

Ternary Biopolymeric Films Based on Collagen Gel, Polyethylene Glycol and Hydroxyapatite

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The aim of this study is to obtain and characterize a range of hybrid polymeric films with different collagen content and synthetic poly-alcohol as polyethylene glycol (PEG). Films were obtained using a "casting solution method" by a cross-linking process at room temperature. The new collagen hybrid biofilms are devoted to cell growth and proliferation.

Keywords: collagen gel, hydroxyapatite, ternary hybrid biofilms

A wide variety of hybrid biopolymers compositions and structures based on natural and synthetic polymers with different properties as a function of application can be obtained [1-5]. In tissue engineering the biopolymers represent very important components. From the point of view of bioartificial polymers with collagen content, these were obtained as allograft from collagen tissue, prosthetic materials from synthetic polymers with collagen extracts, carrier system for active biologic substance on collagen extracts support [6-8]. The collagen is an important protein which is found in skin, conjunctive tissue, extra-cellular matrix, blood vessel, tendons, and cartilages and in other parts of the body. The scaffold biomaterials based on collagen facilitate a normal development of cells on small surface of destroyed tissue (skin, bone), and this could lead to the reconstruction of the tissue [9, 10]. Synthetic polymer, polyethylene glycol (PEG) is one of the polymers used in many biomedical applications [11-15]. The toxicity of the PEG is very small, and this decrease with the increasing of the polymer's molecular weight. In the case of the PEG with molecular weight smaller than 1000, this is absorbed into the intestine and then is eliminated by urine and excrements. An important decrease in the intestinal absorption is observed with the increasing of polymer's molecular weight [11].

This paper contains many hybrid polymeric films based on natural polymer –collagen- and synthetic polymer-polyethylene glycol - with different hydroxyapatite content in order to cell growth and proliferation. Hydroxyapatite, the predominant mineral component of bone is used in biomedical industries due to its highly attractive biologic profile.

Experimental part

Materials and methods

For the obtained biopolymeric films devoted to biomedical applications the following materials were used:

- collagen gel (2.08% dry substance/collagen gel) extracted from bovine skin (Research – Development National Institute for Textile and Leather – Bucharest);
- two types of polyethylene glycol (PEG) with $M = 400$ [g/mol] (PEG 400) and $M = 4000$ [g/mol] (PEG 4000) were purchased from Merck;^w

- ceramic material: Commercial Fluka hydroxyapatite $\text{Ca}_5\text{HO}_{13}\text{P}_3$ (HAP);
- cross-linking agent: formaldehyde (CH_2O) (FA);
- glycerine as lubrication agent.

The obtained process of ternary biopolymeric films consists in the combining of collagen gel and aqueous solutions of PEG 4000 and PEG 400 respectively in aqueous suspension of hydroxyapatite at room temperature. When a homogeneous mixture is obtained, the cross-linking agent is added at the room temperature. All this components are mixed until the solution becomes a gel with $\text{pH} = 3.5$. The gel is removed in glass Petri dishes and the room temperature is maintained for 144 h, and then the biopolymeric film is washed with distilled water in order to remove the cross-linking agent trace [4]. The obtained biopolymeric films are uniform, elastic and insoluble in water.

This study is devoted to types of biofilms as following:

- type A which contains PEG 4000, collagen gel and hydroxyapatite, where the gravimetric ratios are: collagen gel : PEG 4000 : hydroxyapatite 1:1: 4.3;
- types B based on PEG 4000, collagen gel and hydroxyapatite, where the gravimetric ratios are: collagen gel : PEG 4000 : hydroxyapatite 1:1: 3.2;
- type C which contains PEG 400, collagen gel and hydroxyapatite, where the gravimetric ratios are: collagen gel : PEG 400 : hydroxyapatite 1:1: 4.3;
- types D based on PEG 400, collagen gel and hydroxyapatite, where the gravimetric ratios are: collagen gel : PEG 400 : hydroxyapatite 1:1: 3.2;

The physico-chemical parameters and the biocompatibility of the polymeric films were analysed using the following techniques:

- infrared analysis in KBr pellets using FT-IR – Shimadzu spectrometer for pure PEG samples and FT-IR 620 (Jasco, Japonia) for collagen gel, hydroxyapatite and biopolymeric films;
- biological evaluation using cytotoxicity test for fibroblast growth and proliferation;
- image analysis using Sigma Scan program for the cell growth images;
- statistical evaluation with MedCalc program.

