Preparation, Characterization, and *In Vitro* Application of Composite Films Based on Gelatin and Collagen from Natural Resources

A. A. Haroun,[1] H. H. Beherei,[2] M. A. Abd El-Ghaffar[3]

[1]*Chemical Industries Research Division, National Research Centre, Dokki, Cairo, Egypt*
[2]*Biomaterials Department, National Research Centre, Dokki, Cairo, Egypt*
[3]*Polymers and Pigments Department, National Research Centre, Dokki, Cairo, Egypt*

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ABSTRACT: This work aims to prepare and characterize a new generation composite films with gelatins extracted from bovine and bird bones (G_b and G_c, respectively) in addition to collagen, hide powder, (HP) which are blended with copolymerized polyethylene (MPE) and low density polyethylene, using polymer melt technique, which may be suitable for application as a base material for dental and orthopedic implants with better biological response. The bone-bonding ability of the composite films was evaluated by examining the ability of apatite to form on their surface in a simulated body fluid (SBF) with ion concentrations nearly equal to those of human blood plasma. The examination of apatite formation on the composites after immersion in SBF for 7 days was carried out by Fourier transform infrared spectroscopy and scanning electron microscopy with energy dispersive X-ray. The results showed the formation of thick apatite layer on the surface of the biocomposite films in special way on these containing G_b/MPE and collagen/MPE. Promising results have been achieved indicating that these novel biocomposites have unique bioactivity properties, which can be applied in bone implants and tissue engineering applications as scaffolds in future. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 116: 2083–2094, 2010

Key words: blending; bone bioactivity; collagen; gelatin; SBF; polyethylene

INTRODUCTION

The rising number of scientific publications and patent applications dealing with the preparation of synthetic/natural polymer blends can be considered as strictly connected to the utilization of agricultural overproduction and agroindustrial waste, as renewable resources for the production of biodegradable ecocompatible plastic items. Many types of films and composites have been prepared by using starch, cellulose, lignin, or other natural polymers that in some cases can be considered to hold the rank of waste materials.[1–3] Proteins of animal origin can be itself transformed into biomaterials[4] or blended with synthetic and natural polymers.[5] This latter approach represents the aim of this article. The study of the tendency to cast films from gelatin and PVA and/or lignocellulosic materials biodegradation under soil burial simulation conditions was investigated in previous work.[6] The resistance of polyethylene to biological attack is related to its hydrophobicity, water repellency, and high molecular weight and its lack of functional groups recognizable by microbial enzymatic systems. All of these properties limit applications in which biodegradation is a desirable attribute. Major strategies to facilitate polyethylene depolymerization and subsequent biodegradation were focused on the direct incorporation of carbonyl groups within the backbone or on their *in situ* generation by pro-oxidants. The direct incorporation of natural biodegradable polymers to enhance the potential biodegradability of polyethylene has also been evaluated.[7] The major degradation effect promoted by the microbial assimilation of the natural polymers in the blends was the increase of the surface area of the synthetic bulk material. Hence, this is an only example of indirect oxidative degradation of polyethylene afforded by the biodegradation processes of natural polymers.[8] Thermoplastics like polyethylene and polypropylene are semicrystalline materials. Both materials have glass-transition temperatures (T_g) much less than room temperature and crystalline melting temperatures (T_m) much higher than room temperature. The chemical compatibility between the fiber and polymer must be achieved to adhere and spread over the molten polymer to the fiber during melt processing.[9] Gelatin represents a typical renewable material from natural resources of animal origin. It is made up of macromolecules consisting of amino acids residues in

Correspondence to: A. A. Haroun (haroun68_2000@yahoo.com).

variable relative proportions and distributions along the macromolecular backbone.[10] The quality of a gelatin for a particular application depends largely on its rheological properties, as well as its physico–chemical properties that are greatly influenced not only by the species or tissue from which it is extracted, but also by the severity of the manufacturing method.[11] Gelatin is a high molecular weight polypeptide derived from collagen, the primary protein component of animal connective tissues. Industrial preparation of gelatin involves the controlled hydrolysis of the organized structure of collagen to obtain soluble gelatin. The most important sources of collagen for gelatin production are bovine hide, bone, and pigskin. The source, age, and type of collagen, all influence the properties of the gelatins derived from them.[12] Bone is the most commonly replaced organ of the body, with over 500,000 bone repair procedures performed per year in the United States alone.[13] Biological grafts include autografts, allografts, and xenografts. The application of allografts and xenografts is limited because of disadvantages such as histoincompatibility and the possible transfer of infectious diseases. The autograft, often taken from the iliac crest of the patient, has a success rate of 80–90%, with minimal risks of immune infection, rejection, or disease transfer.[14] Consequently, it is the clinically preferred grafting material for bone repair and regeneration. Ideally, a bone graft should be biocompatible, able to support abundant bone formation (osteoconductive), able to induce bone formation (osteoinductive), able to form a continuous interface with surrounding bone tissue (osteointegrative), able to support angiogenesis, and able to be structurally and mechanically compatible with bone tissue. In the past decade, tissue engineering-based bone grafting has emerged as a viable alternative to biological and synthetic grafts, presenting a possible solution to problems currently associated with autografts.[15] Bone is composed of calcium phosphate (69–80 wt %, mainly hydroxyapatite, HAp), collagen (17–20 wt %), and other substances (water, proteins, etc).[16,17] It is a complex biomineralized material with an intricate hierarchical structure.[18] HAp/collagen composites have attracted significant interest and hold great promise as they possess excellent bioactivity and osteoconductivity, as well as enhanced mechanical properties.[19,20] Kokubo[21] developed a biomimetic process and found out that a HAp layer with the desired thickness could be formed not only on ceramics and metal but also on polymer films.[22] The creation of biomimetic coatings consists of dipping implants into a saturated calcium phosphate solution or into simulated body fluid (SBF), to generate thin bone-like apatite crystals. However, the deposit formed via such processes is mainly composed of octacalcium phosphates or carbonated apatite with various crystallinities.[23] One of the requirements of HAp coating onto organic polymer substrates is good adhesion to the substrates. To enhance the interaction between the inorganic apatite and the organic polymers, some researchers have introduced hydrophilic polar groups such as phosphate, carboxyl, and hydroxyl groups onto hydrophobic substrates.[24,25] It has been reported that organic polymers containing carboxyl groups form apatite on their surfaces in SBF if their carboxyl groups have been previously fully combined with calcium ions, as the carboxyl group induces apatite nucleation and the released calcium ions accelerate apatite nucleation.[26,27] In this study, the characteristics of apatite coating on different gelatin/MPE, gelatin/LDPE, and collagen/MPE films, which contain different functional groups like COOH, OH, and NH_2, formed by the polymer melt process, were studied, and these films were characterized by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and energy dispersive X-ray (EDX). This study is concerned with the development of biomaterials that might have potential applications in bone regeneration.

