

## Solid state synthesis of chitosan and its unsaturated derivatives for laser microfabrication of 3D scaffolds

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**Abstract.** Chitosans with various degrees of deacetylation and molecular weights and their allyl substituted derivatives were obtained through a solvent-free reaction under shear deformation in an extruder. Structure and physical-chemical analysis of the samples were carried out using nuclear magnetic resonance (NMR), ultraviolet (UV) and infrared radiation (IR) spectroscopy. Photosensitive materials based on the synthesized polymers were successfully used for microfabrication of 3D well-defined architectonic structures by laser stereolithography. Study on the metabolic activity of NCTC L929 cultured in the presence of the cured chitosan extracts indicates that the engineered biomaterials could support adhesion, spreading and growth of adherent-dependent cells, and thus could be considered as biocompatible scaffolds.

### 1. Introduction

3D models of various cell cultures that are performed in a volume of polymer matrices of well-defined architectonics have been considered as promising biomimetic constructions for tissue engineering in recent years [1, 2]. Natural polysaccharides, including chitosan and its derivatives, are attractive polymers to create scaffolds of that kind due to high cell affinity alongside with biodegradation capability [3]. These scaffolds should possess an optimum microarchitecture to provide efficient transfer of nutrients and cell growth and proliferation. Laser microstereolithography technique based on two-photon absorption polymerization allows to induce a cross-linking spatial process in a small volume at a beam focus. Thus formed scaffolds possess well-managed reproducibility of the 3D structure with high resolution up to 200 nm [4, 5]. Hydrogels or scaffolds for tissue engineering have been developed through two-photon-induced cross-linking of the photosensitive polymer compositions based on gelatin methacrylate [6], polylactic [7] and hyaluronic [8] acids. Chitosan – natural cationic polyelectrolyte which has a free primary amino group almost in each of polymeric units, has been applied early via “guest-host” scheme to design 3D triacrylate-based structures through two-photon polymerization [9].

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To increase the reactivity of the functional groups of the polymers, which are subjected to cross-linking under laser radiation, it is promising to introduce unsaturated groups into their structure. This study reports the solid-state synthesis of chitosan and its unsaturated ether – allyl chitosan – for the purpose of 3D structures fabrication by laser stereolithography.

## 2. Materials and methods

### 2.1. Solid state synthesis of chitosan and its allyl substituted derivatives

The reactive blends of allyl bromide and chitosan characterized by the predetermined ratios of the components (0.5 mol of alkylation agent per 1 unit of chitosan) were processed in a semi-industrial co-rotating twin-screw extruder (Berstorff ZE40, Germany) in the absence of any liquid dispersion media and catalyst at  $-5^{\circ}\text{C}$ . The extruder is equipped with processing elements which performed compression and shear deformation of the substances in a thin layer. Plastic flow realized at such conditions leads to intense chemical interaction of the blend components up to quantitative yield of the products [10]. Chitosan was synthesized through solid-state alkaline deacetylation of crab chitin (Vostok-Bor, Russia) in accordance with a published procedure [11]. Two samples having different content of residual N-acetylated units, 10 and 30 mol%, and comparatively low molecular weight of approximately 50-90 kDa, were obtained. Allyl bromide (99%) was purchased from Sigma-Aldrich. The products were purified with isopropyl alcohol to remove unreacted monomer before characterization.

### 2.2. IR spectroscopy

Infrared spectra were recorded on a Bruker Vertex 70 spectrometer and treated using the program Bruker Opus (version 7.0). All spectra were normalized with respect to the composite stretching band at  $1060\text{ cm}^{-1}$  of the C–O pyranose cycle used as an internal standard. The following major bands in the IR-spectrum of allyl-modified chitosan were observed: c.a.  $3400\text{ (sh) cm}^{-1}$  – O–H stretching;  $3365, 3357\text{ cm}^{-1}$  – N–H asymmetrical and symmetrical stretching, correspondingly;  $1651\text{ cm}^{-1}$  – Amide-I band;  $1590\text{ cm}^{-1}$  –  $\text{NH}_2$ -groups – deformational mode;  $1500\text{-}1200\text{ cm}^{-1}$  region – multiple peaks typical for C–C and C–N mixed vibrations; c.a.  $1150\text{ cm}^{-1}$  – C–O–C stretching in glycoside bridge; c.a.  $1070\text{ (sh), }1060\text{ and }1055\text{ (sh)}$  – several overlapped bands of C–O–C and C–O(H) stretching of pyranose ring. Only small changes in relative intensity and the shape of complex bands in the spectrum of modified product could be observed as compared with the spectrum of initial chitosan.

