EMULSIFICATION PARAMETERS STUDY ON MICELLES FORMATION FROM HYDROPHOBICALLY MODIFIED CHITOSAN

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Abstract

A series of N,O-*acylated chitosan derivatives were emulsified with different fatty acids. Hydrophobically modified chitosan derivatives were expected to exhibit self-assembly behaviour resulting in micelle formation. Several parameters of the oil-in-water emulsification process were investigated: mixing method, speed and duration, volume oil phase and addition of modifiers. Their influence on micellar Z-average diameter, size distribution and Zeta potential was analysed based on dynamic light scattering measurements. There were various relations between the hydrodynamic behaviour of chitosan derivatives, their chemical structure and the process parameters. Additionally, the obtained micelles could serve as active compound carriers because they encapsulated two model substances, namely ibuprofen and α-tocopherol.*

Keywords: *hydrophobically modified chitosan, emulsification, self-assembly, micelles, amphiphilic properties.*

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1. Introduction

Polymeric micelles are crucial in the biomedical field, a fact reflected in the variety of polymers used and in the diversity of the micelle-based delivery systems. Among all-natural polymers, polysaccharides, especially chitosan and its derivatives, have a lead role in drug delivery systems due to their biocompatibility, biodegradability, non-toxicity and mucoadhesiveness [1]chitosan is not approved by the FDA for any product in drug delivery, and as a consequence very few biotech companies are using this material. This review will aim to provide information on these biological properties that affect chitosan's safe use in drug delivery. The term \"Chitosan\" represents a large group of structurally different chemical entities that may show different biodistribution, biodegradation and toxicological profiles. Here we aim to review research in this area and critically discuss chitosan's potential to be used as a generally regarded as safe (GRAS.

One of the strategies to obtain micelles with required properties, mainly with targeted size and drug loading, is the physical or chemical modification of chitosan to obtain amphiphilic macromolecules. The fact that chitosan possesses amino and hydroxyl groups enables the incorporation of hydrophobic groups either by electrostatic interactions or covalent bonds. Changing the substitution degree of grafted hydrophobic moieties, it is possible to alter the size, loading capacity and drug release behaviour of the micelles. For example, the mean diameter of the self-aggregates of 5β-cholanic acid glycol chitosan derivatives decreased with the increase in the substitution degree of the acid due to the formation of compact hydrophobic inner cores [2]prepared by covalent attachment of 5β-cholanic acid to glycol chitosan, were investigated by using 1H NMR, dynamic light scattering, fluorescence spectroscopy, and transmission electron microscopy (TEM. The mean diameter also decreased with increasing degree of substitution, pH or ionic strength of the medium in the case of deoxycholic acid chitosan derivatives [3]. Nevertheless, this strategy used to modify the micellar properties can be recommended only until the micelles function as just a neutral carrier.

The latest trends in drug-release systems are oriented towards a biologically active micellar surface through a biologically active amphiphilic derivative. This design can bring additional value to the therapeutic treatment. For example, Wu *et al*. [4] modified glycol chitosan by stearic and folic acid and showed that the presence of the folic acid increases the targeting ability of the micelles *in vivo*.

Because the precise-specific modification of the chitosan structure can bring additional biological effects, the structural alteration of the derivative should not be used to alter micellar properties. In such a situation, the required size of the micelles needs to be obtained by selection of the appropriate parameters to prepare micelles.

In this paper, we examined in detail the ability of hydrophobically modified chitosan to create micelles and the influence of different emulsification and encapsulation parameters on the micellar size, distribution Zeta potential and stability. For this purpose, we used chitosan derivatised with stearic, oleic, linoleic or α-linolenic fatty acids (FA). In our previous work, we have shown that these derivatives are biologically active with abilities to self-organise [5, 6]; thus, they can be used in various therapeutic systems. Emulsification process parameters such as mixing method, speed and duration, the volume of the oil phase and the addition of modifiers were investigated and the relation of those parameters and micellar size distribution were defined. The distribution of diameter and Zeta potential of the micelles were measured by dynamic light scattering (DLS). Studies with two active compounds – ibuprofen and α-tocopherol – were performed to determine the ability of the micelles to encapsulate drugs.

