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Colloids and Surfaces A: Physicochemical and Engineering Aspects
2020, vol. 607, December 2020, pp. 1-7

ISSN: 0927-7757

DOI: https://doi.org/10.1016/j.colsurfa.2020.125526

Accepted manuscript

Study of cholesterol's effect on the properties of catanionic vesicular systems: comparison of light-scattering results with ultrasonic and fluorescence spectroscopy

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Abstract

This work is focused on the study of properties associated with the effect of cholesterol levels on the stability of vesicular systems based on the ion pair amphiphile hexadecyltrimethylammonium-dodecylsulphate (HTMA-DS) at laboratory temperature. The HTMA-DS catanionic system was doped with dioctadecyldimethylammonium chloride in a 9:1 molar ratio and cholesterol in the amount of 0, 3, 13, 23, 33, 43, 53, 63, and 73 mol.% was added. In this system, the size distributions were studied using the dynamic light-scattering technique and the zeta potential was determined. These standard techniques were supplemented by ultrasonic and fluorescence spectroscopy techniques. Due to low stability and high opalescence of samples, spectral techniques were used only for the samples with cholesterol content above 23 mol.%. The results from High-Resolution Ultrasonic Spectroscopy and from Fluorescence Spectroscopy are in agreement. They equally point to a change in the amount of hydration water in the membrane, the largest amount of which is present in the samples with 43 and 53 mol.% cholesterol. Using the light-scattering technique, the short-term stability of prepared vesicular systems was also observed over the first 36 days. Obtained results confirmed that the most stable systems are those containing 43 or 53 mol.% of cholesterol.

Keywords:

Ion pair amphiphile; Catanionic vesicles; Cholesterol; High-resolution ultrasonic spectroscopy; hydration water; Fluorescence spectroscopy.

1 Introduction

Catanionic vesicles have a spherical structure with, hydrophilic and hydrophobic domains. Due to this structure, some hydrophilic, as well as hydrophobic substances, can be encapsulated into these domains [1–6]. The formation of catanionic vesicles is a spontaneous process, but they can be formed also by using semi-spontaneous or mechanical disruption methods [1–5,7,8]. Their structure is very similar to the structure of a liposome, but catanionic vesicles are formed from oppositely charged single chain surfactants or ion pair amphiphile (IPA) [1,3,9,10]. The surfactants usually precipitate in IPA when the oppositely-charged surfactants are mixed in an equimolar ratio [1,6,8]. The vesicles are formed through the interactions between polar headgroups of cationic and anionic surfactants in the aqueous phase and when the counterions are removed from this mixture; the residue is called ion pair amphiphile [1,3,9,10]. Catanionic vesicles may be used as an alternative to liposomes, e.g. such as drug delivery carriers [1–6]. Their advantage is lower price and possibility of preparations from a wide variety of surfactant combinations. However, their size, polydispersity and lamellarity are determined by the choice of surfactants [6]. There also exist so-called catanionic drug-surfactant complexes, which are formed from a surface-active drug and an oppositely charged surfactant, when the drug is a part of a membrane. These vesicles can be an alternative to the encapsulation of drugs into catanionic vesicles [4]. The catanionic drug-surfactant complexes have been intensively studied. For example, among the drug compounds, which have been investigated, there are lidocaine, ibuprofen and naproxen [11] or a combination of diphenhydramine and tetracaine with sodium dodecylsulphate (SDS) as an oppositely charged surfactant [12] for drug release from a gel. The study [13] describes the combination of double-chained surfactants with oppositely charged drug tetracaine hydrochloride. Anionic or cationic surfactants can be combined also with phospholipids, e.g. a combination with dipalmitoylphosphatidylcholine (DPPC) [14], a combination of natural phospholipid soylecithin with IPA which was prepared by mixing SDS and HTMAB [15,16]. Some hybrid vesicles were also prepared from soy phosphatidylcholine and IPA from HTMAB and SDS [15].

However, catanionic vesicles prepared only from IPAs usually have very poor physical stability and they can easily fuse or aggregate together [1,7,9,10] The adjustment of intra/inter-vesicle interactions can help increase vesicles stability. One of the strategies for improving physical stability is the addition of some charged double-chained surfactants. Then, catanionic vesicles are same-charged and the repulsive interactions between them prevail, therefore the aggregation and fusion are inhibited. A different double-chained surfactant then indicates different long-term stability of vesicles [7,9,17,18]. Charged catanionic vesicles can also bring another benefit in enabling the interaction with oppositely charged (bio)polymers through hydrophobic and/or electrostatic interactions. It can also improve higher stability and biocompatibility of them [4,19]. Another step to improve stability is the addition of cholesterol [1,8,9]. Cholesterol is known as a regulator of membrane permeability, elasticity and stiffness and it is able to prevent the aggregation and enhance the stability of bilayers. Cholesterol is the most often-used in liposome as a stabilizing agent. Thanks to the hydroxyl group, it is able to form hydrogen bonds and then it is incorporated into the bilayer of vesicles and increases their stability and fluidity [20,21]. Also the presence of cholesterol can cause the increase in size of particles due to the incorporation to the bilayers and their expansion. However, larger amount of cholesterol may cause the crystallization of carbohydrate chains and the destabilization effect on vesicles [20]. Cholesterol can influence the charge character, the physical stability and the molecular packing of vesicles bilayers [1,8,9,22]. Its inclusion would increase the van der Waals attraction and the average area per IPA's molecule, and thus reduce the electrostatic repulsion (decrease of zeta potential) [23,24]. The counterion could be gained by the addition of charged double chain surfactants to improve the stability of IPA

