Chitosan-g-lactide copolymers for fabrication of 3D scaffolds for tissue engineering

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Abstract. Chitosan-g-oligo (L, D-lactide) copolymers were synthesized and assessed to fabricate a number of 3D scaffolds using a variety of technologies such as oil/water emulsion evaporation technique, freeze-drying and two-photon photopolymerization. Solid-state copolymerization method allowed us to graft up to 160 wt-% of oligolactide onto chitosan backbone via chitosan amino group acetylation with substitution degree reaching up to 0.41. Grafting of hydrophobic oligolactide side chains with polymerization degree up to 10 results in chitosan amphiphilic properties. The synthesized chitosan-g-lactide copolymers were used to design 3D scaffolds for tissue engineering such as spherical microparticles and macroporous hydrogels.

1. Introduction

Fabrication of 3D polymeric scaffolds that could serve as temporary supporting structures for growing cells and tissues plays an important role in developing of tissue engineering. These scaffolds should meet a number of design criteria related to material properties and processing conditions. The material should be biocompatible and bioresorbable with the same rate as new tissue form. Scaffolds should possess an optimum structure, e.g. surface topography, porosity and pore interconnectivity to provide efficient transfer of cells and nutrients. At the same time, the used polymer should be processable into scaffolds with the structure and properties tailored for the intended application. To reach a compromise between polymer properties and fabrication technologies as well as to enrich the material with special functions, a modification of polymer chemical structure is widely proposed.

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Among a number of polymers used for biomedical applications chitosan, a linear polysaccharide derived from naturally occurring chitin, is widely investigated for scaffold fabrication, wound healing, drug delivery systems. Due to its biocompatibility, degradability by certain enzymes and positive charge of the macromolecule, chitosan is considered as a promising material for various biomedical applications. However, its unsuitability for processing in the molten state and solubility in limited range of solvents narrows a number of technologies to fabricate based on scaffolds and to modify its chemical structure.

This work aims to synthesize chitosan-g-oligo (L, D-lactide) in environmentally friendly solid-state mode and to prove the copolymer's processability with a range of 3D scaffold fabrication methods, such as oil/water emulsion evaporation technique, freeze-drying and laser microstereolithography based on two-photon polymerization. Chitosan-g-oligo (L, D-lactide) copolymers were synthesized through the solid-state reactive blending of chitosan and L, D-lactide mixtures by co-extrusion at low temperature to promote molecular level of mixing of these two components. Chemical reactions under shear deformation of the solid material induced by applying external mechanical energy in solvent-free mode provide high process effectiveness and ecological benefits. Grafting of hydrophobic oligolactide side chains on hydrophilic chitosan backbone results in copolymer's amphiphilic nature that allows us to extend a range of applicable processing technologies and to control other polymer properties, such as biocompatibility, degradation rate, mechanical characteristic, etc.

2. Materials and methods

2.1. Materials

Chitosan (Mw 60 kDa; acetylation degree 0.1) was prepared by mechanochemical solid-state synthesis as previously reported [1]. L, D-lactide was purchased from PuracBiochem (The Netherlands). The chitosan/L, D-lactide blends (marked as CL samples) were processed at lactide molar ratio of 0.5, 1, or 3 per chitosan unit in a semi-industrial co-rotating twin-screw extruder (ZE 40A×40D UTS, Berstorff, Germany) specially designed for powerful dispersion of solids. The process was run on 300-g batches at temperature range from 90 to 120°C [2]. The synthesis conditions and main macromolecular characteristics of synthesized chitosan-g-(L, D-lactide) copolymers are listed in table 1. Poly (D, L-lactide) (PDLLA) (Mw 76 kDa) used for microsphere preparation as a matrix polymer in oil phase was synthesized in CEIB. All other chemicals were of analytical grade and used without further purification.

Table 1. List of chitosan-g-poly (D, L-lactide) batches: conditions of treatment and the copolymer's characteristics.