2. Materials and Methods

2.1. Materials

Chitosan ChitoClear® 43000 – hqg10 with deacetylation degree ~83%, determined by the proton nuclear magnetic resonance (^{1}H NMR) was purchased from Primex ehf (Iceland). The following reagents were purchased from Sigma-Aldrich Co., Ltd. and used without further purification: stearic acid (SA, 95%), oleic acid (OA, ≥ 99%) linoleic acid (LA, ≥ 99%), α-linolenic acid (ALA, ≥99%), 1-ethyl-3-(3-dimethylamino–propyl)carbodiimide hydrochloride (EDC), concentrated acetic acid (AA), concentrated hydrochloric acid (HCl), sodium tripolyphosphate (TPP), ibuprofen, α-tocopherol and methanol (MeOH). Ethylene glycol (EG) and dimethyl chloride were purchased from POCh, Poland.

2.2. Derivative Synthesis

Five FA chitosan derivatives were obtained by *N*,*O*-acylation of chitosan. The derivatives were produced using the method described in our previous papers [5, 6]. For chitosan modified with LA, additional derivatives containing 17 and 40 wt% of FA were obtained, keeping the same synthesis protocol. The synthesis conditions are presented in Table 1.

2.3. Determination of Substitution Degree – Fourier-Transform Infrared (FT-IR) Analysis

According to the developed method of FA substitution estimation we presented previously [5], the total amount of grafted acid was calculated based on analysis of FT-IR spectra of the derivatives. All spectra were obtained on a Bruker ALPHA spectrometer using the following parameters: 32 scans, 2 cm^{-1} resolution and spectral range of 4000-400 cm^{-1}. Prior to the analysis, all samples were dried at 40°C in a vacuum for 24 h.

2.4. Micelle Formation

Micelles were produced by using the oil-in-water emulsification process with different process parameters (Table 2). Chitosan derivatives were dissolved in 1 wt% AA at 1 mg/ml for 24 h. Next, a proper amount of oil phase – DCM – was added dropwise to the derivative solution, then the oil phase was dispersed by one of the investigated methods. The emulsification process was conducted below 25°C.

Table 1. Chitosan derivative synthesis conditions

Chitosan concentration	1 wt%
Temperature	RT*
Fatty acid and amount	stearic acid (SA, 30 wt%) oleic acid (OLA, 30 wt%) linoleic acid (LA, 17, 30, 40 wt%) α-linolenic acid (ALA, 30 wt%)
Solvent for chitosan	1 wt% AA: MeOH (2:1 v/v)*
Time of fatty acid activation with EDC	3 hours
Product processing/ treatment	• Precipitation with methanol/ammonia solution • Washing with water and methanol • Drying for 48 h under vacuum at 60°C

* different in the case of the synthesis with stearic acid [5]

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Table 2. Emulsification process parameters

Mixing method	Ultrasounds bath laboratory shaker
Mixing time	30 min 60 min
Concentration of oil phase	3-5% (v/v)
Chitosan derivative solvent	1 wt% AA
Modifiers	EG (different volume ratio) TPP (different volume ratio)

For modified systems, if EG was used, it was added to an acidic solution. In the case of TPP addition, it was added after the dispersion of chitosan derivative with DCM was obtained, and then continuing dispersing for 30 min. The pH of the TPP solution (0.5 mg/ml) was adjusted to 5 with concentrated HCl.

2.5. Encapsulation of Active Compounds

Two active compounds – ibuprofen and α-tocopherol, which are amphiphilic and hydrophobic, respectively – were chosen as model substances for the encapsulation process. The substances were dissolved at different concentrations (1-9 wt/v) in DCM using the ultrasonic bath for 1 h. After complete dissolution, encapsulation was performed analogously to micelle preparation. Specifically, the oil phase solution (3 vol% DCM with the model substance) was mixed with chitosan derivative solution (1 mg/ml in 1 wt% AA) for 30 min. Subsequently, reduced pressure was applied to remove DCM from the dispersion.

