The Polyelectrolyte Shells for Immobilization of Cells

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ABSTRACT

In this paper, the polyelectrolyte layered shells for immobilized cells systems produced for biomedical purposes are presented. The polyelectrolyte materials applied for multilayer forming are briefly described. Moreover, the methods of detection of layers and presentation of usability of multi-layered shells for immobilization of cells are described. The methods of cell detection on the polyelectrolyte shells are presented using human neuronal and fibroblastic cells as the model adherent cells. Furthermore, an example of the system allowing for regulated polarity of immobilized cells is demonstrated.

Keywords: Polyelectrolyte; Layer-by layer; Layered shell; Scaffold; Immobilized cells systems

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INTRODUCTION

The aim of this work is to give a brief overview of the polyelectrolyte layered shells for immobilized cells systems produced for biomedical purposes. The recent advances in the development of such systems is mentioned including example polyelectrolyte materials applied for multilayer forming, methods of detection of layers and presentation of usability of multilayered shells for immobilization of cells.

The methods of immobilization of cells within polymeric shells have been explored and developed due to their wide range of possible applications in biotechnology, and in cell-based transplantation therapies. However, the layer by layer (LbL) technique [1-6] has crucial role in forming of layered shell as an element of scaffold for cell immobilization.

It can be noted that for now, all approaches concerning application of LbL method for immobilization of cells are short-term.

The systems involving polyelectrolyte shells for biological processes regulation can be constructed involving different immobilized cells such as mouse mesenchymal stem cells, erythrocytes, yeast, Escherichia coli, Bacillus subtilis, dental pulp stem cells, neural cells, endothelial and fibroblastic cells [7-14].

POLYELECTROLYTE MULTILAYER SHELLS

Mechanism of self-assembly

The technique of layer-by-layer multilayers forming was designed in the first place for macroscopic surfaces [15], then was adapted for immobilization of cells. The layer-by-layer (LbL) self-assembly forming can be driven by different processes: electrostatic interaction between the oppositely charged constituents [16], hydrogen bonding [17-19], covalent bonding [20], hydrophobic interactions [20-22], van der Waals forces [23,24], and/or combined processes which can be engaged [22,25,26]. The forming of LbL mutlilayers with combined processes involvement can be driven by hydrophobic interaction and then reinforced by hydrogen interaction developing ultrathin membranes forming [22], also, electrostatic

interaction and hydrophobic interaction or electrostatic and hydrogen interactions [27] may take apart in self-assembly forming [28]. The hydrogen and van der Waals interactions are the forces that may competitively control the morphology of the single monolayer, and the self-assembled 2D nanostructure is determined by balance of these two interactions [26]. The example polyelectrolyte multilayers formed by combined processes are presented in **Table 1**.

PE layers	Process
Gelatin/Tannic acid (TA)	hydrophobic interaction/ hydrogen interaction
Chitosan-graft-phenyl/poly(aspartic-graft-octadecyl)	electrostatic interaction / hydrophobic interaction
O-carboxymethyl chitosan / alginate	electrostatic interaction / hydrogen interaction
Kahalalide F (KF) an anticancer hydrophobic peptide /alginate	electrostatic interaction / hydrophobic interaction
Alginate modified with protein A and fullerenol/polyethyleneimine	electrostatic interaction / hydrogen interaction

Table 1: Example PE multilayers formed by combined processes.

The most commonly applied electrostatic interaction between the charged constituents allows to deposite oppositely charged polyions onto charged substrats and consequitive layers producing of the polyelectrolyte (PE) multilayer shell [16,29].

Covalent bonding in LbL shell is mainly applied in special case of immobilization - cell encapsulation between the cell and the layer covering the cell and is performed by chemical or enzymatic conjugation of polymer to the cell [30-32]. Moreover, metabolic introduction can be applied. The amphiphilic polymers, e.g. temperature-sensitive amphiphilic polyelectrolyte succinylated pullulan-g-oligo(L-lactide) [33] are reported to ensure hydrophobic conjugation.

