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Journal of Nanostructure in Chemistry 2 (1) (2011) 1-7 Contents list available at JNSC Journal of Nanostructure in Chemistry (JNSC) I.A.U. journal homepage: www.jnsc.ir In vitro bioactivity behaviour of Hydroxyapatite -gelatin nano biocomposites aba M.Meskinfam *, M.S.Sadjadi , H. Jazdarreh

a Department of Chemistry ,Faculty of **Science,** Lahijan **Branch, Islamic Azad University** 1 , Lahijan, **Iran b Department of Chemistry,** Science and Research **Branch, Islamic Azad University,** Tehran, **Iran**

ARTICLE INFO Article history: Received 20 January 2011 Accepted 2 April 2011 Keywords: Nanostructures;

Biomaterial; Template ; Bioactivity A B S T RA C T In situ biomimetic process has been used for synthesis of nano hydroxyapatite (nHAp) crystals in gelatine matrix. In vitro bioactivity of prepared samples

was investigated by their **immersion in SBF**	26

after 7 and 14 days.

X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR)	1

have been used for confirmation of crystalline nHAp formation in SBF after passing time.

Size and morphology of the nHAp **samples were characterized using scanning and transmission electron microscopy**	5

(SEM and TEM). Formation of apatite in a short period of time on nHAp/ gelatin composites after their

immersion in SBF, demonstrates **high in vitro bioactivity** of **the**	9

samples.

1. Introduction Bone is a nanocomposite of minerals and proteins.	7

It consists of 20% collagen, 69%

nano size inorganic phase (HAp) **and** 9% **water. The inorganic** phase **of bone** is **hydroxyapatite (HAp)**	5

by Ca (PO) (OH)) 10 4 6 2 formula. The synthetic type of this mineral has been widely used as a

bone implant and bone cement due to its compositional and biological similarities to 8

the mineral phase of bone and tooth [1]. Use of HAp is limited by its brittleness. One way for solving this problem is making composite by combination of it with polymer. Key point for Corresponding author, Tel: +98 21 44120326 E-mail Address: meskinfam@gmail.com creation of composites by good mechanical properties is the

strong interfacial adhesion between the inorganic fillers and the organic matrix 11

[2-5]. The

proper balance between biocompatibility and biodegradation 7

is gained by investigation of

a lot of non-biological and biological type of polymers. 7

The HAp-polymer mixture is a simple physical mixture so, it has deficiency in mechanical properties, and resultant synthetic bone 7

will face

with difficulty. So, researchers have tried to find materials and techniques to 7

synthesis of composites by appropriate biological properties for the human skeleton replacement [1].

Most promising of these attempts is biomimicking approach for synthesis of artificial bone like composite 1

materials. It is believed that in biomimetic process, biologic systems store and 2 M. Meskinfam et al. / Journal of Nanostructure in Chemistry 2 (1) (2011) process information at the molecular level [1, 6-9].

The interaction between the organic template and the organic **filler**	20

is base of this technique. It leads to

controlled nucleation and crystal growth of the inorganic part to	1

form a higher order hierarchical structure [10]. The collagen-HA composites have been synthesized by using biomimetic method [11,12]. High

cost and the poor definition of commercial sources of collagen **which makes it difficult to follow up on well controlled processing**	13

are practical problems of this material. So, collagen is replaced by gelatin in this study. . Another important factor for the latest biomaterials is their in vivo studies. Interpretations of in vivo experiment results are difficult because of

the complexity of the **various cellular responses.**	3

So, In vitro studies are necessary to indication of in vivo performance of materials. Biomaterials should develop

biologically active hydroxy carbonate apatite by their surfaces **after implantation for** further **establishing** the **bonding with natural bone.**	3

On the other hand, capability

of hydroxy carbonate **apatite** formation **on the surface of** 3

materials in vitro can be sign of their bioactivity in vivo [13]. . Gelatin is a natural biopolymer by functional groups such as amino acids. It can be obtained by thermal, physical or chemical denaturation of collagen [14]. Gelatin-hydroxyapatite composites due to

their similar composition to the hard tissues, good biocompatibility and high 9

osteoconductivity are good candidates for hard tissue repairs[15-20]. Many researchers

have been worked **on the preparation methods, physical and chemical properties** 19

and in vitro studies of its composites [15, 21-26]. So, by this history we have decided to synthesis nano HAp in gelatin matrix via biomimmetic route and investigate their bioactivity via

in vitro process. **2. Materials and experimental procedures** 1

Food grade gelatin

was obtained from Merck. The entire chemical **needed for synthesis of** the **hydroxyl apatite and SBF,** 1

