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Characterization and Bioactivity Evaluation of (Starch/*N*-vinylpyrrolidone)—Hydroxyapatite Nanocomposite Hydrogels for Bone Tissue Regeneration

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ABSTRACT: Hydrogel series composed of starch/*N*-vinylpyrrolidone (starch/NVP) have been synthesized by means of γ-radiation-induced graft copolymerization and crosslinking process. Optimization of the preparation conditions was achieved to ensure the highest gelation degree. The produced hydrogels were characterized using fourier transform spectroscopy technique-, as well as by studying their swelling behavior. *In situ*deposition of hydroxyapatite (HAp) was achieved via alternate soaking technique. The developed nanocomposites nHAp-(starch/NVP) were characterized using energy dispersive X-ray spectroscopy technique, thermogravimetric analysis, X-ray diffraction, scanning electron microscopy. Mechanical study revealed that the compressive strength of the composites increased initially after the first cycle of deposition, and then it decreased gradually by increasing the number of the deposition cycles. *In vitro* bioactivity and blood compatibility tests of the obtained nanocomposites indicated that these nanocomposites were both bioactive and biocompatible. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: hydrogel; radiation; starch; polymer nanocomposites; bone regeneration; hydroxyapatite

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INTRODUCTION

Hydroxyapatites [$Ca_{10}(PO_4)_6(OH)_2$] (HAps), complexes of calcium phosphates, with chemical compositions very similar to bone mineral, have been used widely as bone implant in a variety of situations including the filling of osseous defects resulting from inflammatory periodontal diseases.[1] The most attractive characteristics of HAp are its remarkable biocompatibility that provokes no local or systemic toxicity, the absence of inflammatory or foreign body responses on implantation and its good attachment to bone.[2,3] HAp also possesses osteoconductive activities, provide a physical matrix suitable for the deposition of new bone, and can stimulate bone-tissue repair and growth.[4,5] However, the brittleness and the poor mechanical stability of pure HAp limit its use for the regeneration of nonload-bearing bone defects and tissue engineering applications.

Many efforts have been made toward the development of new bone substitutes materials. Among these attempts, HAp/polymer composites have attracted much attention from material scientists, because of its unique advantages over their conventional constituting components such as biocompatibility and bone-bonding ability. These composites combine the osteoconductivity of HAp with the easy processing ability of polymers. Also, in combination with the wide variety of mechanical properties of polymers, the HAp/polymer composites can be made either for load-bearing or for nonload-bearing purposes. Consequently, such composites fulfill the mechanical properties required for their function as skeleton and teeth. Finally, completely degradable HAp/polymer composites can be achieved by using a biodegradable polymer matrix. Such biodegradable composites have the ability to induce new bone growth and gradually degrade, thus enabling the load gradually to transfer from the material to the newly grown bone.[6–8] HAp/polymer composites have been synthesized by many methods like blending[9]; biomimetic process using simulated body fluid (SBF),[10] *in situ* precipitation,[11] and electrochemical deposition.[12] These processes are either complex or time consuming. The preparation of composites based on the wet synthesis of HAp using alternate soaking method is a simple method for the preparation of polymer/HAp composites,[13] such method does not need high processing temperature or special equipments.

Starch-based copolymers and composites have been introduced as promising biomaterials for orthopedic applications.[14] The

combination of the biodegradable character of starch, with the biostability of vinyl monomers and the incorporation of the well-known ceramic compound HAp, could lead to the development of partially biodegradable and bioactive materials, which can promote bone regeneration.[15] These materials are biodegradable, and when adequately reinforced and processed by nonconventional injection molding routes, they possess stiffness matching that of the bone.[16]

This study aims to synthesize a biocompatible nanocomposite hydrogels and test the hypothesis that such nanocomposites have the bioactivity needed to bond to natural bones. In this connection, starch/*N*-vinylpyrrolidone (NVP) hydrogels and nHAp-(starch/NVP) composites will be synthesized and characterized before and after immersion in SBF. *In vitro* bioactivity and biocompatibility evaluation will be investigated.

MATERIALS AND METHODS

Soluble potato starch was received from HiMedia Laboratories-India. Sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$) and calcium chloride dihydrate ($CaCl_2.2H_2O$) were received from Sigma-Aldrich Laborchemiklien and used as precursors for P and Ca. *N*-vinyl-2-pyrrolidone, 98% (NVP) were received from Sigma-Aldrich. Reagent grade NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2$, $CaCl_2$, and Na_2SO_4 and tris–hydroxymethyl aminomethane $(CH_2OH)_3C(NH_2)$, were purchased from Sigma- Aldrich.

Preparation of (starch/NVP) Hydrogels

(Starch/NVP) hydrogels were obtained by radiation-induced copolymerization of mixtures of different compositions from their aqueous solutions using ^{60}Co γ-irradiation. The mixtures were exposed to a total dose of 20 kGy at a dose rate 4 kGy/h. After copolymerization, the vials were broken, the formed polymeric cylinder were removed and cut into discs. The obtained hydrogel discs were washed extensively with distilled water to remove the unreacted component, then air dried at room temperature for further investigations.

HAp Deposition on (starch/NVP) Hydrogel

(Starch/NVP) hydrogel discs with different compositions were soaked in 50 mL of $CaCl_2$ (Ca solution) at 37°C for 24 h. After being removed from Ca solution and rinsed with distilled water, the hydrogels were soaked in 50 mL of Na_2HPO_4 (P solution) at 37°C for 24 h. The hydrogels were removed from P solution and rinsed with distilled water and dried. Repeating these steps (one deposition cycle) results in the deposition of HAp. The mentioned steps were repeated up to nine cycles.

