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PAPER

Synthetic polyion-counterion transport systems in polymersomes and gels†

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Transport across the membranes of polymersomes remains difficult in part due to the great thickness of the polymer bilayers. Here, we report that dynamic polyion-counterion transport systems are active in fluorogenic polymersomes composed of poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline) (PDMS-PMOXA). These results suggest that counterion-activated calf-thymus DNA can act as cation carrier that moves not only across lipid bilayer and bulk chloroform membranes but also across the "plastic" membranes of polymersomes. Compared to egg yolk phosophatidylcholine (EYPC) lipsosomes, activities and activator scope in PDMS-PMOXA polymersomes are clearly reduced. Embedded in agar gel matrices, fluorogenic PDMS-PMOXA polymersomes respond reliably to polyion-counterion transporters, with high contrast, high stability and preserved selectivity. Compared to standard EYPC liposomes, it cannot be said that PDMS-PMOXA polymersomes are better. However, they are different, and this difference could be interesting for the development of sensing devices.

Introduction

Polymersomes have been introduced as liposome analogs that are made from amphiphilic block copolymers with hydrophobic and hydrophilic domains instead of phospholipids.[1,2] Compared to liposomes, these "plastic" vesicles have thicker, sturdier, yet less organized, more deformable bilayer membranes. The self-assembly of macromolecular amphiphiles into polymersomes is compatible with a broad variety of possible sizes and functionalities. In part for these reasons, polymersomes have received much attention for possible applications as robust drug carriers, sensing devices, or nanoreactors[1,2] Whereas synthetic transport systems have been studied extensively in lipid bilayer membranes,[3] surprisingly little is known about transport across the thicker membranes of polymersomes.[2] Taking advantage of unique possibility to screen large activator collections without much synthetic effort,[4] we here report that several dynamic polyion-counterion transport systems[4–8] could be identified as active in polymersomes composed of poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline) (PDMS-PMOXA, Fig. 1).

Polyion-counterion transport systems are attractive because of their significance in nature[6–8] and their usefulness in differential[4] and aptameric sensing systems.[5] In these systems, amphiphilic counterions are used to activate polyions such as the anionic DNA and RNA[7] or the cationic CPPs (cell-penetrating peptides)[8] to move across bulk or lipid bilayer membranes, enter cells, transduce signals in vesicular sensing systems, and so on.[5,6] In dynamic polyion-counterion transport systems, the amphiphilic counterion

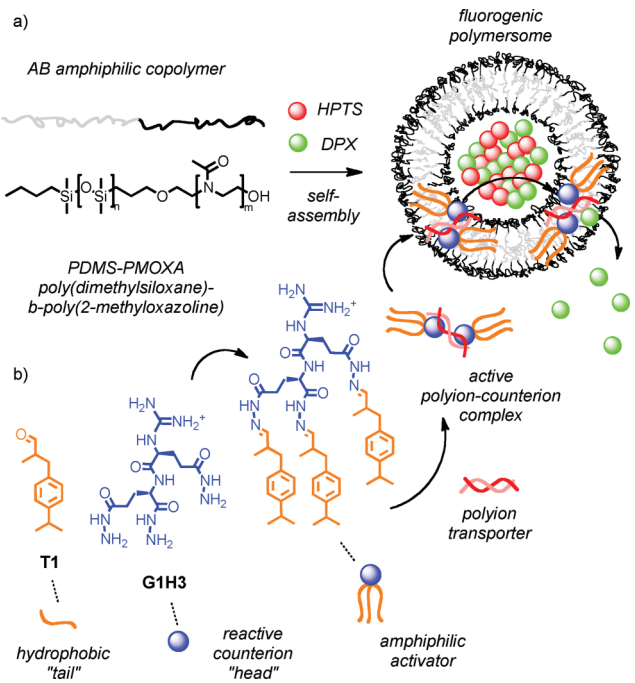

Fig. 1 (a) Preparation of fluorogenic polymersomes and (b) ion transport with dynamic polyion-counterion complexes. Hydrophobic "tails" (*e.g.*, cyclamen aldehyde **T1**) are covalently captured by hydrophilic cations (*e.g.*, trihydrazides **G1H3**) to give triple-tail amphiphiles (*e.g.*, **G1H3T1**) that can activate polyanions (*e.g.*, ctDNA) as transporters in fluorogenic liposomes or polymersomes (here the example for fluorescence recovery in response to the export of trapped cationic quenchers (green) but not anionic fluorophores (red) is shown).

