Studies of doped biomimetic nano-hydroxyapatite/polymer matrix Composites for applications in biomedical field

Hassane Oudadesse, Amany Mostafa, Xuong-Vuong Bui, Yann Le Gal and Guy Cathelineau
SCR, UMR CNRS 6226,
Université de Rennes 1,
263 av. du Général Leclerc, 35042 Rennes cedex, France
hassane.oudadesse@univ-rennes1.fr

Abstract: - Every year, millions of people are suffering from bone defect arising from trauma, tumor or bone diseases. Therefore, there is a growing need for the development of biocomposites with excellent bioactivity and compatibility. In this study, nano-hydroxyapatite was elaborated by using polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) under mild temperature condition. Comparison with pure nano-hydroxyapatite prepared by precipitation method was investigated. The main goal is to highlight the effects of the introduction of polymers on the physico-chemical properties, morphology and on the chemical reactivity and bioactivity for applications in bony surgery. TEM showed a nanosphere hydroxyapatite with an average diameter 45 nm obtained by using PVA. Nano-rods HA with an average dimensions 13 nm width and 156 nm length were obtained by using, PVP. "*In-vitro*" physiological stability and solubility of the investigated samples was performed by soaking powder in Simulated Body Fluid under physiological condition. Characterization by XRD, FT-IR SEM-EDS and ICP-OES were performed to identify phases, microstructure and then the chemical reactivity and bioactivity after soaking in SBF to evaluate the bioactivity kinetics. Crystals on the polymer fibril matrix exhibited certain orientation. Bone like apatite layer onto the surfaces is confirmed after post immersion in SBF by FT-IR, SEM-EDS and XRD. The polymer matrix controlled the dissolution precipitation reactivity with specific rate without change on the pH of the surrounding physiological body fluid.

Key-words: - Nano-Hydroxyapatite, bioactivity, biomimetic, polyvinyl alcohol, polyvinylpyrrolidone, Precipitation, Polymer matix composite, "in vitro" assay

1 Introduction

Bone repair or regeneration is a common and complicated clinical problem in orthopaedic surgery. Recently, a great deal of interest has been directed towards creating bioactive ceramic/polymer composites to be used as bone grafting materials. It is well known that natural bone is an inorganic/organic composite material. Hydroxyapatite (HA) is the main inorganic part in the bone composition and has been used as popular implant materials in the field of bone surgery [1]. There is no single existing material possesses all the necessary properties required in an ideal bone graft. Therefore, there is a growing interest in composite materials. As reported by previous authors, mixture of HA with polymer has been found to be an effective formulation technology to gain the intelligent artificial bone materials [2-4].

Extensive efforts have been made to produce synthetic nano-HA materials. Methods that have been used for preparing nano-HA materials included chemical precipitation [5], sol-gel approach [6], microemulsion [7] and mechanochemical synthesis [8]. while, the biomimetic approach to produce HA powder has the challenge to endues the desired characteristics such as

for example high specific surface area, fine grain size and monodisperse size distribution with very limited agglomeration [9]. Such characterization are directly depended on the process performed. Due to its simple process ; the wet-chemical precipitation route appears as the most talented way for HA powder preparation. Whereas, when HA is used in composite form (HA-polymer) it retains useful bioactive properties as well as enhancement of mechanical properties while showing acceptable osteoconductive properties. Therefore, the essence of the biomimicking process lies in mimicking biological mineralization with organic phase acting as a template for inorganic crystals. This can be used to help in nucleation and growth from supersaturated solution [10].

Several Authors have investigated equivalent materials and the more recent attempts have used polymer additives such as poly(lactic acid) (PLA), poly(acrylic acid) (PAAc), collagen, and gelatin due to the efficiency of contained calcium binding properties [11–14]. It has also been shown that the polar polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) have very interesting applications in the biomedical field [15-18]. In our previous work, we concluded that the effect of polymer

on HA crystal (forming hydrogen bond at earlier stage of reaction). Therefore, it worked on the crystal morphology but not on the phase composition of the product [18]. The purpose of the present work was focused on the investigation of the bioactivity of the previously prepared composites [18] made of nano-HA/polyvinyl alcohol (HA/PVA) and nano-HA/polyvinylpyrrolidone (HA/PVP) through "in vitro" test in (SBF) solutions. We aimed to compare the results obtained of both pure and composite systems. Evaluation of the kinetic rate of dissolution of the composites was calculated using the ICP-OES assay in combination with the physico-chemical characterizations.

