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Synthesis and properties of biocomposites based on collagen/ polyurethane

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Collagen is the most common protein. It is found in all multicellular organisms in the animal world. It is characterized by good biodegradability and biocompatibility, but with unsatisfactory physico-mechanical properties. Polyurethanes are considered to be the most promising class of polymers for *in vivo* studies, and fulfill all criteria for application to medical practice. These materials are biodegradable, characterized with thermal and mechanical stability but there is some limitation on their use due to the lack of biologically active groups. Therefore, collagen and polyurethane based composites, combining the benefits of natural and synthetic material, would be promising biomaterials for tissue engineering, medicine: drug delivery and other areas. In the present work biocomposites based on polyurethane/collagen were synthesized with different amounts of protein (5 and 10 wt.%) as a filler. An attempt was made to explain the mechanisms of interaction between polyurethane and collagen in the composite. FTIR analysis showed the formation of hydrogen and chemical bonds between collagen and polyurethane, as well as hydrogen bonds between urethane macromolecules themselves. DSC analysis confirmed the higher thermal resistance of the composites. *In vitro* tests revealed the formation of apatite layer, which was also confirmed by microscopic analyzes. This would accelerate the healing process in the human body, increasing the growth of osteoblast cells. Microscopic images showed 3-dimensional morphology, different pores with different sizes are observed, the pores are interconnected, allowing the constant transport of nutrients. Thus, the criteria for application to tissue engineering are fulfilled.

Keywords: biocomposites, collagen, polyurethane, biocompatibility

INTRODUCTION

Collagen is the main scleroprotein that builds the extracellular matrix of connective tissue. It is available in large quantities, especially as waste from slaughterhouses and leather production. Collagen can be used in various biomedical fields, especially in tissue engineering due to its biodegradability and biocompatibility. In the 1980s, collagen became the main biomolecule used in many medical fields, especially in the field of bone implant surgery [1-9]. Unsatisfactory mechanical properties are a major disadvantage of natural biomolecules. Collagen and elastin are two of the key structural proteins studied in the extracellular matrix of many tissues [10-12]. These proteins are important modulators of the physical properties of many skeletal and porous structures that affect cell adhesion and cell growth. Collagen can be obtained in various forms: porous sponges, gels, films and more. It is characterized by porous structure, hydrophilicity, and cellular and tissue adhesion, susceptible to combining with other materials (i.e. synthetic polymers).

The term "composite" means having two or more different parts [9]. Most composites are produced to ensure mechanical properties such as strength, stiffness, toughness and fatigue resistance combined with other necessary qualities: biological functions and biocompatibility.

Polyurethanes are synthetic materials, obtained from isocyanates and hydroxyl-containing components, which are considered the most promising class of polymers for *in vivo* studies. Reasons for this are their non-toxicity, tissue compatibility, high mechanical strength, elasticity and aging resistance. Their properties can be modified and various materials can be obtained: from rigid to elastic, coatings, fibers, foams, films and more. They are very important materials in tissue engineering. The properties and porous structure of the polyurethanes allow bone calcification processes to take place. Another advantage is their biodegradability through absorption, but there is some limitation on their application due to the lack of bioactive groups. Therefore, the key factors for their use as biomaterials include the ways to impart biodegradability and biological activity to polyurethanes. The latter can be modified with biomolecules to enhance their biocompatibility. Therefore, collagen and polyurethane composites combining the benefits of natural and synthetic material would be promising biomaterials for tissue engineering, medicine: drug delivery and other fields.

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There are numerous studies on the compatibility of collagen with synthetic polymers and other natural polymers, as well as on its self-application as a biomedical material [1-26].

Radev *et al*. investigated bioactive composites based on collagen-calcium phosphate silicate/ wollastonite [23]. *In vitro* bioactivity studies of hybrids have shown that hydroxyapatite (HA) is formed on the synthesized composite surface. The negatively charged carboxyl groups of the collagen molecule are responsible for the deposition of HA. Hydroxyapatite and other inorganic phosphates have the ability to induce osteoblastic cell growth and consequently bone repair, preventing an inflammatory response.

