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Electroformation of giant unilamellar vesicles in saline solution

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ABSTRACT

Giant unilamellar vesicle (GUV) formation on indium tin oxide (ITO) electrodes in saline solution and from charged lipids has proven to be difficult in the past. Yet the best cell membrane models contain charged lipids and require physiological conditions. We present a way to overcome this problem by using plasma cleaned ITO electrodes. GUVs from zwitterionic lipids, lipid mixtures and even pure charged lipids could be electroformed under physiological conditions and even higher concentrations of NaCl. The hydrophilic ITO surface may facilitate the hydration of the solid lipid film and the formation of lipid bilayers that subsequently bend and form vesicles. The formation of GUVs in saline solution is influenced by different parameters. The influences of the amplitude and frequency of the used AC field, the NaCl concentration, and the temperature were investigated. Finite element analysis simulating the effect of the electric field on GUV formation in saline solution could well explain the experimental results. Frequencies in the kHz-range favored for GUVs formation in saline solution, as they suppress the formation of electric double layer, while higher frequencies could again impair the effect of electric field and impede GUV formation. The diameters of the GUVs increased gradually with NaCl concentration from 0 mM to 200 mM and subsequently decreased from 200 mM to 2 M. High yields of GUVs were also formed in PBS solution and cell culture medium, which indicates this method is a promising way to prepare GUVs on a large scale in physiological relevant conditions.

1. Introduction

Giant unilamellar vesicles (GUVs) are fascinating model cell membrane systems as their size and membrane curvature is similar to live cells [1–3], and individual GUVs can be easily observed with optical microscopes [4,5]. Extensive studies have been performed based on GUVs including membrane-DNA/RNA [6–9], membrane-peptide [10,11], membrane-protein [12,13], and membrane-nanoparticle [14,15] interactions, curvature and elasticity of membranes [16,17], and even their use as micro-scale bioreactors [18]. Methods on GUVs formation include gentle hydration [19,20], electroformation [3,21,22], solid hydration [23], freeze-and-thaw [24], and emulsion-based methods [25,26], *etc.* Among these methods, electroformation is most commonly used because it generates GUVs with high yield and superior quality [27–29]. However the abovementioned methods are typically limited to solutions of low ionic strength ($\leq$50 mM monovalent salt) unless specialized lipid formulations or instruments are used [30–32]. This is a major drawback because GUVs containing physiological strength salt solutions are highly demanded in cell mimic studies [5,33].

The process of GUV formation can be separated into three stages, i.e., the hydration of the solid lipid film, the swelling of the lipid film into liposomes due to forces normal to the bilayers, and the fusion of adjacent liposomes due to mechanical stresses [34]. Solutions with high ionic strength are believed to hinder the separation of lamellae in the first step and increase the required forces in the second step [30,35]. This causes enormous difficulties for GUVs preparation in high strength saline solutions. There are few successful attempts to solve this problem. The addition of PEGylated lipids promotes bilayer separation and GUVs formation at high ionic strengths (up to 2 M) because of steric repulsion by the large PEG moiety [36]. The incorporation of low molar ratio negatively charged lipids can also facilitate the first two steps by electrostatic repulsion [37]. However, special lipid formulations are needed in above methods. Estes [32] used a two-step method to obtain GUVs in solutions of high ionic strength by exchanging isoosmolar glycerol inside GUVs with high ionic strength solution. This procedure is very complicated and time consuming. Mayer [34] introduced a simple, rapid, and reproducible procedure to obtain GUVs in physiological salt solution by hydrating hybrid films of partially dried agarose and lipids. The final products were a mixture of unilamellar and multilamellar

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liposomes, and were also contaminated by agarose molecules. The direct formation of GUVs using electroformation method faced big obstacles in high saline conditions. Yet its advantages, preparing high yields and quality GUVs, over other methods encouraged further research. Montes [5] and Pott [33] overcame the difficulty for GUV formation in saline solution by using high frequencies with two platinum wire electrodes. However, this improved method also had the drawbacks of complicated electroformation parameter setup, low yield (due to the small electrode surface area), and especially, the inability to form GUVs from pure charged lipids and lipid mixtures containing charged lipids. Therefore, formation of GUVs with high purity, high yield and desired lipid compositions in saline solution is still challenging.

Here we demonstrate that high quality GUVs were generated in various high strength saline solutions from zwitterionic lipids as well as charged lipids. We use the electroformation method with a two face-to-face electrode layout of plasma cleaned ITO electrodes. Thereby we demonstrate that we could overcome the two major problems, the failure to form high yields and easily detached vesicles in saline solution, and formation of vesicles from charged lipids. No specialized lipids or instrumentation were utilized for the rapid and large scale formation of high quality GUVs. The method proposed in this paper is promising to prepare high quality GUVs on a large scale in physiological relevant conditions.