### 2.3. UV-Vis spectroscopy

Electronic absorption spectra of 1% polymer solution in 0.1 M HCl were recorded in the 200-420 nm region on a Shimadzu UV 2501 PC spectrophotometer. The measurements were performed in quartz cuvettes with an optical path length of 10 mm.

### 2.4. NMR analysis

$^1\text{H}$  (300 MHz),  $^{13}\text{C}$  (75.5 MHz) and  $^{13}\text{C}\{-^1\text{H}\}$  APT (attached proton test) NMR spectra were recorded on a Bruker Avance II 300 spectrometer at 25 and  $70^{\circ}\text{C}$  in  $\text{D}_2\text{O}$  solution doped with HCl. Signal ascriptions to give a ratio of initial and allyl-substituted pyranose cycles as well as a content of residual N-acetylated units are shown.

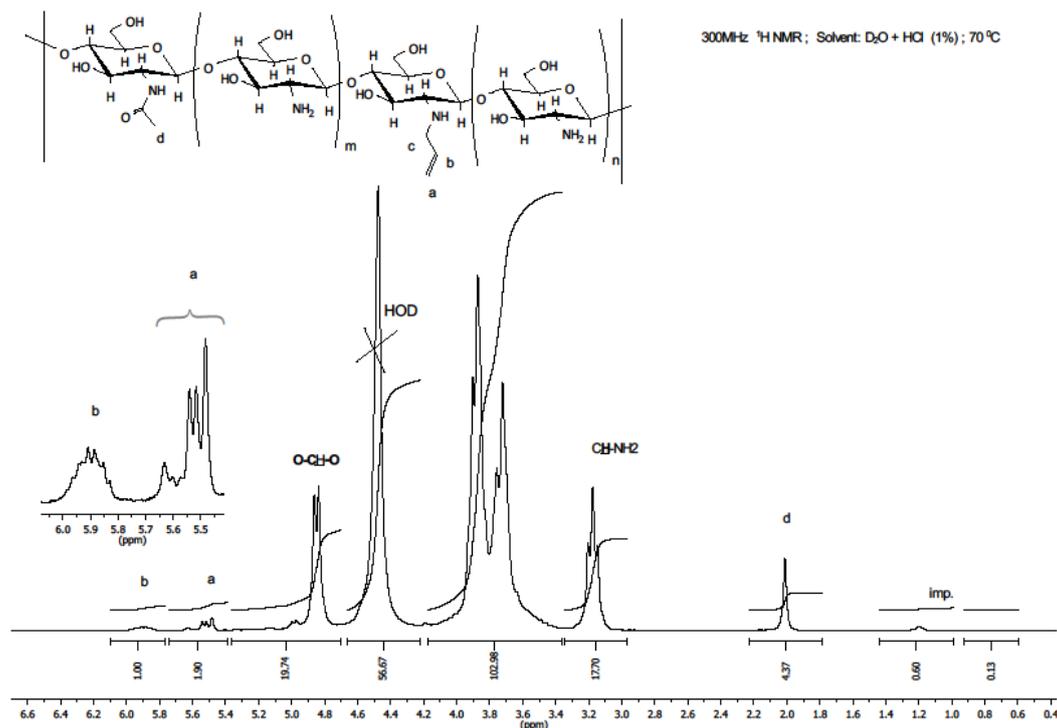
### 2.5. Scaffold fabrication

For scaffold fabrication, 20 wt% highly viscous polymer solutions were prepared in 4 wt% acetic acid. In order to prepare photosensitive material, 100 mg of polymer solution was mixed with 200 mL 1 wt% Irgacure 2959 (BASF Kaisten AG) as well as 20 mg poly(ethylene glycol) diacrylate (PEG-DA, Sigma-Aldrich) for 90 min at ambient temperature.

