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COLLAGEN USE IN BIOCOMPATIBILITY ENHANCE OF POLYETHYLENE GLYCOL

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Abstract: New polymeric materials were prepared by mixing a biodegradable, water soluble synthetic polymer (polyethylene glycol–PEG) with a natural polymer with high degree of biocompatibility (type I collagen from bovine tendon). The blends were processed in the form of membranes by mixing aqueous solutions of components and drying of these mixtures. In vitro biocompatibility of polymer mixtures was evaluated by direct contact method, both qualitative methods (cytochemical staining cells with Giemsa) and quantitative methods (determination of cell viability in a culture of human dermal fibroblasts by MTT assay). Evaluation of these materials interaction with cell culture was also made by analyzing the activity of matrix metalloproteinases (MMP) by zymography. It was found that the mixture variant with the best degree of biocompatibility was PEG: Col 1:1 in combination ratio.

Keywords: synthetic polymers, natural polymers, mixtures, polyethylene glycol, collagen, NCTC cell line, biocompatibility, cell viability, matrix metalloproteinase.

Introduction

Polymer blends or a multicomponent polymer system is of particular interest and a challenge for scientific research. As the development of new mixtures were obtained materials with high performance in terms of physical and mechanical characteristics, machinability, biodegradability and biocompatibility [UTRACKI, 1989] Recent research conducted worldwide have put an increasing emphasis on replacing the synthetic materials used in human and veterinary medicine with biosynthetic (bioartificial) materials. These materials contain at least one natural component that aims to enhance the biocompatibility of the material concerned [LUNGU, 2004]. Natural component of bioartificial materials may be a protein (collagen, fibronectin, elastin), or a polysaccharide of the glycosaminoglycans class (chondroitin sulfate, hyaluronic acid), or a peptide sequence with a role in cell recognition or cell adhesion process. These components are mostly extracellular matrix macromolecules from different tissues. Bioartificial materials containing an extracted and purified natural component of connective tissue typically have a high degree of biocompatibility and can be maintained for longer in contact with the organism [GIUSTI, 1996]. Synthetic polymers are widely used to obtain biomaterials, showing them a series of advantages such as uniform composition, low immunogenicity, suitable mechanical properties, and ability to be sterilized without changing the structure and capacity biodegradation [VERT, 2007]. For a polymeric material can be effectively used in medicine, it must meet the following criteria: not toxic, to be well tolerated by the body, to provide stability in the biological environment. These are features that collagen and elastin proteins carry, being known a series of collagen biomaterials used as implants, haemostatic agents or drug carriers for active principles [VERT, 1996]. Synthetic polymers are characterized by flexibility, strength, thermal stability and biological media actions. Mixtures of synthetic polymers and biological macromolecules are bioartificial polymer materials with enhanced functional properties and convenient characteristics of biodegradability and biocompatibility, at a relatively low price [DUMITRIU, 1994]. The aim of this work is to prepare new bioartificial materials obtained by bioactivation of synthetic polymer (polyethylene glycol) with a natural polymer (collagen). Polyethylene glycol (PEG) is the most important synthetic polymers commercially. It is made by polymerization in suspension of ethylene oxide and marketed in the form of solid or liquid having a molecular weight between 300 and 10000000g/ml. These different forms of polymer are given by the initiator used in polymerization.

Chemical formula is $HO–CH_2–(CH_2–O–CH_2–)_n–CH_2–OH$.

PEG has low toxicity and is therefore

used in the clinical and pharmaceutical field as basis for laxatives, in the composition of face cream and in combination with various medications such as alpha–interferon to treat hepatitis C or filgastrin for treatment of neutropenia. Recent studies have shown the use of PEG for the encapsulation of vectors employed in gene therapy. In our study we used PEG 4000 (SERVA) with molecular weight of 4000 g / ml. Collagen is a protein used to obtain composite materials such as collagen–synthetic polymer for a variety of medical uses, including dialysis membranes, dressings for wounds and artificial skin [AUGER, 1995; PIEPER, 2002]. Collagen is known as a biopolymer that allows cell adhesion and enzymatic degradation [MOLDOVAN, 2004].