EXPERIMENTAL

Materials

Copolymerized polyolefin elastomer (MPE) produced from the copolymerization of ethylene and 1-octene, ENGAGE® (Dow Chemicals, UK), as uniform white pallets, it has melting temperature around 110°C, melting flow rate 5.0 g/10 min (under 190°C, 2.16 kg), and molecular weight 50,000 D. Low density polyethylene (LDPE), supplied by Petrochemical, as uniform granules, it has melting temperature 112°C, melting flow rate 0.4 g/10 min, and molecular weight 30,500 D. Shaved hide powder (collagen, HP) supplied by Merck, it has pH 5.2–5.6, ash content below 0.9 wt %, and total nitrogen content 11.3 wt %.

Extraction of gelatin

Gelatin was extracted from bovine and bird bones in accordance with Ref. 28. Bones used for gelatin extraction were cleared by scraping with a knife to reduce the flesh contamination. They were then demineralized using 3% hydrochloric acid at room temperature for a period of 9–12 days, with the liquor changed after even 3 days, until the bones do not have any hard cores. The demineralized bones were then treated with concentrated sulphuric acid to a pH of 2.5–3.0 for 16 h, until the bones are adequately swollen. The bones were then transferred to beakers covered with warm water and gelatin

TABLE I
The Chemical Composition of the Prepared Composite Films

Sample code	Composition (w/w)
G_c/MPE	G_c/MPE (1 : 1 and 1 : 9)
G_b/MPE	G_b/MPE (1 : 1 and 1 : 9)
G_c/LDPE	G_c/LDPE (1 : 1 and 3 : 7)
G_b/LDPE	G_b/LDPE (1 : 1 and 3 : 7)
H_1	HP/MPE (0.5 : 9.5)
H_2	HP/MPE (1 : 9)
H_3	HP/MPE (2 : 8)
H_4	HP/MPE (4 : 6)

extract (light liquor) and were filtered through compressed cotton wool. The light liquor was then passed through a column of activated carbon and the pH was adjusted to 5.0 using 5% ammonia solution and the extracted gelatin was dried at 40°C.

Preparation of composite films

Different composites were prepared in Brabender plastograph in the following operational conditions: temperature 140°C, rotor speed 50 rpm, and a mixing time of 7 min. HP was blended with MPE and both extracted gelatins were blended with MPE and LDPE at different weight ratios as shown in the Table I. The material was placed in 0.42-mm thick steel frame between two Teflon sheets. The whole system was inserted between the plates of a hydraulic press heated at 140°C and kept without any applied pressure for 7 min allowing a complete melting.

In vitro bone bioactivity test

The bioactivity of the composite films was evaluated by an *in vitro* study. *In vitro* bioactivities studies are much cheaper and quicker than *in vivo* studies. They are also useful in both designing new biomaterials and improving the existing materials for better bioactivity. To study the bioactivity, the samples were soaked in SBF, proposed by Kokubo and Takadama[29] at body temperature 37°C and pH 7.4 for 7 days. The SBF has a composition similar to human blood plasma and has been extensively used for *in vitro* bone bioactivity. It was prepared as shown in Table II. The pH of the solution was adjusted to 7.4 with tris-buffer using 1.0*M* HCl at 37°C. The SBF is metastable calcium phosphate solution because it is supersaturated with respect to apatite, but the super saturation level is not high for nucleation of apatite. A stimulus is required to nucleate apatite from SBF. Only specific materials, or called apatite inducers, can provide such stimulus. The bioactivity behavior provides information for understanding the fundamental aspects of interaction between bone and biomaterials. Each composite film was soaked in SBF for 7 days, then removed and rinsed using deionized water and dried to complete the required investigation. The composites surface after soaking were examined via FTIR and SEM-EDX to confirm the formation of apatite layer. The bioactivity test was carried out three times for each sample to determine its reproducibility.

TABLE II
Ion Concentrations of SBF and Human Blood Plasma

	Ion concentrations (m*M*)	
Ion	SBF	Human blood plasma
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	148.8	103.0
$(HCO_3)^{1-}$	4.2	27.0
$(HPO_4)^{2-}$	1.0	1.0
$(SO_4)^{2-}$	0.5	0.5
pH	7.4	7.2–7.4

Characterization of the composite films

The composite films were tested to determine the effect of composition on elongation and stress (stress/strain) in addition to e-modulus using ASTM D638 tensile test (Tinius Olsen); at 10 mm/min. The tensile test was performed three times for each sample and

TABLE III
Mechanical Properties of the Prepared Composite Films

Sample	Stress at yield (MPa)	Elong. at yield (%)	Stress at break (MPa)	Elong. at break (%)	E-modulus (MPa)
MPE	1.75	29.15	8.66	765	12.07
G_b/MPE (1 : 1)	0.42	10.5	0.48	229	6.3
G_c/MPE (1 : 1)	2.7	29	3.8	256	21.7
LDPE	10.5	65.2	11.8	385	15.2
G_b/LDPE (1 : 1)	5.1	32.5	3.2	98.9	8.4
G_c/LDPE (1 : 1)	8.3	40.8	7.3	120.1	19.7
H_1	1.5	22.4	2.3	168	15.1
H_2	1.4	14.2	2.1	111.3	14.7
H_3	1.3	13.1	1.9	88.4	9.3
H_4	0.88	11.1	1.5	63.3	8.3

