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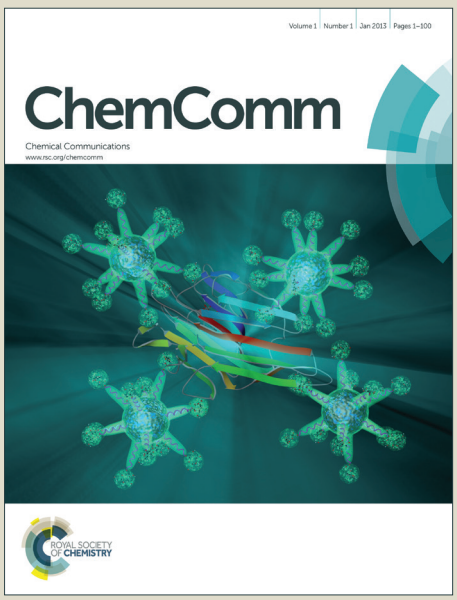

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Temperature-responsive telechelic dipalmitoylglyceryl poly(N-isopropylacryla- mides) vesicles : real-time morphology observation in aqueous suspension and in the presence of giant liposomes

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Telechelic α,ω-di(twin-tailed poly(N-isopropylacrylamides)) form polymersomes in water that increase in size by fusion when the water temperature exceeds the polymers cloud point temperature. Hybrid vesicles form in mixed suspensions of giant phospholipid liposomes and polymersomes by adsorption/fusion, and undergo further transformations, such as fission.

Phospholipid bilayer membranes are of critical importance to life sustainment, acting both as the shields of living cells and as their gate keepers in response to intracellular and environmental stimuli. They exhibit dynamic structural conversions associated with cell fusion, fission, and transitions from vesicular to tubular structures.[1] Materials scientists, in their quest for responsive and addressable systems, are involved actively in understanding how biological membranes undergo stimuli-triggered transformations and in emulating the cell membrane properties in artificial constructs.[2] The primary building blocks of artificial membranes are liposomes, obtained by self-assembly of low molecular weight amphiphiles, such as dialkyl phospholipids.[2] In the 1990's, several groups demonstrated that amphiphilic polymers added to pre-formed liposomes can incorporate into the liposome membrane, affecting their curvature, transport properties, and overall stability.[3] With the advent of polymersomes,[4] it became possible to obtain hybrid membranes by mixing directly preformed polymersomes and liposomes, or by mixing lipids and block copolymers prior to vesicle formation.[5] The resulting hybrid membranes vary often display phase separated domains as a result of the inherent immiscibility between the lipid and polymer components. The ultimate objectives in this field are to create vesicles that combine the robustness of the polymersome membrane and the biofunctionality of liposomes and, ultimately, to obtain artificial multicompartment cells.[6]

In most studies, the thickness of the hydrophobic part of polymersomes membranes (≥ 10 nm) is significantly larger than the phospholipid bilayer (5 nm). This thickness mismatch is believed to be one of the factors controlling the morphology of the membrane of hybrid vesicles.[5] The consequences of this mismatch on the morphology and functions of hybrid membranes remain poorly understood, as they are mitigated by other parameters, such as the polymer/lipid molar ratio and the relative lengths of the polymer blocks. To address this issue, we set about to prepare polymersomes by self assembly of amphiphilic polymers bearing twin-tailed termini analogous to phospholipid tails and to use them to create hybrid polymer/liposome assemblies matched in terms of bilayer thickness and composition. (Fig. 1a).

The amphiphilic copolymers, α,ω-dipalmitoylglycerylpoly (N-isopropylacrylamide) (tDPG-PNIPAM) and α,ω-dimyritoylglyceryl-poly(N-isopropylacrylamide) (tDMG-PNIPAM) were prepared by Michael addition of methacryloyl-oxyethyl derivatives of DMG or DPG to α,ω-dithiol-PNIPAMs (Mn 8, 12, 21, and 29 kg/mol) (Fig. S1, ESI), themselves obtained by reversible addition fragmentation chain transfer (RAFT) polymerization of NIPAM in the presence of a difunctional chain transfer agent (See ESI for synthetic procedures).[7] The degree of end functionalization was ~ 98 %, as determined by integration of the signals in the polymers ^{1}H NMR spectra corresponding to the methyl protons of DMG or DPG and the methine proton of the isopropyl amide repeat unit (Fig. S4 in ESI). Both tDMG- and tDPG-PNIPAM self-assembled into vesicles upon rehydration/sonication of a preformed polymer thin film. The hydrodynamic diameter (D_h) of the vesicles, determined by dynamic light scattering (DLS)

