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# ELECTROFORMATION OF NEUTRAL AND NEGATIVELY CHARGED PHOSPHOLIPID GIANT VESICLES UNDER PHYSIOLOGICAL CONDITIONS

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**Abstract**

We propose a new simple and easy electroformation method for preparation of neutral and negatively charged giant unilamellar vesicles in different aqueous solutions that are of great importance in medicine, biology and electrochemistry. We manage to produce repetitively a subsequent amount of vesicles without any visible defects in pure phosphate buffer solution, pure physiological solution and in mixed sugar-buffer solution. The bending elastic modulus for one of the studied systems is preliminarily calculated on the base of 4 different vesicles, formed by the proposed electroformation procedure. For negatively charged liposomes cardiolipin is used in two different mass ratios. In this short communication we briefly describe our preparation procedure and the biological importance of our achievement.

**Key words:** electroformation, charged phospholipidis, giant vesicles

**1. Introduction.** The giant unilamellar vesicles are the simplest model of the biological cell. They are used for investigation of physical, chemical and electrical, etc. properties of bio-membranes. That is why it is really important to have clear, easy, quick and repetitive procedure for their formation.

Nowadays there are many known methods for preparation of vesicles of different sizes. Sonication is used for small unilamellar vesicles (SUVs) [1, 2], extrusion for large unilamellar vesicles (LUVs) [2]. The most important vesicles are giant unilamellar vesicles (GUVs), which can be observed with optical microscope and

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have similar size as living cells. For preparation of GUVs one of the first methods used was spontaneous swelling [3]. The problem with this method is the wide size and form distribution as well as its time consumption. Very popular method for formation of GUVs is the so-called electroformation method [4,5]. It speeds up the process of swelling of the lipid film by applying an electric field. This method narrows the size distribution and produces a big yield of giant quasi-spherical unilamellar vesicles in some cases with long micro and nano tubular protrusions [6]. It can be found in the literature that many research groups find problems with formation of GUVs in salt solutions at high ionic strength [7,8].

Recently, some research groups succeeded in formation of vesicles in some ionic strength solutions using complicated methods [9,11]. Our electroformation procedure is also applicable to use just zwitter ionic lipids; there is no need to preform the aqueous vesicle suspension; we do not add salts to change ionic strength (osmolarity) of the buffer solution and the pH of the buffer is around 7.4 which is relevant for physiological processes.

The aim of this work was to modify a regular electroformation method in a way to be used for forming giant unilamellar vesicles in human physiological relevant conditions. Our new established procedure is fast, repetitive and easy to apply.

**2. Theory.** The first theoretical models for the mechanical properties of lipid membranes proposed by Helfrich [12] and Evans [13] describe the elastic energy per unit area of lipid membrane, $F_c$ by the expression

$$F_c = \frac{1}{2} k_c \left(c_1 + c_2 - c_0\right) + \bar{k}_c c_1 c_2, \tag{1}$$

where $c_1$ and $c_2$ are the membrane principal curvatures, $c_0$ is the spontaneous curvature, and $k_c$ and $\bar{k}_c$ are bending and saddle bending elastic moduli of lipid bilayer, respectively. The spontaneous curvature of a symmetric membrane in a symmetric environment vanishes.

After the first detailed theoretical model of thermally induced shape fluctuations has been proposed by Milner and Safran [14], the experimental procedures based on the analysis of thermally induced shape fluctuations of quasi-spherical vesicles were developed for the precise measurements of the bending elastic modulus [15,16]. The fundamental expression used by the authors in [14] is

$$\langle |U_n^m(t)|^2 \rangle = \frac{k_B T}{k_c} \frac{1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]}, \tag{2}$$

where $\langle |U_n^m(t)|^2 \rangle$ is the mean square amplitude of the spherical harmonic $Y_n^m(\theta, \phi)$, $k_B$ is the Boltzmann's constant, $T$ is the absolute temperature, $n$ is the mode number and $\bar{\sigma} = \sigma R^2/k_c$ (or $\bar{\sigma} = \sigma R^2/k_c + 2c_0 R + c_0^2 R^2/2$, if $c_0 \neq 0$) is the dimensionless membrane tension.

In fact what is measured in an experiment of fluctuating quasi-spherical giant vesicle is the equatorial cross section radius. It is shown in [15] that its time

averaged angular autocorrelation function is a sum of Legendre polynomials with amplitudes $B_n$, related to the mean squared amplitudes of spherical harmonics

$$B_n = \frac{2n+1}{4\pi}\langle |U_n^m|^2 \rangle, \tag{3}$$

where the factor $2n+1$ is due to the $2n+1$ different $m$-modes for a given $n$ all of them having the same mean square amplitude and $4\pi$ comes from the different normalizations of Legendre polynomials and spherical harmonics.