system. Overall reduction of vesicles zeta potential due to the incorporation of cholesterol leads to the decrease of counterion binding tendency [1,9].

This work discusses the influence of varying cholesterol content on the physical stability and properties of catanionic vesicles. They were composed of IPA and positively charged double chained surfactant dioctadecyldimethylammonium chloride (DODAC). IPA was prepared by mixing hexadecyltrimethylammonium bromide (HTMAB) with oppositely charged sodium dodecylsulphate (SDS). A schematic diagram of the molecular packing of HTMA-DS/DODAC with cholesterol is shown on Figure 1. The average size of vesicles was measured by dynamic light-scattering (DLS) and the zeta potential was measured by electrophoretic light scattering (ELS). These are standard methods that are very often used to describe catanionic vesicles [1,9]. The membrane properties have been studied as well by two methods. One is based on solvent relaxation and the effect on a Laurdan fluorescence probe. Laurdan has been reported many times to be the probe suitable for determining the phase transition of membranes via the interaction of its excited state with polar mobile molecules that penetrate to the vicinity of fluorophore [25]. Due to its hydrophobic tail, the probe is near the polar heads of membrane constituents [26]. The solvent relaxation leads to a bathochromic shift in the fluorescence emission spectra, where the emission maximum in rigid membranes is localized around 440 nm and moves to 490 nm when the system becomes fluid [27–29]. The shifts in the Laurdan emission spectrum are quantified by calculating the generalized polarization (GP) value. This value is highest in the case of a rigid system and lowest and also negative in the case of a fluid system [30]. The second method for describing the membrane properties is high-resolution ultrasonic spectroscopy (HRUS). HRUS is an efficient technique suitable for the analysis of colloidal systems [31–34]. It is based on precision measurements of the velocity and the attenuation of high-frequency (ultrasound) sound waves propagating through analysed samples. This technique allows direct non-destructive investigation of the organization of molecules and intermolecular forces in the sample [35]. Also, many properties of surfactants' activity have been examined using ultrasonic spectroscopy, mainly their interaction with oppositely charged polymers [36–39].

2 Experimental methods

2.1 Materials

Hexadecyltrimethylammonium bromide (HTMAB) (purity ≥ 98.0%), sodium dodecylsulphate (SDS) (purity ≥ 98.5%) and cholesterol (purity ≥ 99.0%) were purchased from Sigma, USA. Dioctadecyldimethylammonium chloride (DODAC) (purity ~ 97%) was supplied by Alfa Aesar, USA. Chloroform was obtained from Penta s.r.o. A Laurdan fluorescence probe (6-dodecanoyl-N,N-dimethyl-2-naphthyalamine, purity ≥ 97.0%) was obtained from Sigma, USA and 5-Hexadecanoylaminofluorescein (purity ≥ 95.0%) was supplied from ThermoFisher, USA. Pure water was purchased from ELGA LabWater.

2.2 Preparation of catanionic vesicles with double-chained surfactant

The preparation of IPA has been described in detail in previous literature, e.g. in [1]. Briefly, IPA was prepared by mixing equivalent volumes, 500 mL, of 20 mM aqueous solution of HTMAB and SDS. This mixture was allowed 1 day for complete precipitation. The precipitate was obtained by centrifugation and filtration. The precipitate was further washed on the filter to remove the counterions. After the counterions were removed from this mixture, the residue was denoted as $HTMA^{+}DS^{-}$ (hexadecytrimethylammonium-dodecylsulphate). The composition was checked and confirmed by the balance of nitrogen and sulphur from Elemental Analysis.

To this IPA powder the double-chained cationic surfactant DODAC was added and both were dissolved in chloroform. These components were mixed in the molar ratio of 9:1 and then varying amounts of cholesterol were added. The total amount of cholesterol was set to 0, 3, 13, 23, 33, 43, 53, 63 and 73 mol.%. Chloroform was evaporated to form thin films on glass beads. The thin film was then hydrated with 60 mL pure water and this sample was dispersed through the sonification process using an ultrasonic dispersion device (model HD 3 200, Bandelin Electronic GmbH & Co. KG) with an amplitude of 50% and sonification energy of 25 kJ.