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Results and discussion

Infrared analysis

The IR spectra were registered for pure samples—collagen gel, PEG 400 and PEG 4000 and also for the obtained biopolymeric films.

The IR spectra for collagen gel are presented in figure 1. The main characteristic band for collagen gel is amide I at about 1644 cm^{-1} , which corresponds to stretching vibration of $\text{C}=\text{O}$ ($\nu_{\text{C}=\text{O}}$) and stretching vibration of $\text{C}-\text{N}$ ($\nu_{\text{C}-\text{N}}$). The bands corresponding to amide II and amide III at 1552 cm^{-1} and 1237 cm^{-1} respectively are also registered. The amide II is due to deformation vibration of $\text{N}=\text{H}$ ($\delta_{\text{N}=\text{H}}$) and stretching vibration of $\text{C}-\text{N}$ bond ($\nu_{\text{C}-\text{N}}$), and also to stretching vibration of $\text{C}-\text{C}$ ($\nu_{\text{C}-\text{C}}$). Another principal band appears at 3329 cm^{-1} , corresponding to stretching vibration of associate $\text{N}-\text{H}$ and $\text{O}-\text{H}$ from inter and intramolecular associated structures by hydrogen bonding.

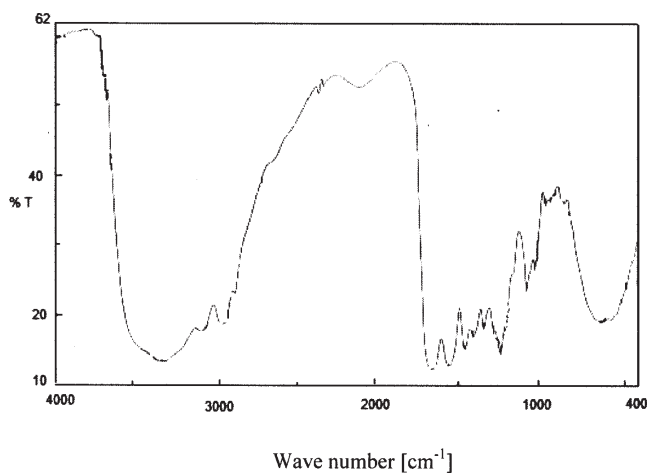


Fig. 1. The IR spectrum for collagen gel

According to IR spectrum from figure 2, as characteristic absorption bands hydroxyapatite presents: absorption band at about 3570 cm^{-1} and 3423 cm^{-1} corresponding to stretching vibration of $\text{O}-\text{H}$ group (OH free and OH associated); absorption band at 1033 cm^{-1} corresponding stretching vibration of asymmetric $\text{P}-\text{O}$ bond from PO_4^{3-} ; there are also presented the absorption band from 629 cm^{-1} corresponding to stretching vibration of symmetric $\text{P}-\text{O}$ bond and the absorption bands at 603 and 565 cm^{-1} corresponding to $\text{O}-\text{H}$ bonds and to stretching vibration of $\text{O}-\text{P}-\text{O}$ from PO_4^{3-} .

