VILNIUS UNIVERSITY

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CATIONIZED AND POLY(ETHYLENE GLYCOL) MODIFIED CHITOSAN DERIVATIVES AND NANOPARTICLES

Summary of doctoral dissertation

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VILNIAUS UNIVERSITETAS

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1. INTRODUCTION

Relevance of the work. As natural biomaterials, polysaccharides are highly stable, safe, non-toxic, hydrophilic and biodegradable. In addition, polysaccharides have abundant resources in nature and low cost in their processing. It is common opinion that the use of polysaccharides in chemical industry should grow rapidly in the near future. It is important to point that polysaccharides can be used for production of environmentally friendly biosynthetic polymers with desirable properties.

Chitosan is a cationic natural biopolymer produced by alkaline *N*-deacetylation of chitin, the most abundant natural polymer after cellulose. Chitosan and its derivatives are used in various fields, such as biomedicine, cosmetics, food industry, agriculture, etc. However, applications of chitosan are limited by poor solubility. It is soluble in acidic aqueous solutions only where the amino groups are protonated. Chemical modifications of chitosan are widely used to obtain its derivatives suspecting that the derivatives will preserve original physicochemical and biochemical properties of chitosan and get new properties depending on the nature of the introduced groups.

Recently prepared comb-like chitosan derivatives containing methoxy poly(ethylene glycol) (MPEG) grafts may find application in household and personal care products, they are interesting as dispersing agents, solubilization aids, surface conditioners, and drug carriers. However, purification of chitosan-MPEG graft copolymers from unreacted oligomeric MPEG is a serious problem since neither dialysis nor gel-filtration gives good results. The use of “click” chemistry which has been used for a variety of selective conversions in the recent years, can help avoiding these problems. “Clicking” of MPEG on chitosan should allow obtaining graft copolymers with predetermined composition and likely reduce the problems related to purification of the products. Additional cationization of chitosan introducing quaternary ammonium groups is one of methods to prepare water soluble chitosan derivatives carrying permanent charges. Cationization of chitosan through hydroxyl groups should allow obtaining cationized chitosan derivatives containing primary amino groups or chitosan derivatives with very high density of quaternary ammonium groups. The cationized chitosan has high moisture-retention capacity, superior bioadhesive properties, permeation enhancing effects and antimicrobial properties even at neutral conditions.

Nanofibres webs from cationized chitosan derivatives are excellent candidates for many applications: in medical textile (wound dressing, medical prothesis, drug delivery), filters, composites, protective clothes, etc. Nanoparticle carriers made of chitosan could prolong the residence time and therefore increase the absorbance of loaded drugs.

The main aim of this work was to synthesize water-soluble chitosan – methoxy poly(ethylene glycol) graft copolymers and cationized chitosan derivatives of varying degree of substitution and desirable structure, and to study their properties.

The objectives of the research are the following:

- to synthesize chitosan-MPEG derivatives by "click" chemistry method;
- to study chitosan-MPEG copolymers and estimate correlation between synthesis pathway, structure and properties of the copolymers;
- to prepare cationized chitosan and chitosan-*N*-MPEG copolymers, and produce nanofibers from the cationized chitosan; to prepare nanoparticles of chitosan and modify their surface with a RAFT chain transfer agent.

Scientific novelty and practical value of the work. Chitosan – methoxy poly(ethylene glycol) derivatives containing triazolyl moiety (chitosan-TMPEG comb copolymers) were prepared for the first time by "click" chemistry. Several new schemes of the synthesis of chitosan-*C(6)*-TMPEG and *C(6)*-cationized chitosan derivatives were suggested based on protection of amino functionality by using chitosan-dodecyl sulfate complexes. *N*-Phthaloyl chitosan derivatives containing azide or propargyl moieties at C(6) position of glucosamine units were synthesized for the first time useful as precursors for modification of chitosan via "click" chemistry reactions. Additional cationization of partially cationized chitosan through its hydroxyl groups in alkaline media enabled to prepare *N,O*-cationized chitosan derivatives with very high charge density. A method of enzymatic degradation of the cationized chitosans was proposed which allowed a tenfold decrease of the molecular weight of the chitosan derivatives.

The results presented in the dissertation enable to defend the following most important ***statements***:

- Comb copolymers chitosan-TMPEG with degree of substitution of chitosan equal to degree of azidation of chitosan were synthesized using "click" chemistry significant breakdown of chitosan backbone took place under this reaction, however.

High degree of cationization of chitosan can be achieved by two-step procedure: cationization of chitosan in acidic medium should be followed by similar procedure in alkaline medium. ***Approbation of the research results***. The results of the research have been presented in 15 scientific publications including 2 papers in the journals from the ISI Web of Science list and 1 paper in the reviewed Lithuanian journal. The results of the work have also been reported in 2 national and 10 international conferences.

Structure of the dissertation. The dissertation consists of introduction, tree chapters, conclusions, list of references and the list of original publications. The material of the dissertation is presented in 153 pages including 37 figures, 35 schemes and 20 tables.

2. EXPERIMENTAL

Main materials. Chitosan (Chs) (M_r 400 000, degree of deacetylation 72%) and poly(ethylene glycol) monomethyl ether (MPEG) (M_r 2000) were purchased from ALDRICH or FLUKA. Propargyl bromide (80 wt.% in toluene), sodium azide and sodium dodecyl sulfate (SDS) were obtained from FLUKA. Sodium hydride (60% dispersion in mineral oil), 2,3-epoxypropyl trimethyl ammonium chloride (Glycidyl trimethyl ammonium chloride) (EPTMAC) (technical, ≥90%) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from ALDRICH. Tris-(hydroxymethyl) aminomethane (TRIS) was obtained from APPLICHEM. All other reagents and solvents were of analytical grade and used without further purification.

Methods. Chitosan derivatives and intermediate products were examined using FT-IR (*PERKIN ELMER Spectrum BX*) spectrometer under a dry air at 20 °C by a KBr pellet method. The ^{1}H NMR spectra were acquired on *UNITY INOVA VARIAN* spectrometer at 300 MHz and 29 °C. The copolymer samples were prepared in D_2O/DCl.

Polymer molecular weights were estimated using size exclusion chromatography (SEC) instrument: Deltachrom pump (Watrex Comp.), autosampler Midas (Spark Instruments), two columns with PL gel MIXED-B LS (10 μm), a light-scattering photometer DAWN DSP-F (Wyatt Technology Corp.), a modified differential viscometer Viscotek model TDA 301 and a differential refractometer Shodex RI 71. Acetate buffer was used as a mobile phase at flow-rate 0.75 cm^3/min. The injection-loop volume was 0.1 cm^3.

Homogeneous solutions of the cationized chitosans were irradiated by 250 W UV lamp varying concentration of the cationized chitosan, volume of the sample and a distance from the lamp to the quartz tube.