**3. Materials and methods.** For vesicle preparation we used the lipids: 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1′,3′-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (cardiolipin) purchased from Avanti Polar. All the lipids were dissolved in chloroform in concentration 1 mg/ml.

A simple electroformation cell used for all experimenal procedures is shown and described in Fig. 1. The frame of the cell was made out of electrotechnical sheet getinaks (micarta). It is a laminated material obtained by hot pressing of paper impregnated with a thermosetting phenol-formaldehyde- or epoxy-based binder. Glasses, coated with transparent conductor, indium tin oxide (ITO; thickness of 100 ± 20 nm, resistivity of 100 Ω/□) acting as an electrode, were glued on both sides to the cell's frame. The electrodes were 3 to 4 mm apart (d=3 or d=4 mm). Over the electrodes an ac voltage is applied while the electroformation process is started.

Small drops of the lipid (lipid mixture) solution were put over the ITO glass electrode. We used three different lipid mixture solutions: pure SOPC lipid solution, POPC or SOPC:Cardiolipin lipid solution in 9:1 mass ratio and POPC or SOPC:Cardiolipin lipid solution in 4:1 mass ratio. The electroformation cell was put in vacuum for 15–20 min to evaporate the organic solvent (chloroform). After entire evaporation the cell was assambled. Through one of the siringe inlets we pour the solution in which the vesicles were formed.

We performed electroformation of GUVs in three different aqueous solutions which are of great importance for medicine and electrochemistry:

1. Pure phosphate buffered saline (PBS) – a buffer solution with osmolarity of 280 mosmol/l;

2. Pure physiological solution with osmolarity of 308 mosm/l (molarity of 154 mmol/l);

3. Mixed isoosmolar solution of sucrose and PBS in 70:30 vol% with osmolarity of 280 mosm/l.

The PBS buffer was prepared from tablets (Sigma-Aldrich, P4417). The tablet was dissolved in 200 ml deionized or double distilled water. The buffer includes: 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M

Table 1

Electroformation method procedure for forming neutral (SOPC) GUVs in pure PBS buffer, pure physiological solution and mixed solution 70:30 vol% of sucrose and PBS buffer

| ac voltage [V] | ac electric field [V/m] | frequency [Hz] | time duration [min] |
|---|---|---|---|
| 1 | 250–333 | 10 | 5 |
| 2 | 500–667 | 10 | 5 |
| 3 | 750–1000 | 10 | 135 |

sodium chloride, pH 7.4, at 25 °C. The physiological solution (normal saline solution) was the most commonly-used term solution of 0.91% weitght/volume of NaCl (9 g/l) with molarity of 154 mmol/l and pH 4.5–7 (Braun).

For security reasons, not to short circuit the output of the function generator (Hameg/HP) and to limit the current flow through the circuit and consequently through the electroformation cell, we added a 50 Ω resistor in series.

The preparation procedures (applied ac voltages and their time durations) for GUVs formations are summarized in Table 1 and in Table 2, respectively.

All the experiments were done at room temperature.

**4. Results and discussion.** We developed and applied an easy and fast electroformation procedure for forming a sufficient amount of neutral and negatively charged giant unilamellar vesicles without visible defects in pure PBS buffer solution, pure physiological solution and mixed 70:30 vol% sugar:PBS buffer solution.

For all the electroformation procedures the same electroformation cell as the previously used for formations in sugar solutions was used.

In Figure 2 are shown neutral (A) and negatively charged (POPC:Cardiolipin=4:1 mass%) (B) GUVs electroformed in pure PBS buffer (1) and pure physiological solution (2).

In scientific experimental research it is needed to mimic real natural environmental conditions as much as possible. For in vitro experiments it is necessary to have the same conditions as in vivo systems have. For modelling of real biological cell it is very important to establish human physiological conditions (pH of 7.4, ionic strength of 150 mM of NaCl, osmolarity of about 300 mosm/l) for model

Table 2

Electroformation method procedure for forming negatively charged GUVs composed of POPC or SOPC:cardiolipin mixture in pure PBS buffer, pure physiological solution and mixed solution 70:30 vol% of sucrose and PBS buffer

| ac voltage [V] | ac electric field [V/m] | frequency [Hz] | time duration [min] |
|---|---|---|---|
| 0.5 | 125–167 | 10 | 15 |
| 1.0 | 250–333 | 10 | 30 |
| 1.5 | 375–500 | 10 | 60 |

