Compatibility and cytotoxicity testing of some polyvinyl alcohol and hydrolyzated collagen based blends

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Abstract

The aim of this paper is to characterize some bioartificial materials based on polyvinyl alcohol (PVA) and hydrolyzed collagen (HC) and to test in vitro biocompatibility on human dermal fibroblasts. The biological analysis (cell proliferation, viability and morphology) showed that bioartificial materials present a good biocompatibility, fibroblasts maintaining their normal cellular phenotype in the presence of polymers. These blends may find applications in cosmetics, pharmacy, agriculture and medicine, as ecologically expedient seeds polymeric covers, as matrix for controlled drug release of bioactive substances and plants' protection.

Keywords: polyvinyl alcohol, hydrolyzed collagen, blends, biocompatibility

Introduction

Testing the *in vitro* biocompatibility on cell cultures offers precious information on *in vivo* behaviour. [1] Some of newly employed cell cultures reproduce *in vivo* conditions that permit to normal cells to grow in medium culture. A polymer can be used in medicine or/and pharmacy if it is biocompatible with blood and tissues, is well tolerated by organism, is physically and chemically stable in biological medium, and it is not toxic.

Polyvinyl alcohol (PVA) is well recognized due to its many utilizations like: suture fibers, protection colloid, adhesive, drug controlled release matrix, skin replacer, soil fertilizer, cosmetic products. [2] All these applications of PVA are based on its properties such as: water solubility, thermal stability, film forming capacity, emulsifier, viscosity controlling agent, it is nontoxic and an excellent barrier against odors, oils and fats, being biodegradable both in aerobic and anaerobic conditions. [3-5]

Because of its hydrophilic characteristics that allows for good compatibility, blending with natural polymers such as starch, chitin, gelatin, silk fibroin, cyclodextrin and lignocellulosic fillers, get low cost of final materials, they show good properties, they are easy to process and are recyclable. [6-12]

Collagen and its hydrolyzates (HC) receive nowadays a great attention in many scientific fields due to the variety of the applications in the orthopedic implants coatings, transdermal patches, sutures, burning healing, in cosmetics, based on the high biocompatibility comparing with other biopolymers. By the point of view of interaction with living cells, all animal cells types specific to conjunctive tissues, like epithelial or endothelial cells, have the capacity to be absorbed at the native collagenic fibrous matrix in living tissues, so that cellular adhesion, proliferation and cell physiology will be different by the *in vivo* situation. Studies on collagenic materials interaction with living cells followed extracellular reconstruction matrix. [13-15]

The collagen addition in blends in different proportions increase the biodegradation rate, enhance adhesion of epidermal cells to the polymer surface and prevent failure in case of implants. Understanding the factors that influence collagen interactions with solid surfaces is of great importance in medicine and pharmacology. [16-18]

In a previous study has been established that the polyvinyl alcohol presents a good compatibility with hydrolysated collagen. Both DSC and IR-spectroscopy evidenced the physical and chemical interactions between the functional groups. It has been established the compatibility "window" and specific interactions

between components. On the basis of the obtained data can be concluded that optima compatibility ratios are 95-70 PVA wt%/5 – 30 wt% HC**.** [19]

Future researches on these blends will aim sanitary applications like: water soluble containers, materials for enzyme immobilization in biosensors, controlled release systems, water treatment, fertilizers, environment degradable packaging, etc. In respect with this aim this paper establishes the influence of polymer blends on cellular parameters like proliferation and cells morphology to evidence the *in vitro* biocompatibility.

Materials and Methods

Materials. PVA with a number-average molecular weight of 71 000 Dalton, a polymeric degree of 1600 and a degree of saponification of 95.8 mg KOH/g was obtained from SA Romacril Râşnov- România.