2.6 Characterisation of the Micelles

DLS was used for size and Zeta potential evaluation. A Zetasizer NanoZS (Malvern Panalytical Ltd., Malvern, United Kingdom); a helium–neon vertically polarised laser operating at 633 nm was used as the excitation source in these studies. Measurements were carried out at 25°C with the backscatter detection system at 173°. All data are presented as mean value of Z-average diameter (Zetasizer Software, Malvern Panalytical) from a minimum of three measurements. According to the Malvern Panalytical definition, the Z-average is the intensity-weighted mean hydrodynamic size of the ensemble collection of particles measured by DLS. Before the DLS measurement, the samples after 1-month storage at 4°C were hand shaken for 3 min and incubated for 180 s inside the DLS apparatus.

3. Results and Discussion

Figure 1 presents the relative amount of FA grafted on the chitosan chains (substitution degree [SD]) depending on the type and amount of FA used (SA, OLA, LA or ALA). There were differences in the SD among the derivatives of various FA. The highest SD was for chitosan modified by OA, whereas the lowest was for chitosan modified by LA or ALA.

The differences in the SD of the derivatives could lie in the chemical structure and thus spatial conformation of the FA. The saturation (the amount of the double bonds) in the FA molecule changes the hydrodynamic behaviour of the derivative. More double bonds in the chain increases the intrinsic viscosity of the derivative solution, most likely due to some steric hindrances of the brushed macromolecules of the derivatives [5]. Hence,

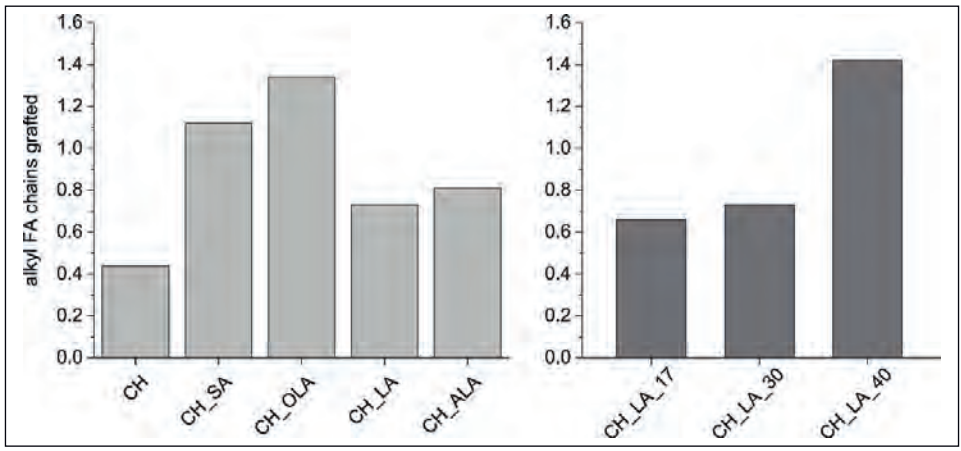

Figure 1. Relative amount of alkyl groups represented by bands ratio for: different fatty acids (FA) derivatives with constant theoretical amount of 30 wt% and derivatives with different amounts of linoleic acid (LA)

micelle formation for various derivatives could be quite different, and the characteristics of the micelles formed would also be different.

The influence of the type of force used to produce the oil-in-water dispersion, simply called the 'mixing method', and its duration were investigated (**Table 3**). As was expected, in the specific process conditions (e.g. using a laboratory shaker for 30 min), the diameter of the micelles of the series of derivatives ranged from about 100 to 380 nm. The size of the obtained micelles changed by applying a longer mixing time, a different mixing method and different derivatives.

Specifically, for CH_SA_30 the use of laboratory shaker or ultrasonic bath (for 30 min) yielded micelles with practically the same diameter (251 nm). Extending the procedure time slightly increased the micellar diameter in the case of the ultrasonic bath. For CH_OLA_30, both types of mixing and its duration influenced the micellar diameter. For the laboratory shaker, the micellar diameter slightly increased with a longer duration, an outcome that was the opposite of the ultrasonic bath, where the longer time reduced the micellar diameter. Interestingly, besides the fact that the micelles of CH_LA_30 had a smaller diameter than CH_OLA_30, they followed the same trend with the longer process duration. CH_ALA_30 exhibited a similar trend as CH_SA. Note that there were high standard deviations of micellar diameter obtained by the method employing the laboratory shaker for 30 min. This outcome meant there were no statistically significant differences compared with the ultrasonic bath. Hence, it was not clear whether the longer duration of the laboratory shaker in a bigger diameter.

