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*Dedicated to prof. dr. I. C. Popescu
on the occasion of his 70th anniversary*

BIOCOMPOSITES FOR ORTHOPEDIC APPLICATIONS

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ABSTRACT. A complex nano-hydroxyapatite (HAP) with.0.6 wt% Mg, 0.2 wt% Zn and 0.2 wt% Si was synthesized by the precipitation method. Composites of HAP with collagen (HAP-COL), HAP with chitosan (HAP-CHI) and HAP-COL-CHI were also prepared. The obtained biomaterials were characterized by X-ray diffraction (XRD), FTIR and FT-Raman spectroscopy, and by transmission electron microscopy (TEM) imaging. These biocomposites are potentially useful for hard tissue repairs and orthopedic applications.

Keywords: *nano-hydroxypatite, collagen, chitosan, composites.*

INTRODUCTION

Bones are formed mainly from hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (HAP) and collagen, mostly type I collagen (COL). Therefore, HAP-COL composites are able to mimic skeletal bones and consequently, they are suitable to be used as bone substitutes [1]. The properties of such biocomposites depend on the manufacturing conditions and crosslinking agents [2]. Chitosan (CHI), a polysaccharide containing N-acetyl glucosamine and glucosamine units can also be used in bone tissue engineering, due to its biodegradability, biocompatibility, capacity to form structures suitable for cell ingrowth and osteoconduction [3]. Moreover, it is shown that COL and CHI as well as multi-substituted HAP significantly inhibit the growth of bacterial pathogens, which is the major cause of prosthesis related infections [4].

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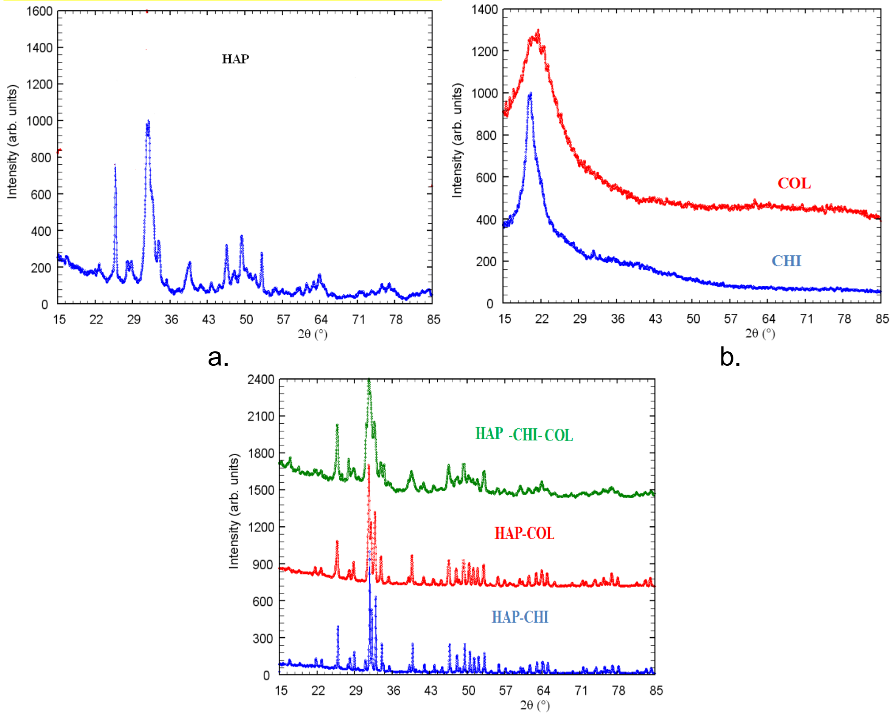
Consequently, this work is focused on the development of novel biocomposites for orthopaedic applications, with ability to promote specific cellular response at molecular level with minimally invasive surgery. In the following, we present an improved method to build homogeneous biocomposites made of HAP and COL (HAP-COL), HAP and chitosan (HAP-CHI) and HAP-COL-CHI, for different weight ratios. The used hydroxyapatite is a substituted nano-hydroxyapatite (HAP), with.0.6 wt% Mg, 0.2 wt% Zn and 0.2 wt% Si, which possesses an improved bioactivity, leading to a better osteointegration [5-10].