Material and methods

Obtaining of conditioned mixtures from synthetic and natural polymers

A solution of 10% polyethylene glycol (PEG) was prepared and was maintained under stirring on water bath at 65°C for 24 hours. After complete dissolution, it was made mixtures in different ratios of combination with a natural polymer: collagen–Col, obtained in our laboratory from bovine tendon, in the form of a gel with pH 5.5, with 0.57% dry weight. Once prepared biodegradable synthetic polymer–biopolymer mixtures, they are left overnight at 4°C to allow air bubbles formed while the homogenization of blends to disappear. PEG mixtures were cast on glass plates covered with polyethylene film, then dried in the oven at 33°C for 12h. The results of conditioning were elastic membranes of different sizes.

Biocompatibility testing of mixtures

Biocompatibility refers to the behavior of (bio)materials in different contexts. This term refers to the specific properties of a material, without specifying where or how to use it. Biocompatibility is reflected in how biomaterial interacts with a body (human or animal) and how this interaction determines the effectiveness of medical devices (pacemakers, hip prostheses, catheters *etc.*). Modern medical devices and implants are usually made of a single material, just from the need to increase their biocompatibility.

In vitro biocompatibility testing

The obtained membranes were prepared for in vitro testing in order to evaluate qualitatively their biocompatibility. These membranes were processed in 5mm^2 sections and sterilized by exposure to UV radiation for 24 hours. Biocompatibility testing was done by direct contact method. For direct contact it was added one piece membrane of 5 mm^2/500µl cell suspension. Tests were performed on NCTC cell line, derived from connective tissue of mouse (*Mus musculus*), clone 929 obtained from The European Collection of Cell Cultures (ECACC) and maintained in MEM supplemented with 10% fetal bovine serum (SFB), 1% PSN (penicillin, streptomycin, neomycin) in an incubator at 37°C and 5% CO_2 humid atmosphere.

Morphology and cell growth were monitored using an inverted microscope with phase contrast.

Biocompatibility of biopolymers was assessed both by qualitative methods (cytochemical staining cells with Giemsa) and quantitative methods (MTT assay). For cell morphology analysis, cells were seeded in 24–well plates with standard density 5x10^4 cells/ml. After adherence (24h), culture was brought into contact with the samples. At 24h and 48h, the cells so grown and treated, were washed with PBS, fixed with cold methanol (–20°C), stained with Giemsa solution and photographed using an optical microscope Zeiss Observer Axio Vision. Cell viability was determined by MTT assay. This spectrophotometric method is based on the conversion of bromide–2–difeniltetrazolium dimetiltiazol (MTT) to insoluble purple formasan crystals under the action of mitochondrial dehydrogenases in living cells. Formasan quantity produced is proportional to the number of living cells and is determined spectrophotometrically after dissolving the crystals in a suitable solvent. For cell viability experiment cells were seeded at a density of 5x10^4 cells/ml in 24–well plates and incubated at 37°C in humid atmosphere with 5% CO_2 for 24 hours to allow the cells adhesion. After incubation, medium was carefully removed

and replaced with fresh culture medium; sections were placed over the substrate cell. After 24h and 48h, medium was removed and MTT solution was added (0.25 mg/ml) and the cells were incubated at 37°C for 3 hours. Later formasan crystals formed were dissolved with isopropanol. Dissolved dye absorbance was measured at 570 nm using a Tecan plate reader.

Analysis of the activity of matrix metalloproteinases (MMP)

Evaluation of these materials interaction with cell culture was also made by analyzing the activity of matrix metalloproteinases (MMP–s) responsible for controlling tissue remodeling process [PAUL, 1997].

MMP–s analysis was done by zymography. Zymography was performed on SDS–polyacrylamide gel electrophoresis of 8% with 1mg/ml gelatin (Type A from porcine skin, Sigma). Samples were treated with non reducing buffer and wells were loaded with equal amounts of protein (2.5µg protein / well).

Zymography ran at 13 mA in gel concentration and at 17 mA in the migration gel. After migration the gel was washed in Triton X–100 solution (Sigma). Washing stage is followed by incubation in developing buffer TRIS–HCl 50mM, pH 8, $CaCl_2$ 5mM at 37°C, 18h. After developing the gel was stained with Comassie Blue R solution 0.2%, enzyme activity being detected by presence of characteristic transparent bands. Reading and analysis of gels was performed with a Lourmat Vilber densitometer (France).