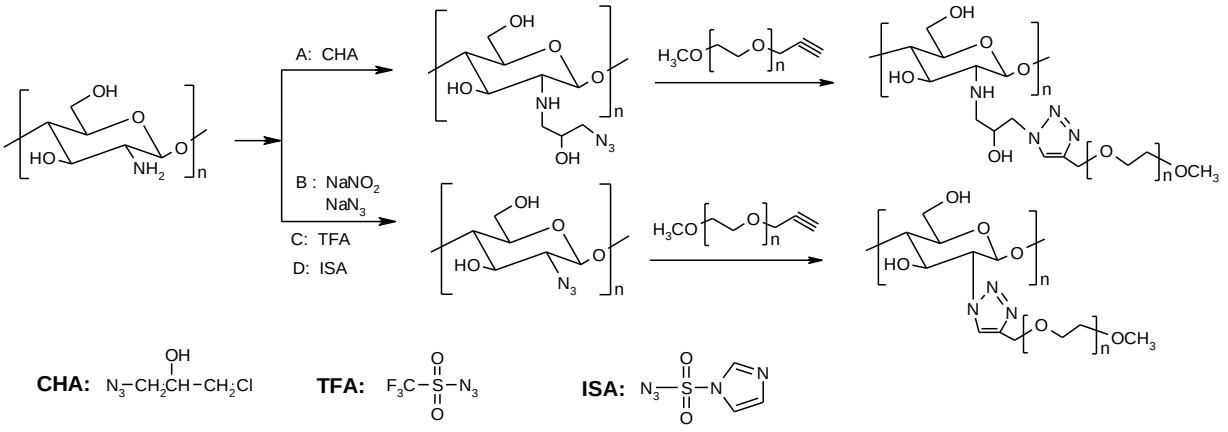
pH-Potentiometric titrations were carried out in aqueous solutions using a CyberScan pH6000 pH-meter with a glass electrode. Intrinsic viscosity of copolymer solutions in distilled water and in acetate buffer (aqueous 0.5M CH_3COOH/0.5M CH_3COONa) at 25 °C was measured using dilution type Ubbelohde viscometer.

3. RESULTS AND DISCUSSION

3.1. „Activation" of chitosan and MPEG

To exploit the copper-catalyzed Huisgen reaction, either chitosan or MPEG needs to contain azide moieties, and the second reagent needs to contain alkyne moieties.

N-Azidation of chitosan. *N*-azidated chitosan was prepared by four different methods: using chlorohydrin azide (CHA) (I), sodium azide and sodium nitrite (II), triflyl azide (TFA) (III) or imidazole-1-sulfonyl azide hydrochloride (ISA) (IV) (Scheme 1). Exploring all the methods, large excess of an azidation reactant was used in order to achieve maximal degree of azidation (DA) of chitosan.



Scheme 1. *N*-MPEGylation of chitosan via "click" chemistry reactions

Azidation of chitosan by the use of chlorohydrin azide (CHA) (I) resulted in the products which were soluble in acidic water only. The presence of azide moiety in the azidated chitosan was confirmed by the presence of absorption band at 2110 cm^{-1} in FT-IR spectra. Regardless of the ratio of the reactants, DA of chitosan was close to 25% (Table 1).

Azidation of chitosan according to the second pathway includes the reactions with sodium azide and sodium nitrite which, unfortunately, is a common depolymerizing agent. Although very small concentrations of sodium nitrite were used and the reaction was carried out at low temperature and for short time, depolymerization of chitosan apparently took place. Depolymerization of chitosan was evaluated by intrinsic viscosity which dropped more than 4 times from 8.40 dL/g for chitosan to 2.07 dL/g for azidated chitosan.

Table 1. Azidation of chitosan by various reagents

Azidating reagent	N, %	NH_2, %	DA (N), %	DA (NH_2), %	DA (FT-IR), %
CHA	11.5	3.5	25.7	27.4	28.4
NaN_3 and $NaNO_2$	8.9	5.8	5.2	8.6	-
TFA	13.7	2.7	40.1	41.1	40.3
ISA	-	-	-	-	64.4

Azidation of chitosan according to the third pathway is based on the use of trifluoromethanesulfonyl (triflyl) azide (TFA). The reaction between freshly prepared TFA and chitosan proceeded at room temperature in the presence of Cu (II) as a catalyst (Scheme 1). The absorption band at 2110 cm^{-1} in FT-IR spectra of chitosan azidated with TFA was more intensive than that of chitosan azidated with CHA (Fig. 1), and the degree of azidation was over 40% (Table 1). Nevertheless, full azidation of chitosan was not reached despite optimization of the azidation conditions.

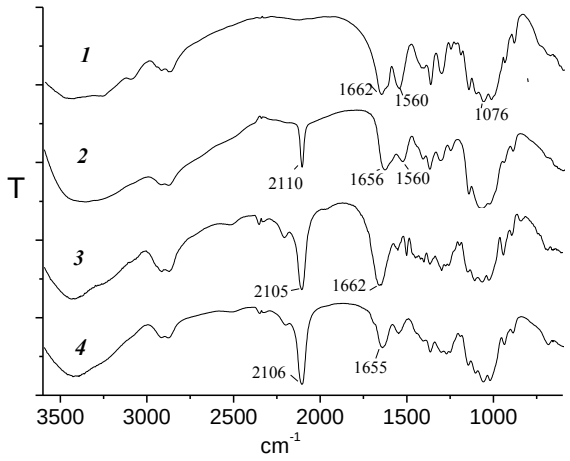


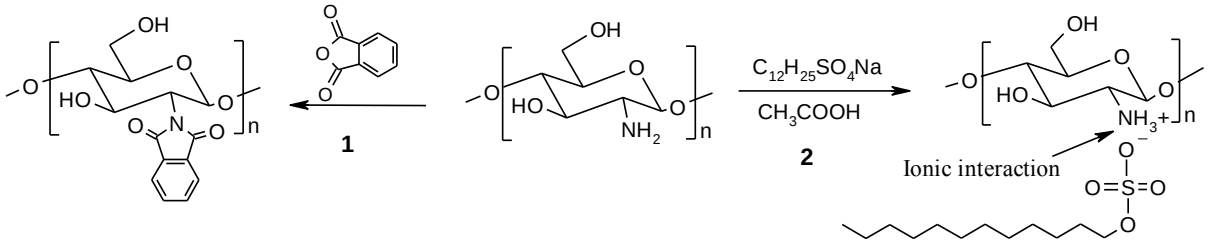
Fig. 1. FT-IR spectra of chitosan (1), chitosan azidated with CHA (DA 27%) (2), TFA (DA 41%) (3) and ISA (DA 65%) (4)

Azidation of chitosan according to the procedure D is based on the use of imidazol-1-sulfonyl azide hydrochloride (ISA). Azidation of chitosan by ISA depends on

excess of this reactant as well as nature and amount of the alkaline compound regulating basicity of the medium. According to FT-IR spectra, maximal degree of azidation of chitosan reached by the use of ISA was 65%. Unfortunately, highly azidated chitosan derivatives were insoluble (or partially soluble) in water at various pH and in common organic solvents. They were soluble in 5% LiCl solution in *N*-methyl-2-pyrrolidoneonly.

Protection of chitosan functionalities. Higher reactivity of amino groups present in the mainchain of chitosan requires protection to limit their participation in reactions under modification of chitosan. This was done by the use of phthalic anhydride or chitosan – dodecyl sulfate complexes (CDC) (Scheme 2). CDC were prepared by simply mixing acidic aqueous chitosan and sodium dodecyl sulfate solutions at equimolar amounts of the components.

In both cases, chitosan derivatives with full protection of amino functionalities soluble in several organic solvents were obtained, and these intermediate compounds were suitable for *O*-modification of chitosan.