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activators are produced *in situ* by covalent capture of hydrophobic molecules with reactive charged heads.[4] This approach is attractive for the generation of larger activator libraries to rapidly screen for transport activity[4–6] or cellular uptake[7,8] and to generate patterns for differential sensing applications.[4,9]

Results and discussion

To explore the activity of dynamic polyion-counterion complexes in polymersomes, PDMS-PMOXA was selected as most promising candidate. The self-assembly of these amphiphilic polymers into large unilamellar vesicles (average size ≈ 100 nm) has been demonstrated, their fluid, highly deformable but ultrastable membrane appeared most promising for transport experiments (Fig. 1).[10]

Fluorogenic PDMS-PMOXA polymersomes were prepared by swelling of a carefully dried PDMS-PMOXA film in water containing not only buffers for pH (10 mM Tris, pH 7.4) and osmotic stress (72 mM NaCl) but also an anionic fluorophore (5 mM HPTS, 8-hydroxy-1,3,6-pyrenetrisulfonate) and a cationic quencher (16.5 mM DPX, *p*-xylene-bispyridinium bromide).[11] The obtained PDMS-PMOXA LUVs were extruded through a polycarbonate membrane and external fluorescent probes were removed by size exclusion chromatography over a sephadex column. Fluorescence recovery in this assay can originate from the export of cationic DPX, anionic HPTS or both from intact vesicles, or from the destruction of the vesicles.[4,6] Counterion-activated DNA transporters have been shown to transport DPX but not HPTS across bulk chloroform membranes, and to move across intact lipid bilayer membranes.[6b] In the ideal case, export of the DPX quencher could be observed as an increase in the emission of the anionic fluorophore HPTS that is left behind (λ_{ex} = 413 nm, λ_{em} = 510 nm).

A focused collection of amphiphiles was used in this study (Fig. 2). Peptidic mini-dendrons with guanidinium cations and two (**G1H2**), three (**G1H3**) or four reactive hydrazides (**G1H4**) were selected as head groups because they have been best to activate calf-thymus (ct) DNA as cation transporter in EYPC liposomes.[4b] **G1H1** and **G1H6** were inactive because of insufficient and excessive hydrophobicity, respectively, the ammonium analogs were consistently less active.[4c] With charge-inverted CPPs, carboxylate amphiphiles were better than phosphates, and gemini amphiphiles with two heads and two tails were best.[4d]

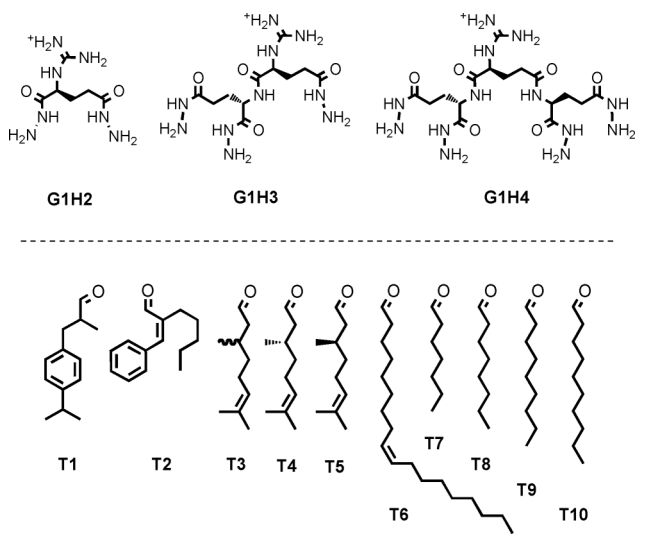

Fig. 2 Reactive counterions **G1H2**, **G1H3** and **G1H4** composed of one guanidinium cation (**G1**) and two to four hydrazides (**H**) for *in situ* reaction with hydrophobic tails **T1–T10** to yield amphiphilic hydrazones that can activate DNA as transporter in fluorogenic polymersomes (see Fig. 1).

