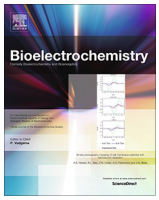
Bioelectrochemistry 143 (2022) 107988

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem



Calcium ion effect on phospholipid bilayers as cell membrane analogues

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ARTICLE INFO

Article history:
Received 9 August 2021
Received in revised form 22 September 2021
Accepted 23 October 2021
Available online 28 October 2021

Keywords:
Calcium electroporation
Liposomes
Planar lipid bilayers
nES GEMMA

ABSTRACT

Ion attachment can modify stability and structure of phospholipid bilayers. Of particular importance is the interaction of phospholipids with divalent cations, such as calcium ions playing an important role in numerous cellular processes.

The aim of our study was to determine effects of calcium ions on phospholipid membranes employing two cell membrane analogues, liposomes and planar lipid bilayers, and for the first time the combination of two instrumental setups: gas-phase electrophoresis (nES GEMMA instrumentation) and electrical (capacitance and resistance) measurements. Liposomes and planar lipid bilayers consisted of phosphatidylcholine, cholesterol and phosphatidylethanolamine. Liposomes were prepared from dried lipid films via hydration while planar lipid bilayers were formed using a Mueller-Rudin method. Calcium ions were added to membranes from higher concentrated stock solutions.

Changes in phospholipid bilayer properties due to calcium presence were observed for both studied cell membrane analogues. Changes in liposome size were observed, which might either be related to tighter packing of phospholipids in the bilayer or local distortions of the membrane. Likewise, a measurable change in planar lipid bilayer resistance and capacitance was observed in the presence of calcium ions, which can be due to an increased rigidity and tighter packing of the lipid molecules in the bilayer.

1. Introduction

Effects of metal cations on biological membranes are of considerable interest since ion attachment can alter the stability and structure of phospholipid bilayers [1,2]. One particular important biophysical question is the interaction of phospholipid membranes with divalent metal cations, such as calcium. Calcium is an important and ubiquitous second messenger involved in the regulation of a variety of different cellular processes. In addition, it plays a crucial role in the maintenance of cell homeostasis [3]. Therefore, the concentration of calcium ions in the intracellular and extracellular environment is highly regulated [4,5]. Changes in calcium concentration can lead to various events such as cell fusion, remodeling of the cell membrane and increased leakage of cellular components [6–8]. Excessive influx and uptake of calcium in cellular storages, signifies cell stress and can lead to a cellular overload, which consequently causes cell death [5,9].

Calcium has recently also been used for medical treatments, such as calcium electroporation. Electroporation is a process of electrically induced increase in permeability of the cell membrane for molecules which otherwise cannot diffuse across the membrane barrier [10]. Calcium electroporation is a novel anti-tumor treatment where large quantities of calcium are internalized by the cell during an electroporation process [11]. Calcium electroporation has been shown to induce tumor necrosis associated with ATP depletion [12–15]. What is more, calcium electroporation also considerably inhibits cell migration capabilities [14]. The anti-tumor effectiveness of calcium electroporation has been demonstrated *in vivo* [12,16,17], *in vitro* [17–19] and in clinical trials [20–23].

Effects of calcium ions on cell membrane stability, structure and cellular processes have been investigated extensively and a large number of different studies have been conducted to better understand the interactions between calcium and phospholipids forming the cell membrane: (i) It was observed that binding of calcium ions causes a conformational change of the phospholipid polar headgroups [24,25] leading to a reduction in phospholipid area per molecule [26,27]. (ii) It was also demonstrated that calcium ions, when adsorbed onto a membrane, reduce the surface charge density of the phospholipids [28] and cause partial dehydration of the bilayer [1,29]. (iii) Significant increase in membrane thickness, and membrane rigidity was observed as well [30,31]. (iv) What is more, several molecular dynamic (MD) studies show a sequential

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https://doi.org/10.1016/j.bioelechem.2021.107988
1567-5394/

binding of calcium ions to the phospholipids leading to structural changes of the lipid membrane [32,33], namely likewise a decrease in area per lipid.

Hoeljolt and colleagues demonstrated that the addition of calcium ions could also influence the transition temperature of the lipids, namely increasing it [34]. Calcium ion effects on phospholipids has been studied on various simplified cell membrane models, such as liposomes and planar lipid bilayers in the past [1,2,35–39]. Liposomes are spherical vesicles surrounded by a phospholipid double layer encapsulating an aqueous lumen. Their resemblance to a biological cell membrane exceeds the planar lipid bilayer, which is considered as a small fraction of a total cell membrane applied for electrophysiological measurements. Planar lipid bilayers consist of two phospholipid sheets with the hydrophobic tails pointing toward the center of the sheet, shielded from the aqueous environment by their hydrophilic phospholipid headgroups. Since planar lipid bilayers are accessible from both sides, their analysis is relatively simple, straightforward [40] and occurs in a well-controlled environment [41,42].

In our study, liposomes and planar lipid bilayers are used as models of biological membranes to investigate the effects of calcium ions on phospholipid bilayers in more detail by comparing two analytical setups. A change in liposome size due to calcium ion addition was observed and quantified via gas-phase electrophoretic measurements. Gas-phase electrophoresis was first described by Kaufman and colleagues in 1996 [43]. Samples are electrosprayed from a volatile electrolyte solution. Subsequently, drying of droplets and charge equilibration in a bipolar atmosphere occurs. Single-charged, surface-dried, polydisperse aerosol is then size-separated in a high laminar-sheath flow and a tunable electric field. By variation of the electric field strength, monomobile aerosol particles are obtained which are then assessed via particle number-based detection as recommended by the European Commission for nanoparticle characterization (2011/696/EU from October 18th, 2011). Relating the electric field strength necessary for the monomobile aerosol to pass the size separator, and hence ultimately the particle size versa particle count values yields the corresponding GEMMA spectrum. Such a setup is known under different names – nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analyzer (nES GEMMA), nES Differential Mobility Analyzer (nES DMA), LiquiScan ES, Scanning Mobility Particle Sizer (SMPS) or MacroIMS – and has already been used in the past for liposome analysis [44–46] or analysis of lipid-containing structures [47–49]. Subsequently, electrical measurements on planar lipid bilayers were carried out, to determine changes in the bilayer properties induced by calcium ion addition. Even small calcium ion concentration related changes in the phospholipid head-group structure could alter the electrical properties of the phospholipid surface and produce measurable changes of the lipid bilayer's properties. We aimed to gain a better insight on interactions between calcium ions and membrane phospholipids comparing two analytical setups and indeed, we observed an effect of calcium ions on liposomes and planar lipid bilayers as cell membrane analogues in both setups.

