Towards *in vitro* centrosome positioning in vesicles under physiologically relevant conditions

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December 10, 2008
Abstract

The position of the centrosome *in vivo* plays a critical role in e.g. cell signaling and cell division. Here we develop an experiment with giant unilamellar vesicles (GUV) to investigate the influence of membrane stiffness on centrosome positioning. *In vitro*, GUVs are formed under physiological conditions by electroformation. We show that this method allows us to introduce micrometer sized beads and small proteins into the GUVs. The beads are intended to function as microtubule nucleation centers, mimicking the *in vivo* centrosome.
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Chapter 1

Introduction

In a eukaryotic cell the cytoskeleton gives stability and shape to the cell and facilitates movement. This cytoskeleton consists of three different types of protein filaments, namely microtubules (MTs), intermediate filaments and actin filaments. In this project we are specifically interested in MTs and their interaction with the cell membrane.

Microtubules are protein polymers than consist of tubulin subunits, arranged in a cylindrical-helical shape with a diameter of about 25nm, where each ring consists of twelve tubulin subunits. Each tubulin subunit is a dimer of an α-tubulin and a β-tubulin, both have a molecular mass of around 55kDa.

One of the very interesting properties of MTs is their dynamic instability. Dynamic instability is unpredictable behavior, where sometimes the MT experiences a catastrophe and begins to shrink. MTs are polarized in structure, with so called + and a − ends. The + end has a higher growth rate [1]. It is known that MTs can exert a force against a barrier [2] and that they can deform vesicles [3].

In vivo MTs radiate outwards towards the cell membrane from a MT organizing center, the centrosome, which is located in the center of the cell. Because MTs are constantly growing and shrinking, they push against the cell membrane and repostition the centrosome. The position of the centrosome determines the centered position of the nucleus inside the cell. MTs and the centrosome also play an important role in cell division. During cell division the centrosome duplicates and MT pushing forces contribute to the two daughter centrosomes being pushed away from each other eventually forming the new cores of the two daughter cells.

In order for MTs to be able to exert pushing forces, there must be a barrier for them to push against. In vivo this barrier is a combination of the cell cortex (a matrix of actin filaments) and the cell membrane. The cell membrane of any cell exists of a lipid bilayer of around 10nm thickness, with the hydrophobic ends of the lipids facing each other and the hydrophilic ends facing either the interior or exterior of the cell. Cells can range from 1µm in diameter to hundreds of mm in length, such as a human neuronal cells.
Chapter 2

An in vitro system to mimic the cytoskeleton.

In our attempt to fully understand the interaction between Mts, the centrosome and the cell membrane it is convenient to make a model system, which should as simple as possible. In this research we will focus on the interactions between MTs and the cell membrane. On the left side of figure 2.1 an in vivo image of the cytoskeleton is shown. The nucleus is shown in blue, next to it the centrosome resides (which is not explicitly shown), in green the MTs are shown, radiating outwards from the centrosome toward the cell membrane. The cell membrane is covered with actin filaments, which are shown in red. On the right side of the figure we see a schematic view of the experiment. The black lines represent lipid layers, that form vesicles. The blue dots represent beads that are (partially) encapsulated by the vesicles. Once the vesicles are formed we start the nucleation of MTs, shown in green.

Figure 2.1: On the left an in vivo image of a cell the nucleus is shown in blue, the actin filaments in red and the MTs in green. The centrosome itself which is positioned next to the centrosome is not marked. Image taken from [4]. On the right the idea of the experiment is shown schematically.
Chapter 3

Results & Discussion

3.1 MT in GUVs

3.1.1 GUV formation under physiological relevant conditions

Various methods to produce vesicles have been developed. Several of them are summarized below with their respective advantages and disadvantages.

- **Spontaneous swelling** is the most basic method for vesicle growth. Lipids dissolved in a chloroform solution are dropped on surface and dried. Then a buffer is added and vesicles will automatically swell form the surface. The spontaneous swelling method is rather slow and results in unilamellar and multilamellar vesicles with great variance in size. Typical sizes are around 10 µm.

