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Aggregation of PEGylated liposomes driven by hydrophobic forces

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ABSTRACT

Polyethylene glycol (PEG) is widely used to sterically stabilize liposomes and improve the pharmacokinetic profile of drugs, peptides and nanoparticles. Here we report that ammonium sulfate (AS) can evoke the aggregation of PEGylated vesicles in a concentration-dependent manner. Liposomes with 5 mol% PEG were colloidally stable at 0.7 mM AS, but above this concentration they precipitated to form micron sized aggregates with irregular shape. While aggregation was reversible up to 0.9 M AS, above 1 M fusion occurred, which irreversibly distorted the size distribution of liposomes. Zeta potential of liposomes markedly increased from -71±2.53 mV to 2.02±0.45 mV upon raising the AS concentration from 0 to 0.1 M, but no considerable increase was seen during further AS addition, showing that the aggregation is independent of surface charge. There was no aggregation in the absence of the PEG chains, and increasing PEG molar % shifted the aggregation threshold to lower concentrations. Modifications of infrared spectrum of PEGylated vesicles suggests that AS dehydrates PEG chains. Other kosmotropic salts also led to aggregation, while chaotropic salts did not, which indicates a general kosmotropic phenomenon. The driving force behind aggregation is likely to be the hydrophobic effect due to salting out the polymer similarly to what happens during protein purification or Hydrophobic Interaction Chromatography. Since liposome aggregation and fusion may result in difficulties during formulation and adverse reaction upon application, the phenomena detailed in this paper may have both technological and therapeutical consequences.

Keywords: liposome; PEG; aggregation; fusion; hydrophobic effect; ammonium sulfate; kosmotropic salts

Introduction

Polyethylene glycol (PEG), which is a linear polymer of [–CH_2–CH_2–O]– monomeric unit, is now routinely used in drug delivery to modify pharmacokinetic properties of active agents. Attaching one or more PEG chains to small molecules, peptides, proteins, oligonucleotides or nanoparticles may effectively reduce their enzymatic degradation and immunogenicity, diminishing side effects and prolonging their circulation time in blood [1]. An important example is liposome PEGylation, which provides "stealth" characteristics to liposomes helping them to evade the phagocytosis by macrophages, which extends their lifetime in the body and results in dose-independent pharmacokinetics (except for very low doses) [2, 3]. Liposome therapy made it possible to increase the bioavailability of drugs that are poorly absorbed (like amphotericin-B), to reduce side effects of highly cytotoxic anti-cancer agents (such as doxorubicin) and to open new routes to generate site-selective effect (e.g.: photodynamic therapy in macular degeneration) [4]. However, new benefits may be accompanied by new risks: e. g., it turned out that liposomes can induce complement activation related pseudoallergy (CARPA), a new type of drug-induced acute immune toxicity [5]. It is suspected that one of its causes could be the presence of liposomal aggregates in the formulated product [6].

Here we report that ammonium sulfate (AS) and other kosmotropic agents may elicit the aggregation and even the fusion of PEGylated liposomes. The aggregates are formed by hydrophobic interactions due to the solvophobic effect of increasing salt concentration [7]. This phenomenon is similar to the salting-out method regularly used in protein fractionation and purification [8, 9]. An understanding of the mechanism of aggregate formation could provide invaluable information for successful drug formulations where liposome aggregation could be either prevented or controlled.

Materials and methods

Materials

Cholesterol, mono PEGylated 1, 2-distearoyl-glycero-3-phophoethanolamine (mPEG-2000-DSPE), and hydrogenated soybean phosphatidylcholine (HSPC) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Ethanol, isopropanol, histidine, sucrose, ammonium sulfate (AS), sodium sulfate, magnesium sulfate, sodium citrate, magnesium chloride, guanidine chloride (GdmCl) were purchased from Sigma Aldrich Kft. (Budapest, Hungary). Salsol infusion (TEVA Hungary Zrt., Debrecen, Hungary) was obtained from the University Pharmacy, Purified water was produced by a Milli-Q Integral 3 Water Production Unit (Merck Millipore, Billerica, MA, USA).

Liposome preparation

Liposome suspension with a lipid and buffer composition similar to the FDA-approved and marketed Doxil® was prepared with the extrusion method [10]. The lipid composition was cholesterol, mPEG and HSPC (see molar ratios in Table I.). The lipids were solubilized in

ethanol-isopropanol mixture (50:50), then the solution was added dropwise to 0.25 M AS containing 0.9% saline (SALSOL) solution. Large, heterogeneous lipid particles were extruded four times through 80 nm Whatman Nuclepore (Track-Etched Membranes) membrane filters (Whatman, Maidstone, UK) by means of a Lipex™ Extruder (Northern Lipids Inc., Burnaby, B.C. Canada) at 50 bars and 70 °C to achieve uniform particle size distribution. The liposomes were then dialyzed against 10mM histidine buffer (pH=7.5) containing 10 w/w% sucrose to remove AS and organic solvents. The total phospholipid concentration was approximately 15.9 mg/ml (cca. 21.4 mM). The liposomal stock solutions were stored at 4°C protected from light and used within 2 weeks. The stock solution was further diluted as dictated by the experiments. The degree of dilution and corresponding lipid concentrations are given in the text and figure captions.

Table I. Liposome compositions

PEG molar %	molar ratio (Cholesterol : mPEG : HSPC)
0	38.4 : 0 : 61,6
2	38.7 : 2: 59.3
5	38.6 : 4.6 : 56.8
10	38.4 : 10 : 51.6

Mixing PEGylated liposomes with different salts

For turbidimetry, light scattering and zeta potential experiments 20 µl of PEGylated liposomes were mixed with 980 µl salt solution of appropriate concentration (50 x dilution). For phase contrast and atomic force microscopy experiments PEGylated liposomes were diluted either 200x or 500x with salt solution of appropriate concentration. For 0 M concentration physiological saline solution (Salsol) was used for dilution. The actual salt and lipid concentrations are given in the text and figure captions.

