3D printing biodegradable scaffolds with chitosan materials for tissue engineering

K N Bardakova¹,²,⁴, T S Demina³, E A Grebenik², N V Minaev¹, T A Akopova³, V N Bagratashvili¹ and P S Timashev¹,²

¹ Institute of Photonic Technologies, Federal Research Centre “Crystallography and Photonics,” Russian Academy of Sciences, 2 Pionerskaya Str., Moscow 142190, Troitsk, Russia
² Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, 8-2 Trubetskaya st., Moscow, 119991, Russia
³ Enikolopov Institute of Synthetic Polymer Materials, Russian Academy of Sciences, 70 Profsoyuznaya Str., Moscow 117393, Russia

E-mail: arie5@yandex.ru

Abstract. Chitosan-g-oligo (L,L-lactide) copolymer was synthesized through a solvent-free reaction in an extruder. Three-dimensional scaffolds based on photosensitive composition contained the synthetized copolymer were formed by two-photon polymerization. The optimum ratio of components, methods of preparation of photopolymerizable mixtures, parameters of the laser structuring and procedure of washing from unbound crosslinkers have been optimized. Chitosan scaffolds were non-cytotoxic and might therefore be a suitable candidate for treating spinal cord injuries and other neuronal degenerative diseases.

1. Introduction

One of the principal socially important tasks of regenerative medicine is to develop methods for correcting violations of the integrity of the pathways of the spinal cord caused by exchange degenerative, traumatic, inflammatory and other processes, followed by reduction of its functional activity [1]. In recent decades, special carriers of cells or scaffolds are actively being developed to solve the problem of stimulation the reparation processes in the body.

The choice of material for scaffold fabrication is of primary importance. Its main property is a compatibility with the spinal cord environment. The classification of materials for spinal cord is enormous - it can be various natural and synthetic materials, hydrogels, various functionalized materials (surface charged, drug-delivery) [2]. Chitosan is widely used for preparing scaffolds for regenerative medicine due to its ability to biore sorption and biocompatibility. There are known works in which lyophilized chitosan scaffolds were successfully tested as candidates for spinal cord regeneration [3] It should be noted that such a technology scaffolds fabrication - freeze drying - has one significant drawback: an insufficient rigidity of the scaffold obtained and the impossibility to tailor mechanical characteristics. As is known, depending on the hardness of the material, the phenotype of mesenchymal stem cells changes toward a certain lineage [4]. Also, an additional fixation of the spine is required in a surgical operation using such a scaffold [5].

In the present work, we examine chemically modified photosensitive chitosan-based materials for their suitability for microstructuring via two-photon polymerization (2PP) and fabrication of scaffolds for spinal cord regeneration. The natural polymer was used in combination with a biodegradable synthetic polymer - polyethylene glycol diacrylate. This approach allows to adjust the physico-chemical properties of the scaffold (e.g., mechanical properties, biodegradation rate) and could enhance the 2PP processing time. Also, 2PP has numerous advantages including the ability to produce structures with
well-defined, user-controlled geometries, structure with high—yet scalable—resolution, and application of a variety of biocompatible materials [6].

2. Materials and methods

2.1. Materials
Chitosan-g-oligo (L,L-lactide) copolymer was prepared by solid-state synthesis in a semi-industrial corotating twin-screw extruder (Berstorff ZE40, Germany) by reactive blending of solid powders of chitosan and oligo (L, L-lactide) at 55 °C according to the protocol of Demina et al. [7].

The resulting graft copolymer of chitosan and oligo (L, L-lactide) was used as the main component of a photosensitive composition for forming hydrogel scaffolds. The synthesized chitosan-co-oligo (L, L-lactide) was dissolved in 3 vol.% acetic acid for 12 hours with constant stirring at room temperature to obtain 4.9 wt.% copolymer solution. The insoluble fraction was separated by centrifugation (40 minutes, 9000 rpm), after which the solution was filtered through a membrane with a pore size of 0.45 μm. Polyethylene glycol diacrylate (PEG-DA, M2000, Sigma-Aldrich, Germany) was further added to the copolymer solution as an additional cross-linking agent and a biocompatible Irgacure 2959 (BASF Kaisten AG, Germany) as a photoinitiator (see Figure 1). Stirring of the photosensitive system was carried out for 20 hours at 35°C until a homogeneous solution was obtained. The ratio of components in the final photosensitive composition was 5/5/1 for chitosan-g-oligo (L,L-lactide), PEG-DA and Irgacure 2959, respectively. The storage time of the photosensitive composition is 3 days.

![Chemical structure of chitosan-g-oligo (L,L-lactide) copolymer unit](image)

Figure 1. Chemical structure of chitosan-g-oligo (L,L-lactide) copolymer unit (a), polyethylene glycol diacrylate (b) and Irgacure 2959 (c).

