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Nanotubes from gelly vesicles

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Abstract. - We study hydrodynamic extrusion of tethers from giant unilamellar vesicles (GUV) enclosing a poly-N-isopropylacrylamide (polyNIPAM) gel. The collapse of the gel upon heating induces a deswelling of the GUV, showing that the membrane is linked to the polymer network. The gelly vesicle is attached to a micro-rod and submitted to a flow (velocity U). Above a threshold velocity ($U > U_c$) a tether is extruded and reaches a stationary length $L_\infty \simeq \tau_0 U$, in a characteristic time τ_0 . The vesicle behaves like an entropic spring, with a tether length L_∞ proportional to the Stokes friction force. Compared to viscous "sol" vesicles, gelly vesicles are much stiffer: L_∞ and τ_0 are hundred times smaller. We conclude that the mobility of lipids is reduced, only a small portion of the vesicle area is free to flow into the tube.

Introduction. – Living cells are small factories protected from the external world by a thin skin, the cellular membrane. This membrane, a phospholipid bilayer decorated by a multitude of proteins, is liquid and is sustained by the cytoskeleton, a complex structure made of actin filaments (cortex) and microtubules. The membrane is vital for the communication of the cell with the external medium, which governs the cellular activity. The cytoskeleton is a dynamical structure: at short times (few seconds), it behaves like a gel and at long times (few minutes), it flows like a viscous liquid. To transfer a signal, the membrane has to be anchored by specific proteins to the actin cortex. If the membrane detaches, blebs start to grow and the cell dies (). To understand the mechanical properties of cells, a first step is to study only the skin. Giant unilamellar vesicles (GUVs) are the most simple model for cells, made of a phospholipid bilayer enclosing an aqueous solution. Because lipids are insoluble in water, they are packed in a bilayer to minimize the surface area. This is why the tension in the membrane is extremely small ($\sigma \simeq 10^{-6} - 10^{-5} N.m^{-1}$). The amplitude of the thermal fluctuations is huge and the membrane is wrinkled. A second step, studied in this letter, is to enclose a polymeric globule inside the vesicle to model the cytoskeleton and get "gelly". We have encapsulated poly-NIPAM and crosslinkers in GUV's. In the absence of

crosslinkers, the vesicles behave as viscous droplets (long time viscous regime for cells). With crosslinkers, we form a poly-NIPAM gel (short time elastic regime). The rigidity of these gelly vesicles can be varied in a controlled way by adjusting the crosslinker ratio. The elastic modulus (measured by micropipet aspiration) ranges between 0.1 and 20 kPa, which compares to the typical values of elastic moduli reported for living cells; indeed micropipet aspiration of chondrocytes (ref Thret), endothelial cells (ref Sato) or ... give values of respectively ..., .., .. Pa for the elastic modulus. We have demonstrated that gelly vesicles are responsive systems triggered by the temperature: they shrink below a critical temperature and re-swell upon decreasing temperature. Membrane is coupled to the gel during the volume transition. This interaction between the membrane and the internal matter is crucial in living cells. Our aim here is to study the membrane cytoskeleton complexity from tube extrusion. When a local force is applied on the cell's membrane, above a threshold force f_c , the membrane deforms into a thin tube. In the technique of hydrodynamic extrusion, cells or vesicles are anchored to a micro stick, or a small adhesive patch, and submitted to a flow. For giant vesicles, extrusion and retraction dynamics of a tube are governed by the bilayer elasticity (). For cells, and red blood cells, extrusion and retraction are governed by membrane-cytoskeleton interactions, and

can be modulated by ATP which regulates the dynamics of transient links() or by drugs acting on the cytoskeleton (). If the laws of tube extrusion are predictable for GUV (), cell extrusion are more complex. In order to better understand cellular nanotubes, here we study the hydrodynamic extrusion of tethers from gelly vesicles, a first step of complexity to understand living cells. We compare our results to the extrusion of vesicles enclosing a poly-NIPAM solution.

Materials and methods. – *Chemicals for preparation of composite Giant Unilamellar Vesicles.*

Phospholipids used to form vesicles 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 99%, Sigma-Aldrich). The gel precursor mixture (pre-gel) encapsulated by the vesicles was prepared from N-isopropyl-acrylamide (NIPAM, Acros Organics), N,N'-methylene-bis-acrylamide (MBA, Sigma-Aldrich) and 2,2-diethoxyacetophenone (DEAP, Acros Organics). All compounds were used as received. Chloroform (Sigma-Aldrich), methanol (Normapur), sucrose (99%, Sigma-Aldrich), glucose (99%, Sigma-Aldrich) and all other reagents are of analytical grade.

