JOURNAL OF OPTOELECTRONICS AND ADVANCED MATERIALS Vol. 10, No. 4, April 2008, p. 942 - 947

# Preparation and characterization of new biocompatibilized polymeric materials for medical use

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The aim of this study was to prepare 4 new composite biomaterials based on polyurethane (PU) blended with different mixtures of collagen (COL), elastin (EL) and glycosaminoglycans (GAG). Bioactivated polymeric materials were structurally characterized by light and electron microscopy and their biodegradability and *in vitro* biocompatibility were comparatively assessed. The biomaterials presented smooth surfaces and the porosity decreased when natural polymers were added. All polymeric blend variants allowed cell proliferation and adhesion, with a more extensive growth on PU-COL variant. In conclusion, it was demonstrated that these new composite materials were biocompatible and good candidates for medical device fabrication.

(Received November 13, 2007; accepted April 2, 2008)

*Keywords*: Biocompatibility, Biodegradability, Collagen, Elastin, Glycosaminoglycans, Polyurethane

## 1. Introduction

A present research trend is the development of biodegradable synthetic polymers used for development of new materials with good biochemical and biomechanical properties, needed in emerging technologies [1]. Polymeric biomaterials fulfilled several medical uses as artificial organs, prostheses, in dentistry, bone repair, drug delivery systems and recently in regenerative tissue engineering [2-4]. In order to be used in patients, their biocompatibility is a key demand because it represents the major limiting factor. Polymeric composites prepared by mixing synthetic and natural polymers or by covering the material surface with natural products offer several advantages, such as improved mechanical properties and biocompatibility. The characteristics of the material surface, like surface chemistry and energy, viscosity and topography play an important role in cell adhesion [5-7].

In the last decade, a broad variety of synthetic polymers like polyvinyl chloride (PVC), polyethylene (PE), polypropylene (PP) and siliconic polymers (PS) have been used in biology and medicine. Because of their nonthrombogenicity, biocompatibility and degradability, the polyurethanes (PU) are recommended as adhesives, membranes for dialyse, in cardio-vascular surgery as vascular protheses, artificial heart components, orthopaedic protheses, etc. Their mechanical properties – elasticity and porosity, are close to those of sanguine vessels [8-10]. In order to improve the biological characteristics of the materials used in contact with blood and tissues for long periods, natural polymers like collagen (COL), fibrin and glycosaminoglycans (GAG) were more often used than other natural products [11-12]. Recent studies showed that elastin (EL) represented a better contact surface than COL due to its antithrombogenic properties [13].

The aim of this study was to prepare and characterize 4 new composite materials based on PU blended with different mixtures of COL, EL and GAG in order to be used as biomaterials for medical device fabrication.

## 2. Experimental

***Polymer fabrication.*** PU was synthesized using a two-step polyaddition reaction, as previously described for other PU preparation [14]. The materials used were diphenylmethane 4,4' diisocyanate (MDI) fresh distilled, from Merck, poly (ethylene glycol) adipate (PEGA, purity 97 %, MW = 2000) and ethylene glycol (EG) (purity 95 %) from Fibrex SA Savinesti-Romania. PEGA was dried at 120 $^{o}$C, for 3 h to remove residual water before use. Briefly, PEGA reacted with MDI at 90 $^{o}$C, under nitrogen atmosphere, for 1 h. In the second stage, the isocyanine groups of the polymer reacted with the chain extender (EG) at 60 $^{o}$C. The molar ratio PEGA:MDI:EG was 1:5:4. The obtained PU was precipitated in water and dried under vacuum for several days.

♣ paper presented at Symposium on Biomaterials, Cluj-Napoca, Romania, October 18-20, 2007

Hydrolyzed collagen (COL) was prepared in our lab from bovine derma by acid hydrolysis followed by a spray-drying process using a BUCHI 190 Mini Spray Dryer. COL is a peptide mixture with the elemental composition: 42.7 % C, 10.8 % H, 12.2 % N and 34.3 % O; the average molecular weight was 99,000 Da, as determined by GPC and its polydispersity was 1.66.

EL was prepared from insoluble elastin powder (calf ligament, Sigma) by stirring in KOH 1M (in ethanol, 1:4, v/v), at 30 $^{o}$C, for 48 h, neutralizing with acetic acid and dialysis against bidistilled water [15]. The final solution has 1.3 % dry weight and 68.7 % protein content.