2. Materials and methods

2.1. Chemicals

Ammonium acetate (NH_4OAc, $\geq$ 99.99 %), ammonium hydroxide (ACS reagent), n-decane (ReagentPlus, $\geq$ 99.00 %), hexane (ACS reagent), calcium chloride ($CaCl_2$, anhydrous, $\geq$ 96.00 %) were purchased from Sigma Aldrich (Steinheim, Germany). Chloroform (Spectronorm quality) was obtained from VWR BDH Chemicals (Roncello, Italy), methanol (LiChrosolv), potassium chloride (KCl) and 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) from Merck (Darmstadt, Germany). Nitrogen and CO_2 gas was from Messer (Gumpoldskirchen, Austria). The lipids l-α-phosphatidylcholine,hydrogenated (Soy) (HSPC), 1, 2-dioctadecanoyl-*sn-glycero*-3-phosphoethanolamine (18:0 PE, DSPE), cholesterol (Chol) and 1-palmitoyl-2-oleoyl-*glycero*-3-phosphocholine (POPC) were from Avanti Polar Lipids (Alabaster, AL, USA obtained via Instruchemie (Delfzyl, The Netherlands)).

2.2. Buffers and electrolytes

NH_4OAc (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.

KCl (0.1 M) and HEPES (0.01 M) were mixed together in a 1:1 volumetric ratio and applied as an aqueous electrolyte in the measurement chamber for planar lipid bilayer measurements. $CaCl_2$ solutions were prepared at indicated concentrations by dissolving $CaCl_2$ in either NH_4OAc solution for liposome measurements or KCl/HEPES solution for planar lipid bilayer measurements.

2.3. Liposome preparation

Liposomes from HSPC:Chol:DSPE (4:3:3 M ratio) or POPC:Chol:DSPE (4:3:3 M ratio) were prepared from dried thin lipid films via hydration [50]. Lipids were dissolved in methanol:chloroform (1:3 mixture [v:v]) prior to formation of a thin, regular film under a constant stream of nitrogen gas. The film was additionally dried for approximately 2 h in a desiccator following its hydration with 1 mL NH_4OAc solution. This yielded a dispersion of 10 mM total lipid concentration. Vortexing and heating in a water bath at about 65°C lead to detachment of the lipid film from the flask surface. Subsequently, small unilamellar vesicles were prepared via extrusion with 21 passes through two pre-wetted 100 nm pore size, polycarbonate membranes (Avanti Polar Lipids) applied in the same membrane orientation. Finally, liposome stock solutions were stored overnight in brown glass vials at 4°C prior to analysis.

2.4. Liposome nES GEMMA measurements

Gas-phase electrophoresis was carried out on a TSI Inc instrument (Shoreview, MN, USA): A nES aerosol generator (model 3480) equipped with either a ^{210}Po α-particle source or an alternating corona discharge unit [51], a nano differential mobility analyzer (nDMA, model 3080) and a n-butanol-based ultrafine condensation particle counter (CPC, either model 3025A or a similar model, namely 3776C) were applied. The samples were introduced to the nES unit via a 25 μm inner diameter, fused silica capillary with a homemade tip [52], generating a stable Taylor cone. A fresh capillary was employed for each day of measurement in order to exclude cross-contaminations. 4.0 lb per square inch differential (psid, approx. 28 kPa) and 0.1 L per minute (Lpm) CO_2 and 1.0 Lpm compressed, particle-free air were employed for transport of particles from the capillary through the neutralization/charge reduction chamber and to the nDMA unit. The applied air was additionally dried (Donaldson Variodry Membrane Dryer Superplus, Leuven, Belgium) to facilitate drying of nES derived nanodroplets. The covered size range of the nDMA was from 4.85 nm to 180.0 nm by application of a sheath flow of 2.5 Lpm. All of the experiments were carried out at room temperature (~21 °C). Every sample was measured four times, for 180 s each, corresponding to 150 s of voltage adjustment and a 30-second window for the instruments to return to idle state of low voltage again.

Prior to gas-phase electrophoresis, six different $CaCl_2$ concentrations in NH_4OAc solution were added (0 mM, 5 mM, 10 mM,

20 mM, 30 mM and 40 mM) to the liposome solutions during spin filtration employing 10 kDa molecular weight cut-off filters (MWCO, polyethersulfone membrane, VWR, Vienna, Austria) leading to a 1:10 [v:v] dilution of the initial stock. The spin filtration of liposomes was carried out in a centrifuge using 9300 g for approximately 7 min. For liposome samples containing $CaCl_2$, the last spin filtration round was always performed in pure NH_4OAc in order to remove unbound, non-volatile calcium ions.

2.5. Planar lipid bilayer formation

Planar lipid bilayers were formed using a Mueller-Rudin method (Mueller et al. 1962). In short, the corresponding amount of POPC or a mixture of HSPC:Chol:DSPE (4:3:3 M ratio) dissolved in chloroform were dried under a N_2 stream and subsequently dissolved in n-decan to obtain a final 20 mM lipid concentration. All of the experiments were carried out at room temperature (~21 °C). A Delrin measurement chamber from Warner Instruments (Hamden, CT, USA) with 150 μm diameter aperture separating the two cuvettes was used to form planar lipid bilayer. The aperture was pretreated with 20 mM lipids dissolved in hexane. After evaporation of hexane, each compartment was filled with 4 mL of KCl/HEPES electrolyte solution. For planar lipid bilayer formation, a small drop of lipids dissolved in n-decane was applied to the aperture using a glass rod. A stable planar lipid bilayer was formed after approximately 30 min and subsequent the capacitance was measured to be around 0.5 $\mu F/cm^2$ for POPC and 0.4 $\mu F/cm^2$ for mixed (HSPC:Chol:DSPE) bilayers.

2.6. Planar lipid bilayer resistance and capacitance measurements

Electrical measurements on planar lipid bilayers were performed using a LCR meter E4980A from Keysight (Santa Rosa, CA, USA) connected to four Ag/AgCl electrodes (In vivo metric, Healdsburg, CA, USA) immersed in the electrolyte solution in the Delrin measurement chamber (Warner Instruments, USA). The LCR meter was set to measure resistance *Rp* and capacitance *Cp* in parallel. The AC voltage was set to 20 mV and the frequency to 2 kHz. Data points were acquired each quarter of a second to obtain measurements of the resistance and capacitance over time. The LCR meter was connected through an Ethernet connection and controlled with MATLAB R2019a (MathWorks, Natick, MA, USA) Toolbox using SCPI protocol. The measured data, capacitance and resistance of each planar lipid bilayer were also processed with MATLAB R2019a.