Sample	Conditions of synthesis		Macromolecular features of the copolymer batches			Degree of chitosan amino group substitution	Average polymerization degree of the side lactide chains
	Lactide/ chitosan molar and weight ratio, mol (g/g)	Temperature (°C)	Relative amount of the reacted lactide (%)	Grafting degree (%)	Molar content of lactic acid units		
CL-0.5	0.5/(31:69)	120	57.1	26	0.58	0.19	3.0
CL-1	1/(47:53)	90	85.3	76	1.69	0.41	4.1
CL-3	3/(73:27)	120	59.3	160	3.58	0.37	9.7

2.2. 3D scaffolds fabrication and characterization

2.2.1. *Spherical microparticles via oil/water emulsion evaporation technology.* Microspheres were prepared by single oil/water emulsion solvent evaporation technique. Two different approaches to chitosan-g-oligo (L, D-lactide) copolymer application have been realized. As a first one, an oil phase

obtained by dissolving of PDLLA in the solvent mixture (CH_2Cl_2: acetone = 9:1 v/v) at concentration of 8 wt-% was rapidly added to the aqueous phase contained 2 wt-% solution of the CL sample in mQ. The oil/water dispersion was mechanically stirred by four-blade propeller at 500 rpm for 1.5 hrs. The system contained as a water phase the 2 wt-% solution of non-modified chitosan in 2% acetic acid was prepared for comparative purpose. The second approach to CL application was based on their additional to an oil phase. Thus, the oil phase contained 30% of CL sample and 70% of PDLLA have been stirred within water phase consisted of mQ without any emulsifier. Temperature mode during the process was keep as 15°C within first 15 min of agitation then increased up to 30°C in order to promote the evaporation of organic solvents from the oil phase and to transform liquid-in-liquid dispersion into solid-in-liquid one. The microspheres were washed several times with mQ, settled by standard sieves with apertures of 500, 250, 125 μm and freeze-dried. Morphology of the prepared microspheres was studied by scanning electron microscopy (SEM) using JEOL-840M (Japan).

2.2.2. Macroporous hydrogels via freeze-drying technology. Water solutions of the chitosan-g-oligo(L,D-lactide) copolymers (1.5 wt-%) were poured into plastic tubes (15×25 mm) and frozen at -15°C, then freeze-dried. To cross-link the polymer and to convert the obtained sponges into insoluble form, the samples were heated in oven at 150°C for 3 hours. The control sample made of non-modified chitosan was prepared from 1.5% chitosan solution in 0.1 nHCl.

Morphology of the hydrogels in dried and swollen states were evaluated by SEM using Jeol JSM-5300 LV (Japan) and confocal laser scanning microscopy (CLSM) using Nikon TE-2000 inverted microscope equipped with an EZ-C1 confocal laser (Japan), respectively. Zeta-potential measurements were performed using PALS Zeta Potential Analyzer V. 3.54 (Brookhaven Instruments Corp., USA). Adsorption of bovine serum albumin (BSA), a major component of fetal bovine serum (FBS) used for cell cultivation, was measured by Bradford protein assay. Degradation of the samples into sterile solution of lysozyme (2 mg/mL) in PBS (pH 7.4) at 37°C was measured during 1 month by monitoring of weight loss. An ability of the hydrogels to support adhesion and growth of mouse fibroblasts L929 cell line was assessed using optical microscopy (Reichert Microstar 1820E, Germany) and MTT colorimetric assay.

