Chitosan-based nanocapsules of core-shell architecture*)

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Abstract: *N*-dodecyl derivative of cationically modified chitosan was used to prepare core-shell nanocapsules templated on liquid cores. Surfactant-free method based on ultrasound-assisted direct emulsification of aqueous solution of polysaccharide with oleic acid was applied. Formation of spherical capsules was confirmed by scanning and transmission electron microscopies. Dynamic light scattering measurements were used to determine physicochemical parameters of the obtained particles as well as to follow the process of multilayer shell formation. Confocal microscopy was applied to examine the ability of encapsulation of hydrophobic compounds inside the cores of the nanocapsules. Performed studies confirmed that hydrophobically modified cationic chitosan provides long-term stabilization of oil-in-water emulsion for biomedical applications as no toxic effect was observed in acute oral toxicity studies.

Keywords: chitosan, core-shell nanocapsules, nanocontainers, amphiphilic polymers, encapsulation.

Nanokapsuły typu rdzeń-otoczka na bazie chitozanu

Streszczenie: Do przygotowania nanokapsuł na ciekłych rdzeniach stabilizowanych bez użycia małocząsteczkowych surfaktantów użyto *N*-dodecylowej pochodnej zmodyfikowanego kationowo chitozanu. Kapsuły otrzymano w procesie wspomaganej ultradźwiękami bezpośredniej emulsyfikacji fazy wodnej zawierającej modyfikowany polisacharyd oraz kwas oleinowy. Powstawanie sferycznych kapsuł potwierdzono za pomocą skaningowej oraz transmisyjnej mikroskopii elektronowej. Obrazowanie z użyciem mikroskopii konfokalnej posłużyło natomiast do zbadania zdolności do enkapsulacji hydrofobowych barwników w rdzeniach chitozanowych nanokapsuł. Stosując technikę dynamicznego rozpraszania światła wyznaczono fizykochemiczne parametry nanoemulsji oraz stwierdzono powstawanie wielowarstwowych otoczek. Przeprowadzone badania dowiodły, że zastosowanie hydrofobowo zmodyfikowanej kationowej pochodnej chitozanu pozwala na uzyskanie stabilnych w czasie emulsji typu olej w wodzie. Wykazany brak toksyczności układów w warunkach *in vivo* pozwala na ich zastosowanie do celów biomedycznych.

Słowa kluczowe: chitozan, nanokapsuły typu rdzeń-otoczka, nanozbiorniki, polimery amfifilowe, enkapsulacja.

The development of the techniques of preparation of nanocontainers has attracted particular attention in recent years. Although many fields take advantages of significant progress of nanotechnology, large share of the works have been focused on biomedical applications. Nanocontainers can especially accommodate lipophilic active substances of low bioavailability such as antineoplastics, anti-inflammatories, immunosuppressants, antigens, hormones, antivirals, antibacterials, antifungals, diuretics, antipneumocystics, and vitamins [1–6]. They may protect encapsulated compounds against degradation, reduce their toxicity by decreasing the adverse effects towards tissues and provide prolonged release of the compound at the site of action. Importantly, that may lead to an improvement of bioavailability of poorly soluble active compounds that is highly desired for pharmaceutical applications.

Since the delivery systems are expected to have properties tailored to the nature of cargo as well as to target tissue it is important to choose appropriate material for their preparation. Recently, increased efforts have been made to prepare oil-in-water emulsion-based polymeric cap-

