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Solid-state synthesis of unsaturated chitosan derivatives to design 3D structures through two-photon-induced polymerization

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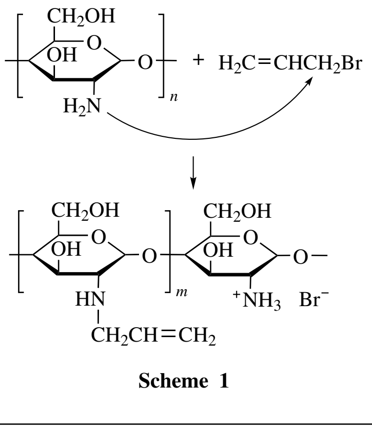
Solvent-free N-allylation of chitosan with allyl bromide was performed under shear deformation in an extruder and the obtained chitosan derivatives were successfully used for the microfabrication of 3D structures by laser stereolithography.

Chitosan is a natural cationic polyelectrolyte which has a free primary amino group in almost each of polymeric units. It is widely used to design materials for biomedical applications such as films, fibers, microbeads and nanoparticles.[1–3] The 3D models of various cell cultures in the polymer matrices of well-defined architectonics have been considered as promising biomimetic constructions for tissue engineering in recent years.[4] Natural polysaccharides, including chitosan and its derivatives, are attractive polymers to create scaffolds of that kind due to high cell affinity alongside with biodegradability.[5–8] Laser microstereolithography based on two-photon absorption polymerization allows one 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 400 nm.[9–11] In order to increase the reactivity of the functional groups of polymers, which are subjected to cross-linking under laser radiation, it is necessary to introduce unsaturated groups into their structure. Hydrogels or scaffolds for tissue engineering have been developed through the two-photon-induced cross-linking of photosensitive polymer compositions based on gelatin methacrylate[12] or polylactic[13] and hialuronic[14] acids. Scaffolds of N-substituted gelatin methacrylate possessed biodegradability, however, they well preserved their microstructures under conditions of cell cultivation within a few weeks. Interaction of methacrylic anhydride with a primary amino group was used to introduce unsaturated moieties into a gelatin structure with degrees of substitution up to 65%.

Chitosan has been applied to design hybrid 3D structures through two-photon polymerization being unaltered included *via* a guest–host procedure into triacrylate cross-linked systems.[15] The aim of this study was to perform a solid-state synthesis of unsaturated ether of chitosan – allyl chitosan – for the construction of 3D structures.[†] In our opinion, as compared to the vinyl monomers, which are widely applicable to synthesize unsaturated chitosan derivatives, allyl bromide is a more suitable alkylation agent for a variety of reasons. Side reaction of the unsaturated C=C bond and free amino groups of chitosan chain, that leads to decrease in the desired products yield, is not intrinsic to the allyl group. It would allow one to avoid an undesirable cross-linking of the polymer before the laser-induced formation of a required scaffold structure. It was reported[19] that the graft polymerization of vinyl monomers onto allylchitosan proceeds with higher reaction rate and monomer conversion, as compared with a non-modified polymer. Therefore, it can contribute to targeted alteration of the properties of chitosan-based 3D scaffolds, in particular to impart them thermoplasticity, directly in the course of their formation.

The amino groups of chitosan are much more reactive than hydroxyl ones in the course of an non-catalyzed nucleophilic reaction, while an alkaline catalyst leads to the formation of mixed O- and N-substituted allyl derivatives.[20,21] NMR and IR spectroscopic data showed that substitution occurs mainly *via* amino groups of chitosan under the given conditions in accordance with Scheme 1.[‡]

As compared to the spectrum of initial chitosan, the following changes evidence that the process of modification involves NH_2



Scheme 1

[†] The reactive blends of allyl bromide and chitosan characterized by the predetermined ratios of the components (0.5 mol of alkylation agent per chitosan unit) were processed in a semi-industrial co-rotating twin-screw extruder (Berstorff ZE40, Germany) in the absence of liquid dispersion media and catalyst in a temperature range of –2 to –5 °C. The extruder was equipped with processing elements, which performed the compression and shear deformation of substances in a thin layer. Plastic flow under such conditions leads to the intense chemical interaction of blend components up to the quantitative yield of products.[16,17] Chitosan was synthesized in accordance with a published procedure.[18] Allyl bromide (99%) was purchased from Sigma-Aldrich. The products were purified with isopropyl alcohol to remove unreacted monomer before characterization.

groups: appearance of 3260 (sh); weakening of 1590 cm^{-1} band which in turn results in appearance of the Amide-II peak as a shoulder at ~1550 cm^{-1}; several insubstantial changes in the region 1500–1200 cm^{-1} of mixed C–C and C–N modes. The participation of methylol groups in reaction with allyl bromide according to FTIR spectra is considerably lower than that of NH$_2$ groups. Only very small changes in the relative intensity and shape of a complex band in the region 1200–800 cm^{-1} are observed.

^{13}C-{^{1}H} APT spectrum of allyl chitosan sample is shown in Figure 1. Chemical shift of carbon in allyl methylene group (53 ppm) indicates its bonding with the nitrogen atom; therefore, N-alkylation mainly proceeds under the conditions of the solid-state synthesis. Due to relatively low degree of substitution (0.08–0.10) found, chitosan modified with hydrophobic allyl moieties retains solubility in acidic aqueous media, which is characteristic of the initial polymer.

