Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 24, supplement 1, 2014, pp. 61-66

EVALUATION OF BIOCOMPATIBILITY OF COMPOSITE BIOMATERIAL WITH ANTI-INFLAMMATORY ACTION, AND STIMULATING TISSUE RECOVERY PROCESS

Catalin Iordachel[1]*, Elena Berteanu[1], Adina-Lidia Zuav[1], Mihaela-Ionica Enache[1], Ana-Maria Dobre[1], Sorin Manoiu[1], Maria Paraschiv[1], Luminita Tcacenco[1]
[1] Department of Biomaterials & Bioproducts, National Institute R&D for Biological Sciences, Address: 296 Splaiul Independentei, 060031-Bucharest, Romania

ABSTRACT. The paper refers to a bioactive matrix in membrane shape, with a thickness of 0.5 – 1.0 mm, and consisting of gelatine, chitosan, and α-chymotrypsin, with or without addition of glutaraldehyde as a cross-linking agent. The bioproduct is a biomaterial with biomedical applications as biocompatible membrane with anti-inflammatory action due to coupling of the proteolytic enzyme. Bioproducts have complex implications in the treatment of traumatized tissue, accelerating the pus cleansing of infected wound, blood clots and other cellular debris, secretions liquefaction and cleansing of necrotic tissue, and also stimulating tissue regeneration and a faster healing of open infected wounds. In order to establish the influence of cross-linking process and membrane's thickness on mechanical properties, ultrastructural studies were carried out on obtained biomaterials by electron microscopy analysis. To demonstrate the biological qualities of new enzymatic biomaterials, the membrane's biological effect was analyzed in a cell culture model (mouse fibroblasts NCTC).

Keywords: biomaterial, chitosan, gelatine, proteolytic enzyme, anti-inflammatory composite.

INTRODUCTION

This study refers to the development of bioproducts with complex implications in the treatment of traumatized connective tissue for accelerating the pus cleansing of infected wound, blood clots and other cellular debris, and for annihilation by proteolysis of some enzymes that can interfere with the action of antibiotics. Chymotrypsin (EC 3.4.21.1) is a protease that preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond (the P_1 position) is a large hydrophobic amino acid (tyrosine, tryptophan, and phenylalanine) (Campbell & Farrell, 2013). This enzyme also hydrolyzes other amide bonds in peptides at slower rates, particularly those containing leucine and methionine at the P1 position. Chymotrypsin has ingredients that reduce swelling and tissue destruction. By coupling gelatine with chitosan, we aimed to obtain a biomaterial with a very good biocompatibility and a low antigenicity.

Chitosan is a natural polymer that can be used as support for drug immobilization and delivery or tissue engineering, polysaccharides reacting with biologically active substances to form stable product, and being non-toxic and pharmacologically inert. Generally, drugs chemically linked to polysaccharides modified structures tested *in vitro* and *in vivo* showed an improvement of therapeutic properties by effect extension, toxicity decrease, water solubility increase, changes on its distribution in the body and an increasing stability. Many pharmaceutical and biomedical applications of chitosan are due to its nontoxic properties, large biocompatibility and biodegradability.

As in chitosan composition are found both amino and hydroxyl reactive groups, which can be changed physically or chemically, turns out to have a high potential for use in tissue engineering applications. One of the most interesting effects of chitosan on healing wounds is the formation of granulation tissue with angiogenesis. In the literature it is known that this polysaccharide induces interleukin release by fibroblasts, which is involved in the migration and proliferation of fibroblasts. Meanwhile, chitosan possesses haemostatic properties, independent of "clotting cascade". It is biocompatible, biodegradable, haemostatic, fungistatic (Rajendran & Anand, 2006), non-toxic and can be successfully used as gels, films and fibers. This polymer also shows antibacterial properties and possesses good wound healing properties (Rigby *et al.*, 1997; Muzzarelli *et al.*, 1999). It has many applications as a wound dressing, drug delivery device and as scaffold for tissue engineering (Khor *et al.*, 2003).

