Cytotoxicity study of novel water-soluble chitosan derivatives applied as membrane material of alginate microcapsules

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Abstract: The majority of cell encapsulation systems applied so far are based on polyelectrolyte complexes of alginate and polyvalent metal cations. Although widely used, these systems suffer from the risk of disintegration. This can be partially solved by applying chitosan as additional outer membrane. However, chitosan can be dissolved in water only at a low pH, which limits its use in the field of bioencapsulation. In this study, novel primary and tertiary amine chitosan derivatives have been synthesized, which may be dissolved at pH 7.0, and retain the ability to effectively form additional membrane on the surface of alginate beads. As aqueous solutions tertiary amines dimethylamino-1-propyl-chitosan and dimethylethyl-amine-chitosan with linear hydrochloride aliphatic chains had the lowest toxicity, whereas dimethylpropylamine-chitosan, diethylaminoethyl-chitosan, and diisopropylaminoethyl-chitosan with branched hydrochloride aliphatic were cytotoxic to the majority of tested cells. When applied as polyelectrolyte complexation agent on the surface of alginate beads, none of the derivates had any negative effect on the metabolic activity of encapsulated beta-cells. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 1907–1914, 2013.

Key Words: chitosan, cytotoxicity, beta-cells, encapsulation, microcapsules

INTRODUCTION

Microencapsulation involves the immobilization of particles or cells in semi-permeable membranes to protect them from their harmful external environment.[1] In recent years, there is a growing interest in research on novel polymers that are compatible with the encapsulated cells. This is confirmed by the increase in the number of scientific publications and patents related to novel materials for cell encapsulation.[2]

The majority of systems that are applied for cell encapsulation so far are based on polyelectrolyte complexes of alginate and polyvalent metal cations, mostly calcium. Although this system does not interfere with the functional survival of cells,[3] it has one major drawback. It is not mechanically stable, as the polyvalent cations, which act as crosslinker within the alginate network, tend to be gradually replaced by monovalent ones, leading finally to dissolving and disintegration of the capsules.[4] The importance of this instability issue for the application of encapsulation is illustrated in fermentation and production of microbiological relevant substances such as lactic acid, where disintegration of the capsules is associated with the requirement to apply costly purification steps of the product. By increasing the stability of the capsules, longer fermentation times can be achieved and, consequently, increased productivity and reduced final costs.[5] Similar issues of disintegration of capsules have been reported in applications such as in transplantation of immunoisolated cells.[6]

A higher mechanical stability of the capsules can be achieved by applying an additional, more stable outer membrane. This can be done by the formation of a polyelectrolyte complexes by interpolymer ionic interaction between negatively charged alginate and cationic polymers such as chitosan.[7] Chitosan holds some advantages for cell encapsulation as it has a low toxicity to various types of mammalian cells[8] and can be applied using various techniques of polyelectrolyte complex microcapsule formation.[9] However, the application of chitosan in cell encapsulation is limited up to now because of its low solubility at a pH higher than 6.0. The low pH is not compatible with biological activity of most of the cells applied for cell encapsulation. This is the reason why so many attempts to modify chitosan have been performed[10] to allow complexation with anionic macromolecules at a physiological pH, that is around 7.0. Recently, some groups[11] have shown that by adjusting the molar mass of oligochitosan in the range of 1000–10,000 g/mol it is possible to dissolve chitosan in the physiological pH range.[12] Unfortunately, such unmodified oligochitosan has only primary amino groups, which results in a chitosan at

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physiological pH with only 10% in electrolyte reactive protonated form.[13] Hence, only 10% of the groups can interact with alginate. As a consequence, the final complex between alginate and unmodified oligochitosan is very weak and mechanically instable during long-term storage in buffer solutions of pH close to the physiological range.[14]