Dilution of precipitates for fusion and aggregation-reversibility studies

500 µl of PEGylated liposomes were mixed with 500 µl of AS solution to produce a stock of precipitated samples of the desired AS concentration (from 0.8 M to 2.0 M). After 15 min incubation time, 20 µl of these stocks was mixed to 980 µl AS solutions of appropriate concentrations (down to 0.1 M). The actual salt and lipid concentrations are given in the text and figure captions.

Turbidimetry

The aggregation of PEGylated liposomes was followed by measuring the apparent optical density of the solution. Briefly, a 4 µl sample of the well-vortexed solution was pipetted onto the pedestal of a NanoDrop 2000 UV-VIS spectrophotometer (Thermo Scientific Ltd., Wilmington, DE), and the optical density was recorded at 250 nm. Because the lipid concentration was kept constant, an increase in optical density corresponds to an increase in

light scatter caused by the appearance of larger particles due to aggregation. For comparability, identical lipid concentrations were used in the different samples.

Dynamic light scattering measurement

The size distribution of liposomes and aggregates were characterized by dynamic light scattering (DLS) on a Zetasizer Nano S instrument (Malvern Instruments Ltd, Malvern, UK). From the intensity fluctuations of a 633-nm laser light scattered at high angle from the freely moving suspended particles their diffusion constant was obtained. Size distribution was calculated by using the Stokes-Einstein equation by the built-in algorithms of the instrument's software. Light scattering was measured at 25±1°C. Z-average values are displayed throughout the article, which represent the primary and most stable parameter produced by DLS technique [11] and recommended for quality control reports (ISO 22412:2008). Z-average values represent a good approximation of hydrodynamic diameter of well dispersed particles with monomodal size distribution (index of polydispersity typically lower than 0.1) and thus are well applicable for PEGylated vesicles. The Z-average, however, does not reflect the real size of precipitated samples that are often heterogeneous in size and may be irregularly shaped. In the latter case Z-average was used only for rough estimation of particle size, which enabled us to follow liposome aggregation without exact determination of aggregate dimensions. Since different experiments may have done using different batches of PEGylated liposomes, minor variations are seen in the average size of control vesicles.

Zeta potential measurements

PEGylated liposomes were diluted with AS solution, and 750 µl of this mixture was injected carefully into folded capillary cells (PCT Kft., Mosonmagyaróvár, Hungary) to avoid bubble formation. Zeta potential measurements were performed by using a Zetasizer Nano ZS equipment (Malvern Instruments Ltd., Worcestershire, UK) in which particle velocity is measured according to a light scattering technique based on Doppler effect evoked by a pair of mutually coherent laser beams (4mW, He-Ne laser at 633 nm). From the autocorrelation function of the scattered light intensity the electrophoretic mobility and, via the Henry equation, the zeta potential are calculated. Measurements were carried out in triplicates at 25°C.

Analysis of zeta potential data

Binding of ions to liposomal surface and the concomitant change of surface potential can be described well by Langmuir-Freundlich isotherm [12].

$$\zeta = \zeta_0 + \Delta\zeta_{max} \cdot \frac{(K \cdot c)^n}{1 + (K \cdot c)^n}$$

where ζ is the measured zeta potential, ζ_0 is the zeta potential at zero AS concentration, $\Delta\zeta_{max}$ is the maximal change of zeta potential, K is the binding constant, c is the ligand concentration and *n* is the index of heterogeneity describing the cooperativity of ion binding.

Atomic force microscopy and image analysis

Atomic force microscope (AFM) images were recorded with a Cypher instrument (Asylum Research, Santa Barbara, CA) by scanning the samples in fluid with a gold-coated silicon nitride cantilever (Olympus Biolever, A lever, typical spring constant: 30 pN/nm). 100 µl sample was applied on a cleaned borosilicate glass coverslip and incubated in a vapor chamber at 23±1 ˚C. Non-contact-mode images were recorded at a linescan rate of 0.5-1 Hz. All measurements were carried out at 28±1 °C. Images were analyzed using the built in algorithms of the AFM driving software (IgorPro, WaveMetrics, Inc., Lake Oswego, OR).

Phase contrast microscopy

Micrographs were recorded with a Nikon Eclipse Ti-U inverted microscope (Auro-Science Kft., Budapest, Hungary) equipped with a uEye UI 1220 LE digital camera (IDS Imaging Development Systems GmbH, Obersulm, Germany) using a 40x Nikon S Planfluor phase contrast objective.

Infrared spectroscopy

ATR-FTIR spectra were collected by a Varian 2000 FTIR Scimitar Series (Varian Inc., Paolo Alto, CA) spectrometer equipped with a 'GoldenGate' (Specac Ltd., London, UK) single reflection diamond ATR accessory. The measurements were performed at room temperature: 3 µl sample was mounted on the top of the diamond ATR crystal and a cap was used to avoid sample drying; 128 scans were collected at a resolution of 2 cm^{-1}. ATR correction was executed after each data collection. All spectral manipulations, including subtractions and spectral deconvolutions were performed using the GRAMS/32 software package (Galactic Industries Incorporation, USA). Band positions for curve fitting were determined using the second derivative. Band shapes were approximated by Lorentzian functions. The intensities and the bandwidth of each component were allowed to vary until the minimal χ^2 parameter was reached. After the fitting procedure, the relative contribution of a particular component was calculated from the integrated areas of the individual components.

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Results and Discussion

PEGylated liposomes can be precipitated by ammonium sulfate

The addition of AS to PEGylated liposomes in few molar concentration initiated the rapid increase of opacity of the sample.

