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Solid-Phase Copolymerization of *L,D*-lactide with Chitosan

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Abstract

With the use of solid-phase synthesis in an extruder, chitosan and *L,D*-lactide grafted copolymers were obtained. A mechanism of graft polymerization was proposed, an effect of process conditions exerted on the yield and structure of the target products was studied. Based on the data obtained from fractionation, IR spectroscopy and study of the elemental composition of the fractions, a substitution level has been calculated for the chitosan amino groups that were 0.19–0.41. An average polymerization level for lactide in grafted chains and an effect exerted by the grafting on the solubility of chitosan and physico mechanical characteristics of film materials were determined. Based on the copolymers synthesized, three-dimensional matrices have been obtained for tissue engineering (macroporous hydrogels, spherical microparticles), as well as compatibility and biodegradation rate have been estimated for the materials.

Key words: solid-phase synthesis, chitosan, lactide, grafted copolymers, biocompatible materials

INTRODUCTION

Nowadays, biopolymers obtained from renewable sources attract researchers' attention. They can be used for the development of materials for biomedical applications as coatings for wounds, matrices for tissue engineering and means for targeted medication delivery. Chitosan, a naturally occurring polymeric product of chitin deacetylation, has good film-forming and fibre-forming properties is one of the most promising components of such biologically active systems [1, 2]. Like the most of polysaccharides, chitosan is biocompatible being capable of biodegradation [3, 4]. As the only naturally occurring cationic polymer, chitosan is widely used for the efficient sorption of heavy metal ions, proteins and acidic substances [5, 6].

Despite a high consumer potential of chitosan, its use is restrained by the technological complications in the synthesis of derivatives, particularly in the synthesis of grafted copolymers, and processing them into products. At the same time, unmodified chitosan is insoluble in neutral aqueous medium, because of a developed system of intermolecular hydrogen bonds and the ability of crystallizing [7, 8], which makes it difficult to obtain materials for biomedical applications. Attempts to obtain the grafted copolymers of chitosan and polylactide were made repeatedly. According to [9, 10], chitosan modified with lactide chains of different length, exhibits improved physicochemical and biological properties, whereby it can be successfully applied in biotechnological and pharmaceutical fields. The modification of chitosan with the use of solution technologies is a labour-intensive multi-stage process. The presence of the amino group and two hydroxyl groups in each chitosan link promotes a great contribution of chain transfer to a polymer in the course of lactide polymerization with the formation of short grafted chains. In the course of performing the process in DMSO solution, using a $Ti(OBu)_4$ in a nitrogen atmosphere, there occurs the formation of oligolactide side

chains with the polymerization level of 1–5 at the amino groups of chitosan [10]. The reaction proceeds heterogeneously and affects only the surface of the polymer particles. The reaction product also represents a suspension swelling in DMSO. Carrying out the copolymerization in the same medium, but with the use of triethylamine as the catalyst enabled the authors of [11] to receive the grafted chains with the number of lactic acid monomer units amounting up to seven. The authors of [12] have reported the grafted copolymers to be obtained in the course of lactide polymerization in the toluene suspension of chitosan, using triethylaluminium as a catalyst. At a 40-fold molar excess of lactide there were obtained grafted chains having a level of oligolactide polymerization equal to 10. The method for determining the length of the graft chain is based on calculating the difference between the mass of the modified product and the mass of original chitosan. The graft chain length cannot be determined using the direct method. In case of a catalyzed reaction, especially in the presence of tertiary amine, the reaction of a lactide with the hydroxyl groups of chitosan is more than likely. As a consequence, the length of the grafted chains is even lower than indicated by the authors, although the level of substitution in the chitosan link in this case is higher. The authors of [13] described a two-stage synthesis of polylactide side chains grafted onto the hydroxyl groups of chitosan. At the first stage, the authors carried out the protection of the amino groups of chitosan with the help of phthalic anhydride; at the second stage they performed copolymerization with lactide using 4-dimethylaminopyridine as a catalyst.