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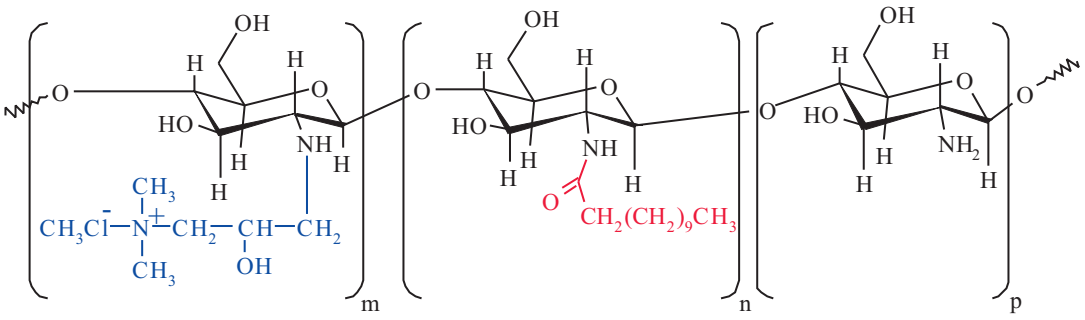
sules serving as carriers of lipophilic active compounds [7–9]. While ultrasound-assisted homogenization of two immiscible phases leads to formation of emulsion, the thermodynamic instability of such systems requires the use of additional stabilizers, which may imply some limitations. Surface-active agents of low molecular weight are commonly used to stabilize oil droplets as they are able to adsorb at the water/oil interface leading to the reduction of the surface tension. It causes a disruption of the droplet surface, reduction of their sizes and prevents their aggregation due to the repulsive electrostatic forces. Such droplets can be further stabilized by ultrathin polymeric films obtained using layer by layer technique (LbL) what leads to formation of nanocapsules templated on liquid cores [10]. Although the use of low molecular weight ionic surfactants allows to cover the oil cores with polymer shells, the dynamic nature of interactions within such micellar systems is a serious limitation as the excessive dilution causes the disintegration of the surfactant layers stabilizing the oil droplets. Another important point is the selection of an appropriate pair of surfactant and polyelectrolyte being the first layer of the shell as the formation of stable interfacial complex is crucial for formation of stable shell of the capsule [11, 12].

While the use of block copolymers seems to be a solution to the problem of instability of micellar systems due to the lower critical micelle concentration (*CMC*) value than for low molecular weight surfactants, variations in the environmental conditions also may lead to destabilization of nanoformulation [13]. Thus, amphiphilic graft copolymers have been introduced [14–16]. They anchor hydrophobic arms in the oil droplets ensuring their stabilization without the need of additional surfactants [17]. Moreover, those polymers play dual role in such nanoemulsions – they act as droplets stabilizers as well as they constitute the first layer of the shell. Moreover, the use of graft polyelectrolytes enables formation of multilayer capsules of enhanced stability and tunable surface charge [15].

For biomedical applications biodegradable and non-toxic polymeric amphiphiles based on natural polysaccharides have been recently studied [18]. The hydrophilic chains of polysaccharides containing several groups of varied molecular weight and chemical composition lead to various physicochemical and biological properties. A group of polysaccharides such as dextran, pullulan, amylose and cyclodextrins have no charged group in their structures, others, such as alginate, heparin, hyaluronic acid, chondroitin and pectin are negatively charged due to the presence of carboxylate or sulfonate groups, while chitosan is positively charged thanks to amine groups. The presence of functional groups in polysaccharides macromolecules enables their derivatization using both, polymer chains, *e.g.*, poly(alkylcyanoacrylates) and poly(methyl methacrylates) [19] as well as short segments of alkyl chains of various length attached to polysaccharide chain by either amino or carboxylate group [20–22]. Dodecane, hexadecane and linoleic acid have been widely investigated for that purpose so far [18]. Moreover, self-assembly of hydrophobically modified polysaccharides in aqueous solution causes the formation of various formulations such as micelles [23], nanoparticles [24], microspheres [25], liposomes [26] and hydrogels [27]. The type of obtained formulation depends on the content of hydrophobic groups covalently linked to the polysaccharide chain [28]. Moreover, it can be chosen depending on the physicochemical properties of the drug to be loaded and the required route of administration. By analogy with the phenomena of micelle formation of small surfactants or lipids, aggregation of amphiphilic polymers is controlled by the balance between the interaction of the hydrophobic groups and the hydrophilic chains [28].

Chitosan, a product of deacetylation of chitin, is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine linked by β(1,4)-glycosidic bonds. The hydrophilic nature of the molecule provides its good solubility in acidic aqueous solutions due to the protonation of amino groups which makes chitosan material of high utility. The presence of charged functional groups and the ability to modify the macromolecules by the compounds having permanent electrostatic charge promote the use of chitosan as a material for the construction of oil-core microcapsules [29] or biocompatible multilayer polymeric films [30, 31] serving as coatings for nanoparticles [32, 33] or protecting the cells during the low temperature storage [34]. Importantly, chitosan is a biocompatible and biodegradable material capable of chelating and binding metal ions and organic compounds, characterized by high adhesiveness to negatively charged membrane proteins [35]. It has been also shown that chitosan exhibits antibacterial and antiparasitic properties [36]. All these features make the number of biomedical publications describing the use of chitosan is growing from year to year.

