CONSTRUCTION OF BIOFUNCTIONAL NANOLAYER ON THE SURFACE OF SCAFFOLDS FOR BONE TISSUE ENGINEERING

Victor KORZHIKOV\textsuperscript{1,2}, Ilia AVERIANOV\textsuperscript{1}, Tatiana TENNIKOVA\textsuperscript{1,2}

\textsuperscript{1} – Institute of Macromolecular Compounds of Russian Academy of Sciences, 199004, St. Petersburg, Bolshoi pr. 31, Russia
\textsuperscript{2} – St. Petersburg State University, Department of Chemistry, 198504, St. Petersburg, Universitetskii pr. 26, Russia
v_korzhikov@mail.ru

Abstract

Incorporation of special biomolecules into the surface of biomaterials in order to control cell-material interactions is of importance for many biomedical applications, bone tissue engineering being one of a number. The authors of this paper propose the strategy for biomaterials biofunctionalization based on application of biocompatible hydrophilic polymers. In the current study the possibility to obtain the nanolayers of hydrophilic polymers on the ceramic and PLA-based scaffolds for bone tissue engineering is demonstrated. For that the physical adsorption techniques as well as covalent reactions were used. The ability of such hydrophilic polymers to couple biomolecules of different origin was proved.

Keywords: biofunctional, nanolayer, hydrophilic polymers, scaffold

1. INTRODUCTION

Surface biofunctionalization, which is implied as attachment of biologically active structures into the material in order to govern its interaction with cells and to control cell growth and function, is of importance for application of many biomaterials \cite{1, 2}, especially for construction of scaffolds for bone tissue engineering \cite{3, 4}. In the latter case the introduction of adhesion motifs, such as RGD-peptides \cite{5, 6}, as well as of growth and differentiation factors \cite{5, 7} is needed for induction of tissue regeneration process.

A number of studies were performed in order to introduce reactive groups directly into the surface of materials \cite{5, 8}. In most cases such approaches allowed only one biomolecules type immobilization \cite{8, 9, 10}. Nevertheless it is very promising to combine several biomolecules type on the scaffold surface in order to have more control over tissue regeneration process.

We propose the strategy for construction of “smart” biofunctional surface which is based on the utilization of hydrophilic polymers bearing a number of reactive groups. Such polymers could be modified by a variety of biomolecules of interest resulting in construction of so-called multifunctional polymer vector. This macromolecular conjugate should be immobilized on the material surface in order to interact with cells.

In this paper the attempt is made to describe how the strategy proposed could be applied for construction of biofunctional nanolayers on the ceramic materials as well as on the hydrophobic surface of poly(lactic acid) (PLA) based scaffolds.

2. EXPERIMENTAL PART

2.1 Materials and instruments

All buffer salts, monomers, biomolecules and other reagents were purchased from Fluka (Buchs, Switzerland) and Sigma (Taufenkiren, Germany). For dialysis and separation of nonreacted ligands from polymer conjugates the spin-columns (VIVASCIENCE, Sartorius Group, Germany) were utilized. For adsorption studies, the macroporous monolithic Sponceram (Sp) (doped ZrO2 ceramic material; pore size 600 μm; surface area ~1.4 m2/g) kindly donated by Zellwerk GmbH (Germany) was used.
The 1H NMR spectra were recorded with Brucker AVANCE-400 instrument. For UV–vis and fluorescence measurements, the UV mini-1240 spectrophotometer (Shimadzu, Japan) and Multiscan Fluorimeter FLUOROSCAN ASCENT (Thermo Electron Corp., UK) were used, correspondingly. The Shimadzu liquid chromatogram consisted of LC-10AD pump, RID-10A refractometric detector and Waters Styragel HMW 6E analytical column was applied for GPC analysis.

2.2 Methods

2.2.1 The synthesis of polymers. The polymerization of 2-deoxy-N-methacrylamidoglucose (MAG) and its copolymerization with O-laurylmethacrylate (LMA) was performed via free-radical reaction according to the previously published procedures [11]. The polymer products yields were 95-97 %. The MWs were estimated to be 20 kDa. In order to introduce the controllable amount of aldehyde groups the synthesized polymers were oxidized with sodium periodate as described earlier [11].

The synthesis of poly-D,L-lactic acid (PLA) for biodegradable matrices formation was performed via typical ring-opening polymerization procedure [12]. The reaction was conducted in the bulk of D,L-lactide in the presence of stannous octoate. The polymer yield was 87 %.

2.2.2 Formation of macroporous matrices via thermally induced phase separation (TIPS) technique. In a glass tube 1 wt.% solution of a mixture of PLA in 1,4-dioxane containing 10 wt.% of water was prepared. The mixture was heated up to 65°C and sustained during 2 hours. After that it was slowly cooled down up to occurrence of liquid-liquid phase separation and quenched in liquid nitrogen. The frozen solvent was removed by freeze-drying, leaving the porous matrix. The pore size and structure of the latter were analyzed by SEM.