2 Materials and Methods

2.1 Elaboration Process of Nano- hydroxyapatite Composites

The "In situ" preparation of nano-hydroxyapatite n-HA has been carried out in the presence of two different types of polymers; polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP). Chemicals used for this study are PVA (Loba Chemicals, India), PVP (Winlab, U.K), calcium chloride dihydrate (AR) (Sisco Research Laboratories , India) and di-ammonium hydrogen phosphate (LR) (Arabian Medical & Scientific Lab. Sup. Co, U.A.E) and sodium hydroxide. Nanohydroxyapatite, without polymer named here after as SHA has been prepared by precipitation [19] and was used for comparison. The composites of n-HA associated with PVA or PVP polymers were denoted as HAV or HAP, respectively [18]. The first step in the synthesis of the composites was the preparation of a 1.388 M calcium chloride dihydrate solution and a 0.833 M solution of di-ammonium hydrogen phosphate (LR). The calcium chloride dihydrate solution was slowly added to the polymer solution under continuous stirring and heating for approximately 10 min at 60 ^{o}C. The pH of the solution was adjusted to 11 by sodium hydroxide. Then, di-ammonium hydrogen phosphate solution was added gradually to the above mixture. A milky white coloration was observed almost instantaneously after the addition of phosphate solution. The pH of the solution was adjusted and maintained at its initial value of pH 11. Then the temperature was rised up to 90 ^{o}C with constant stirring for 3 hours. The suspension was left to age at room temperature for 24 hrs. After decantation the solutions obtained in this manner was centrifugated at 4500 rpm and washed with distilled water then dried in an oven at 60°C over a night.

2.2 Physico-chemical Characterization

The morphology and the size of the powder particles were examined under Transmission Electron Microscope (TEM). Therefore a solution made of a suspension of a few mg of the obtained powder in distilled water prepared then highly dispersed in ultrasonic bath for 15 min. Few drops of the suspensions were put on previously prepared grids covered by a thin film of evaporated carbon. The grids were examined under TEM using a Philips CM-20 equipment operated at 200 kv. Further physico-chemical characterizations have been made using Fourier Transformed Infra Red Spectroscopy (FTIR) (BRUKER EQUIPED 55) and X-Ray Diffraction (XRD) using (Inel Diffractometer). These experiments have been carried out before and after "in vitro" test. FT-IR analysis spectra have been recorded on samples in the form of pellets using KBr as the standard.

2.3 In vitro assays in SBF

In vitro tests have been achieved after soaking 30 mg of powder into 60 ml of simulated body fluid (SBF) with mineral composition nearly equal to those of human blood plasma (Table 1), according to Kokubo's protocol, at 37 ^{o}C in an incubator with shaking at 50 rpm [20].
After soaking in SBF during various ranges of time; short time of immersion (30 min, 9h., 12h. and 2 days) and longer time for (5, 7, 14 and 30 days), the powders were filtered, cleaned with deionized water to stop the reaction and then by ethanol. The drying was achieved in an oven at 60 ^{o}C over a night. The physico-chemical properties of the filtered powders were studied by FTIR, XRD, SEM Jeol with EDS. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), type CIROS vision was employed to determine the ionic exchange between compound and SBF liquid. The ionic concentrations of Ca and P amounts in synthetic physiological liquid (SBF) and their release versus soaking time was highlighted. Also, the pH of SBF was monitored versus the same soaking period of time.

Table 1: Concentrations of the SBF solution

	Inonic concentrations, 10^{-3} mol.L^{-1}						
	Na$^+$	K$^+$	Ca^{2+}	Mg^{2+}	Cl$^-$	HCO$_3^-$	HPO$_4^{2-}$
SBF	142	5	2.5	1.5	148.8	4.2	1
Plasma	142	5	2.5	1.5	103.0	27.0	1

3 Results and Discussion

Various artificial materials including medical polymer, bioceramics, metallic materials and their composites are currently in use for bone repairing. However, none of them can perform as perfectly as natural bone. Therefore, there is a real need for new biomaterials for bone defect repair. As a matter of facts, nano-hydroxyapatite/ polyvinyl alcohol and polyvinylpyrrolidone composites; HA-PVA and HA-PVP respectively have been studied in this objective [15-

18]. Nevertheless, to our knowledge no report has already discussed the kinetic bioactivity and the chemical reactivity of such composites. Investigation of the biological behavior of these biomaterials in SBF is considered as one of the most efficient and economical way to predict their bioactivity in the physiological environment. The "in vitro" formation of an apatite layer on the surface of the materials in the SBF is used extensively for evaluating the bioactivity of bone substitute materials [21].