Hyaluronic acid (HA) (Sigma) was obtained from human umbilical cord and chondroitin sulfate (CS) (Sigma) was obtained from bovine tracheal cartilage.

***Polymer blends preparation.*** Four polymeric blends were prepared as presented in Table 1.

*Table 1. Polymeric blend variants.*

| No. | Composition | Weight ratio |
|---|---|---|
| I | PU:COL | 100:10 |
| II | PU:COL:EL | 100:10:1 |
| III | PU:COL:EL:HA | 100:10:1:0.1 |
| IV | PU:COL:EL:CS | 100:10:1:0.1 |

First, PU was dissolved in dimethylformamide (DMF) to obtain a 30 % (w/w) solution. Then, a suspension of COL in DMF was added into the PU solution at 60 $^{o}$C and stirred for 1 min (I). One quarter of this blend was moved in a capped test tube. The EL solution was added in the remaining blend, under vigorous stirring, at 45-50 $^{o}$C. After 5-10 min, the mixture became homogeneous and cleared (II). One third was separated in a capped test tube. The remaining blend was divided in two and in each part was added the powder of glycosaminoglycan (HA and CS, respectively), under vigorous stirring, at 45-50 $^{o}$C. The 4 blend variants were poured in glass moulds and dried for 15 hours in an air-ventilated drying stove and then under vacuum for 55 hours to obtain polymeric sheets.

***Scaffold characterization.*** Scaffold surfaces and cross-sections were examined by scanning electron microscopy (SEM). The samples were processed in the low vacuum mode and visualized using an ESEM, Quanta 400, FEI, Philips (Holland). Pore sizes were determined using ImageJ and ImageAnalyser soft for digital image processing.

Scaffold porosity ($\varepsilon$) and density ($d$) were determined using the ethanol displacement method previously reported by Guan et al [16]. Briefly, a sample with a known weight ($w1$) was immersed in a beaker holding a known volume of ethanol ($V1$). Then, the total volume of ethanol plus the ethanol-filled scaffold ($V2$) and the residual ethanol volume after the ethanol-impregnated scaffold was removed ($v3$) were recorded. The following equations

$$\varepsilon = (V1-V3) / (V2-V3) \times 100\ \% \quad \text{and} \quad d = w1 / (V2-V3)$$

***Scaffold degradation.*** Dry samples (0.5x0.5 cm) were weighed and immersed in 3 ml Tris-HCl buffer, pH 7.4 containing 2U/ml type I collagenase (Sigma). The degradation was conducted at 37 $^{0}$C in a water bath, for 5 days. The materials were dried and weighed again. The mass loss was calculated as percent from the original weight, at each time point.

***Human dermal fibroblast culture.*** A primary culture of human dermal fibroblasts was obtained from human dermis explants and was used at passage 4. Scaffolds were cut to 10-mm discs and sterilized by exposure to ultraviolet light source in an UV sterilization cabinet (Scie-Plas, England) for 4 h. They were fit into the bottom of a 24-well tissue culture polystyrene plate and 50 µl of cells in DMEM with 10 % fetal bovine serum (Sigma) were added at a density of $1 \times 10^5$ cells/ml. The plate was kept in an incubator for 3h. Then, another 450 µl medium (DMEM) were added to each well. Cell viability was assayed by measuring the mitochondrial dehydrogenase activity using the MTT test [17], after 24 h and 48 h of cultivation, respectively. To analyze cell morphology, cell/scaffold constructs were fixed in Bouin, stained with Hematoxylin and observed using a stereomicroscope (Kruss, Germany) and a Zeiss Axiostar Plus microscope (Carl Zeiss). The photomicrographs were taken by digital camera (AxioCam MRc 5, Carl Zeiss) driven by AxioVision 4.6 (Carl Zeiss) software.

***Statistics.*** Data are expressed as mean ± S.D. Statistical analysis was performed using paired Student's test. Differences were considered significant at $p<0.05$.