Experimental results indicate that the balance between ionic and hydrophobic interactions plays a leading role not only in the construction of the self-assembled system but also in the functional properties of the bioactive interface [34].

Polyelectrolytes

A great deal of attention is payed to investigation of PE gels and complexes for contact with cells [35]. The PE ionic groups enable to create the stable water dispersions where the ionized polymer chain provides the surface active center. Polyelectrolytes classified according to their origin encompass natural and synthetic polymers. The most common synthetic polyelectrolytes are poly (acrylic acid) (PAA), poly(methacrylic acid) (PMA), poly(allylamine) (PAH), poly(styrene sulfonate) (PSS), poly(ethyleneimine) (PEI), poly(vinyl sulfate) (PVS), poly (dimethyldiallylammonium chloride) (PDDA) and poly(N-isopropyl acrylamide (PNIPAM) [36-38]. The group of natural polyelectrolytes includes nucleic acids, proteins, polypeptides e g., poly-L-lysine (PLL), poly-L-glutamic acid (PGA), poly-L-arginine (ARG) and poly-L aspartic acid (ASP), poly-L-glutamic acid (PGA) and polysaccharides such as chitosan (CHIT), cellulose sulfate, carboxymethylcellulose, heparin, hialuronic acid (HA), dextran sulphate, and alginate (ALG) - still ubiquitous in encapsulation of cells [39-41].

The characteristic of the chosen basic natural polymers is shortly presented below

Alginate is a natural anionic polymer isolated from Azotobacter vinelandii, several Pseudomonas species, and algae [42,43]. The sort of utilized algs depends of geographical position where the algae are collected. The algae used in Norway it is Laminaria hyperborea and Ascophyllum nodosum, the algae used in USA it is mainly Macrocystis pyrifera. The chemical composition may slightly differ in dependence of the sort of algae.

Alginic acid is a linear block copolymer of 1,4′-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers in varying proportions combined with glycozyde bindings. The individual polymeric chains may involve the type MM segments or GG or MG connected with hydrogen bindings. The ratio of G and M blocks depends on the source of algae used for alginate extraction. ALG is a polyanion with pKa of about 3.2.

Alginate has a natural ability to create the stable gels in reaction with calcium salts. It's gelling is the effect of conglomeration of the segments of polyguluronic acid in clusters where calcium ions are scattered in the space between the chains ("egg-box model"). Alginates exhibit the higher affinity of the guluronic acid residues for

divalent ions [44] making the alginates with high rate of guluronic acid more stable.

Except of the Ca^{2+} another solutions containing divalent cations, such as Sr^{2+}, or Ba^{2+} can be applied as a crosslinking solution for contact with cells. Alginate's affinity for different divalent ions decreases as follows: $Pb^{2+} > Cu^{2+} > Cd^{2+} > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+}$, Ni^{2+}, $Zn^{2+} > Mn^{2+}$ [45]. A higher affinity of the cation for the alginate residues is associated with a stronger gel [46]. Nevertheless, these cations, however able to crosslink alginate gels are reported to be toxic to the cells [47].

The largest issues with alginate-based systems to date have been purity influencing biocompatibility and stability [48,49]. Alginates are produced by extraction from the algae by EDTA chelation, physical sieving, therefore precipitation with ethanol and KCl, and finally lyophilization. High quality alginates are prepared from algal stipes collected directly from the sea, peeled and treated with antimicrobial agents to reduce the cytotoxic gram-positive debris. Lower-grade alginates are produced from algae which are washed ashore and have significantly higher contamination share, hard to remove from the processed product [50].

Some other basic natural PE of polisaccharides origin involved in cell immobilization can be mentioned like hialuronic acid, chitosan, dextran [51-81].