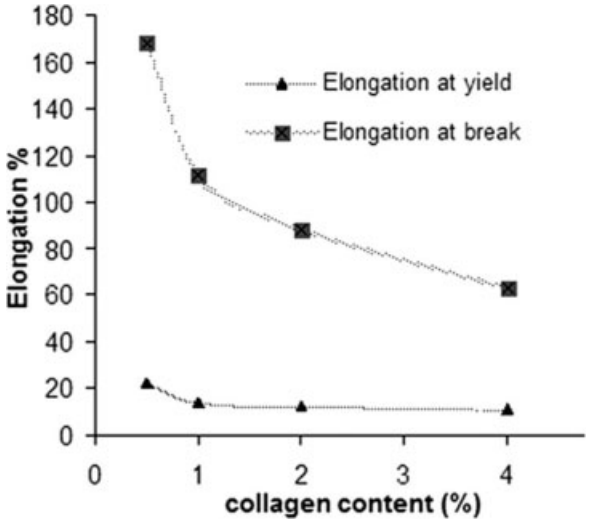

Figure 1 Variation of the elongation at yield and at break versus collagen content for the prepared films.

the data are presented as means. The morphology features of the composite films, before and after immersion in SBF for 7 days, were tested using SEM-EDX, JXA-840, and Electron probe microanalyzer (JEOL-JSM). Before observation, the fractured surfaces were coated with Au with SEM coating device (Edward spotter coater). Three micrographs were taken from different zones of each surface film under investigation. The composite films, before and after immersion in SBF for 7 days, were examined using Perkin–Elmer FTIR under certain conditions such as scan resolution: 4 cm^{-1}; scan rate: 2 mm sec^{-1}; range: 4000–600 cm^{-1}; and mode: transmission. The thermal properties of the composite films were evaluated by thermogravimetric analysis (TGA) using a Perkin–Elmer, seven series thermal analyzer in nitrogen atmosphere at a heating rate of 10°C/min over the temperature range of 50–1000°C.

RESULTS AND DISCUSSION

Processing behavior

In the processing of polymer blend, it is important to know some aspects such as how much energy is required to bring a polymer from an initial state to a homogeneous melt, how much time this process requires, and whether the polymer will degrade under certain conditions. The optimal processing conditions of the obtained blends were in a good agreement with the previous study reported by Dascalu et al.[30] Total weight of each sample is 10 g (100

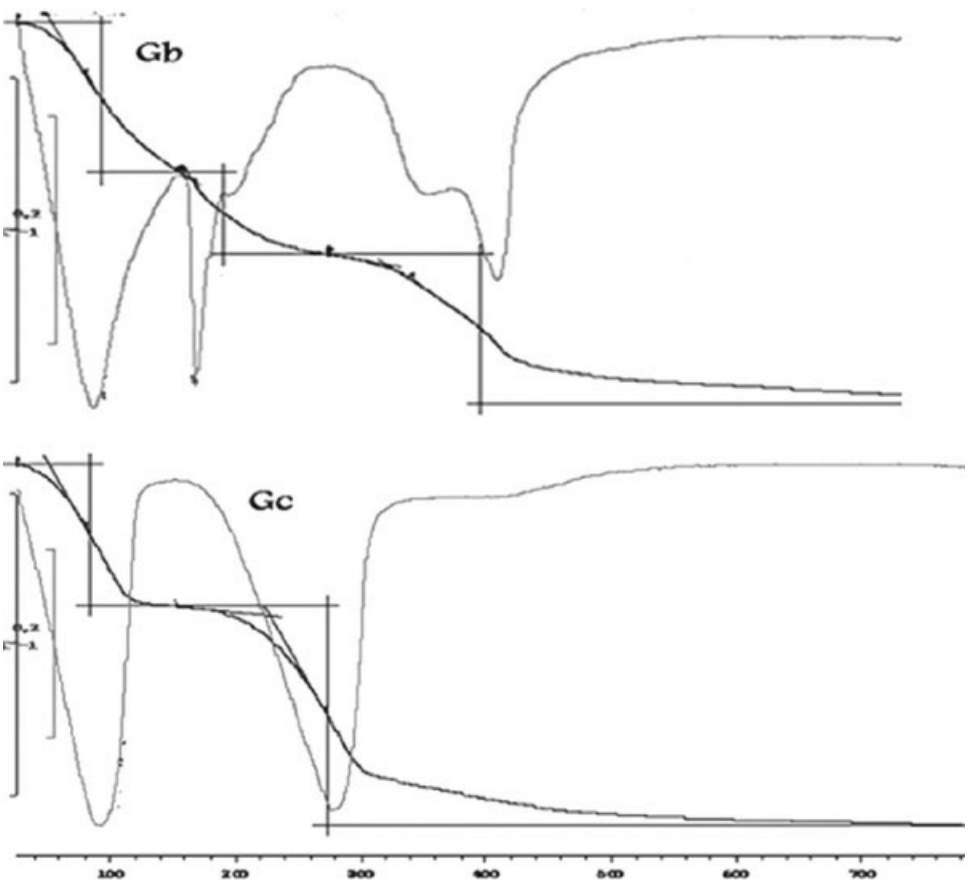

Figure 2 TGA/DTA curves of G_b and G_c.

TABLE IV
TGA Data of the Prepared Composite Films

	Weight loss (wt %)			
Sample	50–155 (°C)	156–221 (°C)	223–310 (°C)	>310 (°C)
G_b	24.9	13.4	24.5	–
G_c	23.5	–	36.4	–
HP	7.3	–	63.7	–
H_1	2.4	–	14.3	78.0
H_2	2.9	–	10.7	60.8
H_3	1.3	–	8.6	87.7

wt %), mixing time is 7 min, temperature about 140°C, and rotor speed around 50 rpm.