2.3 Methods

Size and zeta-potential. Samples were measured one hour after the preparation without dilution or any other modification. For each of them, the average size of particles and the zeta potential were measured using a ZetaSizer Nano ZS (Malvern Instruments Ltd.) with a 10 mW He-Ne laser source (λ = 633 nm). The measurement was carried out at a constant temperature of 25 °C. The zeta potential was measured by electrophoretic light-scattering (ELS) using a dip cell electrode and calculated by Smoluchowski's model for the aqueous environment. Each zeta potential measurement was repeated five times. The average size was measured by dynamic light-scattering (DLS) in a glass cuvette. Each average size measurement was performed in triplicate. The value of average size is obtained from the diffusion coefficient *D* in the Stokes-Einstein equation:

$$D = kT/f = kT/6\pi\eta a. \quad (1)$$

Generalized Polarization. For the measurement of generalized polarization it is necessary to add Laurdan such as fluorescence probe and let it solubilize with gentle stirring overnight. The final concentration of Laurdan in samples was kept at 5 x 10^{-7} M. The samples with high turbidity had to be sonicated in an ultrasonic bath to eliminate the influence of scattering on larger particles. The Laurdan fluorescence emission was monitored with a photon-counting fluorometer FS 5 (Edinburgh Instruments, GB) with temperature controlled by a Peltier module, and the sample homogeneity was maintained by continuous magnetic stirring. A generalized polarization value was calculated from the emission intensities I_{440} and I_{490}, as follows:

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490}). \quad (2)$$

High-Resolution Ultrasonic Spectroscopy. Samples were measured one hour after the preparation without dilution or any other modification. The ultrasonic velocity and attenuation for each sample were measured at six selected frequencies in the range from 2.5 to 14.9 MHz using an HR-US 102T ultrasonic spectrometer (Ultrasonic Scientific, Ireland). This device is equipped with two cells enabling single-cell or differential measurements. The differential mode was used in this work. The temperature was controlled with a Haake PC300 heating bath, which provided a temperature stability of ±0.01 °C. The temperature was recorded by a sensor inserted in the ultrasonic spectrometer by the manufacturer. The measuring and the reference cells were filled with deeply degassed sample and water, respectively. Ultrasonic measurements were accompanied by the measurements of density in order to calculate the compressibility from ultrasonic velocity and density. Each value is the average of three replicate measurements and the standard deviation is expressed by error bars. A DSA 5000 M densitometer (Anton Paar, Austria) was used in this work. When combining the ultrasonic velocity (u) with the density measurement (ρ), it is possible to calculate the adiabatic compressibility (β) of the sample using known Laplace equation (3) [36].

$$\beta = 1/\rho u^2. \quad (3)$$

Turbidimetry. The turbidity of opalescent samples was taken as the apparent absorbance at wavelength 630 nm. It was measured with UV-vis spectrophotometer CARY50 (Varian Inc., USA) and it was calculated similarly as absorbance with the intensity of incident beam I_0, and intensity of that light after it passed through the sample I:

$$\tau = \log(I_0/I). \quad (4)$$

pH measurement. The pH of the samples was measured with SevenEasy pH-meter (Mettler Toledo).

3 Results and Discussion

3.1 Characterization of catanionic vesicles

3.1.1 Initial average size and zeta potential

Most of the samples exhibited significant opalescence, which was problematic for the DLS measurement. Therefore, the turbidimetry was performed using UV-VIS spectroscopy at the wavelength of 630 nm. The turbidity was higher in the sample containing cholesterol from 0 to 23 mol.% and 73 mol.% while the lowest value was reported for the samples with 33, 43 and 53 mol. % of cholesterol as shown in Figure 2 and Figure 3. To obtain comparable results, the samples were diluted to the turbidity value between 0.1 and 0.3. The size and the zeta potential were measured by dynamic and electrophoretic light-scattering. The results are listed in Table 1.

The samples with 0–23 mol.% of cholesterol showed high instability. This resulted in high standard deviation value so repeated measurements did not lead to comparable results. Only the vesicles with 23 mol.% lead to reasonable size results. The aggregation occurs due to system instability, which is in agreement with the turbidimetry measurement. This leads to the formation of polydisperse system, as approved by the polydispersity index value (see Table 1). The generally accepted PDI value for a monodisperse samples is below 0.3 [40]. The instability was confirmed by the measurement of the zeta potential, which is close to instability area. The samples with the cholesterol content of 33 to 53 mol.% had the smallest average size and high zeta potential (see Table 1). With higher cholesterol content (63 and 73 mol.%), the stability decreased, probably due to larger amounts of cholesterol which may cause the crystallization of carbohydrate chains and destabilization of vesicles [20]. All samples showed positive zeta potential due to the addition of positively charged double-chained surfactant (DODAC). These results are in good agreement with other published results [1,9,20].