Formation of a planar lipid bilayer required a 30 min stabilization period, after which a corresponding volume of 9 M $CaCl_2$ solution in KCl/HEPES was stepwise added to one of the compartments in the Delrin measurement chamber to obtain six different $CaCl_2$ concentrations (0 mM, 5 mM, 10 mM, 20 mM, 30 mM and 40 mM).

2.7. Statistical analysis

Datasets for liposome analysis with each calcium ion concentration (raw data obtained from instrument software, MacroIMS manager v2.0.1.0) were combined via their median to yield a corresponding nES GEMMA spectrum. Size precision of the applied nES GEMMA instrument for liposome analysis was ± 0.1 nm. Each spectrum was cut at 40 nm particle diameter in order to blank the low-sized calcium related peaks from the nES GEMMA spectra. Gaussian curve was fitted to each cut spectrum via Matlab R2019a to obtain diameter values and statistical parameters. For comparison of different concentration spectra, a one-way ANOVA on Ranks was used. All pairwise multiple comparisons were made by Tukey's test. We rejected the null hypothesis of analysis if the p-value of the test was less than 0.05 ($p < 0.05$).

Resistance and capacitance measurements of planar lipid bilayers for each calcium ion concentration were combined via their median to obtain the corresponding dataset. For comparison of planar lipid bilayers datasets a one-way ANOVA on Ranks was used. All pairwise, multiple comparisons were made by Tukey's test. We rejected the null hypothesis of analysis if the p-value of the test was less than 0.05 ($p <<< 0.05$).

3. Results and discussion

The effect of calcium ions on phospholipid membranes was studied using two different cell membrane models, namely globular liposomes and planar lipid bilayers. Calcium ions were added to liposomes and planar lipid bilayers in order to observe phospholipid modifications as a function of calcium ion concentration, employing two different measuring methods, nES GEMMA for liposomes and resistance/capacitance measurements on planar lipid bilayers.

3.1. Liposome analysis

Firstly, we focused on liposomes as *in-vitro* cell membrane analogues. In contrast to planar lipid membranes, vesicles exhibit a strong membrane curvature. In addition, the phospholipid surface of vesicles exceeds the section of a planar lipid membrane. Due to the latter, we expected to observe similar effects upon calcium ion addition to liposomes than when employing planar lipid bilayers, yet at higher calcium ion concentrations.

Liposomes encapsulating NH_4OAc solution were applied and calcium ions were added to the vesicles' surrounding medium for incubation during the spin-filtration process. Subsequently, excess small-diameter particles as well as unbound calcium ions were removed via spin-filtration. Other low-sized calcium related peaks were cut out of the spectrum using Matlab R2019a software.

In the absence of calcium ions, the apex of the liposome peak was determined to be at 39.5 ± 2 nm (n = 4 measurements) for POPC:Chol:DSPE liposomes and 71.2 ± 0.7 nm (n = 12 measurements) for HSPC:Chol:DSPE liposomes by fitting the Gaussian curve to the raw data. When calcium ions were added at different concentrations to liposome samples a slight but significant shift in the liposome particle diameter was observed. In a first attempt, liposomes consisting of POPC:Chol:DSPE were analyzed; however, the increased unspecific lipid aggregation rendered them not completely suited for our analysis. As can be seen on Fig. 1A, the liposome peak is hardly distinguishable therefore; the Gaussian curve does not fit well to the data.

Nevertheless, a change in liposome diameter can be observed as seen on Fig. 1B, namely a decrease in particle diameter for 5 mM and 10 mM $CaCl_2$ and an increase in particle diameter for higher calcium concentrations (20 mM, 30 mM and 40 mM $CaCl_2$). An 22% increase in liposome diameter was measured between 0 mM $CaCl_2$ and 40 mM $CaCl_2$ Fig. 2.

In the next set of experiments, HSPC:Chol:DSPE liposomes were used due to their optimal size distribution and ease of analysis using our analytical setup. The liposome peak is easily distinguishable as seen on Fig. 3A and a decrease in liposome size with increasing calcium concentration was observed (Fig. 3B). A 10% decrease in liposome diameter was measured between 0 mM $CaCl_2$ and 40 mM $CaCl_2$. A decrease in the surface-dry liposome volume due to calcium ion binding is also supported by dynamic light scattering (DLS) data [53]. Shrinkage of liposome particles is likely to be caused by a decrease of area per lipid caused by calcium ions as demonstrated by MD simulations [54].

However, this decrease in liposome size is surprising as such, as simple attachment of non-volatile buffer components to analytes

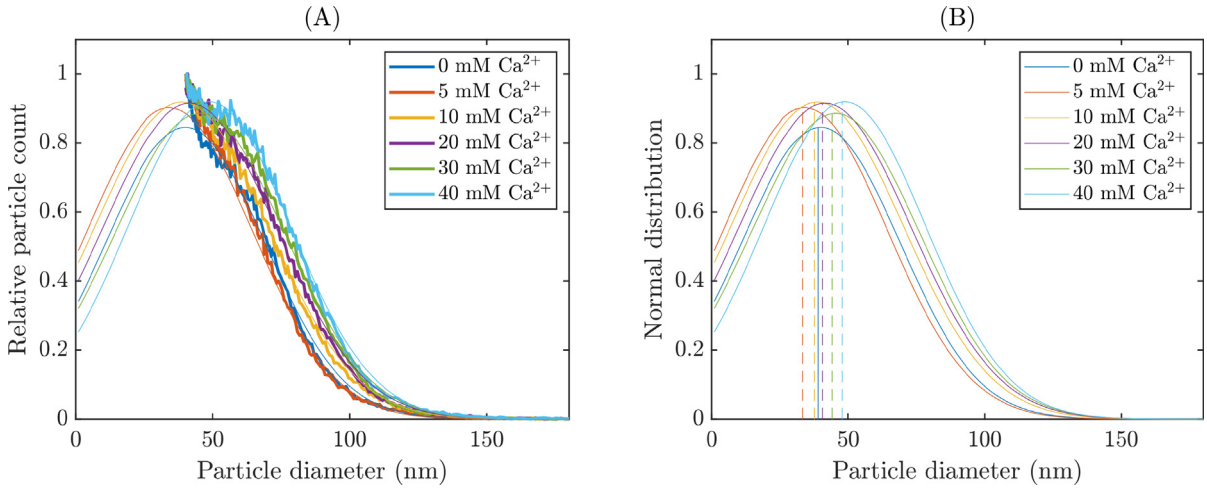

Fig. 1. POPC:Chol:DSPE liposome particle size distribution as assessed via nES GEMMA. (A) Particle size distributions of POPC:Chol:DSPE liposome samples containing 0 mM $CaCl_2$ (N = 4) , 5 mM $CaCl_2$ (N = 4), 10 mM $CaCl_2$ (N = 4), 20 mM $CaCl_2$ (N = 4), 30 mM $CaCl_2$ (N = 4) and 40 mM $CaCl_2$ (N = 4) are depicted. Data is related to the highest peak and is scaled using Matlab rescale function. (B) The fitted Gaussian curve for each specific calcium concentration. Vertical lines represent the liposome diameter for each calcium ion concentration.

