Morphological study of lipid vesicles in presence of amphotericin B via modification of the microfluidic CellASIC platform and LED illumination microscopy

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Abstract. Giant lipid vesicles (liposomes) are the simplest model of the biological cell and can be easily formed from natural or synthetic lipid species with controlled composition and properties. This is the reason why they are the preferred objects for various scientific investigations. Amphotericin B (AmB) is a membrane active drug, used for treatment of systemic fungal infections. In this work we studied the morphological behavior of giant SOPC vesicles in asymmetrical presence of amphotericin B antibiotic in the vicinity of the lipid membrane. The visualization of the vesicles was carried out via inverted phase contrast microscopy. The illumination source was modified in a way that tungsten light bulb was replaced by 10 W white LED chip. All the experiments were performed using CellASIC ONIX Microfluidic Platform. The setup has been modified thus opening new opportunities for a variety of experimental realizations. The performed morphological studies showed strong and irreversible effect on the vesicle shape at the presence of amphotericin B in concentration $10^{-5} \text{g/l}$ in the outer for the liposome’s membrane solution. At concentration $10^{-3} \text{g/l}$ AmB the effect was less visible and in 15-20 minutes the vesicles regained its initial spherical shape.

1. Introduction

Investigations of effects of drugs on cells is mandatory part of creation and verification of medicines. Lipid vesicles are useful model systems, which membranes resemble real cell’s membrane. To study basic interaction mechanisms that are responsible for the structure and function of biological membranes it is necessary to have simple composition of an artificial bilayer to facilitate a detailed examination of the membrane.

Amphotericin B (AmB) is an antifungal polyene antibiotic. It was originally extracted from Streptomyces nodosus, a filamentous bacterium, in 1955 at the Squibb Institute for Medical Research from cultures of an undescribed streptomycete isolated from the soil collected in the Oriente River region of Venezuela. AmB is designated chemically as $(1R, 3S, 5R, 6R, 9R, 11R, 15S, 16R, 17R, 18S, 19E, 21E, 23E, 25E, 27E, 29E, 31E, 33R, 35S, 36R, 37S) - 33 - \text{[(3-amino-3, 6-dideoxy-\beta-D-mannopyranosyl)oxy]} - 1, 3, 5, 6, 9, 11, 17, 37-octahydroxy-15, 16, 18-trimethyl-13-oxo-14, 39-dioxabicyclo [33.3.1] nonatetraconta-19,21,23,25,27,29,31-heptaene-36-carboxylic acid. The structural formula of AmB is shown on figure 1. The cell membrane is the site of action of this antibiotic. AmB is a representative of the group of chemotherapeutics which mechanism of action consists in change of membrane cell permeability. Destroying natural selective membrane
permeability is an effect of incorporation of amphiphilic molecules of membrane-active antibiotic into the lipid phase and their interaction with lipid molecules [1]. Rod-shaped structure of AmB molecule, with the polar head mycosamine, the hydroxyl groups on the one side of the macrolide ring and the polyene fragment on the opposite side, makes it possible to interact both with the polar part of the lipid membrane and acyl chains. The consequence of such a structure of AmB is also a formation of molecular aggregates [2, 3]. Not only formation of porous molecular structures by AmB, but also modification of the physical properties of the lipid bilayers modulates membrane permeability to ions [4, 5]. Such mechanisms can be particularly effective at low concentrations of AmB, at which formation of aggregated molecular structures of the drug within the lipid phase cannot be expected [6].

2. Materials

Amphotericin B, sucrose (purity ≥ 99.5 GC), d- (+) glucose (purity ≥ 99.5 GC) were obtained from Sigma-Aldrich. SOPC (l-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (C18:0/C18:1)) was purchased from Avanti Polar Lipids Inc., USA.

All the chemicals were used without any further purification. The vesicles for the performed morphological studies were prepared via modified electroformation method [7]. The electroformation cell, used for the formation of vesicles contained two glasses, coated with transparent conductor, indium tin oxide (ITO; thickness of 100 ± 20 nm, resistively of 100 Ω/□ acting as electrodes and a PDMS (polydimethylsiloxane) spacer.

