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Effect of Chemical Compositions and Topographical Features of Collagen Biofilms on Cell Response

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The goal of this paper is to characterize new range of hybrid multicomponent polymer biofilms with collagen, synthetic poly-alcohols and hydroxiapatite, changing the components number, the ratio of each component, the open-pore, and the hydrophobic/hydrophilic material character. Films were obtained using a "casting solution method". The cell viability was checked in all cases by the standard trypan blue exclusion text.

Keywords: cell growth, collagen, hydroxiapatite, poly-vinyl alcohol

INTRODUCTION

Developments in medical polymers for biomaterials applications in the last years involved polymers for gene therapy, intelligent materials as polyrotaxane and other new formulations for protein-based polymers [1]. One area of intense recent research activity is the use of biodegradable polymers from natural or synthetic sources for tissue

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engineering applications. As natural polymers the favourite candidates are fibrin, collagen, gelatin, hyaluronan, etc. The selection for synthetic materials includes polymers as polylactic acid (PLA), polyglycolide (PGA), poly(lactide-co-glycolide) (PLGA) [1,2], ethylene oxide block copolymers, or inorganic materials like tricalcium phosphate, calcium carbonate, and hydroxyapatite. Investigating the possibility of manufacturing biodegradable composites for use as fabricating open-pore, biodegradable polymer scaffold for cell seeding, several studies were focused on various polymers devoted to biomedical application as substrates for the culture of cells [3–7]. Taking into account the collagen role in living tissue, there is still a need for new hybrid structure based on collagen and synthetic polymers and this paper is an approach of manipulation of composition, structure and topographical features of biofilms with collagen content. A range of hybrid multicomponent polymer biofilms with collagen synthetic polyalcohols and hydroxyapatite were obtained, characterized and discussed, changing the components number, the ratio of each component, the open-pore, and the surface roughness, taking into account that the material surface properties will influence the initial cellular events on the cell–material biointerface.

EXPERIMENTAL PART

Materials

Neutral (HO_2, HO_8) and acid collagen hydrolysates (HA_4) with 72,000, 16,600 and 11,000 average viscosimetric molecular weight; where 2, 4, and 8 means hydrolysis time (hours); collagen gel (2.08% dry substance/collagen gel); poly(vinyl alcohol) (PVA) with $M_v = 280{,}000$ [g/mol] and $M_v = 420{,}000$ [g/mol]; poly(ethylene glycol) (PEG) with $M_v = 400$ [g/mol] (PEG 400) and $M_v = 4000$ [g/mol] (PEG 4000); Commercial Fluka hydroxyapatite $Ca_5HO_{13}P_3$ (HAP).

In order to obtain binary and ternary hybrid biopolymeric films, two types of cross-linking, by dehydrothermal method and by chemical treatment were lead using for copolymer blends the following systems:

- water soluble synthetic polymer – poly(vinyl alcohol) (PVA) and natural polymer – neutral (HO) or acid (HA) hydrolysates in different volumetric ratios, dispensed in glass Petri dishes by a "casting solution method" for a dehydrothermal treatment, raising the temperature to 120°C in three steps [8,9]. Polymeric matrix based on water soluble synthetic polymer – poly(vinyl alcohol) (PVA) or poly (ethylene glycol) (PEG) and natural polymer – collagen gel and

ceramic reinforcing material – hydroxyapatite (HAP), obtained using a cross-linking agent. (A new patent in processing). The types of films discussed were as follows:

- Types A and B with PVA and neutral collagen hydrolysates HO_2 and HO_8.
- Types C with PVA and acid collagen hydrolysates HA_4.
- Types D and E: PVA and collagen gel without and with hydroxyapatite.
- Types F and G: PEG 400 and collagen gel without and with hydroxyapatite.
- Types H and I: PEG 4000 and collagen gel without and with hydroxyapatite.

Methods

Physicochemical, topographical features and biocompatibility of biofilms were performed using following investigations:

Viscosimetric method for molecular weight for collagen, PVA, and PEG
Liquid porosimetry with Coulter porometer
Surface analysis type Atomic Force Microscopy (AFM) with 2D and 3D visualization of AFM data for roughness determination
Cytotoxicity test for fibroblast growth, proliferation and viability
Image analysis with a Scan programme of microscopic examination by an inverted microscope Nikon (Eclipse TS-100-F).