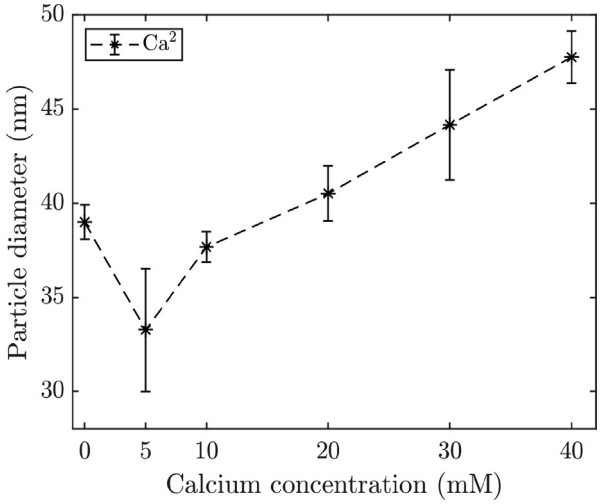

Fig. 2. POPC:Chol:DSPE liposome diameter change as a function of calcium concentration. An increase in POPC:Chol:DSPE liposome size can be observed with addition of $CaCl_2$ to the liposome sample. Raw data was fitted to the Gaussian curve to obtain the diameter points. Represented is the median value of all measurements at specific concentration and standard deviation.

upon nES GEMMA analysis usually results in higher surface-dry particle diameter values [55] as seen for POPC containing liposomes Fig. 4. Hence, a calcium ion dependent decrease in vesicle size might either be related to tighter packing of phospholipid moieties in the bilayer and therefore reduced particle size or local distortions of the lipid bilayer leading to partial loss of encapsulated vesicle components (ammonium acetate, water) upon surface drying during gas-phase electrophoresis. Similar effects (a decrease in vesicle size) was also observed upon addition of a pore-forming peptide (GALA) to liposomes and incubation at acidic pH (unpublished data of a different experimental series).

3.2. Planar lipid bilayer analysis

Subsequently, planar lipid bilayer electrical properties were measured by a LCR meter connected directly to the electrodes in the measurement chamber. As a measure of the influence of calcium ion addition on the lipid bilayer properties, we determined changes in its equivalent parallel capacitance *Cp* and resistance *Rp*. The measured electrical properties can be correlated to the bilayer thickness (d) and permeability using basic equations for resistance (R [Ω]) and capacitance (C[F]) (see equation (1) and (2) below):

$$c = \frac{Cp}{A} = \frac{\varepsilon A}{dA} = \frac{\varepsilon}{d} \tag{1}$$

$$R = \frac{\rho d}{A} \tag{2}$$

A planar lipid bilayer can be represented as a capacitor that is capable of storing charge in an electric field. The bilayer specific capacitance *c* [μF/cm^2] is calculated as measured capacitance divided by the area of the aperture and is as such used to compare capacitance of different measurements setups. *c* is directly proportional to its dielectric constant (ε, [F/m]), and is inversely proportional to the thickness of the bilayer (d, [m]). Resistance of the planar lipid bilayer can also be measured from the values of applied voltage and measured current flowing through the formed bilayer. Resistance of the bilayer is directly proportional to the specific electrical resistance of the bilayer (ρ , [Ωm]) and its thickness (d, [m]), and is inversely proportional to the surface area (A, [m^2]) of the bilayer.

For planar lipid bilayer experiments, in a first attempt POPC lipids were chosen due to their simplicity and ease of bilayer formation. Furthermore, due to the absence of a net charge on POPC we were able to focus primarily on specific lipid–ion interactions. However, since POPC is uncharged, the interaction of calcium ions is expected to be relatively weak compared to charged phospholipids. Nevertheless, even small changes in the lipid head group could significantly alter the electrical properties of the bilayer surface. Therefore, measurement of bilayer's electrical properties was applied as a sensitive technique for determination of calcium binding to the lipid structures.

We measured the changes in resistance (Fig. 5A) and capacitance (Fig. 5B) of the POPC planar lipid bilayer in response to calcium ion addition. A measurable increase in bilayer resistance R and a decrease in bilayer specific capacitance *c* was observed, which we relate to an increase in planar lipid bilayer thickness in the presence of calcium ions. Changes in the bilayer's resistance and capacitance have been reported previously and our results are in accordance with these findings [27,31]. In previous studies,

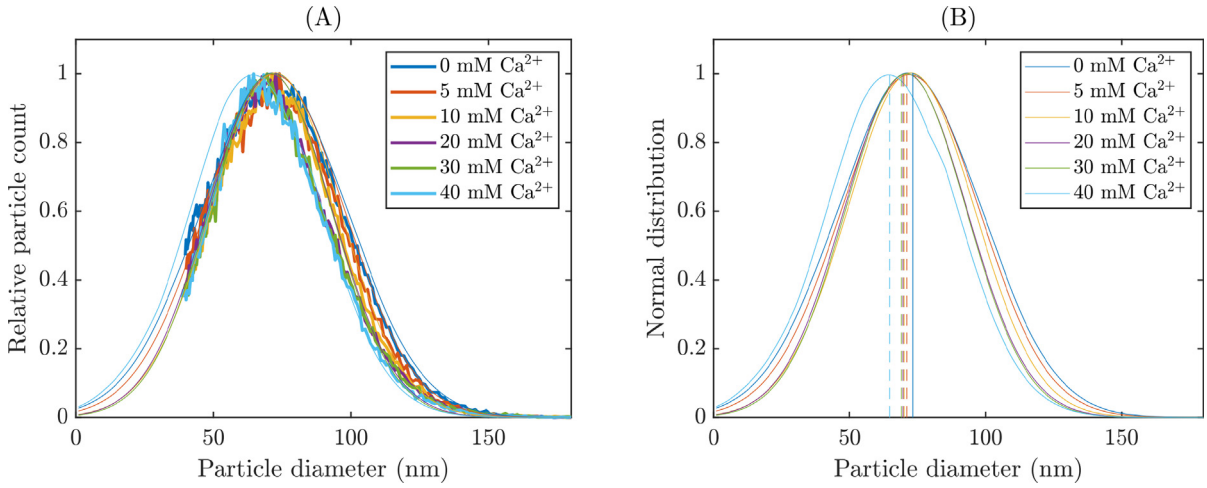

Fig. 3. Liposome particle size distribution as assessed via nES GEMMA. (A) Particle size distributions of HSPC:Chol:DSPE liposome samples containing 0 mM $CaCl_2$ (N = 12) , 5 mM $CaCl_2$ (N = 4), 10 mM $CaCl_2$ (N = 4), 20 mM $CaCl_2$ (N = 8), 30 mM $CaCl_2$ (N = 4) and 40 mM $CaCl_2$ (N = 8) are depicted. Data is related to the highest peak and is scaled using Matlab rescale function. (B) The fitted Gaussian curve for each specific calcium concentration. Vertical lines represent the liposome diameter for each calcium ion concentration.