3.1 Physico-chemical characterization before "in-vitro" experiment

TEM micrographs given in (Fig. 1) show that the particles are aggregated as clusters in the pure sample of HA (without polymer) prepared by precipitation (SHA).
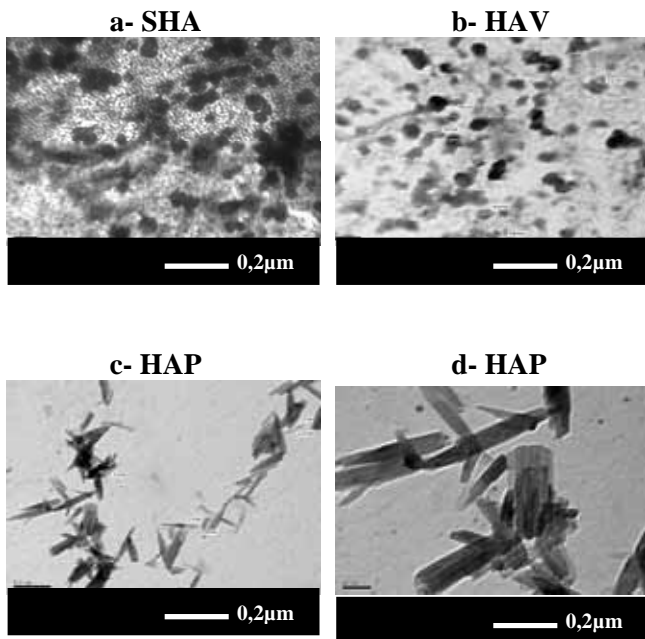

Fig.1, TEM micrographs of the prepared nano-hydroxyapatite samples: (a): without polymer (SHA), (b): hydroxyapatite composite HAV and (c, d): hydroxyapatite composite HAP
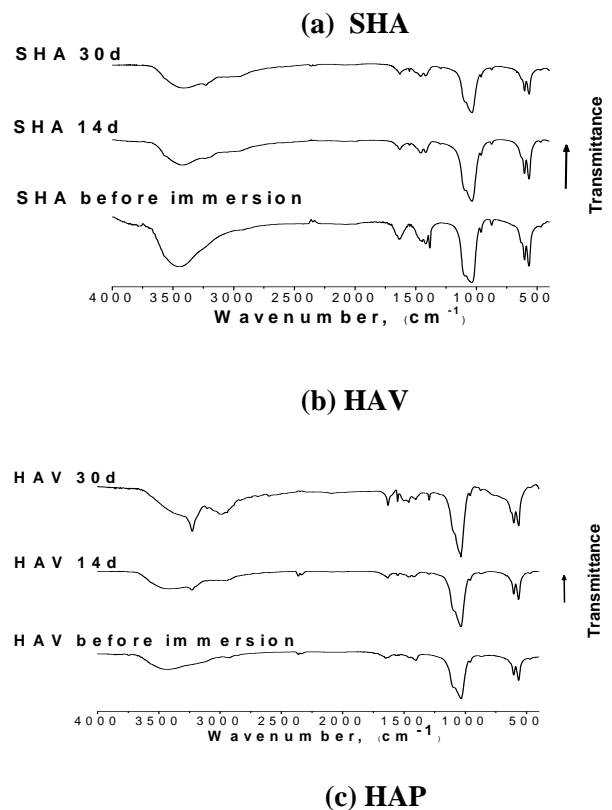
They appear as tiny plate grains, which tend to segregate together with a cross section of about 80.3 nm (Fig. 1-a). It has been shown that on using polymer matrix in composite samples of HA-PVA (HAV) and HA-PVP (HAP) such agglomeration has nearly disappeared. The composite sample HAV seemed to be nearly elongated in shape with smaller particle size than SHA as shown in (Fig. 1-b). While, HAP (Figure 1-c, d) appears like fibers or rod like particles with at least 100 nm in length and about 10-20 nm in diameter. Platy shaped grains occurring as bundles are shown in (Fig. 1-d). Therefore it appears that the PVP polymer greatly facilitated the formation of rodlike HA crystals which

preferentially tend to grow along the <001> direction as described by Zhang and Lu [16].