Hialuronic acid (HA) is a linear non- branched polysaccharide from the group of proteoglycanes. It is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine bound alternate with glycoside bindings in position β-1-3 and β-1-4. The hialuronic acid is a part of extracellular matrix of connective tissue, epithelial, and neural tissues, it can be found in vitreous humour (in the space between the lens and the retina of the eyeball), in skin and umbilical cord. HA is an anionic polysaccharide, and the pKa of its COOH groups is 3-4; thus, at physiological conditions these moieties are ionized. HA is a highly hydrophilic polymer with ability proportional to the polymer molecular weight to bind and adsorb water due to ability of creating the hydrogen bindings of the carboxylic groups and N-acetylic groups of hialuronic acid with solutes of water. HA adsorbing water allows to expand up to 1000 times its solid volume, forming a loose hydrated network.

Another basic natural PE- Chitosan refers to a series of polymers that are deacetylated derivatives of the natural polysaccharide, chitin obtained from the exoskeleton of arthropods as well as from some mushrooms. It is a linear polycation composed of β-1,4-linked glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units) with different degrees of deacetylation (typically between 70% and 95%). The proportions of N-acetyl-D-glucosamine and D-glucosamine residues, provide specific structural changes. The chitosan biodegradability is affected by DD as well as by distribution of acetyl groups [56,57]. Chitosan is positively charged in strong acid conditions. It is characterized with a pKa of about 6.0, thus at pH less than about 6, chitosan's amine groups are protonated providing polycationic behavior to chitosan as well as chitosan aquous solubility. The control of chitin and chitosan-based devices rate of degradation is of great interest since degradation is crutial in tissue regeneration applications and in biologically active molecules release applications.

Finally, another natural PE, polylysine can be mentioned. Poly-L-lysine is an amino acid polymer with approximately one hydrobromide per lysine residue that allows the PLL to be in a crystalline form soluble in water. PLL is a positively charged linear polyelectrolyte characterized with pKa of about 10.5. It is widely used as an attachment factor for cells, promoting cell adhesion by enhancing electrostatic interaction with negatively charged cell membrane.

IMMOBILIZATION OF CELLS WITHIN MULTILAYER SHELLS

Polyelectrolyte Multilayer Scaffolds Shells for Cell Maintenance

Polyelectrolyte shells receive a great deal of attention as the constracts for scaffolds mimicking the extracellular matrix (ECM), supporting cell functioning, adhesion, proliferation, and differentiation for regulation factors delivery and/or new tissue development [82,83].

Immobilization of cells within polyelectrolyte shells can provide a desired concentration of biological material in optimal space; enable directional cell growth for applications in biological processes regulation.

The application of PE shells for scaffolding systems can be dedicated for different cells. E.g. the exploration of PE as the substrats for an optimum surface for exogenous primary neuronal cell growth has been performed [84,85]. Lastly it was reported that the ALG/PLL bilayer scaffolds allowed for survival of rat neurons in culture for over 2 weeks what can find application in controllable regeneration of neural networks in nanomedicine [14]. The NIH-3T3 fibroblasts attachment was reported to be improved due to application as shell material the photocrosslinked hyaluronic acid (HA) hydrogels modified with poly(L-lysine) (PLL) and HA multilayer films made by the layer-by-layer (LbL) technique [86]. Some authors examined interactions between layered shells built of poly-(allylamine hydrochloride) (PAH) and poly-(acrylic acid) (PAA) and the murine fibroblast NIH/3T3 cell line [87]. Silva et al. immobilized human umbilical vein endothelial cells (HUVECs) within polielectrolyte multilayers build of chitosan and alginate and cultured for 24 hours [88].