The hydrophobic tails **T1–T10** are the survivors of an initially much broader screen. Cyclamen aldehyde **T1** and jasmine aldehyde **T2** are branched aromatic aldehydes, citronellals in racemic (**T3**) and enantiopure form (**T4**, **T5**) are classics to study stereochemistry in transport and sensing.[4a,b] The collection is completed by oleyl aldehyde **T6** and the single carbon homologs heptanal **T7**, octanal **T8**, nonanal **T9**, and decanal **T10**.[4d]

The peptidic headgroups **G1H2**, **G1H3** and **G1H4** were synthesized following the reported procedures,[4b] all aldehyde tails were commercially available or very easily prepared. The amphiphilic activators were prepared *in situ* as described previously.[4] For example, incubation of two equivalents of cyclamen aldehyde **T1** per hydrazide in **G1H3** in DMSO for 1 h at 60 °C gave hydrazone **G1H3T1** (Fig. 1), incubation with octanal **T8** gave **G1H3T8**, and so on.

Addition of excess triton X-100 caused full fluorescence recovery, indicating that polymersomes are lysed as completely as liposomes (Fig. 3, t = 200 s). As with liposomes, these conditions could thus be used to calibrate transport experiments in polymersomes to a fractional activity Y = 1.0 with excess triton X-100.

Fluorescence recovery was not observed in response to the addition of **G1H3** to PDMS-PMOXA-LUVs ⊃ HPTS/DPX polymersomes. Addition of either ctDNA or **G1H3T8** alone did not cause an increase in HPTS emission either (Fig. 3b, t < 0). The inactivity of the isolated partners demonstrated that neither polyanion nor countercation alone are capable to mediate the export of either DPX or HPTS from PDMS-PMOXA-LUVs ⊃ HPTS/DPX vesicles. However, the sequential addition of first **G1H3T8** and ctDNA next resulted in fluorescence recovery (Fig. 3, t > 40 s). This finding demonstrated that polyion-counterion transporters are active in polymersomes. The fluorescence intensity just before lysis was taken as fractional activity Y and plotted as a function of activator concentration. The resulting dose response curves were subjected to Hill analysis to yield the Y_{MAX}, the maximal accessible activity under these conditions, the EC_{50}, the effective activator concentration needed to reach 50% of Y_{MAX}, and the Hill coefficient n, which indicates the steepness of the sigmoidal fitting (Fig. 4a□, Tables S1, S2).[11]

Most dose-response curves could not be completed to full saturation because of the onset of either precipitation or lysis at high amphiphile concentrations. In fluorescence kinetics, precipitation was visible by increasing noise from light scattering. Activity of the amphiphiles alone was detectable by fluorescence recovery before the addition of ctDNA. **G1H3T1** for example, the amphiphile obtained from cyclamen aldehyde **T1**, showed outstanding ability to activate ctDNA in polymersomes (Fig. 3a, 4b●). However, at high concentrations, **G1H3T1** caused fluorescence recovery already in the absence of ctDNA (Fig. 3d).

As far as the cationic head groups are concerned, significant activity was only observed for trihydrazide **G1H3**. With dihydrazide **G1H2**, most tested amphiphiles including, **G1H2T8** and **G1H2T9**, were active in polymersomes in the absence of ctDNA and did