It is worth noting that regardless of the FA used for chitosan modification, micelles with a diameter around 400 nm (CH_OLA_30 and CH_LA_30) showed similar diameter changes for the longer duration with both methods, namely an increase with the laboratory shaker and a decrease with the ultrasonic bath. However, micelles with a diameter around 250 nm (CH_SA_30 and CH_ALA_30) underwent similar diameter changes, namely an increase in diameter after extending the ultrasonic bath time.

Analysing the series of the CH_LA derivatives with the increasing amount of FA grafted, there was no trend in the micellar size depending on the process parameters. Importantly, the ultrasonic duration practically did not change the Z-average diameter

Table 3. The Z-average diameter of micelles (nm) depending on the type of fatty acid, mixing method and its duration (constant DCM concentration of 5 vol%; derivative solvent was 1 wt% AA)

	Laboratory shaker		**Ultrasonic bath**	
	30 min	**60 min**	**30 min**	**60 min**
CH_SA_30	251 ± 2	255 ± 2	252 ± 1	274 ± 5
CH_OLA_30	382 ± 4	405 ± 10	325 ± 2	299 ± 3
CH_LA_A_30	370 ± 10	409 ± 10	418 ± 14	391 ± 13
CH_ALA_30	285 ± 25	268 ± 6	224 ± 3	249 ± 4
CH_LA_17	141 ± 21	300 ± 58	231 ± 10	241 ± 7
CH_LA_30	370 ± 10	409 ± 10	418 ± 14	391 ± 13
CH_LA_40	103 ± 1	74 ± 4	200 ± 5	189 ± 2

of the micelles, regardless of the amount of LA in the derivative. Because micelles often needs to be re-dispersed after their preparation (to prepare a solution with therapeutic dosage or to change the medium in which the micelles are dispersed), for this type of derivative (with LA) ultrasonic bath could be used for re-dispersion step.

To investigate the overall stability of the micelles, their size was evaluated after 1 month of storage in static conditions at 4°C. **Figure 2** presents the changes in Z-average values for all obtained systems. For CH_SA_30, CH_OLA_30 and CH_LA_30, the Z-average of the micelles decreased only slightly with time regardless of the mixing method and its duration (**Figure 2a, b**). This positive result indicates that hydrophobic interactions inside the micellar core are sufficient to achieve thermodynamic stabilisation. It is worth emphasising that usually to prevent the agglomeration in an oil-in-water emulsion, low-molecular-weight surfactants are added [7, 8]. However, because the micelles in the presented systems do not agglomerate, the use of additional surfactant is not needed. CH_ALA_30 exhibited different hydrodynamic behaviour. After 1 month, the Z-average diameter increased, suggesting micellar agglomeration, mostly for the system obtained using ultrasonic bath.

Analysing the series with different amounts of LA (Figure 2c and d), there was a tendency towards agglomeration for micelles with the lowest initial diameter. This might suggest that the micellar structure, obtained under shear forces leading to very low diameters (LA_17 and LA_40), are not thermodynamically stable, and thus the initial size is not preserved.

Further analysis led to an unexpected observation. After 1 month, for almost all systems, the Z-average diameter reached the range around 250-300 nm, indicating that this size is most hydrodynamically favourable for chitosan modified with FA, regardless of the mixing type and its duration.

The high Zeta potential of the micelles further confirmed their stability. As presented in Table 4, all systems possessed a positive Zeta potential, and the Zeta potential of the micelles was higher than the Zeta potential of the derivative solutions before emulsification. These data clearly indicate that the micellar form is the most stable spatial conformation of chitosan derivative chains. The observed increase in the positive surface charge of the micelles in accordance with data from the literature leads to stronger interaction of