It can be noted that among different scaffolding materials, polymeric hydrogels characterized with biocompatibility and structural similarity to the tissue play an important role [89]. Unfortunately, hydrogel scaffolds are characterized by weak mechanical strength and lack of biological activity. Thus investigations have been performed on the materials with improved physicochemical properties and / or bioactive features. Recently, a great deal of attention has been paied to incorporation of metallic nanoparticles within biomaterials. It allows to produce nanocomposites with improved mechanical strength involving additional biological features like antibacterial and antiviral activity [90-92]. Some examples of the application of PE shells with additional features for scaffolding systems can be listed. E.g. the poly(hydroxyethyl methacrylate) hydrogel with incorporated AgNPs cytocompatible to mouse embryotic fibroblasts proved anti-bacterial function toward Staphylococcus aureus and Escherichia coli. Moreover, the experiments in vivo showed that the designed material ensured protection to immunological answer [93]. Another based on hydroxyethyl scaffold, hydroxyethyl cellulose with incorporated AgNPs was reported to maintain the growth and proliferation of human fibroblasts [94].

It can be mentioned that besides metallic NPs some other materials may be engaged. e.g. hydroxyapatite (HAP), an inorganic mineral constituting the scaffolding of connective tissue, which is primarily a component of bone tissue, enamel, cementum and dentin [95-97].

The analysis of interaction with eukaryotic and prokaryotic cells of PE shell with layers built on the basis of polyethyleneimine (PEI) and hydroxyapatite indicated the bacteriostatic influence of the PEI-based layer modified with AgNPs but also unmodified (PEI or PEI-Ag layer) in contact with microorganisms, in particular, B. subtilis, on the other hand, indicated that the hydroxyapatite layer is advantageous for contact with eukaryotic cells [98].

Another example is agarose hydrogel with metallic NPs which can serve for cells maintenance with additional features. The agarose hydrogel with incorporated AgNPs coated with chitosan having anti-bacterial activity was reported to maintain the growth of HeLa, MiaPaCa2, and HEK cells for five or 16 days [99].

The collagen based shells are also reported to fulfill the requirements for immobilization of cells. The collagen hydrogel composites with peptide-modified AuNPs and AgNPs were reported to increase proliferation, and the level of connexin-43 under electrical stimulation of rat neonatal cardiomyocytes [100]. Alginate and collagen I composite layered shell involving AgNPs and/or heparineto insert additional biological feature - antithrombotic activity showed maintainance of fibroblastic cells immobilized within the shell during seven day culture [101].

Detection of Multilayered Shells

The recognition of the individual layers presence within the multilayered shell may be performed using different methods.

The analysis of the PE layers can be realized utilizing the microscopic and spectroscopic techniques, and flow cytometry (in case of cell encapsulation within tagged layered shell) supported with biological as well as physico-chemical methods.

The build-up of the individual layers on the support may be confirmed directly using potential Zeta measurements [102,103]. Also, the FTIR, UV-vis spectroscopy can be applied to evidence the nano-thin shells presence allowing to detect the functional groups presence indicating the interaction between the layers. Application of the fluorescently-tagged material to build up the multilayered shell gives opportunity to recognize the shell using the fluorescence microscopy and confocal microscopy [104,105].

The mechanical properties of a shell may be assessed using atomic force microscopy (AFM) technique. Thickness of the layers, Young modulus of the shells and the work of adhesion between the individual layers of a shell can be evaluated, giving the evidence for interaction between the shells in dependence of the material properties [10,106]. However assessment of the mechanical properties of the layers requires AFM technique scanning electron microscopy (SEM) is an efficient tool allowing for analysis of the morphology of the systems layer-immobilized cells. Figure 1 presents an example morphology of PE layers built on the basis of alginate with incorporated hydroxyapatite NPs (Figure 1 B5-B8) or chitosan (Figure 1 C5-C8; D5-D8) with incorporated hydroxyapatite NPs at different proportion. It can be observed that the diminished share of chitosan in proportion with HAP disturbs the integrity of a layer.

Visualization of morphology of immobilized cells and shell-immobilized cells systems

The recognition of the individual cells presence within the shell may be performed using microscopic techniques like light microscopy, fluorescence microscopy, confocal microscopy, skaning electron microscopy.

SEM analysis allowing to examine morphology of the immobilized cell-layered shell systems is presented.

