Effect of calcium ions on liposomes as cell membrane analogues

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Vpliv kalcijevih ionov na liposome kot model celične membrane

Abstract: *Izpostavitev bioloških celic električnemu polju se je izkazala kot uporabna tehnologija za manipulacijo s prepustnostjo celične membrane ter s tem potencialno široko uporabo v številnih biomedicinskih in biotehnoloških aplikacijah. Že kratkotrajna izpostavitev električnemu polju povzroči spremembe na nivoju lipidnih molekul v celični membrani. V odvisnosti od parametrov električnega polja postane membrana začasno prepustna, tudi po zaključeni izpostavitvi električnemu polju, za molekule, ki običajno ne morejo prehajati celične membrane. Pojav imenujemo elektroporacija ali elektropermeabilizacija. Razvita je bila tudi metoda elektroporacije z dodajanjem kalcija za uporabo v zdravljenju rakavih obolenj. Učinkovitost te metode je bila dokazana in vitro, in vivo ter klinični študiji. Kljub temu pa osnovni fizikalni in kemijski procesi pri dodatku kalcija ali kateregakoli drugega dvovalentnega kationa še vedno niso povsem raziskani. Znano je, da določeni ioni vplivajo na elektroporacijo, celične membrane. Liposomi so sferični vezikli, ki jih obdaja lipidni dvosloj. Zaradi njihove preprostosti in podobne geometrijske zgradbe, kot jo imajo celice, ki jih obdaja celična membrana, liposome lahko uporabljamo kot model celične membane. Za preučevanje fizikalnih sprememb smo uporabili plinsko elektroforezo z uporabo naprave imenovane Nano-Electrospray Gas-phase Electrophoretic Mobility Molecular Analyzer (nES GEMMA). Namen naše študije je preučevanje vpliva kalcijevih ionov na strukturne in molekularne spremembe liposomov.*

1 Introduction

Application of an external electric field to biological cells has emerged as a powerful technique for manipulation of cell membrane permeability. Such an electric field, which is briefly applied to cells by applying high voltage electric pulses, induces local distortions and structural rearrangements of lipid molecules in the cell membrane. Depending on the pulse parameters, membranes become either transiently or permanently permeable even after the field has ceased, allowing molecules that are otherwise deprived of transport mechanisms to cross the membrane and reach the cytosol. This phenomenon is often referred to as electroporation or electropermeabilization [1,2].

Calcium electroporation is a new experimental anti-cancer treatment, internalizing calcium into cells by application of short, high voltage pulses. The anti-tumour effectiveness of this approach has been demonstrated *in vitro*, *in vivo* and in clinical study [3]. Additionally, the effect on vasculature has been explored. Calcium internalization induces tumour cell death based on ATP loss through the permeabilized cell membrane. In addition, ATP production is decreased due to the application of high voltage electric pulses [4]. However effects of calcium ions on biological cells has not been fully elucidated.

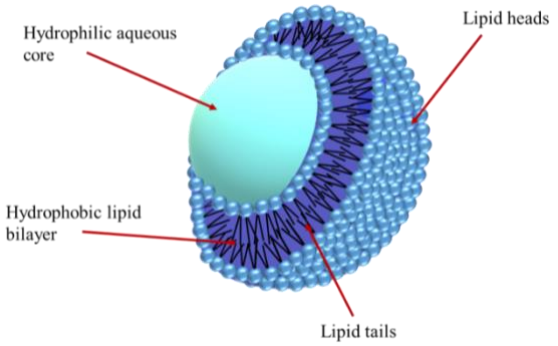

Figure 1: Schematic presentation of liposome vesicle with a hydrophilic core and a hydrophobic lipid bilayer.

