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THE INFLUENCE OF THE COLLAGEN CONCENTRATION ON THE GRAIN SIZE OF HYDROXYAPATITE - COLLAGEN NANOPOWDERS

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Materialele compozite pe bază de hidroxiapatită-colagen prezintă numeroase aplicații în ingineria țesuturilor datorită asemănării compoziționale cu țesutul osos. Deşi aceste materiale s-au preparat prin diferite metode în ultimele trei decenii, încă nu s-a reuşit fabricarea unui material care să prezinte nanostructura, proprietățile mecanice şi răspunsul biologic al osului natural.

Această lucrare are ca scop studiul influenței colagenului asupra dimensiunilor de grăunți ai materialului compozit pe bază de hidroxiapatită şi colagen, prin determinarea experimentală a distribuției de particule, cu ajutorul granulometrului Malvern Zetasizer 3000. De asemenea, stabilitatea suspensiilor apoase pe bază de hidroxiapatită şi colagen este studiată pe baza măsurătorilor potențialului zeta.

Hydroxyapatite-collagen composites have multiple applications in tissue engineering due to their compositional similarities with bone tissue. Although these materials have been prepared through various synthesis methods during the last three decades, obtaining of an artificial material whose nanostructure, mechanical properties and biological response are identical with those of natural bone still has not been reported.

The aim of this paper is to study the influence of collagen on grain size of hydroxyapatite-collagen based composite. Grain size distribution is determined experimentally using Malvern Zetasizer 3000 device. Stability of aqueous suspensions based on hydroxyapatite and collagen is studied through zeta potential measurements.

Keywords: grain size distribution, zeta potential, hydroxyapatite-collagen composites

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1. Introduction

During the last decades, different biomaterials of biological or synthetic origin have been proposed for various applications in bone tissue engineering. One of the most studied materials in this field is represented by hydroxyapatite-collagen (HAp/COL) composite which is designed to simulate bone composition, nanostructure and biological response [1-3]. Melisch et *al*. [4] synthesized a HAp particle/collagen mixture, and tested its biocompatibility by implantation. Miyamoto et *al*. [5] prepared a collagen-reinforced self-setting HAp cement, and reported its high biocompatibility and mechanical properties. TenHuisen et *al*. [6] obtained HAp/COL nanocomposite through the growth of HAp crystallites on collagen fibers.

However, researchers have not obtained yet HAp/COL composites with a real bone-like nanostructure: organic-inorganic hybrid in which inorganic apatite nanocrystals (whose size is 20-40 nm in *c*-axis) are deposited onto organic collagen fibers (one molecule of collagen having 300nm length) into a three dimensional structure [7-9]. Present paper intends to bring its contribution in this field through a study on the hydroxyapatite-collagen composite materials with different chemical compositions, prepared by hydrothermal method in high pressure conditions.

First part of this paper is related to the influence of the collagen concentration on the grain size of composite powders. Optimum Hap: COL ratio is established based on grain size distribution experiments.

The second part of this paper investigates the stability of HAP: COL suspensions for future application in bone tissue engineering as coatings or fillers for collagen based membranes. Preliminary zeta potential measurements are performed for aqueous HAp/COL suspensions without dispersant addition.

2. Experimental. Materials and methods

Experimental work was performed in order to determine grain size distribution (GSD) and isoelectric point of hybrid organic-inorganic nanostructured material. For this purpose, following steps have been done:

a) in situ hydrothermal synthesis of HAp-collagen nanostructured material
b) grain size distribution measurements (GSD)
c) preliminary experiments for isoelectric point test and stability of HAp-collagen suspensions

a) In situ hydrothermal synthesis of HAp-collagen nanostructured material

Hydroxyapatite-collagen nanopowders were synthesized in hydrothermal conditions at low temperature (< 70^0C) and high pressure (40-100 barr) using CORTEST autoclave from INCDMNR institute (Pantelimon, Romania). Natural collagen used in HAp/COL synthesis was previously extracted from bovine bone and hydrolysed at INCDTP institute. Experimental details are presented elsewhere [10]. Based on these powders, HAp/COL aqueous suspensions were further prepared in UNIPRESS institute (Warsaw, Poland), being necessary for GSD and zeta potential measurements.