2.2.3. Microstructured 3D scaffolds via two photon-induced polymerization (2PP). To fabricate microstructured 3D scaffolds 20 wt% CL solutions were prepared in mQ, then 100 mg of the prepared solution was mixed with 200 μL of 1 wt % Irgacure 2959 (BASF Kaisten AG) and 20 mg of poly (ethylene glycol) diacrylate (PEG-DA, Sigma-Aldrich) for 1.5 hours at RT. 2PP fabrication of scaffolds was performed using: ytterbium solid-state femtosecond laser TeMa 1053/100 system (Avesta Project, Russia), which delivers 80 fs pulses at an 69.7 MHz repetition rate. The experimental setup is similar to one that was previous described [3]. Structures were composed of two layers of hollow cylinder arrays. There was a 50 nm offset of cylinder layers, that's why the pores of the scaffold are hierarchically reduced from the top and bottom layer to the scaffold interior. The crosslinking process was followed by structures washing with mQ, 2 wt % acetic acid and aqueous ammonia to remove the uncross-linked material. The electronic absorption spectra of 1% solutions of non-modified chitosan and chitosan-g-oligo (L, D-lactide) copolymer were recorded on a Shimadzu UV 2501 PC spectrophotometer. The measurements were performed in quartz cuvettes with an optical path length of 10 mm.

3. Results and discussion

3.1. Microspheres

Polymeric microspheres are attractive microcarriers for tissue engineering and drug delivery systems. However, synthetic polymers traditionally used for their fabrication, such as polylactide, poly (lactide-co-glycolide), etc., suffer from hydrophobicity and a lack of functionalities needed to promote cell adhesion and proliferation, that limits its usage to design scaffolds for tissue regeneration. Surface

modification of the preformed polylactide microparticles through physical sorption or chemical grafting of bioadhesive polymer has been proposed earlier [4, 5]. This last methodology is particularly tedious, because involving several steps after microparticle preparation to generate active functional groups to their surface before grafting the biomacromolecules. In order to simplify the above procedure but also to avoid non-degradable amphiphilic copolymer typically used to stabilize microparticles during their fabrication, we proposed to use chitosan-g-oligo (L, D-lactide) copolymers for preparation of polylactide-based microspheres.

Grafting of hydrophobic oligo (L, D-lactide) branch chains onto hydrophilic chitosan backbone allowed to obtain copolymers with amphiphilic properties that was clearly demonstrated by samples compatibility with organic solvents. Whilst non-modified chitosan were not dissolved and did not swell in chloroform at all, grafting of lactide chains allowed CL copolymers to swell in chlorinated solvents. At the same time the copolymers remained chitosan's ability to dissolve in diluted acids. Due to copolymer amphiphilic properties they have been applied for fabrication of spherical microparticles by an oil/water solvent evaporation technique using two different strategies. The first one allowed to replace classically used synthetic emulsifier PVA and to enrich the microparticle surface by chitosan moieties, which could positively affect cell attachment and growth on microparticle surface. The second approach relied on CL swelling in organic solvents which allowed to use it for oil phase modification. Therefore, the microparticles contained 30% of the CL sample and 70% of non-modified PDLLA have been prepared. Application of the CL samples in oil phase could provide chitosan moieties distribution over microsphere core and to avoid use of any emulsifier in aqueous phase.

In the frame of first option, water solubility of CL samples allowed us to directly dissolve copolymers in aqueous phase of the oil/water dispersion and use them as emulsifiers for microsphere preparation. As seen in figure 1, use of CL copolymers as the emulsifier in water phase allowed us to prepare spherical microparticles with smooth surface. The type of chitosan-based emulsifier drastically affected a total yield and size distribution of the prepared microparticles. The total microparticle yield was increased when degree of lactide grafting onto chitosan in the CL samples was increased. While application of non-modified chitosan acetic solution led to formation of particles with total yield of 26%, the use of CL-3 sample allowed us to enhance it up to 75% of PDLLA dissolved in oil phase. Better ability of the CL samples to stabilize microparticles during its preparation also appeared as a decrease of the particle size. Thus, the use of CL-0.5 and CL-3 allowed us to obtain the microparticles of 250 μm and 125 μm, respectively. Another approach dealt with 30% replacement of PDLLA in oil phase by the CL sample. It was proposed to fabricate microparticles without any emulsifier in water phase. Analysis of total yield and size distribution of the formed microparticles showed a tendency similar to water phase modification approach. Increase of lactic acid units per chitosan one among the CL-0.5, CL-1 and CL-3 samples led to increase of total microparticle yield: 21, 49 and 80 wt-%, respectively. The use of CL in oil phase gave a rise to smooth microspheres as well. SEM observation