Table 1: Initial average size, polydispersity index, zeta potential and pH of catanionic vesicles HTMA-DS/DODAC with varying molar concentration of cholesterol.

Content of cholesterol (mol.%)	Initial average size (nm)	Polydispersity index (-)	Zeta potential (mV)	pH (-)
0	572 ± 432	0.474 ± 0.351	44 ± 3	6.3 ± 0.1
3	713 ± 817	0.581 ± 0.195	37 ± 4	6.5 ± 0.1
13	927 ± 473	0.809 ± 0.061	35 ± 2	6.5 ± 0.1

23	272 ± 49	0.496 ± 0.055	38 ± 5	6.5 ± 0.2
33	96 ± 1	0.292 ± 0.049	58 ± 6	6.6 ± 0.2
43	91 ± 1	0.265 ± 0.014	57 ± 7	6.5 ± 0.1
53	98 ± 1	0.265 ± 0.008	56 ± 7	6.6 ± 0.1
63	140 ± 2	0.452 ± 0.080	60 ± 2	6.5 ± 0.1
73	196 ± 12	0.736 ± 0.060	30 ± 2	6.5 ± 0.0

The zeta potential depends on the surrounding conditions such as ionic strength, pH, concentration of additives and temperature [41]. For this reason, the pH of all samples was measured, it was relatively the same in all solutions, there were no significant changes (Table 1). This means that increasing the amount of cholesterol did not affect the pH. Another possibility of influencing the zeta potential is the presence of counterions in the solution [42]. Our vesicles were prepared in pure water so no counterions were present. The only ions in our solutions were chloride anions, they were gained from the dissociation of DODAC. This means that positive charge of vesicles could be influenced (decreased) only by anionic counterions. On charged surface the water molecules are predominantly oriented with respect to the charged surface. This results in a strong local decrease of membrane permittivity [43].

3.1.2 Membrane properties

The membrane properties were studied with two approaches. The first one is based on the propagation of ultrasound through a sample and the second one is based on solvent relaxation and the effect on the Laurdan fluorescence probe.

The values determined from an ultrasonic spectroscopy are ultrasonic velocity, adiabatic compressibility and attenuation in cell. In a denser (tougher) medium the ultrasound velocity is higher than in less dense medium. In aqueous solutions, the ultrasound velocity reflects mainly the hydration state of solutes – water in hydration shells is less compressible (denser) than bulk water. The reduction in the relative velocity means decreasing difference between the velocity of the solution and solvent. This may mean either higher mobility of hydration water, decrease in rigidity, or increased elasticity of the vesicles membranes. Water has significantly higher compressibility near hydrophobic groups, on the other hand, if it interacts with charged molecules, it has the lowest compressibility [33]. The measured data in Figure 4 show the relative velocity of ultrasonic waves passing through the sample as a function of the cholesterol concentration. As can be seen, the concentration series show a break in the trend of relative velocity around 43 mol.% of cholesterol, where a shallow minimum is formed, indicating the occurrence of the least rigid structures. The density values at 25 °C were also measured for the samples of the second concentration series so that the compressibility can be calculated according to equation (3).

The graph in Figure 5 shows that the compressibility values of the system increased with increasing cholesterol content which is interesting because cholesterol should stiffen the membranes. The increase is remarkable particularly in the region where the velocity minimum was observed and up to 63 mol.% where it decreased due to the phase separation in the system. In addition to the values of relative velocity and compressibility, the attenuation parameter of ultrasonic waves (Figure 5) was investigated. The attenuation in the presence of cholesterol is almost constant from 3 up to about 60 mol.% of cholesterol with the value of about 0.5 m^{-1}. At the lowest and highest cholesterol concentration there is a significant increase in attenuation values pointing to increased heterogeneity of samples, which is consistent with their observed opacity or even phase separation.

Due to its structure, Laurdan is incorporated into membranes, its fluorophore being located near the polar heads of the membrane components. Its emission spectrum reacts to the presence of hydration

water in its surroundings, which is expressed by the quantity of generalized polarization. The lower the GP value, the more hydration water is in the vicinity of the probe [25].