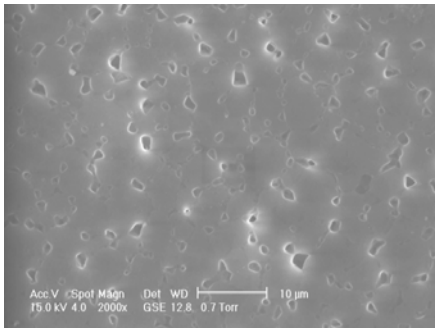
## 3. Results and discussion

### 3.1. Blend morphology

The composite blends obtained by mixing PU with various natural polymers were analyzed by SEM and the morphologies of their surface and cross-section were presented in Fig. 1. On the smooth surface of PU-COL (Fig. 1A), particles having 2-D geometric shapes and 3-D prism shapes were observed. Their statistically calculated average area was 1.73 µm$^2$. The particle size was calculated after twenty measurements and statistic calculation of the average values (Fig. 2). On the surface of PU-COL-EL sample (Fig. 1B), two morphologically different areas were distinguished. One could observe zones of 16,000 µm$^2$ presenting particles of aprox. 20 µm$^2$ and other zones having particles less than 1 µm$^2$. On the surface of PU-COL-EL-HA/CS, areas covered almost completely by polyhedric particles were distinguished (Fig. 1C, D). These areas had 26.000 µm$^2$, while the particle average area was 8.46 µm$^2$ for variant III and 1.32 µm$^2$ for variant IV (Fig. 2).

The SEM images showed that pore sizes decreased after the addition of the natural polymers, in particular for the PU sample mixed with COL-EL (Table 2). PU-COL blend had interconnected pores in cross-section, with an average size of 4.16 µm, smaller than those in the PU sheet (6.06 µm). The pores in cross-section were found to be larger than those on the surface (Fig. 1E-H).

The porosity decreased when EL was added and raised when GAG was present in the mixture. Also, PU-based blends had smaller porosities than the PU sheet. This effect could be explained through interactions between the amide bonds of COL and EL and the urea bonds of PU [16].

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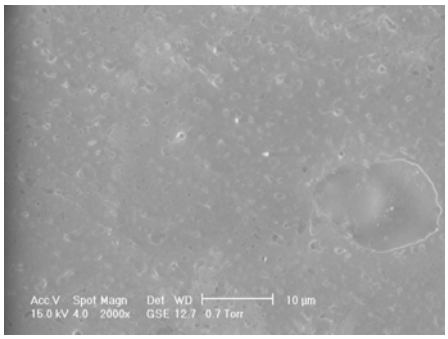


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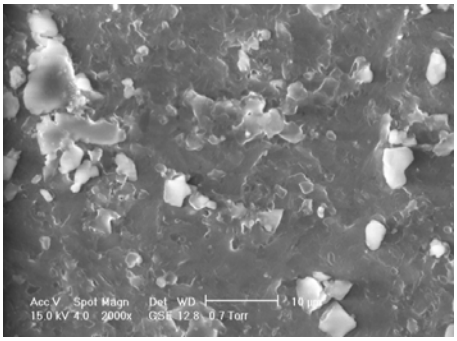


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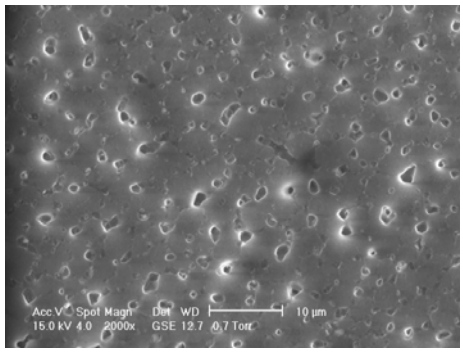


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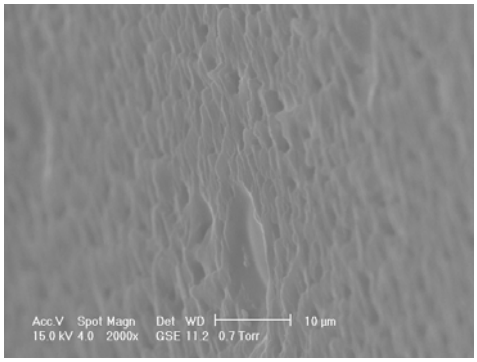


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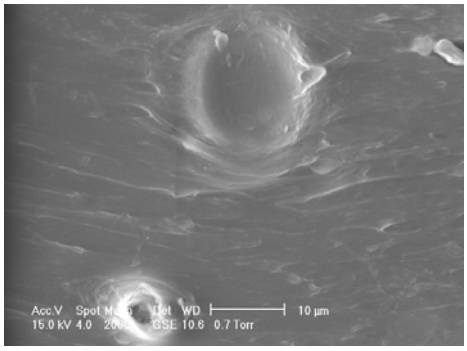