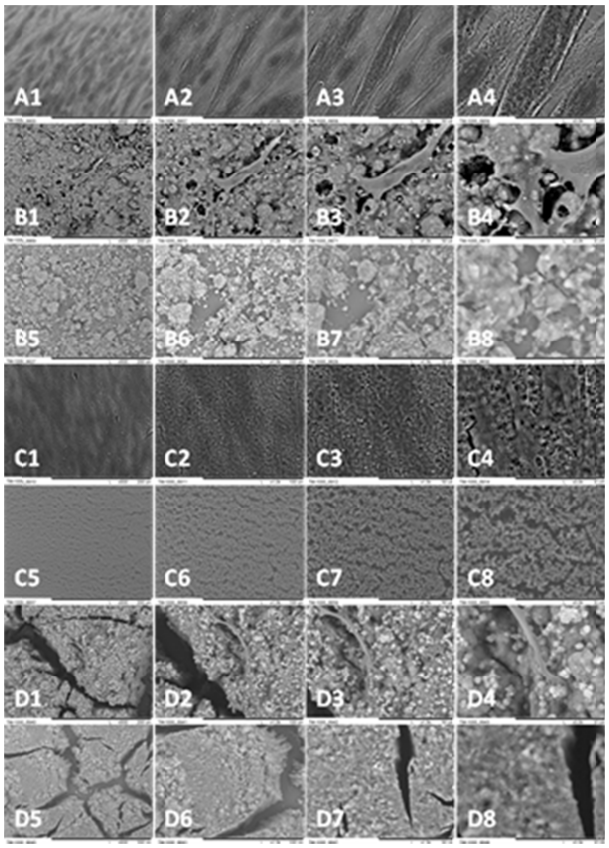

Figure 1: SEM image of alginate-hydroxyapatite or chitosan-hydroxyapatite layered shells; human fibroblasts control or immobilized on alginate-hydroxyapatite or chitosan-hydroxyapatite layered shells after 7-day culture. Magnification from x500 to x3000. A1-A4 - control, B1-B4 - cells immobilized on alginate-hydroxyapatite layer (HAP_ALG 100); B5-B8 alginate-hydroxyapatite layer (HAP_ALG 100), C1-C4 - cells immobilized on chitosan-hydroxyapatite layer (HAP_CHIT 100); C5-C8 - chitosan-hydroxyapatite layer (HAP_CHIT 100); D1-D4 - cells immobilized on chitosan-hydroxyapatite layer (HAP_CHIT 10); D5-D8 - chitosan-hydroxyapatite layer (HAP_CHIT 10). Indexes 1,5 - magnification 500; 2, 6 - magnification 1000; 3, 7 - magnification 1500; 4, 8 - magnification 3000.

Visualization of immobilized neuronal cells

For visualization of the functional structure of neural cells within the scaffolds, the different dyes allowing distinguishing neurons and astrocytes are applied. The Cells are double-stained with anti-microtubule-associated protein 2 (MAP2) that identifies the majority of the proteins of the neuronal skeleton, and anti-glial fibrillary acidic protein (GFAP). Conjugation of the proteins with fluorochromes respectively Alexa Fluor 555 and Alexa Fluor 488 allows to distinguish the objects using fluorescence microscopy. Figures 2 and 3 shows rat neural cells immobilized within the scaffolding bilayer shells respectively alginate/chitosan (ALG/CHIT) or alginate/poly-L-lysine (ALG/PLL) cultured (5% CO_2, 37°C) two weeks in culture medium

(Neurobasal medium supplemented with B27, 0.5 mM glutamine, 12.5 mM glutamate, and a penicillin /streptomycin mixture for first 6 days, then supplemented with B27, 0.5 mM glutamine and a penicillin /streptomycin mixture).

The scaffolds ALG/CHIT; ALG/PLL were built of the basic layer (ALG) of negative charge of potential Zeta. The applied bilayers allowed for glial cells growth, supporting the neurons growth during 2-week culture.

