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Polysaccharide-Stabilized Capsules for Delivery of Indomethacin

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Introduction

Indomethacin (IMC) is a poorly water-soluble non-steroidal andanti-inflammatory drug that is successfully used in the treatment of rheumatoid arthritis or trauma.[1] Besides, recently it has been reported that the drug has a potentially protective effectagain cell damage in Alzheimer's disease therapy.[2-4]

Because of low water solubility (log P 4.27) and pH- dependent dissolution rate, the drug is very amenable to encapsulation. Moreover, the pH-dependent charge density of the drug microcrystals (pKa~ 4.5) allows their encapsulation in core-shell structures by subsequent adsorption of oppositely charged polyelectrolytes (Layer-by-Layer, LbL approach).[5-8] Asan alternative method for encapsulation, it was reported the incorporation of the drug in emulsion droplets.[9]

The encapsulation of poorly water-soluble drugs and bioactive substances in emulsion droplets is a convenient approach to improve their solubility, stability, efficacy of action and protection from the biological environment. Dissolved in a suitable organic solvent (ethanol, chloroform), the substances can be loaded into the oil emulsion droplets with nano- or submicron sizes.[10] The produced dispersions can be applied inpharmacy, medicine and cosmetics.

However, the emulsions are thermodynamically unstable systems because of the unfavourable contact between the oil and water phases. The stability against flocculation, coalescence, creaming or Ostwald ripening depends on the surfaceproperties of the droplets, the stability of the thin liquid film between the droplets and the rheological characteristics of the system.[11] Different approaches can be used for the stabilization of classical oil-in-water emulsions or drug-loaded emulsions: adsorption of low-molecular surfactants or block copolymers,[12,13] deposition of hard particles (Pickering emulsions),[14] adsorption of graft polymers[15] or amphiphilic polysaccharides (chitosan derivates, hydrophobically modified derivates of hyaluronic acid, etc.).[16,17]

The adsorption of emulsifier on the droplet surface improves the stability of the dispersion because of the repulsionbetween the droplets as a result of the steric interactions between the interface surfactant layers and through electro- static interactions due to the repulsion between similarly charged droplets. Previously have been reported that water- soluble surfactants usually lead to destabilization of the emulsion when the oppositely charged polyelectrolyte is adsorbed. Therefore, poorly water-soluble ionic emulsifiers willbe useful for the formation of dispersions.[18]

The stabilization of the droplets by multilayer produced through subsequent adsorption of oppositely charged polyelectrolytes on the surfactant-stabilized droplets is also a convenient method to improve the stability of the dispersion. The influence of the factors affecting the formation and stability of the dispersion has been reported by McClements et al.[19,20] – an effect of the droplets and polyelectrolyte concentration and the experimental conditions (pH and ionic strength). The authors have proposed three different regimes depending on the concentration of polyelectrolyte added to the dispersion. The stability map of the dispersion of particles (or droplets) was derived from a calculation of the critical polymer concentrations required to saturate the surface (used in the saturation method for stabilization of the dispersion), the concentration that can ensure that the polymer adsorption is faster than the interaction between the particles or promote the depletion interactions between them.[21]

Moreover, previously has been reported that stable emul- sion stabilized by polymer adsorption with a minimum excessof polymer in the dispersion can be obtained when the concentration of polymer added to the dispersion correspond to the electrokinetic potential of the droplets with adsorbed layer close to the potential of the same polyelectrolyte in solution.[22]

An alternative approach for the encapsulation of hydro- phobic substances in oil-in-water emulsions is the spontaneous emulsification method. On the basis of the method, Calvo et al.[23] have developed a reproducible

procedure for the formation of chitosan-stabilized oil-core nanocapsules with remarkable stability.

Recently, we have documented the encapsulation of hydrophilic small molecules (caffeine) in chitosan-based shells on oilemulsion droplets[24] and it was obtained a correlation betweenthe physicochemical characteristics of chitosan in the structureof the capsules and the efficiency of drug encapsulation. The investigation of the correlation between the properties of the chitosan and the stability or properties of oil-core capsules wasextended in the present study.