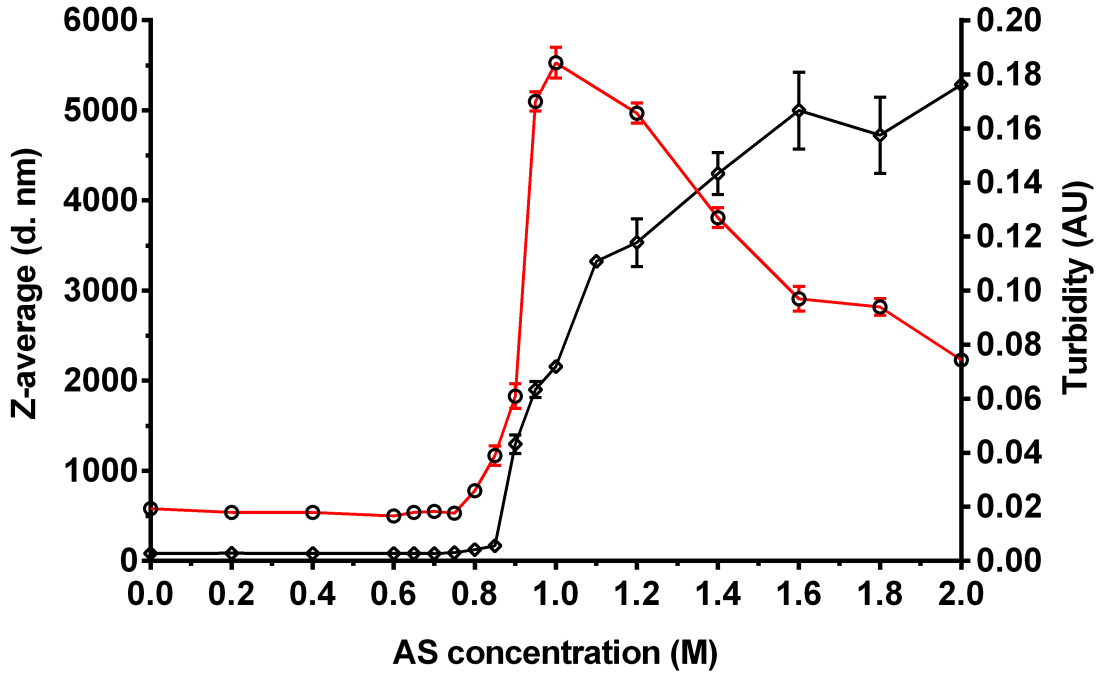

Figure 1. Turbidity (empty rhombi) and average particle diameter (empty circles) of liposomal suspensions as a function of ammonium sulfate (AS) concentration. Average±SD values of three independent measurements are shown. Lipid concentration was kept constant throughout the measurement (50x dilution, 0.318 mg/ml).

To assess the magnitude of liposome precipitation, we measured the turbidity of liposomal suspensions at 0-2 M AS concentrations. According to the turbidity vs. ammonium-sulfate concentration curve (Figure 1.), no precipitation occurs below an AS concentration of 0.75 M. Above 0.75 M, turbidity rises abruptly, then levels off above 1 M to a value an order of magnitude greater than in the absence of AS. From this curve we concluded that precipitation began somewhere between 0.75 and 0.8 M AS concentration. The fall of the turbidimetry curve above 1 M AS concentration may be explained by increasing heterogenity of the system, i.e. the formation of a lipid-rich precipitate and a lipid-poor aqeous phase.

We hypothesized that the abrupt rise of turbidity was related to size increase due to aggregation of vesicles. To follow the change of the size of the precipitates as a function of AS concentration, dynamic light scattering measurements were carried out (Figure 1.) Below a concentration of 0.8 M the mean particle size varied between 83.2 nm and 91.5 nm and it

was independent of the AS concentration. Upon increasing the AS concentration further, however, the particle size rose abruptly to ~2000 nm in the range of 0.8-1 M, then more slowly above 1 M. Upon reaching an AS concentration of 2 M the mean particle size exceeded 5000 nm. Notably, particle size is approximated by Z-average value, which is calculated with the assumption of spherical geometry and monomodal size distribution (see methods section). In case of aggregation, particle shape is likely to deviate from spherical, which, together with growing polydispersity indices measured above 0.75 M AS (data not shown) means that the Z-average parameter may correspond only to an order of magnitude approximation of some average diameter of precipitates. The results of the turbidimetry and dynamic light scattering measurements lead to similar conclusion, namely, precipitation begins upon reaching an AS concentration threshold (0.7-0.8 M) and larger and larger aggregates are formed upon incerasing the AS concentration. Since precipitation takes place instantenously upon mixing the PEGylated liposomes with AS, it is rather difficult to follow the kinetics of aggregation. Size increases rapidly and considerably in the first minute needed to set up a DLS measurement. After this lag time a further continous increase of size was observable (Supplement figure 1), but exact rate and kinetics could not been determined.

To reveal the microscopic details of the precipitation process and assess whether vesicle aggregation takes place indeed, morphological measurements were carried out. Phase contrast microscopy of precipitated samples showed branching objects of irregular shapes apparently formed by smaller clusters (Figure 2.) which resembled electron micrographs of liposomal aggregates seen earlier [13].

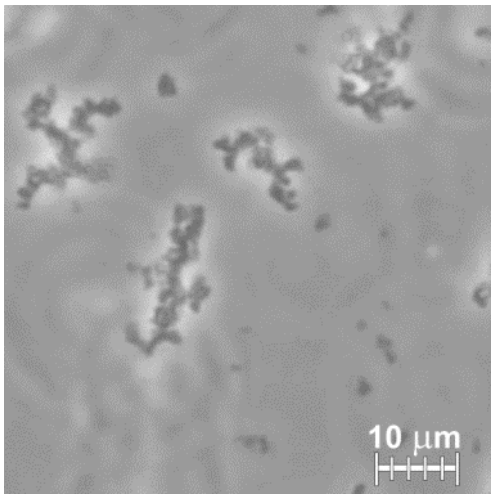

Figure 2. Phase contrast micrograph of liposomal aggregates in 1 M AS. Large branching objects and smaller aggregates are seen (200x dilution, 0.0795 mg/ml lipid concentration).