Chitosan, due to its structural properties, has the ability to heal wounds without scar formation (Usami *et al.*, 1997). Since chitosan is composed of *D*-glucosamine, which is also the component present in the disaccharide subunits of hyaluronic acid, chitosan tries to structurally mimic hyaluronic acid and exert similar effects.

Gelatine is a linear polypeptide, with partially crystalline conformation, consisting of an amino acids sequence (glycine, proline, alanine, valine, hydroxyproline), soluble in water, aqueous electrolytes solutions and in some organic liquids, which, in dissolved state, is able to form gels at temperatures below 30 °C. In the acceptance of most researchers, gelatine is a derivative of collagen that has no property

**Correspondence: Catalin Iordachel, National Institute R&D for Biological Sciences, Department of Biomaterials & Bioproducts, no. 296 Splaiul Independentei, 060031-Bucharest, Romania, Tel./Fax +40 (21)- 220.79.09, e-mail: cataliniordachel@yahoo.com*
Article received: April 2014; published: May 2014

to reorganize in a fiber form and has a molecular weight of about 100,000 Da.

MATERIALS AND METHODS

For experiments were used: chitosan with low molecular weight, M = 150,000 and a deacetylation degree DD = 84.5%, and gelatine, both manufactured by Fluka BioChemika.

The experiments were conducted as follows: 10 ml of chitosan solution (0.5% chitosan in 1% acetic acid) and 10 ml of gelatine solution (0.5%) were mixed at 1:1 ratio, adding the proper amount of enzyme (5-10 mg α-chymotrypsin in 5 ml of distilled water) and stirring for 4 hours.

In order to improve elastic properties, they were cross-linked with 0.5% and 1% glutaraldehyde (pH = 8.7, adjusted with 0.05 M phosphate buffer freshly prepared, 20 minutes, at room temperature).

The mixture was poured into Petri enclosures. The obtained samples were dried in an oven at 37 °C for 24 hours. A semi-transparent elastic membrane was obtained.

Three types of membranes have been synthesized:

M1: chitosan - gelatine, non-cross-linked

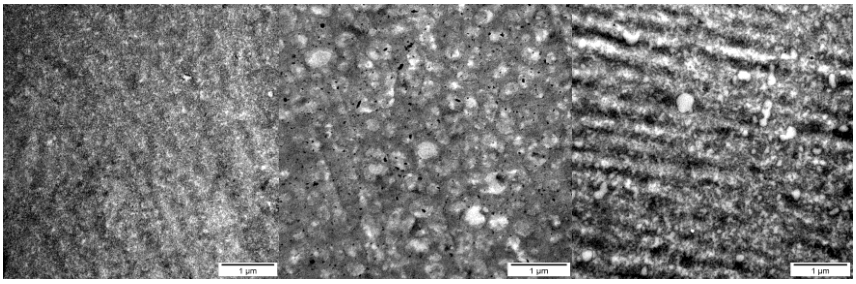
M2: chitosan - gelatine – enzyme, non-cross-linked

M3: chitosan - gelatine – enzyme, cross-linked with glutaraldehyde 0.5%

To characterize the biomaterial, ultrastructural studies were performed and the biological quality assessment was realized by flow-cytometry on experimental models *in vitro*.

Ultrastructural studies

Membrane fragments were included in 0.2 M sodium cacodylate buffer and were fixed in a solution of 4% osmium tetroxide (prepared in sodium cacodylate buffer) at 4 °C overnight. After fixation, the samples were washed, serial dehydrated with ethanol (50%, 60%, 96%), with absolute ethanol, absolute ethanol – acetone mixture, acetone and, then, they were included in Epon 812. After samples' cutting in a Leica ultra-microtome, EMUC-6 type, ultra-fine sections of 7 µm were obtained, which were stained with uranyl acetate and lead citrate. Contrasted sections were fixed on microscopic grids and viewed in a Philips EM 208S electron microscope.



M1 M2 M3

Their electron-microscopic evaluation showed that cross-linking of chitosan-gelatine in membranes (M3) makes their structure to be more uniform and more compact than the non-cross-linked membranes (M2). Thus, with the cross-linking of polymer membrane, the structure becomes more homogeneous, more compact with a low porosity degree and therefore mechanically more resistant.