The oil-core capsules suitable for encapsulation of a hydro-phobic drug indomethacin were produced. The substance was dissolved in the oil phase during the emulsification process. Thecapsules were stabilized by a polysaccharide shell formed through subsequent electrostatic adsorption of oppositely charged k-carrageenan and chitosan.

Carrageenans are a family of gel-forming and viscosifying sulphated polysaccharides which are products of extraction from certain species of red seaweeds. The molecules are composed of alternate units of D-galactose and 3,6-anhydrous- galactose joined by a 1,3 and ß-1,4 glycosidic linkage.[25] Carrageenans are linear water-soluble polymers and typically form highly viscous aqueous solutions. The chemical reactivity of the polymers results from their half-ester sulfate groups which are strongly anionic. Carrageenan is used in food preparation for gelling, thickening or emulsification and in pharmaceutical applications as an anti-inflammatory agent.

Chitosan refers to a family of amino-polysaccharides which are obtained by deacetylation of its parent polymer chitin, a polysaccharide widely distributed in the nature. The molecule isa copolymer of β-(1-4)-2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy- β-D-glucopyranose. Chitosan is a weak cationic polyelectrolyte (pKa ~ 6.5) able to form polyelectrolyte complexes with negatively charged biomacromolecules and to bind to mucosa, cell membranes, and with other oppositely charged particles or small molecules. Given these and other material properties, the polymer is beneficial for various therapeutic and biomedical applications. However, its biological activity strongly depends on the Mw, DA, pH, ionic strength,the concentration of chitosan, biological source (e. g., crustacea or fungi) impurities of lipids and proteins as interferences, surface charge, reaction time and chelating capacity.[26-28]

In order to investigate the influence of the physicochemicalproperties of polysaccharides on the encapsulation of indomethacin, few chitosan samples with different degrees of acetylation and molecular weight were used in the present study.

Materials and Methods

Materials

Indomethacin (IMC) was obtained from Sigma-Aldrich. Chitosan samples (CS), products of Heppe Medical Chitosan HMC+ GmbH (Germany), with different Mw (determined by SEC-MALS-DRI) and DA (determined by HNMR) were used for this study –CS-A (DA 9%, Mw 213 kDa), CS-B (DA 11%, Mw 187 kDa), CS-C-(DA 18%, Mw 208 kDa) and CS-E (DA 15%, Mw 34 kDa). The samples were used as received (the characterization of the chitosan samples was already described in[29]). k-Carrageenan (CAR) with an average molecular weight 660 kDa was provided by CarboMer Inc. and used without any purification. All reagents were of analytical grade from Merck (formerly Sigma-Aldrich). Milli-Q purity grade water was used throughout.

The stock solutions of polysaccharides were prepared at a concentration of 1 mg/ml in water. Chitosan samples were dissolved in 5 % stoichiometric excess of HCl and the solutions were filtered through a 5 µm filter (Minisart®) to remove the possible aggregates. The dissolution of k-carrageenan was performed in double distilled water. Indomethacin (IMC) stock solution (12 mg/ml) was prepared in ethanol.

Methods

Preparation of the Primary Oil-core Capsules

The IMC-loaded oil-core capsules were produced according to the general procedure originally described by Calvo et al.[23] with modifications. Briefly, the oil phase was prepared from 0.512 ml indomethacin solution (12 mg/ml), 0.5 ml ehtanolic lecithin solution (100 mg/ml) (Eikuron 145 V, Cargill texturing solutions Deutschland GmbH & Co. KG, Hamburg, Germany), 0.125 ml Miglyol 812 N ® (Sasol GmbH, Witten, Germany) and 9.5 ml ethanol (without shake). The aqueous phase was 20 ml chitosan solution (0.5 mg/ml). The oil phase was immediatelypoured over the aqueous phase leading to the spontaneous emulsification of the system. After mixing, the ethanol and partof the water were evaporated in a rotary evaporator at 40 °C(10 min at 80 mbar and 25 min at 40 mbar) and the volume

of the final dispersion was 10 ml. In order to estimate the effect of the presence of the drug in the oil core on the properties ofproduced structures, blank capsules without indomethacin were also produced. The oil-in-water emulsions (drug-loaded and unloaded) were also produced according to the same procedure, but the aqueous phase was replaced with ahydrochloric acid solution (pH ~ 4.5).

