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End-capping of amphiphilic nanotubes with phospholipid vesicles: impact of the phospholipid on the cap formation and vesicle loading under osmotic conditions†

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Soft amphiphilic nanotubes are capped with vesicles comprised of either overall neutral, zwitterionic phospholipids, or those that carry a net charge. The phase transition temperature of the zwitterionic phospholipids plays a crucial role in the phase separation that leads to the end-capped nanotubes. The cationic vesicle caps can be loaded into the nanotubes via osmosis whereas the anionic vesicle caps are stable under hyper-osmotic conditions. Furthermore, no additional salt needs to be added for the cationic vesicle caps to induce the loading of the vesicles into the nanotubes due to the presence of counterions.

Self-assembly provides an important platform for the creation of complex, higher-order architectures at the nano/micro scale.^{1–3} However, designing building blocks which can assemble into stimuli-responsive multicomponent systems and as a consequence have adaptive properties remains a major challenge.^{4–6} Responsive amphiphiles provide an interesting approach since they can self-assemble into a large variety of structures such as nanotubes, vesicles, ribbons, sheets or helical structures^{7–11} which can be modified *via* specific stimuli¹² such as light,¹³ pH¹⁴ or reducing/oxidizing agents.¹⁵ Amphiphilic bilayers have the intrinsic property to be permeable to water but not to many solutes like ions. This is the basis for osmotic pressure, a common phenomenon in biological membranes but less frequently explored in amphiphilic bilayers of newly synthesized amphiphiles.¹⁶ Osmosis can nevertheless be an attractive stimulus to achieve a change in self-assembled structures. Only rather recently have responsive polymer membrane capsules,¹⁷ as well as polymersome vesicles,^{18,19} been studied under osmotic pressure and a response similar to phospholipid vesicles under osmotic pressure was reported.²⁰ Osmosis is a very attractive stimulus for

responsive self-assembled nanostructures, which contain semi-permeable membranes, since it is intrinsically simple, robust and potentially applicable to a wide array of structures at the nano/micro scale.

Recently, we obtained hybrid multicomponent nanotubes, capped with phospholipid vesicles, by self-assembly of amphiphilic overcrowded alkene **1** and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) (Fig. 1) and demonstrated the loading of the vesicles into the nanotubes employing an osmotic gradient.²¹ In contrast to earlier studies employing newly created amphiphiles under osmotic conditions,²² osmosis is used as a stimulus to obtain an otherwise unattainable structure like phospholipid vesicles enclosed within a nanotube.

Here we report on the origin of the intriguing phase separation, resulting in a hybrid nanotube-vesicle self-assembled system and the influence of the nature of the cap-forming phospholipids on the process of vesicle loading, which is considered to be of major importance to understand the reorganization of these multicomponent self-assembled systems.

Fig. 1 Structures of overcrowded alkene amphiphile **1** and the phospholipids that are used with their main phase-transition temperatures (T_m). DOPC and DMPC are zwitterionic, DOPS is anionic and EPOPC is cationic.

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Scheme 1 Synthesis of amphiphile **1**. *Reagents and conditions:* (a) CH₃(CH₂)₁₁Br (2.6 equiv.), Cs₂CO₃ (5.0 equiv.), DMF, 110 °C, 16 h, 90%; (b) TsO(CH₂CH₂O)₃H (2.2 equiv.), Cs₂CO₃ (5.0 equiv.), DMF, 100 °C, 24 h, 85%; (c) TBDPSCI (2.5 equiv.), imidazole (3.3 equiv.), DCM, rt, 75%; (d) NH₂NH₂·H₂O (230 equiv.), THF, rt, 10 min, 94%.

Vesicle endcapped nanotubes can be observed when mixing an amphiphilic overcrowded alkene **1**, having an intrinsic photoresponsive function (see Scheme 1 for synthesis), with a common phospholipid (1,2-dioleoyl-*sn*-glycero-3-phospho-choline, DOPC, Fig. 1) in an aqueous solution.⁸ The hydrocarbon tails of amphiphile **1** were found to be interdigitated as was evident from a reduced bilayer thickness of 3 nm of the nanotube compared to a bilayer thickness of 4 nm in the DOPC vesicle cap. Due to the difference in bilayer thickness it is evident that the vesicle-nanotube hybrids are the result of a phase separation between the two amphiphiles, with **1** forming the nanotube and DOPC forming the vesicle cap. The origin of this intriguing phase separation, resulting in a hybrid nanotube-vesicle self-assembled system, as well as the influence of the structure of the cap-forming amphiphile remains unclear, yet are considered crucial to understand the reorganization of these multicomponent self-assembled systems. Phase-separation between phospholipids with widely separated phase-transition temperatures (T_m) is a well-known phenomenon.²³ T_m refers to the melting temperature of the acyl chains which has a large impact on the lipid mobility²³ and vesicle shape.²⁴ The phase-separation of **1** and DOPC in the vesicle capped nanotubes is hypothetically driven by the interdigitation of **1**. We propose that upon hydrating the bilayer to form the nanotubes, initially a mixed bilayer of DOPC and **1** is obtained and sequentially a phase-separation occurs, which results in the formation of vesicle capped nanotubes.