The opportunity of the solid phase synthesis of chitosan and *L*,*D*-lactide copolymers allows to eliminate the use of catalysts, solvents and diluents in the systems, which is attractive from the standpoint of obtaining materials for biomedical applications. The resulting products would not contain toxic components, whose removal from the polysaccharide matrix is completely impossible. The efficiency of the solid state reactions of chitosan amino groups under shear deformation conditions in the extruder was demonstrated in our studies by the example of chitosan acylation with carboxylic acids [14, 15].

The purpose of this work was to study the mechanism of *L*,*D*-lactide copolymerization with chitosan under the conditions of solid phase synthesis as well as in the investigation of the structure and properties of copolymers and materials obtained from them for regenerative medicine.

EXPERIMENTAL

We used chitosan obtained from crab shell chitin, poly[(1→4)-2-amino-2-deoxy-β-D-glucose] obtained at the ISPM of the RAS *via* solid phase synthesis [16]. The molecular mass (MM) of chitosan amounted to $4 \cdot 10^4$; the acetylation level, according to potentiometric titration and elemental analysis, was equal to 0.4. The racemic mixture of *L*- and *D*-lactides (*L*,*D*-lac-

TABLE 1

Process conditions and the yield of reaction products

Sample No.	Molar (mass) ratio lactide/chitosan	Coextruding temperature, °C	Amount of reacted lactide, rel. %	Level of grafting, %	Molar content of lactic acid units
1	0.5 (31 : 69)	90	95.8	43	0.96
2	0.5 (31 : 69)	120	57.1	26	0.58
3	1 (47 : 53)	80	76.0	67	1.49
4	1 (47 : 53)	90	85.3	76	1.69
5	1 (47 : 53)	100	85.7	78	1.74
6	3 (73 : 27)	120	59.3	160	3.58

Note. The level of grafting was calculated from the ratio of relative mass difference between the samples of the copolymer and chitosan in the copolymer to the mass of the polysaccharide in the sample; the molar content of lactic acid units was determined as the product of the level of grafting and the mass ratio of the units equal to 161/72.

tide, *cis*-(±)-3,6-dimethyl-1,4-dioxane-2,5-diol) from PURAC Biochem (the Netherlands) with the melting point $T_{m.p}$ = 124–128 °C was used without further purification. The solid phase synthesis was carried out using an experimental-industrial twin-screw extruder (Berstorff ZE-40, Germany) with the screw diameter of 40 mm and the controlled heating of zones at different componential ratio values and different temperature conditions (Table 1).

The unreacted monomer was removed *via* extraction with chloroform. In order to determine the content of the N-acylated chitosan units (grafted units) the samples were dialyzed for prolonged time against distilled water after the neutralization of the ion-bound products with alkali.

The elemental analysis was performed using the 2000 FLASH 2000 elemental analyzer (Thermo, UK). The calculation of the acylation level (AL) was carried out according to changing the C/N ratio in the samples with respect to the original chitosan. The IR spectra were registered using a FTS40 spectrometer with the resolution of 4 cm^{-1}. The registration and processing of the spectra was performed using a Win-IR version 4 software package (Bio-Rad, Digilab Division). The spectra of all the products under investigation containing chitosan were normalized using a complex band of stretching C–O vibrations in the pyranose ring 1075 cm^{-1} as an internal standard [17].

In order to obtain hydrogels, freezed 1.5–2 % solutions of chitosan and its derivatives, with further dried at −5 °C under vacuum in the frozen state and then heated to obtain insoluble species. The investigation of the in vitro degradation kinetics of hydrogels at 37 °C was carried out in the presence of lysozyme (2 mg/mL).

RESULTS AND DISCUSSION

The work was aimed at evaluating the efficiency of a non-catalytic reaction of the acylation of polysaccharide functional groups in the interaction with lactide, the cyclic dimer of lactic acid. Basing on the difference in the nucleophilicity level of the hydroxyl and amino groups of the chitosan in the absence of catalysis, grafting the *L,D*-lactide to chitosan should occur according to the following scheme proposed (Fig. 1). It can be seen that the reaction consists in the nucleophilic addition of the primary amino group to the lactide cycle and the subsequent polymerization of dilactone owing to joining the substituent hydroxyl group formed it the course of ring-opening to the strained ring of the monomer. The primary amine cannot initiate the anionic polymerization of the monomer, since being already in the transition state it exhibits quickly transferring the proton to outgoing hydroxyl anion.