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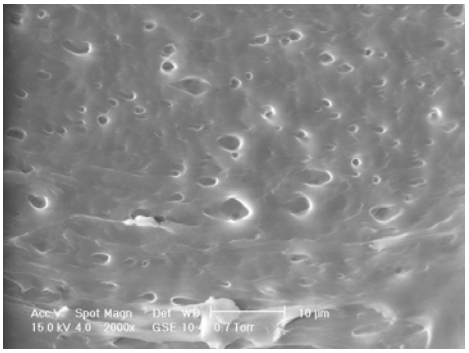


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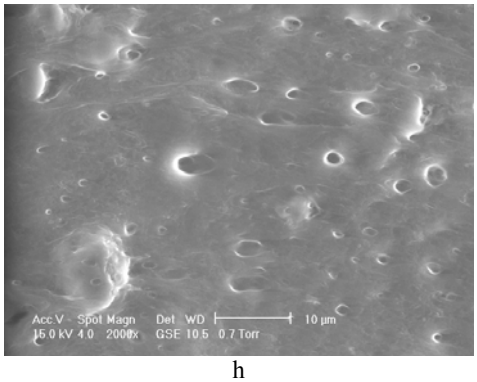



h

*Fig. 1. Scanning electron micrographs of polymeric blend variants presenting the surface (A-D) and cross-section (E-H) morphologies. Scale bars are presented in the lower part of each image. (PU-COL – A,E; PU-COL-EL – B, F; PU-COL-EL-HA – C, G; PU-COL-EL-CS – D, H).*

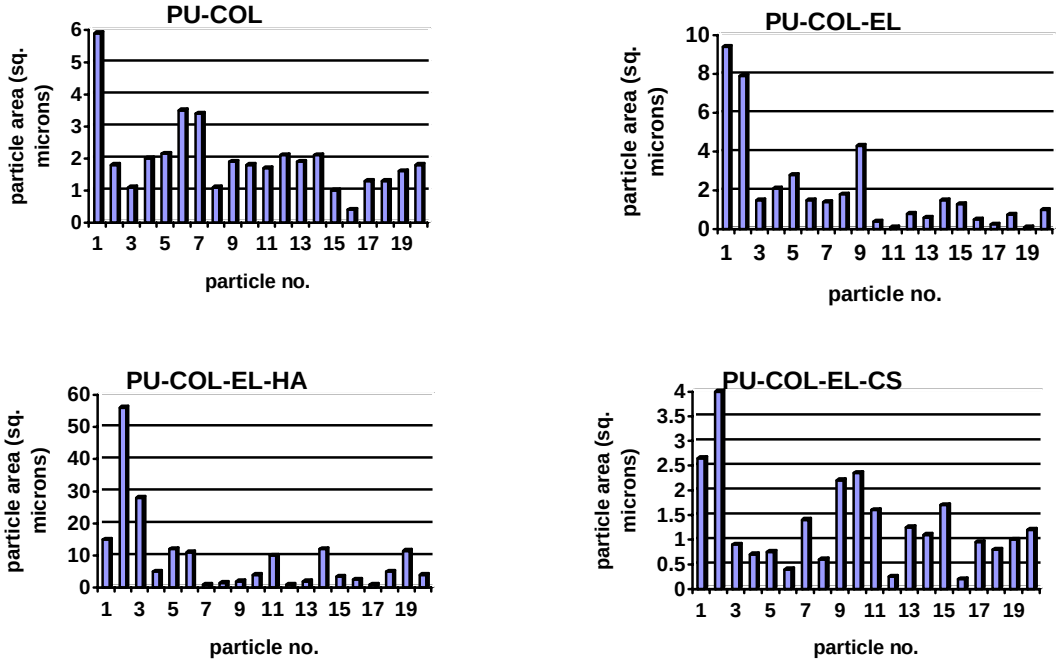



*Fig. 2. Variation of area for particles observed on the polymeric surfaces.*

### *3.2. Physical properties of PU-based biomaterials*

The porosity and density calculated for all blend variants are presented in Table 2.