Scheme 2. Protection of chitosan amino groups by the use of phthalic anhydride (1) and chitosan – dodecyl sulfate complexes (2)

Dodecyl sulfate from CDC could by removed by pouring CDC solution in DMSO into concentrated *Tris* aqueous solution (>5%, pH 8.0) where the complex dissociate completely into its components.

N-Phtaloyl chitosan as a precursors for the synthesis of *C(6)*-derivatives of chitosan has some drawbacks associated with partial destruction of the products under protection-deprotection procedures whereas the complexation/ dissociation processes are expected to be less invasive.

C(6)-functionalized derivatives of chitosan with protected amino groups. *N*-Phthaloyl chitosan derivatives containing azide or propargyl moieties at *C(6)* position of glucosamine units were synthesized for the first time useful as precursors for

modification of chitosan via “click” chemistry reactions. *C(6)*-azidated chitosan was prepared through *C(6)*-tosylation of *N*-phthaloyl chitosan and further reaction with sodium azide. The FT-IR spectrum of *C(6)*-azidated chitosan showed a significant absorption at 2110 cm^{-1} typical for the azide moiety, and the degree of azidation of chitosan was 57%.

Another pathway of the preparation of *C(6)*-azidated chitosan was “activation” of CDC during which the complex was dissolved in 4% LiCl/DMA solution and reacted with excess *N*-bromosuccinimide, toluene-4-sulfonyl chloride (tosyl chloride) or trichlorotriazine followed by azidation of the “activated” CDC. Unfortunately, azidation of CDC was not full which was confirmed by characteristic absorption bands in FT-IR spectra at 1159 cm^{-1} and 815 cm^{-1} attributed to residual tosyl groups.

„Actyvation“ of MPEG. Alkyne containing derivative of methoxy poly(ethylene glycol) (MPEG) was synthesized by reacting MPEG with propargyl bromide in the presence of sodium hydride as a basic catalyst. Degree of alkylation of MPEG (%) according to ^{1}H NMR spectrum was over 95%.

MPEG azide was prepared by mesylation of MPEG followed by nucleophilic substitution using sodium azide. Mesylation of MPEG proceeded with high yields (≈98%). The azidation progress was followed by FT-IR spectroscopy according to the intensity of the absorption band at 2105 cm^{-1} attributed to the azide group. The degree of azidation of MPEG was determined from elemental analysis (nitrogen content) and was 65%.

3.2. Synthesis and study of chitosan-N-TMPEG graft copolymers

A series of chitosan-MPEG derivatives containing triazolyl moiety (chitosan-*N*-TMPEG comb copolymers) differing in degree of substitution (DS) of chitosan were synthesized by reacting *N*-azidated chitosan with acetylene-terminated MPEG at mild conditions (Scheme 1).

Chitosan-*N*-TMPEG copolymers were obtained by coupling via 1,3-dipolar cycloaddition between pendant azide and end alkyne groups of chitosan and MPEG, respectively, using $CuSO_4 \cdot 5H_2O$ as a catalyst and sodium ascorbate as a reductant. The reaction of “clicking” between azidated chitosan and MPEG alkyne was carried out at

40 ºC in the mixture of water and methylene chloride (1/1, v/v). The results of MPEGylation of chitosan via "click" chemistry are summarized in Table 2.

Table 2. The results of the analysis of chitosan-*N*-TMPEG copolymers

No	Azidator of Chs	Molar ratio*		DS, % (NH_2)	DS, % (NMR)	[η], dL/g	$M_w \cdot 10^{-3}$	$M_n \cdot 10^{-3}$	Cu, ppm
		Chs azide	MPEG						
1	CHA (DA 27%)	1	0.2	15	13	1.52	-	-	-
2		1	0.3	21	20	1.41	21.2	8.9	157±3
3		1	0.4	28	26	0.58	18.9	7.9	86±1
4	TFA (DA 40%)	1	0.4	35	32	0.77	44.8	10.5	75±2
5		1	0.5	41	40	0.49	-	-	-

* - Molar ratio of chitosan units and MPEG alkyne in the reaction mixture

^{1}H NMR spectra confirmed formation of chitosan-*N*-TMPEG copolymers (Fig. 2). The presence of a weak singlet assigned to the proton of the triazole ring at δ 7.91 ppm evidenced that the attachment of MPEG to chitosan backbone was realized through triazolyl-containing intermediate.

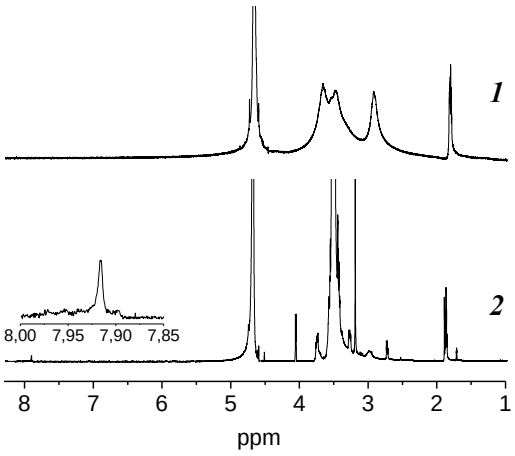


Fig. 2. ^{1}H NMR spectra of chitosan (1) and chitosan-*N*-TMPEG copolymer (DS 26%) (2) in D_2O

Surprisingly, most chitosan-*N*-TMPEG copolymers were insoluble in water irrespective of DS of chitosan but were soluble in acetate buffer (pH 3.7). According to the values of intrinsic viscosity in acetate buffer, the copolymers chitosan-*N*-TMPEG were high-molecular-weight products. SEC measurements of the chitosan derivatives, however, revealed that significant breakdown of chitosan backbone took place under

"clicking" of MPEG resulting in the copolymers with M_w ca $20 \cdot 10^3$-$40 \cdot 10^3$ and M_n about $10 \cdot 10^3$ (Table 2). These values are one-two orders lower than those expected from derivatization of chitosan with M_w $305 \cdot 10^3$. SEC eluograms of chitosan-*N*-TMPEG copolymers were bimodal containing the second peak at about 18.5 ml which corresponded to the fraction with molecular weight $5 \cdot 10^3$-$7 \cdot 10^3$ (Fig. 3). The breakdown of chitosan backbone under "clicking" in the presence of Cu(II)/ascorbate is consistent with recent findings and is rationalized as being mediated by the hydroxyl radicals (•OH) formed *in situ*.

