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Formation of multi-compartmental particles by controlled aggregation of liposomes

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ABSTRACT

The formation of multi-compartmental colloidal particles by controlled aggregation of 100 nm cationic liposomes was investigated. The aggregation was performed under quiescent (perikinetic) conditions and was controlled by the ionic strength of the solution. The time required for obtaining 500 nm particles by liposome aggregation ranged from several hours to a few minutes when increasing the NaCl concentration from 50 mM to 100 mM. It was shown that the aggregation process can be effectively terminated by capping the liposome clusters by a biocompatible non-ionic polymeric surfactant (Poloxamer 188). The clusters were stable in time and resistant to further increase in ionic strength as well as to mechanical agitation. These results demonstrate that multi-compartmental liposome-based carriers with a controlled size can be prepared in a relatively simple and robust way, and used for further studies in environments with variable salinity and hydrodynamic conditions.

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1. Introduction

The formation of compartmentalised colloidal particles is of both fundamental and practical interest and such particles have been used for example as model protocells to study the origins of life [1], as small-scale reactors to investigate the kinetics of enzyme-catalysed reactions in a confined volume [2], or as storage reservoirs for the encapsulation and release of active payload in drug delivery applications [3]. Multi-compartmental particles can in principle be prepared by a top-down approach, whereby the particles are formed from precursor fluid droplets generated e.g. in microfluidic channels [4] or by a drop-on-demand ink-jet nozzle [5], or by a bottom-up approach, whereby the multi-compartmental particles are produced by the aggregation or self-assembly of smaller building blocks [6].

There are several mechanisms by which the controlled aggregation of colloidal particles into larger clusters can be achieved. Commonly this is done by reducing the repulsive interactions between the charged colloidal particles e.g. by increasing the ionic strength or varying the solution pH, which enables attractive van der Waals interactions to dominate [7]. When properly tuned, such mechanism can be even applied for aggregation of primary particles composed out of different materials [8]. Another possibility is the addition of oppositely charged entities, which could be charged polymers (polyions) [9] or oppositely charged colloidal particles [10]. Special aggregation mechanisms, based e.g. on the complementarity of DNA strands attached to the particle surface [11] or on ligand-receptor recognition [12] are also possible.

If the purpose of the multi-compartment particle is to encapsulate and release a chemical payload, liposomes appear to be suitable building blocks due to their core-shell structure and temperature-dependent permeability of the phospholipid bi-layer [13]. In order to use the aggregation mechanisms mentioned above, charged lipids have been included in the liposome bi-layer. For example, Sennato et al. [14] have used positively charged DOTAP (*1*,*2*-dioleoyl-*3*-trimethylammonium-propane) based liposomes and a negatively charged acrylate polymer to form aggregates in the size range in the order of 100s nm. The maximum aggregate size was achieved for a polyion/lipid charge ratio close to 1:1. However, by increasing the salinity of the surrounding solution, the colloidal stability of the system was lost and macroscopic aggregates have formed. Volodkin et al. [15,16] investigated a system based on negatively charged DPPC/DPPG/cholesterol liposomes in combination with a positively charged polymer poly-L-lysine (PLL). The maximum aggregate size was again obtained near a stoichiometric lipid/polyion charge ratio of 1:1, whereby the length of the polymer chain was found to influence the range of lipid/polyion ratios that resulted in the formation of aggregates. However, the addition of excess PLL resulted in the formation of individual PLL-coated liposomes that did not aggregate.

The release of encapsulated carboxyfluorescein (CF) from liposome clusters was investigated as well [16]. The spontaneous leakage of CF from liposome aggregates was found to be higher than from individual liposomes of identical composition. By using phospholipids with a higher phase transition temperature (DPPC/DPPG instead of POPC/

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POPG), the leakage at room temperature was suppressed. The complexation of liposomes into larger clusters by means of a polyion was found to influence the phase transition temperature of the lipid bi-layer. Schwieger et al. [17] investigated DPPG/DPPC liposomes aggregated with PLL and found an increase of the phase transition temperature by up to 8 °C depending on the PLL/lipid ratio and the PLL chain length.

The clustering of liposomes by multivalent ions such as Ca^{2+}, Gd^{3+} or La^{3+} has been shown to result in aggregates with a fractal structure [18,19]. Several special aggregation mechanisms based on specific molecular interactions have also been reported. For example, the aggregation of liposomes decorated by SNARE proteins induced by the addition of Ca^{2+} has been described [20]. Liposome assembly by DNA pairing has been shown to be a versatile mechanism for controlling the aggregate size and structure [21,22]. Liposome aggregation by means of specific ions interacting with gemini peptide lipids incorporated into the lipid bilayer has also been demonstrated [23].

While some of the above-mentioned aggregation mechanisms are highly sophisticated, the presence of biomolecules at the liposome surface may be problematic due to their immunogenicity in the case of using such aggregates in vivo, for example in drug delivery. Simple aggregation based on increasing the ionic strength of the solution [24] may be more appropriate in that case. However, the stabilisation of the aggregates of a specific size to prevent uncontrolled aggregation due to the salinity of physiological fluids or uncontrolled break-up due to shear forces in a flowing fluid has so far not been satisfactorily addressed. Therefore, the aim of the present work was to explore (i) the kinetics of liposome aggregation, (ii) the means of controlling the resulting aggregate size, and (iii) the mechanisms of the aggregate stabilisation at a desired size using a biocompatible non-ionic polymeric surfactant.