A conceivable approach to improve the stability of such membranes is chemical modification of chitosan[15] by introducing permanently charged cationic groups such as quaternary ammonium derivatives or by graft copolymerization.[16] Graft copolymerization is an attractive approach because it allows to modify the chemical structure of biopolymers. Quaternized chitosan derivatives such as trimethyl chitosan chloride (TMC) have been prepared and evaluated as absorption enhancers of hydrophilic drugs at pH values similar to those found in the intestine.[17] TMC polymers proved to increase the intestinal absorption and bioavailability of peptide analogs in both rats and pigs, which illustrates its lack of toxicity and potential application for cell encapsulation. In the modified oligochitosan with quaternary ammonium groups (3-chloro-2-hydroxypropyltrimethylammonium chloride—CHPTMAC), the side chains are permanently charged at a pH range of 2–8. Therefore, the polyelectrolyte complex formed between alginate and modified oligochitosan is less porous and has a higher mechanical stability.[18] It has been proven that this modified oligochitosan is not toxic toward C2C12 myoblast cells and when applied as outer coating of implanted alginate microcapsules it maintained a spherical shape without irregularities at the surface of the membrane, which proves to be a high degree of biocompatibility of such microcapsules.[19]

In this study, we have prepared eight novel modified oligochitosans with the aim to form polymers that should dissolve at physiological pH and could be applied as membrane for alginate/calcium beads. Both synthesis of such modified cationic oligosaccharides and evaluation of their effect on viability and metabolic activity of selected cells have been described.

MATERIALS AND METHODS
Reagents
Chitosan (Yuhuan Ocean Biochemical, China) with a degree of deacetylation in range of 80–85% has been applied as starting material during degradation and chemical modifications. Acetic acid, hydrogen peroxide, sodium hydroxide, acetone, and sodium chloride were purchased from Chempur, Poland. All reactive derivatives of amines, that is 2-chloroethylamine hydrochloride (EA), 3-chloropropylamine hydrochloride (PA), 2-chloro-N,N-dimethylethylamine hydrochloride (DMAE), 2-chloro-N,N-diethylethylamine hydrochloride (DEAE), 3-dimethylamino-1-propyl chloride hydrochloride (DMAP), 2-chloro-N,N-dimethylpropylamine hydrochloride (DMAIP), and 2-(diisopropylamino)ethyl chloride hydrochloride (DIPAE) (Table I) were purchased from Sigma-Aldrich, Poland.

Oligochitosan
Oligochitosan samples with a molar mass of $M_n = 10,000$ g/mol was prepared by controlled radical degradation at 80°C and 2 h reaction time via continuous addition of hydrogen peroxide at a final concentration of 6.2 mmol to 2.5% chitosan solution of starting pH 3.5–4.0. After a concentration process (vacuum evaporator, RVO 200 A, INGOS, Czech Republic) to approximately 15% of the solid content, oligochitosan was precipitated in acetone (1/2 v/v), several times washed with pure acetone, and dried at 50°C for 4 h. The relative molar masses M_n and M_w of unmodified and modified oligochitosan samples were determined using the high-performance liquid chromatography (HPLC)/gel permeation chromatography (GPC) method (HPLC SmartLine system—isocratic pump 1000 equipped with RI Detector 2300—all from Knauer, Germany). For SEC separation, a Tessek Separon HEMA-BIO40 column and 0.33M acetic buffer (pH = 2.5) with 0.1M NaCl as an eluent at a flow rate of 1 mL/min was used. For relative calibration, the dextran standards (PSS Mainz, Germany) were applied.

Chitosan derivatives
Chitosan derivatives were obtained in reactions of oligochitosan dispersed in aqueous alkali solution and different primary and tertiary aliphatic amines of various chemical structures (Table I).

Before modification, 20 g of oligochitosan was introduced in 180 mL of water. The pH was adjusted using 24.5% HCl until the chitosan was completely dissolved. Then, 43.4 g of 31% NaOH was added dropwise to the chitosan solution. In most reactions, 0.04 g of NaBH$_4$[20] was applied as catalyst. It was dissolved in 5 g of water and added to the reaction mixture. The specific amine at a final concentration of 1 mol/mol of chitosan monomeric unit was dissolved in 15 g of water, and then was added to the chitosan solution, heated to 85°C, and maintained for 1 h during continuous mixing at a temperature between 85 and 90°C. Afterward, the reaction mixture was cooled to 40°C and was then neutralized to pH 4.4 by the addition of aqueous 24.5% HCl. In most cases, the chitosan derivatives were purified by precipitation in acetone. Only in one case, that is diethylaminoethyl-chitosan (ultrafiltrated)-DEAE(UF), it has been additionally purified first by ultrafiltration (Labscale TFF System, Millipore, France) with cellulose membranes of cut-off 5000 Da (Pellicon XL, Millipore, France) and then finally precipitated in acetone.