Preparation of the Oil-core Capsules Coated by CAR/CS Bilayer (secondary capsules)

The layer-by-layer procedure[30] was applied for the formation of capsules. For formation of the secondary capsules were used diluted dispersion of primary capsules. The stock dispersion was diluted 1:100 times in a hydrochloric acid solution (pH ~ 4.5).The first layer was formed by adding 9.5 ml from the diluted dispersion of positively charged chitosan-coated oil-core capsules (primary capsules) to the solution (0.5 ml) of negatively charged carrageenan (with concentration 1 mg/ml) and stirringfor 20 min. This procedure was repeated by adding the produced CAR-coated structures to the solution (2.4 ml) ofoppositely charged chitosan (1 mg/ml). In Scheme 1 are presented the subsequent steps in the procedure for preparation of the capsules.

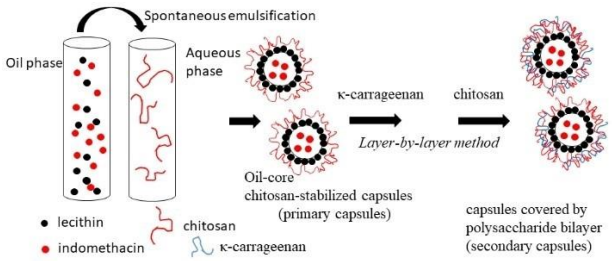
Particle Size and Electrokinetic Properties

The particle size distribution, electrokinetic properties andstability of the capsules were evaluated by dynamic light scattering with non-invasive back-scattering (DLS-NIBS) method.A Malvern Zetasizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) apparatus, equipped with a red laser light output (λ= 632.8 nm) was used for the measurements. The electro- kinetic properties of the capsules were determined by mixed mode measurement phase analysis light scattering (M3-PALS) using the same instrument.

Estimation of the Amount of Encapsulated Indomethacin

The amount of drug loaded in the capsules was determined from the difference between the initial concentration of IMC added to the dispersion and the concentration in the super-natant after centrifugation. The aliquots of the dispersion (4 tubes of 500 µl, Vivacon 500, membrane 2 kDa) were centrifuged (16 000 rpm, 25 000 × *g* for 60 min at 15 °C) by using ultracentrifuge (Mikro 220 R, Hettich GmbH"& Co. KG, Tuttlin-gen, Germany). The emulsion cream (100 µl from each centrifugation tube) was carefully extracted and added to the acetonitrile (3600 µl) to ensure destroying of the capsules. As a result, the encapsulated IMC was released. The solution was filtered (0.2 µm filter (Minisart®) and the concentration of the drug was measured according to the protocol of Bernardi et al.[2] and using a Jasco HPLC system (Jasco GmbH, Gross- Umstadt, Germany) comprising a three-line degasser (DG-2080-53), ternary gradient unit (LG-2080-02S), semi-micro HPLC pump (PU-2085Plus), an autosampler (X-LC™ 3159AS), an intelligent column thermostat (CO-2060Plus) equipped with a C18 reversed phase core-shell silica column (Aeris™ 3.6 µm wide pore XB-C18 200 Å 150 × 2.1 mm, S/N: 698087-3; Phenomenex, Torrance, USA) and UV-vis detector (X-L™ 3075UV). The mobile phase consisted of acetonitrile and water (70 : 30, v/v) adjusted to pH ~ 5.0 (acetic acid). The linear calibration curvewas obtained in the drug concentration in the range of 1–25 mg/ml in acetonitrile. The drug was detected at a wave- length at 267 nm which corresponds to the maximum absorbance peak of the drug. The amount of the drug in solution was calculated using the calibration curve and the encapsulation efficiency (EE%) was estimated by using the relation where the C_{total} is the initial concentration of the IMC added to the dispersion and C_{crean} is the drug concentration in the cream.

$$EE\% = C_{cream} / C_{total} : 100 \quad (1)$$



Scheme 1. Subsequent steps in the procedure for preparation of oil-core capsules coated by CAR/CS bilayer – spontaneous emulsification (accordingto the general procedure originally described by Calvo et al.[23]) and electrostatic adsorption of oppositely charged polysaccharides (Layer-by- layer method[30]).