Characterization of the Prepared Hydrogels and Composites

Gel Content. The dried hydrogels were soaked in distilled water for 24 h at 60°C. The insoluble part corresponding to the gelled part was air dried and weighted. The gel content was determined using the equation:

$$\text{Gel content}\,(\%) = \frac{W_d}{W_0} \times 100$$

where (W_0) is the initial weight of dried hydrogel before soaking and (W_d) is the dried gelled weight of the samples after the extraction of the ungelled parts with water.

Swelling Study. Degree of swelling could be described as water absorptive capability of the hydrogels. The hydrogel samples were immersed in distilled water for 48 h at room temperature until the gel reached the equilibrium state of swelling. After, the excess of water on the surface of the swollen hydrogels was removed with filter paper, the weight was determined. The degree of swelling was determined using the equation:

$$\text{Swelling degree}\,(\%) = W_s - W_0 W_0 \times 100$$

where (W_s) is the weight of the swollen hydrogels and (W_0) is the initial weight of dried hydrogel before swelling.

Fourier-Transform Infrared- Studies. Fourier transform infrared (FTIR) studies of the prepared hydrogels and composites specimens were recorded on Mattson 1000, Unicam, England in the range from 400–4000 cm^{-1}.

Thermogravemetric Analysis. Shimadzu thermogravimetric analysis (TGA) system of Type TGA-50 under nitrogen atmosphere was used to determine the thermal stability and weight loss of the prepared hydrogels and composites as a function of temperature. The temperature ranged from ambient to 600°C at a heating rate of 10°C/min.

X-ray Diffraction. The X-ray diffraction (XRD) patterns of the composites were measured using XRD 6000 diffractometer with Cu target. The XRD runs were carried out over the 2θ ranging from 10 to 80° at a scan speed of 8°/min.

Scanning Electron Microscopy. The surface morphology of the prepared hydrogels and composites was observed by scanning electron microscope (model JSM-5400 JEOL, Japan) at a voltage of 30 kV. The hydrogel samples were allowed to swell till equilibrium, freeze dried, and then the surfaces were precoated with a thin gold layer to reduce charging.

Energy Dispersive X-ray Spectroscopy. Energy dispersive X-ray (EDX) unit microprobe coupled with an electron microscope used to provide a semi scanning quantitative analysis of the chemical composition of the prepared hydrogels and composites.

Mechanical Properties. The compressive strength of the prepared hydrogels and composites after alternate soaking method was measured using a mechanical testing machine (NEXYGEN from Lloyd Instruments). Cylindrical specimens were prepared with dimensions 1 x 1 cm^2. The testing conditions were at room temperature. The crosshead speed was set at 10 mm/min and the load was applied until the sample was fractured. The compressive strength was calculated from the relationship:

$$\text{CS} = \frac{4P}{\pi d^2} \times 100$$

where (P) is the load (N) at the fracture point and d is the diameter (mm) of the cylindrical specimen.[17]

Preparation of Simulated Body Fluid

Standard SBF was prepared according to the formulation and method described elsewhere.[18] Briefly, to prepare 1000 mL of SBF; NaCl (7.995 g), KCl (0.224 g), $CaCl_2.2H_2O$ (0.368 g), $MgCl_2.6H_2O$ (0.305 g), K_2HPO_4 (0.174 g), $NaHCO_3$ (0.349 g), and $Na_2SO_4.10H_2O$ (0.161 g) were added in order into 900 mL of distilled water with a stirring bar into 1000 mL plastic beaker.

Table I. Effect of NVP Content in the Feed Solution on the Gelation Degree and the Equilibrium Swelling Degree in Starch/NVP Hydrogel

NVP content (wt %)	Gel content%	S%
0.09	75.0	300
0.23	94.5	500

The pH of the solution was then adjusted to 7.25 by the addition of Tris/HCl. The total volume was adjusted up to 1000 mL with distilled water.

Bioactivity Assessment in SBF

In vitro bioactivity studies were carried out using standard SBF that contains inorganic ion concentrations similar to those of human blood plasma. The prepared hydrogels and their corresponding composites were placed separately in plastic jars and subsequently 50 mL SBF was added to each jar. During the immersion period, the samples were kept at 37°C in a humidified incubator, and the SBF was refreshed every week. The samples were then collected after 3, 7, 14, 21, and 28 days of incubation, rinsed with distilled water, and allowed to dry at room temperature.

Blood Compatibility Studies

Clot Formation Tests. The blood-clot formation test was done as described elsewhere.[19] In brief, the specimens were equilibrated with saline water (0.9% w/v NaCl) at 37°C for 24 h in a constant temperature bath. To these swollen samples, 0.5 mL of human ACD (acid citrate dextrose) blood was added followed by the addition of 0.03 mL of $CaCl_2$ solution (4 mol/L) to start the thrombus formation. Deionized water (4.0 mL) was then added to stop the reaction. The formed thrombus was separated by soaking in water for 10 min at room temperature and then fixed using 36% formaldehyde solution for another 10 min. The fixed clot was placed in water for 10 min and after drying its weight was recorded. The same procedure was repeated for glass surface and for the composites of varying compositions.