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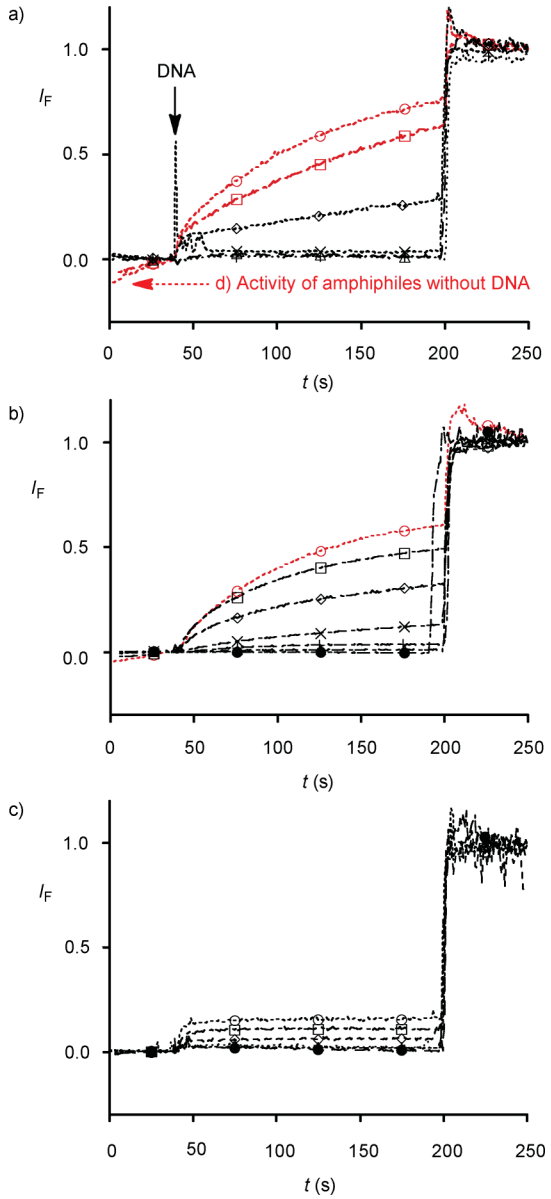

Fig. 3 Changes in fractional fluorescence intensity I_F of HPTS (λ_{ex} = 413 nm, λ_{em} = 510 nm) during addition of a) **G1H3T1** (1.25 (△), 10 (+), 25 (×), 50 (◇), 75 (□), 100 μM (○), final concentrations, t ~ 0 s), b) **G1H3T8** (0.05 (●), 0.50 (△), 2.50 (+), 6.25 (×), 12.50 (◇), 25 (□), 45 μM (○)) or c) **G1H3T7** (0.82 (●), 1.62 (△), 3.25 (+), 6.25 (△), 12.5 (□), 25 μM (○)), ctDNA (1.25 μg ml^{-1} final concentration, t ~ 40 s) and triton X-100 (excess, t ~ 200 s) to PDMS-PMOXA-LUVs ⊃ HPTS/DPX. d) Membrane activity of amphiphiles without DNA.

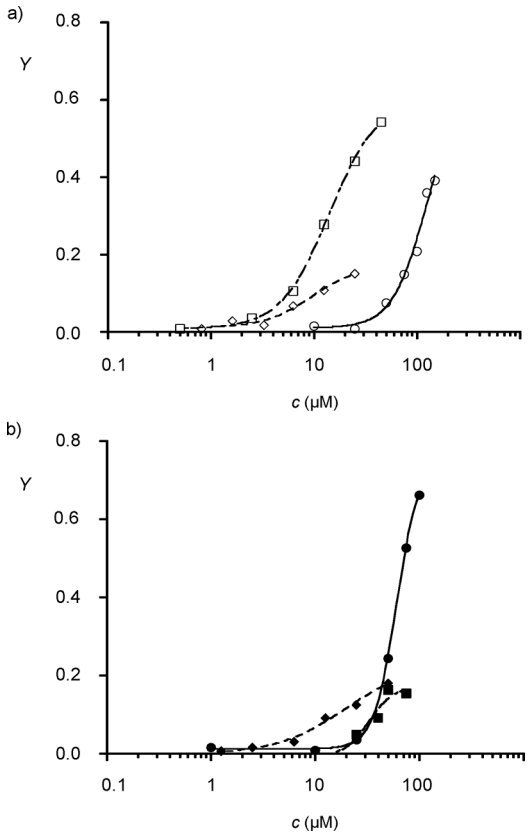

Fig. 4 Dose response curves for ctDNA transporters in PDMS-PMOXA polymersomes activated by a) **G1H3T7** (◇, from Fig. 3c), **G1H3T8** (□, from Fig. 3b) and **G1H3T9** (○). b) Same for **G1H3T1** (●, from Fig. 3a), **G1H3T2** (◆) and **G1H3T6** (■).