According to data presented in Table 1, the reaction product yield, depending on the processing conditions ranges within 57–96 %. Therewith the behaviour of the samples obtained in chloroform traditionally used as a solvent for the polyester, depends on the content of lac-

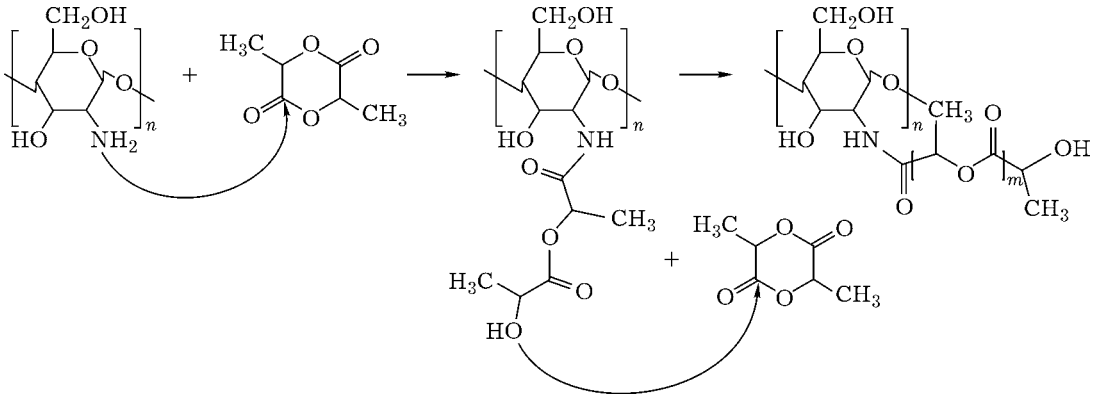

Fig. 1. Reaction scheme of *L,D*-lactide copolymerization with chitosan.

tide in the initial mixture. The samples prepared in the case of a molar deficiency of lactide (samples Nos. 1 and 2) form a relatively stable suspension in chloroform, wherein the original chitosan is insoluble and does not swell. In case of increasing the content of lactide in the mixture up to a 3-fold molar excess (sample No. 6) the sample uniformly swells in chloroform to form a gel. The observed behaviour of the resulting products in the hydrophobic environment means that there occurs profound modifying the chitosan macromolecules with grafting the oligomeric lactide chains under the chosen conditions of solid phase synthesis.

The IR spectra of the samples purified from the unreacted monomer (Fig. 2, *a*), exhibit overlapping the range of amino/amido groups by envelopes those are formed by the superposition of amide I and II bands (1650 and 1550 cm^{-1}, respectively), the bands of deformation vibrations inherent in chitosan amino groups (1600 cm^{-1}) and the stretching vibrations of carboxylate ions. The band at 1383 cm^{-1} belongs to the symmetric vibrations of COO^- groups. Occurring the absorption bands of amide I and II and increasing the intensities thereof with increasing the coextruding temperature indicates the reaction of the aminolysis of lactide cycle ester bonds to proceed efficiently. This is also confirmed *via* changing the C/N ratio, according to the results of elemental analysis (Table 2). The presence of intense absorption bands of the ester group at 1755 cm^{-1} in the sample spectra those could be attributed to the vibrations of carbonyl groups in the side chains of lactide, indicates that the lactide is bound with chitosan is presented at least in the form of a dimer.