b) Grain size distribution measurements (GSD)

Grain size distribution measurements were performed on aqueous suspensions of organic-inorganic powders with different HAp: COL ratios, using Malvern Zetasizer 3000 device from UNIPRESS, Poland. Particles up to 5000 nm in size can be detected with this instrument. Aqueous suspensions were prepared as follows: HAp/COL nanostructured powders were carefully weighted at RADWAG WAA 100/C/1 analytical balance and then dispersed in distilled water.

The suspension was kept in ultrasonic bath Elma Transsonic 460/H for 3-15 minutes in order to break powders' agglomerates resulting fine, colloidal particles dispersed in water.

c) Preliminary experiments for isoelectric point test and stability of HAp-collagen suspensions

Isoelectric point was determined from zeta potential (ξ) measurements of HAp/COL nanostructured powders in pH range = 2 – 10, using Malvern Zetasizer 3000 device.

pH of the suspension after magnetic and ultrasonic stirring was measured with a combined glass electrode attached to Zetasizer. HNO_3 65% and NH_3 25 % were used for pH corection.

Stability of HAp/COL suspensions

Malvern Zetasizer 3000 device does not allow sample stirring during measurements and powder sedimentation may occur, leading to possible errors. To avoid them, ξ is measured three times for each experiment and an average value is calculated representing arithmetic mean. For better results, sedimentation of powder must be avoid. Low concentrations of solid material are required for optimal measurements with Zetasizer 3000.

3. Results and discussion

Grain size distribution (GSD) measurements

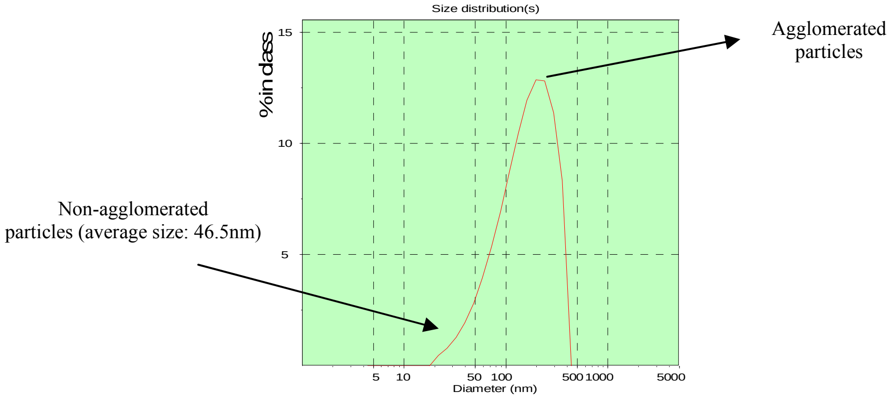
GSD measurements were performed in neutral or weak alkaline conditions for 3 representative samples of organic-inorganic nanostructured materials (A, B and C) with different HAp: COL mass ratio. These ratios have been chosen based on different compositions of bone tissue (cancellous or cortical bone). The influence of collagen concentration on particle size was studied. Average grain size values obtained for each sample are presented in table 1.

Table 1

Grain size distribution for HAp/COL nanopowders

Sample number	Sample name	HAp:COL mass ratio	Z ave [nm]	Observations
1	A	4:1	200.7	Normal distribution, particles agglomeration
2	B	1.5:1	30.2	Binomial distribution
3	C	1:1	21.6	Bimodal distribution

Figures 1 – 3 present grain size distributions for HAp/COL nanopowders with 4:1 (figure 1), 1.5:1 (figure 2) and 1:1 (figure 3) mass ratio, respectively. As one can see in figure 1, an almost normal distribution was obtained for sample A with HAp: COL mass ratio = 4:1. In this case, particle agglomerations predominate over non-aglomerated nanometric particles with average size of 46.5 nm.