- **Electroformation** (EF) is a widely used method to create giant unilamellar vesicles (GUV). It is comparable to spontaneous swelling, but instead of using a typical glass surface a conducting surface is used. By sending a 10 Hz sine-wave signal trough the sample the swelling process is accelerated resulting in GUVs with larger sizes (up to 100 µm in diameter) and a smaller size distribution. The process is also much faster. Normally a sucrose buffer is used and until recently it was widely believed that EF does not work in the presence of salt [5]. However it was shown [6] that GUV formation is possible with an oscillation frequency of 500 Hz. EF in a saline buffer does however yield smaller (around 10 µm) and less regular GUVs than EF in a sucrose buffer. For a comparison with spontaneous swelling see [7].

- **Freeze-thawing** is a much more violent method than EF. The idea is to quickly freeze a lipid solution with liquid nitrogen and then thaw it again
to a temperature above the lipid transition temperature\(^1\). This is repeated several times and results in small unilamellar vesicles which have diameter smaller than 200\(\text{nm}\) and therefore are not useful in our experiments.

- **Other methods** have been developed to form vesicles. Most of them not suited for this research because the vesicles are too small, are not unilamellar or cannot be made in physiologically relevant conditions. One very interesting method however uses a microjet and a bilayer sheet [8]. This is a very delicate method to create unilamellar vesicles of very well controlled size and in a buffer of choice. However it required equipment which was not available.

For this research we found that EF is most useful since it creates large vesicles, works under physiological relevant conditions and does not appear to damage proteins. We used the setup as shown in figure 3.1. ITO coated glass is used as conducting material (see appendix A.6). A PDMS (see appendix A.5) spacer with holes in it was placed between the two glass slides. After the spacer was placed, lipids dissolved in a chloroform/methanol mix were dried onto the bottom glass slide. The chamber was filled with a saline buffer afterward and closed with the second glass slide.

![Figure 3.1: Schematic view and photograph of the EF chamber used in the experiments. Two ITO-coated glasses are separated by a PDMS spacer with holes in it. The two wires attached to the glass are connected to a function generator to create an electric field over the chamber.](image)

In our experiments we used DOPC\(^2\) (and a small percentage of TRITC-DOPC to allow for fluorescence imaging) as lipid, where we added cholesterol to increase the stiffness of the GUVs. DOPC is especially fit for our experiments, because it is an uncharged lipid and it’s transition temperature lies below 0\(\circ\)C. Which allows us to apply EF at a temperature where MTs will not nucleate. As buffer we tried both MRB40 and MRB80. MRB80 is normally used to grow MTs but has a double salt concentration compared to MRB40, so we expected MRB40 to yield better results. Since the lipids are dropped on the surface manually, the exact thickness of the lipid layer and therefore the results

\(^{1}\)Above a certain temperature a lipid bilayer can be described as a 2-dimensional fluid. This temperature depends on the composition of the lipids.

\(^{2}1,2\)-Dioleoyl-sn-Glycero-3-Phosphocholine
vary every time, however MRB40 seems to yield better results in general than MRB80 as can be seen in figure 3.2.

Figure 3.2: GUVs in MRB40 (left) and MRB80 (right). The scale bar in the left image has a size of 10µm.

3.1.2 MT growth conditions

Before we started making GUVs with MTs it was important to check whether or not the tubulin proteins could survive the process of EF. MTs need proper conditions to grow, where the most important factors are temperature and tubulin concentration. There are three phase states [9] as can be seen in figure 3.3. One shows no growth, the second only shows growth from nucleation sites and the third and last shows spontaneous nucleation. In our experiments we want to be in the third phase, since we don’t have nucleation sites in all experiments and we want conditions to be equal.

To test this we added 30µM tubulin with a small fraction of rhodamine labeled tubulin and GTP to the EF chamber. We followed the EF protocol, took the tubulin out, put it in an eppie and heated it in a water bath to start nucleation. Indeed we saw MTs after taxol stabilization as we hoped.

Instead of using GTP, which is present in vivo, it is also possible to use GMPCPP\(^3\), a slowly hydrolyzable analog of GTP [10]. The main advantage is that when MTs are formed with GMPCPP no catastrophe events take place, also MT growth is slower. MTs were grown both with GTP and GMPCPP (method as depicted in appendix A.4). When imaging directly in the EF chamber the high tubulin and hence rhodamine concentration interfered with the contrast between the MT and the background tubulin. However after diluting the MT mix with a taxol solution [1 : 100](the taxol stabilizes the MTs) the contrast was big enough to observe single MTs.