The hydrolyzed collagen was supplied by the National Institute of Research and Development for Biological Science – Bucharest, Romania. It has been obtained by acid hydrolysis of bovine derma. The powdered sample was obtained by a spray-drying process with a BÜCHI 190 MINI SPRAY DRYER. It is a type I and III collagen and it consists of a mass of peptides with the following elemental composition: 42.7 wt% C, 10.8 wt% H, 12.2 wt% N and 34.3 wt% O and has a number average molecular weight determined by GPC on solution in dimethyl formamide of 99 000 Daltons and a polydispersity of 1.66. It is thermally resistant up to 180 ºC. HC is a highly hydrophilic polymer.

Preparing of blends have been done in accordance with the method described in an anterior study [19] There have been carried out 2 solutions of polyvinyl alcohol and hydrolyzed collagen of a 10 wt% concentration. The polyvinyl alcohol solution is white viscous opalescent. The resulted hydrolyzed collagen solution is brown-yellowish and weakly viscous. There have been prepared blends by mixing of the resulted solutions in various proportions and following blends' composition were used in this study: 1HC/9PVA; 2HC/8PVA; 3HC/7PVA; 4HC/6PVA; 5HC/5PVA. The obtained solutions were homogeneous, they present neither separation nor colour changes. Films with a thickness of approximately 0.02 mm have been made from these solutions by solvent casting method. 2 ml of each solution has been laid on polymethyl methacrylate (PMMA) plates of 10x15 cm, which were dried at 60-70 ºC in vacuum oven for 1h. Macroscopic aspects of the obtained films are presented in table 1.

For *in vitro* experiments on primary culture of human dermal fibroblasts, samples were cut in 5x5mm pieces and then sterilized with UV radiation for 8 hours.

Table 1. Macroscopic aspect of the obtained films

Blend	Notation	Films aspect
1HC/9PVA	1	Semitransparent, continuous
2HC/8PVA	2	Semitransparent, continuous
3HC/7PVA	3	Semitransparent, continuous
4HC/6PVA	4	Semitransparent, continuous
5HC/5PVA	5	The film is brittle and transparent
PVA	PVA	Semitransparent, continuous

Methods. Optical microscopy. The obtained films were examined by means of an optical Axiolab.re Carl Zeiss microscope, J/902553, with a magnification of 400 x. The films are homogeneous, this being the first clue for the compatibility of components.

DSC analysis. The DSC analyses were achieved by means of a Mettler DSC 12E instrument. Before starting measuring, the calorimeter was calibrated with indium standard, which has T_m =156.6 ºC. The samples were thermically scanned in two cycles of heating under temperature range between 25-216ºC with a heating rate of 10ºC/min, followed by a cycle of cooling at a cooling rate of 10^{o}C/min. The samples having masses comprised between 6 and 8.2 mg were put into aluminium crucibles. A nitrogen flow of 30 ml/min was passed through the calorimeter. The DSC curves for these blends were drawn for the first heating cycles.

IR analysis. FT-IR spectra of the films of PVA/HC were recorded at 4 cm^{-1} resolution by means of a DIGILAB Scimitar Series FT-IR spectrometer (USA). Processing of the spectra was done by means of Grams/32

program (Galactic Industry Corp.). For the hydrolyzed collagen, the spectra were recorded in KBr pellets because this sample does not form film.

Proliferation and cell morphology. A primary culture of human dermal fibroblasts was obtained from human dermis explant. Tissue sample was washed with PBS (phosphate buffer saline) and minced into small pieces of approximately 2mm.

The fragments were placed into a 60 mm diameter dish and a small volume of DMEM supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml neomycin was added to allow the fragments to adhere to the bottom of the dish. The culture was incubated at 37 ºC in a humidified atmosphere of 5% CO_2. The culture medium was replaced every 3-4 days. In this study were used fibroblasts at passage 15. The morphology and growth of cells were monitored with a phase contrast microscope.

To analyze cell proliferation, human dermal fibroblasts were seeded into 35 mm diameter dishes in DMEM supplemented with 10% fetal bovine serum (FBS) and were incubated at 37ºC for 24h. After this period of time, the culture medium was replaced with a fresh one and test samples were placed in the center of the dish (5 x 5mm pieces). Plates were incubated in the same conditions for 24, 48 and 72 hours. After that, fibroblasts were harvested by tripsinization, stained with a 0.4 % Trypan blue solution and the viable, unstained cells were counted using a Burker-Turk hemocytometer.