Biological quality assessment of new biomaterials by flow cytometry on experimental models *in vitro*

Flow cytometry analysis of the membranes effect on cell model (mouse fibroblast NCTC) was performed by comparing the cell characteristics with a reference fibroblasts culture. The three types of membranes were incubated for 24 hours in mouse fibroblasts (standardized cell line NCTC) (50 000 cells / ml) following some characteristics, namely:

1) Estimation of cell morphology changes by measuring the absorption and scattering of light *(light scatter measurement)*

Comparative analysis of the three types of cytogram obtained by investigating fibroblasts cultured on synthesized membranes and of reference fibroblasts, shown in *Fig. 1 -3*, has led to the following observations:

- there is a change of the fibroblasts content evidenced by YGeoMean values (219 for M1, 314 for M2 and 203 for M3) compared with the reference sample where the YgeoMean value is 153.
- cell size differences are much smaller, but remarkable for M2, where X GeoMean has the lowest value of 170, compared with 234 registered for the reference sample.

The changes are shown in comparative cytograms (*Fig. 1*) and chart (*Fig. 2*) and more detailed in the accompanying statistical analysis (*Fig. 3*)

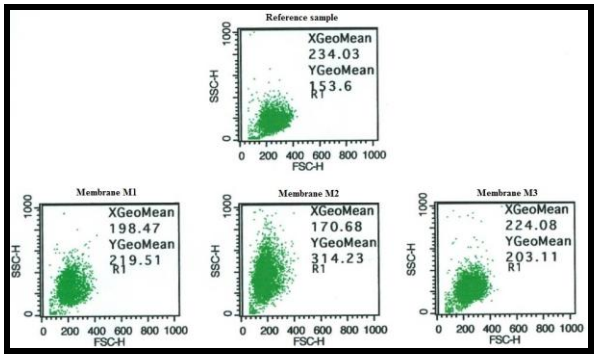


Fig. 1 Comparative analysis of morphological changes in FSC / SSC system

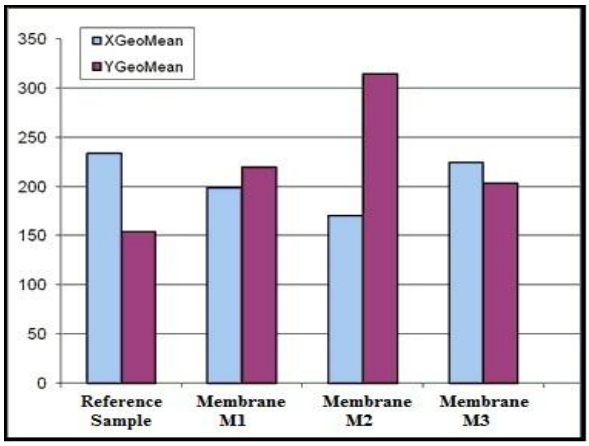
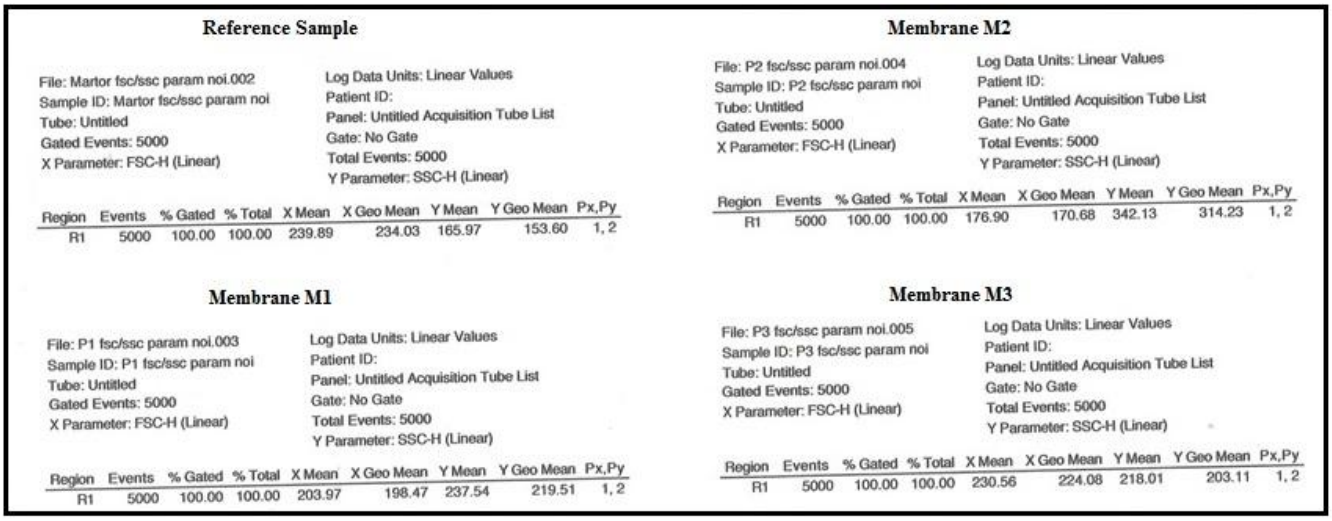