Concentrated sample solutions were subjected to structuring by a microstereolithography setting developed in ILIT RAS and equipped with a TEMA-1053/100 laser (Avesta-Project, Russia).[§]

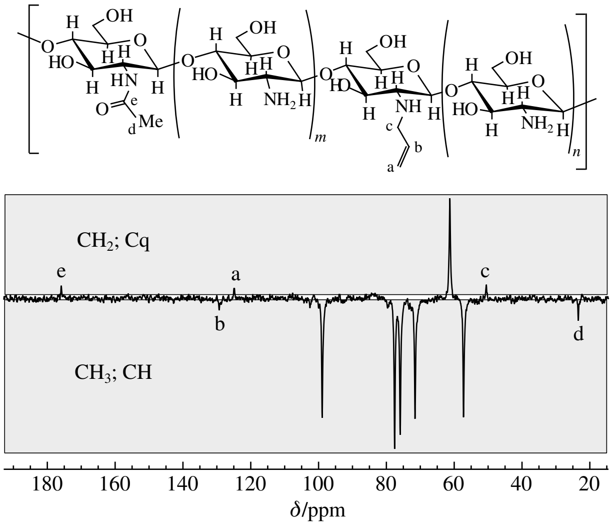


Figure 1 ^{13}C-{^{1}H} APT spectrum of allyl chitosan in D$_2$O solution doped with 1% HCl (75.5 MHz). Signals from the secondary and quaternary carbon atoms are in antiphase with the prime and tertiary ones.

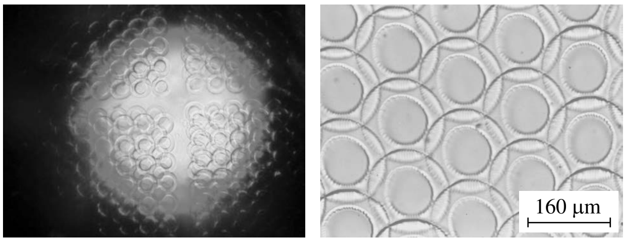


Figure 2 Microscopic images of the structures designed on a microstereolithography setting using the photosensitive polymer compositions based on allyl chitosan, poly(ethylene glycol) diacrylate and Irgacure 2959 as a photoinitiator.

For scaffolds fabrication an Epiplan 20$^\times$ microscope objective (Zeiss, Oberkochen, Germany) was used. The microscopic images (Figure 2) demonstrate that the obtained matrices are the structures of a regular shape, specified partial resolution is transmitted without distortion that indicates the uniformity of cross-linking.

The study on the cytotoxicity of the synthesized allyl chitosan samples to monolayer of mouse fibroblast cell line L929 revealed no significant effects on animal cell viability.[¶]

Thus, the solid-state reaction of chitosan allylation in the absence of any catalysts and organic solvents occurs under the conditions of shear deformation in a semi-industrial extruder and leads to unsaturated chitosan derivatives suitable for designing biocompatible 3D structures with well-defined architecture through two-photon-induced polymerization process.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2015.07.017.

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[‡] IR spectra were recorded on a Bruker Vertex 70 spectrometer and treated using the Bruker Opus program (version 7.0). All spectra were normalized with respect to the composite stretching band at 1060 cm^{-1} of the C–O pyranose cycle used as an internal standard. The following major bands in the IR spectrum of a modified product were observed: ~3400 (sh) cm^{-1}, O–H stretching; 3365, 3357 cm^{-1}, N–H asymmetrical and symmetrical stretchings, respectively; 1651 cm^{-1}, amide-I band; 1590 cm^{-1}, NH$_2$ groups, deformational mode; 1500–1200 cm^{-1} region, multiple peaks typical of C–C and C–N mixed vibrations; 1150 cm^{-1}, C–O–C stretching in glycoside bridge; 1070 (sh), 1060 and 1055 (sh), several overlapped bands of C–O–C and C–O(H) stretching of a pyranose ring. Electronic absorption spectra of a 1% polymer solution in 0.1 M HCl were recorded in the region of 200–420 nm on a Shimadzu UV 2501 PC spectrophotometer. The measurements were performed in quartz cuvettes with an optical path length of 10 mm. The spectrum of allyl chitosan exhibits one strong band at 301 nm and two less intense bands at 264 and 351 nm (with an intensity ratio of 1/0.3/0.1).The main absorption band intensity (at 301 nm) of allyl chitosan increased two times in comparison with that of the initial chitosan sample, while the intensity of other two bands significantly decreased. No significant difference is revealed in absorption spectra of both chitosan and allyl chitosan solutions at concentrations from 0.1 to 1 wt% due to the absence of an aggregation effect. ^{1}H, ^{13}C and ^{13}C-{^{1}H} APT (attached proton test) NMR spectra were recorded on a Bruker Avance II 300 spectrometer at 25 and 70 °C in a D$_2$O solution doped with HCl. For the ^{1}H NMR (300 MHz) spectrum of allyl chitosan, see Online Supplementary Materials.

[§] For scaffold fabrication, 20 wt% highly viscous polymer solutions were prepared in 4 wt% acetic acid. Then, 100 mg of 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 90 min at room temperature. We used a second harmonic of femtosecond laser pulses (80 fs, 69.7 MHz, 1050 nm) for polymer structuring in accordance with a published procedure.[22] After fabrication, the unpolymerized material was washed away with distilled water, 2 wt% acetic acid and aqueous ammonia.

[¶] 100 mg of the sample were incubated in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C for 24 h. Then, the suspension was filtered and the extracts were added to monolayer contained wells of 96-well culture plates. Cell viability was evaluated by MTT assay after cell cultivation in 5% CO$_2$ humidified atmosphere at 37 °C.

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