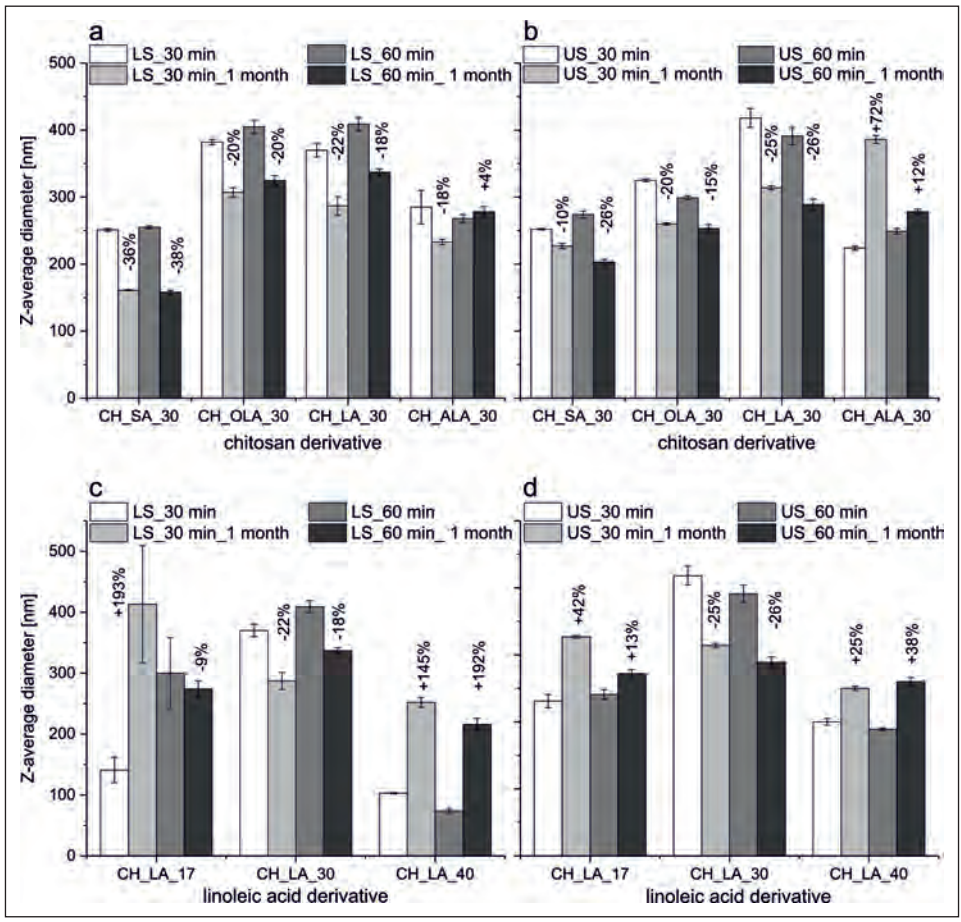

Figure 2. Z-average diameter (nm) for micelles after preparation and 1-month incubation obtained by: (a) laboratory shaker and (b) ultrasonic bath for different fatty acids derivatives and (c) laboratory shaker and (d) ultrasound bath for derivatives with different amounts of linoleic acid (LA)

the micelles with biological negative surfaces like bacteria cell membranes or mucus layer [9, 10], resulting in high applicability of those micellar systems.

Another important process parameter that influences the size of the micelles and drug loading capacity is the volume of the oil phase. The oil phase, apart from initiating the self-assembly of amphiphilic macromolecules, plays the role of a dissolving medium for active substances. As presented in Table 5, regardless of the mixing time, the Z-average diameter of the micelles slightly decreased as the DCM concentration increased. Taking into account that the active substance concentration in the oil phase is constant, a higher amount of the oil phase used in the emulsification process should result in a higher drug load.

The mixing speed was also studied as a parameter that could influence the size of micelles during the emulsification process. Figure 3a and c presents the influence of mixing speed on micellar size distribution. An increased mixing speed resulted in a narrower size distribution of micelles, a phenomenon that can be seen in the greater

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Table 4. The Zeta potentials (ZP) of derivatives solutions and micellar systems

Solution	ZP (mV)
sol CH	61.6 ± 1.5
sol CH_SA_30	74.4 ± 5.1
sol CH_OLA_30	70.4 ± 1.6
sol CH_LA_A_30	55.2 ± 4.2
sol CH_ALA_30	49.3 ± 3.4