Stability of The Capsules in Simulated Saliva Fluid

Simulated saliva fluid (SSF) was prepared according to Peh et al.:[31] 2.38 g Na_2HPO_4, 0.19 g KH_2PO_4 and 8.00 g NaCl were dissolved in one litre of distilled water. The pH of the solution was adjusted ca. 6.7 with phosphoric acid. An aliquot of emulsion or capsules (10 µl) was transferred to cuvettes containing SSF (990 µl) previously equilibrated at 37 °C in an incubator. The variation in the hydrodynamic size (diameter) ofthe different formulations was estimated by using DLS-NIBS as described above. The procedure was repeated using water (pH ~ 4.5, HCl) instead of SSF. The time used for measuring the size of each sample was 60 min with intervals of 5 min betweenthe measurements.

Drug Release in Simulated Saliva Fluid

The dispersion of secondary capsules was centrifugated by using centrifuge tubes (Vivacon 500, membrane 2 kDa, 500 µl) at
16 000 rpm, 25 000 g for 60 min at 15 °C (ultracentrifuge Mikro 220 R, Hettich GmbH“& Co. KG, Tuttlingen, Germany). The cream was carefully extracted. An aliquot (500 µl) of each type of isolated dispersion was transferred to a dialysis tube (D-Tube™ Dialyser Midi, MWCO 3.5 kDa, 500 µl, Sigma Aldrich) and incubated with 25 ml saliva buffer (previously equilibrated in 37 °C an incubator). The drug release from the capsules was estimated in the release medium at 37 °C at gentle stirring. Aliquots (2 ml) were drawn at predetermined time points and the medium was immediately replenished with fresh saliva fluidwith the same volume. The concentration of free drug in samples is estimated by the HPLC system by using appropriate calibration curves.

Results and Discussion

Physicochemical Characterization of Oil-core Capsules loaded by IMC

The first set of analyses examined the influence of the role of chitosan DA and Mw on the hydrodynamic diameter, D, and electrophoretic mobility, U_{ef}, of the drug-loaded or unloaded emulsions and primary capsules. To this end, chitosans of varying DA (9, 15 and 22 %) and comparable Mw (181-287 kDa),respectively, samples CS-A, CS-B and CS-C, and chitosans of similar DA (14-15 %) but approximately an order of magnitude different Mw (28 vs. 181 kDa), respectively, CS-E and CS-B, were used.

The data in Table 1 show that the size of the drug-loaded capsules is invariably higher compared to the unloaded onesand the same dependence is registered for the loaded and unloaded oil-in-water emulsions.

Previous studies have reported that the physicochemical mechanism responsible for the self-assembly process of formation of the capsulesis spontaneous emulsification or solvent displacement mechanism.[32] The process is driven by phase separation and liquid-liquid nucleation in the organic phase, migration of the co-miscible solvent (ethanol) to the aqueous phase, yielding oil-in-water emulsion stabilized by surfactant adsorption at the interface. The main role of the surfactant is to adsorb on the droplet surface, thus reducing the surface tensionand preventing flocculation or coalescence of the droplets by forming a protective interfacial layer around them. During the subsequent step, a water-soluble oppositely charged polymer can be adsorbed on the droplet surface because of the attractive interactions with the lecithin. The final surfactant- polymer membrane improves the emulsion stability.

