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VESICLES WITH TUBULAR PROTRUSIONS IN SYMMETRICAL AND NON SYMMETRICAL CONDITIONS

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ABSTRACT
Experimental study of the behaviour of the shape of vesicles with long tubular protrusions (tethers), connected to them in non-symmetrical isoosmolar and non isoosmolar environment was performed. It is shown that in case of adding a solution with different osmolarity to the outer side of the vesicle with tether, it changes its shape to a vesicle with chain structure of small spherical formations called beads. This shape transformation is reversible in time. After around 40 minutes equilibrium is achieved and the object of interest acquires its initial form. The bending elastic modulus of the lipid membrane, containing tethers is measured using the method of thermally induced shape fluctuations.

Keywords: tubular protrusion, non-symmetrical conditions, lipid vesicle, elasticity

Introduction
The communication between cells and cell compartments determines the proper functioning of multicellular organisms. The intercellular connections provide a route for material and signal transport. Recently a new communication mechanism between cells was discovered (10). Long tubular cell extensions, called membrane tubes provide communication at long distances up to hundreds of micrometers. The transfer of material between organelles is mediated by carrier vesicle that bud from one membrane, travel through tubes and fuse with another membrane (4). Recently the interest towards formation, stability and diversity of membrane tubular protrusions grows (5, 6, 9, 11). In this work we focus on factors influencing the morphology of tubular protrusions (tethers) connected to lipid vesicles, cognition of which can provide us deeper understanding of mechanisms for intercellular material transport in living organisms. The elastic properties of vesicles with tubular protrusions are investigated via thermally induced shape fluctuation method and compared to that of vesicles of the same composition without tethers.

Theory
The first theoretical models for the mechanical properties of lipid membranes proposed by Helfrich (3) and Evans (1) describe the elastic energy per unit area of lipid membrane, F_c by the expression:

$$F_c = \frac{1}{2} k_c \left(c_1 + c_2 - c_0\right)^2 + \bar{k}_c c_1 c_2 \quad [1]$$

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, c_o=0.

After the first detailed theoretical model of thermally induced shape fluctuations has been proposed by Milner and Safran (7), 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 (2, 8). The fundamental expression used by the authors is (7):

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

where $\left\langle \left| U_n^m(t) \right|^2 \right\rangle$ is the mean squared amplitude of the spherical harmonics $Y_n^m(\theta,\varphi)$, 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 the experiment of fluctuating quasi-spherical giant vesicle is the equatorial cross section radius. It is shown in (2) 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 like:

$$B_n = \frac{2n+1}{4\pi} \left\langle \left| U_n^m(t) \right|^2 \right\rangle \quad [3]$$

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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 squared amplitude and 4π comes from the different normalizations of Legendre polynomials and spherical harmonics.

Materials and Methods

GPVs were prepared at room temperature (23^{o}C) by the modified electroformation method from POPC (1-Palmitoy l-2-Oleoyl -sn- Glycero-3-Phosphocholine and SOPC (1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine) (purchased from Avanti Polar Lipids, Inc.). Lipids were dissolved in 2:1 chloroform/methanol mixture, and then small drops of lipid mixture were applied to the electrodes. The solvent was allowed to evaporate in a low vacuum for 2 hours. The coated electrodes were placed in the electroformation chamber, which was then filled with desired aqueous solution (0.2 M or 0.28 M sucrose solution). In our experiments we used sucrose (SigmaUltra® purity, 99.5% (GC), Germany) and PBS tablets (Sigma-Aldrich Chemie GmbH, 53 Buchs, Switzerland) dissolved in double distilled water. All chemicals were used without any further purification. A low frequency (10 Hz) sinusoidal voltage was applied (a step like increase from 0.1V PP (peak to peak) to 1.5V PP) to the electrodes for about 5 hours, which led to the formation of giant vesicles, appropriate for our experiment. The content of the chamber was then carefully taken out of the electroformation cell and used immediately after the formation or stored at 4°C. Vesicles were observed by Zeiss Axiovert 200 inverted microscope in phase contrast mode (objective magnification 100 X) for about 120 minutes and recorded by a Sony XC-77CE video camera.