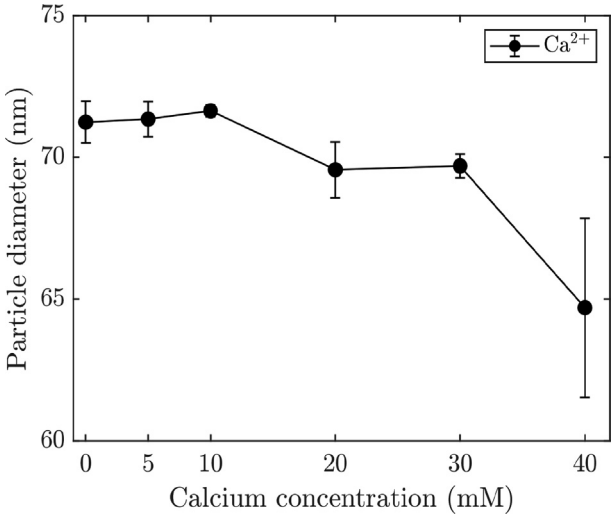

Fig. 4. HSPC:Chol:DSPE liposome diameter change as a function of calcium concentration. A decrease in HSPC:Chol:DSPE liposome size can be observed with addition of $CaCl_2$ to the liposome sample. Raw data was fitted to the Gaussian curve to obtain the diameter points. Represented is the median value of all measurements at specific concentration and standard deviation.

a considerable effect of divalent cations on the packaging order of the lipid molecules was observed as well, and measured as an increase in resistance and a decrease in capacitance. Obtained results can be correlated with a "rigidification" of the planar lipid bilayers in the presence of divalent cations, due to ordering of the lipid molecules and a more tightly packed lateral structure. As mentioned previously, MD simulations [54] show a decrease in area per lipid due to calcium ions, which correlates to an increase in bilayer thickness and is in accordance with our experimental results showing an increase in bilayer resistance and a decrease in capacitance.

In the next step, we measured the changes in resistance (Fig. 5A) and capacitance (Fig. 5B) of the HSPC:Chol:DSPE planar lipid bilayers induced by calcium ion addition. Planar lipid bilayers were formed using a mixture of HSPC:Chol:DSPE lipids for better comparison to nES GEMMA analysis of liposomes. These lipids have a more complex structure in comparison to POPC. Additionally, the surface of PE lipids is distinctively different from that of other phospholipid molecules as DSPE also carries a positive charged head group. It is possible that the chemical structure of the lipids influences the binding properties of calcium ions.

Planar lipid bilayers composed of a complex mixture of lipids, show a different behavior from POPC when calcium is added to the system. Contrary to POPC an increase in resistance as well as a slight increase in capacitance was observed, which proves, that not only the bilayer thickness changes due to calcium, but also other properties of the bilayer as well.

Fig. 5 presents electrical measurements of POPC and HSPC:Chol:DSPE bilayers as a function of calcium ion concentration. Capacitance changes more drastically for POPC lipids, which might be due to no net lipid charge. DSPE carries a positive charge, therefore less calcium comes into proximity of the HSPC:Chol:DSPE bilayers minimizing its effect. For POPC planar lipid bilayer the resistance increased for almost 44% from 0 mM $CaCl_2$ to 40 mM $CaCl_2$. Similarly, the resistance increase was seen for HSPC:Chol:DSPE bilayers where resistance with addition of 40 mM $CaCl_2$ increased for 43%.

Smaller changes were measured for capacitance differences, namely for POPC bilayers the capacitance decreased for almost 8% between 0 mM $CaCl_2$ and 40 mM $CaCl_2$. Whereas for HSPC:Chol:DSPE bilayers capacitance remained almost constant and increased for only about 0,4%.

To sum up, we showed that two different instrumental techniques could be used to detect calcium-induced changes on either liposomes or planar lipid bilayers. However, the choice of the lipid molecules used to build cell membrane models plays an important role in calcium modifications. Differences in measurements between POPC and HSPC:Chol:DSPE bilayers could be due to the fact that the lipid composition greatly influences the membrane's properties, mainly its permeability. What is more, calcium ions increase the phase transition temperature of the lipids and since the permeability of the lipid membrane is highest around the lipid transition phase, the addition of calcium ions will result in higher transition temperatures and planar lipid bilayers will therefore be more tightly packed at room temperature, where our measurements were performed. When calcium ions were added to POPC containing lipid structures, the bilayer's resistance increased while the capacitance decreased which can be correlated to an increased bilayer thickness and decreased area per lipid. Liposome measurements showed an increase in particle diameter, which can be due to the attachment of non-volatile buffer components to analytes upon nES GEMMA analysis or a decrease in area per lipid and consequently increased