Results and discussion

Polymer mixtures consisting of synthetic and natural polymers represent a new class of materials, drawing attention in particular as biomaterials. The success shown by these blends as biomaterials is due to both biological characteristics (high biocompatibility), and suitable mechanical properties, ease of manufacture and low cost price [YANNI, 1995]. Natural polymers have been extensively investigated as a resource for biotechnological and biomedical applications because of their unique properties which include their non–toxicity, degradability and biological compatibility. Mixing a natural polymer with a synthetic one is an alternative way in the preparation of biomaterials with medical applications [MOLDOVAN, 2008].

There have been made mixtures of natural (Col) and synthetic (PEG) polymers in a variety of combination ratios, as shown in *Table 1*.

Table 1

Combination ratios of Col and PEG mixtures

Sample no.	% PEG	% Col
1		100
2	100	
3	15	85
4	50	50
5	30	70

Figure 1. Membranes made of polymers in the folowing mix ratios: A–100% Col; B–85% Col, 15% PEG; C–50% Col, 50% PEG; D–70% Col, 30% PEG.

Obtained membrane elasticity was assessed macroscopically (*Figure 1*), this parameter being essential for further processing of the materials submitted so they can be used as biomaterials. It was observed that membranes obtained from solution of PEG shows no elasticity, they are brittle; in combination with collagen, was acquired membrane elasticity and strength.

Fragments of membranes obtained from samples 1–5 (*Table 1*) were analyzed in terms of biocompatibility by determining cell viability (MTT test). As a result of determinations was established the percentages of living cells in the environment in which the samples were introduced.

The results are presented in *Table 2* that shows cell viability determined by MTT assay after 24 and 48 hours of cultivation in the presence of the analyzed samples.

Table 2

Cell viability for analyzed samples

Sample no.	**MTT 24h (%)**	**MTT 48h (%)**
Cc – control culture	100.00	100.00
1	89.24	91.30
2	80.34	82.55
3	80.99	90.12
4	84.90	96.79
5	81.55	87.03

According to European standard scale cytotoxicity of SR EN ISO 10993–5:2003, was found that although synthetic polymer not mixed with the biopolymer was slightly cytotoxic, mixing it with collagen leads to a significant increase in viability, therefore good biocompatibility was achieved. Thus, PEG's association with native collagen (non–denatured) led to a better cell viability (between 80.99% and 84.90% at 24h and between 87.03% and 96.79% at 48h–samples 3, 4 and 5) than simple PEG (80.34% at 24h and 82.55% at 48h–sample 2). After testing the cytotoxicity by MTT method, it was concluded that the mixture containing the synthetic polymer PEG and natural polymer Col in a ratio of 1:1 gave the best degree of biocompatibility. Samples whose cytotoxicity was analyzed by the MTT quantitative method were subjected to qualitative analysis method: morphology of the cells grown in the presence or absence (Control culture–Cc) of respective polymeric materials was observed. The results obtained from this analysis confirmed the data obtained by MTT method. Cells retained NCTC L929 line characteristic morphology, no apparent changes; the issue of culture was similar to that of control culture. Cell morphology was analyzed by optical microscopy, after Giemsa coloration. The results are presented in *Figures 2–4*.