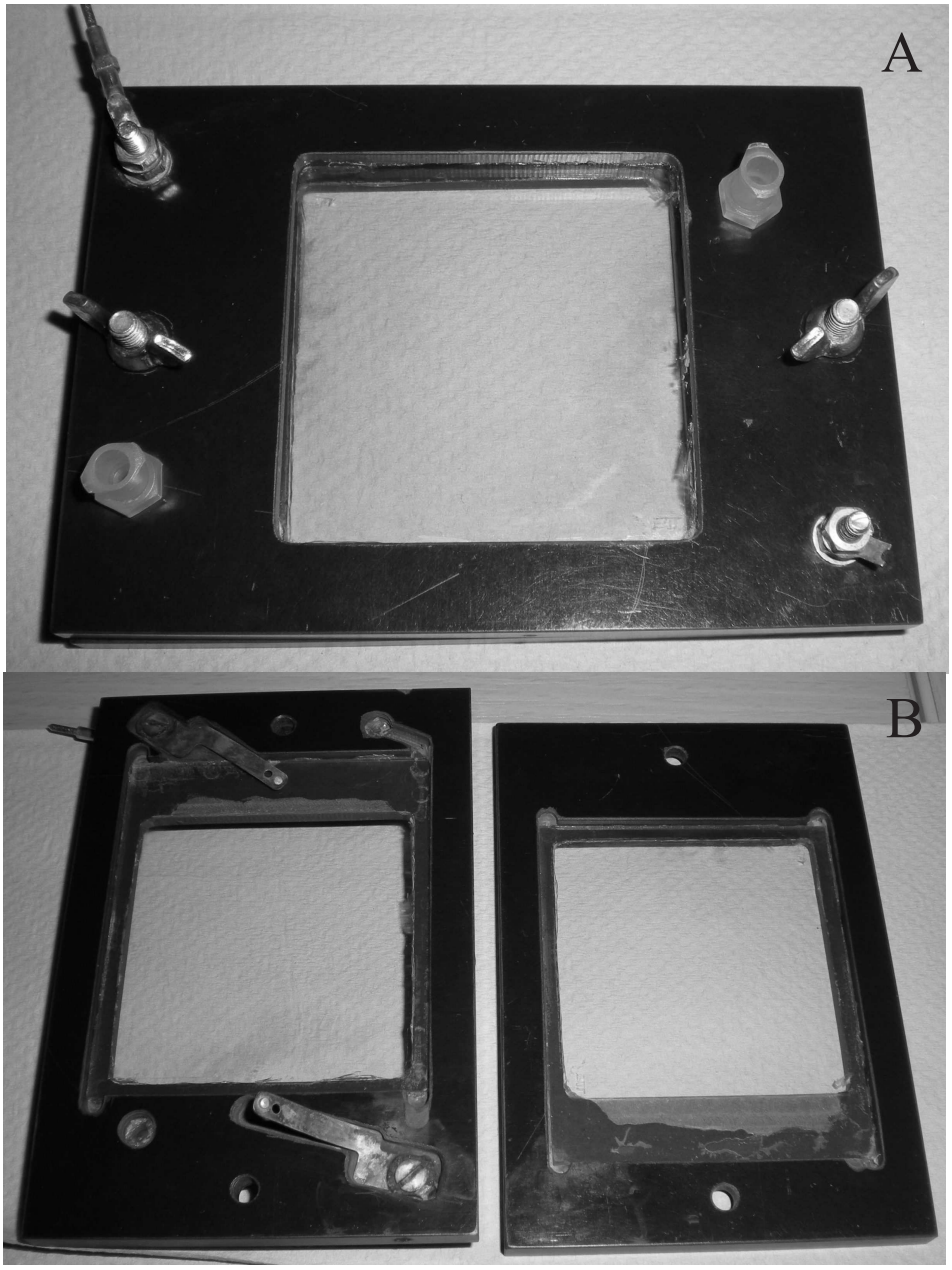


Fig. 1. Photograph of electroformation cell, part A shows assembled cell, part B shows disassembled cell