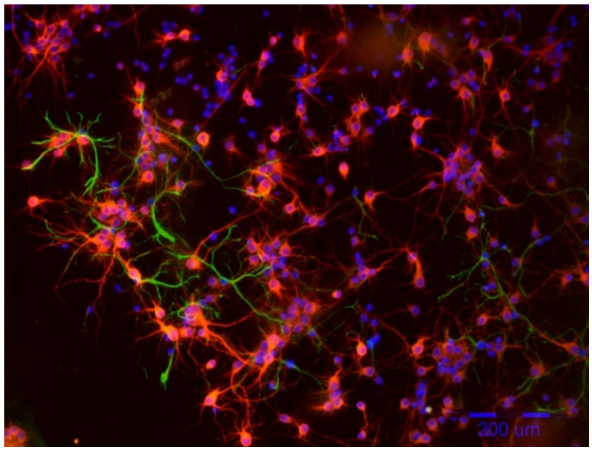

Figure 2: The visualization of neural cells immobilized within bilayered ALG/CHIT shell after 2-week culture. The red fluorescence of Alexa Fluor 555 dye exhibiting neurons presence may be observed, small amount of astrocytes exhibiting green fluorescence can be distinguished. Nuclei of neural cells are dyed in blue with DAPI.

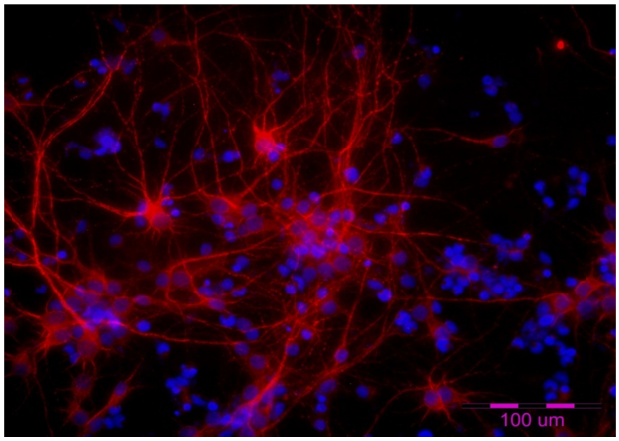

Figure 3: The visualization of neural cells immobilized within bilayered ALG/PLL shell after 2-week culture. The red fluorescence of MAP 2 dye exhibiting neurons presence may be observed, the blue neurons nuclei can be distinguished among the neurons net. Nuclei of neural cells are dyed in blue with DAPI.

Visualization of immobilized fibroblastic cells

Another approach for visualization of the functional structure of the cells is application of phalloidin and 4',6-diamidino-2-phenylindole (DAPI) nuclear stain.

Phalloidin is a toxin isolated from the fungus - Amanita phalloides, which binds directly to filamentous actin (F-actin), which is abundant in fibroblasts. The DAPI-fluorochrome solution specifically staining DNA when added to the cells induce blue fluorescence emittance of cell nuclei under UV light. Blue DAPI fluorescence and red phalloidin fluorescence can be observed at $\lambda = 460 \div 500$ nm and $\lambda = 570$ nm respectively.

There are some examples of visualization of functional structure of immobilized cells using fluorescence microscopy as well as morphology of the system layer-immobilized cells using SEM. Figures 1,4 and 5 show human dermal fibroblasts (HDF) immobilized within the scaffolding layers based on alginate or chitosan with incorporated HAP NPs after one week culture (5% CO_2, 37ºC).