A possible explanation for the results regarding the influence of the drug payload might be that IMC interferes

the emulsification process. The oil-in-water emulsions are stabilized by adsorption of lecithin on the droplet surface and the emulsion droplets bear negative charges conferred by lecithin phospholipids with a net negative charge at pH ~ 4.7.[33] It can thus be suggested that the presence of free IMC (no encapsulated) might have an effect on both the bulk propertiesof the dispersion by modifying the internal part of thesurfactant layer and the emulsification process itself. Therefore, the physicochemical characteristics of loaded and unloaded emulsions could be different. The presence of a free drug can provoke the coalescence or flocculation of the early lipid droplets resulting in larger droplets or flocs covered by lecithin.In the subsequent incubation step, positively charged chitosan molecules from the aqueous phase are deposited on the surfaceby electrostatic and hydrophobic interactions, thus yielding an electrosteric stabilized polysaccharide layer on the oil-core capsules.[34,35] Moreover, the adsorption of chitosan results in areduction in membrane fluidity and increases the stability of the dispersion.[16] The IMC-loaded capsules are positively charged, in keeping with the deposition of a thick chitosan layer on the surface. Clearly, the surface properties strongly depend on the chitosan characteristics (DA and Mw).

Moreover, the results in Table 1 revealed that the size of the unloaded and loaded capsules is greater for structures formed with chitosan with higher DA at almost the same Mw values (samples CS-A, CS-B and CS-C). Unexpectedly, this tendency was more pronounced on the loaded systems. We can attribute these observations to the adsorption of greater amounts of polymer with lower charge density that is required to ensure stabilization of the system, as we have shown in previous studies.[8] As expected, the size of unloaded and drug-loaded capsules formed from chitosans with similar values of DA (samples CS-B, CS-C and CS-E) increases with the molecular weight of the polymer.

Table 1. Hydrodynamic size (diameter), polydispersity index (PDI) and electrophoretic mobility (U_{ef}) of the produced capsules and emulsions.

System		D [nm]	PDI	$U_{ef} \times 10^{-8}$ [$V^2 m^{-1} s^{-1}$]
O/W emulsions	Unloaded	158 ± 1	0.10	-3.09 ± 0.07
	Loaded	178 ± 2	0.11	-2.86 ± 0.07
CS-A capsules	Unloaded	183 ± 2	0.15	+4.57 ± 0.20
	Loaded	185 ± 1	0.19	+3.36 ± 0.08
CS-B capsules	Unloaded	188 ± 2	0.22	+3.04 ± 1.13
	Loaded	208 ± 4	0.20	+3.78 ± 0.27
CS-C capsules	Unloaded	195 ± 4	0.19	+4.84 ± 0.92
	Loaded	300 ± 8	0.27	+4.93 ± 0.18
CS-E capsules	Unloaded	139 ± 4	0.18	+3.40 ± 0.15
	Loaded	166 ± 3	0.10	+3.31 ± 0.05

The secondary capsules were produced by sequential electrostatic adsorption of the oppositely charged carrageenan and chitosan on positively charged chitosan-stabilized capsules. The adsorption of carrageenan molecules results from the electrostatic attraction with the oppositely charged chitosan molecules on the capsules.

The dependences of the electrophoretic mobility and the hydrodynamic diameter of the loaded droplets as a function of the adsorption steps are presented in Figure 1. The results indicate that the overcompensation of the surface charge is achieved after each deposition step but there is no significant difference of the electrokinetic charge (in absolute value) of the capsules covered by different chitosans and carrageenan.

It is interesting to note that in all formulations, the chitosans' DA is below the critical charge density for counterioncondensation (DA~28%) as we have shown in previousstudies.[29] Because of the high charge density of these polymerchains and low surface charge density of the lecithin stabilizeddroplets, it was supposed that the chitosan molecules retain part of the condensed counterions upon the adsorptionprocess. Thus, the measured electrophoretic mobility corresponds to the "effective" and not "real" electrokinetic charge of the droplets covered by a polymer layer.[29] In spite of the registered independency of the surface charge, the hydro- dynamic size of the capsules strongly depends on the chitosan sample even at very close values of DA.