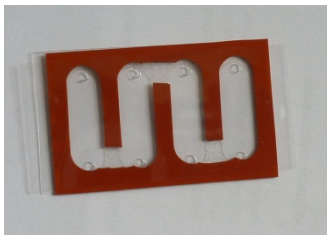


Fig. 1. Experimental cell with semi separated compartments (modified perfusion chamber (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA)

Results and Discussion

In order to study the morphology of vesicles with long tubular protrusion in symmetrical and non-symmetrical conditions we designed an experimental cell with semi separated compartments (see **Fig. 1**). In this way the speed of the flow of the added substance is reduced, which preserves the system from abrupt change and the observation of the object of interest is facilitated.

The experimental cell was filled initially with 180 µl of GUV's suspension in sugar concentration of 0.2 M or 0.28 M. After that we carefully pipetted through a small hole in the cell a volume of 20 µl sucrose (0.2 M or 0.28 M) or PBS (0.28 M) water solution apart from the observation point thus gradually changing the osmolarity of the outer solution of the vesicles not to damage the vesicles. In the case of adding a solution with different osmolarity to the outer for the vesicles environment (0.28 M sucrose or PBS solution to the GUV's formed in 0.2 M sucrose) the vesicle with tubular protrusion (**Fig. 2A**) changed its shape to a vesicle with a long chain of spherical beads (**Fig. 2B**). It was experimentally shown that in most of the cases this change was reversible with time. In about 40 minutes after the transformation the vesicle with tether obtains approximately the initial form (**Fig. 2C**) (vesicle with tether trough vesicle with chainlike structure and back to vesicle with tether).

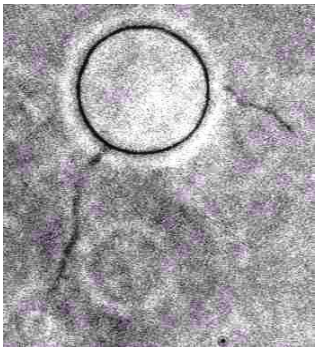
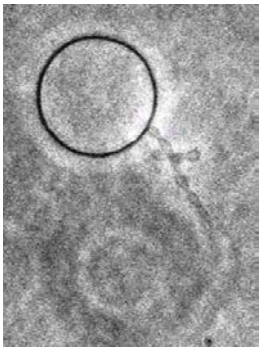
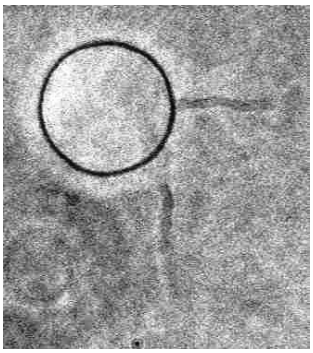
A.  **B.**  **C.** 

Fig. 2. Phase contrast images of a vesicle with tubular protrusions in time: **A.** 0min; **B.** 10-15min; **C.** 40 min

The same effect of shape transformation was observed by changing the temperature of the suspension without changing the composition of the aqueous solution. For this study after the formation the GUV's suspension was stored at 4°C overnight. The experimental cell was filled with cooled vesicles in 0.2 M sucrose solution and placed at room temperature for observation, thus slowly increasing the temperature of the probe from 4°C up to 25°C. In 15 minutes the morphological

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change, described above occurred and in 15 more minutes the object of interest gained its initial form.

In the case of adding to GUV's suspension a solution of PBS with the same osmolarity as the sucrose solution (both 0.28 M) no morphological changes were observed for a period of 180 minutes. The vesicles with tubular protrusion were stable for the observation time.