To resolve the ultrastructure of aggregates and to follow their formation we imaged liposomes with atomic force microscopy (AFM) at various AS concentrations (Figure 3.). At 0 and 0.7 M AS individual, interaction-free liposomes were observed. At an AS concentration of 0.8 M many liposomes were observed in linear assemblies, pointing at the onset of aggregation. At an AS concentration of 0.9 M, large aggregates were clearly seen. In 0.8 M AS (Figure 3. C), beside the vesicles flat patches with a smooth surface and a topographical

height of 5-7 nm were observed. We identify them as bilayers, although they are somewhat thinner than the width of a lipid bilayer covered with a PEG polymer brush at both sides (approx. 12 nm) calculated with a different method [14]. The bilayer patches probably emerge because as osmolality increases, vesicles exhibit a greater propensity to burst on the substrate [15, 16]. Patch formation is a general phenomenon characteristic to liposomal samples. Patches of varying sizes are found in almost all liposomal AFM images throughout the corresponding literature [17-19] and also in Figure 4. Interestingly, patch formation appears to depend on the AS concentration as evidenced by our results shown in Figure 3. While patch formation is only sporadic at lower AS concentrations, it becomes pronounced above 0.8 M (Figure 3. C), and at 0.9 M most of the substrate is covered with a confluent supported lipid bilayer (see the background of clusters and vesicles in Figure 3. D).

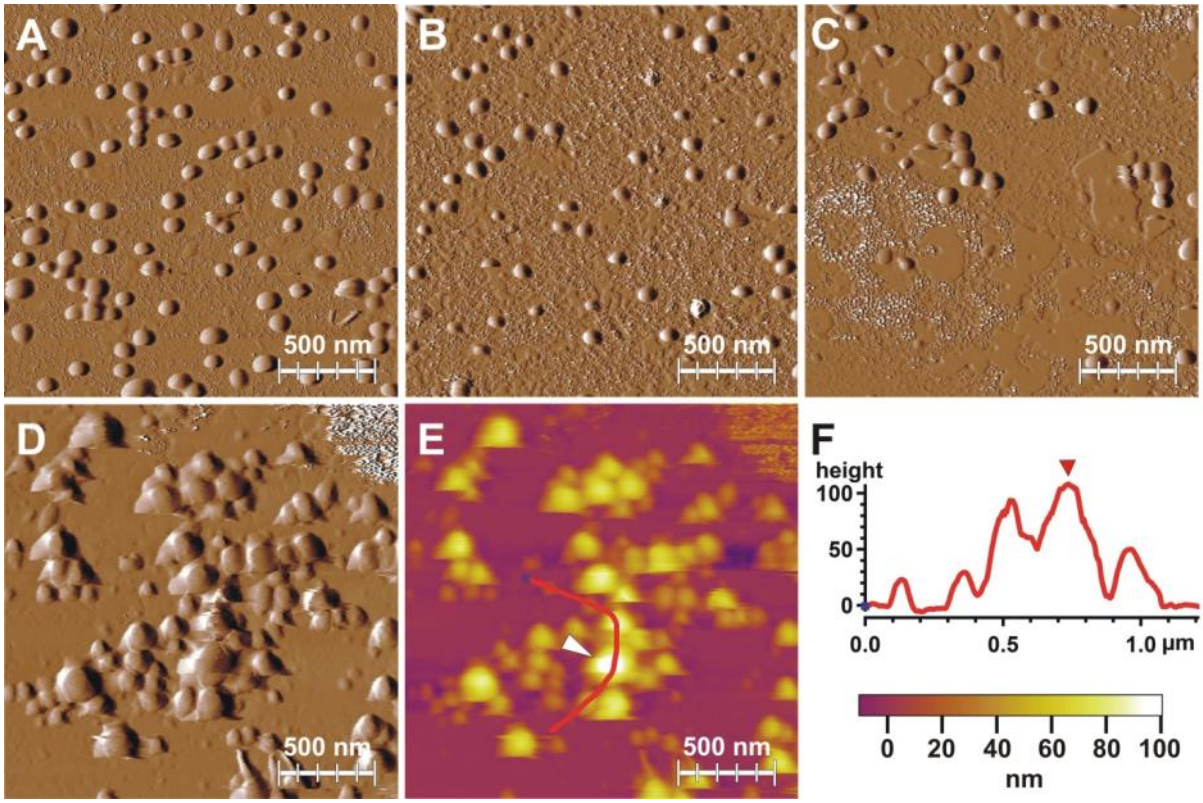

Figure 3. Amplitude-contrast images of liposomes at (A) 0 M, (B) 0.7 M, (C) 0.8 M, (D) 0.9 M AS concentration. Samples were diluted 1 to 500 with their corresponding solution (0.0318 mg/ml lipid concentration). Figure (E) shows the height image of figure (D) color-coded, while (F) represents the height section profile taken alongside the thick red line in (E). The white and red triangles in (E) and (F), respectively points at the highest point of a liposome cluster. The color bar in the lower right corner of the composition codes the height of figure (E).

Liposome clusters observed in 0.9 M AS (Figure 3. D-F) have diverse sizes varying from few hundred nm to few µm, which is in an order-of-magnitude correlation with the DLS data (see Figure 1**Fehler! Verweisquelle konnte nicht gefunden werden.**.) Note that the irregular vesicle shape might be the result of imaging artifacts and not exquisitely of liposomal shape transformations. The image production proved difficult upon raising the AS concentration above 0.8 M, most probably due to the presence of large, flexible aggregates in the sample

that might have been a too wobbly a surface for AFM imaging. Furthermore, the high concentration of electrolytes might have also dramatically altered the charge conditions in the system, interfering with the AFM probe designed to sense electrostatic interactions arising between the tip and the sample surface. The latter may also account for the large background noise (see upper right corner of Figure 3. D and E) resulting in larger apparent substrate roughness compared to images taken at lower AS concentrations. Several attempts have been made to image samples at even higher AS concentrations to find larger aggregates, but unsuccessfully.