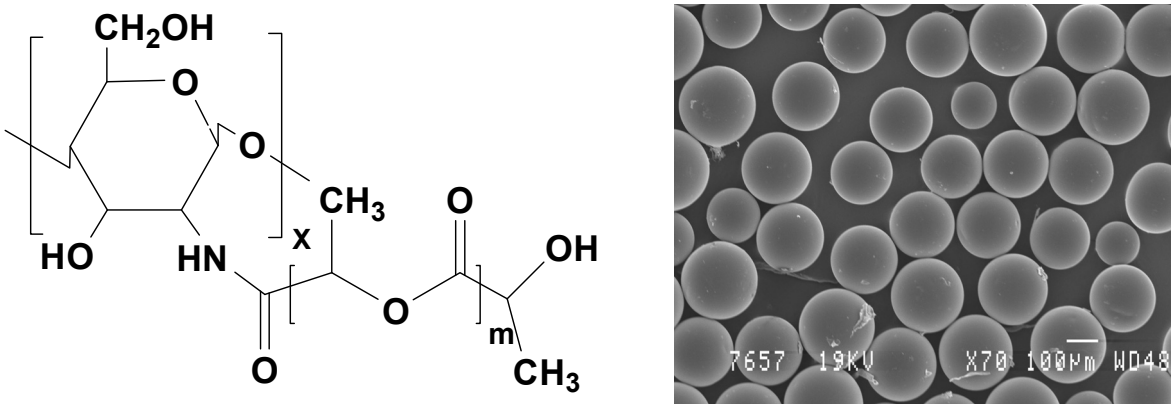

Figure 1. Chemical structure of chitosan-g-oligo (L, D-lactide) copolymer unit (left panel) and SEM image of microspheres made of CL-1 copolymer via oil/water emulsion evaporation technology (right panel).

of the cross-sectioned samples showed that the application of copolymers in oil phase led to formation of highly porous microparticles, an observation which can be explained by the amphiphilic nature of these copolymers.

We suppose that smooth surface could be promising to promote initial cell adhesion, whereas the macroporous internal structure of the microspheres provided the benefits during the microcarrier degradation, i.e. larger surface area for cell anchorage and facilitated diffusion of nutrients and penetration of cells and new tissue. Thus, chitosan-g-oligo (L, D-lactide) copolymers were successfully used for fabrication of microspheres possessed desired size and morphology to serve as drug/cell carriers for biomedical applications.

3.2. Macroporous hydrogels

Freeze-drying technology is a widely used approach for fabrication of macroporous 3D scaffolds for tissue engineering that could serve not only as templates to guide cell growth, but provides void volume for vasculatization, unhindered cell penetration and transfer of nutrients, oxygen and waste products. Freeze-drying allows to prepare macroporous structures as a result of formation of "walls" enclosing empty areas where polycrystals of frozen solvent (e.g. ice in the case of aqueous solutions) originally resided. The application of chitosan-based hydrogels for pharmaceutical applications has been already described in several reviews [6, 7]. Development of a new class of biocompatible and biodegradable hydrogels made of modified chitosan allows to control scaffold properties.

SEM observations of vertically and horizontally cross-sectioned slices of freeze-dried CL samples showed interconnected porous network with a pore mean size in a range of 70-140 μm. Control sample made of non-modified chitosan swell only under forced submergence, while the CL samples swelled up to the limit of their capacity within 1-2 min reaching equilibrium swelling degree up to 43 wt-%. The study of 3D architecture of the samples in swollen state using CLSM showed that their topography was similar to one in dried state, but the pore size had an increase in ~20 % (figure 2). An applicability of the macroporous chitosan-g-oligo (L, D-lactide) hydrogels as scaffolds for tissue engineering was evaluated *in vitro*. Protein adsorption, enzyme degradation rate and mouse fibroblast L929 cell line growth in the non-modified chitosan and CL-based hydrogels have been tested. Before cell/hydrogel interaction several phenomena usually take place, namely surface hydration and protein adsorption. In this research, we evaluated zeta-potential and adsorption of BSA on the control and CL hydrogels. We found that all hydrogels were positively charged, probably, due to the chitosan's cationic nature. The substitution of chitosan amino-groups led to decrease of zeta-potential. Since BSA was negatively charged, its adsorption should be higher on positively charged hydrogel samples. BSA adsorption profiles for various chitosan-based hydrogels were in a good agreement with zeta-potential measurements.