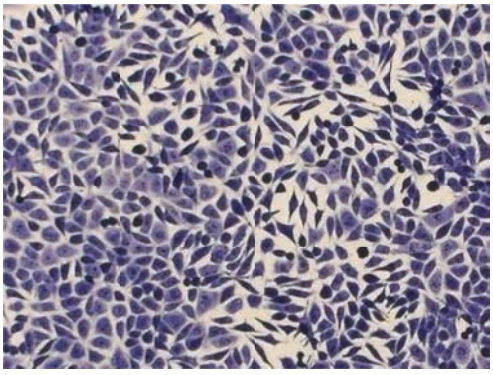

Figure 2. NCTC L929 control culture at 48 hours of cultivation

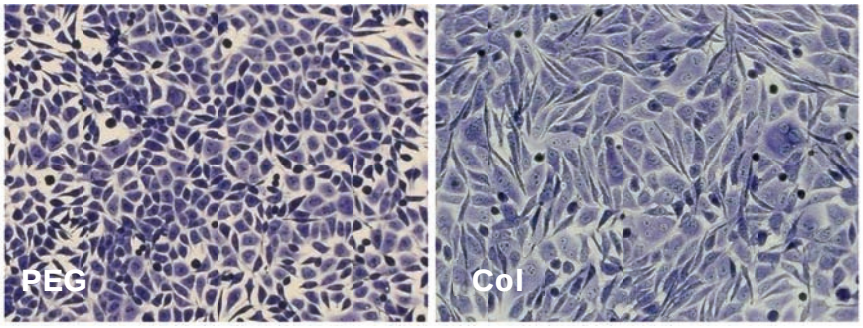

Figure 3. Morphology of cells grown in the presence of natural and synthetic polymer membranes non–mixed, after 48 hours of cultivation. It shows a cellular morphology and appearance of the cell monolayer similar to those in *Figure 2* (Cc).

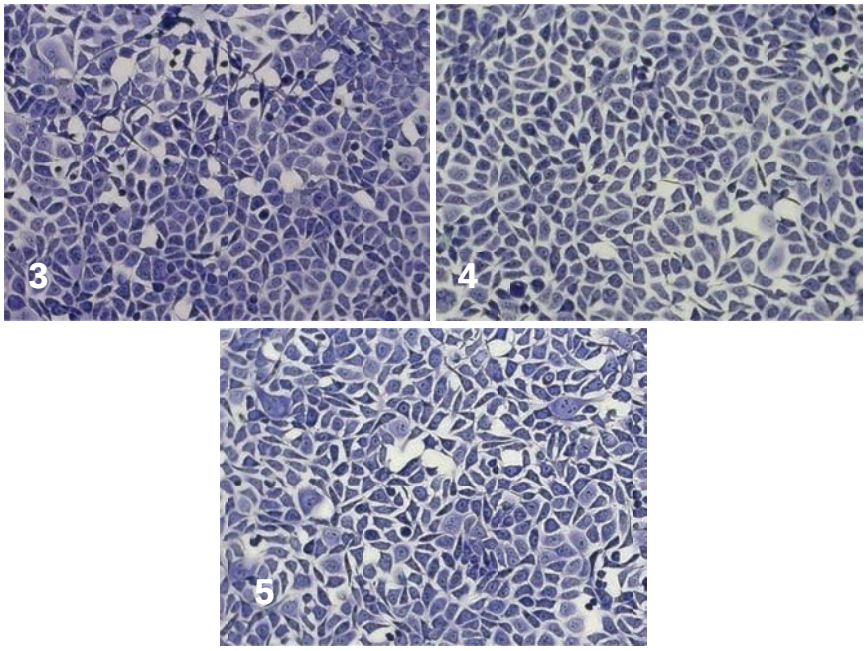

Figure 4. Morphology of cells grown in the presence of PEG membranes and non–denatured collagen (3, 4, 5), after 48 hours. Note that cells do not significantly change their morphology, which shows a good degree of their biocompatibility.

Gelatin zymography shown increased sensitivity in detecting gelatinase activity (MMP2 and MMP9) compared to other MMP's. Therefore, in the analysed samples the representation of the other types of MMP not reflects their level of quantitative rigor. MMP's largest amounts detected in all samples studied were proMMP2 (64–62 kDa), always accompanied by variable amounts of its active forms. The fact is that MMP2 is a MMP–sized member which is equally well expressed: its expression is of physiological nature. The next category as abundant of detected MMP was MMP9 (96 kDa) in its latent form. Latent MMP9 was present in all samples studied. It is also known as a larger quantity of MMP9 is an indicator of the presence of an inflammatory process in those cells [CAMPEAN, 2000]. All three variants of the mixture studied shown a low profile of the amount of MMP9 expressed, with a decrease of 4–5% (*Figure 5*) from simple polyethylene glycol (sample 2). Generally, the active form of MMP9 (84 kDa), which usually accompanies a latent form at 48 hours did not appear on zymography gel or shown inconclusive barely visible traces