Mechanical properties

According to Dascalu et al.,[30] the variation of the tensile strength with the content of the hydrolyzed collagen (HC) was studied. It can be observed that the decrease of the tensile strength with increasing the HC content for LDPE/HC blends. Also, the previous work by Saha et al.[31] reported that the mechanical properties of metallocene-based linear low density polyethylene (mLLDPE)/HP blended films decreased with increases in concentration of the HP, which used as the filler to produce the modified biodegradable polymer film. As expected, the low values of the stress at yield and break for the composite films confirm their brittle behavior, because of a poor adhesion between MPE and gelatin or collagen (amorphous component). The increase of these two properties in some composites, as shown in Table III, can be explained through stronger interface between extracted gelatin (bovine) and MPE, moreover, its high stability under processing conditions. Both stress and elongation are influenced by the content and the type of the used protein. The variation

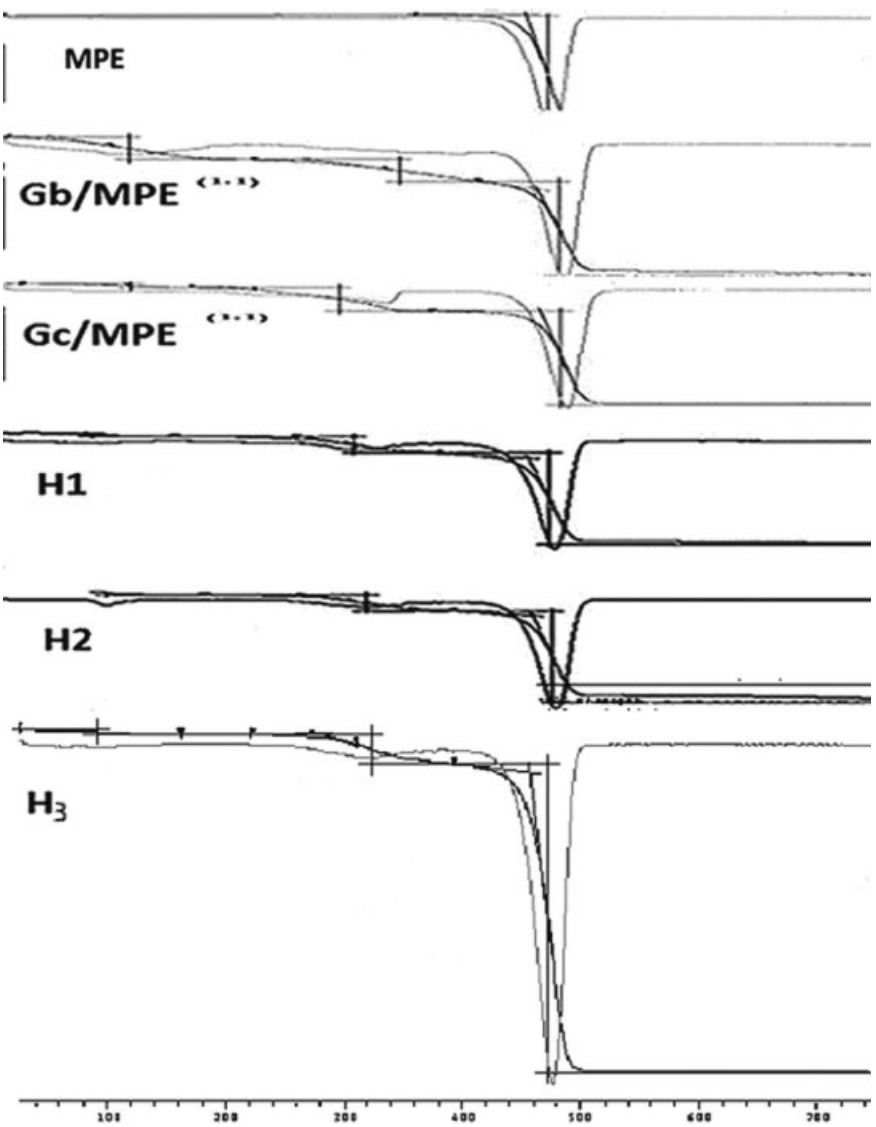

Figure 3 TGA/DTA curves of MPE, G_b/MPE, G_c/MPE, H_1, H_2, and H_3.

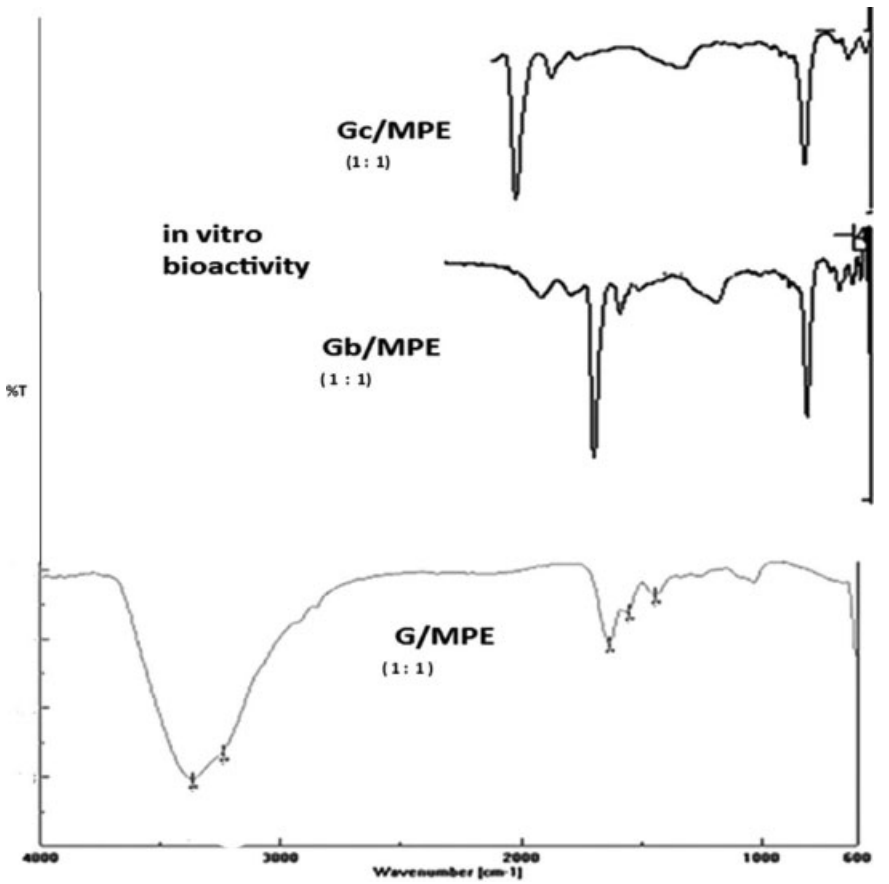

Figure 4 FTIR spectra of G_b/MPE and G_c/MPE composite films before and after immersion in SBF for 7 days.

of the elongation at yield and break with the content of the collagen is presented in Figure 1. It can be observed the decrease of the elongation with increasing the collagen content. At higher content of collagen, the strain is lower, and accordingly, the reactions between the components leading to a rigid material.