not show further increase in activity in the presence of ctDNA (Fig. S1†). With the complementary tetrahydrazide **G1H4**, all tested octopus amphiphiles were inactive in the presence and in the absence of ctDNA (Fig. S2†). Most active in liposomes, the inactivity of octopus amphiphiles in polymersomes presumably originated from the disappearance of these more hydrophobic amphiphiles in the thick "plastic" membranes. With liposomes, a similar inactivation with increasing tail number was observed previously for **G1H6**.[4d]

The screening of tails attached to **G1H3** heads gave with Y_{MAX} = 58.2 ± 3.4% and EC_{50} = 14.4 ± 0.2 μM outstanding activities for octanal **T8**. Compared to **G1H3T8**, the activation of ctDNA transporters by the shortened n-alkyl homolog **G1H3T7** revealed a strongly reduced Y_{MAX} = 16.3 ± 1.2% (Fig. 3c), whereas the interesting EC_{50} ~ 10 μM was preserved (Fig. 4a◇, Tables S1, S2†). With the elongated homolog **G1H3T9**, Y_{MAX} remained satisfactory, whereas the EC_{50} = 124.5 ± 0.6 μM was very high (Fig. 4a○, Tables S1, S2†). The resulting, quite remarkable selectivity for **G1H2T8** in the n-alkyl series with polymersomes was different to the situation with liposomes, where rather similar activities were obtained from pentanal up to dodecanal.

Highest activity Y_{MAX} = 75.3 ± 3.2% could be reached with the branched aromatic tails of cyclamen aldehyde (Fig. 3a, 4b●). The related jasmine aldehyde **T2**, an enone with excellent activity in liposomes, gave poor activity in polymersomes (Y_{MAX} = 22.6 ± 4.5%, 4b◆). Detectable Y_{MAX} = 19.7 ± 2.1% found with oleyl aldehyde **T6** confirmed previous insights from liposomes that central *cis* double bonds can activate long alkyl tails (Fig. 4b■).[9] Racemic citronellal **T3**, (−)-citronellal **T4** and (+)-citronellal **T5** all gave detectable activities with **G1H3** and ctDNA in polymersomes with Y_{MAX} in the range from 6% to 37% and EC_{50}'s from 40 μM to 80 μM (Table S2†). Uniqueness and reproducibility of the data (EC_{50}, Y_{MAX}, n) obtained for **T1–T10** with **G1H3** and ctDNA in polymersomes were confirmed by principal component analysis (PCA).[4a,9] Except for the weakly active heptanal **T7** and jasmine aldehyde **T2**, the obtained 3D score plots showed surprisingly little overlap (Fig. 5). This result confirmed the

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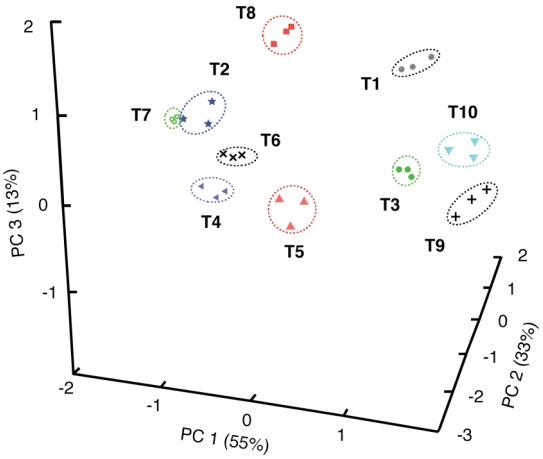

Fig. 5 PCA score plot for the activity of odorants **T1–T10** with **G1H3** and DNA transporters in polymersomes. Data points represent independent experiments (Table S1†).[11]

compatibility of fluorogenic polymersomes with differential sensing applications.[4a,9]