Haemolysis Tests. Haemolysis experiments were performed on the surfaces of the prepared hydrogels and composites as described elsewhere.[20] In brief, dry hydrogels and composite pieces (4 cm^2) were equilibrated in saline water (0.9% w/v NaCl) at 37°C for 24 h and human ACD blood (0.25 mL) was added into the hydrogels and composites. After 20 minutes, 2.0 mL of saline water was added into the specimens to stop haemolysis and the samples were incubated for 60 min at 37°C. Positive and negative controls were obtained by adding 0.25 mL of human ACD blood and 0.9% NaCl, respectively, to 2.0 mL of doubly distilled water. Incubated samples were centrifuged for 45 min, the supernatant was taken and its absorbance at 545 nm was recorded using a spectrophotometer. The percentage of haemolysis was calculated using the following relationship, given in Equation:

$$\text{Haemolysis}\,(\%) = \frac{A_{\text{sample}} - A_{(-)\text{control}}}{A_{(+)\text{control}} - A_{(-)\text{control}}}$$

where A is the absorbance, the absorbance of positive and negative controls was found to be 2.6 and 0.02, respectively.

RESULTS AND DISCUSSION

Preparation of (starch/NVP) Copolymer Hydrogels

Chemical modification of starch via graft copolymerization of vinyl monomers onto it has been studied widely in recent years.[21] The improved properties are becoming more and more important in industrial applications not only because they are low in cost but also mainly because the polysaccharide portion of the product is biodegradable.

The graft copolymers (starch/NVP) hydrogels were synthesized by grafting and self-bridging of NVP onto starch in aqueous medium using gamma rays as clean source for initiation and crosslinking.

Characterization of the Prepared Hydrogels

Gelation and Swelling Behavior (starch/NVP) Hydrogels. The gel content is one of the most important factors that affect the swelling of hydrogels. Table I shows the effect of NVP content in the feed solution on the gelation degree as well as the equilibrium swelling degree of the produced starch/NVP hydrogels. It is clear that, the gelation degree of the prepared hydrogels is in direct proportion with the NVP content. The gelation degree increases by increasing the amount of NVP used during preparation. Such increase in the gelation degree may be attributed to the high tendency of NVP, as a vinyl monomer, for radiation polymerization and crosslinking. The high gelation degree of the prepared hydrogels makes them considerably safe to be used in direct contact with living tissues. Also, it can be noticed that there is a remarkable increase in the value of the equilibrium swelling degree of the obtained hydrogel by increasing the NVP content in the feed mixture solution. Such increase can be attributed to the high gelation degree; consequently well-built network structure that allow the retaining of high amount of water in addition to the high hydrophilic character of NVP monomer molecules.

FTIR Spectroscopy Analysis. The occurrence of graft copolymerization process was confirmed by comparing the FTIR spectrum of starch with that of the obtained graft copolymers.

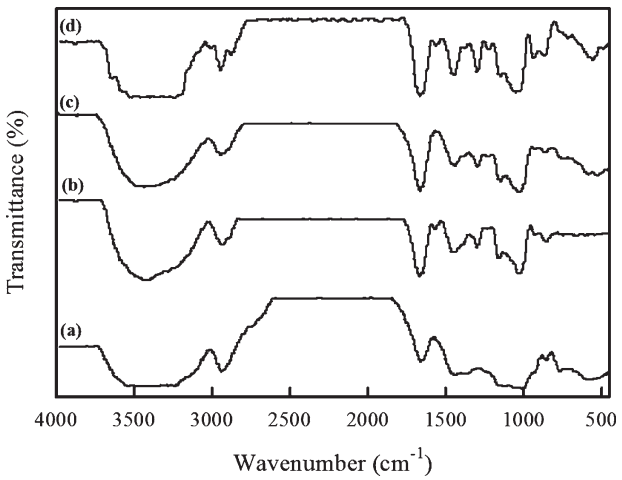

Figure 1. FTIR spectrum of (a) pure starch, (b) starch/NVP hydrogel, (c) nHAp(starch/NVP) composite after five deposition cycles and (d) nHAp(-starch/NVP) composite after soaking in SBF for 28 days.

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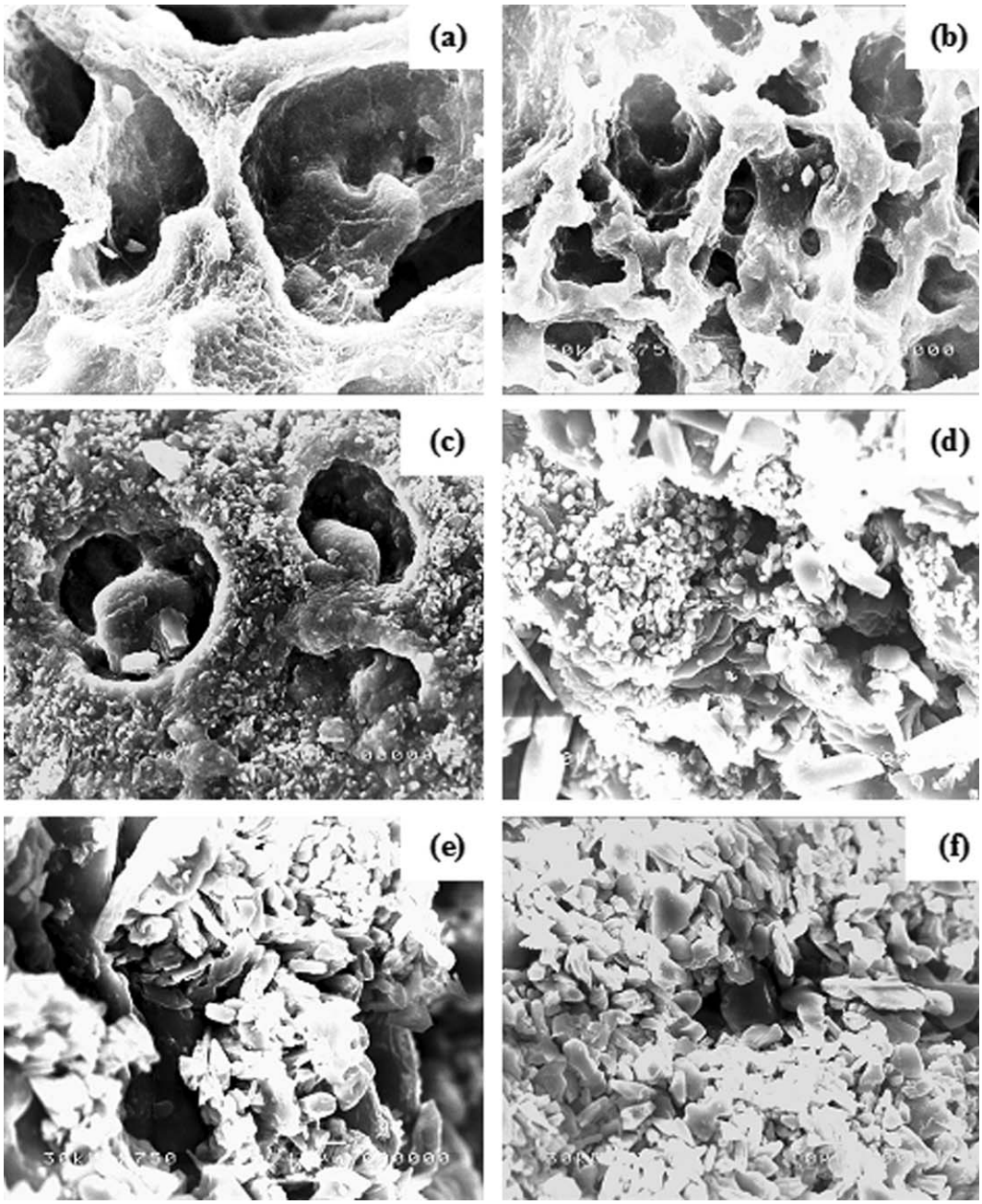