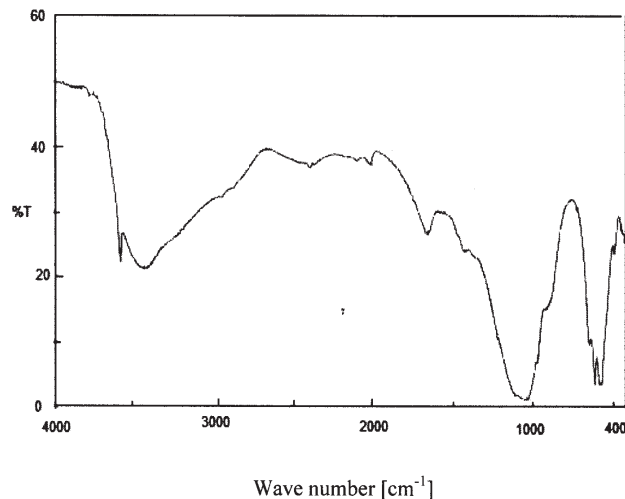


Fig. 2. The IR spectrum for HAP

The IR spectra for synthetic polymer, PEG 400 and PEG 4000 are presented in figures 3 and 4, where are evidenced the next absorption bands: the band at $3384.8 - 3421.5\text{ cm}^{-1}$ corresponding to stretching vibration of $\text{O}-\text{H}$ group, the band at $2877.6 - 2887.2\text{ cm}^{-1}$ corresponding to stretching vibration of CH_2 , and the absorption band at $1114.8 - 1103.3\text{ cm}^{-1}$ corresponding to stretching vibration of $-\text{C}-\text{O}-\text{C}-$ bond.

The spectral assignments for the biopolymeric films are presented in table 1.

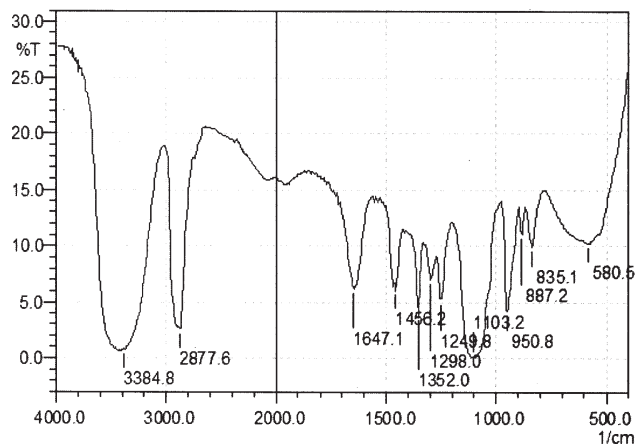


Fig. 3. The IR spectrum for PEG400

Table 1
CORRESPONDING BAND FOR BIOPOLYMERIC FILMS

Wave (cm^{-1})				Band
A	B	C	D	
3570	3570	3570	3569	$\nu_{\text{OH free}}$
3418	3400	3395	3418	$\nu_{\text{NH associate}}$ $\nu_{\text{OH associate}}$
2922	2922	2922	2922	$\nu_{\text{CH}_2 \text{ as}}$
1654	1653	1653	1653	ν_{CO} (amide I)
1547	1544	1545	1557	δ_{NH} (amide II)
1456	1455	-	1456	COO^-
1425	1408	1415	-	δ_{OH} $-\text{Ca}-\text{O}-$
1033	1034	1034	1033	PO_4^{3-}
603	603	603	603	$\delta_{\text{P}=\text{O}}$
565	566	566	565	$\delta_{\text{P}-\text{O}}$

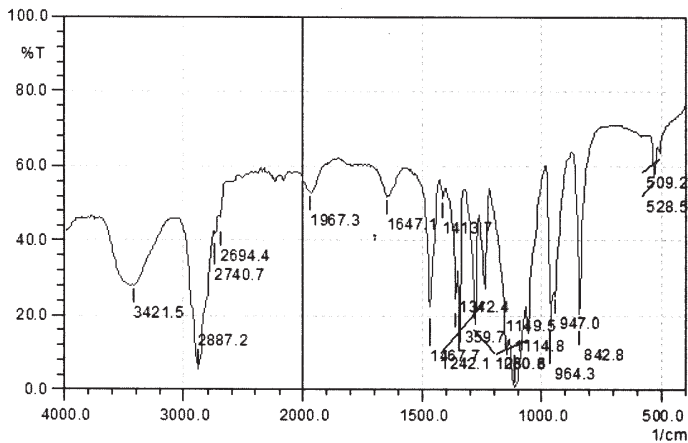


Fig. 4. The IR spectrum for PEG4000

Biological evaluation

A new method used for the replacement of affected or lost tissues is tissue engineering. In order to give back the health to the tissue or to improve the health of the tissue, the *in vitro* cell cultures are realized.