To test the hypothesis that the formation of end-capped nanotubes is initially driven by a phase separation, the formation of nanotubes with zwitterionic dimyristoyl phosphatidylcholine (DMPC) mixed with **1** above and below its phase transition temperature was studied. DMPC (Fig. 1), with a main gel to liquid crystalline phase transition temperature of 24 °C, was used because the T_m of DOPC is far below 0 °C.²⁵ The amphiphilic overcrowded alkene **1** was mixed with an equal amount (w/w) of DMPC in chloroform. After evaporation of the solvent and the subsequent hydration with water below (4 °C) and above (30 °C) the phase transition temperature, the samples were

Fig. 2 Cryo-TEM images: (a) DMPC:**1** prepared at 4 °C, below the phase transition temperature of DMPC. (b) After warming the sample to 30 °C, immediately DMPC and **1** phase separated, forming interdigitated nanotubes of **1**, end-capped with DMPC vesicles. Scale bar 100 nm. (c) Schematic representation of a mixed bilayer of **1** and DMPC (left) and phase separation driven by the interdigitation of **1** at temperatures above T_m of DMPC (right).

imaged using cryo-transmission electron microscopy (cryo-TEM). At low temperatures no end-capped nanotubes but curved mixed bilayer sheets (Fig. 2a) were observed. The excess of DMPC formed edge and rippled vesicles typical for phosphatidylcholine below the main phase transition temperature.²⁴ Upon warming the sample above the phase transition temperature, a phase separation occurs between the amphiphilic overcrowded alkene **1** and DMPC, which results almost instantaneously in the formation of vesicle-capped nanotubes (Fig. 2b and ESI,† Fig. S4b).

When the sample was hydrated with water at a temperature above the main phase transition temperature of DMPC, vesicle-capped nanotubes were formed directly, similar to the previously reported nanotubes with DOPC.⁸ This observation allows us to conclude that mixing **1** in the presence of a phospholipid below its phase transition temperature does not result in a phase separation. This is most likely due to limited lateral movement of the phospholipid in the gel state within the mixed bilayer of **1** and DMPC.²⁶ However, at higher temperatures the tails of the phospholipid become more flexible and an increased lateral movement allows the sample to phase-separate. We can therefore conclude that phase separation is a crucial step to obtain these end-capped amphiphilic nanotubes. Here, phase-separation is most likely driven by the interdigitation of **1** that is hindered when DMPC cannot move freely in the bilayer when it is in the gel state. Above the phase transition temperature, the molecules of **1** interdigitate, maximizing van der Waals interactions in the hydrophobic bilayer of **1** and subsequently pushing DMPC out like a zipper (Fig. 2c).

To extend the nanotube system beyond DOPC as the phospholipid and examine the effect of the nature of the vesicle-forming amphiphile on the nanotube loading, the end-capping of the nanotubes with two different phospholipids was realized. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-ethylphosphocholine chloride salt (EPOPC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DOPS) were chosen due to their close structural relation to DOPC (Fig. 1). However, in contrast to the previously used DOPC, these two lipids are either cationic (EPOPC) or

Fig. 3 Schematic representation of the different behaviour of DOPS and EPOPC when mixed with **1**. Both DOPS and EPOPC form vesicle-capped nanotubes in the presence of **1** after an ageing process of three days. The water loss during the ageing process and the resulting osmotic pressure causes in the case of EPOPC the loading of the vesicle caps into the nanotubes whereas the DOPS vesicles do not get loaded into the nanotubes.

anionic (DOPS), which allowed us to study the influence of the net-charge of lipids on the end-capping and osmotic responsiveness after end-capping a nanotube. All samples were analysed by cryo-TEM (Fig. 3).

In freshly prepared samples only nanotubes with no vesicle caps can be observed (Fig. 4a and ESI,† Fig. S1) for both EPOPC and DOPS mixtures with **1** (each in a 1:1 ratio with **1**, w/w, 1 mg mL⁻¹ each). After ageing the samples for three days at room temperature both mixtures of **1** with EPOPC and DOPS show the formation of end-capped tubes (Fig. 4b and 5a). The presence of net-charged lipid vesicles on the nanotubes opens the possibility for mixing content studies after fusion with oppositely charged vesicles.^{27,28} In our previous studies we observed the instantaneous formation of end-capped nanotubes with DOPC.⁸ A potential origin for the increased time period to form the vesicle caps at the end of the nanotubes might be the fact that both EPOPC and DOPS are net-charged lipids whereas DOPC is a zwitterionic phospholipid. The additional stronger electrostatic repulsion between the phospholipid molecules might cause a higher kinetic barrier for the formation of the end-capped nanotubes. The formation of the vesicle-tube hybrid proceeds *via* solubilisation of **1** with the help of the added phospholipid, followed by a phase separation of the