2.2. UV-Vis spectroscopy
UV-Vis spectroscopy of 5% solution of chitosan-g-oligo (L,L-lactide) copolymer in distilled water and this solution with the addition of 5% PEG-DA were recorded in the 200-500 nm region on a Cary 50 spectrophotometer. The measurements were performed in quartz cuvettes with an optical path length of 1 mm.

2.3. Scaffold fabrication and characterization
Two-photon-induced microstereolithography method was used to form three-dimensional scaffolds from a photosensitive composition based on chitosan-g-oligo(L,L-lactide) copolymer [8,9]. This approach allows the creation of a three-dimensional model with micron resolution using a targeted amount of photosensitive composition without the need to apply separate layers. The photosensitive composition was placed between two glasses and was limited to a silicone spacer to form three-dimensional scaffolds according to a predetermined three-dimensional model. The process of
photopolymerization was carried out under femtosecond laser radiation TEMA-100 (Avesta-project, Russia). Three-dimensional scaffold was formed directly in the bulk of the material. Irradiation was carried out with the use of microscopic planar lens 4X with a small number aperture (n.a. 0.1), which allowed to carry out photocuring process in workspace diameter of 4mm. The volume of the sample was layer-by-layer filled with an array of polymerized "lines" with a small overlapping. The average laser radiation power was 100 mW, the velocity of the laser beam moving along the sample volume was 1 m/s. The scaffold was washed from the non-crosslinked photosensitive composition by cyclic washing with deionized water for 2 hours.

For cell viability experiments the composition based on chitosan-g-oligo(L,L-lactide) copolymer was photocured to form films. The photosensitive composition (100 μl) was placed in a well of an aluminum spacer, and the top and bottom were limited with a release film based on polytetrafluoroethylene. As a source of ultraviolet radiation, a force matrix assembly of ultraviolet diodes with a wavelength of 365 nm (Epileds) was used. It consists of 50 separate diodes, which are placed on a single substrate on a powerful radiator. The total electrical power of the diode assembly was 50 W. Irradiation was performed on both sides for 15 minutes.

2.4. Biocompatibility evaluation

Dissociated rat cortical neurons were cultured on the film surface in the Neurobasal medium (Gibco) supplemented with 2% B27 (v/v) and 0.25% GlutaMAX (v/v) in a humidified incubator in an atmosphere of 5% carbon dioxide at 37°C. After 10 days in culture, the cells were loaded with 2 μM Calcein AM fluorescent probe and then rinsed twice with PBS. The fluorescence signal was accessed using a fluorescence microscope (Olympus, Japan) in order to visualize living cells.

3. Results

3.1. 3D microfabrication

![Figure 2. Electronic absorption spectra of 5 wt % solution of chitosan-g-oligo(L,L-lactide) copolymer (CLL) in distilled water, CLL with PEG-DA (5 wt %) and the final photosensitive composition.](image)

In figure 2 shows the absorption spectra of chitosan-g-oligo (L, L-lactide) copolymer, a mixture of a copolymer with PEG-DA and a photosensitive composition with an introduced photoinitiator. Chitosan-
g-oligo (L, L-lactide) copolymer and its mixture with PEG-DA show no absorption in the UV region at 263 nm (2PP absorption wavelength). This allows us to use them for fabrication copolymer-based hydrogels by two-photon-induced microstereolithography method.

We developed a three-dimensional model for treating spinal cord injuries. It is a truncated cylinder with a length of 1000 μm and a height of 600 μm. There is an array of through holes 30 μm in diameter on the convex side of the scaffold. Each structure required 40 min for fabrication. Then, the scaffolds were washed of unreacted components by cyclic immersion in aqueous medium at various pH. The final scaffolds possessed dimensions increased in 1.8-2 times compared to the pre-set ones due to swelling during the washing process. It should also be noted that there was a slight bending of the obtained scaffold in comparison with the given model that could be caused by the heterogeneity of the cross-linking of the material and the geometric dimensions of the scaffold, as well as the resulting internal stresses associated with the geometry. The swelling behaviour of the hydrogels was preliminary adjusted using the amphiphilic graft copolymers as well as addition of proper amount of synthetic component, i.e. PEG-DA. As a result, we got a well-structured 3D hydrogel with good handling properties, which was of great importance for future in vitro and in vivo experiments.

3.2. Biocompatibility evaluation
It is shown that the films provide a high survival rate of cortical neurons and the formation of neural networks, and thus could be considered biocompatible and suitable for neuroregeneration. The image of Calcein AM-stained cells cultured on the surface of the films is shown in figure 4.

4. Conclusion
In this work, the two-photon-induced microstereolithography method has been used to prepare three-dimensional chitosan scaffolds. Scaffolds produced were shown to be neurocompatible and might
therefore be a suitable candidate for treating spinal cord injuries and other neuronal degenerative
diseases.

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