Many formulations based on amphiphilic polysaccharides have been described so far, however capsules on liquid cores have been rarely described [37]. We have only recently proposed such an approach using derivatized hyaluronate. Surfactant-free technique of stabilization of capsules on liquid cores by hydrophobically modified polysaccharides offers an important advantage in applications of such capsules for controlled delivery of lipophilic compounds in various fields. The results presented herein refer to formation of nanocapsules templated on liquid cores stabilized by *N*-dodecyl derivative of chitosan modified cationically with glycidyltrimethylammonium chloride (CChit-C12). Physicochemical parameters as well as stability of the capsules were examined using dynamic light scattering what confirmed formation of nanoemulsion droplets stable during long-term storage. Moreover, electron microscopies indicated spherical shape and lack of aggregation of the obtained structures. The simplicity and low costs of manufacturing process combine with good stability and large capacity of hydrophobic compounds, low complexity of biocompatible chitosan-based nanocapsules and its resistance to changes



(I)

of ambient parameters such as ionic strength, pH and concentration allow their widespread use as carriers of lipophilic bioactive compounds.

EXPERIMENTAL PART

Materials

Amphiphilic chitosan derivative (CChit-C12) [Formula (I)] containing both, quaternary ammonium (with degree of substitution *DS* = 67.5 %) and *N*-dodecyl groups (*DS* = 2.0 %) was synthesized according to the previously described procedure [38]. Anionic (AChit) and cationic (CChit) derivatives of chitosan were obtained according to the procedures described previously by Bulwan *et al.* [30]. Degree of deacetylation of chitosan was equal to 78 % while the degree of substitution of sulfonate and quaternary ammonium groups in AChit and CChit derivatives were calculated based on ^{1}H NMR integration to be 66.5 and 57.2 %, respectively. Sodium chloride (NaCl, p.a., Lachner), oleic acid (OA, PhEur, Aldrich), *n*-octadecane (p.a., Polyscience Corp.), polyethylenimine (PEI, branched, $\overline{M}_w \approx$ 25 000 g/mol, Aldrich), perylene (Pe, gold label, 99.9 %, Aldrich), hydrogen peroxide (30 % solution, p.a., Avantor Performance Material Poland S.A., Poland), sulfuric acid (H_2SO_4, 96 %, Chempur) were used as received. Deionized water was used to prepare all the solutions.

Methods and testing

To determine physicochemical parameters of capsules by using dynamic light scattering (DLS) Malvern Zetasizer Nano ZS instrument was used. Measurements were performed at 22 °C using instrument working at 173° detection angle. General purpose mode was used as size distribution analysis algorithm and the reported data represent the averages of three series of measurements (10–100 runs each) and their standard deviations (mean ± *SD*, *n* = 3).

Field emission scanning electron microscope (FE-SEM, Hitachi S-4700) and transmission electron microscope (cryo-TEM, TECNAI F20 TWIN) were used for imaging the nanocapsules.

Confocal micrographs were collected using inverted microscope Nikon Ti-E with objective Plan Apo 100x/1.4 Oil DIC and confocal system Nikon A1.

Steady-state fluorescence spectra were recorded at room temperature using an SLM Aminco 8100 spectrofluorimeter with a 450 W xenon lamp as a light source. Nanocapsules were prepared using ultrasonic bath (540 W, Sonic-6, Polsonic).

Preparation of chitosan-based capsules templated on liquid cores

Chitosan-based nanocapsules were obtained in ultrasound-assisted emulsification of mixture of CChit-C12 (1 g/dm^3 in 0.15 mol/dm^3 NaCl) and oleic acid (100:1 volume ratio). The mixture was firstly homogenized using vortex shaker and then sonicated for 30 min at room temperature in the ultrasonic bath.

Multilayer shells were obtained *via* layer by layer (LbL) saturation technique without intermediate rinsing with water. Solution of the anionically or cationically derivative of chitosan (1 g/dm^3 in 0.015 mol/dm^3 NaCl) was added to the suspension of capsules and stirred at 400 rpm. The applied volumes of the polysaccharide solutions were optimized based on the simultaneous measurements of zeta potential and particle size distribution.