To analyze cell morphology, dermal fibroblasts cultivated in presence of polymeric blends, for 24 h, were washed with PBS, fixed with cold methanol (-20ºC), stained with Giemsa solution and photographed using an inversed-phase microscope (Nikon, Japan). The morphology and cellular raising were monitorized with an). in phase contrast.

In all experiments the control sample was represented by dermal fibroblasts cultivated in the absence of polymers.

MTT assay. Cell viability was measured by MTT test. Fibroblasts were seeded into 24-well plates at a density of $3{,}5x10^4$ cells/ml, and after 24 h of incubation at 37ºC the polymeric samples were added. After 24 h and 48 h, the culture medium was discarded, 50 µl of MTT solution (5 mg/ml) dissolved in the culture medium were added in each well and then, cells were incubated at 37ºC for 3h. Water-insoluble dark blue formazan crystals formed in viable cells were solubilized with isopropanol and the absorbance of every well was measured at 570 nm using an UV-VIS spectrophotometer (Jasco V-650, Japan). Concentration of converted dye directly correlates to the number of metabolically active cells in the culture. Cell viability was calculated by comparison with control sample (fibroblasts cultivated in absence of polymers), considered to be 100% viable.

Results and Discussion

Physical and chemical characterization of polymeric blends

The DSC curves of PVA, HC and their 2 and 5 blends are given in figure 1where it may be observed that there is a wide temperature interval where physically absorbed water is released, this process being dependent by the chemical nature of components. Corresponding peak for this process is between 90 ºC for HC and 130 ºC for APV. Temperature peak for the blend 80 % APV/ 20 % HC composition is shifted to lower temperatures, and the peak shape is characteristic to every blend, being thinner than for the components. The new arrangements between functional groups increase components compatibility, due to the different interactions with adsorbed water.

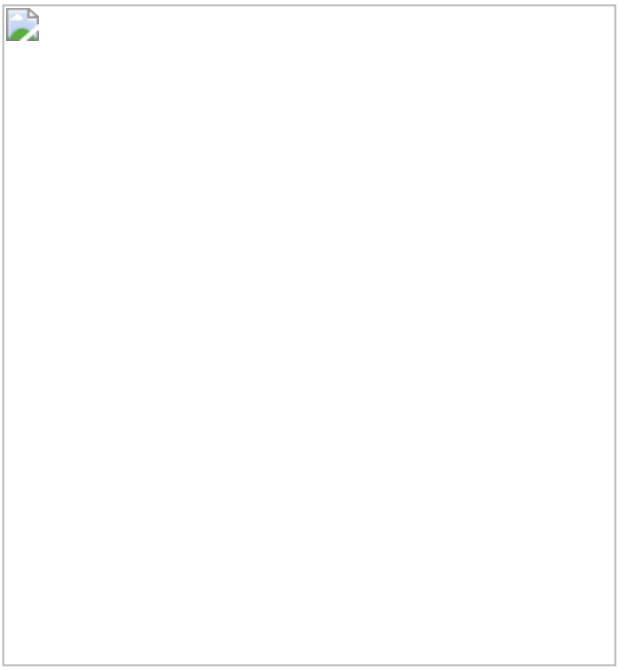


Figure 1. DSC curves of PVA, HC and their blends in the first heating run – are useful to evidence adsorption/desorption of water.

IR spectra for APV, HC and of 2HC/8 APV and 5 HC/5 APV blends are presented in figure 2.