Figure 2. SEM images of starch/NVP hydrogels of different NVP content; (a) starch/NVP (1 : 0.23 wt %), (b) starch/NVP (1 : 0.09 wt %) (c) starch/NVP (1 : 0.23 wt %) after single deposition cycle (d) starch/NVP (1 : 0.09 wt %) after single deposition cycle (e) starch/NVP (1 : 0.09 wt %) after five deposition cycles, and (f) starch/NVP (1 : 0.23 wt %) after nine deposition cycles.

Figure 1 (a) shows the FTIR spectrum of the pure starch. A broad hydroxyl band appears at 3223–3540 cm^{-1} due to the stretching vibration of O—H, also the bands associated with C—H stretching vibration appears at 2928 cm^{-1} and C—O stretching vibration appears at 1000–1155 cm^{-1}. On comparing the FTIR spectra of starch with that of (starch/NVP) hydrogel, additional peaks appeared in the spectra of the prepared hydrogels.

Figure 1(b) shows the FTIR spectra of (starch/NVP) hydrogel. The grafting of NVP monomer is confirmed by the appearance the characteristic absorption bands at 1657 cm^{-1} due to C= O stretching vibration, and at 1431 and 1300 cm^{-1} due to C—N stretching and bending vibrations of monomer molecule.

Development and Characterization of nHAp-(starch/NVP) Composites

The polymer – apatite composite is expected to be useful not only as bone substitute materials but also as soft tissue adhesive materials. Bone-like apatite could be formed in the desired amount on/in the three-dimensional hydrogel network structure at normal temperature and pressure *in vitro* using alternate

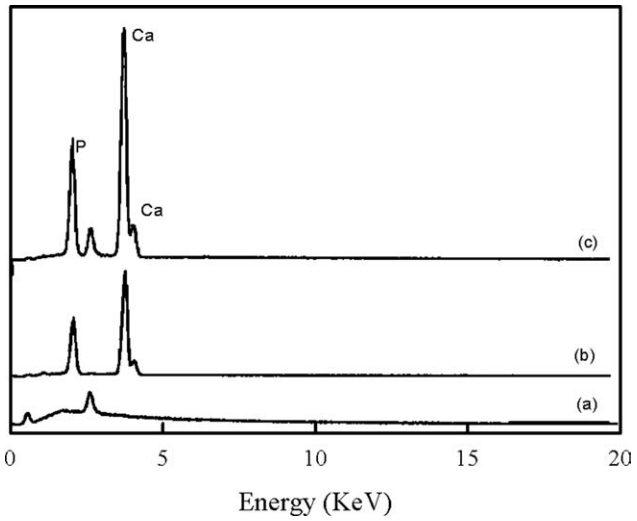

Figure 3. EDX spectra of (a) starch/NVP (1 : 0.23 wt %) copolymer hydrogel in comparison with its nHAp composites produced after (b) one and (c) nine deposition cycles.

soaking process.[22] Using this process, apatite formation can be controlled by changing reaction cycles and the apatite formation not only will be on the surface of the hydrogel but also within it. The rate of the apatite formation on/in the hydrogel is affected by the increase in the swelling ratio.[23] The apatite formation on/in a hydrogel matrix is greatly affected by its specific chemical groups.

Formation of HAp by means of alternative soaking method takes place via three major steps; complexation of Ca ions to the prepared reactive (starch/NVP) hydrogel,[24] assembling of the complexed Ca ions with $(PO_4)^{3-}$ ions to form HAp nanocrystal, which results in a coprecipitation process and a dispersion nucleation and finally, formation of chemical bonding between hydroxyl and amino groups available in the hydrogel and P—O and O—H groups of HAp crystals, resulting in firmly and homogeneously attached HAp crystals.