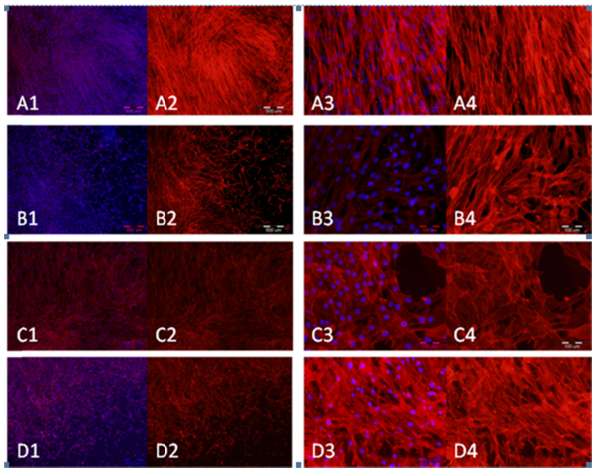

Figure 4: Fluorescence microscopic image of human fibroblastic cells control (immobilized directly on a glass support) or immobilized on alginate-hydroxyapatite or chitosan-hydroxyapatite layers after 7-day culture. (A) control: A1 - cells stained with DAPI (blue) and phalloidin (red), objective magnification 4x; A2 - cells stained with phalloidin, 4x objective magnification; A3 - cells stained with DAPI and phalloidin, 20x objective magnification; A4 - cells stained with phalloidin, 20x objective magnification; (B) cells immobilized on alginate-hydroxyapatite layer (HAP_ALG 100): B1 - cells stained with DAPI (blue) and phalloidin (red), 4x objective magnification; B2 - cells stained with phalloidin, 4x objective magnification; B3 - cells stained with DAPI and phalloidin, 20x objective magnification; B4 - cells stained with phalloidin, 20x objective magnification; (C) cells immobilized in chitosan-hydroxyapatite layer (1: 100) (HAP_CHIT 100): C1 - cells stained with DAPI (blue) and with phalloidin (red), 4x objective magnification; C2 - cells stained with phalloidin, 4x objective magnification; C3 - cells stained with DAPI and phalloidin, 20x objective magnification; C4 - cells stained with phalloidin, 20x objective magnification; (D) cells immobilized in chitosan-hydroxyapatite layer (1:10) (HAP_CHIT 10): D1 - cells stained with DAPI (blue) and with phalloidin (red), 4x objective magnification; D2 - cells stained with phalloidin, 4x objective magnification; D3 - cells stained with DAPI and phalloidin, 20x objective magnification; D4 - cells stained with phalloidin, 20x objective magnification.