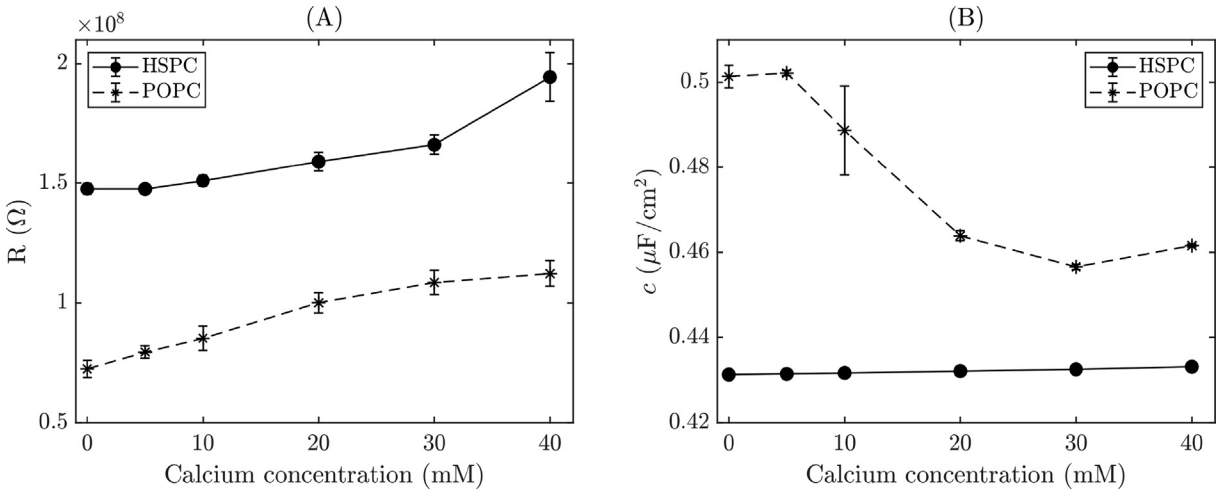

Fig. 5. Difference between POPC and HSPC:Chol:DSPE bilayers. (A) It can be seen that the resistance increases with increasing calcium concentration for POPC (N = 27) and HSPC:Chol:DSPE (N = 23) planar lipid bilayers. (B) Capacitance on the other hand decreases for POPC planar lipid bilayers and slightly increases for HSPC:Chol:DSPE planar lipid bilayer showing a more complex interaction of HSPC and DSPE lipids with calcium ions.

bilayer thickness. A more complex behavior was observed for HSPC and DSPE containing cell membrane analogues. With HSPC:Chol:DSPE planar lipid bilayers, an increase in resistance was observed, which can again be correlated to an increased bilayer thickness and decreased area per lipid. However, the capacitance did not change significantly. HSPC:Chol:DSPE liposomes showed a decrease in particle diameter with increasing calcium ion concentration, which could be due to a decreased area per lipid and consequent tighter packaging of the molecules in the lipid bilayer. This is in line with the results presented by Hoejholt et al [34], where they showed that lipid composition and heat capacity of the membrane can influence the effect of calcium electroporation.

4. Conclusion

Two different cell membrane analogues were used to observe the effect of calcium ions on phospholipids using for the first time the combination of two different measurement techniques i.e. gas-phase electrophoresis of liposomes and electrical measurements of planar lipid bilayers.

We observed an increase in POPC:Chol:DSPE liposomes and a decrease in HSPC:Chol:DSPE liposome diameter due to calcium ion addition upon gas-phase electrophoresis. Therefore, we conclude that calcium ions cause structural changes in the phospholipid membranes. Calcium binds to the phosphate group of the phospholipid molecules thus creating a conformation change in the lipid head group. These small changes can also be observed on planar lipid bilayers via electrical measurements. An increase of bilayer's resistance and a decrease in bilayer's capacitance was observed due to calcium binding to POPC lipids. When a complex mixture of HSPC:Chol:DSPE lipids was employed for bilayer formation, different results were obtained as both resistance and capacitance increased due to calcium binding. This leads us to believe that not only area per lipid and therefore membrane thickness change but also other properties of the phospholipid bilayer are influenced by calcium ion attachment.

Declaration of Competing Interest

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.

Acknowledgements

This study was funded by Slovenian Research Agency (ARRS) with grant P2-0249, and Junior Researcher funding for KB. Partial funding in the scope of Erasmus + is acknowledged. Experiments were partially performed within Infrastructure Program: Network of research infrastructure centers at University of Ljubljana (MRIC UL IP-0510).

References

[1] H. Binder, O. Zschörnig, The effect of metal cations on the phase behavior and hydration characteristics of phospholipid membranes, Chem. Phys. Lipids. 115 (1-2) (2002) 39–61, https://doi.org/10.1016/S0009-3084(02)00005-1.
[2] A. Averbakh, V.I. Lobyshev, Adsorption of polyvalent cations to bilayer membranes from negatively charged lipid: estimating the lipid accessibility in the case of complete binding, J. Biochem. Biophys. Methods. 45 (1) (2000) 23–44, https://doi.org/10.1016/S0165-022X(00)00072-5.
[3] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (7) (2003) 517–529, https://doi.org/10.1038/nrm1155.
[4] H. Bader, Intracellular Calcium Regulation, Manchester University Press, 1986.
[5] Z. Dong, P. Saikumar, J.M. Weinberg, M.A. Venkatachalam, Calcium in cell injury and death, Annu. Rev. Pathol. 1 (1) (2006) 405–434, https://doi.org/10.1146/annurev.pathol.1.110304.100218.
[6] D.E. Leckband, C.A. Helm, J. Israelachvili, Role of calcium in the adhesion and fusion of bilayers, Biochemistry. 32 (4) (1993) 1127–1140, https://doi.org/10.1021/bi00055a019.
[7] R. Bagur, G. Hajnóczky, Intracellular Ca2+ sensing: role in calcium homeostasis and signaling, Mol. Cell. 66 (2017) 780–788, https://doi.org/10.1016/j.molcel.2017.05.028.
[8] M. Brini, E. Carafoli*, Calcium signalling: a historical account, recent developments and future perspectives, Cell. Mol. Life Sci. CMLS. 57 (3) (2000) 354–370, https://doi.org/10.1007/PL00000698.
[9] T. Batista Napotnik, T. Polajžer, D. Miklavčič, Cell death due to electroporation – A review, Bioelectrochemistry. 141 (2021) 107871, https://doi.org/10.1016/j.bioelechem.2021.107871.
[10] T. Kotnik, L. Rems, M. Tarek, D. Miklavčič, Membrane Electroporation and Electropermeabilization: Mechanisms and Models, Annu. Rev. Biophys. 48 (1) (2019) 63–91, https://doi.org/10.1146/annurev-biophys-052118-115451.
[11] S.K. Frandsen, M. Vissing, J. Gehl, A Comprehensive Review of Calcium Electroporation —A Novel Cancer Treatment Modality, Cancers. 12 (2) (2020) 290, https://doi.org/10.3390/cancers12020290.
[12] S.K. Frandsen, J. Gehl, M.M. Alonso, Effect of calcium electroporation in combination with metformin in vivo and correlation between viability and intracellular ATP level after calcium electroporation in vitro, PLOS ONE. 12 (7) (2017) e0181839, https://doi.org/10.1371/journal.pone.0181839.
[13] E.L. Hansen, E.B. Sozer, S. Romeo, S.K. Frandsen, P.T. Vernier, J. Gehl, B. Rubinsky, Dose-Dependent ATP Depletion and Cancer Cell Death following Calcium Electroporation, Relative Effect of Calcium Concentration and Electric Field Strength, PLOS ONE. 10 (4) (2015) e0122973, https://doi.org/10.1371/journal.pone.0122973.