RESULTS AND DISCUSSION

Redefining his initial definition of biocompatibility [10] in a recent article, Williams [11] suggested that the need to differentiate the type of application lead to modification of the definition according to the concept that biocompatibility is a two-way process and its evolution over time is not only the host response to material, but also a material affecting host. However, biocompatibility of many polymeric new biomaterials such as hybrids composites films has not stood the test of long-term behaviour. Hybrid biofilms devoted to cell growth and proliferation, being matrix for a tissue engineering product could be evaluated according to a different concept of biocompatibility which refers to the ability to perform as a substrate that will support the cellular

TABLE 1 The Porosity of the Biofilms

Sample	Natural polymer [g]	Synthetic polymer	Average pore radius [μm]
C	0.6	PVA	0.17
	1.2	PVA	2.80
D	0.6	PVA	0.01
	1.2	PVA	0.08
F	1.1	PEG 400	19
H	1.1	PEG 4000	23

activity without inducing injurious effects on biological system as a cell response. For *in vitro* experiments the discussions are related usually in such cases at the moment, to the existence of a correlation between biomaterials properties and a desirable cell growth and viability.

Porosity of the Biofilms

The porosity of binary and ternary biofilms was determined using a Coulter Porometer as a function of type and ratio of collagen, and average viscosimetric molecular weight. The results indicate a direct correlation between type and collagen content and average pore radius. In Table 1 the variation of average pore radius for natural and synthetic polymers is presented. The values are in the range $0.01 \div 23\,\mu m$.

According to experimental data, in the case of C samples when collagen content is increasing the average pore radius increases, but in the presence of collagen gel (sample D) average pore radius decreases. Also an increase in average molecular weight lead to a slight increase in pore dimensions as we can see from H sample with PEG 4000 and F sample with PEG 400 data. The porosity data support the hydroxyapatite effects as an inductor of porosity. The influence of synthetic polymers is very important taking into account that average pore radius value for D sample – PVA and collagen gel and H sample – PEG 4000 and collagen gel is approximately 10^3* times greater.

Cell Proliferation and Viability

Cytotoxicity test for fibroblast growth was performed using direct contact method with secondary cultures of human skin fibroblasts (HSF) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin; MTT Test [12] measures the cell

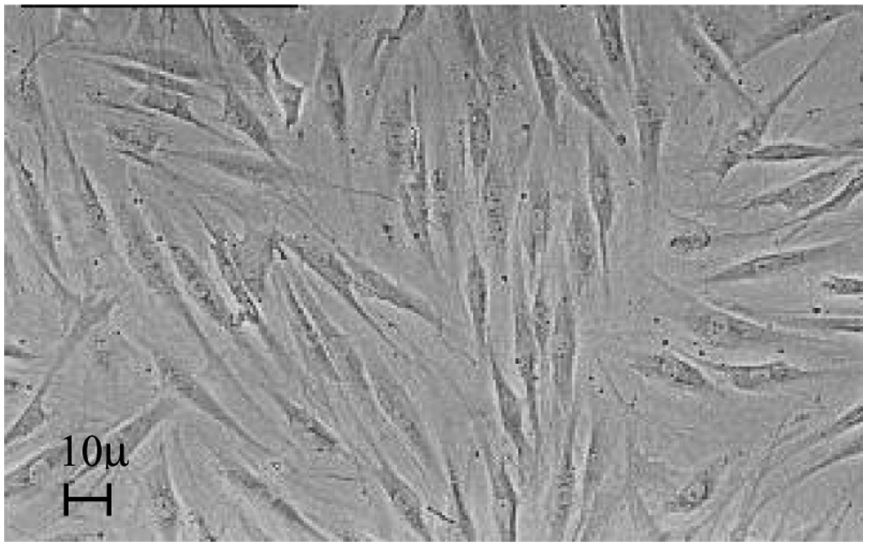

FIGURE 1 Control sample.

activity, proliferation and cell viability. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to the corresponding blue formazan. Human derma fibroblasts (HDF) were treated in 24-well plates and incubated with 40 μL/well of MTT (0.2 mg) in medium for 4 h. The reaction was stopped by addition of 1 mL dimethyl sulphoxide (DMSO) per well, and the formazan released from the cells by incubation at 37°C for 5 min was measured. Absorbance of the supernatant was measured at 550 nm. Absorbance values that are lower than those for the control cells indicate a reduction in the cell activity. Conversely a higher absorbance rate indicates an increase in cell activity/proliferation. Figures 1–5 are the experimental results of cytotoxicity test for fibroblast growth, Figures 1–4 being cells culture on control, sample, and on various biofilms. Figure 5 is a representation of fibroblast cell viability.