The ICP-OES technique was employed to determine the amounts of Ca and P in the prepared samples before immersion in SBF liquid. Data are presented in our previous work [18]. These data show that the molar (Ca/P) ratio of pure stochiometric hydroxyapatite SHA equal to 1.70. Furthermore, the addition of polymer in the composite samples HAV and HAP was found to increase this ratio up to 1.85 near that of biological apatite [19]. Where, bone-apatite is characterized by molar Ca/P ratio varying from 1.37 to 1.87. Therefore, the internal crystal disorder and ionic substitution within the apatite lattice results in the presence of significant levels of additional trace elements within bone mineral [22, 23]. Then, this plays great roles in the overall performance of human bones [24].

3.2 Bone-like apatite structure characterization

There is a great challenge to characterize the newly-formed bone-like apatite on the calcium phosphates with FT-IR spectroscopy, XRD analysis and SEM-EDS composition analysis due to the structure and composition similarities of the newly-formed layer and the CaP substrates.
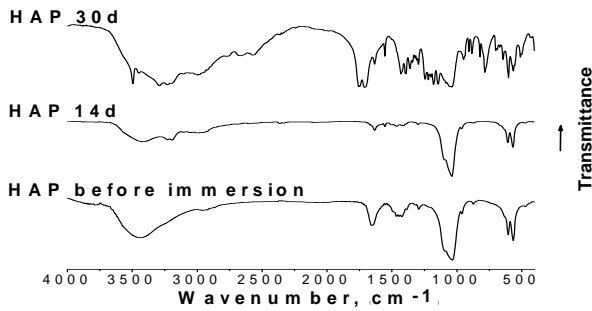

(a) SHA

(b) HAV

(c) HAP

Fig.2, FTIR of the prepared nanohydroxyapatite samples a) without polymer SHA, b) hydroxyapatite composite HAV, c) hydroxyapatite composite HAP; before and after immersion in SBF at 14 days and 30 days

Figs 2-a, b and c show typical FT-IR spectra recorded for the samples SHA, HAV and HAP before and after immersion during 14 and 30 days, respectively. Before immersion all samples present the disordered character (broad bands) related to the nanocrystalline size of HA [25]. All samples showed bands of carbonate (around 1417 and 1456 cm^{-1}) and of phosphate (stretching vibration: 1070, 1020, 980 and 930 Cm^{-1}), (bending: 725, 605, 560 and 490 Cm^{-1}) in the region at (500-1050 Cm^{-1}) [26]. The presence of carbonate inclusions might have come from air carbon dioxide taken during process from air [25]. The region below 1000 cm^{-1} can be interpreted according to the attribution given by Rey et al [27]. The band at 570 cm^{-1} is due to apatitic phosphate, PO_4^{3-}. This peak was resolved that assigned to PO_4 and HPO_4^{2-}. This is in agreement with the results of Jager et al [25] that confirmed through NMR investigation that the nanocrystals consist of a crystalline hydroxyapatite core covered by a disordered surface layer of HPO_4^{2-}. Other bands of carbonate appeared at 868 cm^{-1}. In the spectrum of HAP sample (Figure 2-c) before immersion there is a new band at 1250 cm^{-1} attributed to polyvinylpyrrolidone polymer. Its effect has been also shown in the bands localized at nearly 1400 cm^{-1} and 1600 cm^{-1}. Urch et al [28] referred to the peak presented at 1659 cm^{-1} to the presence of polymer PVP but here we referred this peak to that of carbonate as it is presented in all samples even that without polymer maybe overlapping with it. There is a change in the O.D. of the phosphate groups assigned before and also the OH group near 3500 cm^{-1} after immersion for 14 as well as 30 days. This is clearly appeared for samples SHA and HAV (Fig. 2 a and b) compared to that before immersion. This proves the effect of immersion and their involvement in the biolayer formation. Fig. 2c of sample HAP shows that the O.D. of phosphate groups and OH groups overlapping with the polymer bands. They are enhanced after immersion compared to that before immersion. This proves the deposition of some ions from

the SBF onto the surface of powder of HAP. This is happened as a result of interaction between PVP via imide group N-C=O and the phosphate ions in SBF. In addition, the O.D. of these bands is slightly increased denoting the presence of PVP polymer that hydrolyzes to form carboxyl group resulting in apatite nucleation [29]. Therefore, the introduction of polymer create more adhesion with physiological liquid and consequently enhance the bioactivity of the composite. These important results showed that this stage characterize the direct consequence of the interaction and binding between the orderly arranged side groups like N-C=O characteristic of the PVP polymer and the nucleating HA nanocrystals.