Thermogravimetric results

The thermal properties of gelatin, which was obtained from collagen by heating above the helix-coil transition temperature, have been extensively studied in the past.[32,33] When such heating is carried out in solution, it causes a collapse of the rod like three stranded collagen unit (tropocollagen molecule) into a random coil. Moreover, there is a partial disaggregation of individual chains leading to α (one chain), β (a covalently bonded pair chains), and γ (three covalently bonded chains) coiled polymeric units. Dry gelatin samples have two glass-transition temperatures (T_g), one at 120°C and the other in the temperature range 180–190°C associated with a sharp drop in the shear modulus of dry gelatin. The low transition temperature is assigned to be the diversification of the soft blocks (α-amino acids with glycine at every third position), whereas the high transition temperature is attributed to the diversification of the rigid blocks (imino acids such as proline and hydroxyproline with glycine at every third position) of the copolymer chain.

TGA/DTA curves of the extracted gelatin from bird and bovine bones are shown in Figure 2 and Table IV, it can be observed that two and three stages decomposition patterns, respectively. The first stage starts at about 50–155°C with a weight loss of about 23.5% and 24.9% for bird and bovine bones, respectively, and the second stage (G_b) starts at 156–221°C with a weight loss of about 13.4%. While the third stage starts at 223–310°C with a weight loss of about 36.4% and 24.5%, respectively. We can say that extracted gelatins from bovine bones G_b is more stable than that in case of bird bones G_c. The thermograms of MPE after blended with G_b and G_c, in addition to collagen hide powder (H_1, H_2, and H_3) are shown in Figure 3 and Table IV. In the thermal degradation of the collagen, it was envisaged that the weight loss at 320°C is due to the partial cleavage of peptide bonds with the formation of peptide fragments of low molecular weight.[34] The degradation of the hide powder alone starts at 280°C whereas the degradation of the hide powder after

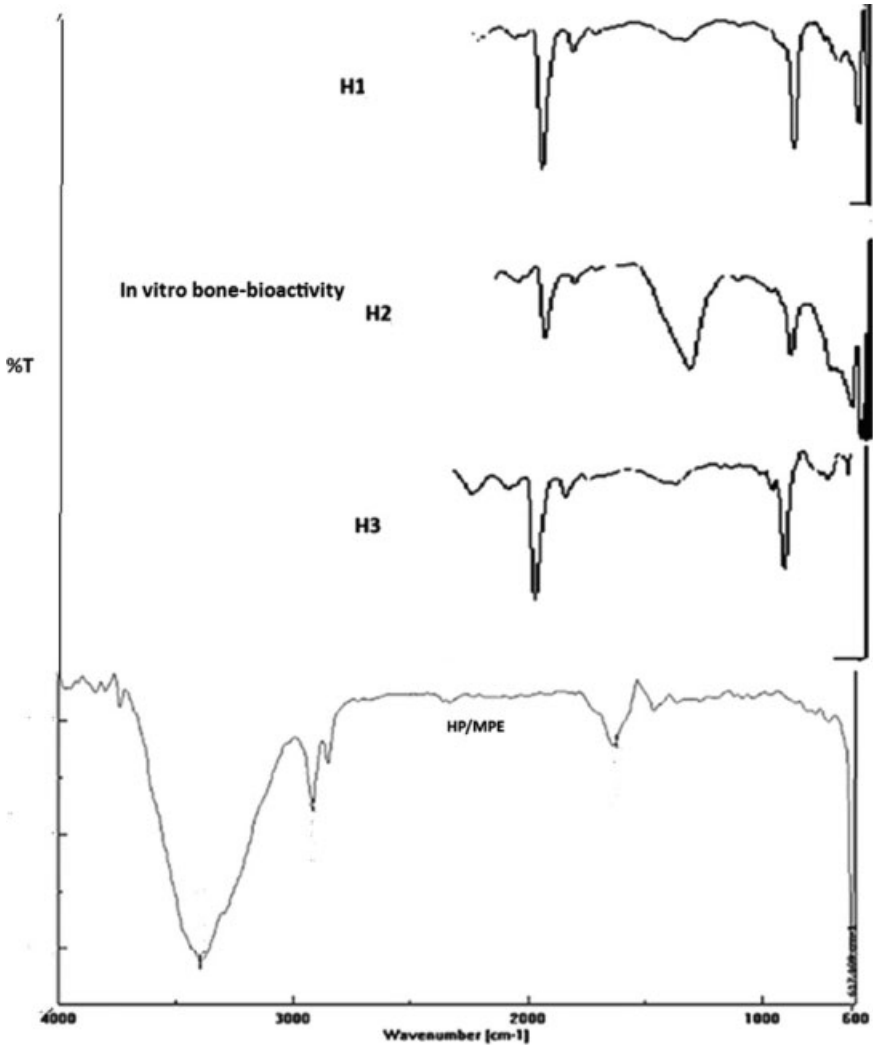

Figure 5 FTIR spectra of composite films before (H_1) and after (H_1, H_2, and H_3) immersion in SBF for 7 days.

blending with MPE was shifted to the range 448–454°C. It might be expected that the formation of lower peptide fragments in thermal-degraded hide powder and collagen occurs similarly. We can conclude that the copolymerized polyethylene (MPE) minimizes the thermal degradation rate of hide powder collagen. Moreover, from the thermal behavior and phase separation of the synthetic polymers, the results obtained make possible to use hide powder collagen as MPE modifier for producing the high quality biodegradable composite films.