Physical processes and molecular-scale mechanisms involved in addition of cations to the cell membrane can be studied using artificial biological membrane models such as planar lipid bilayers or more complex structures such as liposomes. Liposomes are spherical vesicles consisting of lipid molecules, which can form one or more lipid bilayers (Figure 1). Due to the amphiphilic nature of lipid molecules, liposomes are able to self-assembly in aqueous solutions. Furthermore, they encapsulate part of the aqueous solution into their hydrophilic core. The hydrophobic lipid bilayer structurally represents a simplified model of a cell membrane. Therefore, liposomes can be used to study changes occurring to this cell membrane analogue. The first closed, spherical lipid bilayer was described in 1965 by Alec Bangham and his colleagues [5]. Liposome properties, such as size, differ with the lipid composition and method of preparation. Furthermore, chemical modifications can occur that induce changes in the liposome particle size. Gas-phase electrophoresis of single-charged particles enables analysis of vesicles according

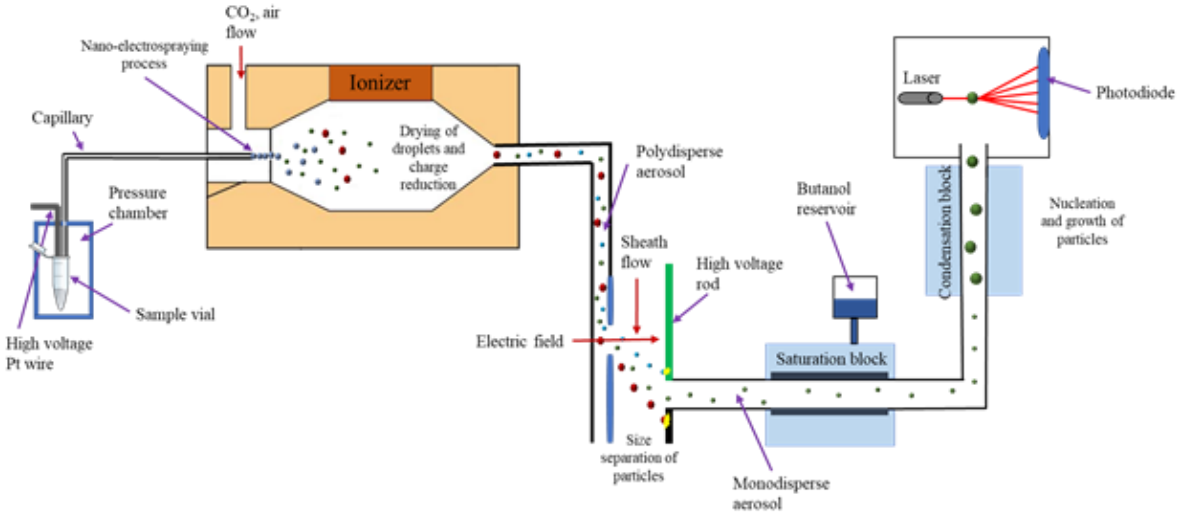

Figure 2: Schematic presentation of nES GEMMA working principle. The first part consists of a nano electrospray based aerosol generator where aerosol is generated from our sample. Polydisperse aerosol then flows through the nano-differential mobility analyser where the particles get classified based on their size. In the end, monodisperse particles are detected by a condensation particle counter.

to the surface-dry particle size [6]. With a nES GEMMA instrument, we are able to obtain a size-distribution analysis and determine a particle-number concentration (Figure 2).

Surface-dry, single-charged particles are obtained after a nano-electrospray process with subsequent drying of droplets and charge equilibration. Next, polydisperse surface-dry analytes are separated in a constant, high laminar sheath flow of particle-free air and an orthogonal, tunable electric field inside a Differential Mobility Analyzer (DMA). By variation of the field strength, a monodisperse aerosol is generated at the exit slit of the DMA. Following size-separation, nanoparticles are detected in an ultrafine Condensation Particle Counter (CPC) enabling particle-number concentration based detection in accordance with recommendations of the European Commission for nanoparticle characterization (2011/696/EU from October 18th, 2011) [7].

Aim of the study was to determine the effects of calcium ions on structural rearrangements and molecular physical changes of liposomes.

2 Materials and methods

2.1 Chemicals

The lipids L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC),1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine (18:0 PE, DSPE) and cholesterol (Chol) were from Avanti Polar Lipids (Alabaster, AL, USA). Ammonium acetate (NH4OAc, ≥ 99.99 %) from Sigma Aldrich (Steinheim, Germany). Chloroform (Spectronorm quality) was obtained from VWR BDH Chemicals (Roncello, Italy), Methanol (LiChrosolv) from Merck (Darmstadt, Germany). Nitrogen gas was from Messer (Gumpoldskirchen, Austria).