The cytotoxicity test is very much used in separation of the reactive or non-reactive materials in order to put in evidence the biocompatibility of the material. This type of test is used in comparison of different materials for various applications, and also to identify the major changes appeared in the synthesis process of hybrid ternary films.

The biopolymeric films based on collagen gel, poly(ethylene) glycol with different molecular weight and hydroxyapatite were used as polymeric support for fibroblaste growth.

The fibroblasts are dispersed cells in the body's conjunctive tissue where these are able to synthesize and secrete different components which contribute to the extracellular matrix forming (MEC). In fact the fibroblast function is to synthesize, organize and to maintain the conjunctive tissues in the increase process being related to the answer of the fibrotic lesions and diseases. The natural extracellular medium of skin fibroblasts contains collagen as following: collagen type I, III and V – able to form fiber; collagen type VI – microfibrils collagen; uncollagenous proteins and proteoglican. When the tissue presents a lesion, the migration of fibroblast cells to the situs of the lesions and the proliferation occurs leading to obtaining of a matrix reach in collagen type I and /or type III which can isolate and repair the tissue.

The fibroblast from different anatomic regions, maintained "in vitro" after cultivation shows special characteristics.

The ternary biopolymeric films were cut in small specimenes (1cm²) placed in Petri deashes (35 mm) and