2.2.3. Adsorption Studies. The study of adsorption was performed at static conditions. All experiments were carried out via immersion of pre-weighted matrix pieces into solution of polymers in 0.01 M sodium phosphate buffer, pH 7.0. The adsorption proceeded at 25 °C and slight stirring (300 rpm).

2.2.4. PLA-based matrix aminolysis. To the 50 mg of PLA-based matrix the 1.5 ml of ethylenediamine solution in 0.01 M NaOH was added. The reaction proceeded at slight stirring for 1.5 h. Then the matrix was isolated, washed with water and ethanol, and finally dried at room temperature. The quantity of amino groups introduced was determined by dissolution of pre-weighted piece of matrix in chloroform and subsequent analytical reaction with 1-fluoro-2,4-dinitrobenzene. The adduct quantity was UV/Vis measured.

2.2.5. Polymers modification. The construction of multifunctional polymer conjugate containing RNase, pLL, and RGD-peptide was performed via “step-by-step” addition of ligands to the polymer solution and using the quantitative methods described above. All reactions were carried out in 0.01 M sodium borate buffer, pH 10.0.

2.2.6. Cell adhesion. The analysis of the adhesion enhancing potential of the conjugates was carried out in the Institute of Technical Chemistry of University of Hannover (group of Prof. Cornelia Kasper) [13]. MC3T3-E1 cells were used. After 1, 4 and 24 hours of cultivation the cells were fixed with ice-cold ethanol for at least 20 min. After that they were incubated in DAPI solution for 15 min at 37°C. The fluorescence signal was measured after washing twice with PBS in the fluorescence reader at 460/360 nm.

3. RESULTS AND DISCUSSION

The proposed by authors scaffolds biofunctionalization strategy (Fig. 1) consists in covering of scaffold surface with hydrophilic polymer which was preliminary coupled with special biomolecules: non-specific adhesion motifs (like polylysine) [4], specific adhesion factor – RGD-peptide [10] and growth factor [9]. The role of the latter molecules is to give the signal for the progenitor cells to attach to the surface as well as to initiate their growth and differentiation into specific cells to form a new specialized tissue. These processes are of crucial importance in the case of scaffolds for bone tissue regeneration.

In the current paper we are trying to show that such strategy could be applied both for inorganic and polyester-based scaffolding materials.
3.1 Polymers synthesis and macroporous matrices fabrication

3.1.1 Hydrophilic polymer synthesis. For the proposed strategy realization the choice of appropriate hydrophilic polymer as well as of biomolecules covalent coupling chemistry is of crucial importance. In that case the use of a new type of macromolecules known as polyvinyl saccharides (PVS) [11, 14], which are carbon backbone polymers containing carbohydrate residues as side groups, looks very promising.

The presence of saccharide groups in such polymers gives the possibility of forming a structure that may induce a significant increase of specific interactions of cells with a scaffold surface [13]. As the reactions of biomolecules covalent coupling should proceed at mild conditions without yielding toxic byproducts and considering the fact that proteins and peptides contain amino groups, aldehyde chemistry was chosen for this purpose. In the current study, the polymers based on 2-deoxy-\textit{N}-methacrylamido-D-glucose (MAG, Fig. 2) with a controllable amount of aldehyde groups were obtained similar to earlier published procedure [11]. The copolymer of MAG with \textit{O}-laurylmethacrylate (pMAG-LMA, Fig. 2) was synthesized in order to enhance the affinity of the polymer to the surface of hydrophobic PLA-based matrix.

The content of aldehyde groups in obtained polymers was evaluated via reaction with Schiff reagent. The samples of oxidized pMAG contained 20 and 30 mol\% [CHO], while oxidized pMAG-LMA possess 7 and 17 mol\% [CHO]. The LMA content in pMAG-LMA copolymer was estimated via integration of 1H NMR signals, corresponding to terminal methyl group of lauryl residue (0.84 ppm), and was found to be about 11 mol\%.

3.1.2 Macroporous matrices. In this study commercial ceramic support Sponceram® as well as PLA based macroporous matrix were tested as supporting matrices. The biodegradable matrix was obtained via thermally induced phase separation (TIPS) of PLA solution in dioxan-water (d/w=9/1) system (Fig. 3). PLA with molecular weight 72 000 (GPC) was synthesized via ring-opening polymerization of D,L-lactide in the presence of stannous octoate.

The morphologies of macroporous matrices utilized in this study are presented on Fig. 4. One can see, that Sponceram possess larger pores than PLA-based material. Moreover it should be noted that Sponceram...
surface has slightly negative charge due to presence of phosphate groups of hydroxyl apatite, while PLA-based matrices have no charge and quite hydrophobic.

**Fig. 3** Fabrication of macroporous matrices via thermally induced phase separation.

**Fig. 4** Macroporous matrices used as scaffolding materials: A - Sponceram® (Zellwerk, average pore size determined by SEM – 600 μm); B – PLA-based matrix (average pore size determined by SEM – 25 μm).