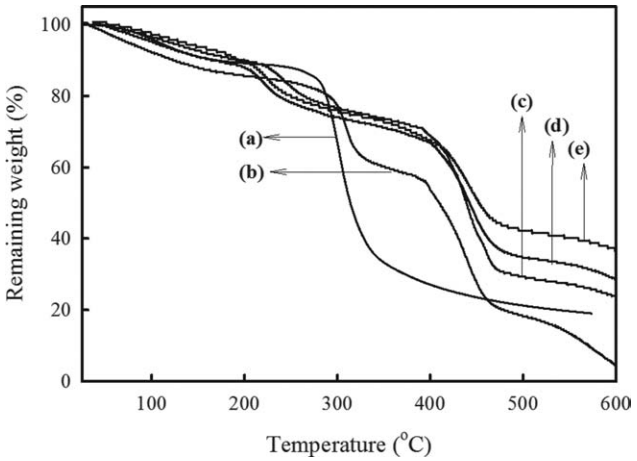

Figure 4. TGA curves of (——) starch, (...) starch/NVP (1 : 0.23 wt %)hydrogels in comparison with nHAp-(starch/NVP) (1 : 0.23 wt %)composites produced after (----) 1, (–••–••–) five and (–––) nine cycles of alternate soaking.

Table II. The Compressive Strength (MPa) of nHAp-(starch/NVP) (1 : 0.23 wt %) Composites after one, five, and nine Cycles of Alternate Soaking Process Compared with Blank Hydrogel

No. of deposition cycles	Compressive strength (MPa)
0	8.8
1	25
5	18.8
9	15.7

FTIR Spectroscopy. FTIR was used to confirm the deposition of HAp on the prepared hydrogel after alternate soaking process. Figure 1(c) displays the FTIR spectrum of nHAp-(starch/NVP) composite after the fifth cycle of alternate soaking. The spectrum shows the characteristic peaks of HAp, PO_4^{-3} bands appearing at 530, 580 and 1074 cm^{-1}. Finally OH broad band appears at 3359 cm^{-1}.

Scanning Electron Microscopy. Porosity is one of the most important factors affecting the morphological properties of biomaterials scaffold in bone regeneration process. Higher porosity favors tissue in-growth, bone formation, and forming biological fixation with surrounding tissue. Bone in-growth requires high levels of interconnected porosity.[25] Scanning electron microscopy (SEM) images shown in Figure 2 illustrate the topographical properties of starch/NVP copolymer hydrogel of different compositions and its HAp composites after alternate soaking in Ca and P solutions at different deposition cycles.

It is clear from Figure 2 (a,b) that (starch/NVP) hydrogels have interconnected irregular porous structure and by increasing the NVP content, the size of the existence pores was reduced. Such structure suggested the prepared hydrogel as a suitable material for bone tissue regeneration. Figure 2 (c,d) shows the starch/NVP copolymer composites with different NVP content after single deposition cycle. It is clear that the HAp was deposited on all the possible surfaces of the hydrogel whereas the hydrogel almost kept its surface structure. Also, it can be seen that, the HAp deposited layers increased by increasing

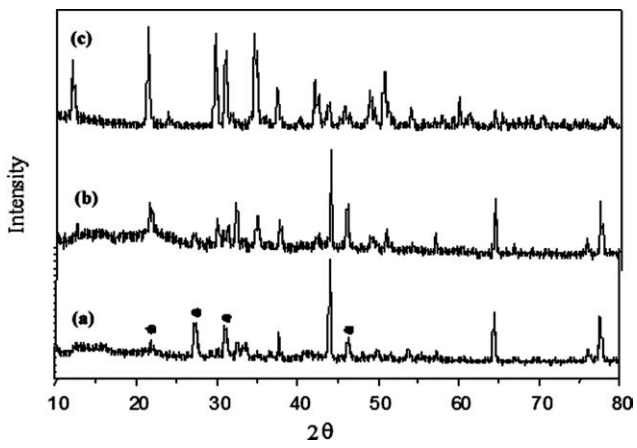

Figure 5. XRD patterns of nHAp-(starch/NVP) (1 : 0.23 wt %) composites after (a)one, (b)five, and (c) nine cycles of alternate soaking process.

Table III. Average Crystallite Size of nHAp in nHAp(starch/NVP) Composites with Different NVP Content (wt %) at Different Deposition Cycles of Alternate Soaking Process

	Crystal size (nm)	
No. of cycles	0.09 wt % NVP	0.23 wt % NVP
1	21.6	44.7
5	37.9	31
9	14.8	33.4

NVP content on the prepared hydrogels. On further deposition, the deposited HAp particles grow both in number and in size and the hydrogel surface was completely covered with rod like HAp as shown in Figure 2 (e,f). The SEM studies revealed that, the prepared composites could be competent for nutrient diffusion, metabolite elimination, cell migration, and bone growth into the interior of composites *in vivo*.