Molar masses of all samples were determined using HPLC/GPC methods as described in **Oligochitosan** section. For final determination of the degree of substitution, ^{1}H NMR spectroscopy, in DCl–D$_2$O (Armar, Switzerland), has been applied (BrukerAvance DPX 400 MHz, Germany) by the method described by Badawy et al.[21]

Cell cultures and cytotoxicity assay
For a screening on toxicity, we applied a total of 1×10^6 Monomac cells. They were cultured in RPMI 1640 containing 10% bovine calf serum (BCS) and gentamicin. The cells were incubated 48 h with the polymer. After the incubation, the cells were washed by centrifugation at 500g for 5 min. After centrifugation, the cells were resuspended in 2 mL of medium with addition of 160 nM of DiOC6 to stain dead

TABLE I. Chemical Structures of Primary and Tertiary Amines and Purification Conditions Applied in Modification of Oligochitosan

No. of Sample	Name (Purification Conditions)[a]	Chemical Structure
1	2-Chloro-N,N-dietyhylethylamine hydrochloride (ultrafiltrated + precipitation in acetone) DEAE(UF)	
2	3-Chloropropylamine hydrochloride PA	
3	3-Dimethylamino-1-propyl chloride hydrochloride DMAP	
4	2-Chloro-N,N-dietyhylethylamine hydrochloride (without catalyst) DEAE (WC)	
5	2-Chloro-N,N-dimethylethylamine hydrochloride DMAE	
6	2-Chloroethylamine hydrochloride EA	
7	2-(Diisopropylamino)ethyl chloride hydrochloride DIPAE	
8	2-Chloro-N,N-dimethylpropylamine hydrochloride DMAIP	

[a] Modified chitosan samples 1–3, 5–8 were purified by precipitation in acetone.

cells. The cells were incubated with DiOC6 for 30 min at 37°C in the dark. Then, cells were centrifuged at 500g for 5 min and resuspended in 408 µL of medium containing 200 ng of propidium iodide to stain viable cells. Finally, 50,000 cells were acquired with the Calibur (Becton Dickinson, USA). Analysis was performed using Win list 32 (Verity Software House, Topsham, ME). The cytotoxicity assay for each examined chitosan derivative was repeated three times.

The Beta-cell cell-line RinM5F, used for immobilization, was cultured in RPMI 1640 containing 13% BCS and gentamicin. All cells were cultured at 37°C in atmosphere with 5% of CO_2.

Alginate capsule formation

After culturing, RinM5F was suspended in sterile filtered (filtration, 0.2 µm) Intermediate-G sodium alginate (~43% guluronic acid, Keltone® LVCR, International Specialty
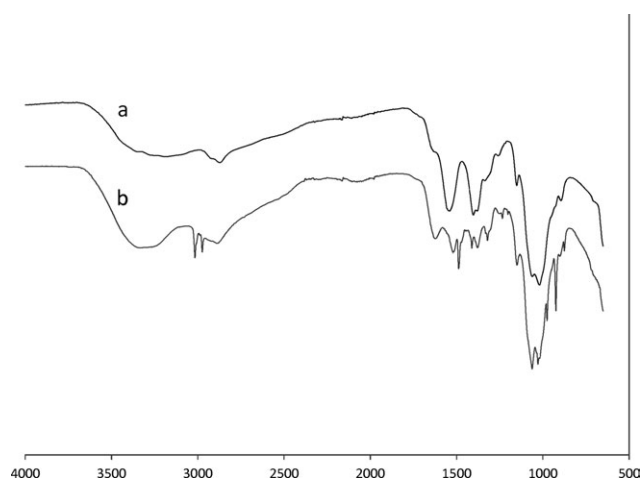