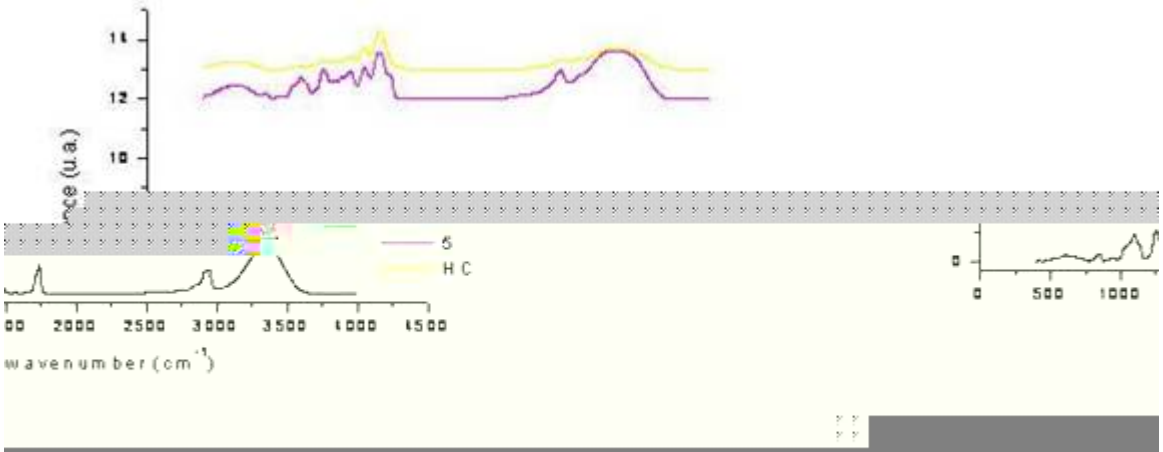


Figure 2. FT-IR spectra of PVA, HC and their blends.

The band from 3350 cm^{-1} region, typical for polyvinyl alcohol, presents a maximum intensity for the 2HC/8PVA blend and its intensity considerably decreases as the HC content rises.

The band from 2939 cm^{-1} region, typical for C-H bond, presents a shoulder for the blends with a high content of PVA, shoulder that disappears in the case of 5HC/5PVA blend. The intensity of this band decreases as the HC content rises.

The band from 1651 cm^{-1} region (typical for terminal groups interactions) appears in the spectra of both blends and it is more obvious at high collagen content.

The band from 1541 cm^{-1} is typical for HC and rises in intensity as the collagen content increases. The changes in bands' intensities normalized for the same film thickness indicate the presence of the interactions between components.

On the basis of the obtained data can be concluded that optima compatibility ratios are 95-70 PVA wt%/5 – 30 wt% HC**.**

PVA / HC polymeric blends biocompatibility

The results on cell proliferation presented in table 2 indicate the number of adherent dermal fibroblasts after 24 h, 48 h and 72 h of cultivation in the presence of polymeric variants. It was observed a continuous increase of fibroblasts number at 48h and 72h, 6PVA/4HC, 7PVA/3HC blends and PVA having proliferation degrees (93.04 %, 82.60 % respectively 80.58 %) closest to the control sample (100%). The other samples had proliferation degrees smaller than 80%

Table 2. Cells number grew in presence of polymeric variants after 24 h, 48 h and respectively 72 h of cultivation

Sample	24 h (cell /ml)	48 h (cell /ml)	72 h (cell /ml)
Control	22.500	51.250	86.250
PVA	25.000	41.250	69.500
9PVA/1HC	26.250	41.250	68.750
8PVA/2HC	18.700	46.250	57.250
7PVA/3HC	18.750	45.000	71.250
6PVA/4HC	21.250	52.500	80.250

The effect of polymeric materials on human dermal fibroblasts was evaluated by MTT test, a simple, rapid and economical assay for measurements of cellular viability, proliferation and cytotoxicity. The method is based on the conversion of the tetrazolium salt [MTT: 3-(4,5-dimethiltiazol-2-il)-2,5-difeniltetrazolium bromide] to water-insoluble dark-blue formazan crystals, by mitochondrial succinate-dehydrogenase in viable cells.

The results presented in figure 3 showed that PVA, 7PVA / 3HC and 6PVA / 4HC samples supported cell proliferation with cell viability values higher than 80%. For PVA polymer, number of viable cells was close to control sample (100%). For 9PVA / 1HC and 8PVA /2HC films values of cell viability were smaller than 80%.