The addition of cholesterol to the vesicles causes an increase in the surface and a decrease in charge density, which will reduce the willingness to interact with countercharged molecules (see Figure 4) [9]. This could mean that there is a decrease in the number of interactions with water (charge-dipole of water), and also the ordered arrangement of water molecules is likely to decrease, due to lower charge density of vesicles [44]. This leads to a decrease in the amount of rigid water, which causes the reduction in the relative velocity. After exceeding a certain concentration of cholesterol in the sample (43 mol.%), this effect is outweighed by the effect of cholesterol on the organization of hydrocarbon chains inside the membrane [9,45]. Due to high opacity of samples with 0, 3, and 13 mol.% of cholesterol, which was also reflected in the measurement of ultrasonic attenuation, because especially in the case of the lowest and the highest cholesterol content in the sample, there was a significant increase in ultrasonic attenuation, indicating increased heterogeneity of the sample. Therefore, the GP values were determined only for the samples with 23–73 mol.% of cholesterol. These values also exhibited a minimum plateau with the cholesterol content of 33 and 43 mol.% related to higher amount of water in the membrane. This plateau is followed by increasing values of GP and decreasing amount of water, which correlates with the results from HRUS. The vesicles with the lowest cholesterol content have the lowest compressibility values, and the membrane compressibility increases with increasing cholesterol content, which is in accordance with the findings of Sarvazyan et al. [33] on charged molecules and their interactions with water dipoles.

3.2 Short-term stability of catanionic vesicles

The stability of the systems was studied during the first 36 days after the preparation. During this time, the size distribution and the zeta potential were monitored by DLS and ELS measurements. The system instability is manifested by increasing size and polydispersity index values [1,9]. The increase in aggregate size can also be easily monitored using the turbidity measurements.

The stability of samples with cholesterol content from 0 to 23 mol.% and 73 mol.% was very poor as was mentioned above. For the sample with 33 mol.%, higher stability was predicted due to high value of the zeta potential, but unfortunately this sample was stable only for one day. On the second day the turbidity and average size increased (see the Supplementary Information), the sample was very polydisperse and the zeta potential decreased. The sample with 63 mol.% of cholesterol was stable for 5 days, despite high value of the zeta potential, and after those 5 days the average size of particles was increased by about 30 nm.

The samples containing cholesterol with molar concentration of 43 and 53 mol.% were stable over 36 days (Figure 6). The average size of particles of those samples was between 80 and 100 nm. The value of zeta potential was between 40 and 60 mV. Some small changes in the average size of particles and zeta potential may have been due to the deviation of measurement. The turbidity of the samples over 36 days was checked by UV-VIS spectroscopy (see the Supp. Inf.). The turbidity slightly increased through the time in the case 43 mol. % (Figure S7) sample, but there are no significant changes in 53 mol. % sample (Figure S9).

4 Conclusions

The obtained results show an interesting comparison of methods monitoring the overall parameters of the vesicular system, such as size, zeta potential and methods monitoring outer membrane properties,

such as compressibility and the presence of water. One way to influence the overall system stability is the addition of cholesterol. The system with the cholesterol content from 33 to 53 mol.% showed the highest stability. Within short-term stability the most suitable samples appeared to be those with additions of 43 and 53 mol.% In these cases, there were no significant changes in vesicle size, no precipitation was observed and the vesicle system was stable. This was also confirmed by the measurement of zeta potential, which was in stable positive area. The results from high-resolution ultrasonic and fluorescence spectroscopy measurements show that increasing amounts of cholesterol at room temperature do not have a monotonous trend in observed specific quantities. The most stable systems are those where in the outer part of membrane, the highest amount of water is present and exhibits the lowest value of generalized polarization (33, 43 and 53 mol.%). These vesicles membranes are also the most elastic compared to lower or higher contents of cholesterol.

As can be seen, this is in a good agreement with short-term stability and confirms the findings from previous work [1,9]. The membrane elasticity, the configuration of water molecules in the solvated part of membrane, and the quantities monitored successfully by high-resolution ultrasonic spectroscopy and fluorescence spectroscopy directly correspond to the expectations about the stability of these vesicles.

It is well-known that different amount of cholesterol could influence the phase transition temperature of vesicles and alter their properties e. g. fluidity, stability and leakage of encapsulated compounds [20,46,47]. Below the phase transition temperature, the membrane is in the solid ordered phase and above this temperature, the membrane is in the liquid disordered phase [48]. Thanks to the inclusion of cholesterol, new phase is formed – liquid ordered [49]. Better understanding of this phase behaviour could help design the most suitable composition of IPA's vesicles. These are the reasons why we plan to extend these experiments with more detailed description of behaviour at different temperatures.

Acknowledgement

This work was supported by the Czech Science Foundation, project No. 19-14024J (GACR), and Ministry of Science and Technology, Taiwan, project No. MOST108-2923-E-006-006-MY3.