The IR spectra of the samples after prolonged dialysis (see Fig. 2, *b*) exhibit a weak shoulder within the range inherent in the ester group (1735 cm^{-1}), that is absent in the spectrum of the original chitosan, which indicates that the reaction of grafting proceeds partially *via* O-acylation route. However, the contribution of this reaction in the formation of the synthesis products is negligible. The intense band of Amide I in the spectra of the products does not allow resolving the band of the ester bonds to a complete extent. Thus, under the purification conditions chosen, an almost selective hydrolysis of the ester groups in grafted chains occurs, which allowed us to calculate the content of the amide bonds those representing the main units of grafting (see Table 2). It has been found that these values are minimal, since the

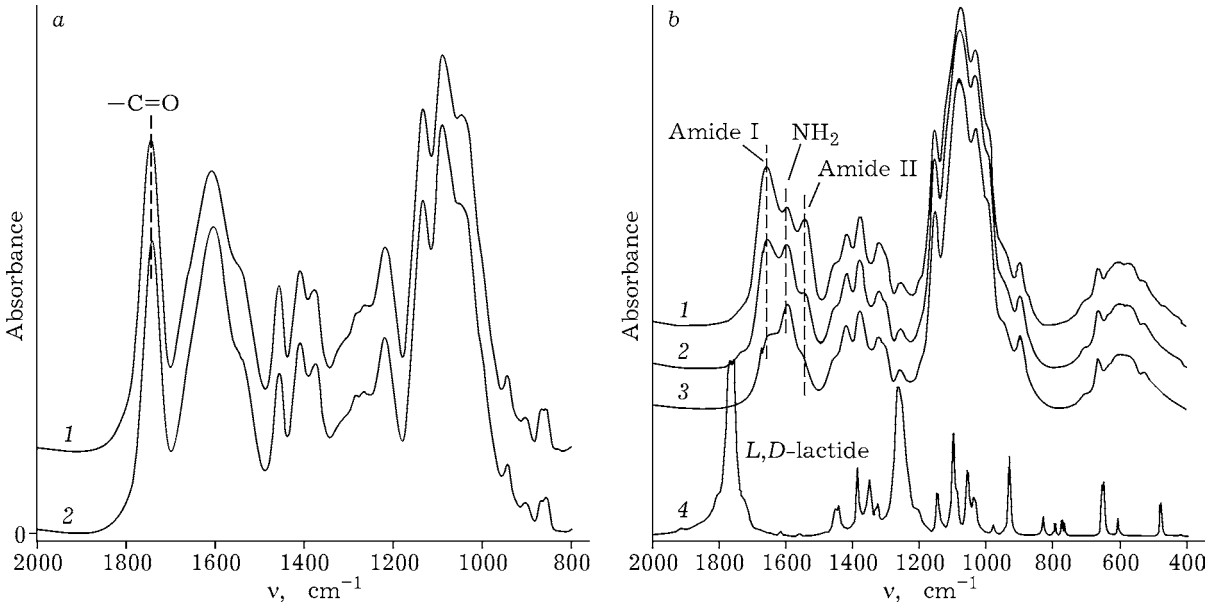

Fig. 2. IR spectra of chitosan/lactide samples obtained at an equimolar ratio between the components at 80 and 90 °C after extracting the unreacted lactide with chloroform (*a*) and after dialyzing against distilled water (*b*): *1*, *2* − the samples after the treatment at the temperature of 90 and 80 °C, respectively, *3* − chitosan, *4* − *L*,*D*-lactide.

TABLE 2

Elemental analysis data and the level of acylation of the amino groups in chitosan

Sample No	Concentration, at. %			C/N	Content of lactic acid units, mass %	Acylation level of the chitosan amino groups	Average PL of grafted chains
	C	H	N				
Chitosan	44.70	6.8	8.70	4.57	0	–	–
1	44.26	6.83	7.86	5.63	9.7	0.19	5.0
2	44.39	6.87	7.91	5.61	9.1	0.19	3.0
3	43.81	6.88	7.89	5.55	9.3	0.19	7.8
4	41.92	6.79	6.65	6.30	23.5	0.41	4.1
5	42.86	6.61	6.65	6.44	23.5	0.41	4.2
6	44.66	6.75	6.84	6.53	21.1	0.37	9.7

Note. PL – polymerization level.

competitive hydrolysis of the amide groups in the course of dialysis cannot be avoided.