The pre-gel aqueous solution was made from sucrose (0.1 M), NIPAM (0.3 M), DEAP (4.85 mM) and MBA (27 mM) corresponding to 9% of the monomer molar fraction. Oxygen dissolved in the pre-gel solution was removed by bubbling dry Argon for 30 minutes before the experiments. The pre-sol solutions were made from sucrose (0.1 M), NIPAM (0.1 M) and DEAP (4.85 mM), a mixture without the crosslinker.

GUVs enclosing all the compounds required for gel (resp. sol) polymerization are prepared using the standard electroformation technique (ref Angelova). 20 μL of 1 mg/mL lipid in chloroform solution was spread on two metallic (ITO) glass plates, and then allowed to dry in primary vacuum for 3 hours. The two plates were assembled by Sigillum wax (Vitrex, Copenhagen, Denmark) spacer to form a closed chamber filled with the pre-gel (resp. sol) solution. A 10 Hz alternative electric field is applied according to the following protocol: 20 min at 0.2 V, 1 h at 0.4 V, 3 h at 0.6 V and finally 1 h at 4 Hz, 0.6 V. The GUVs are then removed from the growth chamber and kept in the dark in an Eppendorf vial filled with Argon.

The preparation of the gel and sol-filled Giant Unilamellar Vesicles (gel-GUVs and sol-GUVs) follows closely the preparation method previously described (ref). All the steps leading to the gel (resp. sol) formation are performed under controlled Argon atmosphere inside a glove box that contains also one UV-B irradiation lamp (Sunlight-Erythema, Harvard Apparatus) and the other required material. Irradiation is performed through a band pass filter (320 nm-400 nm). The solution holders are three glass containers resting on ice packs and covered with a quartz window. The band-pass filter prevents phospholipids damage. The ice pack controls the solutions temperature during photo-polymerization and the quartz window allows transmission of the UV radiation. In

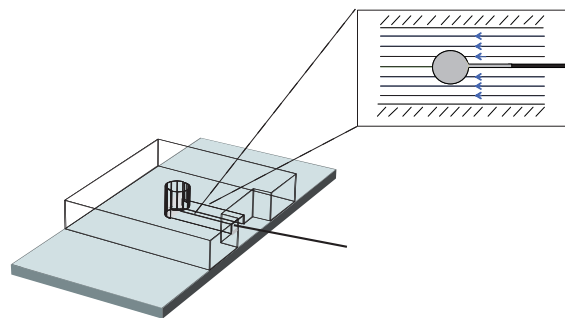


Fig. 1: Experimental setup: chamber consisting a micro-channel formed between the PDMS piece and the glass slide. Zoom: vesicle anchored to a micro-rod inside the channel subjected to a flow U .

the case of the gel-GUVs, the three containers are filled with identical solutions that combine i) 1 mL of the so-called dilution solution, similar to the pre-gel mixture but without the crosslinker and with saccharose replaced by glucose and ii) 50 μL of the pre-gel GUVs solution described above. In the case of the sol-GUVs, the three containers are filled with 1 mL of the pre-sol GUVs solution. All the samples were irradiated for 5 min, removed from the glass containers and stored at 4 C.

Micro-rods and flow chamber.

Micro-rods are made from glass rods using a horizontal laser pipette puller (P-2000, Sutter Instrument Co.) and by breaking off the tips with a microforge at the desired diameter (1 – 5 μm). Tips are immersed in a 0.1% w/v polylysine solution (Sigma Diagnostic Inc.) for few minutes before use. Polylysine is a polyelectrolyte charged positively and it is known that vesicles's membranes are slightly charged negatively (ref??); this allows the vesicle to stick onto the rod thanks to electrostatic forces.