The surfactant chosen for the stabilisation of liposome aggregates was a block co-polymer of poly(ethylene glycol) and poly(propylene glycol), known under the commercial names Poloxamer 188, Pluronic F-68, Lutrol F-68 or Kolliphor P-188. This surfactant is often used in pharmaceutical formulations for the stabilisation of nanoparticles or emulsions, but also in industrial applications such as the coating of cells [25] in bioreactors to prevent unwanted aggregation and improve the cell resistance to shear forces by eliminating the attachment of cells to the rising bubbles [26,27]. The rationale for choosing this surfactant was the similarity between the composition of cell membranes and the lipid bi-layer. The aim was to sterically cap the liposome aggregates by a non-ionic polymer and protect them from further influence of small ions in the solution. It will be shown that the aggregates are stable in time and resistant not only to hydrodynamic forces but also to further salt-induced aggregation.

2. Materials and methods

2.1. Chemicals

The positively charged lipid 1,2-dipalmitoyl-3-trimethylammonium-propane chloride (DPTAP) was purchased from Avanti Polar Lipids (USA). Methanol, sodium chloride, Kolliphor P-188 were purchased from Sigma, chloroform was purchased from Lach-Ner (Czech Republic). Tris–HCl buffer was prepared from 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), purchased from Aldrich, and hydrochloric acid (Lach-Ner, Czech Rep.). Nile Red (Sigma-Aldrich) was used for the fluorescent labelling of liposomes and their aggregates. Deionized water (specific conductivity 1.1 μS/cm) filtered by Aqual 25 (Aqual, Czech Republic) was used for the preparation of all aqueous solutions.

2.2. Preparation of liposomes

Liposomes were prepared by the hydration method followed by extrusion. 10 mg of DPTAP was dissolved in 1 mL of chloroform/methanol solution (9:1, v/v) in a round-bottom flask. In the case of fluorescent liposomes, approximately 0.1 mg of Nile Red (excitation and emission wavelengths $\lambda_{ex} = 514$–550 nm and $\lambda_{em} = 633$ nm, respectively) was added to the lipid solution. The organic solvent was then removed by a rotary evaporator at 60 °C. The pressure was decreased slowly to 20 mbar in order to avoid bubbling. Finally, the flask with the dried lipid layer at the bottom was evacuated in a desiccator for 2 h. The lipid layer was hydrated at 60 °C by 1 mL of 1 mM Tris–HCl buffer (pH = 7.4) while mixing and shaking. The dispersion was then treated by repeated extrusion (at least 20 passes) through a polycarbonate membrane with a pore size of 100 nm at 60 °C using the Avanti MiniExtruder (Avanti Polar Lipids, USA).

2.3. Measurement of aggregation kinetics

The size distribution of liposomes and their aggregates was measured by dynamic light scattering (DLS) (Malvern ZetaSizer Nano ZS, Malvern Instruments, UK). DLS data were process both with and without the CONTIN method, without any significant difference. In a typical experiment, 10 μL of the liposome sample was dispersed in 1 mL of 1 mM Tris–HCl buffer in a cuvette, resulting in a lipid concentration of 0.1 mg/mL. The refractive index for liposomes was set to 1.45 for the purpose of DLS measurement [28]. Aggregation was initiated by the addition of 1 M NaCl stock solution to achieve NaCl concentrations in the cuvette ranging from 0 to 100 mM. Before use, both the buffer and the NaCl stock solution were filtered by a 400 nm syringe filter. After the addition of NaCl, the evolution of the particle size distribution was followed by DLS for up to 8 h. For the study of liposome aggregate stabilisation, 5 μL of 100 mg/ml aqueous solution of Kolliphor P-188 was added to the cuvette containing the liposomes or their clusters, resulting in a final Kolliphor concentration of 0.5 mg/mL. All measurements were performed at 25 °C. The shape and structure of the liposome aggregates were observed by laser scanning confocal microscopy (Olympus Fluoview FV1000) using a 66× oil immersion objective, enabling spatial resolution up to 180 nm. The excitation laser of wavelength 514 nm and an emission filter of 575–675 nm were used.

2.4. Evaluation of aggregate structure

An important parameter of the clusters was not only their equivalent hydraulic diameter measured by DLS, but also their structure — especially their “compactness”. This was evaluated from laser scanning confocal microscopy images of the aggregates using the Shape Filter plug-in for the image analysis software ImageJ [29]. After thresholding and noise removal, the morphological parameters of a large number of aggregates (between 290 and 1145 individual aggregates taken at four different confocal planes separated by at least 5 μm) were evaluated from each sample and summarised in the form of histograms. Two parameters were evaluated — solidity, and the fractal box dimension. Solidity is defined as the ratio of the area of an aggregate in a given confocal plane to the area of its convex envelope in that plane [29]. The more compact the aggregate is, the closer to 1 is the value of solidity. An alternative measure of the compactness of the aggregates is their fractal dimension. As described by Thill et al. [30,31] for such open aggregates their internal structure can be obtained from the analysis of 2D images. Fractal dimension was evaluated by applying the box counting algorithm, which is implemented within the ImageJ software [32].

3. Results and discussion

3.1. Aggregation kinetics

Knowledge of the kinetics of salt-induced liposome aggregation is a necessary prerequisite for the control of aggregate size. To investigate the aggregation kinetics, 100 nm DPTAP liposomes were exposed to NaCl solutions of different concentrations and the size distribution

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