\(^3\)guanylyl-(alpha, beta)- methylene-diphosphonate
3.1.3 Heating the setup

To get MTs to grow we needed to heat the setup (see figure 3.3), however the temperature should not go too high because it can denature the tubulin proteins. To get the setup to the right temperature we used a heating foil. This foil was placed on top of the sample holder and a current was sent through the foil to heat it. To get the right temperature a thermocouple probe was placed inside the chamber to measure the temperature. The data as plotted in figure 3.4 were best fitted by a quadratic function $T = a + b \times V^2$, where $a$ is equal to the room temperature, which was at that time around 23°C (zero voltage means no heating), $V$ is the voltage across the heating foil and $b$ is a constant. Later we made a new sample holder and a new measurement gave comparable data. Note that since the quadratic behavior was already known and we are only interested in temperatures between 35 − 38°C, low voltages were omitted in the second set.
of measurements.

### 3.1.4 MT induced GUV deformation

Since it was not possible to see MTs without diluting them, we were only able to observe them indirectly. The growth and shrinking of MTs caused deformations over time as can be seen in figures 3.5, 3.6 and 3.7.

![Figure 3.5](image)

**Figure 3.5**: A GUV deforming due to MT growth. MTs are grown with GTP at a temperature of 38°C. From left to right, top to bottom we see the GUV with intervals of 60 seconds. We find a growth rate of 2.5 µm/min. The scale bar has a size of 5 µm.

In figure 3.5 we see an example of a GUV deformed by GTP MTs at 38°C. We measured a growth rate of 2.5 µm/min which is agreement with literature [9], which predicts growth rates between 1.6 and 2.3 µm/min. The slightly higher value we find is probably due to a higher temperature or a higher tubulin concentration used. The rate of depolymerization is harder to describe. Figure 3.6 shows a GUV apparently relaxing in the direction of depolymerizing MTs. The retraction however is not regular, as can be seen the GUV is pulled for 10 seconds, pauses for 7 seconds and then is pulled again. Averaging the speed over those periods shows respectively a shrinking of about 5 µm/min and 7.5 µm/min. However a closer examination of the data shown that in the first second the GUV actually displaced 1.8 µm which corresponds to a retraction of 111 µm/min. Initially the MT growth caused stress against the GUV membrane and the shrinking MT, removes the force and allows the GUV to relax again. This also explains the irregular contraction, the MT itself might shrink very regularly but the GUV might adapt slowly resulting in a stepwise movement of it.

Apart from nucleating MTs with GTP we also used GMPCPP as shown in figure 3.7. The main difference is that they grow much more slowly (27 nm/min), about 1% of the speed of GTP grown MTs.

Having shown that MTs still nucleate after EF means that the tubulin proteins survive EF. This suggest that this method also leaves other proteins intact and can also be used to study other proteins in GUVs. Furthermore we see that MT growth affects the GUVs in our experiment differently than shown in for example [3], where MTs penetrate the membrane and form φ-shaped GUVs. We suggests that the main difference is the excess of lipids near the GUV in our case. GUVs are able to extract lipids from nearby lipid structures and therefore
Figure 3.6: A MT catastrophe causes the GUV to relax against the shrinking MTs. From left to right, top to bottom we see images taken at 0, 10, 17 and 25 seconds. The speed at which the GUV retracts is around $6\mu m/\text{min}$. Note that between 10 and 17 seconds the GUV doesn’t move at all. The scale bar has a size of $5\mu m$.

Figure 3.7: GUVs with GMPCPP MTs growing inside at room temperature. The second picture was taken 60 minutes after the first one, which corresponds to a growth of $27nm/\text{min}$. The colored circles show clear deformation of the GUVs due to MT growth. The scale bar has a size of $5\mu m$.

allow the MT to grow without necessarily buckling (we did however observe buckling of MTs so it is not impossible, just less likely).