The viability value for type H biofilm with PEG 4000 is higher than in the case of control sample, in comparison with G and I biofilms with hydroxyapatite where the viability value is smaller than in the case of control sample. The benefit of hydroxyapatite effect in the biofilms based on PEG is an argument for cell phenotype modification in ternary biofilms, this phenotype becoming a normal one (Figs. 3 and 4).

FIGURE 2 Cells on type C film (normal phenotype).

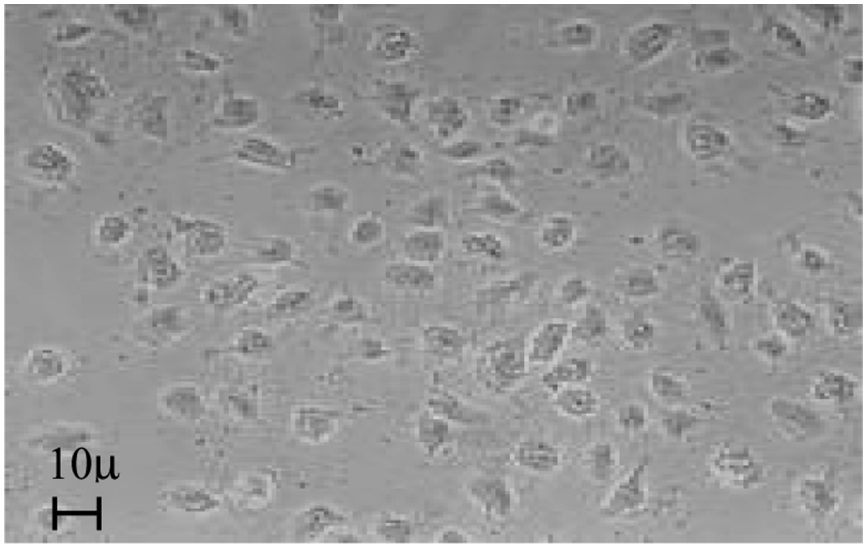

FIGURE 3 Cells culture on type F film with a modified phenotype.

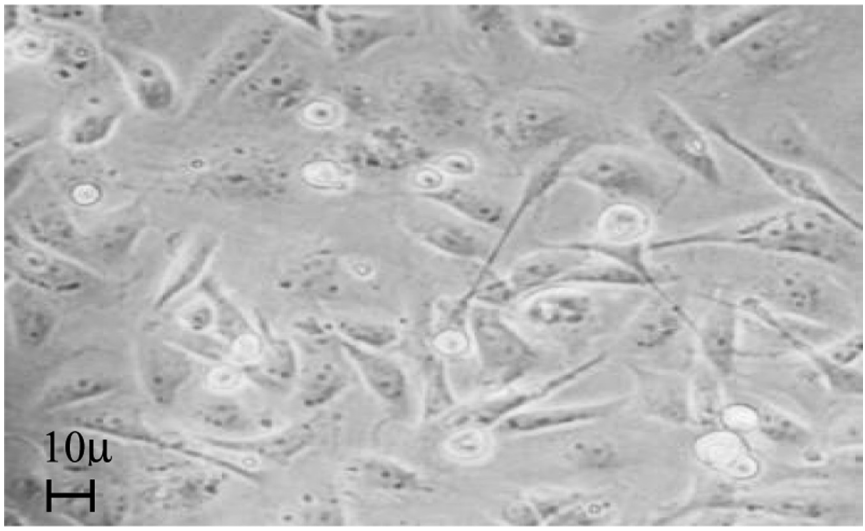

FIGURE 4 Cells culture on type H film with a normal phenotype.