Ca(NO 3) 2. 4H2O, Na3PO 4, NH4OH, NaCl, NaHCO 3,KCl, K2HPO 4.3H2O, MgCl 2. 6H2O, Na2SO 4, (CH2OH) 3CNH 2 and

HCl were also supplied from Merck and used without any further purification. 1

In situ synthesis of spherically hydroxyapatite was carried out in the matrix of food grade gelatin. For comparison, pure HAp nano particles were also prepared in the absence of gelatin. Different concentration of 5

gelatin (3.1, 4.2 and 5.6g of gelatin in 70ml

water were prepared. At first, for preparation of HAp in the presence of gelatin, the 1

required amount of food grade gelatin was added to 70ml of double-distilled water and mixed continuously using a magnetic stirrer at its maximum speed with the simultaneous application of heat 4

about 50ºC. After stirring about 2 hours, the solution of calcium nitrate (80ml, 0.1M) was slowly added to biopolymer solution. After finishing the addition of calcium nitrate, heating was stopped. After stirring for 1 hour, proper amount of sodium phosphate (48ml, 0.1M) was introduced drop by drop to the obtainedmixture. The maintained mixture was

stirred at ambient temperature. NH OH solution was added to maintain 4 and control pH at 8

about 10 in the solution. The quantities of the reactants for formation of HAp were selected so that provides

a Ca/P molar ratio of 1.67. Obtained suspensions (with and without biopolymer) were 15

filtered on buchner funnel,

and then washed with double-distilled water. The resultant precipitate was dried overnight at 8

room . M. Meskinfam et al. / Journal of Nanostructure in Chemistry 2 (1) (2011) 3 temperature. The products

were soaked in simulated body fluid (SBF) for 23

studying their bioactivity behaviour.

Reagent grade chemicals of NaCl, NaHCO , KCl, K HPO .3H O, MgCl 18

.6H2O, Na SO 3 2 4 2 2 2 4 and (CH OH) CNH were dissolved in distilled 2 3 2

water and buffered with HCl to pH 7.4 at 37 °C for preparation **of SBF.** 1

Ionic composition of SBF is very similar **to human blood plasma which has been** 1

shown in table1 [13, 27]. Each sample in the form of Powder was immersed in 30ml SBF

at 37 °C **for 3, 7 and 14 days under static** 1

condition.

After soaking in SBF, samples were washed by double distilled water and dried. As dried products before and after immersion in SBF. 1

The as dried products

were characterized using a Fourier transform **infrared spectroscopy (Thermo Nicolet Nexus 870), X-ray Powder** diffractometry **(XRD; Seisert Argon 3003 PTC using nickel-filtered XD-3a** 1

CuK a radiations (l= 0. 154 nm)), Scanning Electron Microscopy (SEM; Philips electron microscope)

and transmission electron microscope (TEM;

Philips EM208 and microscope operated at 100 kV). 22

3. Results and discussions FT-IR spectra for (a) gelatin, (b) HAp in absence of biopolymer, (c

)synthesized HAp in the presence of 1

4.2g gelatin, synthesized biocomposite

(d) after 7 days and (e) after 14 days immersion in SBF 16

are shown in fig.1. Here, we just investigate one sample IR spectra because of similar changes of all samples. . The –1 FTIR spectra

of gelatin shows peaks at 3450 cm –1 and 3423 cm due to -NH stretching of secondary –1 –1 6 amide, C=O stretching at 1700 cm and 1640 cm , –1 –1 -NH bending between 1550 cm and 1500 cm , -NH –1 out-of-plane wagging at 670 cm , and C-H stretching –1 –1 at 2922 cm and 2850 cm

[28]. In

FT-IR spectra -1 of HAp in the absence of 5

biopolymer, The 470 cm band belongs to the u phosphate mode. 2 Fig. 1. FT-IR spectra for (a) gelatin, (b) HAp in absence of biopolymer, (c) synthesized HAp in the presence of 4.2g gelatin, synthesized biocomposite