Fig. 2 XGeoMean and YGeoMean values graph



Reference Sample

File: Martor fsc/ssc param noi.002
Sample ID: Martor fsc/ssc param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: SSC-H (Linear)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	5000	100.00	100.00	239.89	234.03	165.97	153.60	1, 2

Membrane M2

File: P2 fsc/ssc param noi.004
Sample ID: P2 fsc/ssc param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: SSC-H (Linear)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	5000	100.00	100.00	176.90	170.68	342.13	314.23	1, 2

Membrane M1

File: P1 fsc/ssc param noi.003
Sample ID: P1 fsc/ssc param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: SSC-H (Linear)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	5000	100.00	100.00	203.97	198.47	237.54	219.51	1, 2

Membrane M3

File: P3 fsc/ssc param noi.005
Sample ID: P3 fsc/ssc param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FSC-H (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: SSC-H (Linear)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	5000	100.00	100.00	230.56	224.08	218.01	203.11	1, 2

Fig. 3 Statistical analysis of morphology changes

2) Discriminative analysis of viable fibroblasts, in apoptosis or death, with Annexin-V-FITC/Propidium Iodide

Comparing the % of Annexin V-FITC negative cells (LLquadrant) meaning the normal cells with that of the Annexin V-FITC positive cells (LR quadrant) representing cells in apoptosis and of Annexin V-FITC positive and Propidium Iodide positive cells (UR quadrant) that are the dead cells, it was found that:

- the percentage of viable cells closest to the reference sample (95%) is obtained by culturing fibroblasts on membrane M1, followed by fibroblasts cultured on membranes M3(88.5%) and M2 (76%), as seen in *Fig. 4* and the statistical analysis obtained by the quadrants technique (*Fig. 5*).

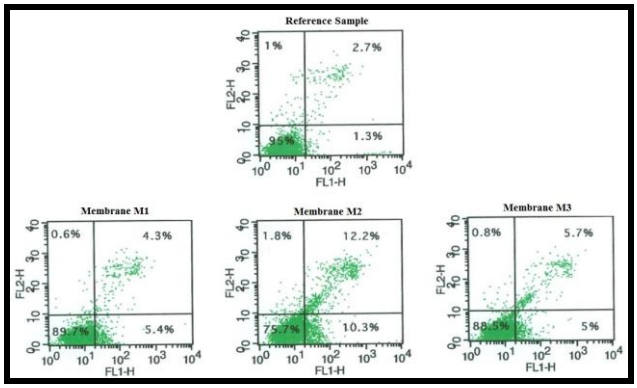


Fig.4 Discriminative analysis of viable fibroblasts, in apoptosis or death, with Annexin-V-FITC/Propidium Iodide

Reference Sample

File: Martor Ann/PI param noi.006
Sample ID: Martor Ann/PI param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)
Quad Location: 19, 9