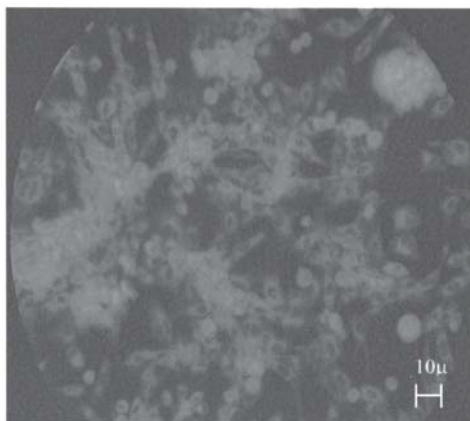


Fig. 4. The IR spectrum for PEG4000

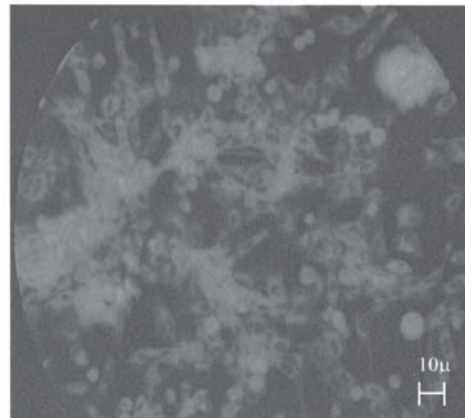


Fig. 5. Fibroblast cells culture on type A biofilm

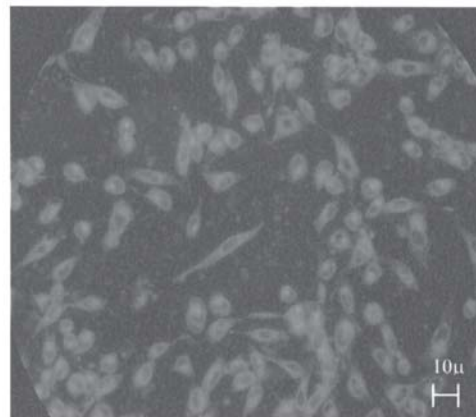


Fig. 6. Fibroblast cells culture on type B biofilm

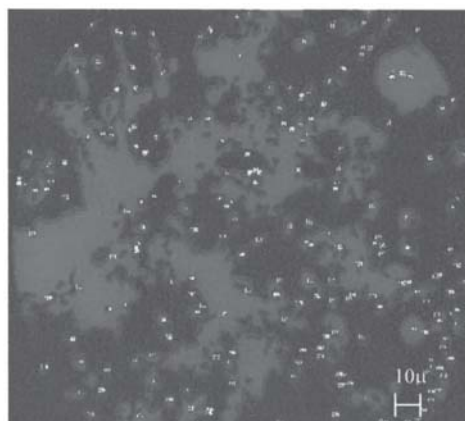


Fig. 7. The image of type A biofilm obtained with Sigma Scan program

sterilized in UV for 6 hours. Cytotoxicity test for fibroblast growth using direct contact method with secondary cultures of human skin fibroblasts (HSF), obtained, grown, and subcultured at 37°C in a humidified incubator equilibrated with 95% O₂ and 5% CO₂.

Experimental cell culture medium (Sigma) was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin. After 20 h the fibroblasts have started to proliferate around the explants. The density of the cells grown on the film was $1 \cdot 10^5$ cells /mL.

The cytotoxicity tests denote a normal phenotype of fibroblast cells grown on all four types of ternary hybrid biofilms based on collagen gel/ polyethylene glycol/ hydroxyapatite. The fibroblast cells grown on type A and B biofilms are presented in figures 5 and 6.

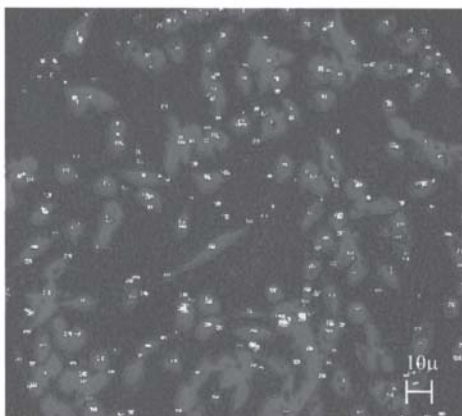


Fig. 8. The image of type B biofilm obtained with Sigma Scan program

Image analysis

The aim of this method consists in qualitative and quantitative analysis of microscopic images in order to identify and to obtain some results concerning the structures, morphology, required characteristics in evaluation of investigated biologic medium. From the point of view of quantitative analysis the results are obtained using Sigma Scan program. This program allows the evaluation of many parameters like area, perimeter, and volume, but the most important interest is the area of segmented cells and the cell spreading. Cell spreading represents the ratio between the area of cells and the area of the host region. Taking into account that entire image of ternary polymeric biofilm A is $206423.04 \mu\text{m}^2$ and the area of the cells is $73555.04 \mu\text{m}^2$ can be evaluate the cell spreading as a ratio between these two areas. The cell spreading for type A biofilms is 35.63%. From the point of view of type B ternary biopolymeric film the area of the image is $235814.4 \mu\text{m}^2$, and the cells area is $53392.64 \mu\text{m}^2$ leading to a cell spreading around 22.64%. These results indicate that an increasing in cells spreading value is obtained when hydroxyapatite is used.

The images of type A and type B films obtained with Sigma Scan program are presented in figures 7 and 8.

Conclusions

The average molecular weight of the components used in obtaining of hybrid composites, the cross-linking, solubility and the roughness of the films are important factors in cell growth and viability.

The cytotoxicity tests indicate a normal phenotype of fibroblast cells grown on polymeric supports based on collagen gel-PEG- hydroxyapatite.

A good way to optimize the biointerface is the manipulation of structure and composition of micro and nanofilms obtained as support for cell growth.

The image analysis permitted the quantitative evaluation of different parameters like area, perimeter, volume of segmented cells and also the cell spreading. The presence of more quantity of hydroxyapatite leads to increase of cell spreading value.

The results of this study permit a better good understanding of cells/polymeric support interface and a better selection of the films with specific applications.

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