Energy Dispersive X-ray Spectroscopy (EDX). The Ca/P molar ratio is one of the most important characteristic of biomaterial to be used for bone substitution, that it scales its phase purity, chemical homogeneity, and solubility. Figure 3 shows the EDX spectra of (starch/NVP) (1 : 0.23 wt %) hydrogel in comparison with its composites produced after one and nine deposition cycles of alternate soaking. The EDX spectrum of the (starch/NVP) (1 : 0.23 wt %) blank hydrogel shows no signal in the region of both Ca and P range. After alternate soaking, the material formed on the hydrogel was found to be calcium phosphate. It can be seen that the Ca/P ratio of HAp deposited within the starch/NVP hydrogel increased by increasing the number of deposition cycles. Ca/P ratio of the deposited HAp was found to be ranged from 1.3 to 1.56 which is slightly lower than the theoretical value of stochiometric HAp 1.67. Although the naturally occurring stoichiometric HAp has calcium to phosphorous ratio of 1.67, the physiologically occurring HAp is always calcium deficient, due to the incorporation of hydrogen phosphate ions. Calcium phosphate ceramics with a Ca/P ratio of either 1.67 (stoichiometric HAp) or 1.5 (calcium-deficient HAp) have been reported to be biocompatible.[26]

Thermogravimetric Analysis. TGA analysis was carried out with the objective to verify the thermal stability of the prepared composites, as it will be applied as a biomaterial, it is necessary to be highly steady not only in the temperature of the human body but also mainly in higher intervals of temperature, which involve sterilization processes.

Figure 4 compares TGA curves of starch and (starch/NVP) (1 : 0.23 wt %) hydrogel and its composites after one, five, and nine cycles of alternate soaking. It can be seen that, starch/NVP hydrogels started to degrade at about 293°C. The weight loss increases with increase in temperature from 293 to 330°C and thereafter decreased gradually. The degradation step in the temperature range 400–485°C is the main degradation step of poly(vinylpyrrolidone), leading to the formation of esters as a consequence of the scission of the N—C—O bonds at 480°C.[27]

Also, it can be noticed that, (starch/NVP) (1 : 0.23 wt %) hydrogel started its initial thermal decomposition very near to that observed for pure starch. These results indicate that, after the copolymerization process, the copolymer maintains the thermal stability characteristic of its backbone structure even with high grafting yields.

The obtained data, Figure 4, reveal an improvement in the thermal stability of hydrogel after alternate soaking process. The total weight loss due to the thermal decomposition of hydrogel composites decreased as the inorganic content (HAp) in the composites increased. This is due to the higher thermal stability of HAp. After alternate soaking, the initial degradation temperature was shifted slightly to lower temperature which indicates lower weight loss.

Mechanical Analysis. Among the mechanical properties to be considered in orthopedics, compressive properties are the most relevant for replacement of cancellous bone.[28] Compressive strengths of human bones vary between 2 and 12 MPa for cancellous bone and between 100 and 200 MPa for cortical bone.[29] Therefore, the measurements of compression strength values of the prepared composites are very important.

Table II shows the compressive strength in (MPa) of the obtained nHAp-(starch/NVP) (1 : 0.23 wt %) composites after one, five, and nine cycles of alternate soaking compared with that of pure hydrogel. It is noticed that the compressive strength of composites increased initially after the first cycle, then it decreased gradually with increasing the number of deposition cycles. The observed compressive behavior can be explained by the degree of adhesion between the HAp particles and the hydrogel matrix. HAp nanoparticles behave as load carriers leading to good mechanical properties if they are present in small amounts and distributed homogeneously in the hydrogel matrix. If the proportion of the HAp particles increases, this could lead to nonhomogeneous distribution and, therefore, aggregation of particles may occur. This may cause phase segregation and nonhomogeneity in the structure and poor adhesion to the matrix leading to a decrease in the compressive strength.[30]

The compressive strength for nHAp-(starch/NVP) (1 : 0.23 wt %) composites recorded comparable values compared to the cancellous bone(2–12 MPa).

X-ray Diffraction Analysis (XRD). XRD used to determine the phase(s) of mineral crystals (or layer) formed on the hydrogel after alternate soaking process. Figure 5 shows the XRD patterns of nHAp-(starch/NVP) (1 : 0.23 wt %) composites after one, five, and nine cycles of alternate soaking process. The XRD pattern after one cycle shows the main peaks of HAp appears at 2θ values 21.7, 27.3, 30.9, and 46.6° which are characteristic peaks of HAp.

It is noticed that with increasing the number of deposition cycles, the samples showed sharp and well- defined peaks indicating the growth of apatite crystals in/on the hydrogels and the further increase of crystalline nature of composites with increasing HAp deposition. The shift and increase in intensity of each peak of HAp after increasing the deposition cycles clearly