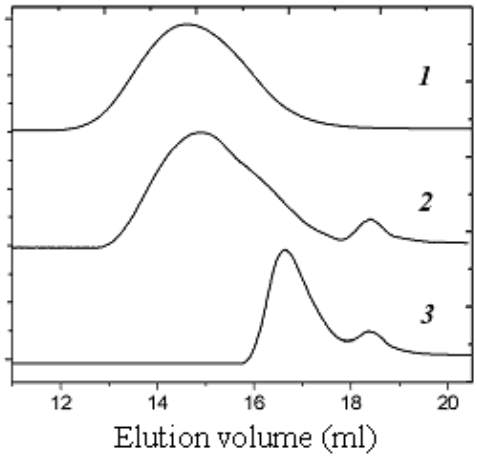
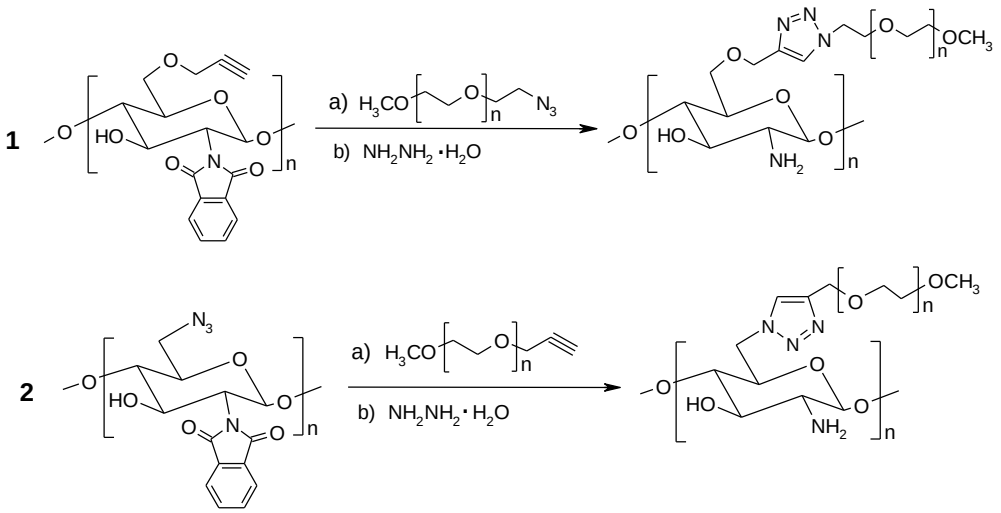


Fig. 3. SEC traces of chitosan (1) and chitosan-*N*-TMPEG copolymers (2, 3) obtained by using TFA (2) and CHA (3) azidated chitosan

Chitosan-*N*-TMPEG copolymers prepared in the present study contained moderate amount of Cu (Table 2). Nevertheless, even this residual amount of Cu could be a drawback when using chitosan-*N*-TMPEG copolymers in biotechnology or in biomedicine.

3.3. Synthesis and study of chitosan-C(6)-TMPEG copolymers

A series of chitosan-*C(6)*-TMPEG comb copolymers differing in DS of chitosan were synthesized by "click" chemistry. Copolymers were obtained by coupling via 1,3-dipolar cycloaddition between *C(6)*-azidated chitosan and propargyl-terminated MPEG or *(C6)*-propargyl chitosan and azidated MPEG at mild conditions as shown in Scheme 3.



Scheme 3. Synthesis of chitosan-*C(6)*-TMPEG copolymers by "click" chemistry using *C(6)*-propargyl chitosan (1) and *C(6)*-azidated chitosan (2)

The results of MPEGylation of chitosan via "click" chemistry are summarized in Table 3. DS of chitosan in its MPEGylated derivatives synthesized via the use of *C(6)*-azidated chitosan was in the range 20 to 64% while intrinsic viscosity of these copolymers varied between 0.102 and 0.132 dL/g. Characteristics of the reaction products are similar despite different precursors used in the two pathways. ^{1}H NMR spectra confirmed formation of chitosan-*C(6)*-TMPEG copolymers.

Table 3. Parameters of chitosan-*C(6)*-TMPEG copolymers prepared via "click" chemistry

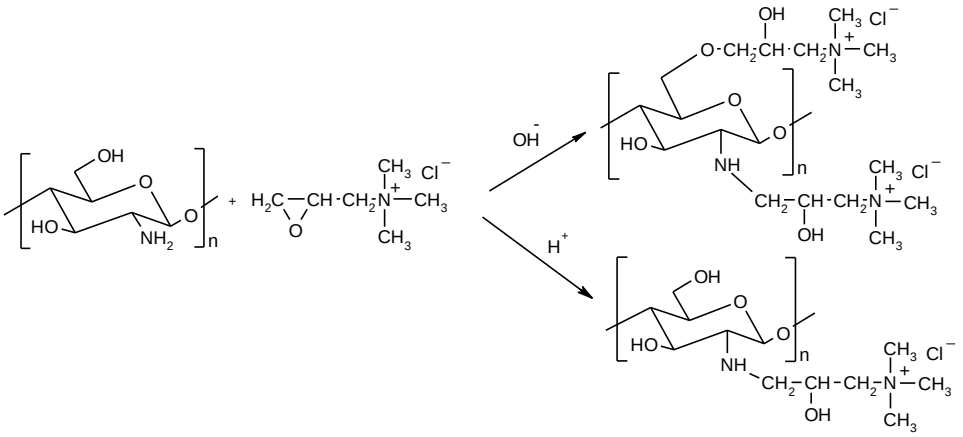
No	Molar ratio		NH_2, %	DS, %		[η], dL/g
	C(6)-Azido-Chs (DA 57%)	MPEG alkyne (DS 93%)		NH_2	NMR	
1	1	0.5	1.10	42	39	0.126
2	1	1	0.80	61	59	0.109
	C(6)-propargyl Chs (DS 90%)	MPEG azide (DA 65%)				
3	1	0.4	1.72	24	20	0.132
4	1	1	0.75	66	64	0.102

According to the values of intrinsic viscosity in acetate buffer, the copolymers chitosan-*C(6)*-TMPEG were low-molecular-weight products. Chitosan-*C(6)*-TMPEG copolymers prepared using "click" chemistry reactions and isolated through rotary evaporation and vacuum drying were insoluble in water irrespective of DS of chitosan. All the samples were soluble in acetate buffer (pH 3.7).

An alternative pathway of the synthesis of chitosan-*C(6)*-TMPEG copolymers via "click" chemistry involving azidation of chitosan – dodecyl sulfate complexes was unsuccessful giving virtually no graft copolymers.

3.4. Cationization of chitosan and chitosan-N-MPEG copolymers

Cationization of chitosan by the use of EPTMAC. The reaction of cationization of chitosan was carried out in acidic, neutral or alkaline media at various ratios of (2,3-epoxypropyl)trimethylammonium chloride (EPTMAC) to chitosan and resulted in *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) (Scheme 4).



Scheme 4. Cationization of chitosan by EPTMAC

The degree of cationization of chitosan (DC) depends on the reaction conditions such as reaction time, reaction temperature, and molar ratio of EPTMAC to amino groups of chitosan. Maximal DC of chitosan reached in acidic conditions was about 70%. Successful cationization of chitosan was confirmed by elemental analysis and FT-IR and ^{1}H NMR spectra of the products.

Table 4. The results of cationization of chitosan by EPTMAC

No	Reaction medium	EPTMAC :Chs, mol	NH_2, %	DC_{NH2}, %	Cl, %	DC_{Cl},	η_{red}*, dL/g
1	Acidic	1	2.65	35	5.61	36	10.4
2		6	0.24	68	9.06	72	3.69
3	Neutral	1	3.04	30	4.86	29	11.4
4		4	0.61	62	8.16	61	6.32
5	Alkaline	4	5.08	11.5	0.94	4.8	4.22
6*		4	-	-	14.5	185	3.41
7*		4	-	-	9.9	84	4.68

* – C = 0.3%

6* Additional cationization of *N*-HTCC (No 2 in Table 4)

7* Additional cationization of *N*-HTCC (No 4 in Table 4)

Though acetic acid usually acts as a catalyst, the cationization of chitosan in aqueous dispersions at neutral pH was successful as well, and sufficiently high DC of chitosan was reached during relatively short time (Table 4). Reduced viscosity η_{red} of *N*-HTCC prepared from commercial medium molecular weight chitosan varied from 11.4 to 3.69 dL/g declining at higher DC (Table 4).