Aggregation may promote vesicle fusion

To test whether liposome aggregation is followed by fusion and to determine the threshold concentration of fusion, liposomal samples were precipitated in various concentrations of AS solutions and 15 min later diluted to 0.1 M AS. DLS data showed no considerable change of average size at 0.8 and 0.9 M precipitating concentration. A slight rise was observed at 1.0 and 1.1 M followed by a more significant and monotonous increase from 1.2 M (Figure 4. A). AFM images of these samples revealed, that the increase of average, size experienced by DLS measurements, may be attributed to the occurrence of fused liposomes. Both size and relative amount of fused vesicles raised with AS concentration: at 1 M oversized liposomes appeared sporadically (Figure 4. C), while at 2 M large, irregular shape vesicles predominated (Figure 4. D). This is in a good accordance with size distributions shown in Supplement figure 2., the averages of which are shown in Figure 4. A. Up to 1.1 M a slighter, than from 1.2 M a more significant upward shift is seen due to occurrence of oversized objects. In addition to this, from 1.5 M a population of several micron sized particles occurred, which was not observed in AFM images possibly due to their small number.

Taken all together it seems that PEGylated liposomes may not only aggregate, but also fuse upon AS addition. The rate of fusion is concentration dependent, the threshold concentration seemed to be 1 M at this particular incubation time. This also means that AS-driven aggregation of the vesicles can be reversed depending on AS concentration and incubation time. To disrupt aggregated liposomes they should be diluted well below the AS concentration at which they started to precipitate (Supplement figure 3.).

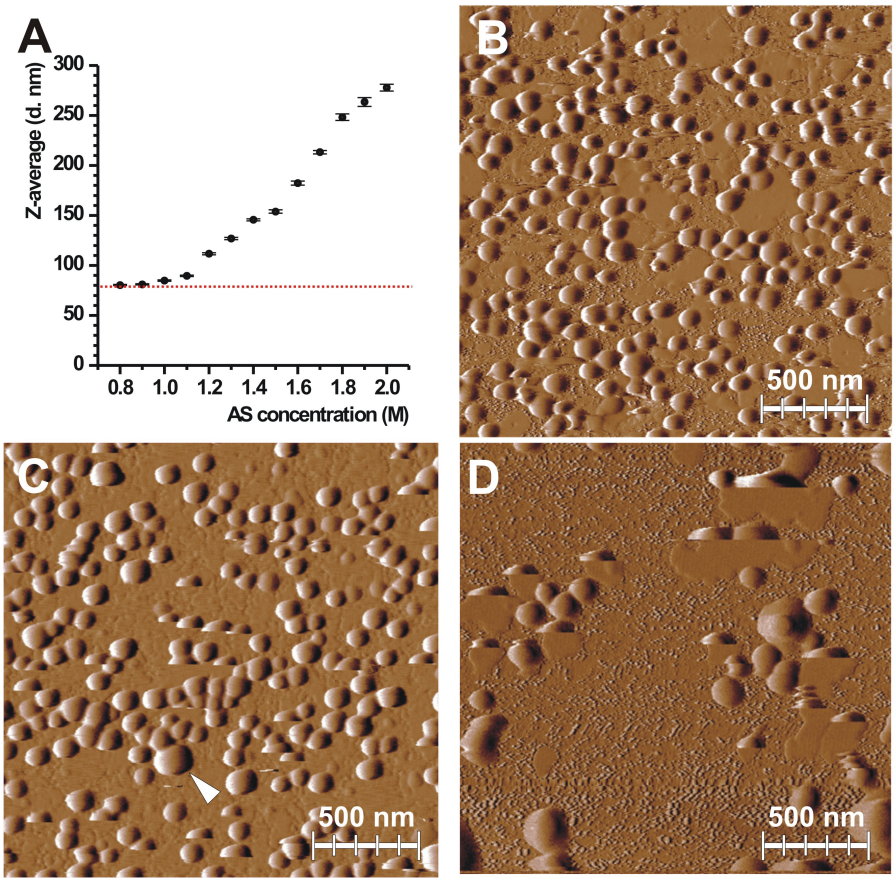

Figure 4. (A) Size of liposomes (Z-average±SD) diluted from various precipitating AS concentrations (indicated on x axis) to 0.1 M. Red dotted line shows the size of control liposomes. (B) Amplitude-contrast AFM image of the liposomal suspension in physiological saline solution. (C) and (D) Amplitude-contrast AFM images of a 1 M and 2 M AS-aggregated sample diluted back to 0.1 M AS concentration. White arrowhead indicates an oversized vesicle in panel C. In panel D few of the larger vesicles ruptured during the scan to form bilayer patches on the surface. Lipid concentration was identical in the samples (200x dilution, 0.0795 mg/ml lipid concentration).

Precipitation does not depend on surface charge relations

Physical stability of colloidal vesicles depends largely on their surface charge density, approximated with the electric potential difference (or zeta potential) between the bulk solution and slipping plane of ions associated to the vesicular surface. It is widely accepted that a zeta potential lower than 30 mV makes colloidal dispersions prone to aggregate [20]. Since the surface charge relations and thus physical stability of liposomal vesicles can be largely affected by ions [21], modification of the liposomes' zeta potential by AS addition might be a key factor evoking aggregation. To assess the charge-modulating effect of AS on PEGylated vesicles, zeta potential measurements were carried out.

Zeta potential of the control sample was -71.7±2.53 mV, which implies a strongly negative surface potential and corresponds to an extremely high colloidal stability [20]. Adding AS led to a massive increase of the zeta potential value at even relatively low concentrations (Figure 5.), which may be explained by the association of NH_4^+ ions to the originally negative

vesicular surface. Coordination of NH_4^+ cations to the ether oxygens was proposed by Bailey and Callard [22]. An alternative explanation is that structural modifications of the charge-altering PEG chains (discussed later) may have led to the observed charge increase.

The Langmuir-Freundlich isotherm fits well to the data points suggesting monolayer absorption to a heterogeneous surface. The adsorption constant of 1001 M^{-1} points at outstanding affinity of NH_4^+ ions to the PEG-covered surface and 0.73 as index of heterogenity indicates negative cooperativity of the ions.

The pronounced surface-charge altering effect of AS levels off at as low concentration as about 0.1 M, which is 7-8 times lower than the concentration needed to evoke aggregation. Furthermore, no considerable change of zeta potential is seen reaching the concentration regime of aggregation (above 0.75 M, see Figure 1.). These together suggest that AS mediated increase of zeta potential cannot be the primary reason for liposome aggregation.