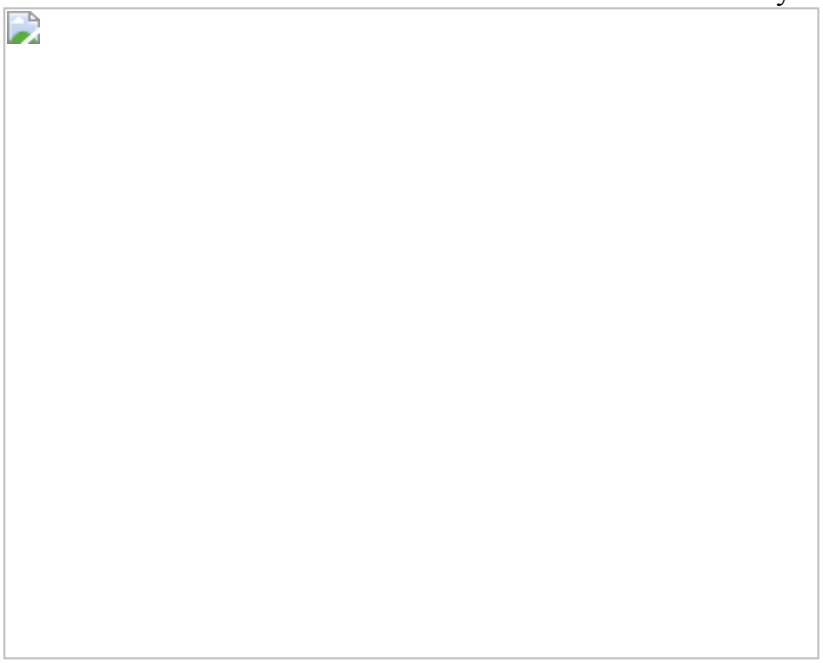


Figure 3. Effect of polymeric blends on dermal fibroblasts viability after 24 h and 48 h of cultivation, evaluated by MTT assay (1 – control; 2 –PVA; 3 - 9PVA / 1HC; 4 - 8PVA / 2HC; 5 - 7PVA / 3HC; 6 - 6PVA / 4HC).

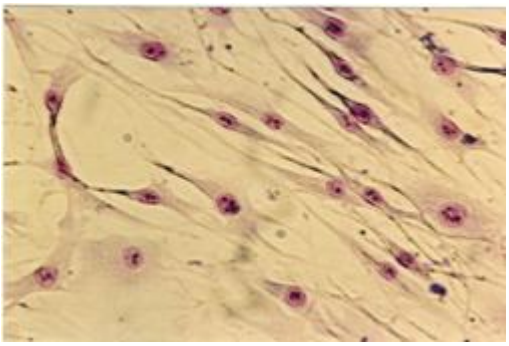
Cell morphology

Light microscopy images showed that all five tested polymeric samples caused no morphological modifications of human dermal fibroblasts. After 24 h of incubation, the control and cells cultivated in presence of polymeric materials have retained their specific characteristics. Cells presented the elongated morphology of

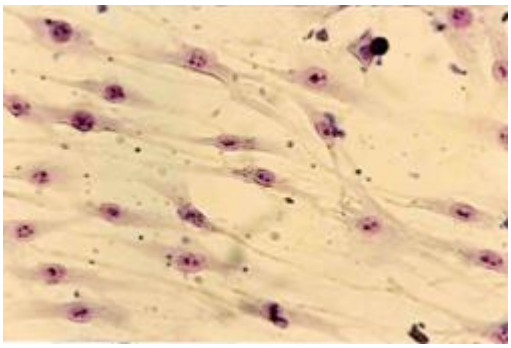
normal fibroblasts with euchromatic nucleus, 1-3 nucleoli, with thin cytoplasmatic extensions and a fine granular cytoplasm.