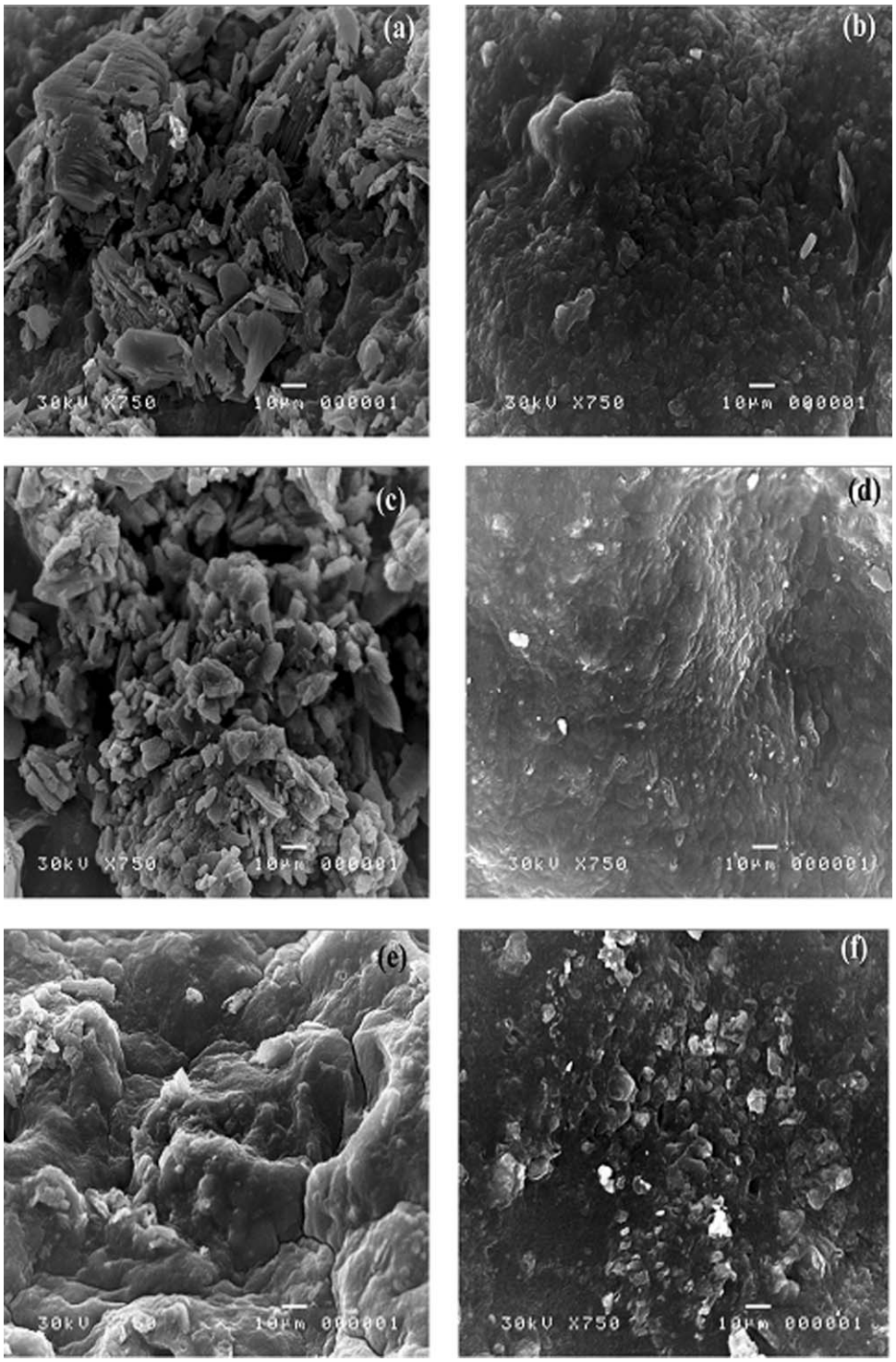

Figure 6. SEM images of nHAp-(starch/NVP) (1 : 0.23 wt %) hydrogel composites after soaking in SBF; (a) one deposition cycle after soaking in SBF for 3 days (b) one deposition cycle after soaking in SBF for 28 days, (c) five deposition cycles after soaking in SBF for 3 days (d) five deposition cycles after soaking in SBF for 28 days.

indicate the presence of bonding between HAp particles and hydrogels.

The average crystallite size of the obtained nHAp was calculated from the broadening in the XRD pattern according to the Scherrer's equation:

$$L = \frac{K\lambda}{\beta \cos\theta}$$

where L is the average crystallite size, β is the full width of the peak at half of maximum intensity (rad) (FWHM), λ is the

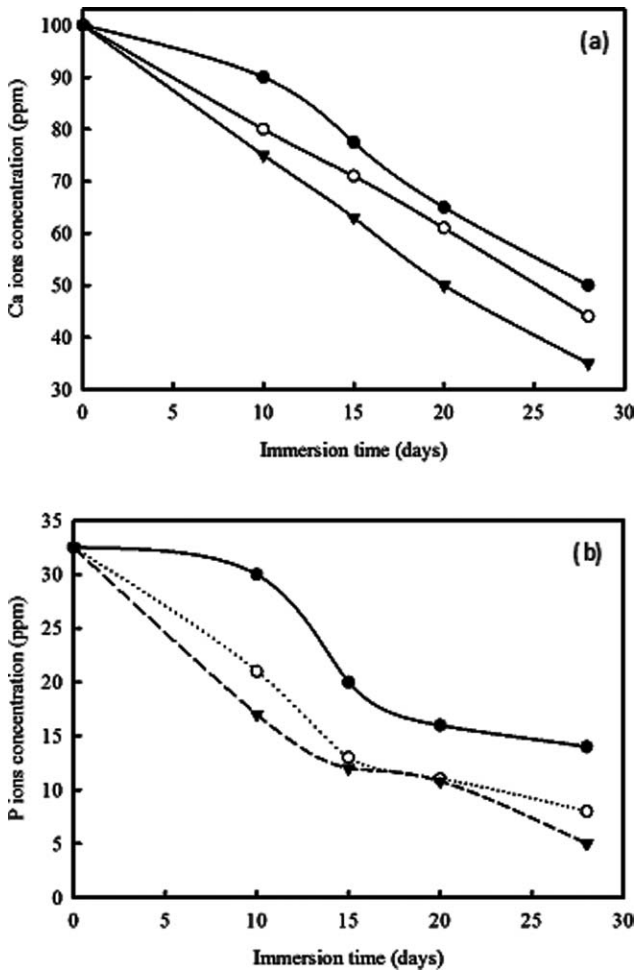

Figure 7. The change of ionic concentration of (a) calcium and (b) phosphorous of SBF as a function of immersion time of nHAp(starch/0.23 wt % NVP) composite after (●)one, (○)five, and (▼)nine cycles of alternate soaking process.

wavelength of monochromatic X-ray beam radiation Cu radiation (λ = 1.5406 Å), θ is the peak diffraction angle (Bragg's angle), K is a Scherrer constant defined as the crystallite shape and is approximately equal 0.9.