The flow chamber is made from a channel-shaped piece of PDMS sheet (Silgard 184, Dow Corning) between two clean glass cover slides (fig. 1). The channel (section $\sim 150.10^3 \mu\text{m}^2$, length 1 cm) is filled with a glucose solution (100 mOsm, $\eta = 10^{-3}$ Pa.s). Then, vesicles are suspended in a reservoir connected to one end of the channel, where the micro-rod is introduced. A vesicle is picked up with the tip of the polylysine-coated micro-rod and brought in the channel. The micro-rod is set in the center of the channel, which allows to avoid any wall effects and minimizes uncertainty on velocity in the Poiseuille flow inside the channel. The other end of the channel is connected to a syringe that pumps the liquid at a given velocity. In our geometry, the Poiseuille flow velocities ranges from 0 to a few 1000 $\mu\text{m}/\text{s}$, so that the Reynolds number is very small ($Re \ll 1$). A step of flow velocity U is applied. When $U > U_{c0}$, the gelly vesicle is stretched and a tube is ex-

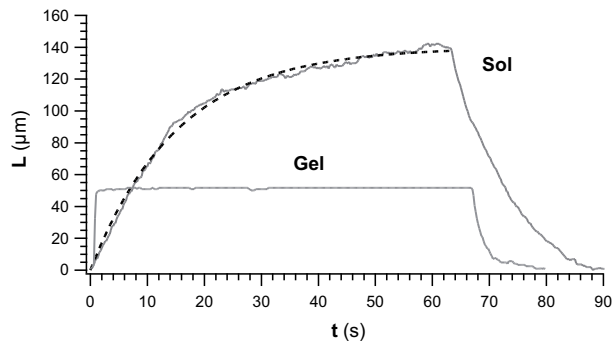


Fig. 2: Tube extrusion and retraction dynamics on a sol-filled vesicles ($R \simeq 8 \mu\text{m}$; NIPAM concentration of 600 mM) (**sol**) and a gel-filled vesicle ($R \simeq 19 \mu\text{m}$; crosslink ratio of 9 %) (**gel**) submitted respectively to a flow velocity $U \simeq 25 \mu\text{m/s}$ and $U \simeq 130 \mu\text{m/s}$. Extrusion and retraction dynamics of sol-vesicle are fitted respectively with equation (5) and (6) (dashed line).

tracted. Successive steps of increasing velocities can be applied.

Tube extrusion dynamics are observed using a microscope (Axiovert S 100, Zeiss) under bright field illumination and monitored with a numerical camera (Photometrics cascade 512B). Sequences of interest are recorded and analyzed using a tracking program (metamorph, software). We have in fig. 2 a typical plot of the length of the tube L versus time for "sol" and "gelly" vesicles.

Reminder: hydrodynamic extrusion of tethers. –

We remind here the main results of hydrodynamic tube extrusion first from simple vesicles and then from cells. The extrusion of a tube can be seen as a first order transition at a threshold force $f_c = 2\pi(2\kappa\sigma)^{1/2} = 2\pi\kappa/r$, where $\kappa \simeq 4 \times 10^{-20}$ J is the bending rigidity of the membrane, σ its tension and r the tube radius $r = (\kappa/2\sigma)^{1/2}$. Under a flow velocity U , the force on an immobile vesicle (radius R) is the Stokes friction $f_v = 6\pi\eta UR$ (water viscosity η). The equilibrium $f_v = f_c$ leads to the threshold velocity U_c :

$$U_c = \frac{(2\kappa\sigma)^{1/2}}{3\eta R} = \frac{\kappa}{3\eta Rr}. \quad (1)$$

In our experiments, the initial tension σ_0 is not imposed and ranges from 10^{-7} to 10^{-5} N/m. From eq. (1), σ_0 defines the initial threshold velocity U_{c0} . If $U > U_{c0}$, a tube is extruded at a velocity $\dot{L} = dL/dt$ given by the force balance:

$$U - \dot{L} = U_c. \quad (2)$$

As the tube grows, the excess surface area of the vesicle decreases, and the vesicle membrane tension σ increases.

The relative area extension $\Delta A/A$ is related to the membrane tension σ by [4, 5]:

$$\frac{\Delta A}{A} = \frac{kT}{8\pi\kappa} \ln \frac{\sigma}{\sigma_0}. \quad (3)$$

For a tube length L and radius r , $\Delta A = 2\pi Lr$ and $A = 4\pi R^2$. As L grows, σ increases and, according to eq. (1), U_c increases. The growth stops when $U_c = U$, which fixes the membrane tension σ_∞ from eq. (1). Inserting r and σ into eq. (3) leads to:

$$L_\infty = \tau_0 U \ln \frac{U}{U_{c0}}, \quad (4)$$

$$\frac{L}{L_\infty} \simeq 1 - \exp \frac{-t}{\tau}, \quad (5)$$

where the characteristic time $\tau_0 = \frac{3kTR^3\eta}{2\pi\kappa^2}$ strongly depends upon the size (R) and the curvature modulus (κ) of the vesicle and $\tau \simeq \tau_0(1 + \ln(U/U_{c0}))$ weakly depends upon U .