Incubation of polymeric films in presence of human dermal fibroblasts led to solubilisation of all the five membranes, which does not affect proliferation or cell morphology.

Figure 4. Morphological aspect of the control after 24 h of cultivation (Giemsa staining, **x100**)

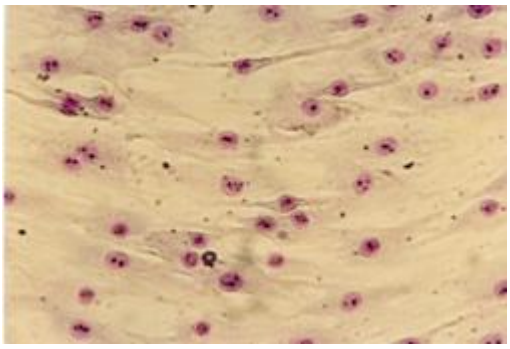


PVA

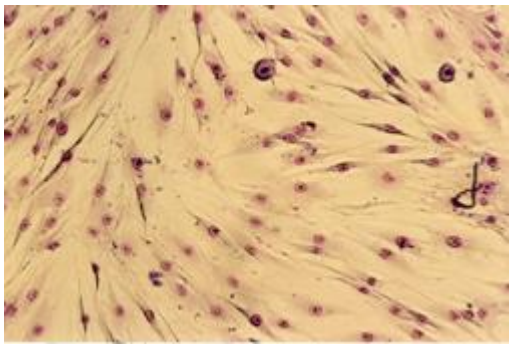


9PVA/1HC

Figure 5. Morphology of dermal fibroblasts cultivated in presence of PVA and 9PVA / 1HC at 24 h (Giemsa staining, **x200**)

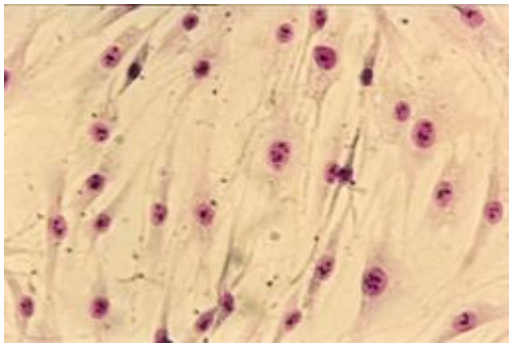


8 PVA / 2HC



7PVA / 3 HC

Figure 6. Morphology of dermal fibroblasts cultivated in presence of 8 PVA / 2HC and 7PVA / 3 HC at 24 h (Giemsa staining, **x200** - 8 PVA / 2HC; **x100** - 7PVA / 3 HC)



6PVA / 4HC

Figure 7. Dermal fibroblasts cultivated in presence of 6PVA / 4HC at 24 h (Giemsa staining, **x200**)

Conclusions

Both DSC and IR-spectroscopy evidenced the physical and chemical interactions between the functional groups. It has been established the compatibility "window" and specific interactions between components; the higher compatibility was obtained for 95-70% PVA with 5-30% HC.

Morphological analysis of dermal fibroblasts cultivated in presence of PVA/HC blends indicated a good biocompatibility of polymers, fibroblasts keeping their normal cellular phenotype. Regarding the cellular viability, the biomaterials caused no cytotoxic effect *in vitro*, the higher percentage, over 80%, being observed for PVA, 6PVA / 4HC and 7PVA / 3HC.

All the tests carried on this polymer blends indicate the possibility for using them to produce biomaterials with applications in medicine and pharmacy as matrix for controlled release of drugs or bioactive substances and in agriculture as ecologically expedient seeds polymeric covers, and chemical means of plant protection.