FIGURE 1. Attenuated total reflectance–FTIR spectra of (a) unmodified chitosan and (b) DMAE-chitosan.
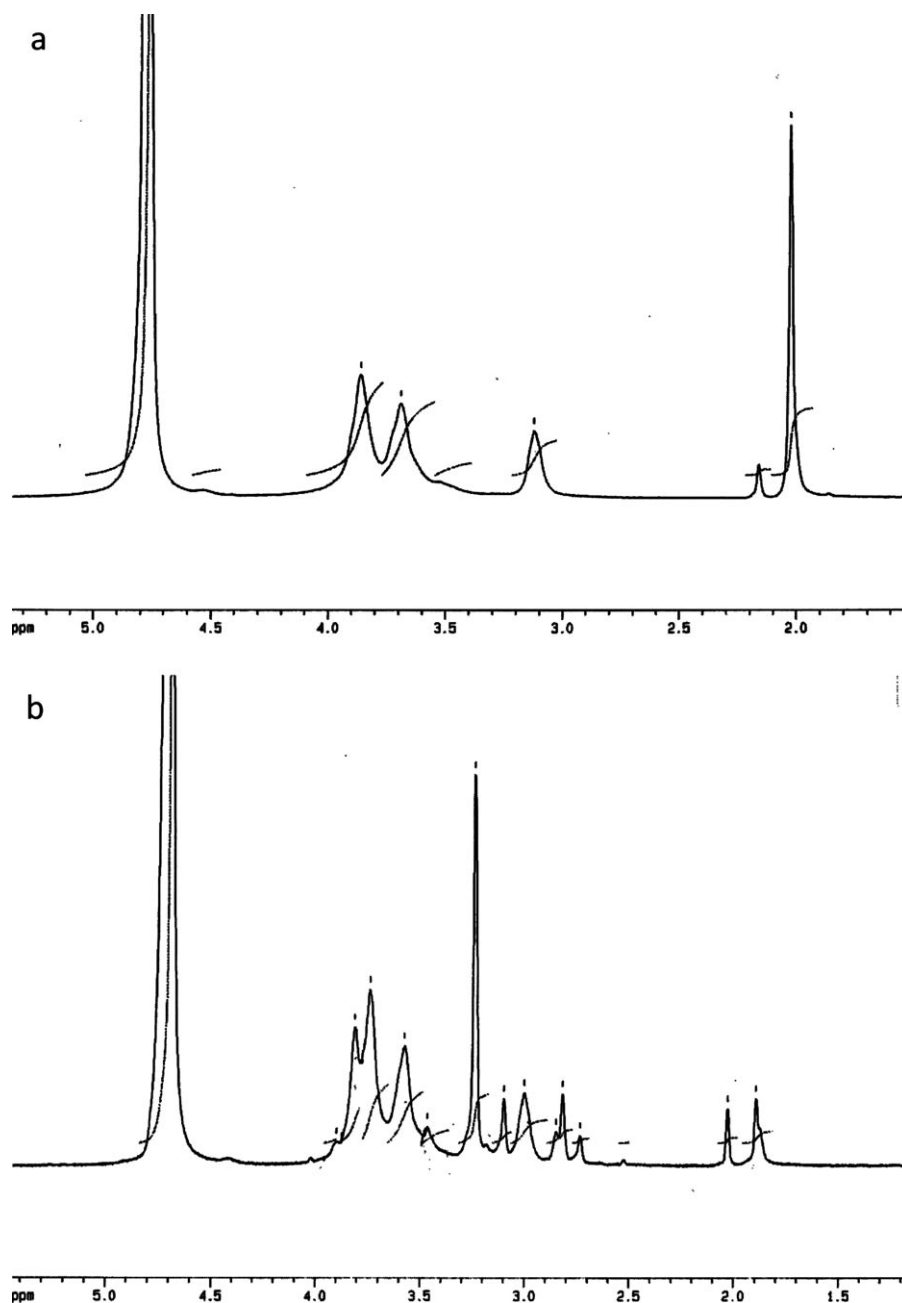
FIGURE 2. ^{1}H NMR spectra of unmodified chitosan (a) and DMAE-chitosan (b).

Products, United Kingdom) solution. We applied 10^7 cells/mL. Alginate solutions were extruded from a 23G needle using a syringe and a coaxial air stream to produce droplets.[22] The alginate droplets were immersed in a 100 mM CaCl$_2$ solution and allowed to gelate for 5 min after extrusion of the last droplet. The extrusion process never lasted longer than 4 min. Gelled calcium alginate beads were 650–675 μm in diameter. Subsequently, the beads were suspended for 3 min in Krebs Ringer Hepes (KRH) containing 2.5 mM CaCl$_2$ as described previously.[23] The outer membrane was formed by suspending the alginate beads for 10 min in 1% Ca^{2+}-free KRH solution containing 135 mM NaCl and 1% of the different oligochitosan derivatives. After the

coating process, the beads were washed two times with Ca^{2+}-free KRH containing 135 mM NaCl. The morphology of beads and capsules was studied under the microscope (Bausch and Lomb BVB-125, and 31-33-66). This was also done for studying the formation of the membrane when chitosan was added to calcium beads. All capsules were cultured and stored in RPMI1640.