Surface Analysis by Atomic Force Microscopy (AFM)

AFM images were acquired in standard contact mode, in following conditions: close loop (height mode) at scan sizes ranging from $20 \times 20\ \mu m^2$

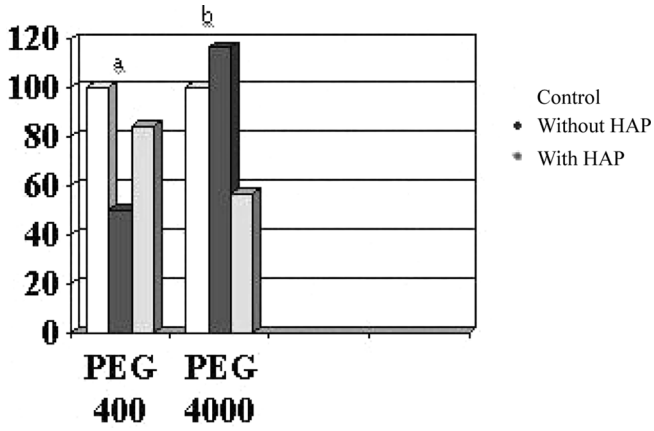

FIGURE 5 Fibroblast cell viability as a function of components number from biofilms: a) control sample; binary and ternary films based on PEG 400 without and with HAP; b) control sample; binary and ternary films based on PEG 4000 without and with HAP.

to $2 \times 2\,\mu m^2$. Delay ranged from 3000–8000 μs. The program provides 2D and 3D visualization of AFM data.

A and B films with the same collagen content, but with different hydrolysis time (2 and 8 hours), indicate a decrease in the root-mean-square roughness from 16 nm to 10 nm with increasing hydrolysis time. Hydroxyapatite effect is an increase in roughness data, in all studied cases the values being between 16–20 nm.

Image Analysis

The processing of data with Sigma Scan program referring to a representative image as a control, permitted a quantitative evolution of various parameters, such as: area, perimeter, volume, and the relative estimator cell spreading, which is the ratio between the total surface occupied by cell and the surface of total host area. The cell spreading or the binary biofilm type F is 10.93%, for the ternary type G is 17.61%, and for control 57%. These data are arguments for hydroxyapatite effect in increasing cells spreading. Taking into account that hydroxyapatite presence in biofilms is a porosity inductor, it is to point out a direct relation between porosity and cells spreading. Regarding area, perimeter, and volume of segmented fibroblast cells, the histograms of Figures 6–11 present a comparison for biofilms F and G.

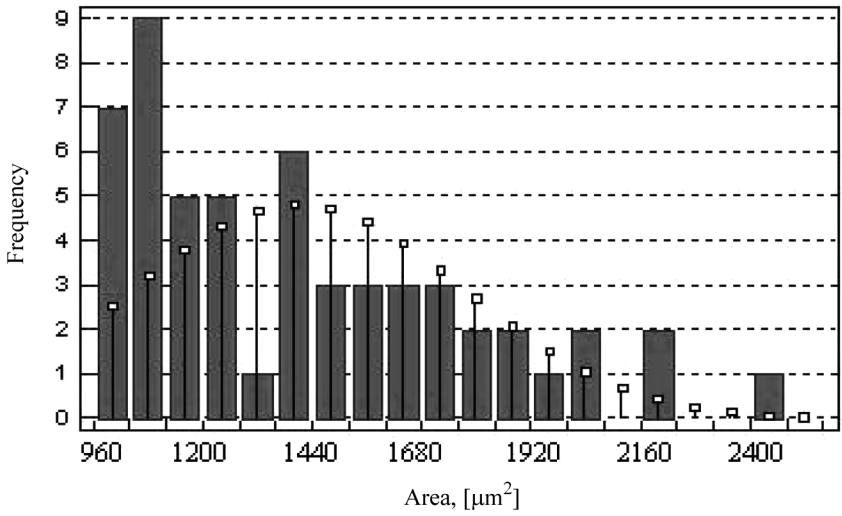

FIGURE 6 Histograms of area values of segmented cells from binary film – F.