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Figure 1

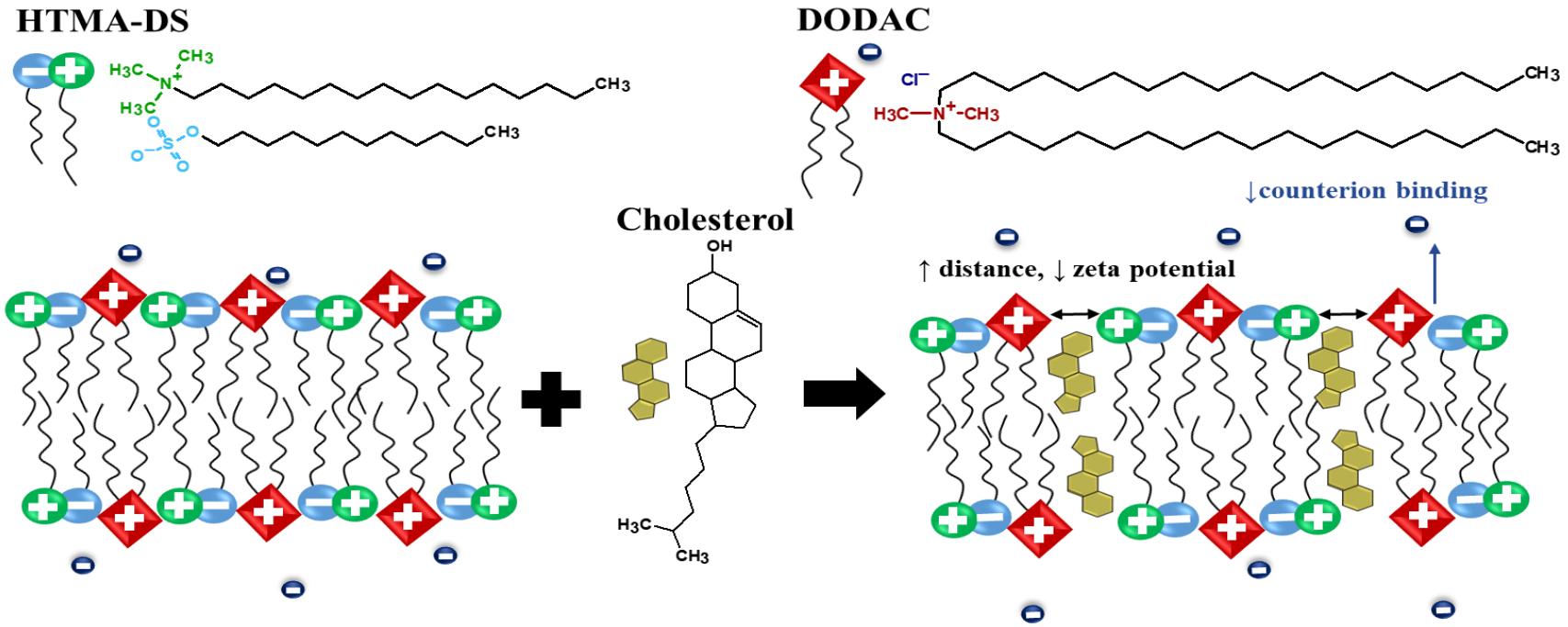
HTMA-DS
DODAC
Cl⁻
↓counterion binding
Cholesterol
OH
↑ distance, ↓ zeta potential

Figure 2

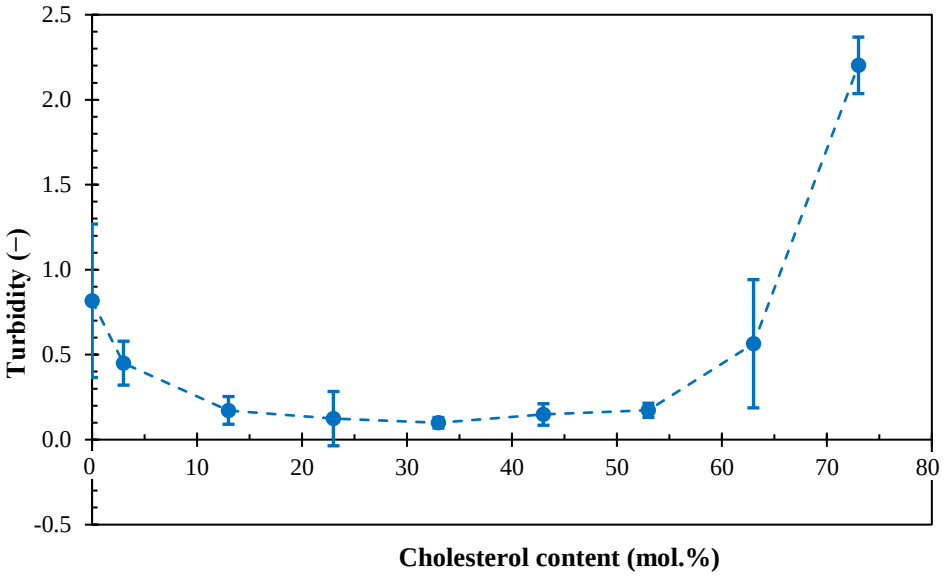
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Turbidity (–)
0
10
20
30
40
50
60
70
80
Cholesterol content (mol.%)