2. Materials and methods

2.1. Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine(DOPC), 1-hexadecano-yl-2-(9Z-octadecen-oyl)-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipal-mitoyl-*sn*-glycero-3-phosphocholine(DPPC), 1,2-dioleoyl-*sn*-glycero-3-phospholserine, sodium salt (DOPS), 1,2-dioleoyl-3-trimethylammonium-propane, chloride salt (DOTAP) were purchased from Avanti Polar Lipids (USA). Texas red-labelled 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (TR-DHPE) and fluorescence-labeled 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD PE) were obtained from Invitrogen (China). DMEM and RMPI 1640 cell culture mediums were obtained from Gibco (China). Sodium chloride, monopotassium phosphate, disodium hydrogen phosphate and chloroform were purchased from Sigma (China). Polymerase Chain Reaction (PCR) buffer was obtained from TransGen Biotech (Beijing, China). Glass slides coated with indium tin oxide (ITO, sheet resistance ≈8–12 Ω, thickness ≈ 160 nm) were purchased from Hangzhou Yuhong technology Co. Ltd (China). Ethanol (analytical grade, purity >99.5%) was purchased from FuYu Chemicals (China). Millipore Milli-Q water with a resistivity of 18.0 MΩ/cm was used for solution preparation in the electroformation experiments.

2.2. Preparation of GUVs

ITO-coated glass slides (1.5 × 3 cm) were cleaned by sonication in ethanol and water for 15 min each, dried by a N_2 stream, and plasma cleaned for 30 s to obtain a clean and hydrophilic surface. To evaluate the influence ITO electrode surface on GUVs formation, trimethoxyoctadecylsilane (ODS) and (3-aminopropyl)triethoxysilane (APTES) functionalized ITO electrodes were obtained by incubation in ODS-toluene (1:100, *v*/*v*) or APTES-toluene (1:100, *v*/*v*) solution for 4 h. GUV formation on bare ITO electrode, ODS and APTES functionalized electrodes before and after plasma cleaning were investigated. Lipid thin films were prepared on ITO electrodes using the flat-coating method as described in detail elsewhere [38,39]. Briefly, solutions of lipid at a concentration of 5.0 mg/mL composed of phospholipid (DOPC, POPC, DMPC, DPPC, DOPS or DOTAP) and TR-DHPE at a 99.5:0.5 mass ratio, or DOPC and NBD-PE at a 95:5 mass ratio were prepared in chloroform. A drop of lipid solution was deposited onto ITO electrode surface using a needle to spread carefully back and forth 5 times. For DOPC, POPC, DMPC, DPPC, and DOTAP GUVs, 2.5 μL of lipid solution was used for each side of the ITO electrode, while for DOPS GUVs, 5.0 μL of lipid solution was used. Finally, the lipid coated ITO electrode was dried in a vacuum desiccator for 2 h. The experimental setup for GUV formation have been mentioned elsewhere [38,40] and is schematically depicted in Fig. 1. Two slides of ITO electrodes both coated with lipid thin films were separated by a 3 mm thick spacer with a 10 mm diameter hole.

The electroformation cell was filled with sodium chloride solution, PBS solution, PCR buffer or cell culture medium. The assembled sample block was placed on a hot plate (YS-300S, China) for temperature control. A sinusoidal AC electric field was applied with a signal generator (Aim-TTi TGA12104, England) to generate GUVs.

2.3. Observation and analysis

The formed GUVs were observed under a fluorescence microscope (Nikon 80i, Japan). The size of GUVs for each preparation was calculated from 300 randomly selected GUVs from 6 fluorescence images. GUV population density was also obtained from these fluorescence images. Three batches of GUVs were prepared for each electroformation parameter. To prove unilamellarity of the formed GUVs, we applied an already-published method [41], based on the quantification of the intensity of the incorporated fluorescently labelled lipids in the membrane. The intensities of 100 free floating vesicles was measured and plotted as a function of the liposome size and their occurrence.

2.4. Simulation

The electric field distribution is simulated combining the AC/DC module and chemical species transport module of COMSOL Multiphysics 4.3. The vector equation of electric potential in NaCl solution can be written as the Poisson's equation.

$$\nabla \cdot \left[(\sigma + i\omega\varepsilon) \nabla \tilde{\phi} \right] = \rho \tag{1}$$

where σ is the electric conductivity, ε is the permittivity, ω is angular frequency, ϕ is electric potential, and ρ is the charge density depending on the ion concentrations. The ion fluxes N_i are described by Nernst-Planck equation.

$$N_i = -D_i \nabla c_i - u_i z_i F c_i \nabla \tilde{\phi} \tag{2}$$

with D_i, u_i, z_i, c_i are the diffusion constant, mobility, charge and concentration of ions, respectively. F (96,500 C/mol) is Faraday's constant. The diffusion coefficient of ions in bulk solution is 2.0×10^{-9} m^2/s [42]. The diffusion coefficient of ions in lipid membrane with a value of 1.0×10^{-15} m^2/s was determined through electrochemical impedance spectroscopy referring to the method proposed by Tanaka [43] (Supporting information, Fig.S1). The mobility u_i was obtained from the Nernst-Einstein relation.

$$u_i = \frac{D_i}{RT} \tag{3}$$

where R (8.314 J K^{-1} mol^{-1}) is the gas constant and T (318.15 K) is the temperature.