In vitro bone bioactivity

The composite films were investigated postimmersion for 7 days after their withdrawal from SBF by FTIR and SEM-EDX to confirm the formation of apatite layer onto the composite surface. The interaction between the surface of composites and calcium phosphate solution may be responsible for the apatite nucleation. This theory is supported by SEM observation at high magnification, which shows a close association of apatite with the surface of composites. The composites might possess a strong affinity to both calcium and phosphate. The characteristic peaks of the extracted gelatins are illustrated at 1631, 1550, and 1238 cm^{-1}, according to the peptide bond vibration, correspond to amide I, amide II, and amide III, respectively. The amide I component has been attributed to random coils of peptide chains and some changes in secondary structure that occurred during the conversion of the triple-helix collagen to the denatured form (gelatin). The results of FTIR (Figs. 4 and 5) show that the composite reveals different behavior of these materials, when they are in contact with physiological fluid SBF for 7 days. The characteristic peaks of bone-like apatite are illustrated at 1462–1640 cm^{-1} (stretching), 963–1107 cm^{-1}, and 721–850 cm^{-1} (bending) corresponding to asymmetric hydroxyapatite carbonate $(CO_3)^{-2}$. The characteristic peaks at 960–1168 cm^{-1} (stretching) and 558–721 cm^{-1} (bending) corresponding to

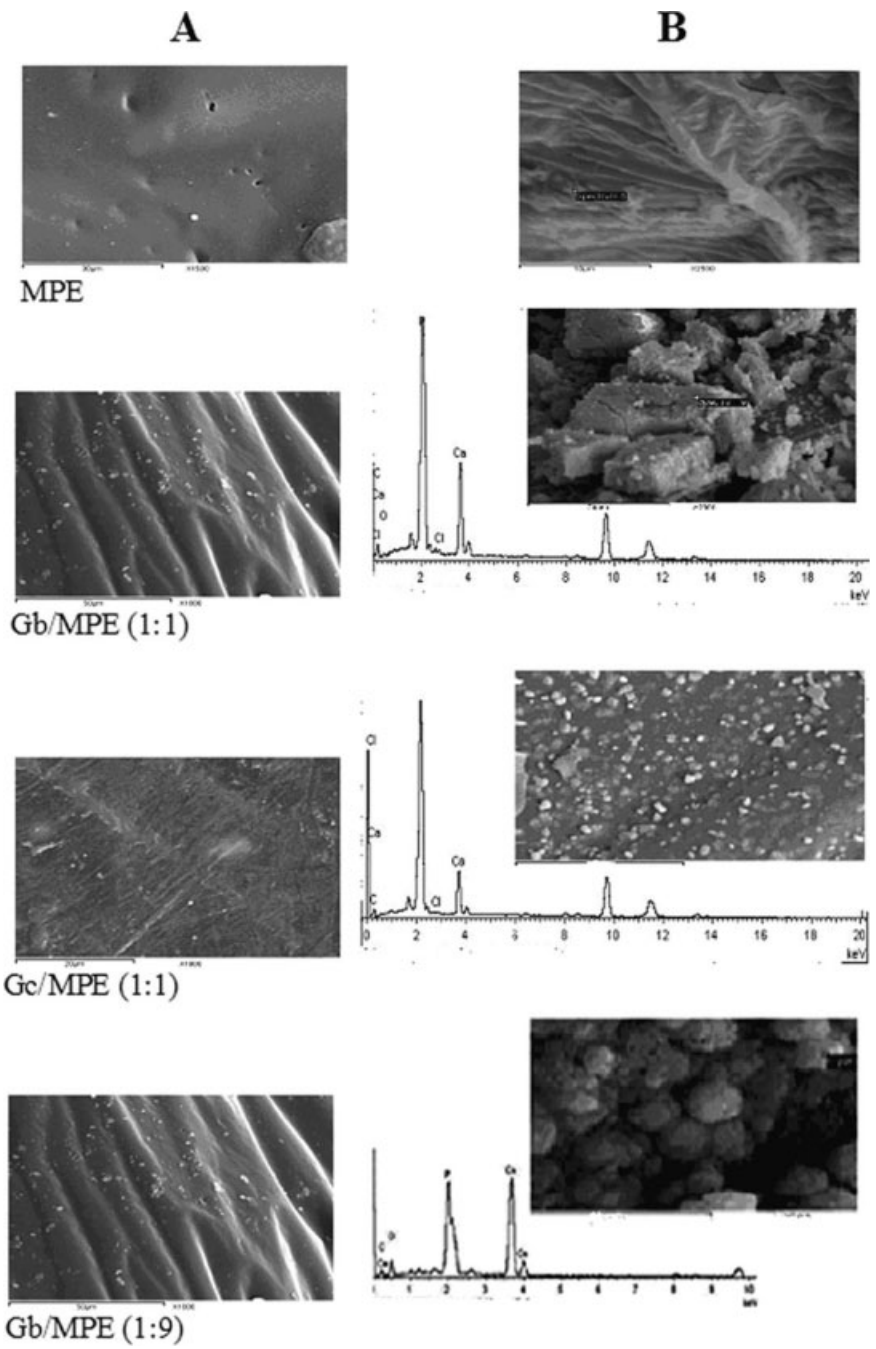

Figure 6 SEM images and EDX-spectra of extracted gelatins/MPE composite films (A) before and (B) after immersion in SBF for 7 days.

phosphate $(PO_4)^{-3}$. While the characteristic peaks at 638 cm^{-1} (stretching) and 600 cm^{-1} corresponding to labile phosphate and OH apatite, respectively. In addition, their intensities are enhanced with increase of extracted gelatin content till 50%. The carbonate band at 873 cm^{-1} indicating the formation of the B-type carbonate. Therefore, the highly precipitated new carbonate hydroxyapatite (C-HA) on the surface denotes the biomineralization from SBF. The interaction between the surface of composites and calcium phosphate solution may be responsible for the apatite nucleation. This theory is supported by SEM observation at high magnification, which shows a close association of apatite with the surface of composites. The composites might possess a strong affinity to both calcium and phosphate. Also, SEM observation of apatite formation on the coating after the composites were immersed in SBF shows a dense carbonate–apatite layer formation after soaking composite in SBF for 7days. SEM-micrographs of the prepared composite films before and after soaking in SBF for 7 days at 37°C are shown in Figures 6–8. A layer