Unfortunately, cationization of chitosan in alkaline media did not prove itself since DC of chitosan hardly exceeded 10% (Table 4).

An attempt was made to increase DC of chitosan by additional cationization of *N*-HTCC synthesized in acidic or neutral conditions. Additional cationization of *N*-HTCC enabled to increase significantly DC of chitosan (up to 190%). Since degree of deacetylation of chitosan was 72%, one could expect that in both cases partial cationization of chitosan through hydroxyl groups took place resulting in *N,O*-[(2-hydroxyl-3-trimethyl-ammonium)propyl] chitosan chloride (*N,O*-HTCC). Thus, additional cationization of *N*-HTCC through its hydroxyl groups is an efficient method to be used for the cationized preparation of chitosan derivatives with very high charge density.

O-cationization of chitosan. Cationization of chitosan exclusively through its hydroxyl groups requires protection of more active amino groups of chitosan. For protection of amino functionality of chitosan, chitosan – sodium dodecyl sulfate (SDS) complexes (CDC) were used which were solubilized in DMSO and reacted with excess amount of EPTMAC. SDS from the cationized chitosan was removed by the use of a strong base of *TRIS* which acted as a decomplexing agent.

Table 5. Cationization of chitosan by the use of SCC as an intermediate

No	Reaction medium	EPTMAC: CDC, mol	Cl, %	DC_{Cl}, %	DC_{NMR}, %	[η], dL/g
1	DMSO/H_2O (1:0,8 v/v)	4	3.33	19	37	4.01
2	DMSO/ 2-PrOH (1:0,8 v/v)	4	3.34	19	32	4.03
3		6	4.59	28	42	3.88

In ^{1}H NMR spectrum of *O*-HTCC (Fig. 4), the characteristic signals at 0.86, 1.25 and 3.69 ppm assigned to ($\underline{CH_3}$), (-($\underline{CH_2}$)$_9$-) and (-$CH_2\underline{CH_2}$-O) protons of SDS, respectively, disappeared confirming that SDS was removed completely. The appearance of strong signals at 3.0–3.2 ppm and at 4.4 ppm attributed to the protons in methyl

groups of the quaternary ammonium and –CH(OH)- protons in residue of EPTMAC, respectively, confirmed successful *O*-cationization of chitosan. Calculation of DC of chitosan was possible from the ratio of the signal at 4.4 ppm attributed to the protons from the –CH(OH)- proton to the signal at 1.8-1.9 ppm attributed to the protons in residual acetyl group of chitosan. Usually, DC of chitosan calculated from NMR analysis was larger than that from elemental analysis.

The results of *O*-cationization of chitosan are summarized in Table 5. According to relatively low values of DC, this method of additional cationization of chitosan did not prove itself. Moreover, *O*-cationized chitosan was insoluble in neutral water.

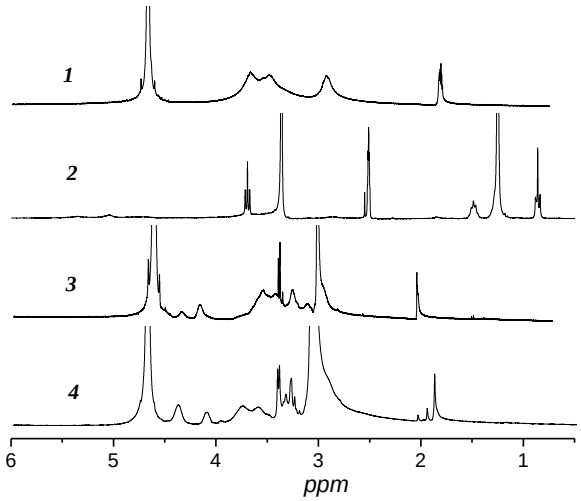


Fig. 4. ^{1}H NMR spectra of chitosan (1), CDC (2), *O*-HTCC (DC 32%) (3) and *N*-HTCC (DC 61%) (4). The spectra of chitosan, *N*-HTCC and *O*-HTCC were recorded in D_2O while that of SCC in DMSO-d_6

Cationization of chitosan-N-MPEG copolymers. Chitosan-*N*-MPEG graft copolymers with DS 20 to 43% synthesized via EDC mediated reaction were cationized with EPTMAC. Despite three-fold tmolar excess of EPTMAC, DC was very low (5%). Likely, long chains of MPEG connected to chitosan impede access of the cationization reagent to the amino groups allocated at the mainchain.

Partial destruction of the cationized chitosans. Viscosity of polymer solutions plays a major role in the production of nanofibers and is one of the most studied parameters in electrospinning. What concerns spinning of chitosan, for the production of

continuous and uniform fibers, optimal concentration of the solutions is 7-7.5% with viscosities ranging from 4.8 to 5.9 P.

It was determined that dynamic viscosity (DV) of 7% solution of *N*-cationized chitosan with DC 60% in 0.5% acetic acid was 338 P while that of the same chitosan derivative with DC 28% even 2930 P. Required viscosity at about 5 P can be achieved by dilution of the above solutions to 2.0-2.8% which is apparently low concentration for the production of nanofibers. It is obvious that the molecular weight of the cationized chitosans is too high for getting solutions with required viscosity.

Partial destruction of the cationized derivatives of chitosan was carried out by UV irradiation or enzymatic hydrolysis. It was determined that UV irradiation helped to decrease DV of the solutions of the cationized chitosans with high DC but cannot be applied for partial destruction of the cationized chitosans in their solutions if degree of cationization is relatively low (DC < 50%) (Fig. 5).

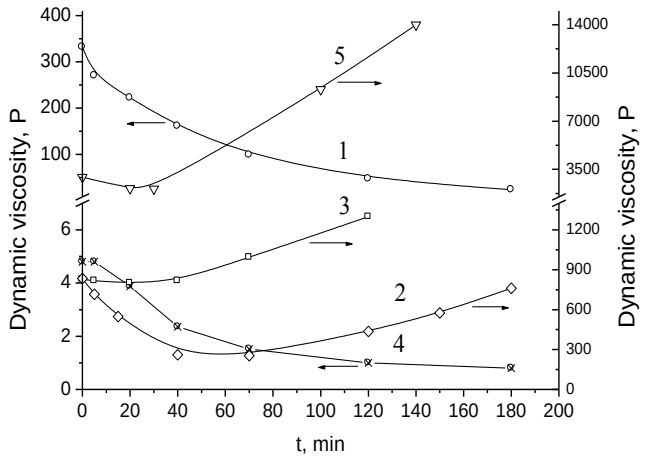


Fig. 5. UV irradiation of 7% solution of *N*-cationized chitosan with DC 50% (1), 40% (2, 3), 68% (4) and 30% (5). The distance from the lamp to the quartz tube was 24 cm (1, 2, 4, 5) and 35 cm (3)

Enzymatic hydrolysis of the cationized chitosans was carried out by the use of pectinase from *Aspergillus niger* or cellulase from *Trichoderma reesi*. Although both enzymes were efficient, a few better results were achieved using cellulase. Enzymatic hydrolysis by cellulase during 2 hrs enabled to reduce DV of 7% solutions of the cationized chitosans with DC 50% to 5 P (Fig. 6).