From Table 2 one can see that an increase in the treatment temperature by 10 °C only (samples Nos. 3 and 4) results in the fact that the yield of the products of N-acylation reaction is doubled, which is consistent with the analysis of the IR spectra of the samples. The same effect is exerted by increasing the concentration of lactide in the reaction mixture at the same temperature of deformation (sample Nos. 1 and 4). Therewith, increasing the content of lactide up to a 3-fold molar excess (sample No. 6) does not lead to further increasing the acylation level (AL) of amino groups in chitosan. Increasing the synthesis temperature over 90–100 °C results in reducing the yield of target reaction products and the level of efficient reagent utilization (see Table 2, samples Nos. 1 and 2, Nos. 4–6). Thus, the efficiency of mechanochemical process is reduced in the case of a relatively high content of "mechanically soft" thermoplastic component. The effective temperature of the synthesis is approximately equal to $0.7T_{m.p}$ of lactide.

In order to attain a high level of lactide grafting to the chitosan, the synthesis of sample No. 6 was carried out in two stages: first, the formation of grafting units at a molar deficiency of lactide (sample No. 2) occurred, then the treatment was performed with the additional calculated amount of the reagent at the temperature close to the melting point thereof, which is required in order to create local areas with a high concentration of the lactide in the reaction mixture. The gel-chromatographic analysis of the sample fractions soluble in chloroform demonstrated the presence of a broad shoulder within the range high molecular mass values, that is absent in the chromatographic profile of the original lactide. This indicates that the process of the lactide homopolymerization occurs at elevated mechanosynthesis temperatures. As a consequence, the level of grafting the lactide units onto chitosan exhibits an increase up to 160 %, and the length of the grafted chains increases.

The calculation of the average chain length from comparing the data concerning the molar content of the lactic acid units in the fractions purified of unreacted lactide and amide bonds in hydrolyzed fractions (see Table 2) demonstrates that the level of polymerization (LP) of the grafted chains is low. Thereby the LP appropriately decreases at lower lactide content in the starting mixture and with an increase in the AL of amino groups in chitosan. It is obvious that an excess of chitosan amino groups in reaction mixtures, a significant contribution is drawn by the reaction of graft chain aminolysis, which results in a two-fold increase in the level of substitution, and in a two-fold shortening of the side chain. However, since the six-membered ring of lactide is strained (excess energy as compared to linear analogs being equal to 10 kcal/mol), the aminolysis reaction rate constant for the substituent formed is lower than that of the target reaction of polymerizing the lactide onto chitosan.

It follows from the data concerning the fractional analysis in aqueous media, that increasing the treatment temperature up to 100 °C in

case of equimolar ratio between the components does not affect the yield of the reaction product (sample No. 5). However, it leads to the loss of the sample solubility (up to 37 % with respect to the total mass and 70 % as calculated for the chitosan content in the samples) due to the processes of the polymer branching and partial crosslinking. Samples Nos. 1 and 2 obtained with a molar deficiency of lactide also contain a water-insoluble fraction (38 %), but its presence is associated with a low AL of amino groups in the chitosan. This is confirmed by the fact that the samples exhibit an almost complete dissolution in case of acidifying the medium up to pH 6. Increasing the level of grafting the lactide units onto chitosan in the sample No. 6 results in the fact that the modified chitosan swells in chloroform, thereby its solubility in aqueous media, both at the neutral and the acidic pH values appropriately decreases. The content of the water-insoluble fraction in sample No. 6 is equal to 62 % with respect to the mass of chitosan in the sample.

All experiments revealed a general pattern: the lipophilicity level of the system increases with increasing the level of substitution. However, owing to the fact the system of copolymer grafted chains is characterized by a complicated statistical length distribution of the original and grafted molecules, as well as grafting sites distribution in the macromolecules of substrate, the properties of such molecular systems are rather manifold. Among them one could consider the following: solubility and swelling in chloroform; solubility in acidic aqueous environments; the presence of a fraction soluble in neutral water (therewith containing chitosan). The ability of dissolving and swelling in chloroform is provided by the length of the ester linkage to the chitosan and by the number of grafted chains per one chitosan chain. The solubility in neutral water indicates the formation of relatively short chitosan chains containing lactic acid residue (2-hydroxypropionyl). These data confirm the reaction of substituent aminolysis to occur.