In our previous study [24], polyurethane (PU)/ bioglass (BG) composite materials were synthesized with different contents of BG (10 and 20 mol.%) as a filler. It has been proved that hydroxyapatite deposition induces osteoblast cell synthesis.

The solubility of collagen in acetic acid enables it to be mixed with other water-soluble polymers [1-3]. The biocomposites based on collagen and water-soluble synthetic polymers have been investigated in medical practice, namely: polyvinylpyrrolidone (PVP); polyvinyl alcohol (PVA); polyethylene glycol (PEG); polyethylene oxide (PEO) [15-22]. The composites can be processed into a variety of forms including porous sponges, thin films, hydrogels, coatings, etc. Other synthetic polymers used are: polyurethanes (PU); polyglycolic acid (PGA), polylactic acid (PLA), poly (DL-lactide-glycolide) (PLGA).

Jianjun *et al*. developed a flexible, biodegradable scaffold structure for cells transplantation in the form of a composite material based on biodegradable poly(esterurethan) urea and collagen type I [16]. Poly(esterurethan) urea was synthesized from poly(caprolactone), 1,4-diisocyanatebutan and putrescine.

The synthesis and properties of a microporous composite based on polyurethane and collagen were investigated (collagen content: 0-15 wt.%). The resulting two-phase structures are characterized by good mechanical properties, pores of the required dimensions and biocompatibility, which are important indicators for biomedical application [17].

Collagen coatings have been successfully applied to numerous hydrophilic polymer scaffolds to improve cell adhesion [18].

The regeneration of damaged or lost tissue requires some functional repair cells to assemble three-dimensionally around and inside the surrounding porous structure [22]. Collagen-based nanofibers and functionalized thermoplastic polyurethane (TPU/collagen) have been successfully obtained by electrospinning technique to produce biomedical material with a porous structure.

A variety of hybrid systems have been developed and optimized to obtain optimal polyurethane structures, some of which serve to aid the healing process, others are applied in the form of implants, others are for drug delivery, for enhancing cell adhesion, cell growth, and more.

The purpose of the present study is to synthesize biocomposites based on polyurethane and collagen, to evaluate the *in vitro* bioactivity of the resulting biocomposites and to analyze application options in tissue engineering and other fields. The other aim is to explain the mechanisms of interaction between collagen and polyurethane in the composite.

EXPERIMENTAL

Materials

Collagen type I (extracted from the skin of bovine lyophilized tissue) was supplied by the Leather-Footwear Research Institute (Bucharest, Romania). Collagen was added as a filler in composite compositions.

The components used for the synthesis of polyurethane foam are as follows: 4,4'-methylenebis (phenylisocyanate) (MDI), from Alfa Aesar, Germany, with a melting point of 40°C, density 1.230 g/cm^3; polypolyol (Lupranol 2095) from BASF - highly reactive trifunctional polyether, molecular weight 4800 g/mol, hydroxyl number 35 and viscosity 850 mPa.s; 1,4-butanediol (BD) as a chain extender; water as a foaming component.

Polyurethane synthesis

The synthesis of polyurethane foam (PUR) was carried out by the reaction of 2-step polyaddition of polyesterpolyol (Lupranol 2095) and 4,4'-methylenebis (phenylisocyanate) (MDI), by extending the chain with 1,4-butanediol, and adding the foaming component.