Encapsulation studies

The ability of encapsulation of hydrophobic compounds was examined using fluorescent probe perylene, which is only sparingly soluble in water ($c = 1.6 \cdot 10^{-9}$ mol/dm^3) and has negligible fluorescence in an aqueous solution [39]. The dye was dissolved in oleic acid and the solution was further used as an oil phase in the process of the capsules preparation. The concentration of perylene in the nanocapsules cores was *ca*. 10^{-5} mol/dm^3.

Preparation of capsules for SEM imaging

The chitosan-based capsules for SEM measurements were obtained according to the procedure described above. *n*-Octadecane was used as a core instead of oleic acid and sonication was performed at 32 °C. After sonication the capsules with liquid *n*-octadecane cores were cooled down to room temperature which led to solidification of *n*-octadecane. The capsules were then deposited on a silicon wafer coated by cationic polyelectrolyte [immer-

sion in PEI solution (1 g/dm^3) and sonication for 5 min] for stronger interactions with the negatively charged capsules. A droplet of the emulsion was placed on the surface that was carefully rinsed with water and dried in a stream of argon. The silicon substrates were, prior to the deposition of PEI, cleaned carefully using "piranha" solution (a mixture of 96 % H_2SO_4 and 30 % H_2O_2 solutions with volume ratio of 1:1), which is a highly corrosive and oxidative mixture.

Acute oral toxicity determination – fixed dose procedure

Mature, 9 week-old, nulliparous and non-pregnant adult female Wistar rats were used to determine acute oral toxicity of the capsules according to OECD 420 guideline. Experiments were approved by the Local Committee for Experiments with the Use of Laboratory Animals in Poznan, Poland. Animals were obtained from Department of Toxicology of Poznan University of Medical Sciences. Rats were acclimatized for 14 days before the test and randomly selected for the study. Conditions during quarantine and experiment were maintained in accordance with SPT-T/VIVO/11.

Stepwise procedure was used to assess the health consequences after administration of single dose of CChit--C12 capsules according to OECD TG 420. In the preliminary study nanocapsules were administrated by gavage using a stomach tube at dose of 300 and 2000 mg/kg bw, respectively, with 24 hours break between. Based on the results from the preliminary test, the 2000 mg/kg bw dose was used in the main experiment in which the other four animals were administered the capsules and observed for 14 days. The weight of each animal was measured on the day of dose administration and after 7 and 14 days. After 14 days of observation the animals were sacrificed and postmortem examined including external surface of the body, all orifices, cranial, thoracic and abdominal cavity together with its contents examination.

RESULTS AND DISCUSSION

Structure of *N*-dodecyl derivative of cationically modified chitosan

Chitosan modified with dodecyl chains and quaternary ammonium groups (CChit-C12) [see Formula (I)] was used for stabilization of liquid droplets in an emulsion. The degree of substitution of the hydrophobic side chains was calculated based on the ^{1}H NMR spectrum to be *ca*. 2 %, while the degree of substitution with quaternary ammonium groups was equal to *ca*. 67.5 % as determined also from ^{1}H NMR spectrum [38].

Chitosan-based nanocapsules

Chitosan-based nanocapsules templated on liquid cores, as oil droplets stabilized by thin polymer coatings, were prepared by mixing the aqueous solution of CChit-C12 and hydrophobic liquid phases followed by sonication of the mixture. Formation of the nanocapsules was followed using DLS measurements as well as electron microscopy. The capsules for cryo-TEM (shown in Fig. 1) and DLS measurements were templated on oleic acid cores while for SEM *n*-octadecane served as the core that solidifies at room temperature and such capsules did not disintegrate under vacuum and could be easily manipulated maintaining their properties. Spherical particles of diameter between *ca*. 200–550 nm were clearly observed in both, SEM and cryo-TEM images.