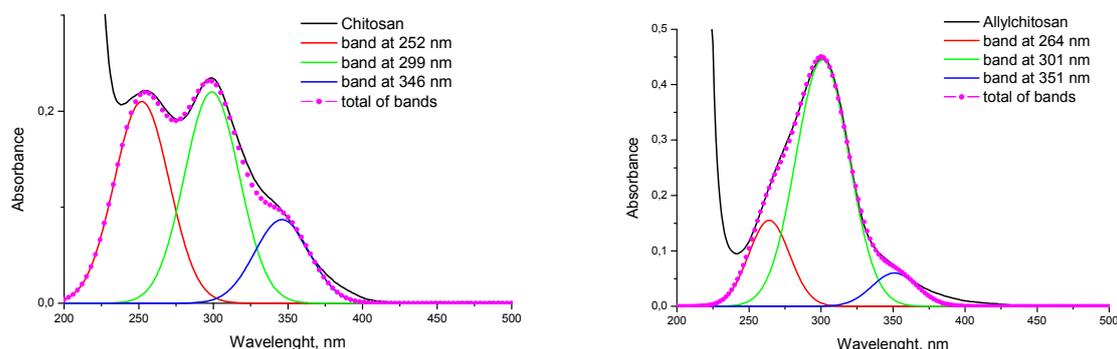
## 3. Results

### 3.1. Polymers characterization

$^1\text{H}$  (300 MHz) spectrum of allyl chitosan sample is shown in figure 1. And 8-10 mol% of allyl-modified units and similar content of residual N-acetylated units were found in accordance with the data. Chemical shift of carbon in allyl methylene group (53 ppm) in  $^{13}\text{C}$ - $\{^1\text{H}\}$  APT NMR spectra indicates its bonding with nitrogen atom, therefore N-acylation mainly takes place at the given conditions of the solid state synthesis. Since relatively low degree of amino-groups substitution (about 20 mol% in total) was found, chitosan modified with hydrophobic allyl moieties retains solubility in acidic aqueous media which is characteristic of initial polymer.



**Figure 1.**  $^1\text{H}$  NMR (300 MHz) spectrum of allyl chitosan in  $\text{D}_2\text{O}$  solution doped with 1% HCl.



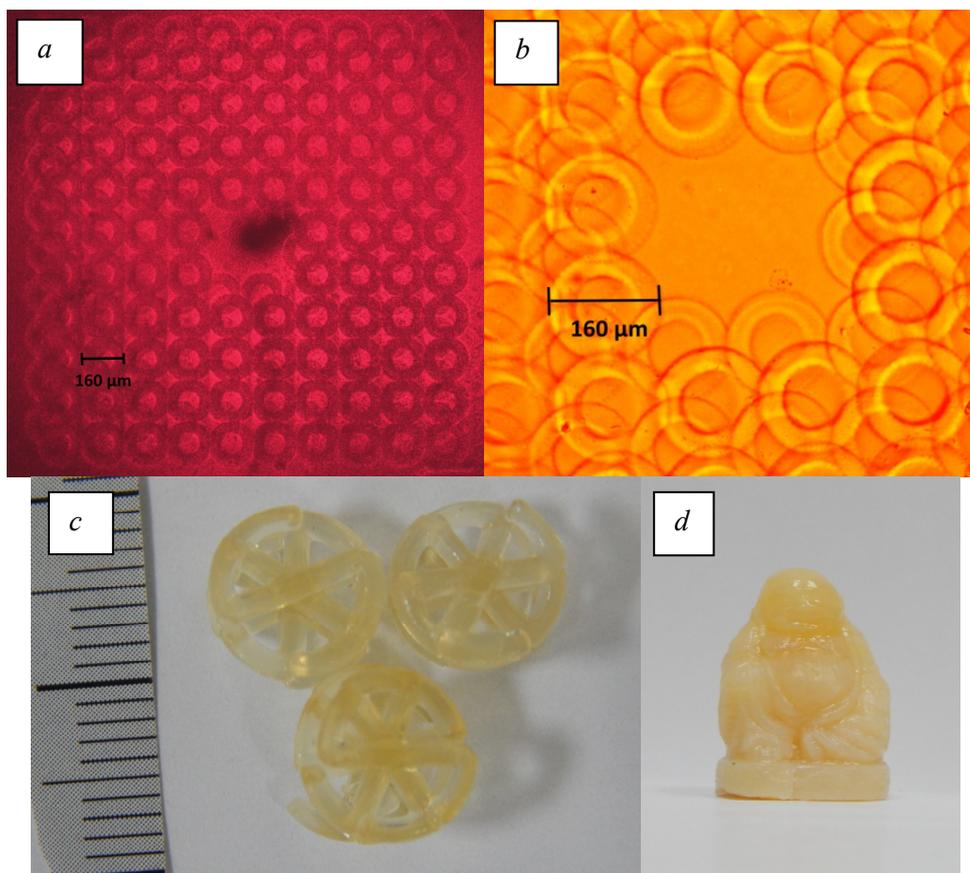
**Figure 2.** Electronic absorption spectra of 1% polymer solution in 0.1 M HCl in a range of 200-420 nm: initial chitosan (left) and allyl-modified chitosan (right) samples.