Figure 3

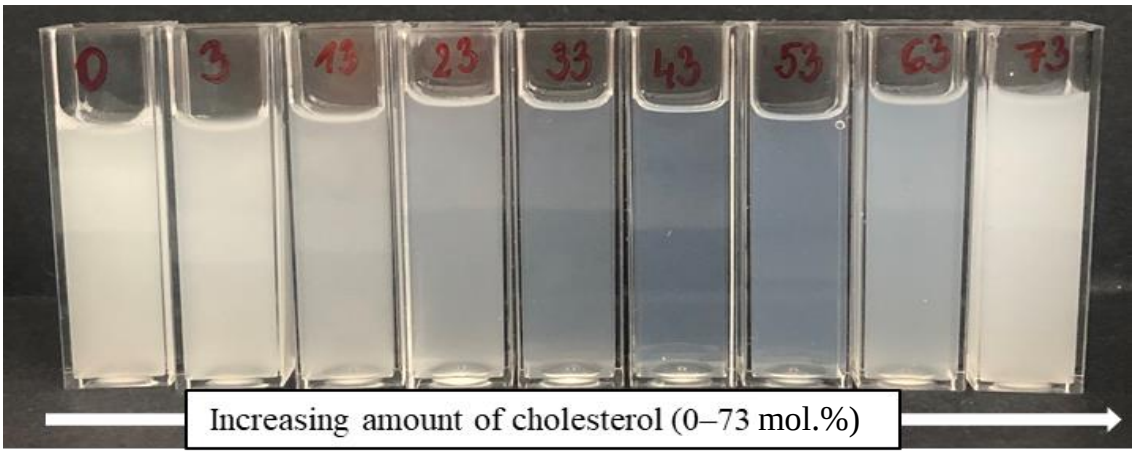
0
3
13
23
33
43
53
63
73
Increasing amount of cholesterol (0–73 mol.%)

Figure 4

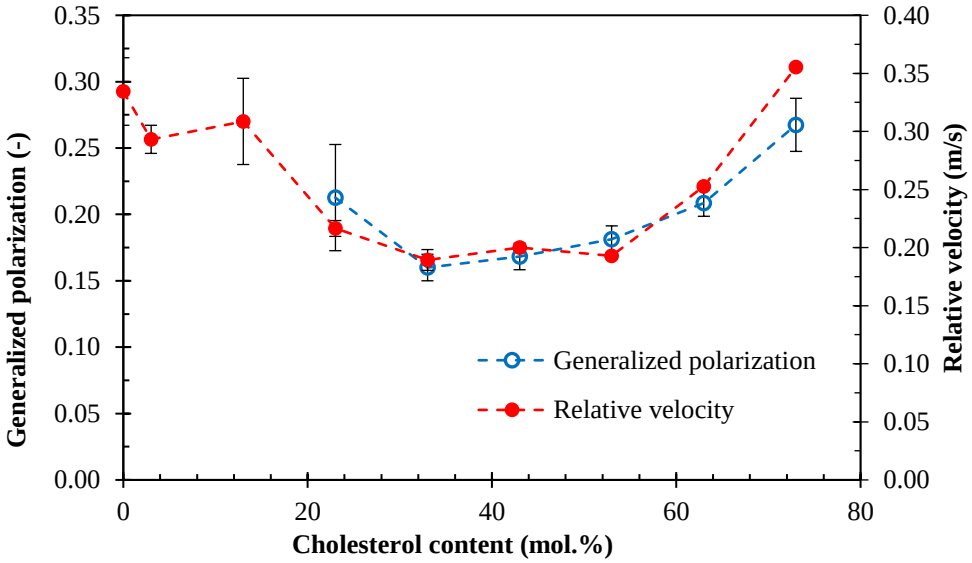
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
80
Generalized polarization (-)
Relative velocity (m/s)
Cholesterol content (mol.%)
Generalized polarization
Relative velocity