To assess whether chitosans influence the functional capacity of the cells, we applied the cell proliferation reagent WST-1 (Roche, Mannheim, Germany). The tetrazolium salt in this test is cleaved by metabolically active cells to formazan (dark red), which is quantified by a scanning multiwell spectrophotometer by measuring the absorbance of
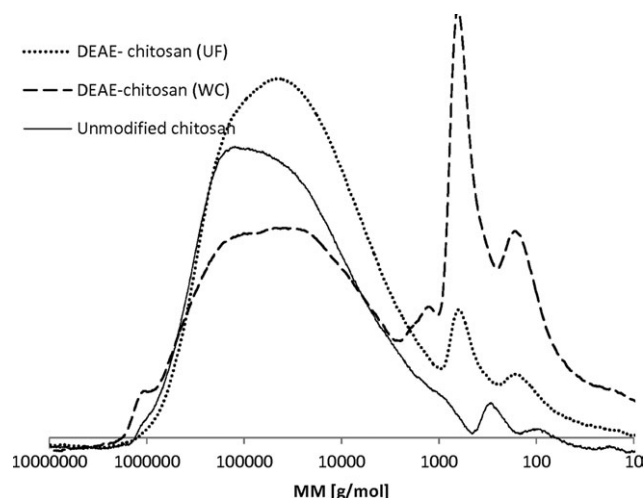
FIGURE 3. HPLC/GPC chromatograms of chitosan DEAE derivative purified by two methods: ultrafiltration and precipitate in acetone (DEAE(UF)) and precipitate without ultrafiltration (DEAE(WC)).

the dye solution at 450 nm. The higher the absorbance the more living cells in the solution.

Microencapsulated RinM5F beta-cells were cultured in RPMI1640 supplemented with 13% BCS. The alginate beads were coated with chitosan derivative (DIPAE, DMAP, and DEAE(UF)) and WST-1 was added after 48 and 72 h of incubation. After 48 h, the measurements were taken at time points of 0, 1, 2, 4, and 24 h. After 72 h, new WST-1 solution was added and the measurements were collected again at 0, 1, 2, 3, and 4 h.

RESULTS
Characterization of modified oligochitosan
The eight oligochitosans (Table I) were engineered and characterized as described in the **MATERIALS AND METHODS** section. All these modified chitosans can be dissolved at a physiological pH of 7.0, which qualify the molecules for application in encapsulation of cells. All the polymers were tested as complexation agent on the surface of alginate beads. They all could be successfully dissolved and readily formed membranes around the capsules.

The Fourier transform infrared (FTIR) spectra of chitosan and DMAE derivative are shown in Figure 1. The peaks at 1058 and 1022 cm^{-1} are corresponding to the stretching vibration of the C—O—C in glucose units, whereas the peaks at 1150 and 895 cm^{-1} are characteristic of β (1–4) glucoside bonds of chitosan.[24] The additional peaks at 3015 and 2973 cm^{-1} are corresponding to C—H stretching of —CH$_3$ group and support the presence of the substitution. In case of the primary derivatives (2-chloroethylamine hydrochloride (EA), 3-chloropropylamine hydrochloride(PA)) additional peaks were observed at 1615 cm^{-1}, which correspond to primary amine N—H bending vibrations.

Figure 2 shows the ^{1}H NMR spectra of unmodified and DMAE-chitosan. In the unmodified chitosan, the peak of NHAc is at 2.0 ppm (*A*), of H-2 of GlcN unit at 3.2 ppm (*B*), and the signals from protons H-3, 4, 5, 6 of GlcN and H-2 of GlcAc unit are in the range of 3.5–4.1 (*C*). Based on these

TABLE II. Molar Masses and Polydispersity of Chitosan Derivatives (GPC Results)

Chitosan Derivative	M_n	M_w	Polydispersity
DEAE(UF)	18,000	113,000	6.3
PA	17,000	165,000	9.7
DMAP	17,000	247,000	14.5
DEAE (WC)	19,000	290,000	15.3
DMAE	17,000	185,000	10.9
EA	17,000	139,000	8.2
DIPAE	19,000	140,000	7.4
DMAIP	19,000	196,000	10.3