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References

1. C.N. ALLEN, E.S. HARPUR, T.J.B. GRAY, B.H. HIRST, Toxic effects of non-steroidal anti-inflammatory drugs in a human intestinal epithelial cell line (HCT-8) as assessed by the MTT and neutral red assays. *Toxicology in vitro*, **5**, 183-191 (1991).
2. F. CRIVOI, C. VASILE, Aplicatiile hidrolizatelor de colagen şi ale alcoolului polivinilic în cosmetică. Simpozionul Naţional de Cosmetică şi Produse Aromatizante, Iaşi, 29 mai-1 iunie 2007; *RSCC Magazine*, **7** (2-3), 57-67 (2007)
3. C.M. HASSAN, N.A PEPPAS. Structure and applications of poly(vinyl alcohol) hydrogels produced by conventional crosslinking or by freezing/thawing methods, *Adv. Polym. Sci*., **153**, 37-65 (2000).
4. N.K. MONGIA., K.S. ANSETH., N.A. PEPPAS, Mucoadhesive poly(vinyl alcohol) hydrogels produced by freezing/thawing processes: applications in the development of wound healing systems, *J Biomater Sci Polym Ed.*, **7**(12), 1055-1064, (1996).
5. T. OKADA, D.H BARK, M.R MAYBERG., Local anticuagulation without systemic effect using a polymer heparin delivery system, *Polymer Delivery System*, **19** (12), 1470-1476, (1988).
6. E. CHIELLINI, P. CINELLI, SH. IURAM, L.MAO, Composite films based on biorelated agro-industrial waste and polyvinyl alcohol. Preparation and mechanical properties characterisation; *Biomacromolecules* , **2,** 1029-1037, (2001).
7. S. PATACHIA, PVA şi amestecuri ale acestuia cu polimeri naturali sau sintetici, cap. XIX , *Polimeri Biodegradabili şi Biocompatibili*, C. Vasile, A. Chiriac, L.E. Nita eds. Tehnopress, 2006, pp. 400-423.
8. X.J. WANG, R.A. GROSS, S.P. McCARTHY, Rheological study of biodegradable blends of starch and polyvinyl alcohol, *J. Envrionm. Polym. Degrad*. **3,** 161–167, (1995).
9. J.L. LAWTON, G.F. FANTA, Glycerol-plasticized films prepared from starch-poly(vinyl alcohol) mixtures: effect of poly(ethylene-co-acrylic acid). *Carbohydr, Polym.* **23**, 275–280, (1994).
10. D.R. COFFIN, M.L. FISHMAN, T.V. LI, Thermomechanical properties of blends of pectin and poly(vinyl alcohol), *J. Appl. Polym. Sci.* **61**, 71–79, (1996).
11. K. TSUKADA, G. FREDDI, J.S. CRIGHTON, Structure and compatibility of poly(vinyl alcohol)-silk fibron (PVA/SF) blend films, *J. Polym. Sci: B. Polym. Phis*. **32,** 243–248, (1994).
12. M. PEESAN, R. RUJIRAVANIT, P. SUPAPHOL, Characterisation of beta-chitin/ poly(vinyl alcohol) films. *Polymer Testing* **22**, 381–387, (2003).
13. E. BELL, H. P. ELRICH, D. J. BUTTLE; A blood vessel model constructed from collagen and cultured vascular cells, *Sience,* **211**, 1024, (1981).

14. R. MONTESANO, P. MOURON, M. AMHERDT; Collagen matrix promotes reorganization of pancreatic endocrine cell monolayers into islet-like organoids, *J. Cell Biol*., **97**, 935, (1983).
15. M. BOUVIER, M. COUBLE, D.J. HARTMANN, J.P. GUTHIER, H. MAGLOIRE, Ultrastructural and immunocytochemical study of bone-derived cells cultured in three-dimensional matrices: Influence of chondroitin-4 sulfate on mineralization;
Differentiation **45** (2), 128–137, (1990).
16. M. LUNGU, A. POGONARU, M. PRECUP, L. MOLDOVAN, A. OANCEA, O. ZARNESCU, Increasing biocompatibility of synthetic polymers for medical use by compounding with collagen; *Roum. Biotechnol. Lett*., **2,** (2), 153-161, (1997).
17. L. MOLDOVAN, W. BUZGARIU, O. ZARNESCU, M. FLAMANZEANU, O. CRACIUNESCU; *In*