RESULTS AND DISCUSSION

The *X-ray diffraction patterns* of HAP powder calcined at 650° C for 6 hours (Fig. 1a) show the high crystallinity degree of this powder while both COL and CHI present an amorphous structure (Fig. 1b). All the composites HAP-CHI, HAP-COL and HAP-CHI-COL showed diffraction patterns (Fig. 1c) and crystallinity similar to the HAP powder.



a. b.

c.

Figure 1. X- Ray powder diffraction patterns of HAP (calcined at 650 ºC) (a), COL and CHI samples (b), and HAP-CHI (93:7), HAP-COL (70:30) and HAP-CHI-COL (68.5 :5.5 :26) powders (c).

FTIR spectra are compared in Fig. 2. The spectrum of the *hydroxyapatite* with 0.2% Si, 0.6% Mg and 0.2% Zn presents the usual phosphate bands; the most intense is the P-O stretching vibration band, v_3 [11] (maxima at 1033 and 1094 cm^{-1}). The P-O bend vibration band, v_4 [12] shows maxima at 564 and 603 cm^{-1}, while the maximum at 633 cm^{-1} is assigned to the structural OH (libration vibration). In the 4000-3000 cm^{-1} region, we find absorption bands originating from H-O vibrations: the broad band (maximum at 3434 cm^{-1}) due to absorbed water with hydrogen bonding O-H…O [13], overlapped with the band of structural OH (from hydroxyapatite) at 3570 cm^{-1} [14]. The band with the maximum at 1636 cm^{-1} is also due to absorbed water [14].

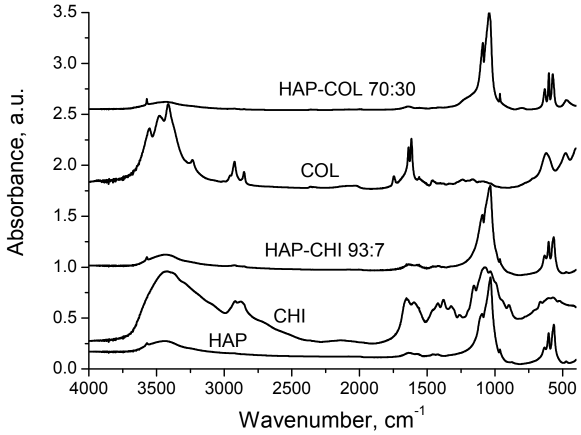


Figure 2. FTIR spectra of HAP, CHI, HAP-CHI, COL, and HAP-COL

Chitosan has a backbone similar to cellulose, but containing NH_2 groups which partially substitute the OH groups. It can also contain N-acetyl groups, $-NH-CO-CH_3$, if the deacylation of chitin was not complete. The large band at 3000-3500 cm^{-1} is due to hydrogen bonded O-H and N-H stretching vibrations (maximum at 3425 cm^{-1}). The peaks at 2917 and 2877 cm^{-1} are characteristic of the C-H vibration [15]. The peaks at 1648 and 1597 cm^{-1} correspond to the amide I (C=O stretching) and amide II (N-H bending) bands respectively [16] from the residual $-NH-CO-CH_3$ groups. The amide III band, assigned to C-N stretching and C-N-H bending, appears at 1324 cm^{-1} [17]. The peak at 1377 cm^{-1} is the characteristic band of CH_3 symmetrical deformation mode. The broad band with peaks at 1073 and 1154 appears from the C-O-C stretching vibration characteristic for polysaccharide structure of chitosan [18-20].