DEAE(UF), diethylaminoethyl-chitosan (ultrafiltrated); PA, propylamino-chitosan; DMAP, dimethylamino-1-propyl-chitosan; DEAE(WC), diethylaminoethyl-chitosan (without catalyst); DMAE, dimethylethylamine-chitosan; EA, ethylamine-chitosan; DIPAE, diisopropylaminoethyl-chitosan; DMAIP, dimethylpropylamine-chitosan.

data, the degree of substitution was calculated using the equation DS = $6D/n(B + C)$, where D is the peak area of substituent (1.9, 2.7–3.1, and 3.8) and n is the number of hydrogen atoms per substituent.

Chromatograms of diethylaminoethyl-chitosans show that by ultrafiltration it is possible to remove most of the low-molar-mass substances with molar mass below 1000 g/mol, where precipitation with acetone is also quite effective to remove such low-molar-mass impurities (Fig. 3). Results of GPC characterization also illustrate that the sample that was first purified by ultrafiltration with membranes of 5000 Da cut-off is less polydisperse in molar mass (Table II).

All degrees of substitution analyzed by NMR were in the range of 0.19–0.52, where the theoretical value is 3.0, which corresponds to potential substitution of three of the side chain groups—two hydroxyl groups and one amino group (Table III).

Cytotoxicity
The different chitosans have different degrees of cytotoxicity when coincubated with Monomac cells. DMAE- and DMAP-chitosans have shown to have the lowest toxicity as 77.9 and 76.8%, respectively, of the cells (Fig. 4) and they were still viable after 48 h of incubation. This was completely different with DMAIP derivatives in which only 31.7% of the cells were still alive after 48 h of exposure. This

TABLE III. Degree of Substitution of Chitosan Derivatives Determined by ^{1}H NMR

Chitosan Derivative	Degree of Substitution
DEAE(UF)	0.19
DMAP	0.46
DEAE (WC)	0.37
DMAE	0.52
EA	0.21
DIPAE	0.49
DMAIP	0.20

DEAE(UF), diethylaminoethyl-chitosan (ultrafiltrated); PA, propylamino-chitosan; DMAP, dimethylamino-1-propyl-chitosan; DEAE(WC), diethylaminoethyl-chitosan (without catalyst); DMAE, dimethylethylamine-chitosan; EA, ethylamine-chitosan; DIPAE, diisopropylaminoethyl-chitosan; DMAIP, dimethylpropylamine-chitosan.
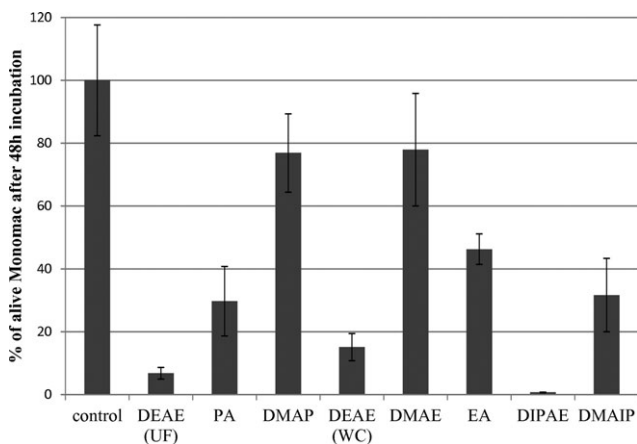
FIGURE 4. Cytotoxicity of chitosan derivatives forward Monomac cells. (DEAE(UF), diethylaminoethyl-chitosan (ultrafiltrated); PA, propylamino-chitosan; DMAP, dimethylamino-1-propyl-chitosan; DEAE(WC), diethylaminoethyl-chitosan (without catalyst); DMAE, dimethylethylamine-chitosan; EA, ethylamine-chitosan; DIPAE, diisopropylaminoethyl-chitosan; DMAIP, dimethylpropylamine-chitosan. The cytotoxicity assay for each examined chitosan derivative was repeated three times.

demonstrates the high toxicity of this derivate. The most pronounced toxicity was observed with DIPAE. After 48 h of incubation with this derivative, about 99.3% of the cells did not survive. Polymers obtained in the reaction with primary, ethyl, and propyl amines (EA, PA) kill 46.3 and 29.8% of the cells, respectively.