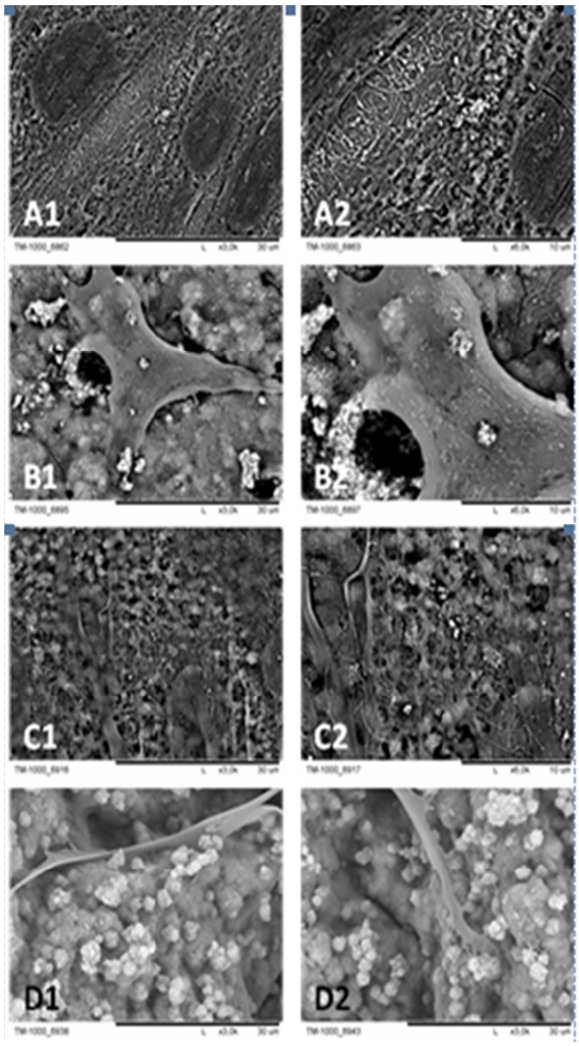

Figure 5: SEM image of human fibroblasts control or immobilized on alginate-hydroxyapatite or chitosan-hydroxyapatite layered shells after 7-day culture; alginate-hydroxyapatite or chitosan-hydroxyapatite layered shells. x3000 (left column), x6000 (right column). A1-A2 - control, B1-B2 - cells immobilized on alginate-hydroxyapatite layer (HAP_ALG 100); C1-C2 - cells immobilized on chitosan-hydroxyapatite layer (HAP_CHIT 100); D1-D2 - cells immobilized on chitosan-hydroxyapatite layer (HAP_CHIT 10).

For producing the shells a 30% aqueous solution of hydoxyapatite and solutions of natural polyelectrolytes-2.5% acetic acid) were used. The shells were made of a alginate (0.05% in 0.9% NaCl) and chitosan (0.1% in mixture of a hydroxyapatite solution with an alginate solution in the ratio 1: 100 (HAP_ALG100 layer) or a hydroxyapatite solution with a chitosan solution in the ratio 1: 100 (HAP_CHIT100 layer) or in a ratio of 1:10 (HAP_CHIT10 layer). The solutions were deposited on the coverslips and dried at room temperature for 24 hours. After one week, the cultures were analysed usimng fluorescence microscope or SEM.

Visualization of morphology of shell-immobilized cells systems

SEM analysis allows to examine morphology of the system of cells immobilized within shell. Figure 1 shows SEM image of human fibroblasts control (immobilized directly on a glass support) or immobilized on alginate-hydroxyapatite or chitosan-hydroxyapatite layers after 7-day culture. Figure 5 is a derivative of Figure 1, showing the control or immobilized on the shells cells at x3000 and x6000 magnification.

The SEM image in Figure 1 A1-A4 (control) shows the spindle-shaped characteristic form of fibroblastoid cells. The cells obtain generally a spherical shape on the membranes, which can be seen in Fig. 1 B1-B4, C1-C4, D1-D4 as well as Figure 5 B1-B2, C1-C2, D1-D2.

It can be noted that the HAP_CHIT10 layer ensures higher cell growth comparing with HAP_CHIT100 layer. However, the layer HAP_CHIT10 characterized by impaired integrity.

The fluorescence microscopic observations revealed morphological differences between the control (cells grown on coverslips) and the cells grown on the prepared scaffolding shells (Figure 4). In the case of control, the fibrillar forms of actin that make up the fibers are arranged in one direction, and cell growth is intense (Figure 4 A1, A2, A3, A4). In the case of the HAP_ALG 100 scaffold, the cell growth is slightly weaker, individual cell nuclei can be distinguished (Figure 4 B1, B3). The direction of the fibers arrangement is generally uniform (Figure 4 B4). When HAP_CHIT 100 is used, the structure becomes three-dimensional, polarized in many directions (Figure 4 C1, C2, C3, C4). Reducing the proportion of chitosan in the HAP_CHIT10 scaffold weakens this effect, allowing to obtain a three-dimensional structure, but more ordered, polarized in one direction (Figure 4 D3), showing stronger cell growth comparing with control and the other shells. The rapid growth of cells is confirmed by the SEM microphotograph (Figure 1 D3, D4 and Figure 5 D1, D2) showing the characteristic clusters formed by proliferating cells. Regulated polarity of cell growth can be an important element in scaffold technology for controlled cell growth.

CONCLUSIONS

Polyelectrolyte shells as elements of the scaffolds for immobilization of cells give ability to adjust cells Interaction with material at material-host interface for tissue engineering purposes. It can be noted that layered shells combine some problems with material stability. The PE materials may undergo deterioration by endocytosis. Also, dissociation to the surrounding medium or body fluids may occured. Thus, only the temporary usability of the systems may be considered.

It can be assumed that the future work on the systems of layered shells with immobilized cells will concentrate on functional materials to improve effectiveness of cell function maintaince and to involve biological activity as well as novel cell types.

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