Micelles	Laboratory shaker		Ultrasounic bath	
	30 min	60 min	30 min	60 min
CH_SA_30	87.3 ± 2.9	90.6 ± 2.8	101 ± 2.7	100 ± 3
CH_OLA_30	65.6 ± 2.6	66.7 ± 1.8	67.3 ± 1.8	67.2 ± 2.8
CH_LA_30	18.8 ± 0.5	47.8 ± 0.7	66.6 ± 1.3	61.8 ± 1.4
CH_ALA_30	66.2 ± 0.9	61 ± 2.4	63.5 ± 1.5	63.9 ± 2.1
CH_LA_17	59.9 ± 2.9	56.8 ± 1.1	59.3 ± 0.9	61.2 ± 1.9
CH_LA_30	18.8 ± 0.5	47.8 ± 0.7	66.6 ± 1.3	61.8 ± 1.4
CH_LA_40	61 ± 0.7	61.6 ± 3	60.2 ± 2.2	59 ± 1

Table 5. The Z-average diameter of micelles (nm) obtained with different concentration of DCM at two mixing times (CH_LA_30, 1 mg/ml solution, laboratory shaker)

DCM concentration [vol. %]	Mixing time	
	30 min	60 min
3%	496 ± 8	550 ± 2
4%	503 ± 7	513 ± 6
5%	370 ± 10	409 ± 10

number of the micelles in the range of 122-394 nm. Higher rotation speed increases shear force in the dispersion, a change that probably results in more homogenous size of DCM droplets. This dependence is even more pronounced for the dispersion system modified by the addition of ethylene glycol, where the micellar size distribution in the analysed range increased significantly from 65% to 92% (Figure 3b and d).

The changes observed for the system containing EG probably result from specific properties of EG molecules, which can act as a co-solvent and structure-breaking solute in micellar systems [11-13]12-,s-12,2Br- where s = 3-5 methylene groups, has been investigated in water-ethylene glycol, EG, mixtures with weight percentages of EG up to 50%. Subsequently, effects of the addition of the organic solventen the micellar growth of these surfactants and on the surfactant concentration range where sphere-to-rod transitions occur were studied by means of steady-state and time-resolved fluorescence quenching and spectroscopic measurements. Results show that an increase in the weight