The *HAP-CHI composite* sample presents absorption bands arising from HAP and CHI, while the peaks due to CHI are rather small, since the proportion of CHI in the composite is low. For instance, the phosphate peak is shifted from 1094 to 1092 cm^{-1} due the superposition of the C-O-C peak of chitosan (1073 cm^{-1}). Since TEOS was used as crosslinking agent for chitosan/collagen and hydroxyapatite, the small absorption peak at 769 cm^{-1} could be related to the formation of hydrogen bonds between silanol (Si-OH) groups of the silica network and amide- and oxy-groups of chitosan. Other small peaks in the region of 1223 cm^{-1} show characteristic absorption for the Si–O–Si band [15].

Collagen, a protein composed mainly from the amino acids glycine, proline, and hydroxyproline, also presents the large band at 3300-3600 cm^{-1} for hydrogen bonded O-H and N-H vibrations (the highest peak at 3414 cm^{-1}). The C-H stretching vibrations in CH_3 and CH_3 groups are responsible for the peaks at 2924 and 2853 cm^{-1} [16, 21]. Amide I band appears at 1638, amide II at 1569 and CH_2 wagging and deformation at 1617 cm^{-1} [22, 23]. The absorption at 1084 cm^{-1} arises from C-OH stretching vibrations [22].

As in the case of HAP-CHI, the *HAP-COL composite* presents a FTIR spectrum similar to that of HAP, with slight shifts in the maxima, due to the interactions with COL. The new band appeared at 800 cm^{-1} indicates the presence of Si-OH groups in the hybrid system HAP-COL. The FTIR spectrum of HAP-COL-CHI composite (not presented) is similar to the spectra of the HAP-COL and HAP-CHI composites.

The *FT Raman spectra* (Fig. 3) complete the information furnished by FTIR spectroscopy, since some vibrational modes inactive or weakly manifested in FTIR spectra are more visible in Raman spectra. For *HAP*, the most intense band is that corresponding to the symmetric stretching mode (v_1) of the PO_4 group, which was inactive in FTIR; it is situated at 969 cm^{-1}. The v_3 mode, dominant in the FTIR spectra, is here represented by two weak peaks at 1015 and 1087 cm^{-1}. Other vibration modes of the PO_4 group are present: v_4 with three distinct peaks in the domain 546-610 cm^{-1}, and v_2 at 405 and 438 cm^{-1}.

The Raman spectrum of *chitosan* presents a low-intensity doublet at 1589 and 1601 cm^{-1}, assigned to the scissoring vibration of NH_2 groups. The main peak, at 2885 cm^{-1} is attributed to the stretching vibrations of CH groups [24], while that at 3301 cm^{-1} represents the vibrations of N-H bonds. The band with the peak at 1375 cm^{-1} corresponds to the CH_3 symmetrical deformation mode.

In the HAP-CHI composite, the intense phosphate band of apatite, with maximum at 961 nm, is present, while the bands of chitosan are barely visible, due to its small proportion. A new broad high-intensity band appears (maximum at 769 cm^{-1}, with a shoulder at about 700 cm^{-1}).

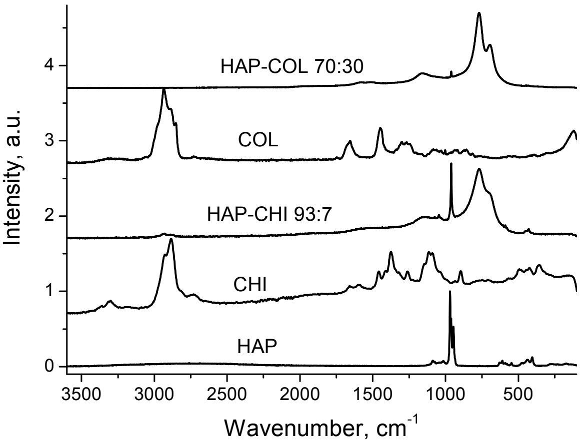


Figure 3. FT-Raman spectra of HAP, CHI, HAP-CHI, COL, and HAP-COL

For *collagen,* in the Raman spectrum, the most important band, at 2935 cm^{-1} is associated with CH_3 and CH_2 deformations, and so is the 1448 cm^{-1} band [25]. N-H stretching contributes to the 3290 cm^{-1} peak. The amide I band appears at 1655, and the amide III band at 1269 cm^{-1} [26].