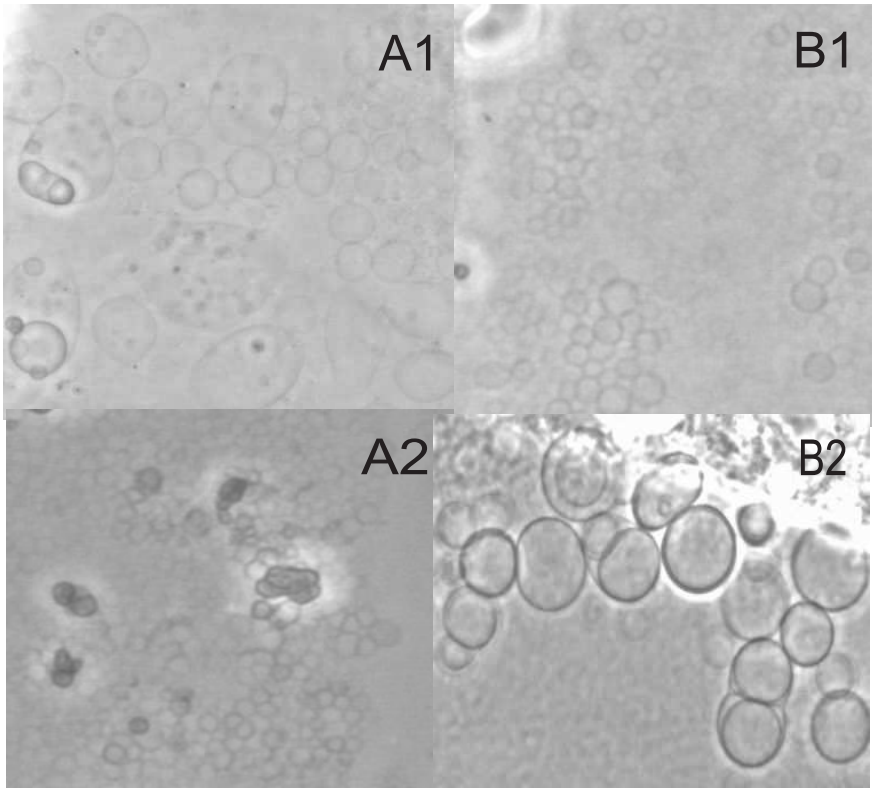


Fig. 2. Micrograph A1 shows neutral (SOPC) GUVs electroformed in pure PBS buffer, micrograph A2 shows neutral (SOPC) GUVs in physiological solution, micrograph B1 shows negatively charged (POPC:Cardiolipin=4:1 mass ratio) GUVs in PBS buffer and micrograph B2 shows negatively charged (POPC:Cardiolipin=4:1 mass ratio) GUVs in physiological solution

lipid membranes. It is known from medicine that most body proteins change their conformation, structure and consequently function when they are taken out of their natural environment. That is why it is very important to ensure buffer or physiological aqueous solutions for model lipid membranes when their interactions with human body proteins are studied.

For all theoretical and experimental work in electrochemistry science, it is necessary to ensure controlled pH and ionic strength of the studied experimental solutions. This could be achieved by using buffer or salt solutions.

The cardiolipin molecule has two negative charges in its headgroup, so the liposomes containing 10 mass% of cardiolipin have on average 20% of negative charge in their lipid membranes, which is relevant at physiological conditions [17, 18].

The procedure for negatively charged (mixture of POPC or SOPC : Cardiolipin) GUVs is similar to the neutral (SOPC) GUVs preparation procedure, but the final voltage should be smaller to prevent lipid degradation.

The electroformation cell filled with pure PBS buffer or pure physiological solution has a resistivity in the range of few hundred Ohms which is much smaller compared to the resistivity of the same cell with pure sucrose solution, which is in the order of mega Ohms.

Maximal applied voltage for neutral lipids was 3V (the electric field should be less than 1000 V/m) and maximal applied voltage for lipid mixture with negatively charged lipids was 1.5 V (the electric field should be less than 500 V/m) to avoid lipid mixture degradation for both cases.

The analysis of thermally induced shape fluctuation method was used to estimate the bending elastic modulus $k_c$ of the membrane of the giant unilamellar vesicles, formed by the described electroformation method for one of the studied systems, i.e. pure SOPC vesicles in mixed isoosmolar solution of sucrose and PBS in 70:30 vol% with osmolarity of 280 mosm/l. The value for the bending elasticity modulus as described in section 2, was calculated as a weighed average value of 4 different vesicles is $k_c = 0.75 \pm 0.07 \cdot 10^{-19}$ J. This value is in very good agreement with the previously obtained values for the bending elastic modulus of SOPC membrane in presence of sucrose [19–21]. In all experimental data for measuring $k_c$ provided in this work the stroboscopic illumination was used [22]. Stroboscopic illumination presents an instant picture of the observed object to the observer and removes the artifacts due to the video camera integration time.

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