Fig. 5 Cryo-TEM images of (a) EPOPC:**1** (1:1, w/w, 1 mg mL⁻¹ each) after 3 days vitrified at 100% humidity, showing end-capped nanotubes, and (b) the same sample after 3 days vitrified at 80% humidity, showing vesicles inside the nanotubes (highlighted with black arrows). Scale bars in (a) and (b) 100 nm.

lipids, which results in the formation of the end-capped nanotube. We postulate that the phase separation is likely slowed down due to the enhanced electrostatic interactions between the net-charged phospholipids.²⁶ Since the phase transition temperature of both DOPS and EPOPC are below 0 °C (Fig. 1)²⁹ it is not likely that the phase transition temperature influences the end-cap formation.

End-capped nanotubes and inclusion of vesicles inside the nanotubes can be observed for EPOPC containing samples after three days (Fig. 5b). Instead of osmotically shocking the system by the addition of a solution of high ionic strength,²¹ evaporation of water was used to create an osmotic pressure. Due to the evaporation of water during the vitrification process, the chloride counterion concentration on the outside of the capped nanotubes increases with respect to the concentration of chloride ions on the inside of the capped nanotubes. Therefore the induced osmotic pressure is sufficient to induce the shape transformation of the vesicle caps, which results in the inclusion of vesicles into the nanotubes. The formation of stomatocyte shapes^{30,31} was also found for EPOPC vesicles made in pure water upon the evaporation of water during the vitrification process (ESI,† Fig. S5) and was previously observed for the end-capped nanotubes.²¹ The decrease in film thickness due to the evaporation of water

Fig. 4 Cryo-TEM images of DOPS:**1** (1:1, w/w, 1 mg mL⁻¹ each) (a) after 1 day showing the nanotubes and (b) after 3 days showing end-capped nanotubes. Scale bar in (a) 200 nm and in (b) 50 nm.

during the vitrification process was previously measured to be 20 nm s^{-1} at 20°C and 80% humidity.³² In contrast to the previously reported inclusion of DOPC vesicles into nanotubes, addition of NaCl is not necessary to induce the formation of an osmotic gradient and hence the loading of the vesicle caps. We here employ the chloride counterion of the cationic lipid as the source for creating an osmotic gradient. Hence, the strategy presented herein to induce osmosis does not rely on the addition of further amounts of salt to induce the concentration gradient necessary for loading of the vesicles into the nanotubes. Potentially the sole presence of counterions is very useful for applications where additional amounts of salt are not feasible.

Evaporation of water to induce an osmotic gradient to load vesicles into nanotubes could also be employed for samples with DOPC end-capped nanotubes. We observed that for a sample containing DOPC:1 (1:1, w/w, 1 mg mL^{-1} each) in 10 mM aq. NaCl solution, the evaporation of solvent³³ during the vitrification process for cryo-TEM at 80% humidity is sufficient to induce the inclusion of the vesicles (ESI,† Fig. S2). This further supports our understanding that an osmotic gradient is indeed the source of this unique behaviour of vesicles attached to the end of a nanotube.

Remarkably, for DOPS no included vesicles can be observed either after the ageing process or after addition of further amounts of NaCl. After three days, only vesicles capping the end of the nanotubes are seen. Even upon adding aq. NaCl solutions at various concentrations (10 mM, 100 mM and 400 mM in a 5:1 ratio v/v) no inclusions of vesicles are observed. Control experiments (ESI,† Fig. S3) of free DOPS vesicles under hyperosmotic conditions show the expected shape deformation into so called stomatocytes,²² which indicates that a shape deformation for DOPS vesicles due to osmotic pressure is possible. However, attempting to load the DOPS vesicles into the nanotubes appears to be not possible, likely due to the increased geometric restraints a vesicle has to undergo in the process. Previously, similar curvature restraints were found for negatively charged phospholipids.³⁴ The size of the nanotube requires a strong bilayer curvature for enclosed vesicles, which is apparently not possible for DOPS. The resulting vesicle caps are thus stable under osmotic conditions and therefore allow for the creation of end-capped nanotubes.

In conclusion, the phase separation between the amphiphilic overcrowded alkene 1 and the phospholipid to form end-capped nanotubes occurs only when the phospholipid is in the liquid crystalline state, *i.e.* above the phase transition temperature. Furthermore, we were able to cap the nanotubes with two new net-charged phospholipid vesicles by ageing the samples for three days. The cationic vesicle caps are responsive to osmotic stimuli and can be loaded into the nanotubes due to the osmotic gradient caused by the evaporation of water during the ageing process. It was discovered that no additional source of salt is necessary to induce the loading of vesicles into the nanotubes. In contrast to the cationic vesicle caps, the anionic vesicle caps are stable under osmotic conditions. The choice of lipids to end-cap the nanotubes therefore allows for control over the ability to be stimuli-responsive to an osmotic gradient in these nanotube-vesicle hybrid self-assembled objects.

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