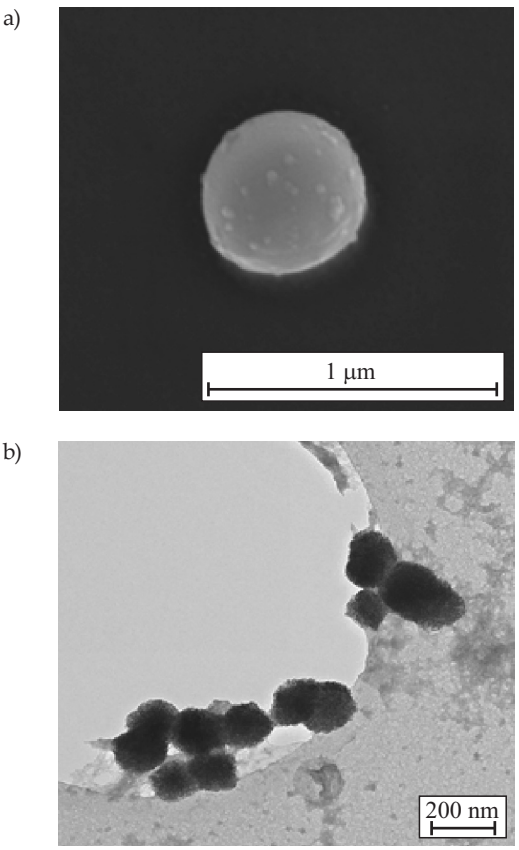


Fig. 1. The images of chitosan-based nanocapsules obtained using: a) SEM, b) cryo-TEM

The results of DLS measurements, presented in Fig. 2, confirmed formation of the particles of diameter equal to *ca*. 320 nm and low dispersity ($Đ$ = 0.2). High absolute values of zeta potential (ζ = +24 mV) measured for the capsules immediately after sonication indicated stability of the nanoemulsion. Importantly, capsules remained stable even upon 400-fold dilution as no significant variations in parameters were observed during DLS measurements. Moreover, colloidal stability was found to be pH-independent. Nanocapsules of diameter between 250 nm and 350 nm were observed for the suspensions at pH varied from 3 to 9, however capsules obtained at pH = 3 exhibited the highest stability described by ζ equal to +34.0 ± 0.8 mV while at pH = 9 it did not exceed +18 mV.

Detailed studies on stability of the capsules stored at room temperature showed that neither size nor ζ changed

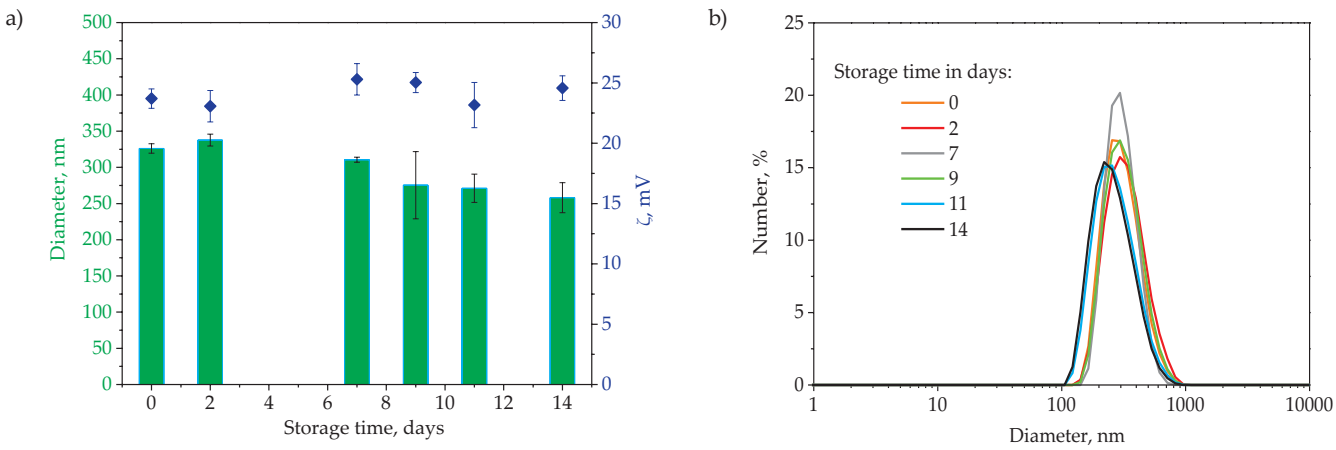


Fig. 2. The effect of storage time at room temperature of the capsules stabilized by CChit-C12 on values of: a) number-weighted hydrodynamic diameters and zeta potential (ζ), b) particle size distribution

over the first two weeks after preparation. Further measurements carried out once a month during prolonged storage of the capsules confirmed their long-time stability (at least 15 months while stored at 4 °C). Importantly, size distribution of the particles stored for several months was found to be narrow and did not vary in time. It indicates great potential of such hydrophobically modified ionic derivative of chitosan for the long-term stabilization of the oil cores of nanocapsules. Hydrophobic arms seem to be stably anchored in the oil droplet thus prevented aggregation and disintegration of the capsules during prolonged storage.