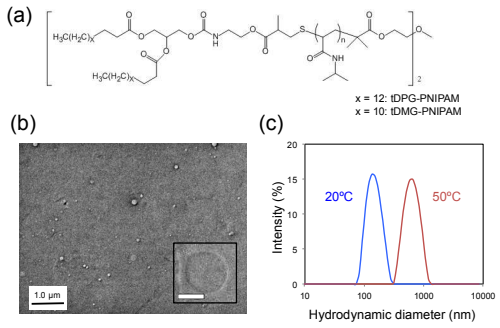

Fig. 1 (a) Chemical structures of tDPG-PNIPAM and tDMG-PNIPAM. (b) TEM images of tDPG-PNIPAM10K polymersomes. (Inset: cryo-TEM image of tDPG-PNIPAM10K, inset scale bar = 100 nm). (c) Hydrodynamic diameter and size distribution of tDPG-PNIPAM10K polymersomes at 20ºC and 50ºC in water.

Table 1 Characteristics of tDPG-PNIPAMs and tDMG-PNIPAM in pure water

Polymer	[a]M_n x 10^3 g mol^{-1} (M_w/M_n)	[b]T_{CP} (°C)	[c]D_h (nm)		[d]Anisotropy	
			20°C	50°C	20°C	50°C
tDMG-PNIPAM8K	7.6 (1.06)	30.9 ± 1.2	147.3	178.2	0.27	0.06
tDPG-PNIPAM10k	11.7 (1.10)	36.5 ± 1.8	117.3	442.3	0.22	0.13
tDPG-PNIPAM20k	21.0 (1.05)	33.9 ± 0.6	131.6	278.3	0.27	0.14
tDPG-PNIPAM30k	28.8 (1.08)	33.4 ± 0.7	106.2	337.7	0.22	0.12

[a] Molar mass data of the corresponding precursors (tTTC-PNIPAM) obtained by SEC-MALS measurements (see ESI for details)
[b] Cloud point temperatures monitored by turbidity measurements (λ: 550 nm, heating rate: 0.5 °C/min, temperature corresponding to 50 % turbidity).
[c] Determined by DLS measurements.
[d] Fluorescence anisotropy was determined using 1,6-diphenyl-1, 3, 5-hexatriene as a hydrophobic probe.

for aqueous suspensions (1.0 g/L) at room temperature, ranged from 106 to 147 nm (Table 1).

This size exceeds by far the size of flower micelles formed by telechelic PNIPAMs with single alkyl chain end groups, which feature D_h values of 21 nm and 26 nm, respectively, in the case of t(*n*-octadecyl)-PNIPAM of Mn 12 and 22 kg/mol (20 °C, 1.0 g/L).[8] The vesicular morphology and size of the self-assembled particles was confirmed by transmission electron microscopy (TEM) and Cryo-TEM examinations of tDPG-PNIPAM aqueous suspensions (Fig. 1b) and by static light scattering (SLS) and DLS studies of polymer suspensions (0.2 g/L) in water (Fig S5 in ESI). Radius of gyration to hydrodynamic radius ratios of ~ 1.0 were obtained in all cases. The fluidity of the polymersomes membrane was evaluated by measuring the fluorescence anisotropy of diphenylhexatriene (DPH) entrapped in the polymersomes membrane. The fluorescence anisotropy of DPH in tDPG-PNIPAM vesicles at 20°C ranged from 0.217 to 0.270 (Table 1). Under the same conditions, the fluorescence anisotropy of DPH in dipalmitoyl phosphatidylcholine (DPPC, gel phase) liposomes at 20 °C is 0.329.[10] The enhanced fluidity of the DPG bilayer in polymersomes, compared to liposomes, indicates that the tight packing of DPG characteristic of the gel phase cannot be achieved in polymersomes, presumably due to steric interference of the tethered polymer chains.

Suspensions of the polymersomes heated past ~ 33 °C became turbid, as anticipated in view of the thermo-responsiveness of PNIPAM in aqueous media.[11] Concurrently, tDMG-PNIPAM and tDPG-PNIPAM vesicles underwent a significant increase in size, with D_h values increasing from ~ 110 nm (20 °C) to 300 nm or more (50 °C) (Table 1 and Fig. 1c). The swelling/shrinking behavior of the vesicles was reproducible over several heating/cooling cycles. The fluorescence anisotropy of DPH in the polymersomes membrane decreased significantly upon heating, revealing an increase of the polymersome membrane fluidity with temperature (Fig. 2d). The largest change in fluorescence anisotropy occurred from ~ 32 °C to 38 °C, a temperature range that corresponds to the dehydration/collapse of the PNIPAM chains and subsequent increase in the polymersomes size.