### 3.2 Beads in GUVs

#### 3.2.1 Bead inside GUVs

We were able to encapsulate both dynabeads (see appendix A.7) and polystyrene beads, both with a diameter of around $1\mu m$ (the polystyrene beads are slightly bigger with a diameter of $1.04\mu m$) inside GUVs. We used the polystyrene beads for a proof of principle and later changed to dynabeads, which can specifically bind histidine-tagged proteins. Before starting EF we waited 10 minutes, during that time all beads settle on the lipid-coated surface. Resulting in a density of
about 1 bead per square 10\(\mu m\). It was possible to get beads inside as shown in figure 3.8 but unfortunately the chances of success were slim. It appeared that beads only became encapsulated when there were big GUVs (size greater than 10\(\mu m\)) present.

![Figure 3.8: A polystyrene bead encapsulated in a GUV. The scale bar has a size of 10\(\mu m\).](image)

It is interesting to note that polystyrene beads can be moved with optical tweezers, which would allow to measure forces on the GUV and also the GUV stiffness itself.

### 3.3 AMTOCs

To create centrosome we used artificial MT organizing centers (AMTOC), which in this case are dynabeads covered with the Xenopus Microtubule associated protein 215 (XMAP215) [11]. We used the protocol as depicted in appendix (A.4). To check AMTOC formation they are stabilized by dilution in a taxol solution (1 : 100) to be able to examine them under a fluorescence microscope. As seen in figure 3.9 the MTs radiate out of the dynabead as was expected. It is interesting to mention that the MTs prefer to nucleate from the bead, but that since the tubulin concentration is high MTs also nucleate spontaneously.
Figure 3.9: An XMAP215 coated bead with MTs radiating outwards. The MTs are stained with rhodamine and are stabilized with taxol. The scale bar has a size of 10µm.
Chapter 4

Conclusion & future outlook

In this research we showed it is possible to encapsulate μm sized beads and proteins in GUVs while using EF under physiologically relevant conditions. These proteins were still active after EF since we were able to grow MTs from them. We were not able to see MTs nucleating from beads that were encapsulated in GUVs, but we showed that all necessary steps are there. In future research it would be very useful to be able to see both the GUVs and the MTs at the same time and to be able to distinguish between them. Furthermore tubulin concentrations have to be decreased to increase contrast and the temperature should be more uniformly controlled.
Appendix A

Materials & methods

A.1 Optical setup
Al images were made with a WAT-902H camera mounted on a Zeiss Axiovert 40 CFL microscope. The microscope was equipped with a Zeiss oil immersion objective. For the fluorescence imaging we used an mercury lamp.

A.2 GUVs
GUVs were obtained with EF as described in the following protocol.

1. Make lipid cocktail or take from \(-80^\circ C\).
   - Add 1000\(\mu l\) chloroform to a 0.1mg TRITC-DOPE aliquot for the TRITC suspension.
   - Put 15.9\(\mu l\) chloroform into an aliquot with 0.5mg DOPC.
   - Add 60\(\mu l\) cholesterol suspension [0.1mg/100\(\mu l\)].
   - Add 4.1\(\mu l\) TRITC suspension to the DOPC.
   - Add 20\(\mu l\) methanol.

2. Clean ITO glass slides and spacer before use
   - Rinse with \(H^2O\).
   - Rinse with \(EtOH\).
   - Rinse twice with isopropanol.
   - \(N^2\) dry.

3. Gently press the PDMS spacer onto the bottom ITO slide. Make sure that there are no air bubbles and that there is a good seal.

\(^1\)http://www.aegis-elec.com/
4. Drop 1\(\mu l\) lipid cocktail onto ITO slide so that the lipid droplet lies in the circle made by the PDMS spacer but does not touch the PDMS. Dry lipids and beads on a 50\(^\circ\)C hotplate under \(N^2\) flow for at least 30 minutes.

5. Clean Dynabeads:
   - Vortex Dynabeads.
   - Mix 20\(\mu l\) Dynabeads with 180\(\mu l\) PBS/0.01\% Triton.
   - Sonicate beads for 1 minute.
   - Centrifuge and remove PBS.
   - Vortex vigorously.
   - Wash 2 times.