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	48	0.96	0.96	8.96	7.69	93.57	35.95
UR	128	2.56	2.56	144.52	98.37	379.03	236.32
LL	4756	95.12	95.12	4.82	4.34	1.77	1.66
LR	68	1.36	1.36	426.93	62.69	2.14	1.75

Membrane M2

File: P2 Ann/PI param noi.008
Sample ID: P2 Ann/PI param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)
Quad Location: 19, 9

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	92	1.84	1.84	12.04	10.41	21.34	15.32
UR	611	12.22	12.22	199.93	109.09	157.13	76.00
LL	3787	75.74	75.74	7.87	6.98	2.39	2.11
LR	510	10.20	10.20	83.49	32.68	2.75	2.25

Membrane M1

File: P1 Ann/PI param noi.007
Sample ID: P1 Ann/PI param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)
Quad Location: 19, 9

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	30	0.60	0.60	11.16	9.65	36.43	19.98
UR	214	4.28	4.28	161.08	108.44	256.56	156.30
LL	4486	89.72	89.72	7.16	6.37	1.71	1.60
LR	270	5.40	5.40	58.16	30.82	2.15	1.82

Membrane M3

File: P3 Ann/PI param noi.009
Sample ID: P3 Ann/PI param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)
Quad Location: 19, 9

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000
Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	38	0.76	0.76	9.76	8.04	22.83	17.24
UR	287	5.74	5.74	219.97	114.28	143.16	70.33
LL	4423	88.46	88.46	7.13	6.38	2.02	1.85
LR	252	5.04	5.04	41.14	31.54	2.43	1.99

Fig. 5 Statistical analysis obtained by the quadrants technique of viable fibroblast, in apoptosis or death

3) Fibroblast viability estimated by measuring intracellular esterase activity with Calcein-AM

From samples statistical annex, the compared analysis of FL1 histograms (fluorescence intensity of calcein) for M1 regions (region of viable cells), both as a percentage and as MFI (Mean Fluorescence Intensity) showed that:

- there is no significant toxic effect on fibroblasts growing on the three types of tested membranes, and for fibroblasts grown on membrane M1 even a little higher percentage of viable cells is noticed compared to the reference sample (90, 89% from 90.48 %).

- also the other two studied membranes have a high percentage of viable cells, nearly identical to the reference sample, as shown in *Fig. 6 - 7*.

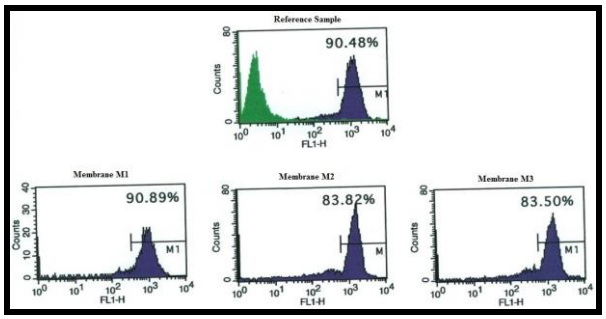


Fig.6 Highlighting cell viability with Calcein-AM in analysis system by FL1 histogram

Reference Sample

File: Martor calceina param noi.010
Sample ID: Martor calceina param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	5000	100.00	100.00	1068.76	856.65	45.49	1055.45	1218
M1	470, 9910	4524	90.48	90.48	1159.57	1090.69	35.78	1104.00	1218

Membrane M2

File: P2 calceina param noi.012
Sample ID: P2 calceina param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	5000	100.00	100.00	1219.82	860.81	49.73	1263.46	1
M1	610, 9910	4191	83.82	83.82	1415.40	1351.18	31.39	1369.99	1567

Membrane M1

File: P1calceina param noi.011
Sample ID: P1calceina param noi
Tube: Untitled
Gated Events: 1680
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 1680

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	1680	100.00	100.00	855.33	650.58	48.80	824.17	691
M1	340, 9910	1527	90.89	90.89	928.55	864.09	39.06	858.21	691

Membrane M3

File: P3 calceina param noi.013
Sample ID: P3 calceina param noi
Tube: Untitled
Gated Events: 5000
X Parameter: FL1-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: No Gate
Total Events: 5000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	5000	100.00	100.00	1139.88	884.09	50.01	1154.78	1420
M1	573, 9910	4175	83.50	83.50	1312.99	1244.61	34.28	1252.15	1420

Fig. 7 Statistical analysis of cell viability

The cell viability

In parallel with cytofluorimetric analysis, being one of the most used testing methods of biomaterials cytotoxicity, cell viability measure was also used.