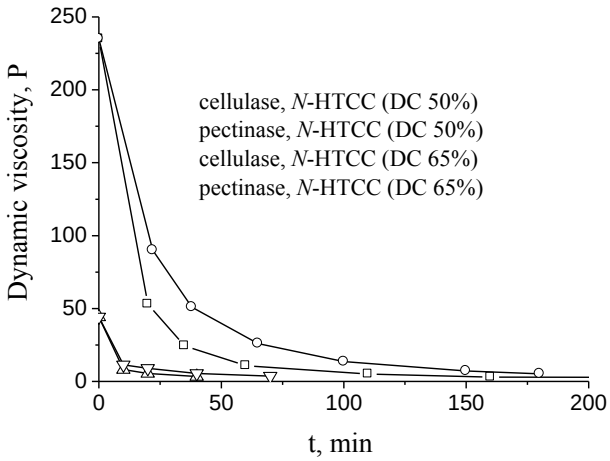


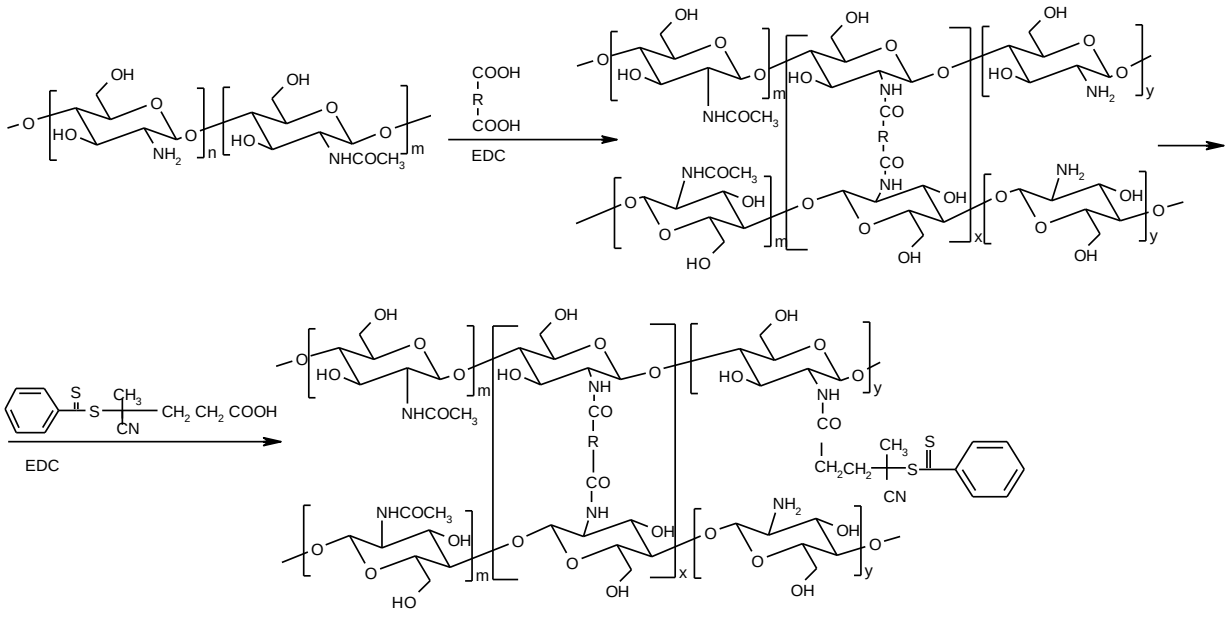
Fig. 6. Dynamic viscosity of 7% solution of *N*-cationized chitosan in acetate buffer versus enzyme type and time of enzymatic hydrolysis at 18 °C

Enzymatic hydrolysis of the cationized chitosan by pectinase or cellulase do not lead to the monomeric units of glucosamine or other low molecular fragments, but it helps a lot in getting polymer solutions with the viscosity prerequisite for electrospinning.

Solutions of enzymatically degraded *N*-cationized chitosan were tested for production of nanofibers by electrospinning. The electrospinning solutions were prepared by mixing 8 wt.% solution of poly(vinyl alchohol) (PVA) with 8 wt.% solution of the cationized chitosan (DC 30%) at weight ratios HTCC:PVA=15:85 to 25:75. More uniform nanofibers were formed from the compositions containing lower content of the chitosan derivative.

3.3. N-modification of chitosan with carboxylic acids resulting in nanoparticles

N-modification of chitosan was carried out by a covalently cross-linking EDC mediated condensation reaction between amino groups of chitosan and carboxyl groups of dicarboxylic (tartaric acid (TA), adipic acid (AA)) or tricarboxylic (citric acid (CA)) acids. Modification of chitosan was done under mixing and in an excess of amino groups over carboxyl groups in order to avoid full cross-linking of the chitosan. Chitosan nanoparticles were further *N*-modified by water soluble RAFT chain transfer agent (4-cyanopentanoic acid)-4-ditiobenzoate (CPAD) (Scheme 5).



Scheme 5. *N*-modification of chitosan with carboxylic acids and CPAD

Chemical structure of the partially cross-linked chitosan derivatives was confirmed by FT-IR and ^{1}H NMR spectroscopy, and DS of chitosan was evaluated by ^{1}H NMR spectroscopy and elemental analysis (N content).

Chitosan-carboxylic acid derivatives were soluble in water when DS of chitosan was between 7 to 22%. At higher DS of chitosan the products were cross-linked and insoluble. Intrinsic viscosity of the soluble chitosan derivatives was less than that of the initial chitosan but still high enough.

Table 6. Results of chitosan modification with carboxylic acids

No	Derivative of Chs	Chs:carboxylic acid, mol	DS (NMR), %	[η], dl/g	$M_w \cdot 10^{-3}$
1	Chs-*N*-AA	1:0.11	10	3.12	294
2	Chs-*N*-AA	1:0.23	15	3.24	502
3	Chs-*N*-TA	1:0.18	22	4.26	189
4	Chs-*N*-CA	1:0.05	7	3.45	289
5	Chs-*N*-CA	1:0,1	17	2.44	434
		Chs*:CPAD			
6	Chs-*N*-AA (DS 10 %)	1:0.18	12	2.12	111
7	Chs-*N*-CA (DS 17 %)	1:0.25	15	-	-
8	Chs-*N*-TA (DS 8 %)	1:0.05	5	2.42	-

Chs* chitosan in its derivative with carboxylic acid

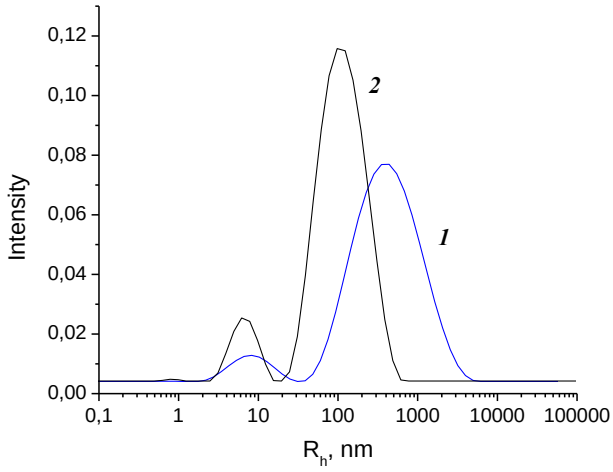


Fig. 7. Size distribution of Chs-*N*-AA (DS 10%) (1) and Chs-*N*-AA (DS 10%)-*N*-CPAD (DS 12%) (2)

Controlled *N*-modification of chitosan by di- and tricarboxylic acids resulted in nanoparticles which aqueous solutions were clear or opalescent stable colloidal systems. Hydrodynamic radius of these nanoparticles determined by dynamic light scattering (DLS) was in the range from 170 to 850 nm (Fig. 7). Beside nanoparticles, colloidal solutions of chitosan – carboxylic acid derivatives contained individual macromolecules with the radius from 8 to 30 nm. Further modification of chitosan – carboxylic acid derivatives by CPAD resulted in nanoparticles with smaller size (Fig. 7).