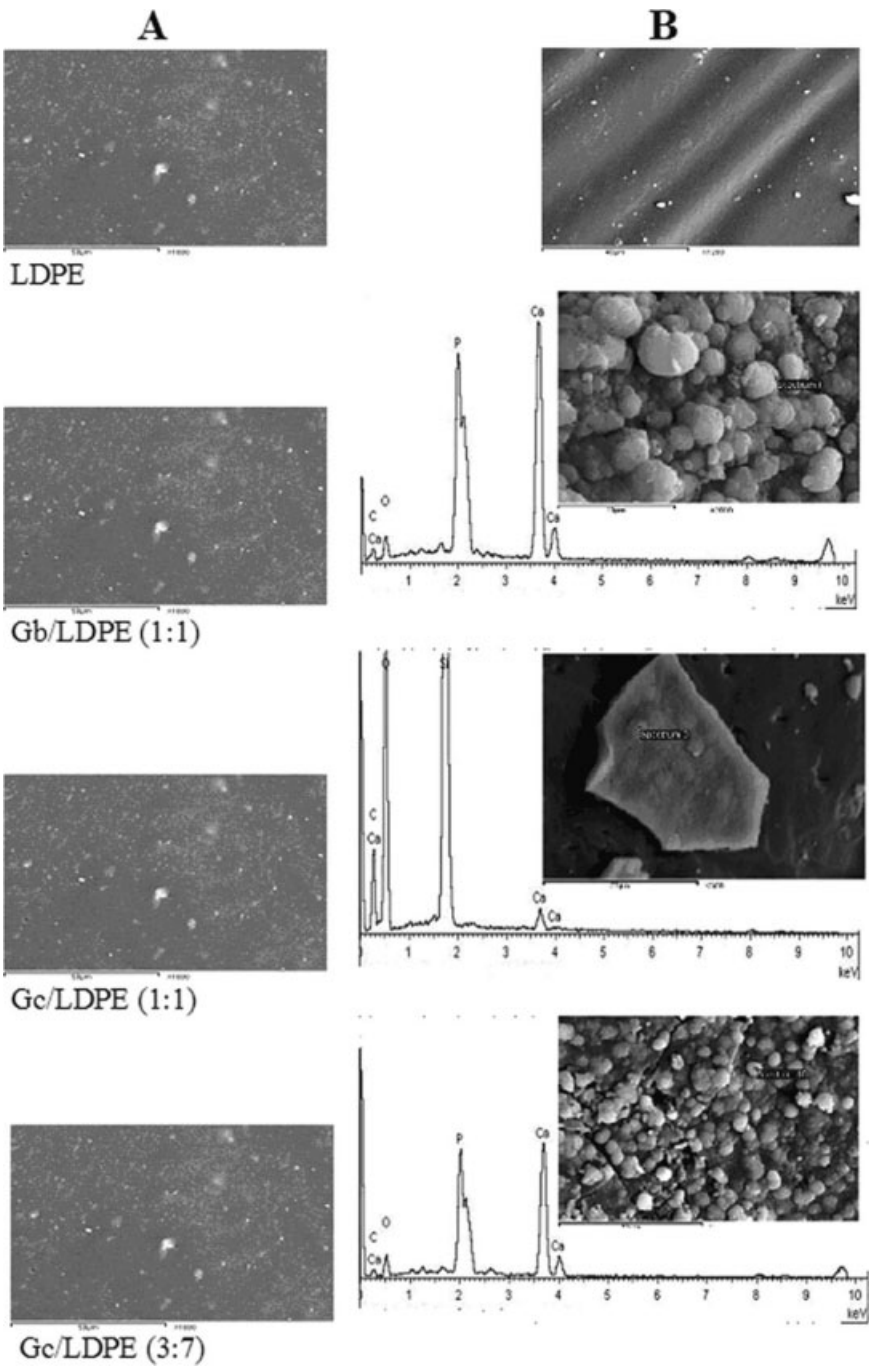

Figure 7 SEM images and EDX-spectra of extracted gelatins/LDPE composite films (A) before and (B) after immersion in SBF for 7 days.

consisting of spongy structure is formed on the whole surface for the composites G_b/LDPE and G_b/MPE characterizing the large shape of apatite layer proving effect of gelatin for the enhancement of apatite formation. Also, it can be observed that the proliferation and growth of bone-like apatite on the surface of the composites. SEM-micrographs show the appearance of large spherical shapes containing a minute pores accumulated to each other, which benefit bone in growth. The island-like features that appeared after the immersion in SBF were assemblies of tiny crystals formed on the surfaces of the collagen composites. The number of apatite islands increased appreciably with composites, which include low collagen content. EDX spectrum shows peaks of high intensity corresponding to the presence of carbon from organic phase, and peaks of low intensity corresponding to the trace elements Cl and Na from the gelatin extraction method and peaks corresponding to the calcium phosphate inorganic