6. To make microtubule mix, gently add the tubulin and MRB40 to the GTP.
   - 7\(\mu l\) tubulin [10mg/ml] (−80\(^\circ\)C)
   - 7.8\(\mu l\) MRB40 (4\(^\circ\)C)
   - 0.7\(\mu l\) GTP [100mM] (−80\(^\circ\)C)
   - 2\(\mu l\) beads

7. Drop microtubule mix on top of dried lipids.

8. Place the top ITO plate on top of the PDMS spacer and press down to seal.

9. Take setup to cold room (4\(^\circ\)C).

10. Wait 10 minutes.

11. Connect contacts to function generator and perform the following series of functions:
   - 30 min at 500Hz, graduate increase from 50V/m to 1300V/m, change every 5 minutes to the following voltages: 30\(\mu V\), 155\(\mu V\), 280\(\mu V\), 405\(\mu V\), 530\(\mu V\), 655\(\mu V\), 780\(\mu V\)
   - Keep at 780\(\mu V\) (1300V/m) and 500Hz for 90 minutes.
   - Gradually decrease frequency from 500Hz to 50Hz while at 780\(\mu V\), change every 5 minutes to the following frequency: 500Hz, 425Hz, 350Hz, 275Hz, 200Hz, 125Hz, 50Hz.

12. Check that microscope and heater are ready for use.

13. Move vesicle chamber to microscope for observation.

14. Heat setup to 37\(^\circ\)C for at least 15 minutes to grow microtubules.
A.3 Microtubules

To grow MTs the following solution is used:

- $35\mu M$ tubulin
- $4mM$ GTP/GMCP
- MRB40/MRB80

A.4 AMTOCs

1. Clean Dynabeads:
   - Vortex Dynabeads.
   - Mix 20$\mu l$ Dynabeads with 180$\mu l$ PBS/0.01% Triton.
   - Sonicate beads for 1 minute.
   - Centrifuge and remove PBS.
   - Wash 2 times with MRB40.
   - Vortex vigorously.

2. Add 2$\mu l$ of cleaned beads to 0.5$\mu l$ of XMAP215 (2$\mu M$).

3. Vortex.

4. Incubate on ice for 20 minutes, vortexing of gently pipetting mix up and down intermittently.

5. Make tubulin mix (see appendix A.3).

6. Incubate 2.5$\mu l$ of prepared beads in an eppie with 10$\mu l$ of free tubulin and 12.5$\mu l$ MRB80 at $37^\circ C$ for 20 minutes.

A.5 PDMS

Polydimethilsilozaan (PDMS) is a silicon based polymer that is optically clear and non-toxic. At room temperature is is fluid but it can be cured to form an elastic substance, ideal to seal of the chamber.
1. Thoroughly mix (by stirring) 1:10 elastomer: curing agent by volume in a plastic container.

2. Degass in an exicator for at least 1 hr.

3. Carefully pour degassed PDMS into a glass petri dish (pour a volume that allows for the desired thickness).

4. Place in the oven at 100°C for 1 hr.

5. Remove from oven and allow to cool on the workbench. The PDMS can now be removed from the petri dish and cut to desired size with a razor blade.

A.6 ITO glass

ITO coated glass of dimensions 25.8 mm × 25.8 mm × 0.2 mm was ordered at Diamond Coatings\(^2\). A metallic strip, with a wire soldered on, was glued onto the conducting side of these glasses to be able to connect the glasses to the function generator.

A.7 Dynabeads

We obtained superparamagnetic beads from invitrogen\(^3\), so called dynabeads TALON. These beads have a diameter of 1 µm.

A.8 Lipids

In our experiments we used mainly DOPC furthermore we used Tritc-DOPE\(^4\) as fluorescence labeled lipids and up to 20% cholesterol to stiffen the GUVs.

![Figure A.1: Chemical structure of DOPC.](image-url)

\(^2\)http://www.diamondcoatings.co.uk

\(^3\)http://www.invitrogen.com

\(^4\)1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl)
A.9 Buffer

MRB 40/80 was used as buffer solution during the experiment.

- 40/80 mM K-PIPES
- 4 mM MgCl₂
- 1 mM EGTA
- pH 6.8

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51,4-Piperazinediethanesulfonic acid dipotassium salt
6Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
Bibliography