Electronic absorption spectra of the initial and modified chitosan samples are shown in figure 2. The spectrum of allyl chitosan sample exhibits one strong band at 301 nm and two less intense bands at 264 and 351 nm (with intensity ratio of 1/0.3/0.1, correspondingly). The intensity of the main

absorption band (at 301 nm) of allyl chitosan is increased two fold in comparison with initial chitosan sample, while the intensity of other two bands is significantly decreased. No significant difference is revealed in the absorption spectra of both chitosan and allyl chitosan diluted solutions at the concentration range from 0.1 to 1 wt%, which indicates the absence of an aggregation effect.

### 3.2. 3D microfabrication

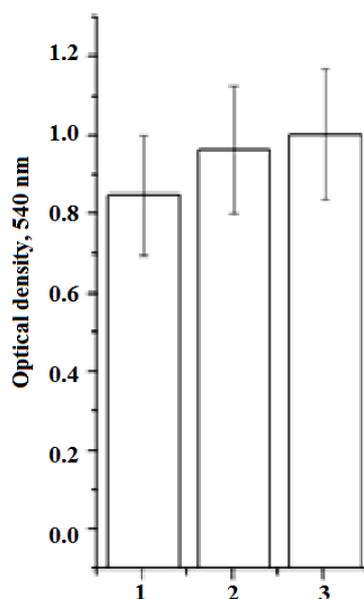
Concentrates of the samples were subjected to structuring by a microstereolithography setting developed in ILIT RAS and equipped with a laser TE MA-1053/100 (Avesta-Project, Russia) and an Epiplan 20× microscope objective (Zeiss, Oberkochen, Germany). Second harmonic of a femtosecond laser pulses (80 fs, 69.7 MHz, 1050 nm) was used for polymer structuring via two-photon absorption (2 PP) method in accordance with a published procedure [12]. Single laser absorption (1 PP) scaffolds were fabricated with LS 120 stereolithography setting (ILIT RAS, Russia), equipped with He-Cd laser with a wavelength of 325 nm and intensity 10 mW. The scanning speed was 1.5 m/sec, a beam diameter - 0.10 mm. After fabrication, unpolymerized material was washed away with mQ, 2 wt% acetic acid and aqueous ammonia. In figure 3, microscopic images demonstrate that the matrices are of a regular shape structures, and specified partial resolution is transmitted without distortion that indicates the uniformity of cross-linking.



**Figure 3.** Microscopic images of the 2PP (*a*, *b*) and 1 PP (*c*, *d*) structures designed on a microstereolithography setting using the photosensitive chitosan-based compositions.

### 3.3. Study of biocompatibility

Study on the metabolic activity of NCTC L929 cultured in the presence of the engineered chitosan extracts indicates that the engineered biomaterials could support adhesion, spreading and growth of adherent-dependent cells, and thus could be considered as biocompatible scaffolds (see figure 4).



**Figure 4.** Metabolic activity of NCTC L929 cultured for 48 hours in 3-day extracts of: 1– chitosan + Irgacure 2959 (0.92%), 2 – chitosan + Irgacure 2959 + PEG-DA, 3 – control. Results of MTT-test are expressed as mean  $\pm$  standard deviation for three replicates.

#### 4. Conclusion

The laser microstereolithography techniques based on non-linear absorption of femtosecond laser pulses allows to fabricate structures of photosensitive materials with a resolution from tens of micrometers down to hundreds of nanometers. These 3D engineering approaches offer numerous opportunities for tissue engineering biomaterial scaffolds that mimic the complex architecture of native tissues and provide mechanical support for the cells. In this study, both 2PP and 1PP techniques are applied to develop precisely defined biocompatible 3D chitosan scaffolds. Chitosan with various degrees of deacetylation and molecular weights and their allyl substituted derivatives are obtained through a solvent-free reaction under shear deformation in the semi-industrial extruder. Photosensitive materials based on the chitosan and its unsaturated derivatives are successfully used for microfabrication of 3D structures by laser stereolithography methods. The study on the cytotoxicity of the fabricated chitosan-based structure reveals no significant effects on animal cell viability.

#### Acknowledgments

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