2.2 Buffers and electrolytes

NH_4OAc solution (40 mM, pH 8.4) filtered through a 0.2 µm pore size syringe filter (surfactant free cellulose acetate membrane from Sartorius, Göttingen, Germany) was used for vesicle preparation and as aqueous electrolyte for nES-GEMMA. 2 mM, 5 mM, 10 mM, 20 mM and 40 mM calcium in NH_4OAc solution (40 mM, pH 8.4) was employed for the experiments.

For all electrolyte preparations, water from a Simplicity apparatus (Millipore, Billerica, MA, USA) with 18.2 MΩcm resistivity at 25°C was applied.

2.3 Liposome preparation

Liposomes from HSPC:DSPE:Cholesterol (4:3:3 molar ratio) were prepared from dried thin lipid films via hydration. Therefore, corresponding amounts of lipids were dissolved in methanol:chloroform (1:3 mixture [v:v]). Subsequently, a thin, regular film was formed under a constant stream of nitrogen gas. The film was further dried in a desiccator for approximately 2 hours. Afterwards, hydration of the lipid film was performed with 1 mL NH_4OAc. This yielded dispersion of 10 mM total lipid concentration. The lipid film was detached from the flask surfaces via vortexing and heating in a water bath (approx. 65°C). Subsequently, small unilamellar vesicles were prepared via extrusion of dispersions (21 times through two pre-wetted 100 nm pore size, polycarbonate membranes (Avanti Polar Lipids) applied in the same membrane orientation). Liposome stock solutions were stored in brown glass vials at 4°C until further use.

2.4 Instrumentation and samples

Gas-phase electrophoresis was carried out on a TSI Inc instrument (Shoreview, MN, USA): A nES aerosol generator (Model 3480) equipped with a ^{210}Po α-particle source, a nDMA (Model 3080) and a n-butanol-based ultrafine CPC (either model 3025A or a similar model, 3776C) were applied. The samples were introduced to the nES via a 25 µm inner diameter, fused silica capillary with a homemade tip generating a stable Taylor cone. In order to exclude cross-contaminations, a fresh capillary was employed for each set of measurements. 4.0 pounds per square inch differential (psid, approx. 28 kPa) and 0.1 liters per minute (Lpm) CO_2 and 1.0 Lpm compressed, particle-free air were employed for transport of analytes from the capillary through the neutralization chamber and to the nDMA unit. The air was additionally dried (Donaldson Variodry Membrane Dryer Superplus, Leuven, Belgium) to facilitate drying of nES derived droplets.

Liposome stocks were simply diluted 1:10 [v:v] in ammonium acetate prior to gas-phase electrophoresis. Different concentrations of calcium electrolyte solution were applied to the external environment of the liposomes during the buffer exchange via spin filtration. Filtration was carried out two times in the presence of calcium ions and once more in electrolyte containing only ammonium acetate in order to remove lower sized material.

2.5 MATLAB analysis

Analysis of obtained number based particle concentrations was carried out using Matlab 2017b. Obtained particle counts were filtered using a zero phase Butterworth filter to ensure a smooth graph, which would enable us to determine the peak apex of the particle size distribution. The cutoff frequency were determined from obtained graphs and is presented as an error distribution.

3 Results

The aim of our study was to measure and observe structural changes occurring to spherical lipid bilayers, e.g. liposomes, due to the addition of calcium cations. We were able to demonstrate that measurable size changes occur to the liposome particles due to the addition of calcium cations to the external environment of the liposomes. Changes were measured using gas-phase electrophoretic analysis employing a nES GEMMA instrument.

With the application of three different lipid components for liposome preparation, HSPC, Cholesterol and DSPE respectively, vesicles with diameter of approximately 76.6 nm were obtained as seen from Figure 3. Mixed lipid composition proved to give optimal measurable changes due to the size of prepared liposomes and the fluidity of the lipid bilayer.

Liposomes were prepared with the dried thin lipid film hydration technique and further diluted. During the dilution process, calcium solutions with different molarities were added to the exterior of the liposomal particles. First, we measured the liposomes via nES GEMMA in the corresponding electrolyte (ammonium acetate) without addition of calcium ions. A size distribution graph with a peak at 76.6 nm particle diameter was obtained as seen on Figure 3.