It can be noticed from Table III that, the average crystallite size ranged between (14 : 44 nm). Also, it can be seen that, by increasing NVP content, the average crystallite size of the deposited nHAp was increased. It should be borne in mind that crystallite size is not necessarily related to particle size; hence particles may be composed of several crystallites.[31]

Bioactivity Evaluation

In vivo studies are essential for biomaterials that are currently developed. However, results obtained from *in vivo* experiments are often difficult to interpret due to the complexity of various cellular responses. On the other hand, *in vitro* studies can provide information that indicates the *in vivo* performance of materials.[32] Bioactivity is a result of the chemical reactions occurring at the surface of a material exposed to body fluids which lead to the formation of a surface layer of hydroxyl carbonated apatite (HCA) after implantation which is essential for establishing bonding with natural bone.[33] Generally, it is believed that the *in vitro* calcification ability of biomaterials has a correlation with the bone-bonding ability *in vivo*. Thus, investigating the biological behavior of bioceramics in SBF is considered to be the most efficient method to authenticate their bioactivity in the body environment.

FTIR Spectroscopy. FTIR was used to give more information on the apatite formed on the surface of composite after its immersion in SBF. Figure 1(d) displays FTIR spectrum of the apatite formed *in vitro* on nHAp-(starch/NVP) composite after immersion in SBF for 28 days. The spectrum showed new small peaks that belong to the bone-like apatite of vibrational bands of carbonate groups. They were $\upsilon 2$ band at the 879 cm^{-1}, $\upsilon 3$ bands at 1550 cm^{-1}. These results suggested that the apatite formed on the surface of composite in SBF was carbonated apatite, which is similar in composition and structure to bone apatite. As bone mineral contains a substantial amount of carbonate (4–8 wt %) in the human body, the presence of carbonate in the composites is advantageous, as it increases the mechanical consistency and bioactivity of the apatite[34] and hence, more osteoconduction and tissue in-growth is expected on implantation. The carbonate ions are part of apatite structure and not as different phases

SEM Microscopy. SEM studies showed the growth of a layer of HCA on the surface of nHAp-(starch/NVP) (1 : 0.23) wt % composite after their immersion in SBF. This apatite layer is bone-like apatite, which was also found to form on various bioactive materials when they were immersed in SBF. Figure 6 (a–f) shows the SEM images of nHAp-(starch/NVP) composites after one, five, and nine cycles of alternate soaking after immersion in SBF for 3 and 28 days. After 3 days in SBF, both the size and the amount of the deposited HAp particles were increased. By increasing the soaking time up to 28 days, the surface becomes smooth and a thick layer of HCA completely covers the composite surface, which revealed high *in vitro* bioactivity for the prepared composites. According to SEM images, as the number of deposition cycles increased, the composite has high ability for formation and nucleation of apatite after soaking in SBF. These results suggested a promising potential bonding ability that facilitates bone ingrowth formation and good osteointegration *in vivo*.

Solution Analysis. In this study, the bioactivity of the developed composites was evaluated by investigating the variation of calcium and phosphorous ions concentration in the SBF solution with time. Figures 7 (a,b) show the change of ionic concentration of Ca and P of SBF, respectively, as a function of immersion time of nHAp-(starch/NVP) composites after one, five, and nine cycles of alternate soaking process.

It is clear that, the Ca and P ions concentrations in SBF decreased gradually as a function of immersion time. Such decrease in the Ca and P ions concentration is attributed to the consumption of the Ca and P ions to form amorphous HCA layer on the surface of the sample. It can be seen that, as the deposition cycles increased, the consumption of Ca and P ions increases, that is, the greater the HAp content, the higher the bioactivity.

Blood Compatibility

For materials that come into contact with blood, the formation of clot is the most undesirable but frequently occurring event

Table IV. Blood Compatibility Parameters of nHAp (starch/NVP) Composites with Different Deposition Cycles of Alternate Soaking Process Compared with Blank Hydrogel

No. of cycles	Haemolysis (%)	Blood clot (mg)
0	0.21	20
1	0.11	≈0
5	0.014	≈0
9	0.001	≈0

that restricts the clinical acceptance of a material to be used as biomaterial. Therefore, certain test procedures have been developed and they need to be used to judge the haemofriendly nature of materials.[35]

Table IV shows the blood compatibility parameters of starch/NVP (1 : 0.23 wt %) hydrogel and its composites after one, five, and nine cycles of alternate soaking process. The obtained results revealed that, (starch/NVP) blank hydrogel possess the highest percent of haemolysis and blood clot formation ability if compared with its composites. Haemolytic and blood clotting activity of the nHAp -(starch/NVP) composites declare that haemolysis percent and blood clotting tend to be zero as the content of HAp increases by increasing the deposition cycles. The obtained results may be attributed to the improved blood compatible quality of the composites.

CONCLUSION

In this study, starch/NVP hydrogels of different compositions have been synthesized by means of γ-radiation-induced graft copolymerization and crosslinking process. Alternate soaking technique was used to develop a biocompatible bone-like HAp layers within the (starch/NVP) hydrogels. SEM studies revealed that the amount of the deposited HAp within the hydrogels increased by increasing the deposition cycles. XRD studies confirmed the formation of the HAp within the composites, TGA and mechanical analysis showed that the prepared composites meet the thermal and the mechanical requirements of these types of materials. The produced nHAp-(starch/NVP) composites were examined by EDX to estimate the Ca-P ratio. FTIR and SEM examination showed the growth of HCA on the surface of HAp- (starch/NVP) composite after their immersion in SBF. *In vitro* blood compatibility studies of (starch/NVP) hydrogel and its prepared composites confirmed the biocompatibility of the developed nHAp-(starch/NVP) composites.

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