Fig. 3 shows XRD patterns of investigated samples before and after immersion in SBF. There are differences in intensity of the peaks for the spectra before and after immersion as it is referred to the structure of apatite. Special refinement of the peaks observed in the spectrum after 30 days of immersion highly imperfect apatite particles formed on the surface in SBF.
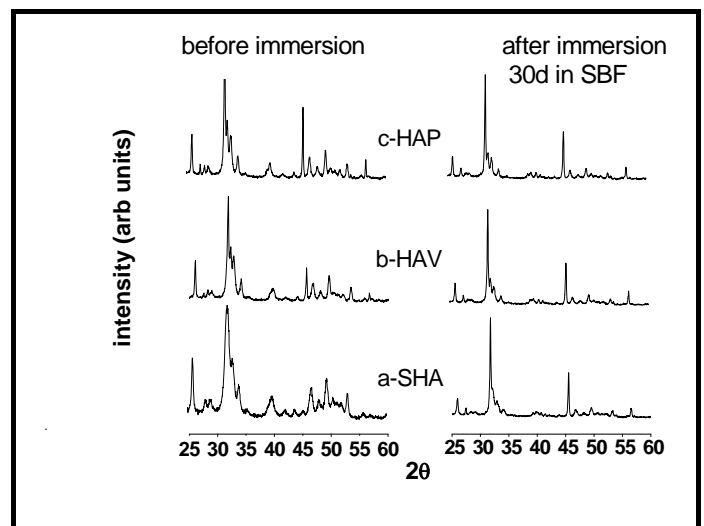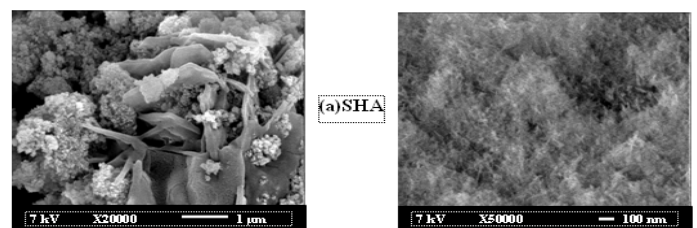

Figure 3: XRD of the prepared nano-hydroxyapatite samples, a) without polymer SHA b) hydroxyapatite composite HAV, c) hydroxyapatite composite HAP; before and after immersion in SBF at 30 days
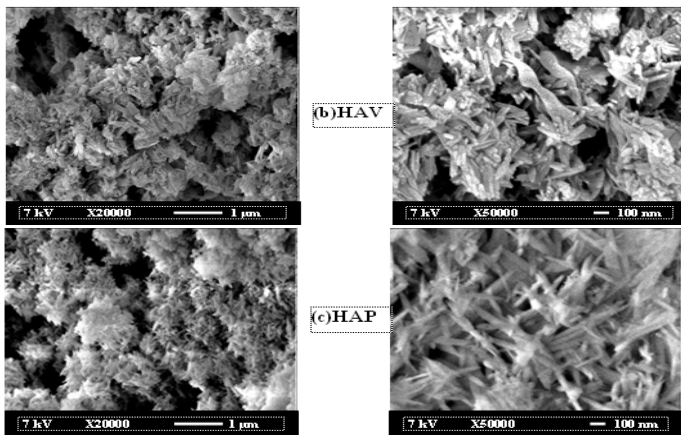
Fig.4, SEM of (a) pure hydroxyapatite SHA, (b) hydroxyapatite-polyvinyl alcohol polymer matrix HAV and (c) hydroxyapatite-polyvinylpyrrollidone HAP; after 30 days of immersion in SBF with different magnifications