### 3.2 Attachment of hydrophilic polymers to the surface: adsorption and chemisorption

Firstly, the construction of biofunctional nanolayer was intended to be formed via physical adsorption of oxidized pMAG and pMAG-LMA on the Sponceram and PLA-based matrix, correspondingly. Nevertheless it was shown, that while in the case of oxidized pMAG/Sponceram system the interaction is quite strong (**Fig. 5**) the adsorption of oxidized pMAG-LMA on PLA-based matrix is insignificant.

**Fig. 5** Adsorption isotherms for oxidized pMAG and pMAG-LMA on the Sponceram and PLA-based matrix, respectively. The adsorption time corresponding to the plateau on the kinetic curve was previously determined and was found to be 2 hours for oxidized pMAG/Sponceram system and 8 hours in the case of oxidized pMAG-LMA/PLA-based matrix. The experiment was performed in 0.01 M sodium phosphate solution, pH 7.0, at 25 °C and at slight stirring, 300 rpm.

Therefore the attempt was undertaken to create the covalent link between oxidized pMAG-LMA and the PLA surface. For that the PLA-based matrix was treated with ethylenediamine, leading to the aminolysis of the polyester molecules located on the surface of the matrix (**Fig. 6**, A). The amount of introduced primary amine groups was evaluated via analytical reaction with 1-fluoro-2,4-dinitrobenzene and was found to be 6 mmol/g. The capacity of activated PLA-based matrix regarding oxidized pMAG-LMA was determined from chemosorption isotherm (**Fig. 6**, B). The calculated value $Q_{\text{max}} = 79.1 \text{ mg/g}$ is five times higher than in the case
of adsorption of oxidized pMAG on the Sponceram surface \( (Q_{\text{max}} = 15.9 \text{ mg/g}) \). Thus it might be concluded that strategy realization depends on the material properties. Nevertheless it is obvious that formation of hydrophilic nanolayers is possible via physical adsorption or by covalent reaction.

Fig. 6 Covalent attachment of oxidized pMAG-LMA: A - scheme of PLA-based matrix surface aminolysis and subsequent polymer coupling; B – chemosorption isotherm.

### 3.3 Biomolecules coupling, adsorption and first cell culture experiments

On the next step of our work we have studied the quantitative parameters of biomolecules coupling process. The Ribonuclease A (RNase, physical model of growth factor BMP-2), polylysine and GRGDSP-peptide were step-by-step coupled with the corresponding hydrophilic polymers. In order to evaluate the amount of ligands attached they were preliminary bound with fluorescent labels. The data obtained are presented in Table 1.

**Table 1** Quantitative parameters of biomolecules coupling process during step-by-step construction of multifunctional polymer vector.

<table>
<thead>
<tr>
<th>Initial aldehyde groups concentration, μmol</th>
<th>Amount of attached ligand, μmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.43</td>
<td>oxidized pMAG</td>
</tr>
<tr>
<td>0.67</td>
<td>oxidized pMAG-LMA</td>
</tr>
</tbody>
</table>

Figure 7. Construction and effect of multibiofunctional surface: A – adsorption of pMAG-based multifunctional macromolecular conjugate on Sponceram; B – cell adhesion on Sponceram (DAPI staining).

One can observe that coupling of biomolecules occur on each stage, resulting in formation of so-called multifunctional polymer vector. The important finding of this work was that the number of reactive functional groups introduced on the polymer synthesis stage determines the amount and number of biomolecules that could be coupled to the polymer. This is the straight way from controlled chemistry to controlled biological properties. It appears that such strategy works like connecting the details in „molecular meccano“.

The obtained multifunctional conjugate was successfully adsorbed on the Sponceram surface (Fig. 7, A). It was also estimated that 34 mg of such conjugate could be coupled to the 1 g of aminated PLA-based matrix. The
first cell culture experiments (Fig. 7, B) allow one to observe that the adsorbed conjugates can distinctly affect the osteoblast progenitor cell adhesion process. Moreover, the synergic effect was observed when both polylysine and GRGDSP-peptide were introduced into biofunctional nanolayer.

CONCLUSION

The strategy of construction of biofunctional nanolayers as based on application of hydrophilic polyvinylsaccharides was realized. It was shown that both adsorption and chemisorption could be utilized for polymer attachment to the surface, depending on scaffolding material type. The hydrophilic macromolecules were modified by different biomolecules and first cell culture experiments indicated the fact that proposed nanolayers could benefit the scaffolds for bone tissue regeneration.

ACKNOWLEDGEMENTS

The work was financially supported by Russian Ministry of Education and Science (Programm: “Scientific and Scientific-Pedagogical Personnel of the Innovative Russia”, Agreement #8471) and Russian Foundation for Basic Research (# 11-03-00829-a).

REFERENCES