It may be argued that low-molecular-mass substances, such as amino reagents left after the reaction and thus present in the final product, could have caused the negative effect on the overall high toxicity of DMAIP- and DIPEA-modified samples. Therefore, in case of DEAE chitosan derivatives, we combined acetone precipitation and ultrafiltration to separate and remove all substances with a molar masses below 5000 Da. Both ultrafiltration and acetone purification did not improve cell viability as more than 85% of them were killed after incubation in DEAE derivative solution.

Metabolic activity

The chitosan derivates should not only be nontoxic, but they should not also interfere with functionality of the cells to qualify as an acceptable polymer for encapsulation. The viability test of encapsulated beta-cells was performed with diisopropylaminoethyl-, dimethylaminopropyl-, and diethylaminoethyl-chitosans only since they successfully passed the cytotoxicity testings. Capsules formed from these derivatives were stable, did not swell during the encapsulation process, and could be kept in culture for weeks without significant disintegration of the chitosan–alginate membrane. The cells were tested after 48 and 72 h by quantifying the mitochondrial activity in the WST-1 assay. As shown in Figures 5 and 6, the presence of the chitosan membrane had no influence at the metabolic activity of the cells as both cells in alginate beads and alginate–chitosan beads had identical metabolic activity.
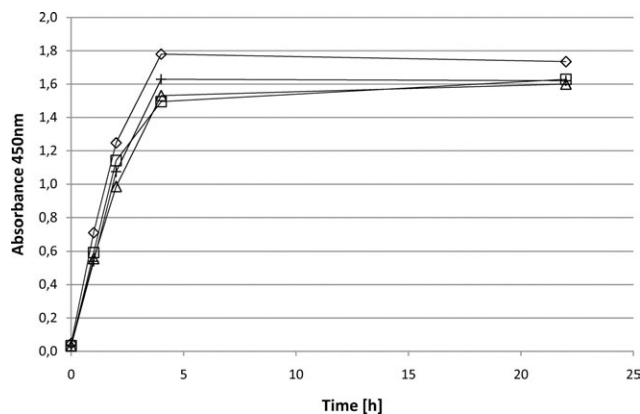

FIGURE 5. Viability of encapsulated beta-cells after 48 h of incubation. (◇ capsule without membrane, + DIPAE membrane, Δ DMAP membrane, and □ DEAE(UF) membrane). DIPAE, diisopropylaminoethyl-chitosan; DMAP, dimethylamino-1-propyl-chitosan; DEAE(UF), diethylaminoethyl-chitosan (ultrafiltrated).

DISCUSSION

In this study, we present eight oligosaccharidic derivatives of chitosan that can be dissolved at a physiological pH of 7.0 and therefore qualify for application in encapsulation methodologies. In contrast to regular chitosan, these polymers can be dissolved at appropriate pH close to neutral without losing the ability to bind alginate. We have shown that modification reactions performed in the alkaline medium allows for substitution of chitosan groups by amino groups of different chemical architecture, which finally leads to improved solubility in water similar to *N*-trimethylene chloride chitosan.[25] All chitosans could be successfully applied as surface electrostatic complexation agent on alginate-based microcapsules and formed stable membranes as illustrated by prolonged survival and mechanical integrity of the capsules at even weeks after culture (data not shown).

Not all chitosan derivatives proved to be nontoxic for cells. It may be concluded that tertiary amines are less cytotoxic than primary ones, as chitosan derivatives containing primary versus tertiary aliphatic amines (EA and DMAE as well as PA and DMAP in Fig. 4), are associated with higher
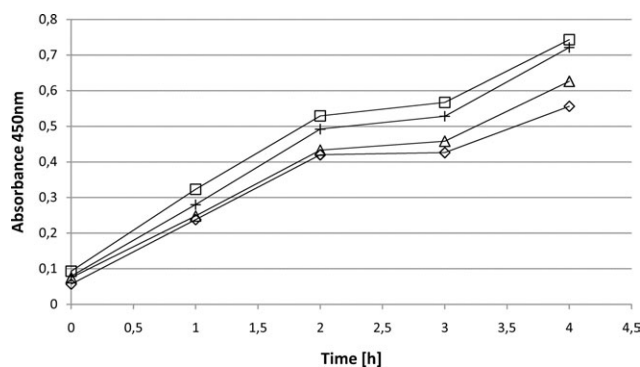

FIGURE 6. Viability of encapsulated beta-cells after 72 h of incubation. (◇ capsule without membrane, + DIPAE membrane, Δ DMAP membrane, and □ DEAE membrane). DIPAE, diisopropylaminoethyl-chitosan; DMAP, dimethylamino-1-propyl-chitosan; DEAE(UF), diethylaminoethyl-chitosan (ultrafiltrated).