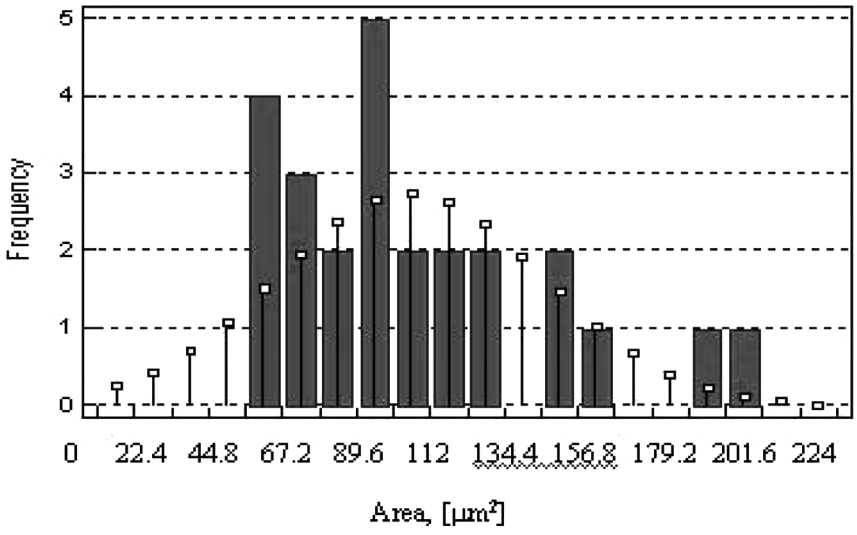

FIGURE 7 Histograms of area values of segmented cells from ternary film – G.

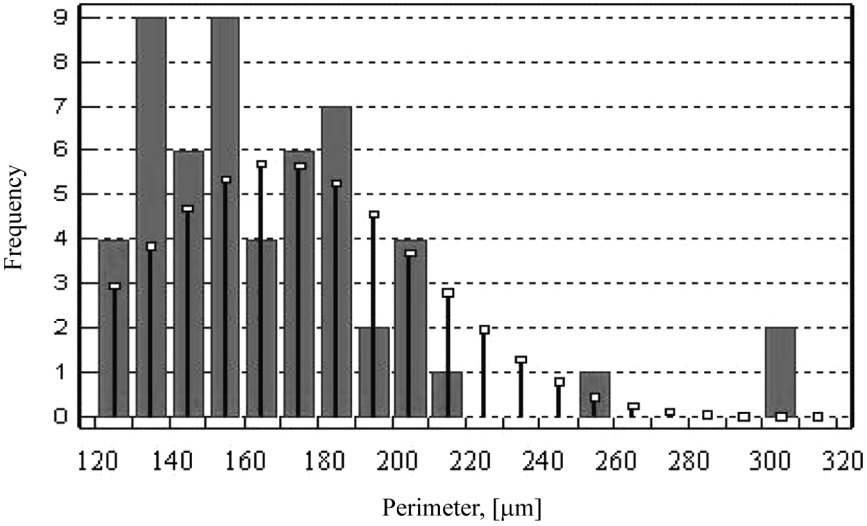

FIGURE 8 Histograms of perimeter values of segmented cells from binary film – F.

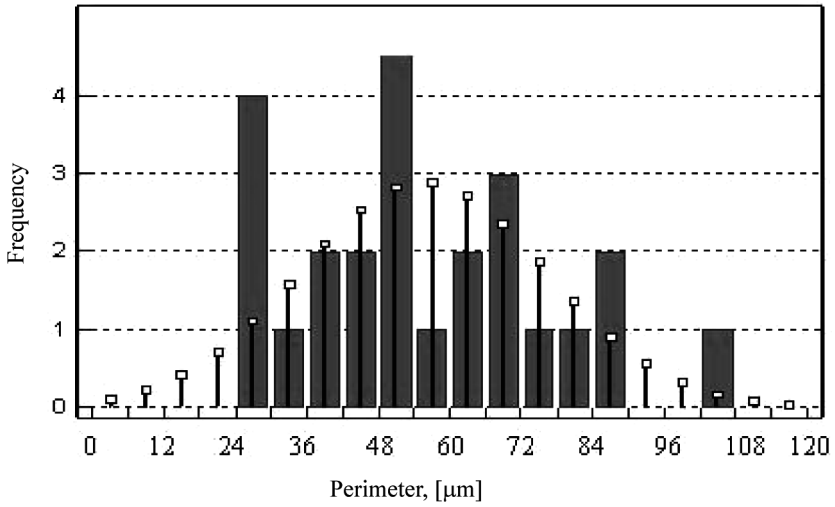

FIGURE 9 Histograms of perimeter values of segmented cells from ternary film – G.