These tests are based on the ability of viable cells to exclude dyes, while nonviable or affected cells lose this selective ability and incorporate dyes as trypan blue or Erythrosine B.

Factors affecting the dye exclusion are staining time and serum concentration, having significantly influence on tissue accuracy.

Vital staining analysis can be automated, since cells stained with trypan present an absorbance at 632 nm.

The usual trypan concentration is 0.4% and dye incorporation is influenced by pH value, the maximum value of pH being 7.5 for trypan and 7 for Erythrosine.

Cells forming monolayer should be well dispersed and homogenized. After adding the dye, the solution is again dispersed and the counting at hemocytometer of stained and unstained cells is done after 10 minutes.

The percentage of viability (% viability) is given by the ratio of unstained cells and the total number of cells. In order to take into account the differences that may occur in subsequent cell disintegration due to exposure to various substances, the "viability index " has been introduced.

To achieve "viability index", cell viability determined by dye exclusion is multiplied to the ratio of total number of intact cells in the tested sample and that existing in reference sample.

$$\text{viability index} = \%\ \text{viability}\ x\frac{\text{Sample intact cells}}{\text{Reference intact cells}}$$

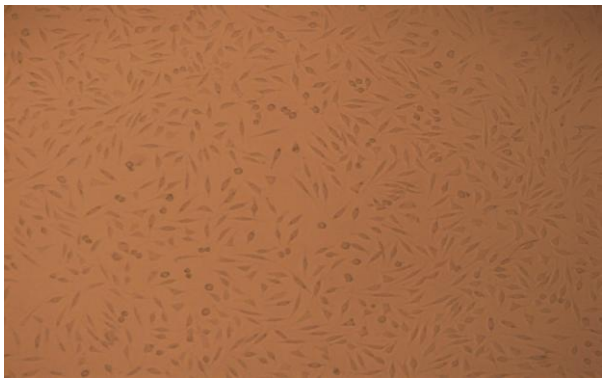


Fig. 8 Reference fibroblasts culture

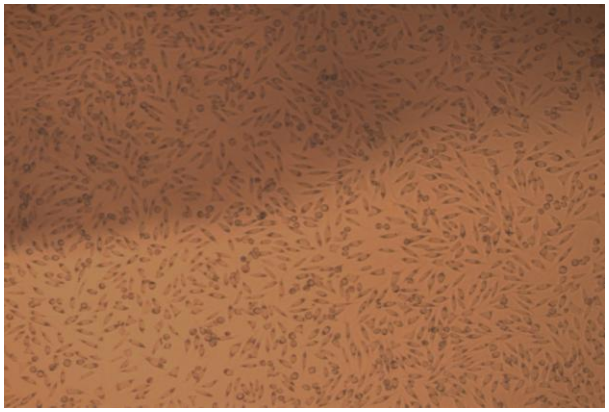


Fig. 9 Fibroblasts culture in the presence of M1

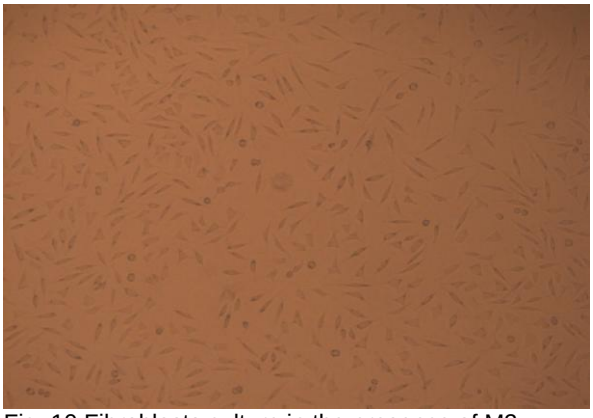


Fig. 10 Fibroblasts culture in the presence of M2

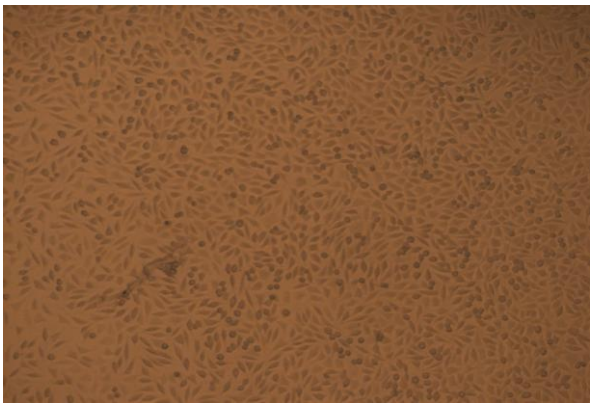


Fig. 11 Fi broblasts culture in the presence of M3

Calculation of cell viability showed that the reference sample (simple culture) exhibited 96.13% of viable cells, while fibroblast cultures in the presence of tested membranes have 95.53% viable cells for M1, 88.36% for M2, and 92.89% for M3.

CONCLUSIONS

The experimental data relates to a biocompatible composite based on chitosan, gelatine and enzyme (α - chymotrypsin), without antigenicity, used for the stimulation of dermal tissue regeneration.