Agglomerated particles

Non-agglomerated particles (average size: 46.5nm)

Fig. 1. Grain size distribution of sample A dispersed in water, HAp:COL=4:1

The presence of non-agglomerated particles could be observed in the left side of the distribution curve, before changing its shape.

A very sharp and narrow distribution (binomial distribution) is obtained for HAp: COL=1.5:1 mass ratio suggesting that particles are well dispersed in solution and no agglomerates are present. Therefore, a homogenous colloidal suspension with average particle size of 30.2 nm is obtained (see figure 2). Smaller particles with 22-25 nm in diameter are also present in sample B and they could be observed in the left side of figure 2, before the shape change of distribution curve.

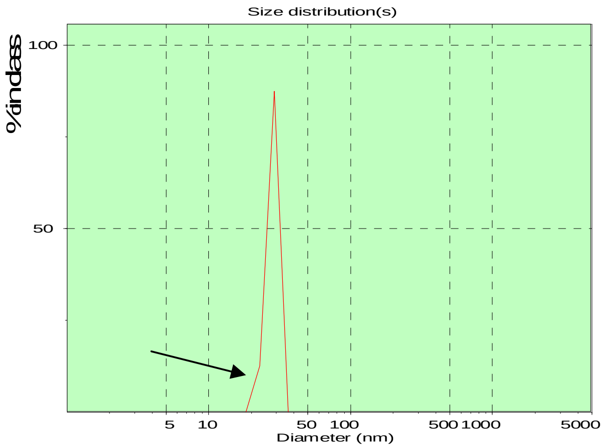


Fig. 2. Grain size distribution of sample B dispersed in water, HAp:COL=1.5:1

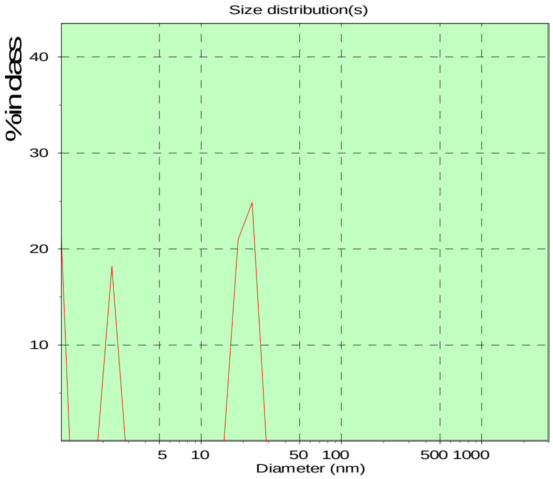


Fig. 3. Bimodal distribution of sample C dispersed in water, HAp:COL=1:1

Bimodal distribution appears in the case of sample C for HAp: COL mass ratio = 1:1. Besides nanometric particles with average size of 21.6 nm, a second distribution of small sized particles (average diameter =2.2 nm) may be observed in figure 3. Average particle size of HAp/COL composite powder decreases while collagen concentration increases. Different types of distributions depicted in figures 1-3 could be a consequence of hydroxyapatite-collagen interactions. It could be supposed that collagen acts as a crystal growth inhibitor for hydroxyapatite particles, but not as a dispersing agent. When collagen concentration is low, as in the case of sample A, particle agglomerations are formed. Grain size distributions become very sharp and narrow and are situated in the nanometric range as the collagen concentration increases.

It is also to be noticed that grain sizes of both sample B and C are similar with those of apatite crystals in a long human bone [2].

Zeta potential measurements. Preliminary stability testing of HAp/COL suspensions

Preliminary zeta potential measurements have been done for sample C (HAp: COL =1:1 mass ratio) in order to prepare stable HAp/COL suspensions for further deposition as biocompatible coatings. Hydroxyapatite-collagen nanostructured powder stabilised as aqueous suspensions could also be used as fillers for biocompatible membranes or as filling materials in bone defects.