In the Raman spectrum of the HAP-COL composite, the bands of HAP are considerably attenuated (e.g. the phosphate band at 961 cm^{-1}). and so are the COL bands. As for the HAP-CHI composite, a new intense band appears, with maxima at 770 and 695 cm^{-1}. These bands are probably due to the cross linking generated by TEOS.

Observations from *TEM micrographs* (Fig. 4) reveal that HAP and its composites with COL and CHI, present similar rod like structures (acicular particles*),* with average width 11 nm, and average length 37 nm.

CONCLUSIONS

A multi substituted nano-hydroxyapatite with Mg, Zn and Si was prepared and presents a characteristic acicular structure. Its binary and ternary composites with collagen and/or chitosan were obtained using TEOS as a binding agent. The acicular aspect of HAP nano-particles was preserved in obtained biocomposites (HAP-COL, HAP-CHI and HAP-CHI-COL), as judged by TEM images. The interactions among the components are revealed by FTIR and FT-Raman spectroscopy. Mechanical and biological tests on scaffolds made from these biomaterials are now under investigation in order to further develop biocomposites to be used as new bone substitutes with improved biocompatibility. In vitro cell culture studies are the centre of our future research to continue the biocomposites development, for dental and orthopaedic applications.

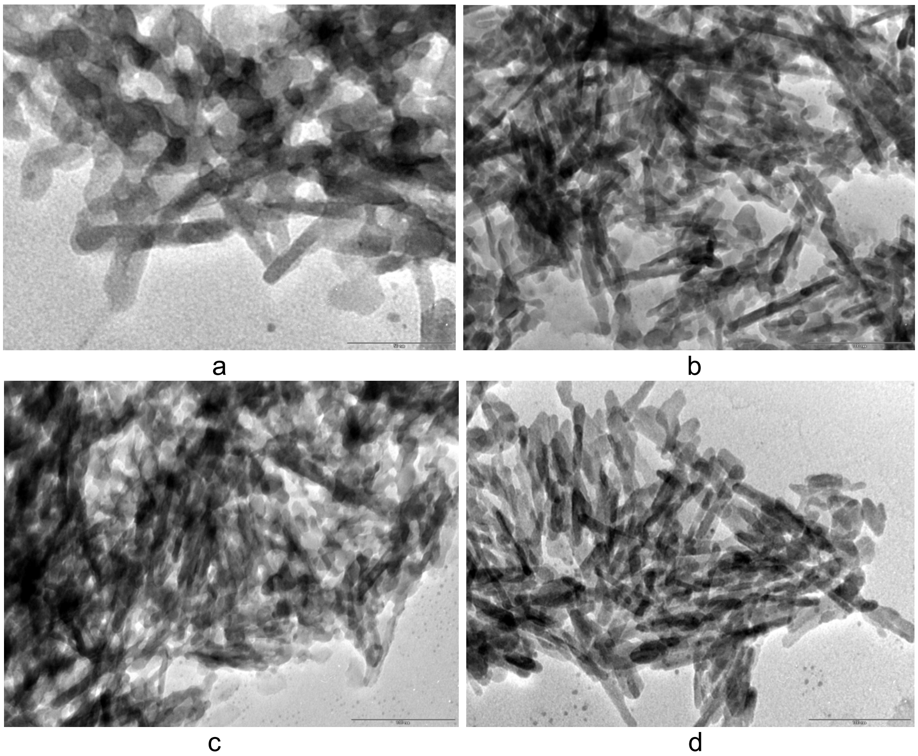


Figure 4. Representative TEM images of HAP powder after thermal treatment at 650°C (a); HAP-CHI (b); HAP-COL (c) and HAP-CHI-COL (d).