With activity of polyion-counterion transport in fluorgenic polymersomes confirmed in solution, experiments with polymersomes in gels were envisioned next. Transport experiments with vesicles in gels would be attractive for the construction of solid or, more precisely, semi-wet devices.[12] With fluorogenic EYPC liposomes, transport and sensing experiments in gels have met with little success so far. To elaborate on transport experiments with polymersomes in gels, the polysaccharide agar-agar, a undisruptive gel widely used from food industry to electrophoresis, cell culture and so on, was selected.[13]

To gelate polymersomes, agar suspensions were boiling in buffer (10 mM Tris, 107 NaCl, pH 7.4) and then allowed to cool down. Fluorogenic PDMS-PMOXA-LUVs ⊃ HPTS/DPX vesicle suspensions were added at 35 °C, just before gelation, and the mixture was transferred to a Petri dish (10 cm diameter) and allowed to cool down. With a 50 µl micropipette, 19 small holes were punched in regular repeats into the obtained gel (Fig. 6a).

Interestingly, Triton X-100, most powerful in solution (Fig. 3), was incapable to permeabilize PDMS-PMOXA polymersomes in gels. For calibration, the detergent Tween 20 was used instead and placed in rows 1 and 5 (Fig. 6a). Rows 2, 3 and 4 were loaded with 4 µl of 1 : 3 mixtures of ctDNA with **G1H3T3**, **G1H3T1** and **G1H3T9**, respectively, at increasing concentrations from 625 µM to 10 mM. Gratifyingly, the contrast of the fluorescent spots obtained after 5 min of incubation at room temperature compared to the background was sufficient to take the pictures without removal of the gel from the plate.

Transport experiments with fluorogenic polymersomes in gels were quantified with the Java-based image processing program ImageJ (Fig. 6b). The values obtained for integrated fluorescent areas were normalized against maximal fluorescent area covered with excess surfactant (Fig. 6a, rows 1 and 5) and reported as fractional activities Y. The resulting dose response curves with polymersomes in gels (Fig. 7a and 8a) were compared to results for the same polyion-counterion complexes with polymersomes in solution (Fig. 7b and 8b), liposomes in gels (Fig. 6c, 7c and 8c) and liposomes in solution (Fig. 7d and 8d).

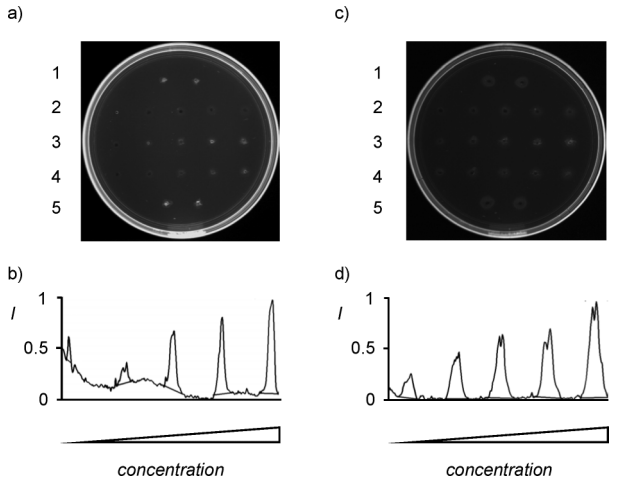

Fig. 6 (a) Fluorescence image of an agar gel plate loaded with PDMS-P-MOXA-LUVs ⊃ HPTS/DPX after the addition of 4 µl of 1 : 3 mixtures of ctDNA (1 mg ml^{-1} Tris (10 mM, pH 7.4, 107 mM NaCl) and (2) **G1H3T3**, (3) **G1H3T1** and (4) **G1H3T9** (DMSO; 0.6, 1,25, 2.5, 5.0, 10 mM, left to right) or (1, 5) 4 µl of 50% aqueous Tween 20. b) ImageJ scan of row 3 in (a) (cyclamen amphiphile **G1H3T1**). c, d) Same for EYPC-LUVs ⊃ HPTS/DPX, with 1.2% Triton X-100 in (1) and (5).