The SEM micrographs coupled with EDS of samples of pure-HA (SHA), HA-polyvinyl alcohol (HAV) and HA-polyvinlpyrrolidone (HAP) after 30 days of immersion in SBF, with different magnifications are presented in Fig. 4 and 5. More precisely Fig. 4-a shows the coli flower precipitation of hydroxyapatite on its higher magnification showed very fine nature of nanohydroxyapatite precipitation layer. For both composites HAV and HAP the SEM figures show significant differences between pure SHA and that of the composites samples. Incorporation of HA with these polymers induce a great modification of their grains. There are presented in a particular geometrical form elongated needle shaped of about 500 nm. The scanning electron microscopy analysis suggests the existence of strong molecular interaction between each type of polymers PVA and PVP networks, leading HA to be dispersed uniformly in the composite. EDS spectra (Fig. 5) showed the presence of Ca, P, Na and Cl in all samples. The phosphocalcic ratio Ca/P after 30 days of immersion in SBF (examination over the whole surface) is nearly equal to the stoichiometric apatite as it is equal to 1.73, 1.78 and 1.79 for SHA, HAV and HAP, respectively. This explains the formation of bone like apatite. The results obtained from XRD and SEM coupled with EDS are complementary and confirmed that the layer formed over the pure nano-HA or over nano-HA/polymer composite are bone like apatite according to its phosphocalcic ratio and its crystallization.
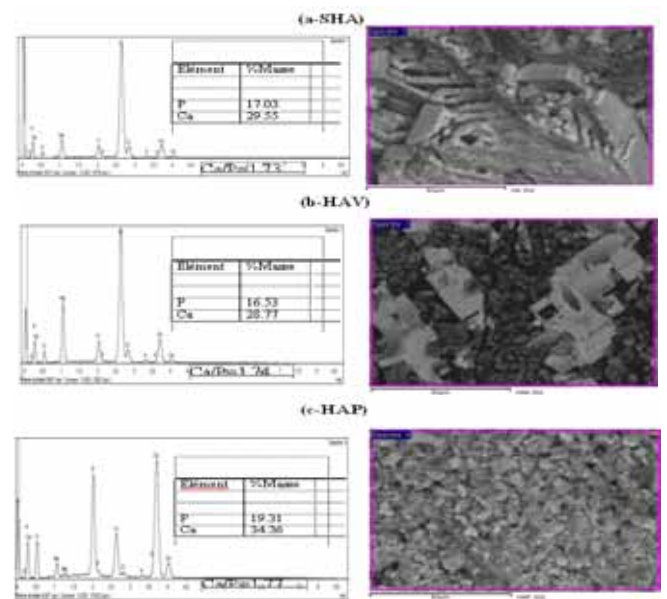

Fig.5, EDS elemental analysis of (a) pure hydroxyapatite SHA, (b) hydroxyapatite-polyvinyl alcohol polymer matrix HAV and (c) hydroxyapatite-polyvinylpyrrolidone HAP; after 30 days of immersion in SBF.

3.3 Bioactivity test

To evaluate the ionic exchanges and then the chemical reactivity of our investigated samples SHA, HAV and HAP, the synthetic physiological liquid (SBF) was systematically analyzed after the withdrawal of powder samples. Different delays of soaking ; short time intervals (30 min, 9 hrs, 12 hrs and 2 days) and long time intervals (5 days, 7 days, 14 days and 30 days) were chosen. Ionic exchange between pure nanohydroxyapatite SHA and composite samples HAV and HAP with SBF solution was evaluated.
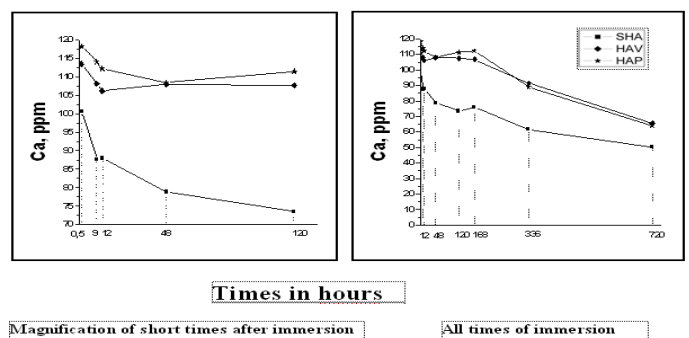

Fig.6, Evolution of elemental concentrations of Ca in SBF measured by ICP, Versus soaking time

Times in hours

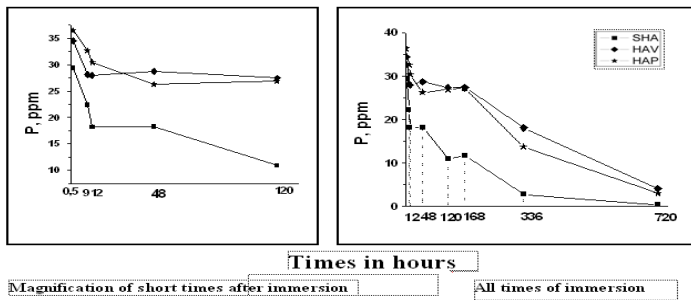Magnification of short times after immersion   All times of immersion

Fig.7, Evolution of elemental concentrations P in SBF measured by ICP, Versus soaking time.