The SOPC lipid was dissolved in chloroform 1 mg/ml. A number of small droplets of the lipid solution were laid on the glasses of the electroformation cell and put under vacuum. After the entire evaporation of the solvent the experimental cell was assembled and filled with 180 mM sucrose solution in double distilled water. A low frequency (10 Hz) sinusoidal alternative voltage was applied (1.5 Vpp) to the conductive glasses overnight. This procedure leaded to the formation of vesicles, appropriate for our experiment. After the formation the vesicles were taken out of the formation cell to a flask and 180 mM glucose solution in double distilled water was added to the flask. The flask was then carefully shaken and left for 1-2 hours so that the vesicles precipitate to the bottom of the flask. For the experiment the sample of the vesicles are taken from the bottom of the flask. We have chosen giant (diameter of the order of 20-40 μm) spherical vesicles for the study of the morphology due to the addition of antibiotic Amphotericin B. AmB was dissolved in 180 mM water solution of glucose in concentration $10^{-5}$ g/l and $10^{-3}$ g/l.
3. Methods

3.1. LED illumination

In this work we present a modification of Nikon TMS inverted microscope’s illumination. The recent advances and mass production of LEDs has brought prices of LED chips down to affordable level. LED power has increased drastically, thus their light intensity. LEDs are of compact size, consume less electrical energy, dissipate less heat and have very long lifetime compared to incandescent bulbs. In visual spectrum, LEDs do not emit in UV and IR spectrum, which reduces health risks, omits the need of filters and improves the light throughput of the system. LEDs output intensity can be easily and precisely controlled. When lowering the LED’s drive current (dimming), there are no noticeable changes in emission spectrum and LED maintains practically the same colour temperature over a range of output intensity [8, 9, 10, 11]. All these advantages of the LED illumination motivate the implementation of LED in this experimental study.

In our case an incandescent light source as seen on figure 2 has been replaced with a white light emitting diode (LED) from SYNCHRO as seen on figure 3. The LED has a 10 W of electrical power with 850 lm luminous flux and has a colour temperature of 4000 K. To narrow down the emitted beam of light we used a collimating lens, which was put in front of the LED chip. Therefore we achieved tighter spot and brighter illumination (increase in light intensity).

The adaptation made to the inverted microscope is applied to phase contrast (Ph) microscopy. Lipid vesicles in liquid medium can be visualised and studied via Ph microscopy.

![Figure 2. Incandescent light source of inverted microscope.](image1)

![Figure 3. White LED light source with its power supply atop.](image2)

3.2. CellASIC microfluidic setup - modification

Phase contrast microscopy technique has been bundled with Onix CellASIC Millipore microfluidic system. Microfluidic (MF) setup as shown on figure 4 has been obtained for
conducting experiments with cells or cell-like objects. In our study we only focus on cells’ model membranes, the vesicles.

The CellASIC ONIX Microfluidic System uses microfluidic technology to enable continuous live imaging with or without media flow. The proprietary design allows objects of interest (cells, vesicles) to be exposed to different solutions and conditions via pressurised flow channels controlled by user-specified time intervals and flow rates.

The microfluidic plate can be used with typical inverted microscopes. The CellASIC ONIX Microfluidic System connects to the microfluidic plate via a pneumatic manifold (F84-DL3-015 regular manifold type) that uses pressurised air to pump objects of interest and liquids from the plate wells into the microfluidic cell culture chambers. A vacuum seal created between the manifold and the microfluidic plate ensures that each well is independent and that flow rates and fluid switching are accurate. A modification of the standard microfluidic setup is accomplished. A syringe is screwed to the vacuum outlet to ensure the proper working of the setup. Controls of the pressure appliance are managed through a USB connection to a computer on which a CellASIC software program is run [12]. MF setup uses special well plate (in our case M04S-03 type microfluidic plate was used as shown on figure 6) which has microchannels stretching from inlet wells to culture chamber and later on connecting it to outlet wells. It is possible to load vesicles into observation chamber via inlets 1 or 6. For experiments we can supply up to four different solutions (inlets from 2 to 5) e.g. proteins, antibiotics, etc., to the culture chamber and observe the response of vesicles’ membranes. The desired pressure is applied to microchannels

Figure 4. Picture of an original (non-modified) microfluidic setup.

Figure 5. Modification of the microfluidics setup.

Figure 6. Picture of the well-plate (M04S-03) commonly used in our experiments.