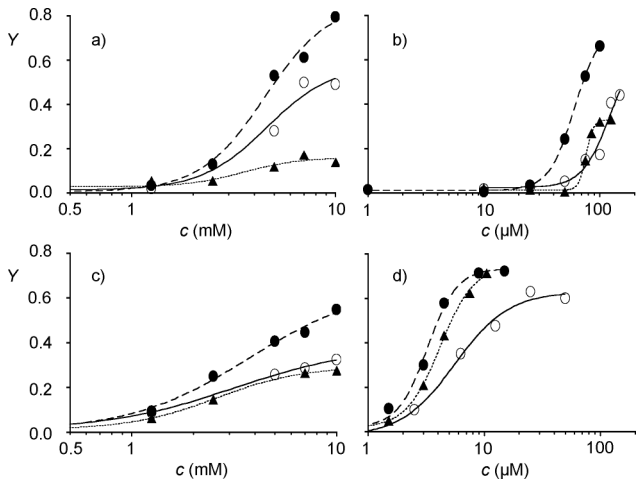

Fig. 7 Dose response curves for **G1H3T3** (◆), **G1H3T1** (●) and **G1H3T9** (○) with DNA and (a) polymersomes in gels (from Fig. 6a, 2–4), (b) polymersomes in solution, (c) liposomes in gels (from Fig. 6c, 2–4), and (d) liposomes in solution. The concentrations in a) and c) refer to the concentrations of the 4 µl stock solutions added to the gel.

In general, the fluorescence responses of polymersomes and liposomes in gels were remarkably different (Fig. 6a *vs.* 6c). Transport experiments with fluorogenic polymersomes in gels gave small and bright spots that translated into sharp peaks with single maxima in ImageJ scans (Fig. 6a, 6b). Liposomes gave larger, more diffuse spots, often with broad doublets in ImageJ scans (Fig. 6c, 6d). These distinct differences are consistent with the lower EC_{50}s found with EYPC LUVs compared to those with polymersomes, both in solution (Fig. 7b *vs.* 7d).

Compared to transport experiments in solution, large diameters of spots in gel assays should correspond to low EC_{50}, whereas high intensity and good contrast in gels should correspond to high Y_{MAX}, perhaps supported by large Hill coefficients.

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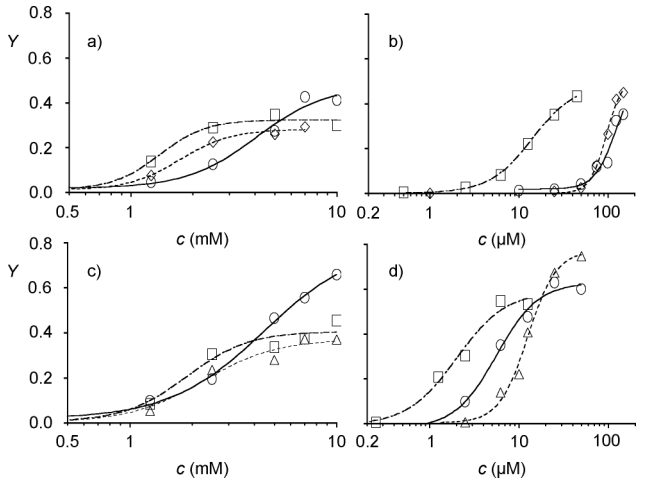

Fig. 8 Dose response curves for **G1H3T8** (□), **G1H3T9** (○), and **G1H3T10** (◇) with DNA and (a) polymersomes in gels, (b) polymersomes in solution, (c) liposomes in gels, and (d) liposomes in solution. The concentrations in a) and c) refer to the concentrations of the 4 μl stock solutions added to the gel.

In the series **G1H3T1** > **G1H3T9** > **G1H3T3**, characterized by different Y_{MAX}, high contrast with fluorogenic polymersomes in gels coincided with high Y_{MAX} in solution (Fig. 6a, 7a, 7b). Cyclamen amphiphile **G1H3T1** with highest Y_{MAX} in solution emerged as most powerful activator in gels (Fig. 7a, 7b, ●). Citronellal amphiphile **G1H3T3** with poor Y_{MAX} in solution performed also poorly in gels (Fig. 7a, 7b, ◆). Whereas the differences in Y_{MAX} were decisive for the outcome with fluorogenic polymersomes in gels, small differences in EC_{50} appeared almost irrelevant in this series (Fig. 7a, 7b).