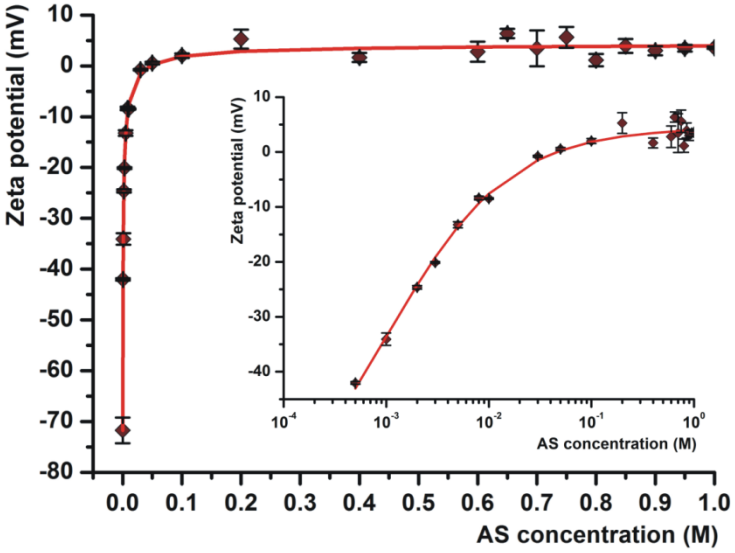

Figure 5. Zeta potential of PEGylated liposomes as a function of AS concentration. Red line shows Langmuir-Freundlich isoterm fitted to the data. Zeta potential was measured in triplicates, error bars show the standard deviation. Zeta potential at 0 AS concentration was -71.7±2.53 mV. Inset shows the data plotted on a logarithmic x axis. Dilution: 50x (0.318 mg/ml lipid concentration).

Precipitation is PEG-related

To elucidate the role of PEG chains in liposome aggregation, liposomes with different amounts of PEG chains on their surface were produced and mixed with AS. DLS data revealed that conventional liposomes (i.e., ones without PEGylation) showed no size increase up to 2 M AS (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This indicates that no precipitation takes place in the absence of the PEG brush on the liposomal surface and highlights that the aggregation evoking effect of AS must be mediated through the PEG polymer layer but has no such an effect solely on the phospholipid head groups. Upon increasing the PEG coverage from 2 to 10 molar %, the precipitation curves shifted to the

left, meaning that lower concentrations of AS were enough to elicit aggregation. It again underpins that precipitation is PEG-related. Considering that modification of surface charge does not affect the aggregation of liposomes (see above), we hypothesize that some structural transitions of the surface polymer chains must have led to the higher propensity to association.

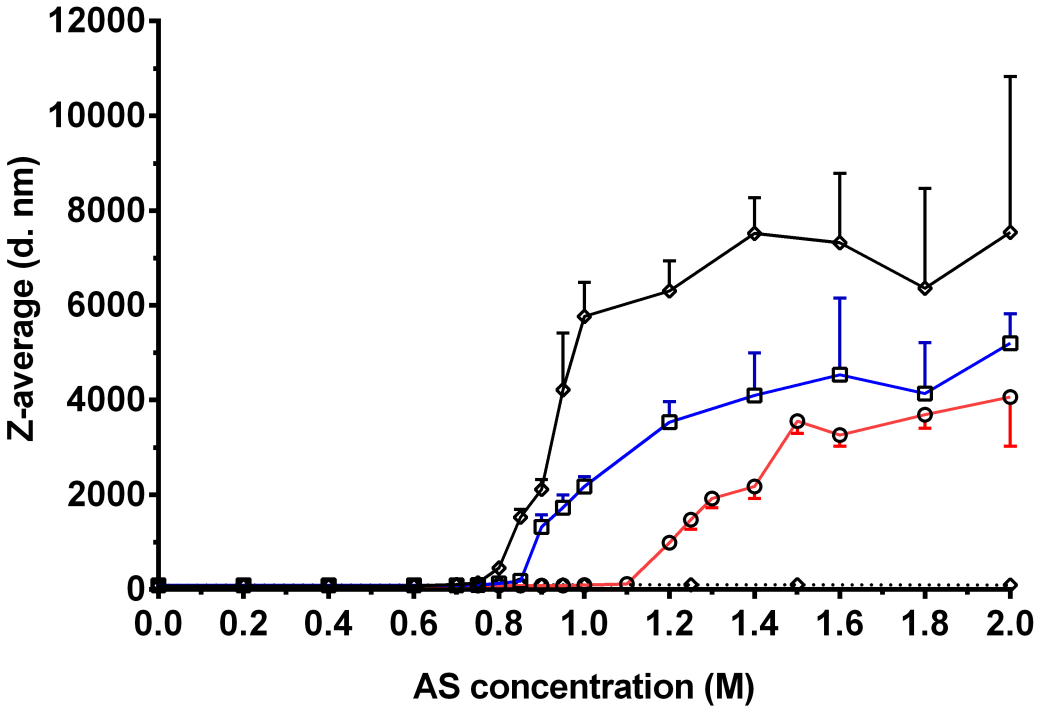

Figure 6. Effect of PEG concentration on the AS concentration dependence of liposomal aggregation. Dilution: 50x (0.318 mg/ml lipid concentration). Broken line: 0% mPEG; red line: 2% mPEG; blue line: 5% mPEG; black line: 10% mPEG

AS dehydrates PEG chains

Taken that precipitation was found PEG-related and not dependent on zeta potential alterations, it seemed plausible that AS, which is a kosmotropic agent [23, 24] dehydrated PEG polymers and this led to aggregation of the PEG covered vesicles through hydrophobic intractions. For the estimation of the hydration level of PEG layer attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) combined with curve fitting procedure was applied. The method was proposed by Varga et al. to characterize the PEG-layer of stealth liposomes based on the ratio of *trans* and *gauche* conformations of C-O-C groups of PEG chain [25]. Briefly, based upon literature, the complex stretching vibration C-O-C band around 1100 cm^{-1} of the PEG chain can be decomposed in five band components due to non-interacting vibrations. The band around 1093 cm^{-1} is related to C-O-C groups of PEG in *trans* conformation, while the one around 1113 cm^{-1} belongs to *gauche* conformation relative to C-C bond [26]. The two extreme components with smaller intensities around 1139 and 1029 cm^{-1} can be assigned to $\delta(-CH_2-)$ deformation and ν(C-C) stretching vibrations, respectively. The relative intense band component around 1068 cm^{-1} might be related to the ν(C-OH)

bands. A higher extent of intramolecular H-bonding of the C-O-C groups of the PEG moiety to a neighboring etheric oxygen results in a greater proportion of the more constrained *gauche* conformer, with an appropriate increase of relative intensity. Thus, the ratio of the *trans* and *gauche* conformations can be a marker to characterize the PEG layer structure and, indirectly, an indication for its hydration state [25].