TABLE IV. Structure and Number of Carbon Atoms in Various Chitosan Derivatives vs. % of Alive Monomac Cells

Type of Chitosan Derivative	Total Number of Carbon Atoms—Derivative Side Chain	Number of Carbon Atoms Direct Bound to N-atom	Number of Carbon Atoms Between Chitosan Backbone And Nitrogen Atom (Linear/Branched)	Number of Carbons That Mask The Nitrogen Atom	% of Alive Monomac Cells
EA	2	1	2(2/0)	0	46.3
DMAE	4	3	2(2/0)	2	77.9
DMAIP	5	3	3(2/1)	2	31.7
DEAE(UF)	6	3	2(2/0)	4	6.9
DIPAE	8	3	2(2/0)	6	0.7
PA	3	1	3(3/0)	0	29.8
DMAP	5	3	3(3/0)	2	76.8

survival rates especially in case of amines with low number of carbon atoms in the range of 2–5 (Table IV), which is in agreement with results obtained by Jung-Kul et al.[29] A higher cytotoxicity toward the Monomac cells was observed for tertiary amines, which might be explained by an increase in numbers of carbons that mask the nitrogen (DMAE, DEAE, and DIPAE). In case of tertiary amines with two methyl groups as substituent, the increase in numbers of carbon atoms from 2 to 3 between the chitosan backbone and the nitrogen atom (DMAE vs. DMAP) did not lead to significant change in their cytotoxicity. In this case, the structure of the carbon chain seems to be more important, whereas for more branched structures a higher cytotoxicity of amine derivatives (DMAIP vs. DMAP) was found. This is in line with the statement[26] that cytotoxicity increases with higher hydrophobicity as higher numbers of aliphatic groups make the molecules more hydrophobic. Our data, therefore, suggest that the higher number of hydrophobic interactions between the chitosan derivatives, for example DEAE and DIPAE and functional residues on the cell surface should be hold responsible for cell death induced by chitosan.

We took beta-cells as an example for testing effects of chitosan on metabolic activity. This should not be interpreted, however, as a suggestion that these chitosans are being proposed for only this field of application. They are potentially applicable in any system where dissolving and disintegration of the capsules[4] have been reported. This varies from implantation of mammalian cells, microencapsulation of probiotic bacteria to application in continuous fermentation bioprocesses including bioconversion of agrochemical byproducts to green-chemistry products. Chitosan can be produced at much lower cost than many of the other crosslinking polyamides and are associated as shown in this study with high survival rates of the encapsulated cells, where no interference with the metabolic activity of the enveloped cells has been observed. The capsules are stable, rigid, and can be easily produced.

We have noticed, as also described by Prokop et al.,[27,28] that even the chitosans that were found to be toxic did not necessarily interfere with the metabolic activity of cells when they were being applied as coating polymers for capsules. This should be explained by the fact that the molecules are readily adsorbed by the alginate surface of the capsules and efficiently crosslinked, and do not reach high enough concentrations in the core of the capsules to have detrimental effects on the cells. Although we would prefer the applications of chitosans that as dissolved molecule has minimal to no toxic effects, our data show that the detrimental effects in our cellular toxicity assay should not exclude the application of the molecules as they can safely be applied when adequately bound by the surface of the capsules. Future study should lead to testing the efficacy of these chitosan derivates in the large field of bioencapsulation from biomedicine to biotechnology.

CONCLUSIONS

In this study, primary and tertiary amine chitosan derivatives were obtained and tested for their applicability in the formation of alginate-/Ca-coated microcapsules applied for cell encapsulation. As single molecules, tertiary amines DMAP and DMAE with linear hydrochloride aliphatic chains and small amine substituent (methyl) had the lowest toxicity, whereas DEAE and DIPAE with larger than methyl amine substituent or DMAIP with branched hydrochloride aliphatic chain killed the vast majority of the tested cells. However, all these chitosan derivatives complexed on the surface of alginate microcapsules did not had any negative effect on the metabolic activity of beta-cells.

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