The origin of differences in maximal activity Y_{MAX} is often unclear. Y_{MAX} refers to the percentage of vesicles involved in transport at saturation with transporters, the occurrence of $Y_{MAX} < 100\%$ has been associated with transporter aggregation and precipitation from the media at high concentration, with hindrance of intervesicular transfer by irreversible partitioning, and more. Presumably due to their increased fragility, the results with liposomes in gels in the series **G1H3T1** > **G1H3T9** > **G1H3T3** were clearly less satisfactory (Fig. 6c, 7c).

The series with alkyl homologs **G1H3T8** > **G1H3T9** ~ **G1H3T10** is characterized by similar Y_{MAX} but more different EC_{50} for both polymersomes and liposomes in solution (Fig. 8b, 8d). In this situation, the outstanding EC_{50} of **G1H3T8** with polymersomes in solution was at least partially preserved in gels (Fig. 8a, 8b, □). However, the clear trend **G1H3T8** > **G1H3T9** > **G1H3T10** for liposomes in solution was lost in gels (Fig. 8c, 8d). In this series, trends in gels were overall less significant and contained more complex relationships between Y_{MAX} and EC_{50} (Fig. 8a, 8c).

Hill coefficients seemed to generally decrease in gels. This effect is likely to originate from the exposure of the vesicles to transporter gradients. These reduced Hill coefficients are thus unrelated to transport mechanisms, the larger concentration range accessible can be viewed as a potential advantage for sensing applications.

Conclusions

The bottom line is that polyion-counterion transporters are active in fluorogenic polymersomes that are assayed in solution and in gels. This conclusion is reached based on experimental evidence secured with ctDNA as polyion, PDMS-PMOXA amphiphiles for polymersomes, and internal DPX and HPTS as probes. Dynamic hydrazone counterions are used as DNA activators because covalent capture of hydrophobic aldehydes with cationic hydrazides provides facile access to large libraries.

Compared to standard EYPC liposomes, it cannot be said that PDMS-PMOXA polymersomes are better. They are different. For instance, PDMS-PMOXA polymersomes gave higher EC_{50}'s and higher selectivity. These differences are in agreement with an overall increased barrier to be overcome while moving across the polymer membrane. Among the tested head groups, only peptide dendrons with one guanidinium cation and three hydrazides (*i.e.*, **G1H3**) were active. Best results were obtained after covalent capture of cyclamen aldehyde and octanal, activity was detectable for at least ten different hydrophobic aldehydes. Conjugates of hydrophobic aldehydes with **G1H2** were surfactants, and conjugates with **G1H4** were inactive.

Roughly reproducing the selectivity in solution, activity of fluorogenic PDMS-PMOXA polymersomes in gels was also best for **G1H3** with cyclamen aldehyde, followed by nonanal and octanal. For these experiments, a simple solid support device for pattern printing was developed using agar gelation of fluorogenic vesicles solutions. With high EC_{50}s and variable Y_{MAX}, polymersomes in gels gave bright fluorescent spots with high contrast and small diameter. The low EC_{50}s and high Y_{MAX} of liposomes produced diffuse spots with large diameter and low contrast in gels. Compared to liposomes, polymersomes in gels also showed better matching with the selectivity sequences observed in solution.

These results do not only provide experimental evidence for the activity of polyion-counterion transporters in fluorogenic polymersomes. They also demonstrate compatibility with differential sensing in solution. The compared to liposomes superior performance of polymersomes in gels is of particular interest for the development of semi-wet differential sensing devices.[12] Attractive future directions include the screening of membranes formed by other di- or tri-block copolymers,[1,2] charge inversion experiments to study counterion-activated cell-penetrating peptides (CPPs) in polymersomes,[4c] or the study of different gelators to improve activity, selectivity, contrast and stability.[12a,14]

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