[14] B. Staresinic, T. Jesenko, U. Kamensek, S. Krog Frandsen, G. Sersa, J. Gehl, M. Cemazar, Effect of calcium electroporation on tumour vasculature, Sci. Rep. 8 (2018) 1–14, https://doi.org/10.1038/s41598-018-27728-z.
[15] S.K. Frandsen, H. Gissel, P. Hojman, T. Tramm, J. Eriksen, J. Gehl, Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis, Cancer Res. 72 (6) (2012) 1336–1341, https://doi.org/10.1158/0008-5472.CAN-11-3782.
[16] A. Szewczyk, J. Gehl, M. Daczewska, J. Saczko, S.K. Frandsen, J. Kulbacka, Calcium electroporation for treatment of sarcoma in preclinical studies, Oncotarget. 9 (2018) 11604–11618, https://doi.org/10.18632/oncotarget.24352.
[17] S.K. Frandsen, L. Gibot, M. Madi, J. Gehl, M.-P. Rols, B. Rubinsky, Calcium Electroporation: Evidence for Differential Effects in Normal and Malignant Cell Lines, Evaluated in a 3D Spheroid Model, PLoS ONE. 10 (12) (2015) e0144028, https://doi.org/10.1371/journal.pone.0144028.
[18] H. Falk, P.F. Forde, M.L. Bay, U.M. Mangalanathan, P. Hojman, D.M. Soden, J. Gehl, Calcium electroporation induces tumor eradication, long-lasting immunity and cytokine responses in the CT26 colon cancer mouse model, OncoImmunology. 6 (5) (2017) e1301332, https://doi.org/10.1080/2162402X.2017.1301332.
[19] A. Zielichowska, M. Daczewska, J. Saczko, O. Michel, J. Kulbacka, Applications of calcium electroporation to effective apoptosis induction in fibrosarcoma cells and stimulation of normal muscle cells, Bioelectrochemistry. 109 (2016) 70–78, https://doi.org/10.1016/j.bioelechem.2016.01.005.
[20] H. Falk, L.W. Matthiessen, G. Wooler, J. Gehl, Calcium electroporation for treatment of cutaneous metastases; a randomized double-blinded phase II study, comparing the effect of calcium electroporation with electrochemotherapy, Acta Oncol. Stockh. Swed. 57 (3) (2018) 311–319, https://doi.org/10.1080/0284186X.2017.1355109.
[21] H. Falk, S. Lambaa, H.H. Johannesen, G. Wooler, A. Venzo, J. Gehl, Electrochemotherapy and calcium electroporation inducing a systemic immune response with local and distant remission of tumors in a patient with malignant melanoma – a case report, Acta Oncol. 56 (8) (2017) 1126–1131, https://doi.org/10.1080/0284186X.2017.1290274.
[22] C.C. Plaschke, J. Gehl, H.H. Johannesen, B.M. Fischer, A. Kjaer, A.F. Lomholt, I. Wessel, Calcium electroporation for recurrent head and neck cancer: A clinical phase I study, Laryngoscope Investig. Otolaryngol. 4 (1) (2019) 49–56, https://doi.org/10.1002/lio2.233.
[23] D. Ágoston, E. Baltás, H. Ócsai, S. Rátkai, P.G. Lázár, I. Korom, E. Varga, I.B. Németh, É. Dósa-Rácz Viharosné, J. Gehl, J. Oláh, L. Kemény, E.G. Kis, Evaluation of Calcium Electroporation for the Treatment of Cutaneous Metastases: A Double Blinded Randomised Controlled Phase II Trial, Cancers. 12 (2020) 179, https://doi.org/10.3390/cancers12010179.
[24] M.F. Brown, J. Seelig, Ion-induced changes in head group conformation of lecithin bilayers, Nature. 269 (5630) (1977) 721–723, https://doi.org/10.1038/269721a0.
[25] H. Akutsu, J. Seelig, Interaction of metal ions with phosphatidylcholine bilayer membranes, Biochemistry. 20 (26) (1981) 7366–7373, https://doi.org/10.1021/bi00529a007.
[26] D. Huster, K. Arnold, K. Gawrisch, Strength of Ca(2+) binding to retinal lipid membranes: consequences for lipid organization, Biophys. J. 78 (2000) 3011–3018.
[27] M. Karabaliev, V. Kochev, Interaction of thin wetting films of lecithin with some divalent cations, Bioelectrochemistry. 63 (1-2) (2004) 177–181, https://doi.org/10.1016/j.bioelechem.2003.10.018.
[28] U.R. Pedersen, C. Leidy, P. Westh, G.H. Peters, The effect of calcium on the properties of charged phospholipid bilayers, Biochim. Biophys. Acta BBA - Biomembr. 1758 (5) (2006) 573–582, https://doi.org/10.1016/j.bbamem.2006.03.035.
[29] R.J. Alsop, R. Maria Schober, M.C. Rheinstädter, Swelling of phospholipid membranes by divalent metal ions depends on the location of the ions in the bilayers, Soft Matter. 12 (32) (2016) 6737–6748, https://doi.org/10.1039/C6SM00695G.
[30] G. Pabst, A. Hodzic, J. Štrancar, S. Danner, M. Rappolt, P. Laggner, Rigidification of Neutral Lipid Bilayers in the Presence of Salts, Biophys. J. 93 (8) (2007) 2688–2696, https://doi.org/10.1529/biophysj.107.112615.
[31] M. Karabaliev, Effects of divalent cations on the formation and structure of solid supported lipid films, Bioelectrochemistry. 71 (1) (2007) 54–59, https://doi.org/10.1016/j.bioelechem.2007.02.003.
[32] R.A. Böckmann, H. Grubmüller, Multistep binding of divalent cations to phospholipid bilayers: a molecular dynamics study, Angew. Chem. Int. Ed Engl. 43 (8) (2004) 1021–1024, https://doi.org/10.1002/anie.200352784.
[33] J.M. Boettcher, R.L. Davis-Harrison, M.C. Clay, A.J. Nieuwkoop, Y.Z. Ohkubo, E. Tajkhorshid, J.H. Morrissey, C.M. Rienstra, Atomic View of Calcium-Induced Clustering of Phosphatidylserine in Mixed Lipid Bilayers, Biochemistry. 50 (12) (2011) 2264–2273, https://doi.org/10.1021/bi1013694.
[34] K.L. Hoejholt, T. Mužić, S.D. Jensen, L.T. Dalgaard, M. Bilgin, J. Nylandsted, T. Heimburg, S.K. Frandsen, J. Gehl, Calcium electroporation and electrochemotherapy for cancer treatment: Importance of cell membrane composition investigated by lipidomics, calorimetry and in vitro efficacy, Sci. Rep. 9 (2019) 4758, https://doi.org/10.1038/s41598-019-41188-z.