The experimental results, obtained in this work can be explained by the change of area to volume ratio of the studied objects, induced by local inhomogeneities of osmolarity of the external for the membrane solution or change of temperature. The membrane permeability towards water (12, 13) allows the significant change of the volume of the studied object at fixed number of molecules, comprising the membrane.

After a sufficient period of time an equilibrium conditions in the whole cell are achieved and the object of interest almost obtains its initial form.

Using thermally induced shape fluctuation method for a quasi spherical vesicle the bending elastic modulus of the membrane of vesicles with tubular protrusions connected to it was evaluated. An important criterion for selecting the vesicles is their stationarity during the experiment. In order to examine the time stationarity of the vesicles, the time dependence of the squares of the amplitudes of the second mode in the autocorrelation function decomposition in Legendre polynomials was plotted for every vesicle. In **Fig. 3**, both possible cases for this dependence are shown. The experimental data were fitted with a linear function of the type $y=a+bn$ ($n=0 \div Nmax$) and the values of constants a and b were obtained.

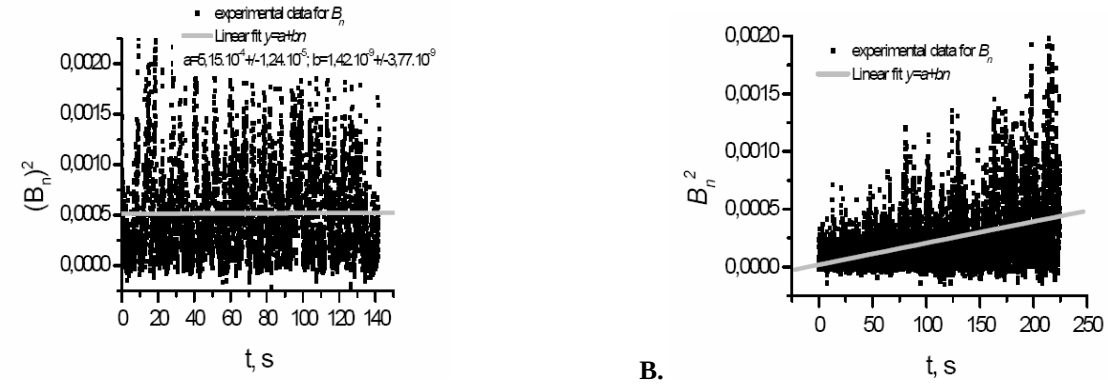

Fig. 3. Time dependence of the squares of the amplitudes of the second mode in the autocorrelation function decomposition in Legendre polynomials, with gray being a linear fit: **A.** stationary liposome; **B.** not stationary liposome

We introduce the following criterion for the time stationarity of the vesicle: $bN_{\max}/\sigma \le 3.5$,

where *Nmax* is the maximal time value at which *Bn* (the amplitude of the second mode in the autocorrelation function decomposition in Legendre polynomials) is acquired and σ is the mean error of the amplitudes of the second mode in the autocorrelation function decomposition.

TABLE 1
Bending elastic modulus of the lipid membrane of vesicles with and without tethers

SOPC lipid vesicles in double distilled water	**Bending elastic modulus** $\overline{k_c}$
Vesicles with tethers	$(2,0 \pm 0,1) \times 10^{-19}\, J$
Vesicle without tethers	$(2,1 \pm 0,1) \times 10^{-19}\, J$

The values for the bending elastic modulus k_c for SOPC lipid membrane in double distilled water were calculated as weighted average over about 10 vesicles. The obtained values for k_c were compared to the value obtained using the same experimental technique for the same system for vesicles without tubular protrusions. The obtained results for both vesicles with and without tubular protrusions, connected to the membrane are shown in **Table 1**. The obtained results show that in the frames of the experimental error there is no difference in the values for the bending elastic modulus for vesicles' membrane, with and without tubular protrusions, connected to it.

Acknowledgements

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