The thickness of the deposited CAR/CS bilayer is estimated by the difference in the size before and after polymer adsorption – CAR/CS-A (ca. 35.6 nm), CAR/CS-B (ca. 56.1 nm), CAR/CS-C (ca. 68.7 nm) and CAR/CS-E (ca. 7.9 nm). Note that the thickness of the bilayer is significantly lower for the chitosan with lower Mw (CS-E). Given that the Mw of CS-A, CS-B and CS-C are similar (181-287 kDa), the results indicate that there is a correlation between the film thickness and DA of the chitosan samples. This observed increase in shell thickness could be attributed, as in the case of the primary capsules, to the greater amount of adsorbed chitosan as the DA increases.

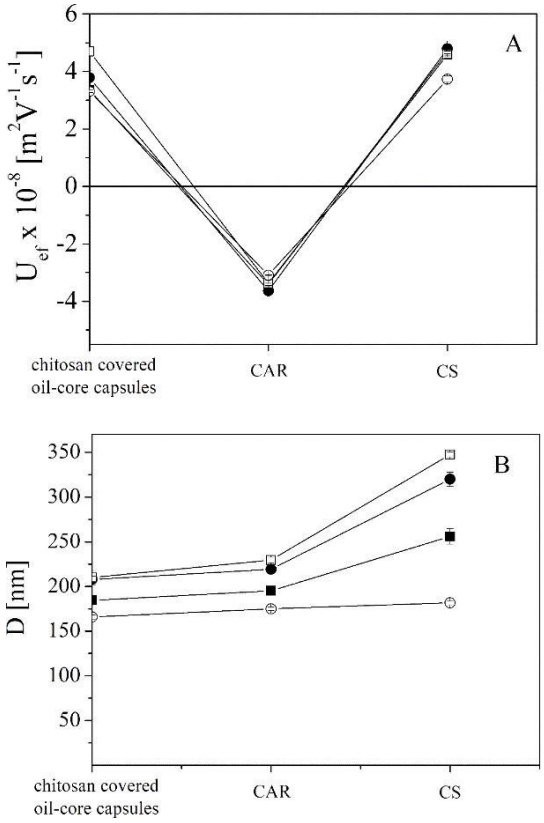


Figure 1. Dependences of the electrophoretic mobility, U_{ef}, (A) and the hydrodynamic diameter, D, (B) of secondary oil-core capsules stabilized by subsequent adsorption of k-carrageenan and chitosan: CS-A (&), CS-B (*), CS-C (&) and CS-E (*).

Indomethacin Encapsulation Efficiency

The data of drug payload encapsulation efficiency for differentcapsules revealed that the EE% for capsules comprising chitosans of high molecular weight is ca. 88 % (CS-A), 90 % (CS-B) and 94 % (CS-C), whereas for capsules of low-molecular chitosan CS-E is estimated ca. 70 % (Eq. 1.). There seems to be a correlation between the hydrodynamic size, hydrodynamic thickness of CAR/CS bilayer and IMC EE% of the capsules: thelarger the size of primary capsules, correlate with a thicker thepolysaccharide film and greater the concentration of the associated drug in the capsules. These multiple relationships may partly be explained by the adsorption of chitosan that ultimately determines the thickness of the polysaccharide shell and the interplay with the association of IMC.

Stability and Drug Release from the Capsules in SSF

The stability of the capsules in SSF was studied according to theprocedure described above. The variation in the diameter of the capsules during incubation in SSF is presented in Figure 2. In spite of the usage of much-diluted dispersion, the results clearly indicate that the stability of the system depends on the type ofchitosan used in the formation of the capsules. According tothe data, the more stable systems are those formed with chitosan sample CS-A that show no variation in size during the incubation in SSF.

The addition of SSF to the dispersion, leading to a drasticincrease of the ionic strength (ca. 3 orders of magnitude) is expected to influence on the morphology of the polysaccharideshell.[36] On the other hand, the bulk properties of the dispersion also will be different. Indeed, the stability of the studied systemin specific media is known to be affected by the variation insurface and bulk properties upon variation of the experimental conditions. Further investigation of the stability of the thin liquid films will provide insight into the emulsification mechanism and can be pursued in future studies.