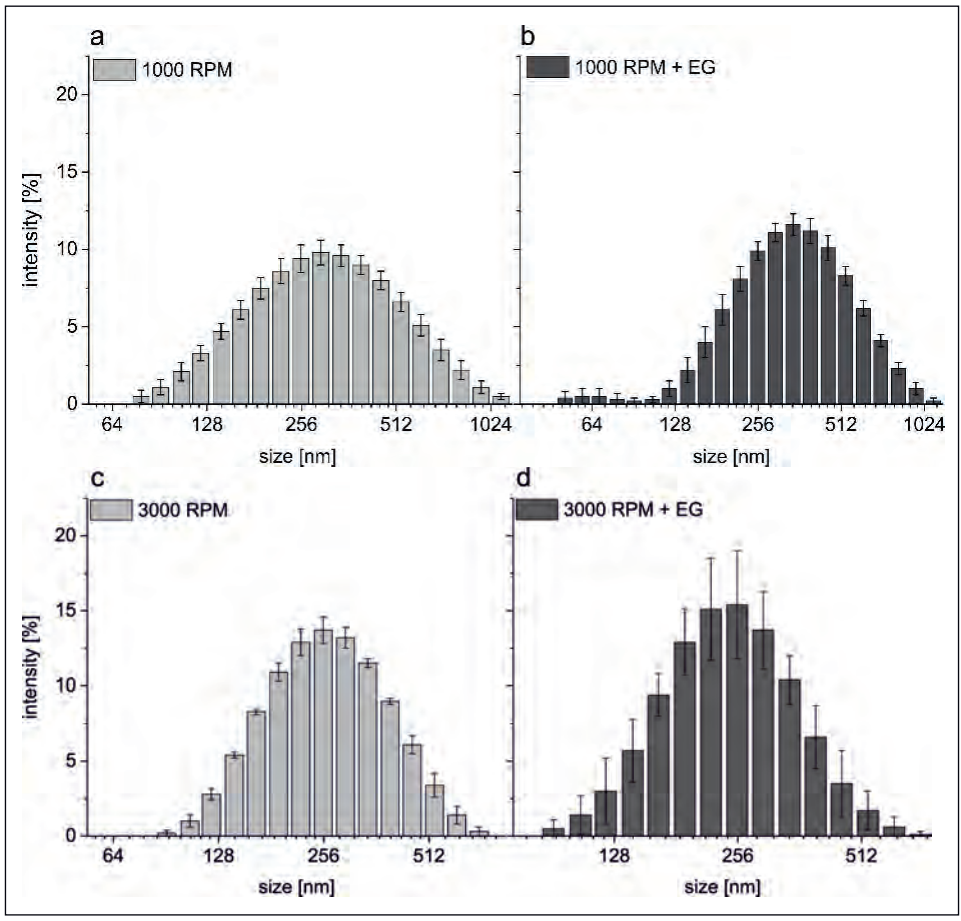

Figure 3. Size distribution of the micelles at selected preparation conditions: 1000 rpm (a) without ethylene glycol (EG) and (b) with EG; and 3000 rpm (c) without EG and (d) with EG (for all cases: CH_LA_30, 1 mg/mL solution, laboratory shaker)

percentage of ethylene glycol added to aqueous 12-s-12,2Br- (s = 3-5. Therefore, by applying higher shear forces (3000 rpm) combined with physicochemical interactions of EG with amphiphilic chitosan derivative, a more uniform size distribution of micelles was achieved.

TPP was used as the second modifier to increase the stability of the micelles. The addition of TPP is one of the common cross-linking method for chitosan based micro- and nanosized micelles [14-16]. To increase the positive effect of TPP on micelles formation for this part of the study, the chitosan derivative concentration was reduced to 0.5 mg/ml. Figure 4 presents the size distribution of micelles obtained at different chitosan derivative:TPP ratios (v/v). For the 1:0.25 ratio, there was a broad size distribution of micelles. For the 1:0.3 and 1:0.35 ratios, the distributions were significantly narrower, with more micelles in the range of 50-122 nm.

Ionic interactions between TPP and chitosan macromolecules lead to increased rigidity and hydrodynamic packing density of the chitosan chains in the outer shell of the micelles. As a consequence, the size distribution of micelles becomes smaller and more

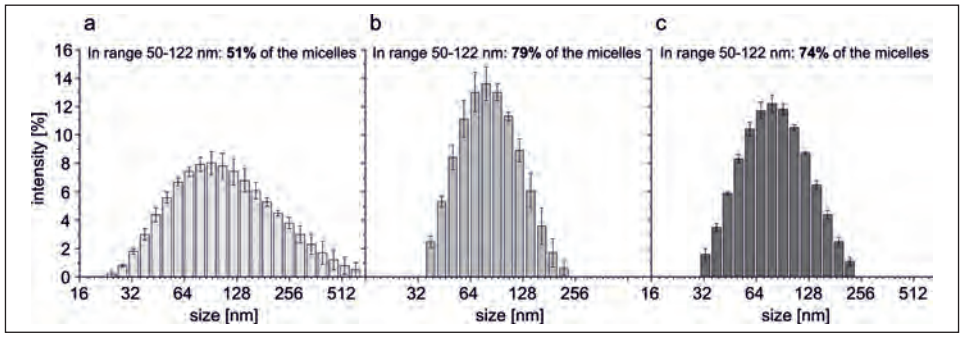

Figure 4. Size distribution of micelles obtained with different CH_LA_30:TPP ratios: (a) 1:0.25 (v/v), (b) 1:0.3 (v/v) and (c) 1:0.35 (v/v)

uniform (here observed for the 1:0.3 ratio). However, a further increase in TPP induced the opposite effect. When no more TPP molecules can interact with protonated amino groups of chitosan chains inside the micelles, they will start to create the outer layer of the micelles. This phenomenon will induce intra-micellar interactions and cause their aggregation and mutual cross-linking.

In biomedical applications, nanometric polymeric micelles can serve as drugs, enzymes or living cell carriers [17, 18]. Due to the specific interactions between encapsulated compound and the micellar core-shell structure, the self-assembled properties of amphiphilic macromolecules could be altered. Therefore, we examined the potential encapsulation of two model compounds, namely ibuprofen and α-tocopherol. Ibuprofen is an anionic, amphiphilic drug, with limited water solubility (21 mg/ml at 25°C, logP = 3.97), whereas α-tocopherol is hydrophobic and insoluble in water compound (logP = 12.2). Figure 5 presents the Z-average diameter of micelles with different amounts of drugs after preparation and after 7 days of incubation at 37°C.