ATR-FTIR spectra of the PEG-liposomes with 1-2 wt.% concentration are dominated by the strong water absorption bands; so, as a first step of spectral evaluation the subtraction of water background (AS solution) was performed. Since the ν(C-O-C) vibration bands overlap with the phosphate stretching vibrations (νPO_2^-) of the lipid components, the reference spectrum of pure hydrated HSPC (hydrogenated soy phosphatidylcholine, main lipid component for PEGylated liposomes) was carefully subtracted from the spectra of PEGylated liposomes. Typical deconvolutions of the band around 1100 cm^{-1} after spectral subtraction are presented in Figure 7.

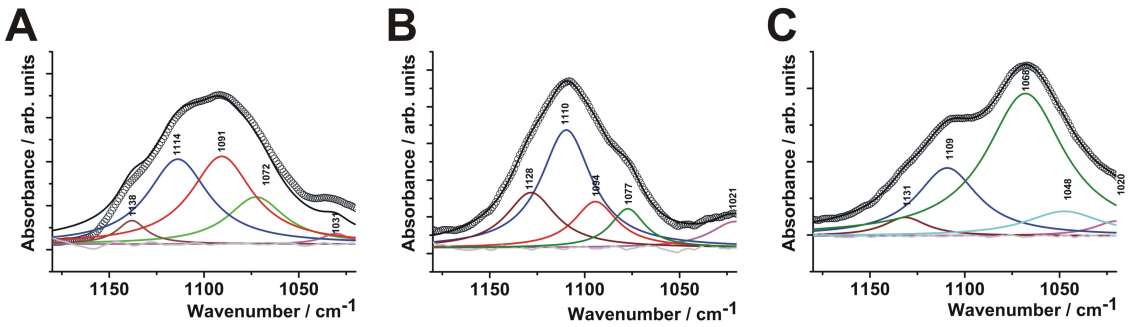

Figure 7. Deconvolution of the band around 1100 cm^{-1} after spectral subtractions: A) PEGylated liposomes in water, B) PEGylated liposomes in 1M AS solution, C) PEGylated liposomes in 2M AS solution. Empty circles denote the measured data points, solid lines represent the fitted spectra, the individual band components and the residuals.

The higher the relative intensity of *trans* conformers the higher the hydration level of the PEG layer. By addition of ammonium sulfate salt (1M concentration), the relative intensity of ν(C-O-C) in *gauche* conformation increases (Figure 8.), indicating that the kosmotropic salt reduces the hydration of the PEG polymer chains. As to the higher amount of AS (2M concentration), however, a new band component at 1068 cm^{-1} dominates the spectrum. This band component might be assigned to ν(C-OH) groups. Interestingly, no band component belonging to ν(C-O-C) *trans* component could be deconvoluted. This may indicate a conformational change more pronounced than the *trans* – *gauche* variation. Similar phenomenon was observed also for micelles formed by pure DSPE-PEG2000 lipid (~10 wt.%) in water (unpublished results).

FTIR data supports the notion that the mechanism behind aggregation of stealth vesicles might be the reduction of hydration of PEG polymer chains due to kosmotropic effect of AS. This is further underpinned by the observation that a chaotropic salt, guanidine chloride (GdmCl) did not affect significantly the ν(C-O-C) *trans* / *gauche* ratio and thus the hydration

level of the polymer (Figure 8., see spectrum, and deconvolution of the bands in Supplement figure 4. and Supplement table I.).

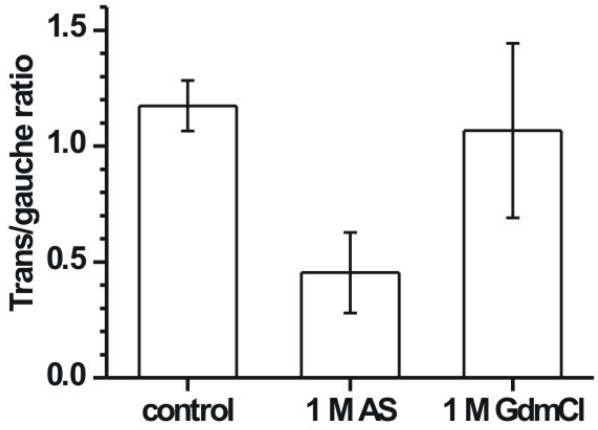

Figure 8. The *trans/gauche* ratio of the ν(C-O-C) band of PEG chains for SSL samples in water, in 1M ammonium sulfate and in 1M guanidine chloride. Averages±SDs of three independent measurements are shown. The high standard deviation for guanidine chloride samples might be caused by the difficulty in guanidine chloride background subtraction.

Other anti-chaotropic salts also lead to precipitation of PEGylated liposomes

We studied the effect of various salts on stealth liposomes to test the hypothesis that PEG-related precipitation of liposomes is not specific for AS but a general kosmotropic phenomenon. All examined kosmotropic salts (sodium citrate, sodium sulfate, magnesium sulfate) led to precipitation of the vesicles (Supplement figure 5.). On the contrary, chaotropic salts (magnesium chloride and guanidine chloride) did not aggregate the liposomes (Supplement figure 6.). Precipitating effect of kosmotropic salts was found concentration dependent, and their threshold concentration followed a series of: sodium citrate < sodium sulfate < magnesium sulfate < ammonium sulfate (Figure 9.). This order is in good accordance with two phase forming capacity of ions in PEG-salt-water systems observed earlier [27].