Previously, Santander-Ortega et al.[34] reported the electro- kinetic behaviour and colloidal stability in biological media of unloaded chitosan-stabilized capsules produced under the same protocol as used in our study. The authors have shownthat the stability of the capsules strongly depends on the physicochemical characteristics

of the chitosan adsorbed on thedroplet surface. The capsules formed from polymers with high Mw are more stable compared to those comprising chitosans with low Mw (at low DA).
However, in the present study, we have found another factor that may also affect the stability of the systems. After the adsorption of each polysaccharide layer, the excess non- adsorbed CAR or CS molecules was not removed from the dispersion. The centrifugation steps of the procedure were eliminated because of the usage of much-diluted dispersion and because the absence of the centrifugation steps enabled control of the amount of each component in the solution during the process of film formation. Therefore, the presence of very low amount of free non-adsorbed polyelectrolyte molecules in the dispersion can also provoke the depletion flocculation.

The released amount and loading capacity are estimated (Table 2.). According to the experimental results, the stability of the systems correlates with the drug release in the buffer.

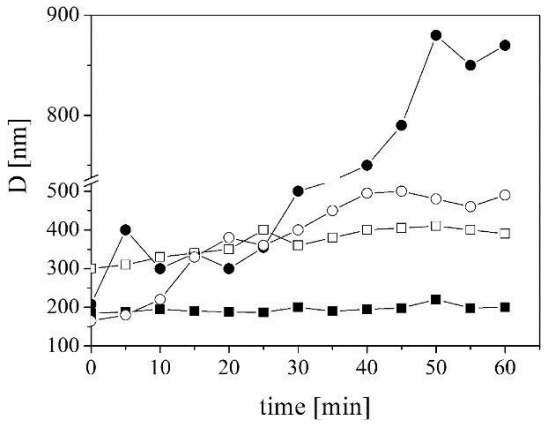


Figure 2. Stability test of secondary oil-core capsules in the presence of simulated saliva fluid (in 1:9 dilution). The variation of the hydrodynamic diameter, D, of the capsules formed of chitosan with different physiochemical properties: CS-A (&), CS-B (*), CS-C (&) and CS-E (*).

Conclusions

Indomethacin was encapsulated in oil-core carriers stabilized through a polysaccharide film. The layer-by layer method was applied for the formation of stable composite structures. In order to investigate the physicochemical properties of the polysaccharides on the drug encapsulation, chitosan samples with different DA and Mw were chosen in this study.

The experimental results indicated that the size (diameter) of the loaded capsules is larger compared to the unloaded ones. Moreover, the size of the loaded capsules depends on the Mw of the chitosan used in their formation even at very close values of DA. The thickness of the deposited CAR/CS bilayer also depends on the physicochemical properties of chitosan (LH increases with Mw and decreases with the DA of the polymer). The estimation of the EE% indicated that the amount of drug loaded in the capsules is in the range of 70 %-94 % for the different dispersions. The variation of the electrokinetic charge was registered after each adsorption step and the results indicated the achievement of overcompensation of the surface charge after chitosan and carrageenan deposition.

The experimental results seem to indicate that among the studied dispersions, the capsules formed with CS-A (DA 9 %, Mw 213 kDa), are more suitable for drug encapsulation because they are more stable in simulated biological media and have the highest EE% of indomethacin (~ 94 %).

The present study offers a feasibility to use composite polysaccharide-coated oil-in-water emulsions, of tuneable size and surface properties, to associate non-steroidal and anti-inflammatory drugs. Future studies can be aimed to establish the biopharmaceutical advantages (e. g. mucoadhesion, enhanced bioaccessibility and bioavailability).

Table 2. Encapsulation efficiency, EE%, loaded amount and released amount of indomethacin in simulated saliva fluid (SSF) (after 24 hours), stabilized by adsorption of polysaccharide film.

chitosan sample	encapsulation efficiency, [%]	loaded amount, [mg/ml]	IMC release in SSF, [%]	IMC release in SSF, [mg/ml]
CS-A	88	10.6	30.7	3.2
CS-B	90	10.8	85.2	9.2
CS-C	94	11.3	50 2	5.7
CS-E	70	8.4	70.4	5.9

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are availablefrom the corresponding author upon reasonable request.

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