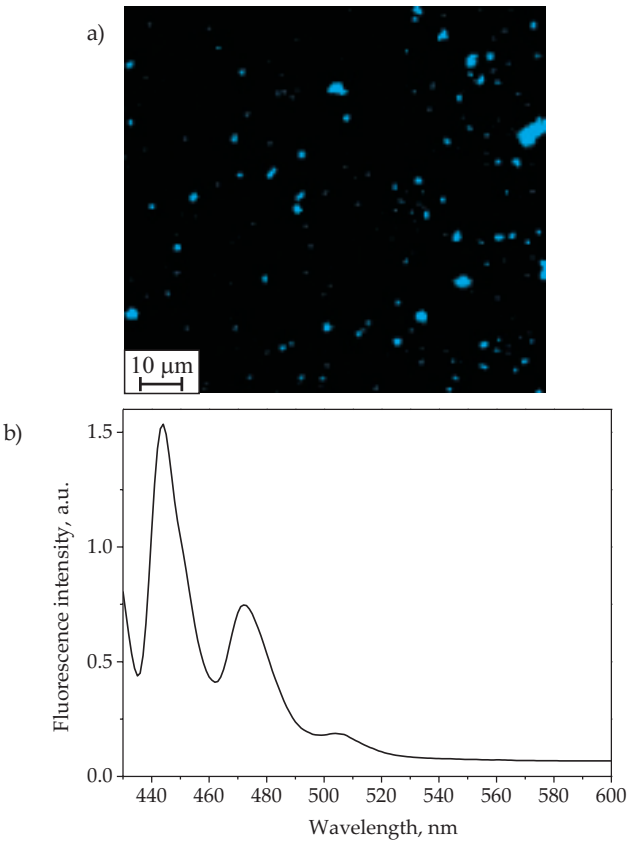


Fig. 3. Confocal micrograph of chitosan-based nanocapsules templated on oleic acid cores with encapsulated perylene using λ_{ex} = 410 nm DAPI filter (a) and steady-state emission spectrum of encapsulated perylene using λ_{ex} = 410 nm (b)

Encapsulation of hydrophobic molecular probes

Perylene was used as the model probe in encapsulation study. Confocal micrograph as well as steady-state emission spectrum, shown in Fig. 3, confirmed that the dye molecules were located in the hydrophobic interior of the capsules since perylene does not fluorescence in an aqueous solution. Moreover, no variations in particle size were observed what indicates that the encapsulation did not affect the morphology of the capsules.

Nanocapsules coated with multilayer chitosan shells

Multilayer shells of the capsules were obtained *via* LbL saturation technique using cationic (CChit) and anionic (AChit) derivatives of chitosan (c = 1 g/dm^3 in 0.015 mol/dm^3 NaCl) as it is shown in Fig. 4. After deposition of two AChit/CChit bilayers on the surface of the initial capsules with diameter of *ca.* 260 nm and ζ of approx. +25 mV, the diameter of the capsules increased to approx. 340 nm while ζ change only slightly to +20.8 mV (see Fig. 5).