EXPERIMENTAL SECTION

Materials. Calcium nitrate hydrated, $Ca(NO_3)_2{\cdot}4H_2O$, diammonium hydrogen phosphate, $(NH_4)_2HPO_4$, magnesium nitrate hexahydrate, $Mg(NO_3)_2{\cdot}6H_2O$, all p.a. products, tetraethyl orthosilicate **(**TEOS*,* 98 wt%**)***,* ethanol, nonylphenol, 4-(2,6-dimethylheptan-4-yl)phenol, and acetic acid were purchased from Merck; zinc nitrate hexahydrate, $Zn(NO_3)_2{\cdot}6H_2O$, purum 99%, sodium silicate (molar Na_2O:SiO_2 ratio of 1:3.2), and ammonia solution (25%), *c*ollagen type I (COL) and chitosan (medium molecular weight), were obtained from Sigma-Aldrich.

Syntheses: Nano-hydroxyapatite modified with silicon, magnesium and zinc (HAP) was prepared by co-precipitation method. A solution containing calcium nitrate, nonylphenol (as surfactant), magnesium and zinc nitrates (for a final 0.6 wt% Mg, and 0.2 wt% Zn content in the HAP powder) was prepared (pH 6.3). Another solution contained $(NH_4)_2HPO_4$, nonylphenol, sodium silicate (Na_2O:SiO_2 molar ratio 1:3.2) for a final 0.2 wt% Si content in HAP; the pH was adjusted to 11.5 by adding 25% ammonia solution. The two solutions with a

Ca/P molar ratio 5/3, corresponding to that in stoichiometric hydroxyapatite, were mixed (2-3 sec) at 60°C under vigorous stirring (800 rpm). The reaction mixture was stirred for 24 h at 70°C for maturation, allowing the calcium phosphates, precursors to HAP, to nucleate and grow, the process being controlled by the presence of nonylphenol. The precipitate was separated by filtration, washed with distilled water until no NO_3^- ions were detected, and dried by lyophilization (Alpha 1-2 LDplus Freeze Dryer) to avoid particles agglomeration and then calcined at 650 °C for 6 h.

HAP-COL composite was prepared from a 2% wt. collagen solution in 1% acetic acid (pH 2.5 – 3), with a TEOS solution in ethyl alcohol, mixed with calcined HAP powder under intense stirring for 24 h (pH 7, adjusted with 10% sodium hydroxide solution). The HAP/COL ratio was 70:30. The precipitate obtained was kept under continuous stirring at room temperature for 24 h and then it was dried by lyophilization. *HAP-CHI composite* was obtained by the same procedure from a 2% wt. chitosan solution in 1% acetic acid (pH 3.5), with TEOS solution and HAP powder (pH 7 adjusted with 10% NaOH solution). The HAP/ CHI ratio was 93:7. The *HAP-CHI-COL biocomposite* was obtained analogously by coprecipitation from a 2% collagen solution (pH 3-3.5), a 2% chitosan solution (pH 3.5) and HAP powder, with TEOS solution and HAP powder (pH 7 adjusted with 10% NaOH solution). The composite contained 68.5% HAP, 26% COL and 5.5% CHI.

Nanopowders characterization. X-ray diffraction measurements were carried out on a DRON 3 type diffractometer, in Bragg-Brentano geometry and applying CuK_α radiation at 25 kV and 20 mA. *FT-IR spectra* were obtained with a JASCO 6100 FTIR spectrophotometer in the 4000-400cm^{-1} spectral domain with a resolution of 4 cm^{-1} by using KBr pellet technique. *FT Raman spectra* were obtained with a the FRA 106/S FT-Raman Module attached to Bruker EQUINOX 55; an Nd:YAG laser was used (wavelength 1064 nm) and a liquid nitrogen cooled germanium detector (D418-T). The spectra were recorded for wave numbers below 3600 cm^{-1} with resolution 2 cm^{-1}. *Transmission electron microscopy* (TEM: JEOL-JEM 1010, Japan) images have been recorded with JEOL standard software.

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