The variation of calcium and phosphorous ionic concentration in the SBF liquid are presented in (Figs. 6 and 7), respectively. A magnification of the short period soaking times of each element up to 5 days (120 h) is also presented. ICP-OES results highlight different behaviors of the three calcium phosphate samples. The behavior of change in the Ca and P concentrations could be divided into three stages ; first stage accompanies by a decrease, second stage accompanies by a stop or slight increase and the third stage accompanies by a continuous decrease again till 30 days of both elements. These stages have a significant differences between pure HA (SHA) sample and that of the composites HAV and HAP. For SHA sample ; the 1st stage accompanies by a decrease in Ca and P concentration is extended up to 5 days (120 h) then the 2nd stage accompanies by slight increase from 5-7 days (120-168 h) and lately the 3rd stage accompanies by continuous decrease again starts from 7-30 days (168-720h). For composite HAV sample the 1st stage accompanies by a decrease in Ca and P concentration happens in the early stage of immersion only up to 12 h then the 2nd stage starts and accompanies by slight increase up to 7 days (12-168h) and lately the 3rd stage accompanies by continuous decrease again starts from 7-30 days (168-720h). Besides, HAP composite sample attain the same behavior as HAV with a slight change in the 1st stage that it is extended up to 2 days (48h) more than 12h in case of HAV. In addition, there is a stability in the second stage without realising an increase The decrease in pure HA (SHA) is much more evident in the beginning than that in case of polymer composites (HAV and HAP). But, after 30 days all samples reached almost to the same point of consumption for Ca and P from SBF. This means that the presence of polymer enhances the bioactivity in the early stages and their involvement in the biolayer formation due to the effect of mineralization. We can conclude that the first stage is accompanied by consuming Ca and P ions from SBF then the second stage was accompanied by equilibrium of the exchange of ions with solution before the last stage that accompanied by these exchange again. These three stages are variables in the three investigated samples these apparently due to the presence of polymer. This could be explained by the rapid saturation of polymer composite HAV and HAP samples network. This means that the equilibrium stage is enhanced in the presence of polymer better than pure HA. So, the chemical composition of SBF is less altered in contact with both the composites samples than the pure-HA sample. This is an important effect and advantageous of the composite samples of hydroxyaptite HAV and HAP as they haven't altered the chemical properties of the surrounding physiological liquid for a long time like in case of pure-HA. This may lead to less inflammation when use in surgery.

We can also calculate the amount of Ca and P consumed from SBF and precipitated on the samples surface after 30 days of immersion. Data are shown in Table 2. From these data we can calculate the phosphocalcic ratio. These ratios are 1.72, 1.63 and 1.66 for SHA, HAV and HAP respectively. This is in a good agreement with the EDS analysis. It is evident that the consumption of both Ca and P amounts after 30 days is nearly the same for all the investigated samples leading to the formation of stoichiometric hydroxyapatite. These results are also confirmed by measuring the pH of the SBF at the same previous time intervals of soaking (Fig. 8). We can notice that the use of composites with polymer matrix of either PVA or PVP did not show a drop in pH upon "in vitro" degradation on immersion in SBF as described by Tadic el al. [30].

The induction of apatite formation on the surface maybe resulted from the dissolution of nano-HA which may lead to the local supersaturation of Ca^{2+} and PO_4^{3-} and may promote the nucleation and growth of apatite crystals. Moreover, the presence of negatively charged carboxylate groups of polyvinylpyrrolidone or OH groups in polyvinyl alcohol surface may attract calcium ions present in the SBF solution and favor calcium phosphate apatite nucleation. Once the apatite nuclei formed, they can grow spontaneously by consuming the calcium and phosphate ions from the surrounding SBF. Substitution of CO_3^{2-} ion in the SBF for the PO_4^{3-} ion in the apatite results in the Ca/P atomic ratio of the apatite increases. This property can probably be extended to other bioactive ions. Nanocrystalline apatites can adsorb much larger amounts of bioactive molecules and proteins than well crystallized apatites for the same surface area [31].

The detonation of nano-hydroxyapatite polymer composite surface is chemically multifunctional (surface OH, COOH, C=C, C-O-C and C=O groups exist), so that the hydroxyapatite is grown both by physical adsorption and chemical interaction. These groups are regarded to play an important role in the hydroxyapatite growth on a composite surface from SBF.

4- Conclusion

The present work has demonstrated the possibility to prepare nano-hydroxyapatite associated with polyvinyl alcohol and polyvinylpyrrolidone polymer composites. The method used is a simple and reproducible biomimetic approach at low temperature. The two types of polymer charge HA negatively and attract Ca^{2+} ions, which in turn attract PO_4^{3-} ions, thus forming apatite nuclei. Considerable efforts have been made to control the shape of the nanocrystals during synthesis owing to their shape-dependent properties. However, generally capping agents/surfactants that preferentially adsorb on different crystal surfaces are used to achieve such shape control [32]. By this approach, some of the demerits of nano HA such as bioresorption and particle migration can be alleviated. The rate of bioresorption of nano HA was controlled and improved by the addition of both types of polymer PVA and PVP. Equilibrium stage starts early in both composite samples might be leading to minimize the effect of inflammation after surgery. It is anticipated that this kind of composites would act as a bone substitute with superior bioactivity and osteoconductivity when they are applied in bony surgery and particularly as bony substitute materials. The above findings pave ways to exploit the use of the investigated composites into bone tissue engineering.