When we stop the flow, the force balance gives $\dot{L} = -U_c$. Equation. (3) leads to:

$$L \simeq \tau U \exp \frac{-t}{\tau} - \tau U_{c0}. \quad (6)$$

This approximate solution well describes the retraction dynamics that start at $L = L_\infty$ and $\dot{L} = -U$ and ends at $L = 0$ and $\dot{L} = -U_{c0}$ after a time $\tau_{\text{ret}} \sim \tau \ln(U/U_{c0})$. Experiments by N. Borghi () on nanotube extrusion from DOPC vesicles have confirmed the laws for L_∞ and the dynamic of extrusion () and relaxation (). For $R = 12 \mu\text{m}$, $\tau_0 \simeq 2\text{s}$, which leads to $\kappa_b \simeq 10kT$. For tubes extruded from cells, the membrane has to detach from the cytoskeleton to flow into the tube. If W is the cortex/membrane adhesion energy, the force of extrusion become $f_c = 2\pi\sqrt{2\kappa(\sigma + W)}$. The threshold force allows us to deduce W_0 .

Hydrodynamic extrusion from gelly vesicles. –

We study the extrusion of tethers from vesicles filled with a) polymer solution, b) reticulated gel. Dynamics of extrusion is illustrated in fig. 2. For a sol vesicle $R = 8 \mu\text{m}$, we reach a stationary state after few seconds. Fitting the curve $L(t)$ with eq. (5) and $L_\infty(U)$ with eq. (6) leads to $\tau_0 \simeq 13 \text{ s}$ $U_{c0} \simeq 20 \mu\text{m/s}$. From the expression of τ_0 , we find $\kappa_b \approx 3 \text{ kT}$. A similar reduction of κ_b has been found by one of us by micro aspiration technics.

We compare these results with data of tube extrusion from vesicles filled with gel of polyNIPAM ($R = 9 \mu\text{m}$) with a crosslink ratio of 9% (fig. 2). Study of the sol-filled vesicles allows a relevant comparison with the gelly vesicles. Indeed, sol-filled vesicles is characterized by interactions between chains of polyNIPAM and membrane without network effect of 3D gel. Hydrodynamic extrusion from these gelly vesicles allows to reach stationary states. However, the main feature appears in the order of magnitude

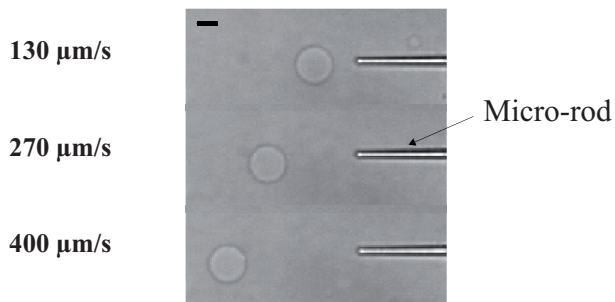


Fig. 3: Video micrographs of tube extrusion sequence from gelly vesicle (crosslink ratio of 9%) under a hydrodynamic flow increasing velocity step by step (130 $\mu\text{m/s}$; 270 $\mu\text{m/s}$; 400 $\mu\text{m/s}$). The tube is attached to the extremity of a polylysine-coated glass micro-rod (bar = 10 μm).

of characteristic time extrusion which is much lower than the time found during the extrusion of tubes from sol-filled vesicles. The stationary lengths also increase with the flow velocity. For a gelly vesicle, the evolution of the tether length $L(t)$ is much faster, and the stationary length L_∞ much smaller.

We study the evolution of $L(t)$ versus U by increasing the velocity step by step (fig. (4)). Sequence of movie is shown in fig. (3). The evolution of stationary length L_∞ with the flow velocity U is linear. The fit of $L(t)$ with eq. (5) and $L_\infty(U)$ with eq. (4) leads to a value of characteristic time about $\tau_0 \simeq 0.12\text{s}$. We also represent the stationary length versus velocity for a gel vesicle with a crosslink ratio of 6%. We find for the characteristic time $\tau_0 \simeq 0.51\text{s}$. For comparison, plot $L_\infty(U)$ for sol vesicle is also represented. We note that for higher crosslink concentration, characteristic time of extrusion τ_0 is larger.