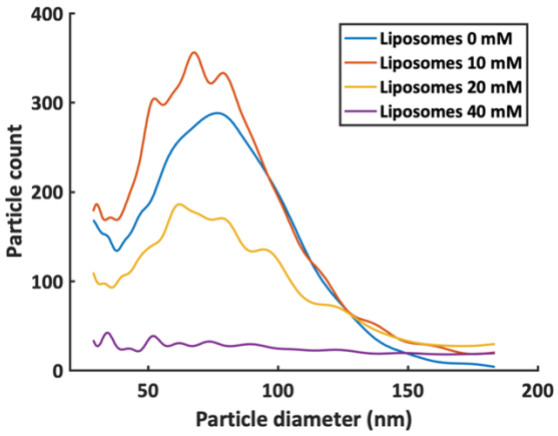

Figure 3: Size distribution of particles in our liposome sample. Liposome particles have their peak at approximately 85 nm. Changes occurring to the particle count and particle size due to addition of different calcium concentrations can be seen. When no calcium was added (blue line) the liposome size distribution had a peak at 76.6 nm particle diameter. When 10 mM calcium solution was added (orange line) the liposome peak shifted to lower particle diameter. The same occurred to the liposomes with 20 mM calcium solution (yellow line). Fort the liposomes with added 40 mM calcium solution (violet line) the liposome particle count is too low to distinguish the liposome peak

Calcium was added to the liposome solution during the spin filtration process. For the 10 mM and 20 mM calcium addition to the liposome exterior, we were able to observe measurable changes in liposome particle size (Figure 4). The size of the liposomes did indeed shift to lower diameter values. For 10 mM calcium addition to 67.6 nm and for 20 mM calcium addition to 62.4 nm particle diameter. With the addition of 40 mM calcium solution the liposome particles became twice smaller, what is more their particle count decreased as well, most probably due to liposome particles to burst and smaller structures are being formed. Structures such as micelles and free calcium cations was detected using nES GEMMA.

The dependence of particle size to the calcium cation concentration is presented on Figure 4. Liposome diameter decreases with increasing concentration of calcium in the electrolyte solution. Calcium ions believed presumably modify the phosphate head groups in the lipid molecules therefore making the lipid molecule smaller, consequently decreasing the area per lipid.

The graph on Figure 4 consists of four points, each representing a different calcium solution concentration. The error bars represent the cut-off frequency of obtained distributions. The particle diameter decreases with increasing calcium concentration. Therefore, calcium cations induce changes to the lipid molecules causing the liposome particles to decrease in size.

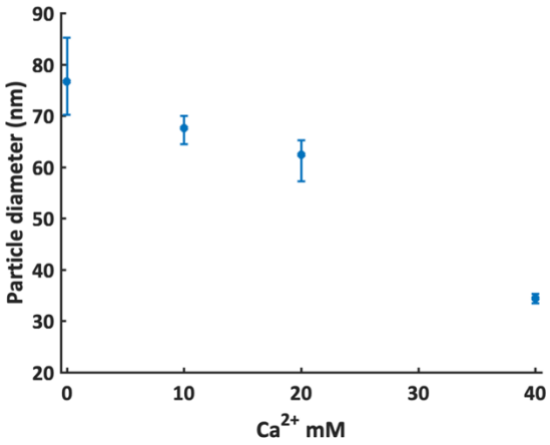

Figure 4: Decrease in liposome particle diameter with respect to concentration of calcium solution added to the liposome sample. The error bars represent the cutoff frequency of the obtained distribution.

4 Conclusion

In our study, we were able to observe and quantify the changes occurring in the lipid structures, such as liposomes, induced by the addition of calcium ions. We were able to demonstrate that with an increasing concentration of calcium ions the liposome vesicles decrease in size. The results of this study are in agreement with studies based on Molecular Dynamics simulations, where binding of the calcium ions to the lipid phosphoryl oxygen interior and reduction of area per lipid were demonstrated with addition of calcium ions into the bulk solution [7].

In the future, we should be able to demonstrate size changes due to calcium ion addition on liposomes with different lipid compositions. Likewise, other divalent cations, such as magnesium could be used to observe changes occurring to the lipid structures.

With our study, we were able to confirm that structural rearrangements do occur on spherical lipid bilayers due to the addition of calcium ions. Furthermore, we plan to observe the behaviour of liposomes with added calcium cations under the electric field application in the future. This would give us a better insight in the possible processes occurring during the electroporation, especially when calcium ions are added to the treated area.

Literature

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