Gradual increase in the size of capsules after each step of LbL indicates alternating adsorption of the oppositely charged chitosan derivatives and lack of undesired aggre-

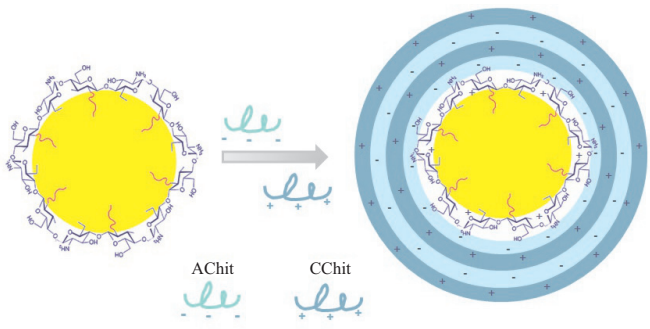


Fig. 4. Preparation of multilayer chitosan-based nanocapsules

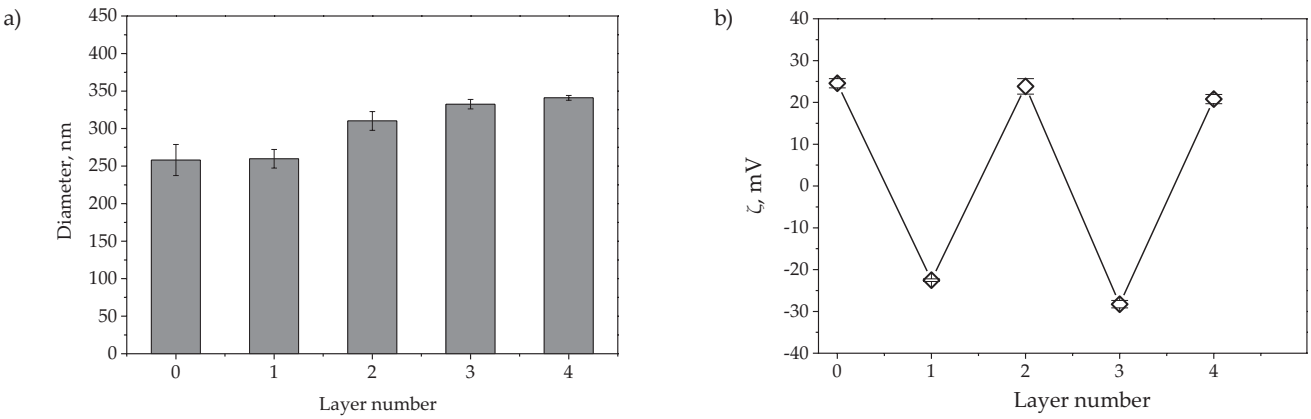


Fig. 5. The effect of the number of AChit and CChit layers on determined for chitosan-based nanocapsules values of: a) number--weighted hydrodynamic diameters, b) zeta potential (ζ)

gation. The typical zigzag shape of the plot of ζ confirmed formation of the multilayer shell and electrostatic stabilization of the capsules. Moreover, some additional stabilizing effect seemed to be observed while using negatively charged chitosan as the absolute value of ζ was found to be greater (layer number 3) than in case of positively charged shell. It may be explained by higher content of the charged groups in the case of AChit than CChit.

Toxicity study

Preliminary study showed no symptoms of toxicity after administration of capsules in dose of either 300 or 2000 mg/kg bw and thus the higher dose was used in further experiment. In the main study, after administration of single dose of 2000 mg/kg bw no symptoms of toxicity were observed. All animals survived whole period of study. After 14 days of observation the gross necropsy was performed. The gross necropsy (included a detailed external examination, as well as all the holes and cavities and detailed macroscopic examination of organs in the cranial cavity, thoracic and abdominal) did not show macroscopic changes during general visual inspection of animal body and examination of internal organs in all cases. The organs were correct structure and size, and on the cross-section, the structure of organs was also correct. There were no deviations that would suggest acute toxic effects of the chitosan-based capsules.

CONCLUSIONS

Surfactant-free method of stabilization of nanocapsules templated on liquid cores has been demonstrated. *N*-dodecyl derivative of cationically modified chitosan served as the stabilizer of the nanocapsules as it is able to anchor the hydrophobic side chains in the oil droplets providing resistance to the changes of the parameters of external environment including pH, ionic strength and dilution. By implementation of cationically modified polysaccharide the surface of the capsules can be easily modified with ionic polymers by applying layer by layer deposition of oppositely charged polyelectrolytes as it was shown for anionically modified chitosan. Such manipulation leads to an improvement in the stability of multilayer capsules and thus provides formation of carriers able to accommodate and protect sensitive hydrophobic compounds for long periods as the performed study indicated long-term stability of the capsules (up to 15 months). Lack of toxicity ensures the opportunity of application of chitosan-based nanocapsules in biomedical field.

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