(d) after 7 days and (e) after 14 days immersion in SBF. -1 The 16

bands at 560, 600 cm are derived from the 2

υ 4 -1

phosphate modes. The peak at 960 cm is assigned to υ 1 phosphate mode. The bands at 1039 and -1 1086 cm stand for the υ phosphate mode. The 3 -1 absorption band at 1639 cm reflects H2O bending -1 mode. The band at 3439 cm band may come from lattice H2O because this band exists in the range of -1 3200– 3550cm . The stretching vibration and bending - -1 modes of the OH appeared at 3569 and 631 cm , -1 respectively. 2

Absorbance band obtained at 3425 cm corresponds to hydrogen bonded -OH stretching band [29]. FT-IR spectra of (c),

give more information about the interaction between the nHAp phase and gelatin 4

matrix. The

typical peaks of phosphate -1 vibration at 1030 cm and amide bands at 1650 and -1 4

1520 cm can be seen in spectra.

Absorption peaks - of asymmetric and symmetric R-COO groups 4

from 4 M. Meskinfam et al. / Journal of Nanostructure in Chemistry 2 (1) (2011) the gelatin amino acid groups appear at 1660 and -1 - 1420 cm , respectively. The COO ,C=O

and amino groups can provide sites for nHAp through binding or chelating oppositely charged ions, calcium and phosphate 4

leading to

formation of nHAp on the surface of gelatin network [29,30]. The 15

broad OH -1 stretching band centered around 3450 cm belongs to hydroxyl group of HAp and-NH stretching of secondary amide in gelatin. Remained items which are belong to Samples after immersion

in SBF after 7 and 14 days (d,e) show the 10

peaks similar senthesized HA in absence of biopolymer. Existence of new -1

small peaks in the range between 1411-1460cm -1 (v 3) and 1

876 cm (u 2)

indicate the presence of a 2- small amount of CO 3 groups 1

[1]. By these results it can be concluded

that the formed apatite on the surface of samples in SBF is carbonated apatite which is similar 3

to bone apatite from composition 3- and structure point of view. Changes in the PO 4 stretching modes due to

distortion of the HAp 2- crystalline structure occurring when the CO 3 ions replace the phosphate groups in the HAp structure 11

can be cause of broadening the bands at -1 960, 1035 and 1088cm . Fig.2 shows

the X-ray diffraction patterns of (a) prepared nHAp, (b) nHAp in 21

presence of 4.2g gelatin, prepared nanobiocomposite after immersion

in SBF for (c) 7 days and (d) 14 days. 10

Here, we just present XRD patterns of one sample because of similar changes of all samples.

As shown in this figure, HAp and HAp in gelatin matrix 1

before and after soaking in SBF 25

have similar XRD patterns which the diffraction peaks can be assigned to monophase low crystalline HAp. 1

There is just one point that

the intensity of apatite peaks increases gradually with immersion time which is the sign of apatite growth on the surface of samples in SBF. 3

Broadening the peaks in XRD pattern implying to

small size and low crystallinity of HAp **similar to natural bone mineral.**	14

Using low temperature procedure may be the cause of

poor crystalline nature of the prepared HA. In comparison **to** pure HAp **the**	14

peaks corresponding to HAp in gelatin matrix are slightly broader

which can be a sign for decreasing the HA **crystallinity in the presence of matrix**	1

[31, 32].

SEM micrographs of the HAp in	1

gelatin matrix

before and after soaking in SBF for 14 days are **shown in Fig.**	12

3a-f.

Nucleation of new mineral particles can **be**	3

observable for samples after soaking

in SBF for 14 days, but the growth of these	3

Fig. 2. XRD patterns of (a) prepared nano **HAp,** (b) nHAp **in** 1

presence of 4.2g gelatin, prepared nanobiocomposite after immersion

in SBF for (c) **7 days and** (d) **14 days.** 10

M. Meskinfam et al. / Journal of Nanostructure in Chemistry 2 (1) (2011) 5 (a) (b) (c) (d) (e) (f) Fig. 3.

SEM micrographs of synthesized HAp in the presence of 1

3.1g gelatin (a)

before and (b) **after soaking in SBF,** presence of **4.** 24

2 g polymer (c)

before and (d) **after soaking in SBF , and** 10

presence of 5.6g gelatin (e)

before and (f) **after soaking in SBF, for 14 days,** 12

respectively. crystals are very slow. It seems that the overall morphology of the obtained powders

before and after immersion in SBF 1

is spherical.