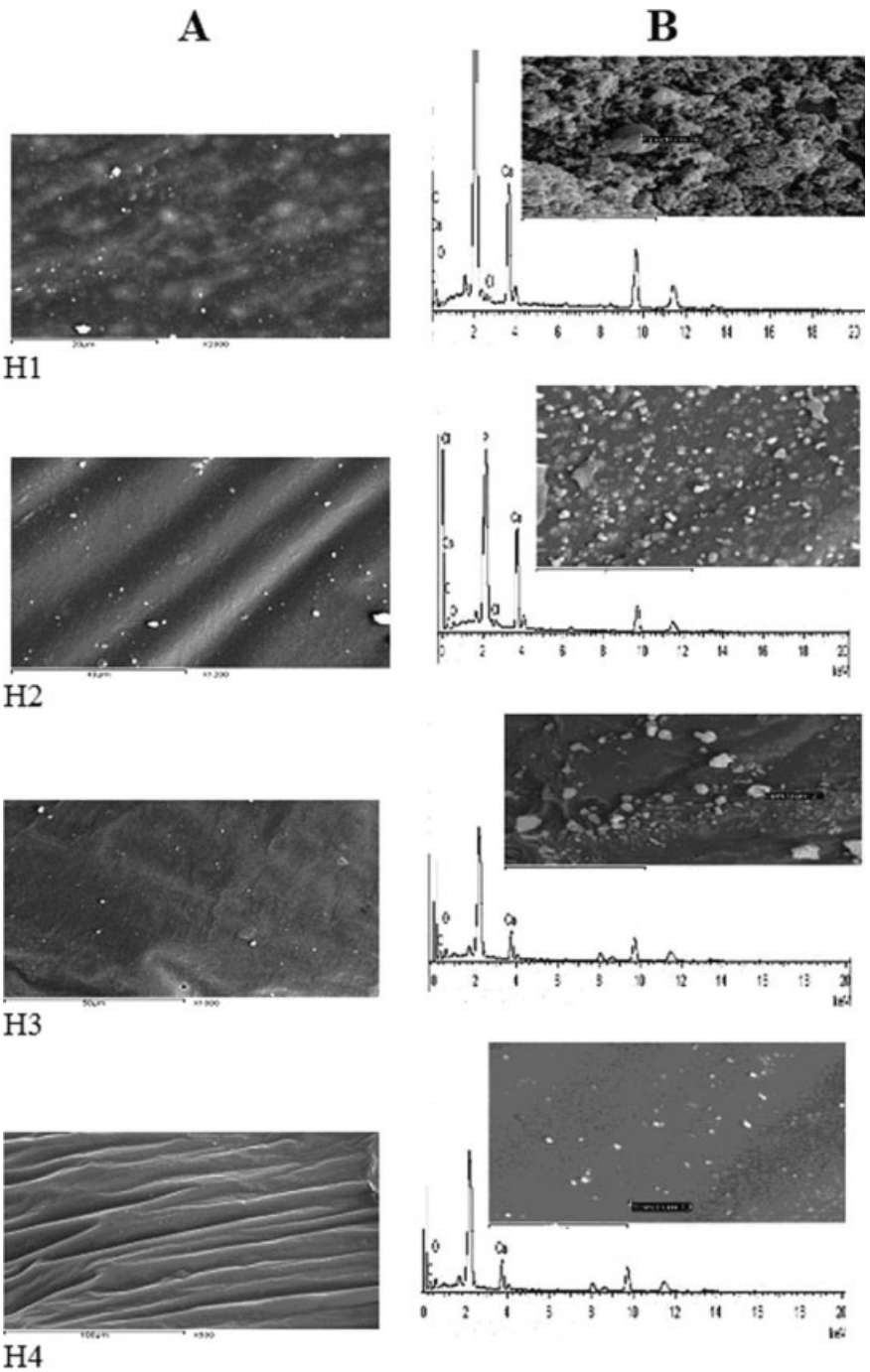

Figure 8 SEM images and EDX-spectra of hide powder-based composite films (A) before and (B) after immersion in SBF for 7 days.

component. The *in vitro* test proves that the high concentration of hide powder (collagen) in the composite films H_3 and H_4 indicates slight chemical bioactivity in SBF whereas the lower concentration of collagen in the composite films H_1 and H_2 exhibits high chemical bioactivity postimmersion, which are revealed by FTIR. These results indicate that the addition of certain amounts of gelatin and collagen to special synthetic polymers, like MPE and LDPE, can be significantly effective as investigated by their accelerated bioactivity. The bioactive mechanism of composites is rapidly enhanced and a positive response can be obtained. Additionally, the results confirm an important role of hide powder (collagen) in HP/MPE composites. It accelerates the formation of apatite layer on the composite surface. It was reported that, when most of bioactive materials are soaked in SBF, a new calcium phosphate phase, which is similar to calcium deficient apatite layer, is formed in the living body (bone-like apatite layer)

on their surfaces. This phenomenon is known as biomimetic formation of apatite layer forming new materials, which can provide an information on their chemical properties in development of biomaterials.[35] Therefore, the deposition was found using FTIR containing carbonate ion, which plays a vital role in the bone metabolism, and they occupy about 3–8% weight of the calcified tissue.[36] From these results, we have shown that all composites, under certain condition, form an apatite layer on their surfaces after immersion in SBF for 7 days. FTIR spectra and SEM-EDX of apatite layer formed on the surfaces of composites are similar to those of apatite in the bone. Both apatites are poorly crystallized, calcium deficient incorporated with carbonate that enters the apatite structure to replace both phosphate and hydroxyl groups. Therefore, it can be concluded that the surfaces of composites which included: H_1, H_2, G_b/MPE, G_b/LDPE, G_c/MPE, and G_c/LDPE stimulates bone-like apatite formation from metastable SBF physiologically relevant calcium phosphate solution. An essential requirement for an artificial material to show bioactivity is the formation of a biologically active bone-like apatite on its surface in body environment. There is an oscillating phenomenon of precipitation and dissolution processes of *in vitro*, which is due to metastable SBF. It was reported that the precipitation and dissolution processes of bone like apatite take place during the immersion of bioactive materials in SBF. Hench[37] reported that there is a good correlation of *in vitro* bone-like apatite formation from SBF and *in vivo* bone-like apatite (calcium phosphate) formation needed to secure bone bonding. On other words, the surface of the composites included: H_1, H_2, G_b/MPE, G_b/LDPE, G_c/MPE, and G_c/LDPE could stimulate bone-like apatite deposition on the surface of bioactive glass by the uptake of calcium and phosphate from body fluid. Hence, the chemical bonding between the surface of those composites and bone is expected to be achieved through the apatite in the same way that bioactive materials bond with bone. These expectations are currently under investigation *in vivo.*

CONCLUSIONS

The results of FTIR and SEM-EDX indicated that the presence of extracted gelatins till 50 wt % enhanced the *in vitro* bone bioactivity of the biocomposite films and their ability to form the carbonate apatite layer after their immersion in SBF for 7 days. HP/MPE composites with HP content till 10 wt % (H_1 and H_2) had high ability to form an apatite layer on the surface compared with composites at higher HP content (H_3 and H_4). It may be suggested that the apatite induction could take place on negatively charged surface with sufficient carboxyl and hydroxyl groups. As expected, the mechanical properties (stress, elongation, and e-modulus) of unblended PE were reduced by blending with extracted gelatins and hide powder collagen due to their biodegradable behavior. In other words, the results illustrated that both of extracted gelatins and collagen (hide powder) play an important role to accelerate the formation of apatite layer on the biocomposite films. This indicates that they are able to induce apatite nucleation. Moreover, blends of extracted gelatin and MPE and/or LDPE up to a content of 50 wt % of gelatin and blends of HP and MPE up to a content of 10 wt % of collagen (hide power) are susceptible to be processed by the melt and provide films that are characterized by good *in vitro* bone activity and satisfactory thermal and mechanical responses. Therefore, these composites could be studied *in vivo* in the future for evaluation as bone implants.

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