CONCLUSIONS

1. Modification of chitosan with methoxy poly(ethylene glycol) (MPEG) by "click" chemistry was studied in detail for the first time. Grafting of MPEG onto *C(2)* amino groups or *C(6)* hydroxyl groups of chitosan resulted in several series of chitosan-MPEG comb copolymers differing in molecular weight, graft density and other properties.
2. *N*-azidated chitosan prerequisite for „click" chemistry was prepared by the use of four different azidating reagents. Trifluoromethane sulfonyl azide and imidazole-1-sulfonyl azide hydrochloride were found to be the most suitable reagents for *N*-azidation of chitosan giving the degree of azidation of chitosan up to 40% and 64%, respectively. The use of the second azidating reagent resulted, however, in insoluble in aqueous media azidated chitosan derivatives.
3. Synthesis of chitosan-MPEG copolymers by „click" chemistry is rather well controllable, and the degree of substitution of chitosan (DS) is equal to the degree of azidation of chitosan. Chitosan-MPEG derivatives containing triazolyl moiety (chitosan-*N*-TMPEG comb copolymers) with DS of chitosan varying from 13 to 40% were synthesized via „click" chemistry. Chitosan-*N*-TMPEG copolymers were soluble in acetic media only. Significant breakdown of chitosan backbone took place under "clicking" of MPEG in the presence of Cu(II)/ascorbate catalyst resulting in comb copolymers with molecular weight one-two orders lower than expected.
4. Novel chitosan-*C(6)*-TMPEG copolymers with different DS (20-64%) were synthesized for the first time via "click" chemistry by exploring *N*-phthaloyl chitosan intermediates containing azide or propargyl moieties at *C(6)* position of glucosamine units. Because of destructive processes occurring during protection-deprotection procedures of amino functionality and "clicking", chitosan-*C(6)*-TMPEG copolymers were low-molecular-weight products.
5. In order to avoid degradation of chitosan backbone, for protection of amino functionality chitosan – dodecyl sulfate complexes (CDC) were used instead of *N*-phthaloylation. Because of solubility in DMSO and full protection of amino groups, CDC were found to be suitable intermediates for *O*-modification of chitosan.

"Clicking" of propargyl-terminated MPEG onto azidated complexes chitosan–dodecyl sulfate was unsuccessful, however, giving virtually no graft copolymer.

6. Cationized derivatives of chitosan (HTCC) with degree of cationization (DC) varying from 30 to 67% were synthesized by the reaction of chitosan with (2,3-epoxypropyl)trimethylammonium chloride (EPTMAC) in acidic or neutral media. Additional cationization of *N*-HTCC in alkaline media enabled to increase DC of chitosan up to 190%. *O*-Cationization of chitosan present in the complexes chitosan – dodecyl sulfate resulted in water insoluble derivatives. Cationization of chitosan-*N*-MPEG copolymers was tenuous resulting in the products with very low DC.
7. In order to decrease dynamic viscosity (DV) of the solutions of *N*-cationized chitosan and make them suitable for production of nanofibers by electrospinning, partial degradation of *N*-HTCC by UV irradiation or enzymatic hydrolysis by pectinase or cellulase was studied. UV irradiation helped to decrease DV of the solutions of the cationized chitosans with high DC (DC > 50%). Enzymatic hydrolysis was even more efficient, especially by cellulase, and helped to decrease DV by tenfold and more in short time. Electrospinning from the solutions containing a mixture of HTCC and poly(vinyl alcohol) resulted in nanofiber webs possessing cationic properties.
8. Modification of chitosan by tartaric, citric or adipic acids yielded partially cross-linked chitosan derivatives. At DS of chitosan 7-22%, alongside individual modified macromolecules, chitosan nanoparticles with hydrodynamic radius ranging from 170 to 850 nm were received. At higher DS of chitosan, insoluble cross-linked derivatives of chitosan were obtained. Further modification of chitosan nanoparticles by dithiobenzendicarboxylate resulted in RAFT macroinitiators as precursors of functionalized nanoparticles.

LIST OF ORIGINAL PUBLICATIONS

Papers in the journals form the ISI Master Journal list:

1.Kulbokaite, R., Ciuta, G., Netopilik, M., Makuska, R. *N*-PEG'ylation of chitosan via "click chemistry" reactions. *Reactive & Functional Polymers*, 2009, V. 69, p. 771-778.

2.Gruškienė, R., Čiuta, G., Makuška, R. Grafting of poly(ethylene glycol) to chitosan at C(6) position of glucosamine units via "click chemistry" reactions. *Chemija*, 2009, V. 20, p. 241-249.

Paper in the reviewed Lithuanian journal:

1. Gorochovceva, N., Kulbokaitė, R., Juškėnas, R., Makuška, R. Synthesis and study of chitosan and poly(ethylene glycol) graft copolymers containing triazine moiety. *Chemija*, 2004, V. 15 (1), p. 22-27.

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1. Kulbokaite, R., Gorochovceva, N., Vaiciulis, A., Makuska, R. Additional cationization of chitosan and chitosan-poly(ethylene glycol) graft copolymers. *Programme and Proceedings of Baltic Polymer Symposium 2006*. Riga, Latvia, 2006, p. 37.
2. Makuska, R., Gorochovceva, N., Janciauskaite, U., Kulbokaite, R., Dedinaite, A., Claesson, P. Brush copolymers of chitosan and poly(ethylene glycol): synthesis and properties of adsorbed layers. *Proceedings of VIIIth International Conference: Modern Perspectives in Chitin and Chitosan Studies*. Kazan, Russia, 2006, p. 37-40.
3. Makuška, R., Kulbokaitė, R. Synthesis and properties of chitosan-poly(ethylene glycol) comb copolymers. In: *„(Bio)degradable Polymers from Renewable Resources". Proceedings of the International Conference, Vienna, November 18-21, 2007*. Series „Conference Proceedings and Monographs, Vol. 8, Vienna, Austria, 2008, p. 220-223.
4. Kulbokaitė, R., Čiuta, G., Makuška, R. Synthesis and characterization of *N*-azidated chitosans. *Polymer Chemistry and Technology: Proceedings of Scientific Conference Chemistry and Chemical Technology*. Kaunas, Lithuania, 2009, p. 83.

