Fig. 5 demonstrates nanocrystalline structure of analyzed powder. Major phase observed is hydroxyapatite.

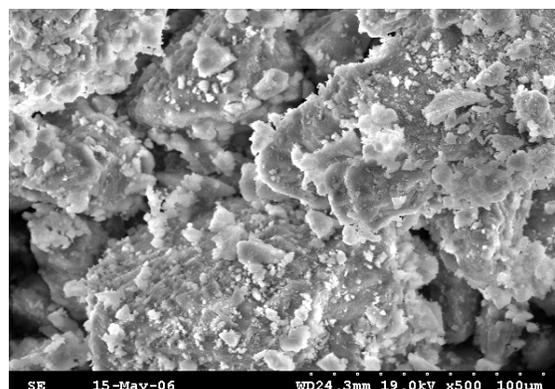


Fig. 3. Scanning electron microscopy of HCl sample (x500 magnification).

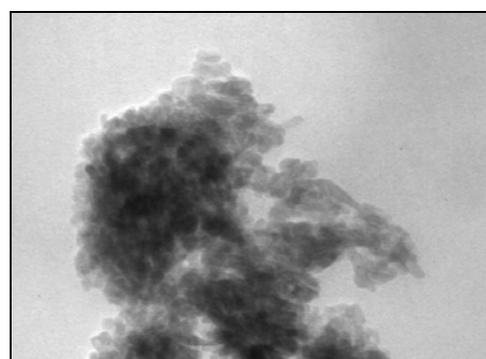


Fig. 4. TEM image of HCl sample (scale 100 nm).

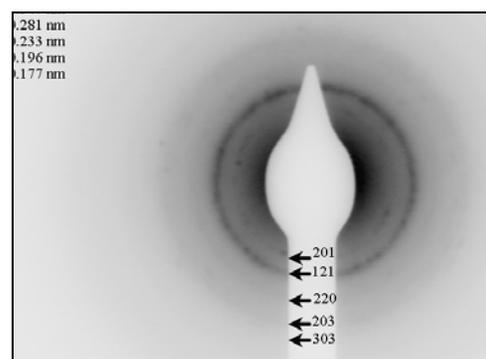


Fig. 5. SAED picture associated to figure 4.

4.2 HAp/COL nanocomposites using jellified collagen

X-ray diffraction patterns for different types of HAp/COL powders couldn't evidence collagen fibrils. As Fig. 6 shows, all XRD peaks correspond to hydroxyapatite phase. Fig. 7 reveals the morphology of HG1 sample (HAp with jellified collagen). Aggregates in submicron range could be observed. Porosity is higher in the case of powders with hydrolysed collagen compared to those with jellified collagen.

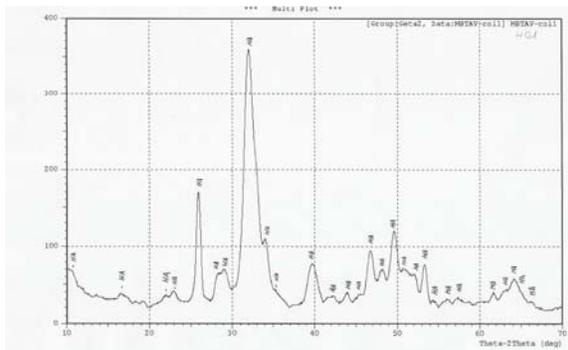


Fig. 6. X-ray diffraction for HG1 sample (hydroxyapatite-collagen gel).

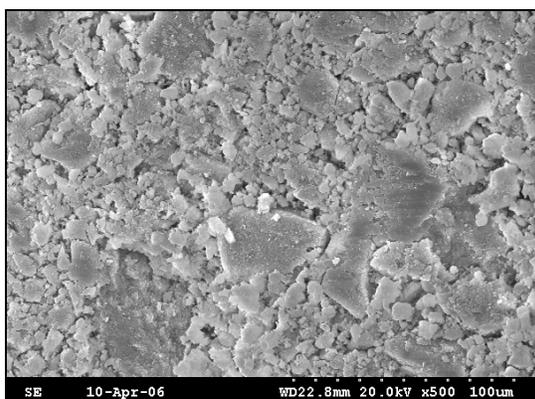


Fig. 7. Scanning electron microscopy of HG1 sample (x500 magnification).

5. Conclusions

Nanostructured composites based on hydroxyapatite as major phase and natural collagen as secondary phase were obtained. High pressure hydrothermal synthesis represents a good method for preparing chemically bonded nanocomposites. Analysis by FT-IR spectroscopy reveals

a chemical bonding between organic and inorganic phase. Further studies are required to examine the strength of this bond. Nanostructure of hydroxyapatite-collagen powders was underlined in TEM images.

Possible applications of nanocrystalline composites thus obtained could be as filling materials for regenerative medicine. As it was mentioned from the beginning, chemical composition chosen for the synthesized powder is similar with that of human bone. In near future, biocompatibility of resulted materials will be tested.

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