This work was also devoted to exploring the potentiality of obtaining different forms of materials from synthesized copolymers for using them in regenerative medicine. For the initial chitosan and the sample No. 4 we chose the conditions for the formation of macroporous polymer hydrogels with pore size sufficient for the diffusion of nutrients and cell growth (80–150 μm in size) (Fig. 3, *a*). It is found that the copolymer hydrogels are completely destroyed within two weeks, whereas the loss of mass of the chitosan sample was equal to 90 % in a month. Therewith, the cell viability of mouse fibroblasts (L929) with the use of copolymer

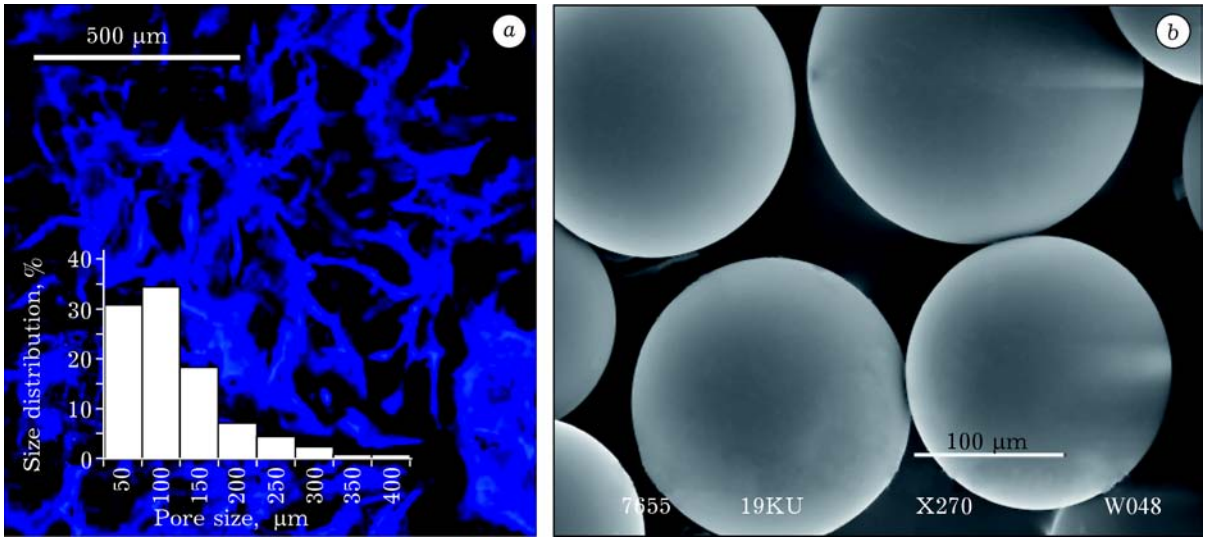

Fig. 3. Different matrix forms for the regenerative medicine based on copolymers of lactide and chitosan: *a* – confocal micrograph of the hydrogel; *b* – micrograph of microparticles obtained using chitosan/lactide samples as an emulsifier.

hydrogels of was higher by (80±4) %. Using the synthesized copolymers as an emulsifier in the aqueous phase have allowed us to obtain spherical polylactide microparticles safe for an organism, those are characterized by a uniform surface and size distribution thereof required for efficient cell growth (see Fig. 3, *b*). They represent some of the most promising types of matrices for tissue engineering such as cell microcarriers.

CONCLUSION

Thus, the interaction between chitosan and L,D-lactide under the conditions of solid phase synthesis leads to grafting the lactide onto the chitosan preferably due to the reaction of chitosan amino group acylation with a level of substitution equal to at least 0.19−0.41, accompanied by polymerizing the lactide and opening the ring. The grafting level of oligolactide in copolymers amounts up to 160 % at the average level of graft chain polymerization amounting to three to ten units. Introducing the short chains of grafted lactide into the chemical structure of chitosan provides the biphility of the system and the chitosan ability of swelling in organic solvents. The resulting copolymers could be successfully used in the development of materials for biotechnology and medicine, since they are characterized by a high biocompatibility level and biodegradation rate.

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