The surface charge modifying effect of kosmotropic salts levelled off at much lower concentrations than that needed for aggregation (Supplement figure 7.), similarly to what was observed for AS (Figure 5.). The adsorption constants of the ions do not correlate with their precipitating ability. Furthermore not only kosmotropic, but also chaotropic salts, which do not aggregate PEG-liposomes, shifted the zeta potential of liposomes from strongly negative values to neutral regime (Supplement figure 7.). These together clearly suggest that aggregation phenomenon is not connected to ion adsorption driven surface charge alteration of the vesicles, but to kosmotropic effect.

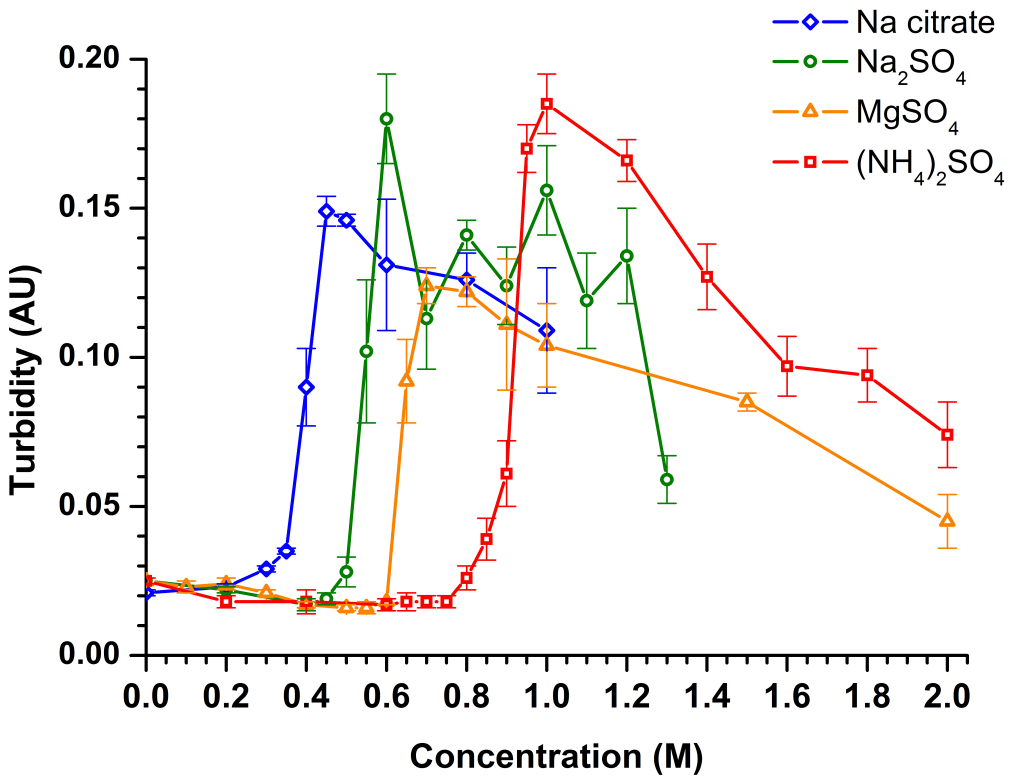

Figure 9. Turbidity of liposomal suspensions as a function of salt concentration. Average±SD values of three independent measurements are shown. PEG concentration was 5 mol% and lipid concentration was kept constant throughout the measurement (50x dilution, 0.318 mg/ml).

Conclusions

Here we presented that ammonium sulfate and other kosmotropic salts has a precipitating effect on PEGylated liposomes. Aggregation takes place above a threshold concentration (Figures 1, 3, and 6.) and leads to the formation of irregular micron-sized aggregates (Figures 2 and 3. D-F). At certain AS concentrations the process is reversible; aggregates can be fully disintegrated by dilution, but higher AS concentrations may evoke the irreversible fusion of vesicles (Figure 4.). Reduction of surface charge does not have any effect on aggregation of vesicles (Figure 5.). At 0 molar % PEG content no aggregation occurs while the propensity of precipitation increases with PEG coverage in the range of 2 to 10 molar % (Figure 6.), which clearly shows that AS-driven aggregation of stealth vesicles is related to the PEG chains. AS leads to the dehydration of PEG polymer chains, while GdmCl does not affect it (Figures 7. and 8.). Other kosmotropic salts (such as Na_3 citrate, Na_2SO_4, $MgSO_4$,) also precipitates PEGylated liposomes (Figure 9.), while chaotropic salts (like GdmCl and $MgCl_2$) do not. We propose that kosmotropic salts may induce a salting out on the polymer chains promoting their hydrophobic interaction [9] and leading to the separation of PEG phase together with the coupled liposomes. Reversibility of aggregation is the simple consequence of the reduction of concentration of the kosmotropic agent, similarly to what happens in case of protein

purification or during the elution phase of hydrophobic interaction chromatography. The observation that high AS concentration results in rapid fusion of the vesicles may be explained by excessive structural alterations of PEG chains due to their hydrophobic modification. Modification of structure and hydration of PEG chains may lead to steric imbalance of the vesicles as it was shown earlier [28, 29].

Since AS (and other kosmotropic agents) may be used during formulation of PEGylated nanoparticles (e.g. remote loading of doxorubicin into liposomes [14]), great care is needed to avoid unwanted aggregation or fusion phenomena, which may take place during either production or storage. There is a hypothesis that complement activation related pseudoallergy (CARPA), observed several cases in the clinical practice when PEGylated liposomes are administered intravenously, may be connected to sporadic aggregation or fusion of liposomes [6]. The results presented above may open the route to produce aggregates or fused vesicles in a controlled method and test their role in pseudoallergic reactions. Reversible aggregation may also be used to separate PEGylated liposomes/nanoparticles from their outer aqueous phase during formulation. However to test feasibility of the method in aforementioned applications further studies are needed.

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Dedication

This article is dedicated to the memory of *Berci (Benedek Kálmán),* a great colleague and friend.

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