*Table 2. Morphological and physical parameters of the blend variants.*

| Polymer | Pore size in cross-section (μm) | Porosity (%) | Density (g/cm$^3$) |
|---|---|---|---|
| PU | 6.06 ± 0.36 | 90.76 | 0.061 |
| PU-COL (I) | 4.16 ± 0.24 | 70.41 | 0.317 |
| PU-COL-EL (II) | < 1 | 52.26 | 0.503 |
| PU-COL-EL-HA (III) | 1.87 ± 0.11 | 71.87 | 0.229 |
| PU-COL-EL-CS (IV) | 1.5 ± 0.09 | 72.61 | 0.166 |

The highest value for material porosity was registered for the PU sheet (90.76 %) and the lowest for COL-containing sample (70.41 %), similar to that of PU-COL-EL-AH/CS but significantly greater than that of PU-COL-EL. In turn, the density varied in an inverse proportion.

### *3.3. Biodegradability of PU-based biomaterials*

*In vitro* degradation of biocompatibilized PU-based polymers was assayed in buffer containing collagenase, over a 5 days period (Fig. 3).

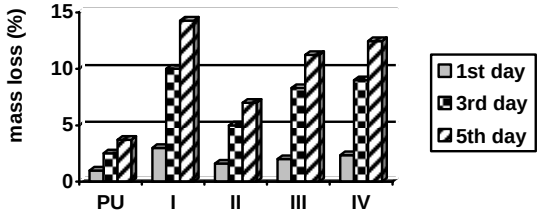



*Fig. 3. Degradation of polymeric blend variants in buffer containing collagenase. (I – PU-COL; II – PU-COL-EL; III – PU-COL-EL-HA; IV – PU-COL-EL-CS).*

Ideally, a composite material would possess sensitivity to both enzymatic degradation and hydrolysis. In the case of PU sheets it was observed a lack of sensitivity to collagenase degradation. The observed mass loss was probably due to hydrolysis of ester and other labile bonds present in the polymer. The degradation of blend variants in collagenase solution was at least 2-folds higher than that of PU alone. The PU-COL-EL blend released a significant smaller protein quantity than PU-COL variant ($p<0.025$). When a GAG was added in the mixture, the degradation was increased to values closer to variant I – PU-COL.

More relevant results might be captured over an *in vivo* study or other experimental models analyzing matrix metalloproteinase concentrations and possible inflammatory processes.

### *3.4. In vitro biocompatibility of polymeric blend variants*

The polymers biocompatibility was assayed using the MTT test, which allows calculating the number of viable cells in a primary culture of human dermal fibroblasts. The results, as quantified by mitochondrial activity assessment, are presented in Fig. 4.

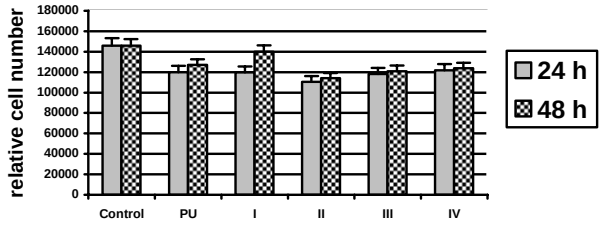
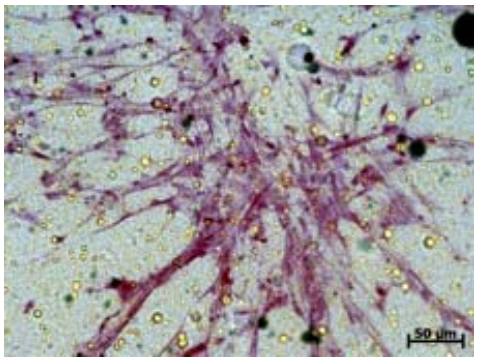



*Fig. 4. Cell proliferation on PU-based polymeric blends after 24 h and 48 h, respectively. Measurements represent mean ± S.D. (n=3) of control (cell culture) and samples (cells on polymers). (I – PU-COL; II – PU-COL-EL; III – PU-COL-EL-HA; IV – PU-COL-EL-CS).*

The calculated values showed that PU-based polymers mixed with natural ones were biocompatible; only the PU-COL-EL blend presented values less than PU sheets. When a GAG was added in the mixture, cell proliferation raised reaching values higher than $1.2 \times 10^5$ cells/ml.

No significant differences in cell number were found at 24 h of cell cultivation. At 48 h after cell seeding, the fibroblasts grew more extensively on PU-COL blends than on other blend variants and PU sheet ($p<0.05$). The dermal fibroblast number increased during the culture period. It was observed that the significantly reduced porosity of PU-COL blend, compared to that of PU, was not a disadvantage in supporting cell growth.