References:

[1] Huang J, Best SM, Bonfield W, et al. J Mater Sci Mater Med 2004;15(4):441-445

[2] Shikinami Y, Okuno M. Biomaterials 2001; 22(23):3197-3211

[3] Wang M, Bonfield W. Biomaterials 2001;22(11):1311-1320

[4] Petricca SE, Marra KG, Kumta PN. Acta-Biomater 2006; 2(3):277-286.

[5] Cüneyt –Tas A. Biomat. 2000; 21(14): 1429-1438.

[6] Chai CS, Ben-Nissan B. J. Mater. Sci. Mater. Med. 1999; 10(8): 465-469.

[7] Bose S, Saha SK., Chem. Mater. 2003; 15: 4464-4469.

[8] Silva CC, Pinheiro AG, Miranda MAR, Goes JC, Sombra ASB. Solid State Sci., 2003;5: 553-558.

[9] Sung Y, Lee J and Yang JJ. Cryst. Growth. 2004; 262:467- 472.

[10] Katti K and Gujjula P. Control of mechanical responses in in-situ polymer/ hydroxyapatite composite for bone replacement, in: Proceedings of the 15th ASCE Engineering Mechanism Conference, Columbia University, New York, NY, June 2–5, 2002.

[11] Chang, M.C., Ko, C.C., Douglas, W.H. Biomater. 2003; 24(17):2853–2862.

[12] Schiller C., Epple Matthias. Biomater. 2003;24(12): 2037–2043

[13] Chian Liou SZ, Chen SY, Liu DM. Biomater. 2003;24(22):3981–3988.

[14] Spanos N, Deimede V, Koutsoukos PG. Biomater., 2002;23(3):947–953.

[15] Mollazadeh S, Javadpour J, Khavandi A, Ceram. Intern., 2007;33:1579–1583.

[16] Yanjie Zhang and Jinjun Lu. Crys Grow and Desg 2008; 8 (7): 2101-2107.

[17] Kobayashi H. Advances in Sci and Tech 2006;53:9-16.

[18] Mostafa AA, Oudadesse H, Mohamed MB, Foad ES, Le Gal Y, Cathelineau G.; Chem. Eng. J. 2009;153:187-192.

[19] Webster TJ, Massa-Schlueter EA, Smith JL, Slamovich EB. Biomater. 2004; 25 (11): 2111-2121

[20] Kokubo T, Kushitani H, Sakka S, Kitsugi T and Yamamuro T. J. J Biomed Mater Res, 1990; 24: 721-734.

[21] Lin XY, Fan HS., Li XD, Tang M. and Zhang XD. Key Eng. Mat. 2005;284-286:553-556.

[22] McConnell D, Apatite, Springer Verlag, Vienna-Heidelberg-New York 1973, p. 111.

[23] Posner AS, Crystal chemistry of bone mineral. Physiol. Rev., 1969,49: 760.

[24] Shi D, Biomaterials and Tissue Engineering, Springer Berlin Heidelberg, New York 2004, p.2.

[25] Jager C, Welzel T, Meyer-Zaika W, Epple M. Magn. Reson. Chem. 2006;44:573-580.

[26] Tadic D and Epple M. Biomater 2004;25(6):987–994.

[27] Rey C, Combes C, Drouet C, Lebugle A, Sfihi H, Barroug A. Mat.-Wiss. U Werkstofftech., 2007; 38: 996-1002.

[28] Urch H, Vallet-Regi M, Ruiz L, Gonzalez-Calbet JM and Epple M, J Mat Chem 2009;19: 2166-2171.

[29] Mohamed KR and Mostafa AA, J Mat Sci Eng C 2008;28:1087-1099.

[30] Tadic D, Peters F, Epple M, Biomater. 2002; 23(12): 2553-2559.

[31] Ouizat S, Barroug A, Legrouri A, Rey C. Mat Res Bull. 1999; 34(14/15): 2279-2289.

[32] Viswanath B, Paromita K, Mukherjee B, Ravishankar N. Nanotechnol. 2008;19:195603.