For the ternary biofilms, the perimeter data around the average value have the highest frequency; for the binary biofilms the highest frequency is related to smallest perimeter value.

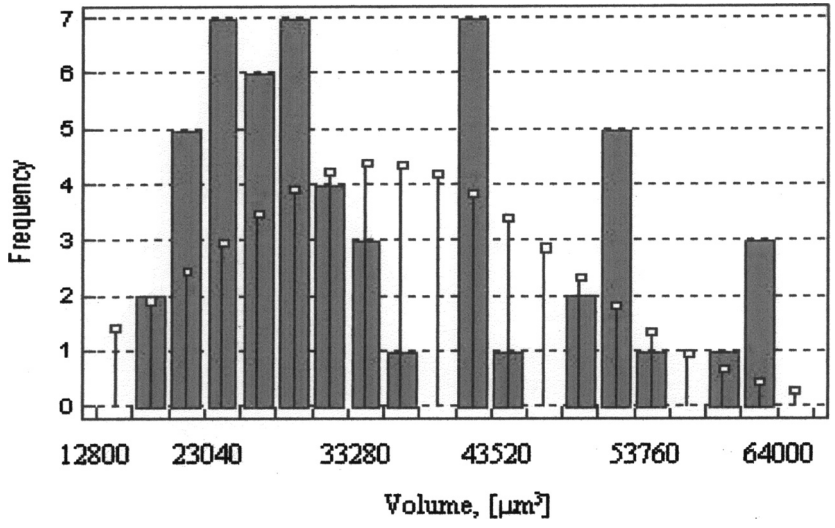

FIGURE 10 Histograms volume values of segmented cells from binary film – F.

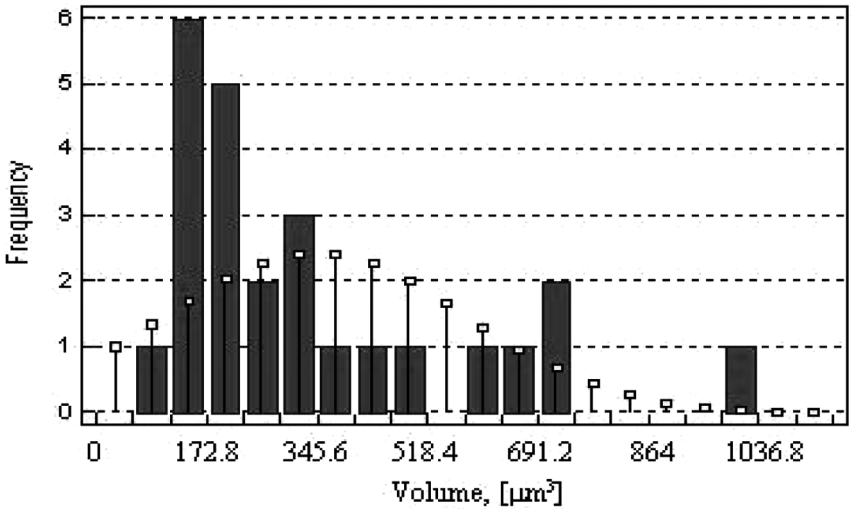

FIGURE 11 Histograms of volume values of segmented cells from ternary film – G.

The same treatment of data indicates that for both binary and ternary types of biofilms the frequency of cell with a volume and area less than average one is significant.

CONCLUSIONS

1. The average molecular weight of the components of hybrid composites, the cross-linking, solubility, and roughness of films are in a specific relationship with cell adhesion.
2. Cytotoxicity test indicates a normal phenotype of fibroblast cell culture on the collagen/PVA blends and a modified phenotype of cell on the collagen/PEG blends; the hydroxyapatite presence in the collagen/PEG blends lead to a normal phenotype too, and this is an argument of hydroxyapatite benefit.
3. The processing of data with Sigma Scan program referring to a representative image as a control, permitted a quantitative evaluation of various parameters, such as: area, perimeter, volume, and cell spreading.

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