Cell morphology is an important factor in carrying out any cellular function, such as proliferation, migration and biosynthetic activity [18]. The morphological characteristics of dermal fibroblasts grown on polymer surfaces were observed after 72 h of cultivation by light microscopy and image processing. Light micrographs showed that cells maintained their normal phenotype, presenting euchromatic nuclei with 1-2 nucleoli and a clear cytoplasm. Moreover, the fibroblasts firmly adhered on the substrate and had cytoplasmic extensions, proving cell migration on the material surface (Fig. 5). For PU-COL-EL variant, it was observed a small population of apoptotic cells, but their number was not significant after 72 h of cultivation (Fig. 5D). The addition of GAG in the mixture of natural polymers improved the biocompatibility of PU-COL-EL blend variant.

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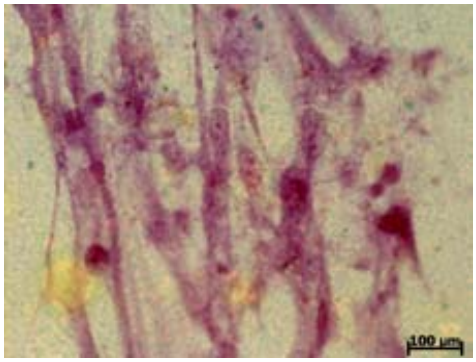


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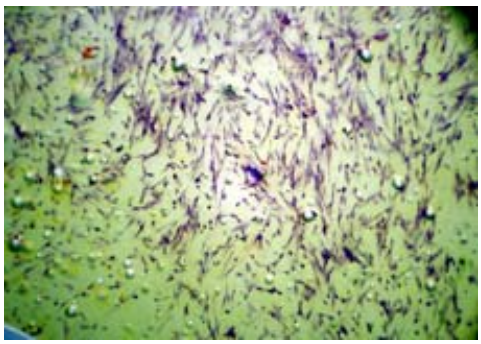

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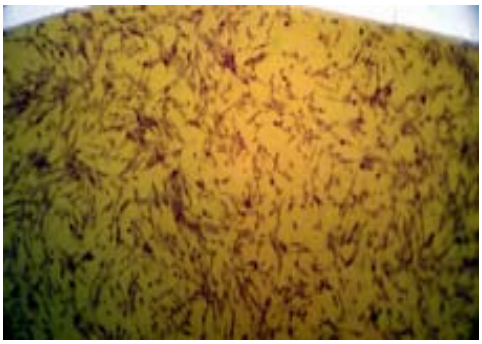

d

*Fig. 5. Light micrographs of human dermal fibroblasts grown in direct contact with blend variants I and III, for 72 h. (A - PU-COL; B - PU-COL-EL-HA). Bright-field stereomicroscope images of dermal fibroblasts cultured on variants II and IV, for 72 h. (C - PU-COL-EL-CS; D - PU-COL-EL). (C, D, x 45) (A-D, Hematoxylin staining).*

The polymeric blends prepared by mixing PU with extracellular matrix molecules (COL, EL, GAG) allowed cell attachment and growth over the culture period and did not interfere with morphological and functional characteristics of the cells, demonstrating a high biocompatibility.

This study aims to extend an earlier work which presented the preparation of PU sheets covered with various mixtures of natural polymers in order to form material sheets with increased biocompatibility and biodegradability [19]. It is known that pure collagen scaffolds do not possess good mechanical properties and are inappropriate to be processed without chemical cross-linking [20]. On the other hand, chemicals used for cross-linking can induce cytotoxicity of the material [21, 22]. Still, many composites based on COL combined with synthetic polymers were prepared and used in biology [23, 24]. We believe that PU with its elastic properties and COL with its high biocompatibility are a promising combination for medical applications. Still, there are some aspects to be clarified, like optimal products and conditions used for synthesis, possible release of toxic products, or the interactions with cells.

## 4. Conclusions

It was demonstrated that blends of PU with different natural polymer (COL, EL, GAG) mixtures could be prepared and processed as biomaterial sheets in the described conditions. The biocomposites were characterized through morphological parameters, collagenase sensitivity and cellular growth assessments, properties required for medical applications. Our results propose them as biomaterials for medical device fabrication. Future investigations regarding *in vivo* biocompatibility are necessary.

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