Figure 7. Well-plate M04S-03 with modifications. Rubber/silicone tubing is used to apply pressure to the well-plate inlets. A syringe ensures vacuum for a proper working of the pressure appliance.
via setup’s eight nozzles. It is possible to decouple nozzles 1 and 2 from the rest of them (nozzles from 3 to 8). Thus a possibility to apply two different pressures at the same time is achieved. Originally, for applying a desired pressure a special manifold is used. It is vacuum sealed to the well-plate every time the experiment starts.

For the application of pressure to the well-plate we used rubber and/or silicone tubings and vacuum was ensured by syringe screwed onto a vacuum nozzle (black colour) as shown on figure 4.

With such modification we achieved three main advantages of the modified microfluidic setup: there is no need to seal a manifold onto the well-plate, which is not so trivial task to accomplish; we can use only one row in well-plate when applying desired pressure to the well-inlets; there is no need to use the whole column. Also this realisation opens up many possibilities for custom microfluidic circuit design with two different pressure inlet options.

4. Results and Discussion

Using a modified CellASIC ONIX Microfluidic Platform morphological study of SOPC vesicles at asymmetrical presence of amphotericin B antibiotic was performed. The setup has been modified thus opening new opportunities for a variety of experimental realizations. A possibility of custom micro-chip design or Millicell μ-Migration Assay usage is achieved. The modified realization of the setup enables the application of two different pressure levels.

![Figure 8. Morphology of lipid vesicles in addition of a) amphotericin B in concentration $10^{-5}$ g/l in the outer vesicle environment; b) amphotericin B in concentration $10^{-3}$ g/l in the outer vesicle environment; c) control with addition of glucose solution.](image)

The visualization of the vesicles was carried out via inverted phase contrast microscopy. The illumination source was modified in a way that tungsten light bulb was replaced by 10 W white LED chip.

For achieving a high contrast images the vesicles for the experiment were formed in 180 mM sucrose solution of double distilled water and afterwards dissolved in glucose solution of the same osmolarity. At the beginning of the experiment all the compartments of the well-plate were thoroughly washed with 180 mM glucose solution. The giant lipid vesicles were added to the observation chamber. The amphotericin B dissolved in 180 mM glucose solution at different concentration ($10^{-5}$ g/l and $10^{-3}$ g/l) was injected to the vesicle sample at pressure level of 0.25 psi for 15 seconds. The change of the shape of the lipid vesicle started right after the addition of the antibiotic. On figure 8 the course of vesicle shape changes, induced by the asymmetrical presence of the molecules of the antibiotic is shown in time. As it is seen on the figure (case a)) a strong and irreversible effect on the vesicle shape was observed at the presence of amphotericin in B in concentration $10^{-5}$ g/l in the outer for the liposomes membrane solution. At concentration $10^{-3}$ g/l AmB (case b)) the effect was less visible and in 15-20 minutes the vesicles regained its initial spherical shape. A control experiment was performed where we added glucose solution at
the same pressure and time (case c) on figure 8) and no effect on the vesicles shape was observed in time. The possible explanation of the visualized effect on the morphology of lipid vesicle is the incorporation of antibiotic molecules in the structure of the outer layer of the liposome thus changing the local membrane curvature. At higher concentration of AmB the antibiotic molecules tend to agglomerate into bigger composites and do not manage to build into the lipid membrane structure.

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References
[1] Wolf B and Hartel S 1995 Biochimica et Biophysica Acta (BBA) - Biomembranes 1238 156
[2] Fujii G et al 1997 Biochemistry 36 4959
[3] Gruszecki W I et al 2002 FEBS Lett 524 92
[4] Baginski M et al 2006 Chem Rec 6 320
[5] Gabrielska J et al 2006 FEBS Lett 580 2677
[6] Gagos M et al 2010 Biochim Biophys Acta 1798 2124
[7] Angelova M I et al 1992 Prog. Coll. Pol. Sci. 89 127
[8] Silk E 2002 Microscope 50 101
[9] Cole R W and Turner J N 2008 Microsc. Microanal. 14 1
[10] Aswani K et al 2012 Microscopy Today 20 22
[11] Wessels J T et al 2012 Cytometry Part A 81A 188197
[12] User Guide CellASIC ONIX Microfluidic Platform