Figure 5

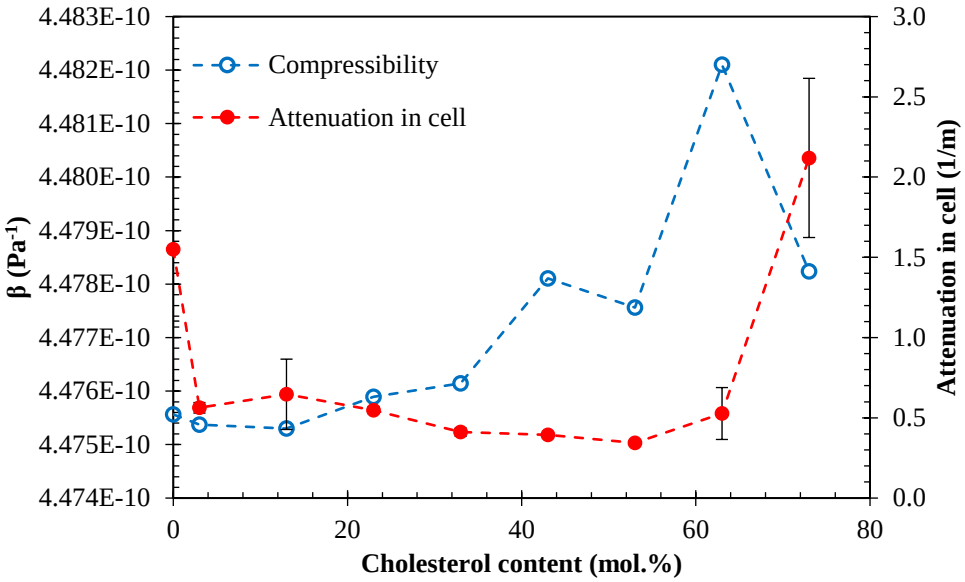
4.483E-10
4.482E-10
4.481E-10
4.480E-10
4.479E-10
4.478E-10
4.477E-10
4.476E-10
4.475E-10
4.474E-10
β (Pa-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Attenuation in cell (1/m)
Compressibility
Attenuation in cell
0
20
40
60
80
Cholesterol content (mol.%)

Figure 6

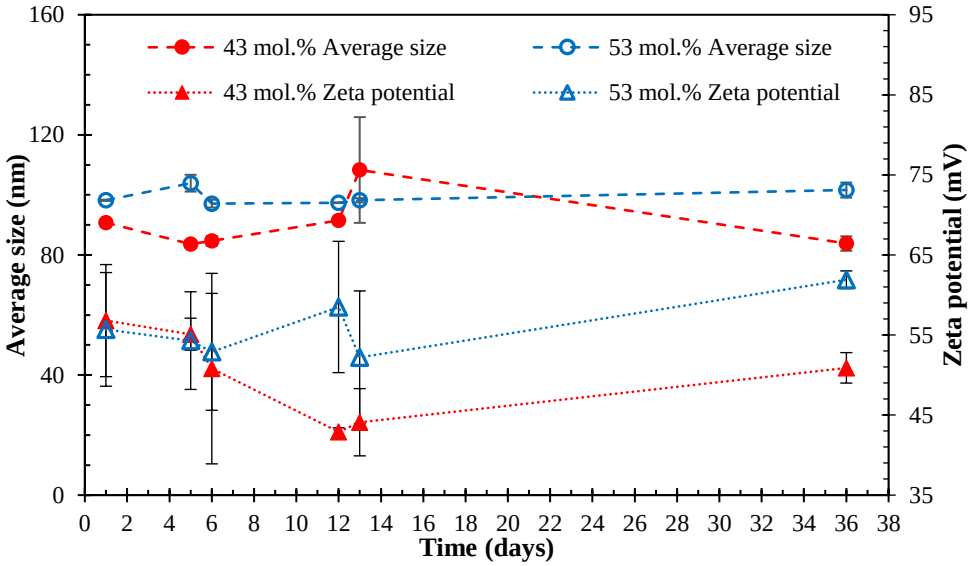
43 mol.% Average size
53 mol.% Average size
43 mol.% Zeta potential
53 mol.% Zeta potential
Average size (nm)
Zeta potential (mV)
Time (days)
0
40
80
120
160
35
45
55
65
75
85
95
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38

Figure Titles

Figure 1: A schematic diagram showing the influence of cholesterol on the molecular packing of HTMA-DS/DODAC. Adapted from [9].

Figure 2: The dependence of turbidity of vesicular system on the cholesterol content 1 hour after the sample preparation.

Figure 3: The picture of samples with different amount of cholesterol (from 0 to 73 mol. %). The lowest turbidity was observed for the sample with 33, 43 and 53 mol.% of cholesterol.

Figure 4: The dependence of generalized polarization and ultrasound relative velocity on the cholesterol content.

Figure 5: The dependence of compressibility and attenuation in cell on the content of cholesterol.

Figure 6: The dependence of average size and zeta potential of particles on the time of measurement for the sample containing 43 and 53 mol. % mol. of cholesterol.

CRediT author statement

Martina Havlíková: Investigation, Writing - Original Draft. **Jana Szabová:** Investigation. **Adam Jugl:** Investigation, Formal analysis. **Ludmila Mravcová:** Conceptualization, Formal analysis, Project administration. **Chien-Hsiang Chang:** Conceptualization, Writing - Original Draft. **Po-Sung Huang:** Conceptualization. **Miloslav Pekař:** Conceptualization. **Filip Mravec:** Conceptualization, Supervision, Funding acquisition, Writing - Review & Editing.

*Declaration of Interest Statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: