Physico-chemical assessment of biomimetic nano-hydroxyapatite/polymer matrix for use in bony surgery

H. Oudadesse, A. Mostafa, X. V. Bui, E. Foad, G. Kamal, Y Legal and G. Cathelineau

***Abstract*—**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.

***Keywords*—**Bioactivity, biomimetic, "in vitro" assay, nano-Hydroxyapatite, polymer matix composite, polyvinyl alcohol, polyvinylpyrrolidone, precipitation.

I. 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 apopular implant materials in the field of bone surgery [1]. There is no single existing material that 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]. However, the biomimetic approach to produce HA powder has the challenge to provide the desired characteristics such as for example high specific surface area, fine grain size and monodisperse size distribution with very limited agglomeration [9]. Such characteristics are directly dependent on the process performed. Due to its being a simple process , the wet-chemical precipitation appears to be the most route for HA powder preparation. When HA is used in composite form (HA-polymer) it retains useful bioactive properties as well as enhances the 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 most recent attempts have used polymer additives such as poly(lactic acid) (PLA), poly(acrylic acid) (PAAc), collagen, and gelatin due to the efficiency of their 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 [18], 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 to focus on the investigation of the bioactivity of the previously prepared composites [18] made of nano-HA/polyvinyl alcohol

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(HA/PVA) and nano-HA/polyvinylpyrrolidone (HA/PVP) through "in vitro" test in (SBF) solutions. The aim is compare the results obtained of both pure and composite systems. Evaluation of the kinetic rate of dissolution of the composites will be calculated using the ICP-OES assay in combination with the physico-chemical characterizations.

II. MATERIALS AND METHODS

A. Elaboration Process of Nano-hydroxyapatite Composites

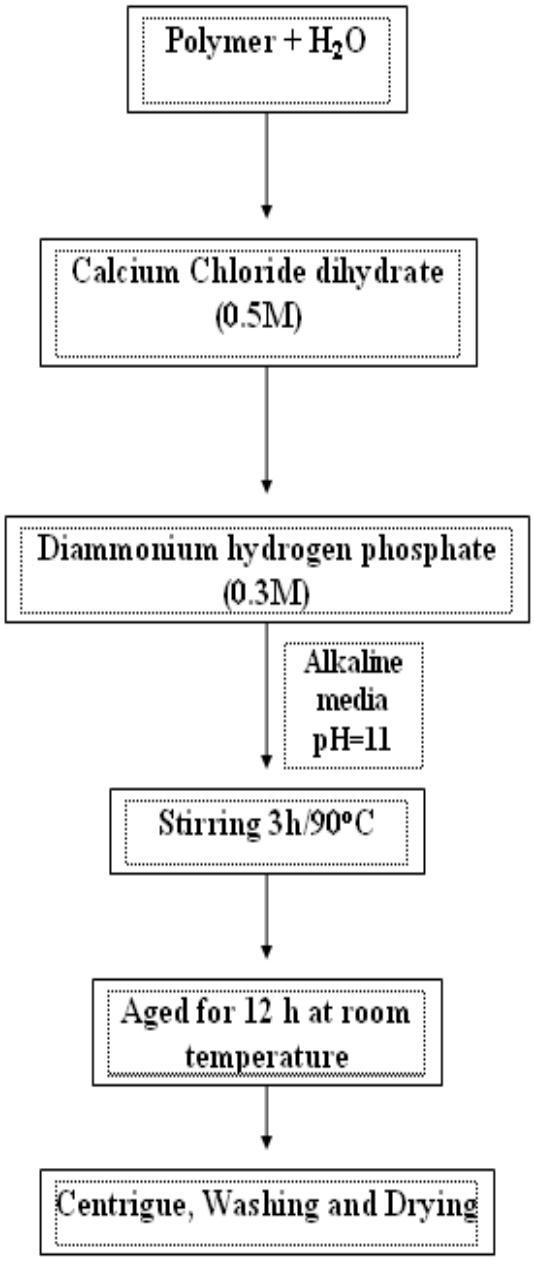


Fig. 1 In situ synthesis of nano-size hydroxyapatite in polymer matrix

The "In situ" preparation of nano-hydroxyapatite n-HA was 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 (Modern Lab. Egypt). Nanohydroxyapatite, without polymer named hereafter 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 is the preparation and 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 adjust and maintained at its initial value of pH 11. The temperature was then raised 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 centrifuged at 4500 rpm and washed with distilled water then dried over night in an oven at 60^{o}C. The composites were elaborated according to the steps presented in figure 1.

B. Physico-chemical Characterization

The morphology and the size of the powder particles were examined under Transmission Electron Microscope (TEM). A solution made up 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 added to the previously prepared grids covered by a thin film of evaporated carbon. The grids were examined under TEM using 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 were carried out before and after "in vitro" test. FT-IR analysis spectra were recorded on samples in the form of pellets using KBr as the standard.

C. In vitro assays in SBF

In vitro tests were performed after soaking 30 mg of powder into 60 ml of simulated body fluid (SBF) with mineral composition nearly equal to that of human blood plasma (Table 1), according to Kokubo's protocol, in an incubator at 37 ^{o}C with shaking at 50 rpm [20].

After soaking in SBF for various durations of immersion, short durations (30 min, 9h., 12h., and 2 days) and longer ones (5, 7, 14 and 30 days), the powders were filtered, cleaned first with deionized water to stop the reaction and then with ethanol. The drying took place over a night in an oven at 60 ^{o}C. 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 the compound and SBF liquid. The ionic concentrations of Ca and P in synthetic physiological liquid (SBF) and their release with soaking time was studied. The pH of SBF was also monitored versus the same soaking period of time.

Table 1: Concentrations of the SBF solution, 10^{-3} mol.L^{-1}

Ion	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}
SBF	142.0	5.0	2.5	1.5	148.8	4.2	1.0
Plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0

III. 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 fact, nano-hydroxyapatite/ polyvinyl alcohol and polyvinylpyrrolidone composites; HA-PVA and HA-PVP, respectively have been studied in this regard [15-18]. Nevertheless, to the best of our knowledge no report has discussed the kinetic bioactivity and the chemical reactivity of such composites. Investigation of the biological behavior of these biomaterials in SBF is considered 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].

A. Physico-chemical characterization before ''in-vitro'' experiment

TEM micrographs given in (Fig. 2) show that the particles are aggregated as clusters in the pure sample of HA (without polymer) prepared by precipitation (SHA).

They appear as tiny plate grains, which tend to a gregate together with a cross section of about 80.3 nm (Fig. 2-a). It has been shown that on using polymer matrix in composite samples of HA-PVA (HAV) and HA-PVP (HAP) such agglomeration nearly disappeared. The composite sample HAV seemed to be nearly elongated in shape with smaller particle size than SHA as shown in (Fig. 2-b). While, HAP (Figures 2-c and 2-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. 2-d). It can thus be deduced that the PVP polymer greatly facilitated the formation of rod like HA crystals 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 is equal to1.70. Furthermore, the addition of polymer in the composite samples of HAV and HAP was found to increase this ratio up to 1.85, a value close to that of biological apatite [19]. 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]. This in turn plays great role in the overall performance of human bones [24].

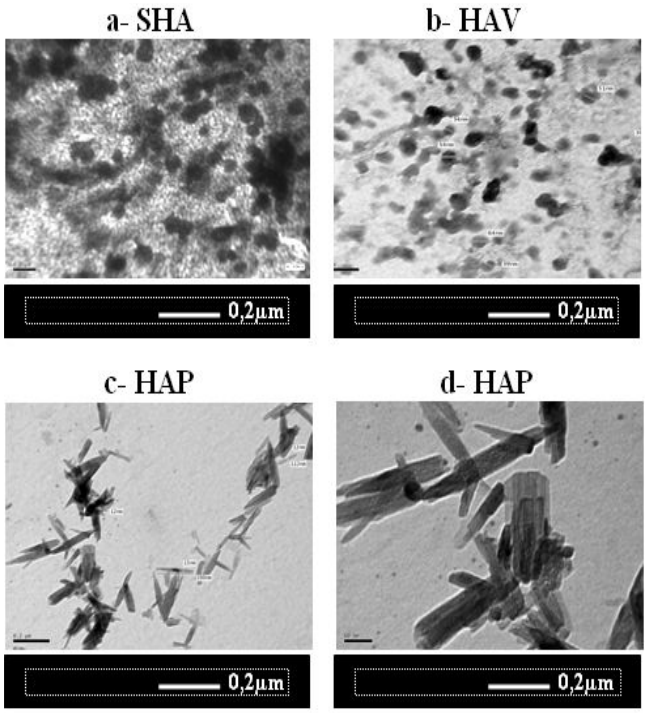


Fig. 2 TEM micrographs of the prepared nano-hydroxyapatite samples: (a): without polymer (SHA), (b): hydroxyapatite composite HAV and (c, d): hydroxyapatite composite HAP

B. 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.

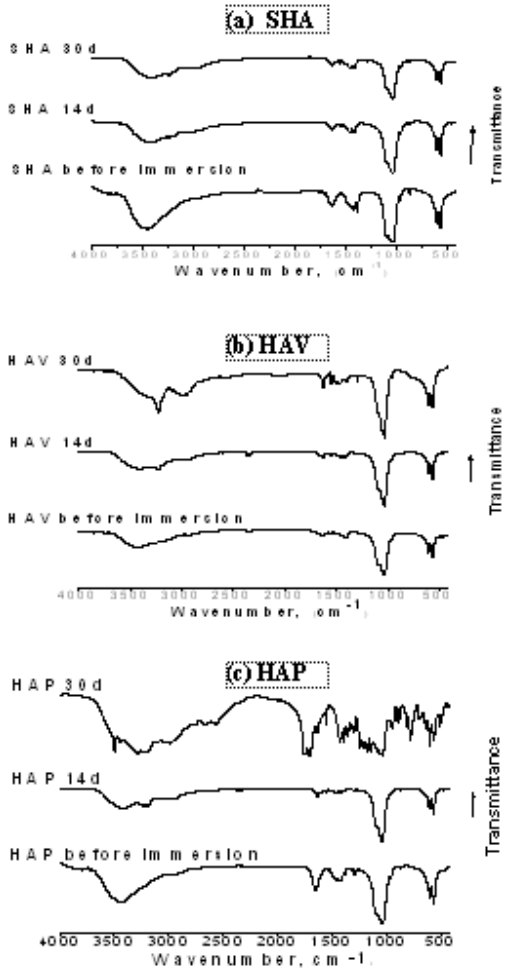


Fig. 3 FTIR of the prepared nanohydroxyapatite samples a) without polymer SHA, b) hydroxyapatite composite HAV, c) hydroxyapatite composite HAP; before and after immersion in SBF at 14 days and 30 days

Figs 3-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 [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}) HA [26]. The presence of carbonate inclusions might have come from air carbon dioxide taken from air during processing [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} could be due to apatitic phosphate, PO_4^{3}. This peak was resolved and 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 before immersion (Figure 3-c), 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] ascribed 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 thougth the peak 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 clear for samples SHA and HAV (Fig. 3a and b) compared to that before immersion. This result shows the effect of immersion and their involvement in the biolayer formation. Fig. 3c of sample HAP shows that the O.D. of phosphate groups and OH groups overlap with the polymer bands. They are enhanced after immersion in comparison to that before immersion. This confirms the deposition of some ions from the SBF onto the surface of the powder of HAP. This occurred as a result of the interaction between PVP via imide group N-C=O and the phosphate ions in SBF. In addition, the O.D. of these bands was slightly increased denoting the presence of PVP polymer that hydrolyzes to form carboxyl group resulting in apatite nucleation [29]. Therefore, the introduction of polymer creates more adhesion with physiological liquid and consequently enhances the bioactivity of the composite. These important results showed that this stage characterizes 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.

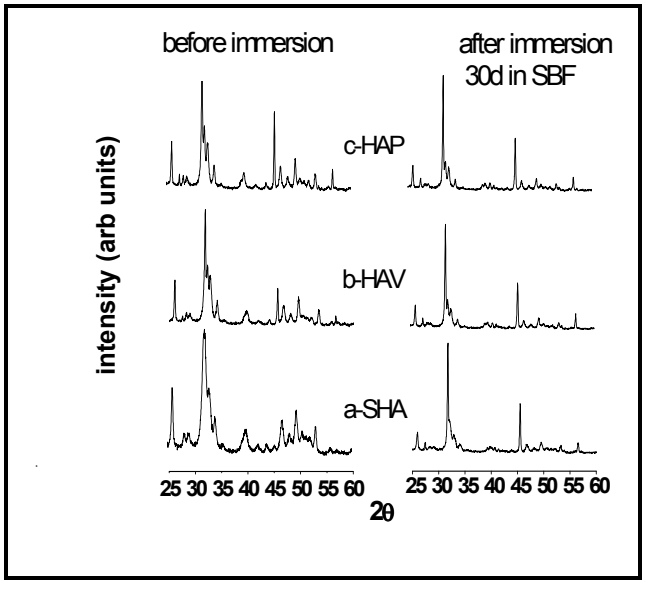


Fig. 4 XRD of the prepared nano-hydroxyapatite samples, a) without polymer SHA b) hydroxyapatite composite HAV, c) hydroxyapatite composite HAP; before and after immersion in SBF at 30 days

Fig. 4 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.

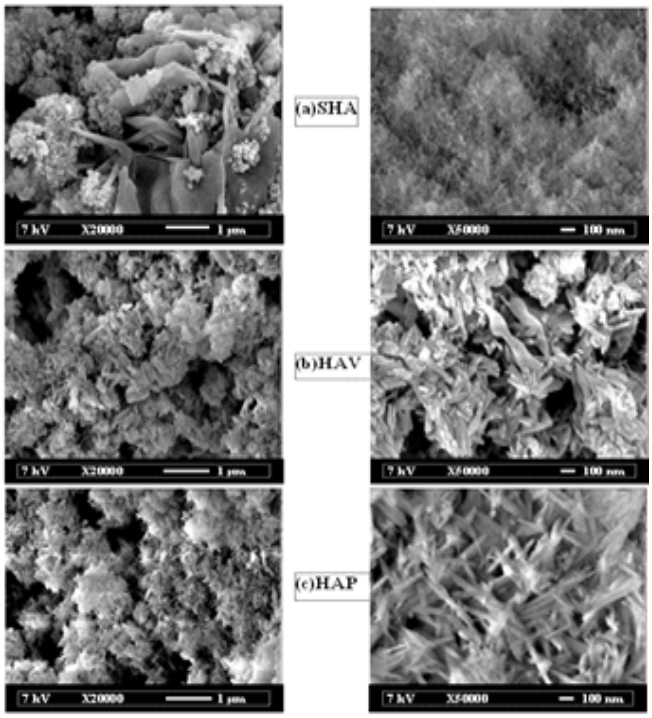


Fig. 5 SEM of (a) pure hydroxyapatite SHA, (b) hydroxyapatite-polyvinyl alcohol polymer matrix HAV and (c) hydroxyapatite-polyvinylpyrrollidone HAP; after 30 days of immersion in SBF with different magnifications

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 Figs. 5 and 6. More precisely Fig. 5-a shows the coli flower precipitation of hydroxyapatite at its higher magnification, the Fig. shows 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 to their grains. They are presented in a particular geometrical form elongated needle shape of about 500 nm. The scanning electron microscopy analysis suggests the existence of strong molecular interaction between each type of polymer PVA and PVP networks, causing HA to be dispersed uniformly in the composite. EDS spectra (Fig. 6) shows 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.

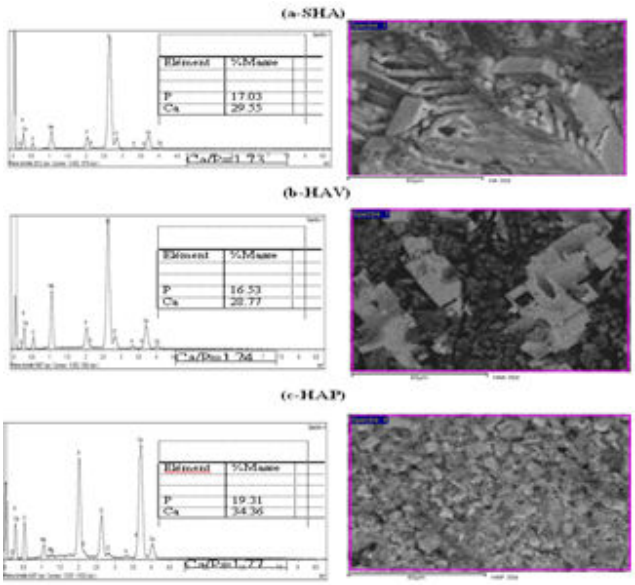


Fig. 6 EDS elemental analysis of (a) pure hydroxyapatite SHA, (b) hydroxyapatite-polyvinyl alcohol polymer matrix HAV and (c) hydroxyapatite-polyvinylpyrrolidone HAP; after 30 days of immersion in SBF.

C. Bioactivity test

To evaluate the ionic exchange capacity and 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 periods of soaking; short time intervals (30 min, 9 h, 12 h 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.

The variation of calcium and phosphorous ionic concentration in the SBF liquid are presented in (Figs. 7 and 8), 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 accompanied by a decrease, second stage accompanied by a stop or slight increase and the third stage accompanied by a continuous decrease again till 30 days for both elements. For these stages, there is a significant difference between pure HA (SHA) sample and that of the composites HAV and HAP. For SHA sample; the 1st stage accompanied by a decrease in Ca and P concentration is extended up to 5 days (120 h) then the

2nd stage is accompanied by slight increase from 5-7 days (120-168 h) and finely the 3rd stage accompanied by continuous decrease again starts from 7-30 days (168-720h). For composite HAV sample, the 1st stage accompanied 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 is accompanied by a slight increase up to 7 days (12-168h) and finely the 3rd stage accompanied by continuous decrease again starts from 7-30 days (168-720h). Besides, HAP composite sample follows the same behavior as HAV with a slight change in the 1st stage that was extended up to 2 days (48h) as opposed to 12h more in case of HAV. In addition, there is stability in the second stage without any 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 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 is accompanied by equilibrium of the exchange of ions with solution before the last stage that is accompanied by these exchanges again. These three stages vary in the three investigated samples apparently due to the presence of polymer, which could be explained by the rapid saturation of polymer composite HAV and HAP samples network. This means that the equilibrium stage is enhanced better in the presence of polymer than in pure HA. So, the chemical composition of SBF is less altered in contact with both composite samples than with pure-HA sample. This is an important effect and is advantageous to the composite samples of hydroxyaptite HAV and HAP as they have not 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 used in surgery.

We could also calculate the amount of Ca and P consumed from SBF and precipitated on the samples surface after 30 days of immersion. The obtained data show the phosphocalcic ratio as 1.72, 1.63 and 1.66 for SHA, HAV and HAP, respectively. This is in 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 may have 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 are 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 increasing the Ca/P atomic ratio of the apatite. 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 from SBF on a composite surface.

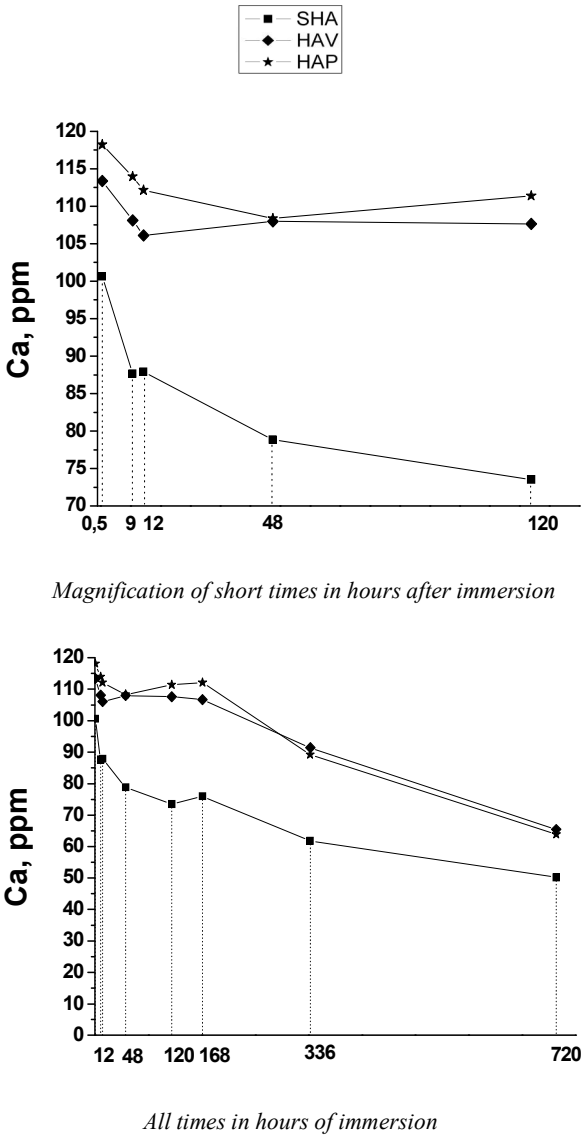
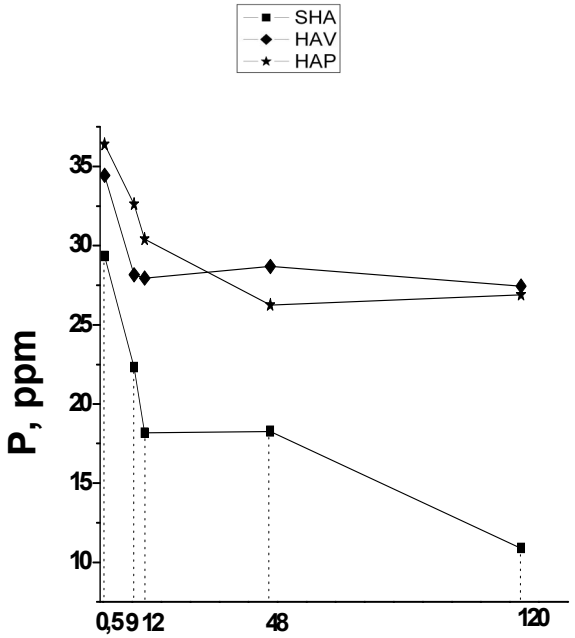
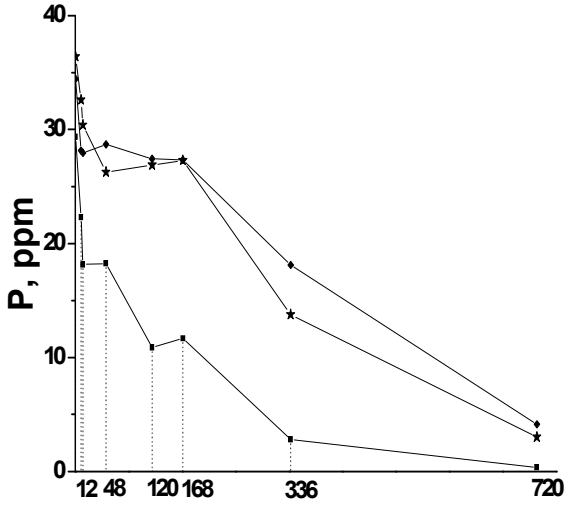


Fig. 7 Evolution of elemental concentrations of Ca in SBF measured by ICP, versus soaking time



Magnification of short times in hours after immersion



All times in hours of immersion

Fig. 8 Evolution of elemental concentrations of P in SBF measured by ICP, versus soaking time.

IV. CONCLUSION

The present work demonstrated the possibility to prepare nano-hydroxyapatite associated with polyvinyl alcohol and polyvinylpyrrolidone polymer composites. The method used is based on a simple and reproducible biomimetic approach at low temperature. The two types of polymers charge HA negatively and attract Ca^{2+} ions, which in turn attract PO_4^{3-} ions, thus forming apatite nuclei. Considerable efforts were 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 could be alleviated. The rate of bioresorption of nano HA was controlled and improved by the addition of both types of polymers; PVA and PVP. Equilibrium stage started early in both composite samples which might have led to minimizing 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 the way to exploiting the use of the investigated composites into bone tissue engineering.

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