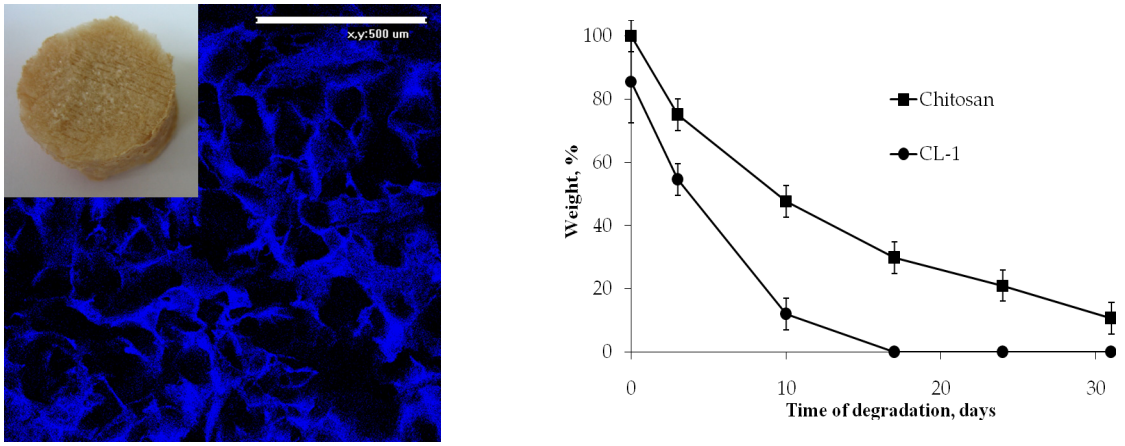

Figure 2. CLSM and photo of macroporous hydrogel made of CL copolymer using freeze-drying technology (left panel) and degradation of CL-1 hydrogels in PBS containing 2 mg/ml of lysozyme at 37°C (right panel).

An important scaffolds characteristic that could affect performance of cell growth, tissue regeneration and host response is a degradation rate. Ideally, scaffolds should degrade in a controllable manner with the same rate as new tissue form, thus, gradually transfer the structural and functional roles to the newly formed tissues. For the degradation study we selected lysozyme that is an enzyme present in the human body and is also produced by macrophages during wound healing. Monitoring of hydrogel's weight loss after incubation in lysozyme solution for various time intervals showed that CL samples degraded two times faster when hydrogels made of non-modified chitosan. It should be also stressed that during the degradation process hydrogels were not fallen into pieces but were found to be porous, very thin and fragile after degradation study. The study of ability the prepared hydrogels to support cell adhesion and growth by MTT-assay showed that a relative viability of L929 in the CL-based hydrogels was up to 80% higher than that in non-modified chitosan hydrogels. Optical observations showed that hydrogel's pore size was large enough for the cells to penetrate. The cells were seen to be mainly attached to the wall borders when to the wall surface. Thus, chitosan-g-oligo (L, D-lactide) copolymers were successfully used for fabrication of macroporous 3D hydrogels possessed enhanced biodegradation rate and the ability to support growth of animal cells.