The process of preparing the polyurethane foam includes two steps: (1) To 10 g of polyol component (Lupranol 2095), a few drops of water are added and the mixture is stirred vigorously. To the polyol mixture a stoichiometrically calculated amount of isocyanate (MDI) - 1 g, is added, followed by vigorous stirring again. The synthesis reaction proceeds at 50°C for 30 min. The next step (2) is the addition of the chain extender (1,4-butanediol) to increase the molecular weight. The

foaming reaction begins seconds after the components are mixed. For PUR synthesis, the reaction with water is important (step 1), in which 2 isocyanate molecules react with 1 molecule of water. The first obtained product is carbamic acid. It is unstable and releases carbon dioxide, which acts as a foaming agent during polycarbamide formation, which leads to the building of the basic macromolecular skeleton. The following process is the reaction of the amine with isocyanate to form urea:

$$R\text{-}NCO + H_2O \longrightarrow R\text{-}NH_2 + CO_2 \xrightarrow{+\ R\text{-}NCO} R\text{-}NH\text{-}CO\text{-}NH\text{-}R$$

The two-step polymerization process is more complex. In the first step, the polyol reacts with excess of diisocyanate, resulting in a prepolymer. This prepolymer is a mixture of elastic segments with terminal isocyanate groups that will form solid segments later. In the second stage, the chain extender is introduced. It reacts with the remaining amount of unreacted diisocyanate, forming solid segments and bonding them to the elastic segments. The material has a two-phase structure, i.e. presence of domains resulting from the agglomeration or crystallization of the various chains. These domains are different for different materials and this predetermines the higher or lower elasticity [25]. In addition to having a two-phase structure, the material also has many functional groups: hydroxyl, ester, ether, urethane -NHCOO-, isocyanate, urea, amide, allophanate, biuret, and hydrogen bonds.

Synthesis of the biocomposites

In the synthesis of PU/Coll (polyurethane/ collagen) composites, collagen was added *in situ* during the polymerization reaction. The reaction proceeds in the same way as for pure polyurethane, except that collagen (Coll) was pre-prepared as an aqueous solution. Collagen in an amount of 5 and 10 wt. % was added to the polyol component. The stoichiometrically calculated amount of isocyanate (MDI) -1 g was then added to the polyol mixture, again followed by vigorous stirring. The synthesis reaction proceeds at 50°C for 30 min. The next step was the addition of the chain extender (1,4-butanediol). The composites are called PU/5Coll and PU/10Coll. In the resulting biocomposite material, in addition to the abundance of polyurethane functional groups, collagen also offers its functional groups: hydroxyl, carboxyl, amide (-NH-CO-), imide (-CO-N=), and polar terminated groups (-COOH, $-NH_2$, -OH).

Bioactivity essay

Bioactivity of the composites obtained was evaluated by examining the apatite formation on their surfaces in SBF solutions. The SBF was prepared from the following salts: NaCl = 11.9925 g, $NaHCO_3$ = 0.5295 g, KCl = 0.3360 g, $K_2HPO_4 \cdot 3H_2O$ = 0.3420 g, $MgCl_2 \cdot 6H_2O$ = 0.4575 g, $CaCl_2 \cdot 2H_2O$ = 0.5520 g, Na_2SO_4 = 0.1065 g, and buffering at pH 7.4 at 36.5ºC with 9.0075 g of *tris* (hydroxymethyl) aminomethane (TRIS) and 1M HCl. A few drops of 0.5% NaN_3 were added to the SBF solution to inhibit the growth of bacteria [27]. After soaking the specimens were removed from the fluid, gently rinsed with distilled water, and then dried at 36.6ºC for 12 h.

In vitro tests of composites in SBF were performed for 7 days under static conditions.

Methods for analysis

Fourier-transform infrared (FTIR) spectroscopy was used for qualitative analysis of the obtained composites. FTIR transmission spectra were recorded by using a Bruker Tensor 27 spectrometer with scanner velocity of 10 kHz. KBr pellets were prepared by mixing ~1 mg of the samples with 300 mg of KBr. Transmission spectra were recorded using MCT detector, with 64 scans and 1 cm^{-1} resolution.