[35] N. Düzgüneş, S. Ohki, Calcium-induced interaction of phospholipid vesicles and bilayer lipid membranes, Biochim. Biophys. Acta BBA - Biomembr. 467 (3) (1977) 301–308, https://doi.org/10.1016/0005-2736(77)90307-8.
[36] S. McLaughlin, Electrostatic Potentials at Membrane-Solution Interfaces, in: F. Bronner, A. Kleinzeller (Eds.), Curr. Top. Membr. Transp., Academic Press, 1977: pp. 71–144. https://doi.org/10.1016/S0070-2161(08)60677-2.
[37] S. Mitaku, S. Aruga, A.L. Copley, Effect of calcium ion on the mechanical properties of lipid bilayer membrane, Biorheology. 19 (1-2) (1982) 185–196, https://doi.org/10.3233/BIR-1982-191-221.
[38] A. Filippov, G. Orädd, G. Lindblom, Effect of NaCl and CaCl2 on the lateral diffusion of zwitterionic and anionic lipids in bilayers, Chem. Phys. Lipids. 159 (2) (2009) 81–87, https://doi.org/10.1016/j.chemphyslip.2009.03.007.
[39] F. Bordi, C. Cametti, A. Gliozzi, Impedance measurements of self-assembled lipid bilayer membranes on the tip of an electrode, Bioelectrochemistry. 57 (1) (2002) 39–46, https://doi.org/10.1016/S1567-5394(02)00005-1.
[40] E. Zakharian, Recording of Ion Channel Activity in Planar Lipid Bilayer Experiments, Methods Mol. Biol. Clifton NJ. 998 (2013) 109–118, https://doi.org/10.1007/978-1-62703-351-0_8.
[41] S. Ohki, The variation of the direct current resistance of lipid bilayers, J. Colloid Interface Sci. 30 (3) (1969) 413–420, https://doi.org/10.1016/0021-9797(69)90410-X.
[42] A. Maček Lebar, D. Miklavčič, M. Kotulska, P. Kramar, Water Pores in Planar Lipid Bilayers at Fast and Slow Rise of Transmembrane Voltage, Membranes. 11 (2021) 263, https://doi.org/10.3390/membranes11040263.
[43] S.L. Kaufman, J.W. Skogen, F.D. Dorman, F. Zarrin, K.C. Lewis, Macromolecule analysis based on electrophoretic mobility in air: globular proteins, Anal. Chem. 68 (1996) 1895–1904, https://doi.org/10.1021/ac951128f.
[44] H. Epstein, E. Afergan, T. Moise, Y. Richter, Y. Rudich, G. Golomb, Number-concentration of nanoparticles in liposomal and polymeric multiparticulate preparations: empirical and calculation methods, Biomaterials. 27 (4) (2006) 651–659, https://doi.org/10.1016/j.biomaterials.2005.06.006.
[45] V.U. Weiss, C. Urey, A. Gondikas, M. Golesne, G. Friedbacher, F. von der Kammer, T. Hofmann, R. Andersson, G. Marko-Varga, M. Marchetti-Deschmann, G. Allmaier, Nano electrospray gas-phase electrophoretic mobility molecular analysis (nES GEMMA) of liposomes: applicability of the technique for nano vesicle batch control, The Analyst. 141 (21) (2016) 6042–6050, https://doi.org/10.1039/C6AN00687F.
[46] V.U. Weiss, K. Wieland, A. Schwaighofer, B. Lendl, G. Allmaier, Native Nano-electrospray Differential Mobility Analyzer (nES GEMMA) Enables Size Selection of Liposomal Nanocarriers Combined with Subsequent Direct Spectroscopic Analysis, Anal. Chem. 91 (6) (2019) 3860–3868, https://doi.org/10.1021/acs.analchem.8b04252.
[47] G. Allmaier, C. Laschober, W.W. Szymanski, Nano ES GEMMA and PDMA, new tools for the analysis of nanobioparticles-protein complexes, lipoparticles, and viruses, J. Am. Soc. Mass Spectrom. 19 (8) (2008) 1062–1068, https://doi.org/10.1016/j.jasms.2008.05.017.
[48] M.P. Caulfield, S. Li, G. Lee, P.J. Blanche, W.A. Salameh, W.H. Benner, R.E. Reitz, R.M. Krauss, Direct determination of lipoprotein particle sizes and concentrations by ion mobility analysis, Clin. Chem. 54 (2008) 1307–1316, https://doi.org/10.1373/clinchem.2007.100586.
[49] V.S. Chernyshev, R. Rachamadugu, Y.H. Tseng, D.M. Belnap, Y. Jia, K.J. Branch, A. E. Butterfield, L.F. Pease, P.S. Bernard, M. Skliar, Size and shape characterization of hydrated and desiccated exosomes, Anal. Bioanal. Chem. 407 (12) (2015) 3285–3301, https://doi.org/10.1007/s00216-015-8535-3.
[50] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1) (1965) 238–IN27.
[51] V.U. Weiss, J. Frank, K. Piplits, W.W. Szymanski, G. Allmaier, Bipolar Corona Discharge-Based Charge Equilibration for Nano Electrospray Gas-Phase Electrophoretic Mobility Molecular Analysis of Bio- and Polymer Nanoparticles, Anal. Chem. 92 (13) (2020) 8665–8669, https://doi.org/10.1021/acs.analchem.0c01904.
[52] A. Tycova, J. Prikryl, F. Foret, Reproducible preparation of nanospray tips for capillary electrophoresis coupled to mass spectrometry using 3D printed grinding device, ELECTROPHORESIS. 37 (2016) 924–930, https://doi.org/10.1002/elps.201500467.
[53] C.G. Sinn, M. Antonietti, R. Dimova, Binding of calcium to phosphatidylcholine–phosphatidylserine membranes, Colloids Surf. Physicochem. Eng. Asp. 282–283 (2006) 410–419, https://doi.org/10.1016/j.colsurfa.2005.10.014.
[54] A. Melcrová, S. Pokorna, S. Pullanchery, M. Kohagen, P. Jurkiewicz, M. Hof, P. Jungwirth, P.S. Cremer, L. Cwiklik, The complex nature of calcium cation interactions with phospholipid bilayers, Sci. Rep. 6 (2016) 1–12, https://doi.org/10.1038/srep38035.
[55] V.U. Weiss, A. Lehner, L. Kerul, R. Grombe, M. Kratzmeier, M. Marchetti-Deschmann, G. Allmaier, Characterization of cross-linked gelatin nanoparticles by electrophoretic techniques in the liquid and the gas phase, Electrophoresis 34 (24) (2013) 3267–3276, https://doi.org/10.1002/elps.201300307.