Since changes in membrane fluidity and increase in polymersomes size occur within the same temperature range, it is tempting to speculate that vesicle fusion (Fig. 2e), rather than vesicle aggregation, underlies the size increase of polymersomes heated above ~ 33 °C. To test this hypothesis, the real time morphological transformations of polymersomes in HEPES buffer containing Mg^{2+} were observed by optical microscopy at 24 °C and 50 °C. The microscope was equipped with a temperature jump device (Fig. S7 in ESI) that allows fast

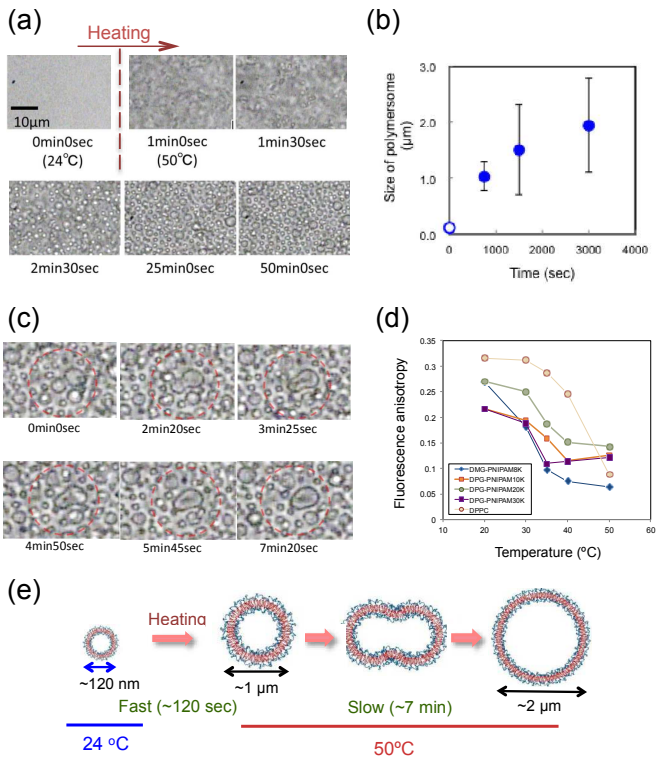

Fig. 2 (a) Microscopic observation of the formation of micrometer-scale tDPG-PNIPAM10K polymersomes by heating a tDPG-PNIPAM10Kaqueous suspension from 24 °C to 50 °C. (b) Changes of the tDPG-PNIPAM10K polymersomes diameter as a function of incubation time at 50 °C; full circles: from analysis of optical micrographs (see ESI); open circle: D_h at 20 °C determined by DLS measurements. (c) Optical micrographs depicting the fusion of tDPG-PNIPAM10K polymersomes at 50°C. Time 0: start of observation; (d) Temperature dependence of the fluorescence anisotropy of DPH in the polymersomes bilayer (HEPES buffer (10 mM, pH 7.5) containing 10 mM $MgCl_2$. (e) Schematic illustration of the growth and fusion of tDPG-PNIPAM polymersomes heated from 24 °C to 50 °C.

(< 60 s) equilibration at a desired temperature. Optical micrographs recorded as tDPG-PNIPAM polymersomes suspensions were brought from 24 °C to 50 °C are presented in Fig. 2a as a function of the time past T-jump initiation. The left-most micrograph, recorded at 24 °C, is featureless, indicating that the size of vesicles formed at room temperature in HEPES buffer is below the detection limit of the microscope. All other micrographs were recorded after the suspension attained 50 °C. Within 60 s after T-jump, objects appear faintly in the field of vision. Distinct vesicles gradually form within the following minute. The vesicles grow rapidly, reaching a median diameter of 1.03 ± 0.25 μm (t = 750 s). The polymersomes size increases slightly over the next 50 min. Their size distribution is broad, with a large fraction of small vesicles (diameter d ~ 1.2 μm) and a small population of very large vesicles (d ~2.7 μm) (Fig. 2b) The vesicles in Brownian motion in the suspension fluid undergo collisions, which in some cases result in the merging of two or three vesicles into a larger one within 3 to 5 min. Micrographs depicting a successful merging event are presented in Fig. 2c. At time 0 (immediately after collision), vesicles are linked by a short bridging tether. Subsequently, the polymer chains near the tether rearrange to allow fusion of the vesicles into a single one. The heat-induced growth of tDPG-PNIPAM vesicles is reversible: All vesicles disappear from the field of vision within 60 s of a T-jump from