Scanning electron microscopy SEM (Jeol, JSM-35 CF, Japan) was used to ascertain the morphology and chemical constituents of the prepared composites before and after immersion in 1.5 SBF for 7 days at an accelerating voltage of 15 kV.

The differential scanning calorimetry (DSC) method was used to determine the changes in enthalpy, thermal and oxidation stability of the samples. STA PT1600 TG-DTA/DSC (STA Simultaneous Thermal Analyses), Messgeräte GmbH, Germany was used, with a temperature range of 20 ÷ 1550 ºC.

RESULTS AND DISCUSSION

Characterization of the biocomposites before in vitro test in SBF

Figs. 1 and 2 show typical infrared spectra of pure polyurethane (PU) and pure collagen (Coll), which will be compared with the spectra of the biocomposites. In Fig. 1, the spectrum reveals the characteristic bands of PU, the stretching vibrations of the N-H bond at 3392 cm^{-1}, the asymmetric and symmetric vibrations of the CH_2 groups at 2924

cm^{-1} and 2858 cm^{-1}, respectively [29]. The other vibrational bands of CH_2 are positioned at 1410, 1373, and 1303 cm^{-1} [29, 30]. The absorption band of amide I is at 1706 cm^{-1} [29, 31]. Strongly expressed strain δ (N-H) and valence vibrations ν (C-H) are recorded at 1510 cm^{-1} [29] and δ (N-H) + ν (C-N) are recorded at 1237 cm^{-1} [29, 32]. The band at 1450 cm^{-1} is characteristic for the elastic segments of PUR. Absorption at 1183 cm^{-1} and 1100 cm^{-1} refers to the ether bonds [29, 33] and the C-O-C stretching is observed at 1012 cm^{-1} [29, 34]. In addition, the spectrum shows bands at 1776-1597 cm^{-1} that refer to the C=O bond in polyurethane [29]. Moreover, the stretching bands at 1665 cm^{-1} and 1706 cm^{-1} are due to the absorption of hydrogen bonded C=O of urethane linkages [29, 35, 36]. In addition, the isocyanate group absorbs strongly at 2275 cm^{-1} [37]. Many functional groups were registered in the structure of polyurethane.

Fig. 2 shows the FTIR spectrum of collagen. Especially, the amide I, II and III band regions of the spectrum are directly related to the polypeptide conformation [38]. The amide I band, with a characteristic frequency in the range of 1600-1700 cm^{-1}, is mainly associated with the stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide chain and refers to peptide secondary structure [39]. The amide I bands are at 1650-1660 cm^{-1}, 1630-1640 cm^{-1}, and 1680-1700 cm^{-1} in the amide I region of the protein. In our case, the amide I band is centered at 1656 cm^{-1}. In the amide II region of proteins there are bands at 1540-1550 cm^{-1}, 1620-1530 cm^{-1}, and 1520-1545 cm^{-1} [40, 41]. In our case, the amide II is centered at 1553 cm^{-1}. For the amide III band of protein, there are bands centered at 1270-1300 cm^{-1}, 1229-1235 cm^{-1}, and 1243-1253 cm^{-1}. From Fig. 2, the amide III bands were assigned at 1240 and 1281 cm^{-1}. Amide B is centered at 3081 cm^{-1} [39]. The two bands visible at 1399 and 1340 cm^{-1}, can be assigned to the presence of COOH and COO- in the spectrum of pure gelatin and collagen [42].

The spectra of the composites PU/5Coll and PU/10Coll are presented in Figs. 3 and 4. The characteristic bands at 3400 cm^{-1} for N-H bonds are observed. The peak at this absorption band is smaller for pure polyurethane than for PU/Coll. This is an indicator of the occurrence of hydrogen bonds between collagen and polyurethane macromolecules, which leads to strengthening or crosslinking of the structure of the composite. The intensity of the peak of the composite at 1600 cm^{-1} increases, which means that the -OH, $-NH_2$, C=O groups of collagen form hydrogen bonds with the C=O and N-H groups of polyurethane. It can be seen that hydrogen bonds are also formed between urethane macromolecules themselves. The improvement of samples properties even in the presence of small amounts of collagen is confirmed by other assays. It also decreases the peak intensity at 2275 cm^{-1} due to the depletion of the isocyanate groups. This may be explained by the formation of new urethane bonds (-NHCOO-) between the OH groups of collagen and the isocyanate (NCO-) groups of polyurethane.

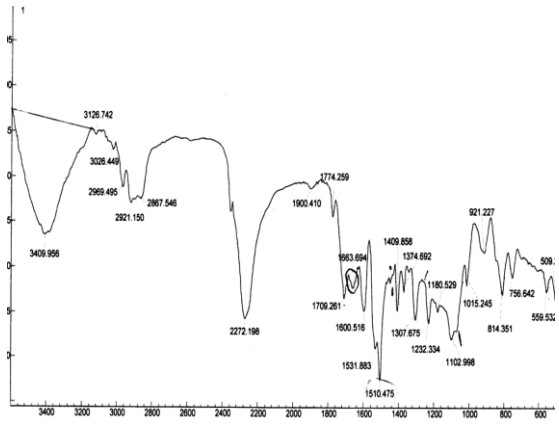

Fig. 1. FTIR spectrum of pure polyurethane (PU)

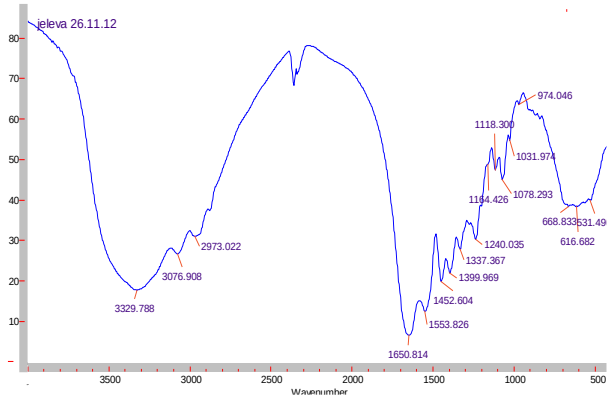

Fig. 2. FTIR spectrum of pure collagen (Coll)

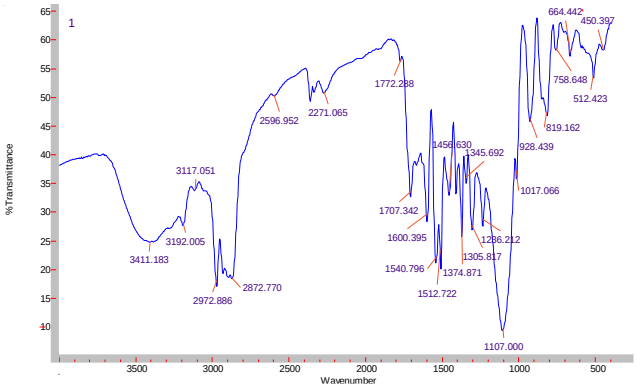

Fig. 3. FTIR spectrum of PU/5Coll composite

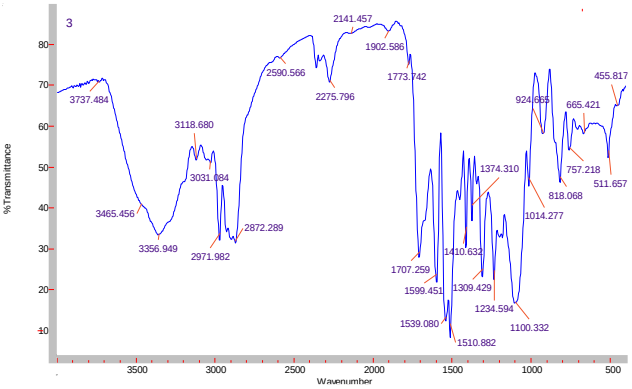

Fig. 4. FTIR spectrum of PU/10Coll composite

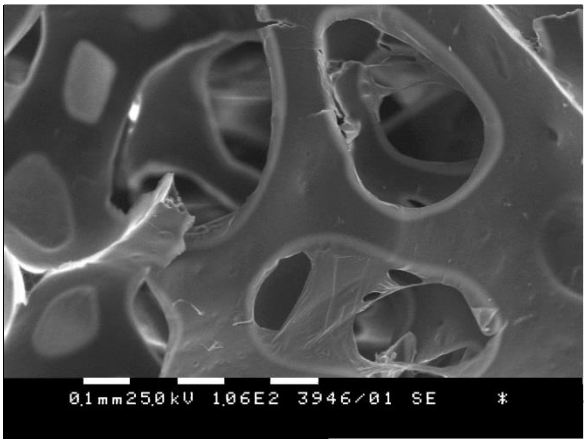

Fig. 5. SEM of PU

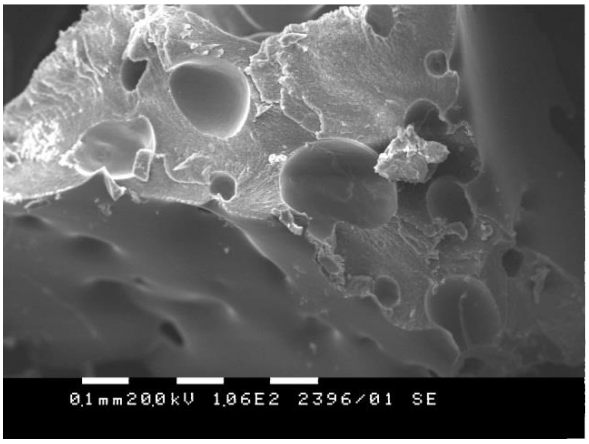

Fig. 6. SEM of the PU/Coll composite

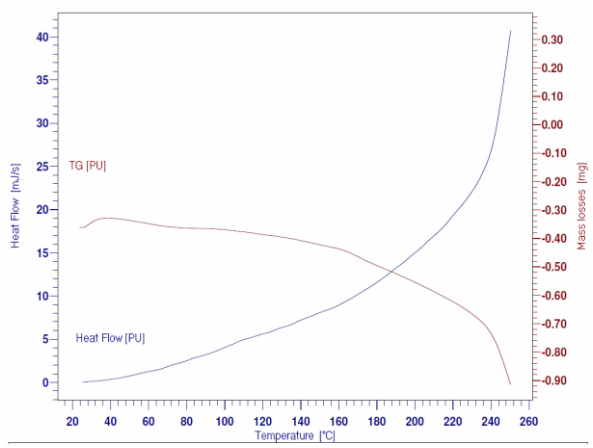

Fig. 7. DSC curves of PU

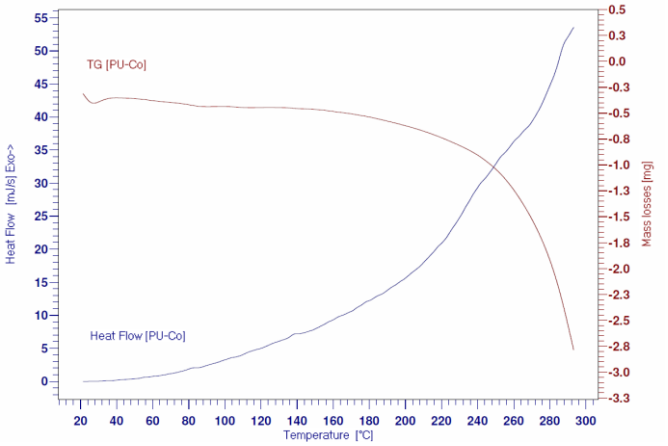

Fig. 8. DSC curves of PU/Coll

The microscopic images (Figs. 5 and 6) show 3-dimensional morphology, interconnected pores with different sizes. The PU/Coll porous scaffolds (Fig. 6) have not only macropores, but also a lot of micropores positioned on the macroporous wall, allowing for the constant transport of nutrients.

The formation of the porous structure can be explained by the gelation mechanism in which the collagen migrates to the surface of the composite films and thus large pores are formed. These pores are randomly distributed and interconnected. The interconnectedness is due to the presence of smaller pores that are adjacent to the collagen fibers. This improves the hydrophilicity of the biocomposites [28].

From the DSC analysis (thermogravimetric curves in Figs. 7 and 8) it can be seen that the transition temperature increases with the addition of the protein and therefore the samples are more thermally stable, increasing the enthalpy. This means that the composites show a higher destruction temperature. Confirmed by other

methods, this is due to the additional cross-linking of structures and the formation of hydrogen and chemical bonds between the molecules of the synthetic and natural polymer. From the thermal analysis, the primary mass loss at 20°C in the TG curve for PU is about ~ 4.5% (Fig. 7) and for the PU/Coll composite it is ~ 1.8% (Fig. 8), which is explained by evaporation of free water and solvents. Between 160-250ºC the mass loss for PU is approximately 8.5% and for PU/Coll between 180 - 300ºC the mass loss is 14%, which also shows the higher thermal destruction of the composite.

Characterization of the biocomposites after in vitro test in SBF

It was observed following feature in each of the samples after soaking in simulated body fluid (SBF). The spectrum of the biocomposite (Fig. 9) shows peaks at 630 cm^{-1} and 560 cm^{-1}, which is an evidence of the formation of hydroxyapatite forms observed in microscopic studies.

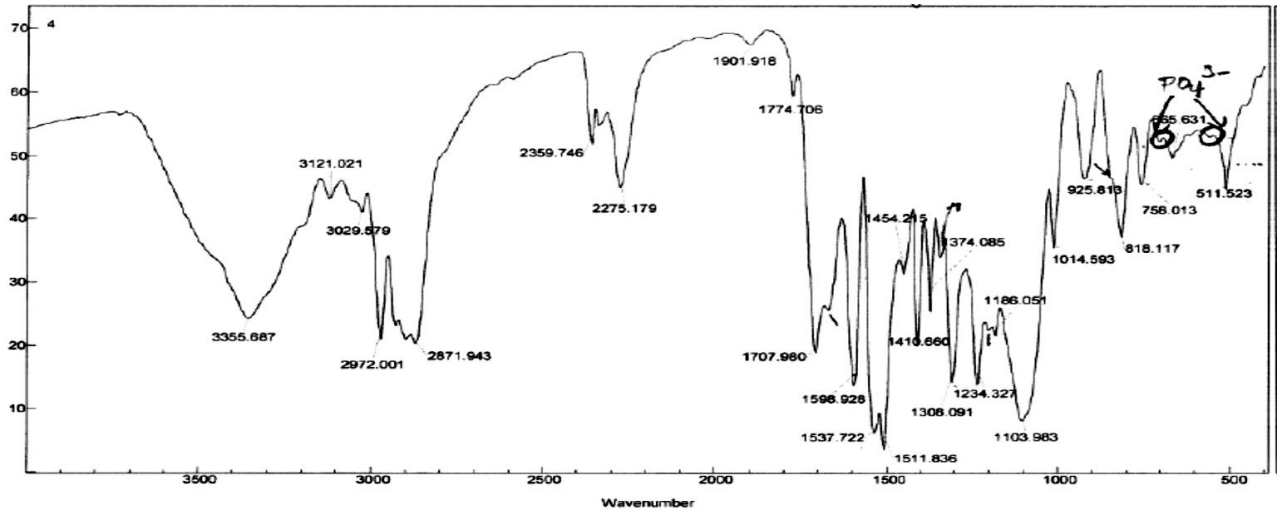

Fig. 9. FTIR spectrum of the PU/Coll composite after *in vitro* test in SBF for 7 days