Fig 4a and b shows the transmission electron micrographs of HAp prepared in the presence of 5

4.2g gelatine

before and after immersion in SBF for 14 days, 1

respectively. Morphology of HAp particles in both situations is spherical and there is no change in it due to soaking SBF. The ultimate particle size of spherical HAp which is reveale by 6 M. Meskinfam et al. / Journal of Nanostructure in Chemistry 2 (1) (2011) TEM is aboutd 10nm and 13 nm

before and after immersion in SBF for 14 days, 1

respectively which indicates nHAp particle growth due to immersion in SBF and its bioactivity. (a) 4. Conclusion Gelatin has been used as a templating agent for synthesis of HAp particles at room temperature via biomimetic route. Formation of the HAp has been confirmed by XRD patterns and

absorption bands in the IR spectra. HAp in 1

starch matrix in comparison to pure HAp showed slightly broader peaks in XRD patterns which may be due to

the small size and low crystallinity of HAp 1

in the presence of biopolymer matrix. 1

Experimental results obtained from SEM and TEM images showed that, the morphology of nanocomposites is spherical and crystal size show growth due to soaking in SBF. Formation of

biologically active carbonate **apatite layer** in **a short** 9

time after soaking the samples

in SBF indicated **high in vitro bioactivity** of **the** 9

composites. So, these samples can be applicable as bioactive material. Acknowledgment The financial and encouragement support provided by Research

vice Presidency of Science and Research branch, Islamic Azad University and Iranian Nanotechnology Initiative 17

(Govt. of Iran). References (b) Fig. 4. TEM images of nHAp in 4.2g gelatine matrix (a) before and (b) after immersion in SBF for 14 days. [1] Myung Chul Chang, Ching-Chang Ko, William H. Douglas , Preparation, structural and mechanical characterization of porous hydroxyapatite-gelatin composite scaffolds for bone tissue engineer. Biomaterials, 24(2003) 2853–2862. [2] Weeraphat Pon-On , Siwaporn Meejoo , I-Ming Tang., Formation of hydroxyapatite crystallites using organic template of polyvinyl alcohol (PVA) and sodium dodecyl sulfate (SDS). Materials Chemistry and Physics, 112(2008) 453–460. M. Meskinfam et al. / Journal of Nanostructure in Chemistry 2 (1) (2011) 7 [3] V.M. Rusa, C.H. Ng, M. Wilke, B. Tiersch, P. Fratzl, M.G. Peter, Size-controlled hydroxyapatite nano- particles as self - organized organic - inorganic composite materials. Biomaterials , 26 (2005) 5414-5426. [4] M.G. Ma, Y.J. Zhu, J. Chang, , Monetite Formed in Mixed Solvents of Water and Ethylene Glycol and Its Transformation to Hydroxyapatite. J. Phys. Chem. B 110(2006) 14226- 14230. [5] K. MiWoo, J. Seo, R. Zhang, P.X. Ma, Suppression of apoptosis by enhanced protein adsorption on polymer / hydroxyapatite composite scaffolds. Biomaterials, 28(2007) 2622-2630. [6] Mann S , Ozin GA , Molecular tectonics in biomineralization and biomimetic materials chemistry,Nature, 365 (1993) 499–505. [7] Mann S, Archibald DD, Didymus JM, Douglas T, Heywood BR, Meldrum FC, Nicholas JR, Crystal- lization at Inorganic-organic Interfaces: Biomaterials and Biomimetic Synthesis. Nature , 382 (1993) 313–318. [8] Muthukumar M, Ober CK, Thomas E.L., Competing Interactions and Levels of Ordering in Self- Organizing Polymeric MaterialsScience , 277 (1997) 1225–1231. [9] Stupp SI, Braun PV. Molecular manipulation of microstructures : biomaterials , ceramics , and semiconductorsScience, 277(1997) 1242–1248. [10] J. Pe˜na, I. Izquierdo-Barba, M.A. Garc´ýa, M. Vallet-Reg., Room temperature synthesis of chitosan/ apatite powders and coatings. Journal of the Euro- pean Ceramic Society, 26(2006) 3631–3638. [11] AL Boskey Will , biomimetics provide new answers for old problems of calcified tissues? Calcif. Tissue Int, 63 (1998) 179–182. [12] JE. Zerwekh, S. Kourosh, R. Schienbergt, Fibrillar collagen-biphasic calcium phosphate

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