3.3. 3D microstructures with well-defined architecture

Fabrication of 3D scaffolds through laser microstereolithography technique based on two-photon absorption polymerization (2PP) allows to achieve well-defined architecture with high resolution and reproducibility. The manufacturing of 3D objects by 2PP is based on a spatially controlled cross-linking process in a small volume at a computer-controlled beam focus. In contrast with single-photon polymerisation 2PP is excited by the nearly simultaneous absorption of two photons with low intensities that together provide enough energy to initiate cross-linking [8, 9]. This leads to high resolution (up to 200-400 nm) and possibility to carry out the scaffold formation without local overheating. Polymeric scaffolds of well-defined 3D architecture obtained by 2PP are considered as promising biomimetic constructions for tissue engineering [10].

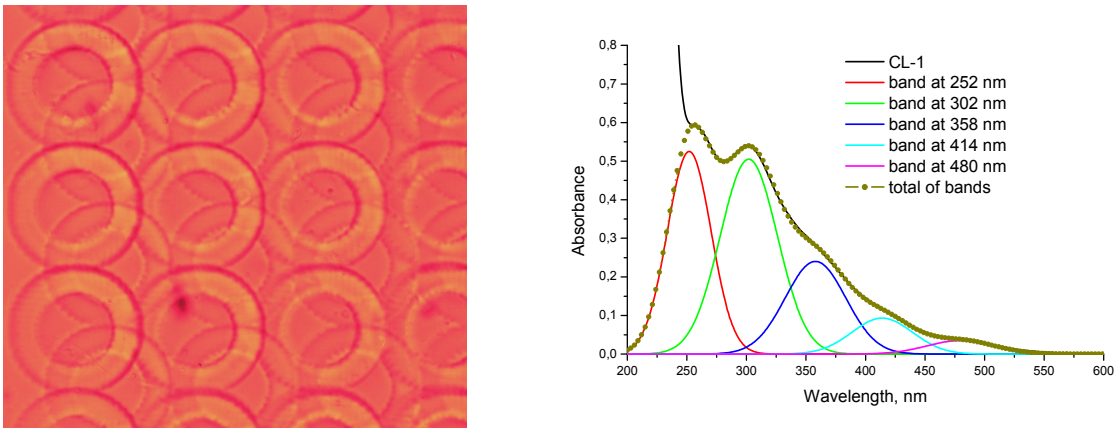

Figure 3. Optical micrographs of 3D microstructures fabricated by two-photon induced polymerization (left panel) and electron absorption spectra of CL-1 sample (right panel).

Here, we investigated the possibility to fabricate 3D scaffolds by 2PP from CL samples. It was found that photosensitive compositions based on CL samples could be successfully subjected to structuring under femtosecond laser beam. To reveal a difference in electron absorption spectra of non-modified chitosan and CL-1 solutions UV-Vis spectroscopy was carried out. Spectra of 1 wt-% non-modified chitosan solution in 0.1 n HCl was in a range of 200-420 nm and was consisted of three intensive bands having a maximum at 252, 299 and 346 nm and the bands intensity ratio as 1/1/0.4, while the chitosan-g-oligo (L, D-lactide) had two additional long wavelength bands with low intensities at 414 and 480 nm (figure 3). The CL sample showed a short wavelength band (< 200 nm) related to lactic acid. No significant differences were revealed in absorption spectra of both non-

modified chitosan and chitosan-g-oligo (L, D-lactide) copolymer diluted solutions at concentration range from 0.1 to 1 wt-% that indicated the absence of an aggregation effect. However, analysis of absorption spectra showed that CL samples have particles with larger size than non-modified chitosan.

The microscopic observations (figure 3) of structures obtained by 2PP from CL samples demonstrate regular shape and definition, which shows applicability of this method for fabrication of CL-based microstructured objects. Although the simplicity of the presented structure, the objects of any shape could by produced with this technique.

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4. Conclusions

Several types of 3D scaffolds, such as spherical microparticles and macroporous hydrogels, based on synthesized chitosan-g-oligo (L, D-lactide) have been successfully fabricated using a variety of technologies. Grafting of oligo (L, D-lactide) branch chains onto chitosan backbone allowed to broaden a number of technologies for fabrication of chitosan-based scaffolds and to improve materials properties.

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