Zeta potential of sample C was measured in the absence of a polymeric dispersant for pH range = 2 – 8. Zeta potential - pH dependence of sample C is depicted in figure 4.

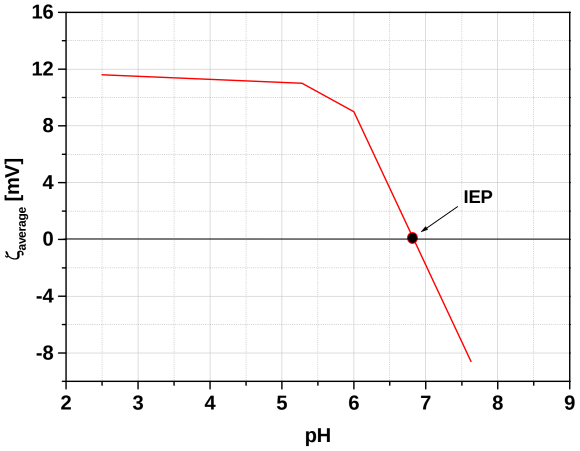


Fig. 4. Determination of izoelectric point (IEP) of sample C (HAp:COL=1:1)

Isoelectric point for 1:1 hydroxyapatite-collagen aqueous suspension is around pH= 6.7 - 6.8. Zeta potential varies between -8.6 and 11.6, being relatively constant in acid range. These values indicate that sample C is not stable without dispersant addition and fast sedimentation may appear. According to theoretical considerations regarding zeta potential, a suspension could be considered stable if zeta potential value (ξ) is above 50 mV or below -50 mV. In this case, mutual electrostatic repulsion between particles hinders their coagulation (formation of larger assemblies of particles) and sedimentation. When ξ is close to zero, particles coagulate and this causes suspension's instability. Further experiments are necessary for ξ measurements in the alkaline range (pH=7-10). A polymeric dispersant will be used to stabilize HAp/COL nanopowders.

4. Conclusions

Grain size distributions of various hydroxyapatite-collagen nanopowders dispersed in water were measured and the influence of collagen concentration on particle size of HAp/COL composite was studied. Average particle size of nanocomposite powder decreases with increase of collagen concentration. Collagen acts as a crystal growth inhibitor but not as a dispersant. Grain size distributions are situated in the nanometric range for composite powders with HAp: COL mass ratio = 1.5:1 and 1:1.

Isoelectric point of hydroxyapatite-collagen nanostructures in the absence of a polymeric dispersant was about pH=6.8. Low values obtained for zeta potential suggest that the suspension is not stable without dispersant addition.

Further experiments are necessary for ξ measurements in the alkaline range (pH=7-10). Based on these results, stability of aqueous suspensions will be investigated using polymeric dispersants.

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REFERENCES

[1] *Kikuchi M. et al.*, Composites Science and Technology, **vol. 64**, p. 819–825, 2004.
[2] *M. Wang,* Biomaterials, **vol.24**, p. 2133–2151, 2003.
[3] *C.V.M. Rodrigues et al.*, Biomaterials, **vol. 24**, p.4987–4997, 2003.
[4] *D.R. Mehlisch et al.,* Oral. Surg. Oral. Med. Oral. Pathol., **vol. 70**, issue 6, p. 685-692, 1990.
[5] *Y. Miyamoto et al.,* Biomaterials, **vol. 19**, p.707-715, 1998.
[6] *K.S. TenHuisenet al.*, J. Biomed. Mater. Res., **vol. 29**, issue 7, p. 803-810, 1995.
[7] *E. Song, et al.,* Biomaterials, **vol. 27**, p. 2951-2961, 2006.

[8] *S. Itoh et al.,* Biomaterials, **vol. 23**, p. 3919-3926, 2002.
[9] *D.A. Wahl,* J.T. *Czernuszka*, European Cells and Materials, **vol. 11**, p. 43-56, 2006.
[10] *L.M. Popescu et al.*, Journal of Optoelectronics and Advanced Materials, **vol. 9**, nr. 11, p. 3354-3357, 2007.