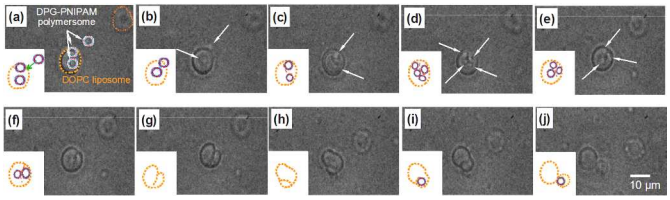

Fig. 3 Interaction between tDPG-PNIPAM10K polymersomes and DOPC giant vesicles at 50ºC. (Inset: schematic illustration of each observation) (a)-(h) are consecutive micrographs recorded as a function of observation time. (a)-(d): Before and after a collision between a DOPC liposome and tDPG-PNIPAM10K polymersomes. (e)-(j): Fission of a DOPC liposome induced by tDPG-PNIPAM10K.

50 ºC to 24 ºC (see Fig. S8 in ESI). Visual examination of the cooled suspension confirmed that the entire suspension was clear with no sign of irreversible precipitation.

In most previous reports, the self-assembly of lipids and copolymers into hybrid giant unilamellar vesicles was achieved by rehydration of lipid/copolymer binary mixtures with sonication or electroformation.[12] We chose instead to insert copolymer bilayer membranes into phospholipid bilayers by fusion of preformed polymersomes and giant DOPC liposomes, which enabled us to follow in real time the formation of hybrid membranes, First, giant DOPC liposomes (diameters: ~ 3 µm to ~ 30 µm) containing Texas Red where prepared at room temperature in a HEPES buffer containing Mg^{2+}. tDPG-PNIPAM polymersomes, prepared in the same buffer, were added to the liposome suspension. The mixture was placed in the sample holder of the microscope described above and observed at 24 ºC. Giant DOPC liposomes were detected by bright field and fluorescence microscopy. Polymersomes were invisible. A T-jump to 50 ºC was initiated. Within ~ 60 s, 1 to 2 µm-sized polymersomes appeared in the field of vision (Fig. 3a). At this temperature, the PNIPAM shells surrounding the bilayer are partially dehydrated. Upon collision, some polymersomes remained adsorbed on the liposomes Others were engulfed within the liposome bilayer (Fig. 3b) and induced large deformations of the liposome membrane, as seen by comparing micrograph (c) in Fig. 3, which depicts a liposome prior collision with polymersomes and micrograph (d) (Fig. 3), which shows the same liposome after incorporation of two polymersomes. Additional polymersomes are engulfed in the liposome bilayer, as seen in micrograph (e) (Fig. 3), up to a point where they promote fission of the original liposome into two new vesicles, as observed in the (f) to (k) sequence of micrographs (Fig. 3 and Fig. S9 in ESI, showing frame 3 (j) observed in the fluorescence mode), which illustrates the progressive growth of a fissure in the membrane leading ultimately to the separation of daughter vesicles. As the temperature of the mixed suspension is brought back to 24 ºC, the polymersomes on the DOPC liposome membrane vanish from the field of vision (Fig. S10 in ESI). Their disappearance is significantly slower than in suspensions of polymersomes alone, which may indicate that the polymer chains fuse in the phospholipid membrane. Their fate cannot be determined from optical micrography alone and additional studies are in progress in order to elucidate this point.

In conclusion, a simple route towards thermoresponsive polymersomes with a membrane commensurate with the phospholipid liposome bilayer was established using the self-assembly of telechelic twin-tailed PNIPAMs. Dynamic interactions of preformed liposomes and polymersomes generate hybrid lipid-polymer vesicles, providing a new entry to this class of materials that may offer new opportunities for fundamental studies of cell models and as delivery systems.

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Notes and References

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† Electronic Supplementary Information (ESI) available: [synthesis, characterisation of polymers, DLS, SLS data, micrographs]. See DOI: 10.1039/c000000x/

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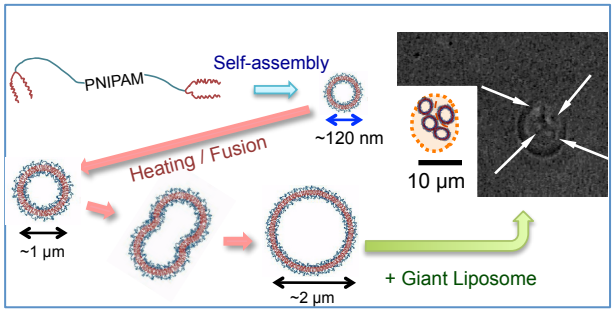

Telechelic dipalmitoylglyceryl poly(N-isopropyl-acrylamide) vesicles fuse above the phase transition temperature of PNIPAM and form hybrid vesicles with phospholipid liposomes