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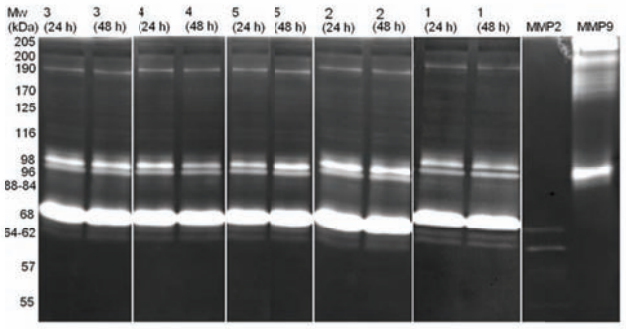

Figure 5. Zymography gel for Col (1), PEG (2), mixtures of PEG–Col (3, 4, 5) and markers used in the experiment: MMP–2 and MMP–9.

The MMP–s activity analysis for studied PEG–Col mixtures shown a lower activity of matrix enzymes (MMP9–latent and active form), which means a lower possibility that these mixtures of polymers to induce an inflammatory effect in a living tissue.

Conclusions

It can be concluded that new bioartificial materials were prepared by bioactivation of a synthetic polymer (polyethylene glycol) with a natural polymer (non–denatured type I collagen).

Mixtures conditioned in form of membranes were tested in terms of biocompatibility and we can indicate the most biocompatible variant of mixture: PEG / Col in 1:1 combination ratio.

Using collagen type glycoproteins is a good option for increasing the biocompatibility of synthetic polymers, aiming to realize new materials with biomedical applications.

References

1. Utracki, L., *Polymer Alloys and Blends*, Hanser Publishers, Munich, **1989.**
2. Lungu, M; Pascu, M.C.; Bumbu, G.G.; Darie, H.; Vasile, C.; Moldovan, L, Bioartificial polymer materials based on PVC/natural polymer blends: binary PVC/ hydrolyzed collagen blends, *International Journal of Polymeric Materials*, **2004**, 53, pp. 525–540.
3. Giusti, P.; Lazzeri, L.; Cascone, M.G., *The Polymeric Materials Encyclopedia*, CRC Press, Boca Raton, Florida, **1996**.
4. Vert M., Polymeric biomaterials. Strategies of the past vs. strategies of the future, *Progres in Polymers Science*, **2007**, 32, pp. 755–761.
5. Yanni, J., Making PVC more Biocompatible, *Medical Device Technology*, **1995**, 20, pp. 1.
6. Dumitriu, S., *Polymeric biomaterials*, Marcel Dekker Inc., New York, **1994**, pp. 1
7. Auger, F.A.; Lo´pez–Valle, C.A.; Guignard, R.; Tremblay, N.; No¨el, B.; Goulet, F.; Germain, L., Skin equivalents produced by tissue–engineering using human collagens, *In Vitro Cell Dev Biol*, **1995**, 31, pp. 432–439.
8. Pieper, J.S.; van der Kraan, P.M.; Veerkamp, J.H.; van Kuppelvelt, T.H., Crosslinked type II collagen matrices: preparation, characterization and potential for cartilage enginering, *Biomaterials*, **2002**, 23, pp. 3183.
9. Moldovan, L.; Buzgariu, W.; Craciunescu, O.; Oprita, E.I.; Oancea, A.; Zarnescu, O., Obtaining of porous collagenous matrices and their interaction with human fibroblasts, *Romanian Biological Sciences*, **2004**, 3–4, pp. 3–10.
10. Paul, R.G.; Tarlton, J.F.; Purslow, P.P.; Sims, T.J.; Watkins, P.; Marshall, F; Ferguson, M.J.; Bailey A.J., Biomechanical and Biochemical Study of a Standardized Wound Healing Model, *Int J Biochem Cell Biol*, **1997**, 29, (1), pp. 211–220.
11. Moldovan L., Craciunescu O., Zarnescu O., Macocinschi D., Bojin D., Preparation and characterization of new biocompatibilized polymeric materials for medical use, *J Optoelectron Adv Mat*, **2008**, 10(4), 942–947.
12. Campean A.; Caloianu, M.; Alexandru D.; Efimov, N.; Buzgariu, W, Effect of interleukin–1β on gelatinolytic activity and cell morphology of human osteoarthritic chodrocytes in culture, *J Med Biochem*, **2000**, 4(2), pp. 113–129.

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