For micelles with ibuprofen, there was no direct relation between the size of micelles and drug concentration. Even more interesting, the smallest micelles (502 ± 15 nm) had the highest ibuprofen concentration. Pereira *et al*. [19] observed that interactions of ibuprofen with chitosan macromolecules involve the ibuprofen C=O and CH-CO groups and the chitosan NH_2 and OH groups. This suggests that as the ibuprofen concentration increases, it can be located in the hydrophobic core of the micelles as well as in their outer shell, which probably could enhance the internal packing of chitosan chains and in consequence decreasing the micelles size.

For micelles with α-tocopherol, the micelles with the highest Z-average diameter (1370 ± 126 nm) had the highest dug concentration; this was the opposite result of ibuprofen. Nevertheless, after the incubation time, the micelles with the highest αTOC concentration had a comparable size with the other systems with a different drug concentration (~400 nm).

Larger diameters of 'as-received' micelles may arise from the type of mixing method: vortex and distribution of shear forces during the emulsification process could result in various spatial structures of micelles. It has been shown that anisometric, rod-like structures are often induced by the shear forces in the flow direction. However, after the flow is stopped those shear-induced spatial structures decay and reform to a more energetically preferable shape (sphere) [20]. Therefore, it is expected that after incubation, micelles possess thermodynamic stability, and thus the Z-average size of micelles, irrespectively of the drug type and concentration, is in the similar range. This hypothesis was confirmed

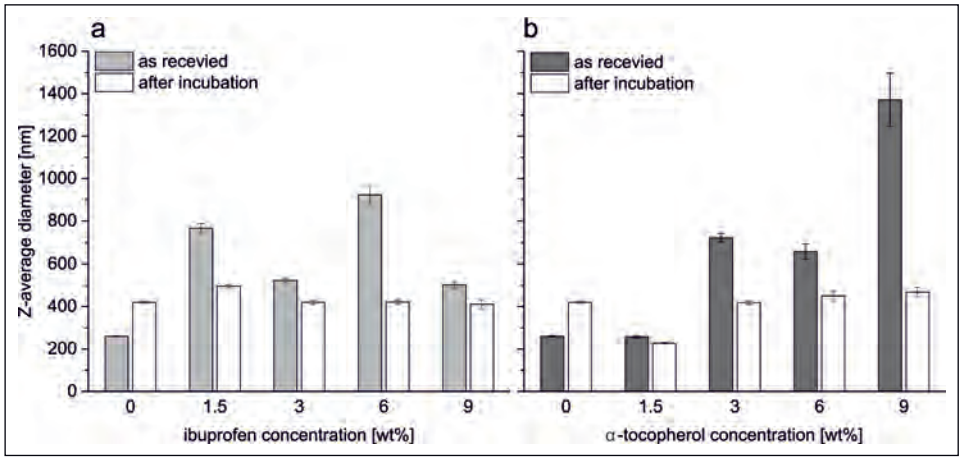

Figure 5. Z-average diameter (nm) of micelles with (a) different concentration of ibuprofen and (b) different concentration of α-tocopherol as received and after incubation (for all cases: CH_LA_30, 1 mg/mL solution, laboratory shaker)

by the lack of differences in the Zeta potential of micelles with drugs. Regardless of the drug concentration, the Zeta potential was similar: 72 ± 2 mV for ibuprofen and 71 ± 1 mV for α-tocopherol.

4. Conclusions

In the presented, we evaluated the influence of various process parameters such as mixing method, its speed and duration, the concentration of the oil phase and the addition of modifiers. The Z-average diameter of the micelles could be regulated by applying various forces to the chitosan derivative solution in the emulsification process. The method rather than the duration had a greater impact on Z-average diameter. Regarding vortex mixing, and thus using shear forces, the speed of mixing seems to be the most important parameter. Zeta potential measurements indicated high stability of all micellar systems. Additionally, the hydrodynamically favourable size was defined: after 1 month of storage, most of the micelles were 250-300 nm in diameter, regardless of the mixing type and its duration. If the targeted size of the micelles cannot be achieved by the applied process parameters, the modifier can be added to the emulsification system. This approach can narrow the size distribution of micelles and/or increase their stability. Synthesised derivatives led to versatility of the formed micelles according to the emulsification process parameters used. Their capability for drug delivery application was proved by successful encapsulation of the model compounds.

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