Soft versus hard spring. – A tethered vesicle under flow behaves like an entropic spring: the equilibrium of extension length L_∞ is proportionnal to the applied force.

$$f_v = 6\pi\eta UR = \kappa_{eff} L_\infty \quad (7)$$

where: $\kappa_{eff} = \frac{(2\pi\kappa_b)^2}{R^2 kT} = \frac{6\pi\eta R}{\tau_0}$ is the effective elastic constant associated to the tube stretching, without logarithmic correlations. The time τ_0 for sol-filled vesicles is 30 times higher than for gelly vesicles. We conclude that gelly vesicles are stiff springs, whereas sol vesicles are soft springs $\kappa_{eff,gel}/\kappa_{eff,sol} \sim 30$. κ_{eff} varies with the curvature modulus of the membrane κ_b and the surface (R^2) of the vesicle. For a simple GUV, the tension is uniform on all the surface of the vesicle (it equilibrates very fast by marangoni flow in a 2D lipidic film). For a gelly vesicle, we conclude that only a fraction of the surface stretched, flow in the tube. If R_e is the radius of the patch of mobile lipids, able to flow in the tube, $R_e^2/R^2 \sim 1/30$. Only a small part of the vesicle is free to flow. For a less cross-linked gelly vesicle (crosslink ratio of 6%), we find $\tau_0 \simeq 0.51\text{ s}$, leading to $R_e^2/R^2 \sim 1/15$. If we

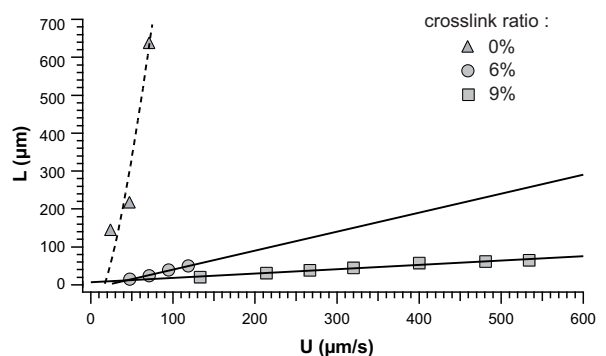
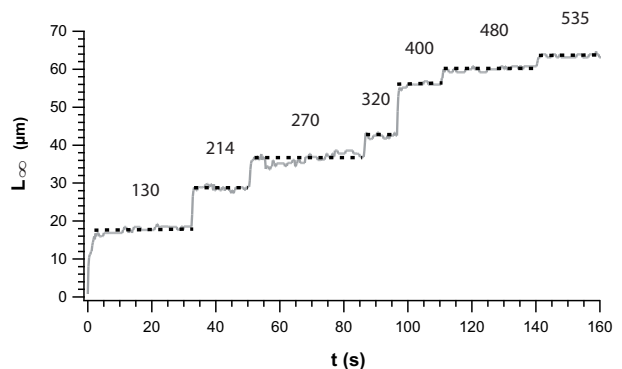


Fig. 4: a) Lengths of the tube L as a function of time with different steps of velocities (velocities appearing in bold); b) Plot representing stationary lengths vs flow velocities: gelly vesicle $R = 9 \mu\text{m}$ (square); $R = 12 \mu\text{m}$ (round) and sol vesicle with NIPAM concentration of 600 mM ($R = 8 \mu\text{m}$) (dashed line). Data are fitted with $L_\infty \simeq \tau_0 U$ (full line).

compare nanotubes from gelly vesicles to nanotubes from cells, we find two major differences: i) in cells, binders between the membrane and the cytoskeleton are point like: lipids are free to flow through the fixed proteins bound to the cytoskeleton. Here, binders are much stronger and lipids are enclosed; ii) in cells, energy of adhesion between membrane and cytoskeleton is much larger than membrane tension, and dominates tube extrusion. Cellular nanotubes do not reach a stationary length (because surface tension increase remains a small effects compared with energy of adhesion membrane-cytoskeleton) but extrusion is done at velocity \dot{L} . Here, in case of gelly vesicle, we observe a plateau.

Conclusion. – This study reveals a behavior relatively different from the gelly vesicles compared with the sol vesicles and even with the vesicle DOPC. For the gelly vesicles, stationary length of tube and characteristic time of extrusion are smaller compared with sol vesicle. Gelly vesicles are stiff spring where only a fraction of the lipids flow in the tube. This particular behavior can be ex-

plained by a nonspecific fastener of the lipids to the gel, with the image of the cells, but on lines which prevents the free flow of the lipids towards the tube. Lipids diffusion measurements can support this theory. Future works is to realize systems with a gel attached specifically to the membrane in a well controlled way.

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