Thesis of international Conferences:

1. Makuska, R., Janciauskaite, U., Kulbokaite, R. Synthesis and properties of chitosan brush polyelectrolytes. *European Polymer Congress 2007: Programme and Book of Abstracts*. Portoroz, Slovenia, 2007, p. 243.
2. Kulbokaite, R., Makuska, R. Synthesis of chitosan-poly(ethylene glycol) comb copolymers employing "click" chemistry. *Baltic Polymer Symposium 2007: Programme and Book of Abstracts*. Druskininkai, Lithuania, 2007, p. 62.
3. Makuška, R., Kulbokaitė, R., Bendoraitienė, J., Adomavičiūtė, E., Milašius, R. Quaternization of chitosan and its use for preparation of cationic nanofibres by electrospinning. *International Conference "Polyelectrolytes 2008". Book of Abstracts*. Coimbra, Portugal, 2008, p. 199.
4. Čiuta, G., Kulbokaite, R., Makuška, R. Azidation of chitosan and "click" reactions for preparation of PEG'ylated derivatives. *1ST Baltchem International Student Conference on Chemistry. Conference Book of Abstracts*. Warsaw, Poland, 2008, p. 31.
5. R. Gruskiene, R. Deveikyte, R. Makuska. Preparation and partial destruction of the quaternized chitosan derivatives. *Programme and Proceedings of Baltic Polymer Symposium 2009*. Ventspils, Latvia, 2009, p. 60.
6. E. Udrenaite, R. Gruskiene. *N*-Modification of chitosan with carboxylic acids by carbodiimide. *Programme and Proceedings of Baltic Polymer Symposium 2009*. Ventspils, Latvia, 2009, p. 103.

Thesis of national Conferences:

1. Janciauskaite, U., Kulbokaite, R., Makuska, R. Breakdown of chitosan backbone under synthesis of its *O*-derivatives. *Chemija 2007: Abstracts of 8th National Lithuanian Conference*. Vilnius, Lithuania, 2007, p. 69.
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KATIJONIZUOTI IR POLIETILENGLIKOLIU MODIFIKUOTI CHITOZANO DARINIAI BEI NANODALELĖS

Santrauka

Didžioji dalis dabartinių polimerų yra sintetiniai. Dažniausiai jie nėra bioskalūs ir mažiau biosuderinami negu gamtiniai polimerai, pvz., celiuliozė, krakmolas, chitinas, chitozanas ar jų dariniai. Yra sričių, kuriose gamtinės kilmės polimerai ypač pageidaujami: tai medicina, farmacija, kosmetika, biotechnologija.

Vienas iš svarbesnių gamtinės kilmės polimerų yra chitozanas, pasižymintis unikaliomis fizikinėmis, cheminėmis, adsorbcinėmis ir biologinėmis savybėmis. Daug chitozano gaminių naudojami ten, kur reikalingas biosuderinamumas, pvz., vaistų pernašai. Platesnį chitozano panaudojimą apsunkina mažas jo tirpumas neutralioje ir šarminėje vandeninėje terpėje bei daugelyje savrbiausių organinių tirpiklių.

Daug dėmesio skiriama įvairiems chitozano modifikavimo būdams, kuriais galima pakeisti chitozano tirpumą, o taip pat jam suteikti naujų vertingų savybių. Katijonizavimas – vienas iš būdų chitozano tirpumui pagerinti, kartu pakeičiant kai kurias jo savybes. Iš katijonizuoto chitozano sudarytos nanodalelės ir nanopluoštai gali būti plačiai taikomi farmacijoje ir medicinoje.

Tarp chitozano darinių ypatingą vietą užima chitozano-metoksipolietilenglikolio kopolimerai ir konjugatai, kurie, kaip ir baltymų-PEG dariniai, jau seniai naudojami medicinoje ir biotechnologijoje. Deja, akivaizdžiai gero metodo chitozano-MPEG darinių sintezei nėra, nes kopolimerų gryninimas nuo nesureagavusio MPEG yra sudėtingas.

Pagrindinis šio darbo tikslas buvo susintetinti vandenyje tirpius norimos struktūros bei skirtingo pakeitimo laipsnio skiepytuosius chitozano-MPEG kopolimerus bei katijonizuotus chitozano darinius ir ištirti jų savybes.

Svarbiausi šio darbo rezultatai, atspindintys jo naujumą, originalumą ir svarbą:

Pirmą kartą chitozano-metoksipolietilenglikolio skiepytieji kopolimerai susintetinti „klik“ chemijos reakcijų pagalba. Naudojant „klik“ chemijos Cu(I) katalizuojamos alkinų-azidų ciklizacijos reakciją, susintetinti įvairaus pakeitimo laipsnio (PL 13-40 %) *N*-pakeisti chitozano dariniai, turintys triazolilliekaną ir tirpstantys tik rūgštinėje vandeninėje terpėje. „Klikinimo“ reakciją katalizuojant Cu(II)/askorbato

sistema, vyksta chitozano grandinės destrukcija, todėl gautų chitozano kopolimerų molekulinė masė yra dešimtimis kartų mažesnė, negu tikėtasi.

Naudojant *N*-ftaloilchitozano darinius, gliukozamino grandžių C(6) padėtyje turinčius azido arba propargilo liekanas, „klik“ chemijos metodu pirmą kartą susintetinti chitozano-*C(6)*-TMPEG kopolimerai, kuriuose chitozano PL siekia iki 64 %. Dėl destrukcinių procesų, vykstančių aminogrupės deblokavimo ir „klikinimo“ metu, gauti mažos molekulinės masės chitozano dariniai. Siekiant išvengti destrukcinių procesų, chitozano aminogrupių apsaugai vietoje *N*-ftalinimo panaudotas chitozano/dodecilsulfato kompleksų (CDK) sudarymas. Tačiau chitozano-*C(6)*-TMPEG sintezės schema, chitozano aminogrupės blokavimui naudojant CDK, nepasiteisino, gauti kopolimerų šiuo metodu nepavyko.

Rūgštinėje ar neutralioje vandeninėje terpėje ištirpintą chitozaną veikiant *N*-2,3-epoksipropil-*N*,*N*,*N*-trimetilamonio chloridu (EPTMAC), susintetinti katijonizuoto chitozano (KChz) dariniai, kuriuose katijonizavimo laipsnis (KL) siekia iki 67 %. KChz darinius papildomai katijonizuojant EPTMAC šarminėje terpėje, gauti *N*,*O*-katijonizuoti chitozano dariniai, KL padidinant iki 190 %.

Siekiant sumažinti susintetintų KChz tirpalų klampą ir padaryti juos tinkamus nanopluoštų formavimui elektroverpimo būdu, ištirta dalinė KChz destrukcija UV spinduliuotės ir fermentų pektinazės ir celulazės poveikyje. Nustatyta, kad poveikis UV spinduliuote yra tinkamas tik tada, kai chitozano KL didelis (KL > 50 %). Fermentinė KChz tirpalų hidrolizė yra efektyvi (ypač, dalyvaujant celulazei); ją naudojant dinaminę KChz tirpalų klampą galima sumažinti dešimtimis kartų.

Chitozaną modifikuojant daugiafunkcinėmis vyno, citrinų ar adipo rūgštimis, susintetinti dalinai tinklinti chitozano dariniai. Chitozano PL esant 7-22 %, greta modifikuoto chitozano makromolekulių susidaro chitozano nanodalelės, kurių hidrodinaminis spindulys yra nuo 170 iki 850 nm. Esant didesniam chitozano PL, susidaro netirpūs tinklinti chitozano dariniai. Prie chitozano ir karboksirūgštimis modifikuotų chitozano nanodalelių prijungus (4-cianpentano rūgšties)-4-ditiobenzenkarboksilatą, susintetintas makroiniciatorius gyvybingajai radikalinei polimerizacijai RAFT metodu.