Results provided by flow cytometry revealed that the cells did not exhibit phenomena signalling cytotoxicity, and there are very small differences in cell viability compared to reference sample. It can be concluded that synthetized biomaterials showed a good biocompatibility, without toxic influence to the cells and can be used for human use.

By the designed bioproduct, we have focused on the good wound healing properties, on the ability to heal wounds without scar formation of chitosan, and, on the other hand, the action of proteolytic enzyme that contributed to the removal of necrotic tissue, pus and other detritus and to the inhibition of some enzymes secreted by the germ to reduce the effect of antibiotics.

Functional qualities of obtained biomaterial, the absence of cytotoxicity, the possibilities of easy administration, compliance of compatibility between biopolymers and injured tissue, association with other biologically active substances, and aggregation

synergistic effects of the components recommend the use of this composite biomaterial.

Socially and economically, the new product can bring a number of clear advantages such as: low cost, shorten time for treatment and recovery without squeals of wounds, and a faster return of patients to active life.

REFERENCES

Campbell MK, Farrell SO, Biochemistry, 8th edition, Cengage learning, USA, ISBN-10: 1-285-42910-9, 2013.

Khor E, Lim LY, Implantable applications of chitin and chitosan. Biomaterials, 24, 2339–49, 2003.

Muzzarelli RAA, Mattioli-Belmonte M, Pugnaloni A, Biagini G, Biochemistry, histology and clinical uses of chitins and chitosans in wound healing. In: Jolles P, Muzzarelli RAA, eds. Chitin and chitinases. Basel: Birkhouser Verlag, Basel, 251–64,1999.

Rajendran S, Anand SC, Contribution of textiles to medical and healthcareproducts and developing innovative medical devices. Indian J. Fibre Text. Res., 31, 215–29, 2006.

Rigby AJ, Anand SC, Horrocks AR, Textile materials for medical and healthcareapplications. J. Text. Inst., 88, 83–93, 1997.

Silver FH, Berg RA, Doillon CJ, Weadock K, Whyne C, Biodegradable matrix and methods for producing same, United States Patent 4, 970, 298, 1990.

Usami Y, Minami S, Okamoto Y, Matsuhashi A, Shigemasa Y, Influence of chainlength of N-acetyl D-glucosamine and D-glucosamine residues on direct and complement mediated chemotactic activities of canine polymorphnuclear cells. Carbohydr. Polym., 32, 115–22, 1997.