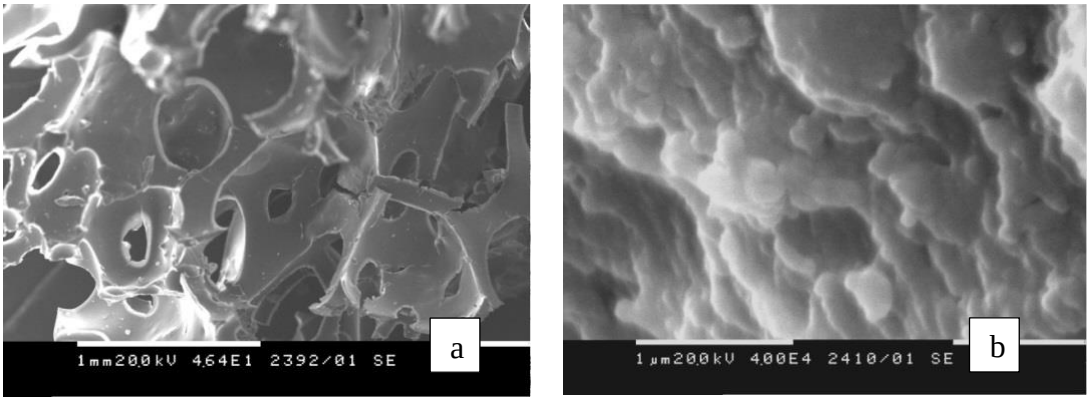

Fig. 10. SEM of the PU/Coll composites after *in vitro* test at different magnifications

The microscopic images (Fig. 10) also show deposition of spherical-sized hydroxyapatite agglomerates after 7-day soaking in SBF. The exact mechanism of calcification is still unclear, but it has been reported that this occurs by the interaction of the polyurethane foam with calcium and phosphorus ions in blood or other body fluid [25, 43]. Bone is a specific form of connective tissue composed of a collagen skeleton impregnated with calcium salts (Ca^{2+}, PO_4^{3-}) [25]. The presence of oxygen is thought to have the greatest influence on calcification. Calcification in hydrophobic polyether urethanes takes place on a surface that is in direct contact with body fluids. In the case of hydrophilic polyether urethanes (as well as our samples), this process takes place both on the surface and through the polymer. For example, acceleration of calcification can be achieved by modification with PEG (polyethylene glycol), which increases the hydrophilicity of PUR [43]. In our composites, collagen, as a natural molecule, increases the hydrophilicity of the biomaterial, imports biological groups, and it would improve cell adhesion and proliferation. PURs swell in the

SBF fluid and increase volume, which increases the direct contact between the material and the tissue [44].

Morphological properties of the PU/Coll samples after soaking in SBF during 7 days, observed by SEM at high magnifications (Fig.10b), show that soaking led to formation of an apatite layer on the surface of the samples. The pores are interconnected to allow continuous flow of nutrients in the scaffold.

CONCLUSION

Biocomposites based on polyurethane/collagen were synthesized with different amounts of protein (5 and 10 wt.%) as a filler. FTIR analysis showed the formation of hydrogen and chemical bonds between collagen and polyurethane, as well as hydrogen bonds between the polyurethane chains. The result is crosslinking and additional reinforcement of the composite structure. *In vitro* tests revealed the formation of hydroxyapatite, confirmed by microscopic analyses. Collagen increases the hydrophilicity of the biomaterial, introduces biological groups, which would lead to improved cell adhesion and proliferation. Microscopic images showed 3-dimensional morphology, different pores with different sizes, and interconnected pores allowing the constant transport of nutrients. Thus, in large part, the criteria for application to tissue engineering are fulfilled. This would accelerate the healing process in the human body, increasing the growth of osteoblast cells.

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