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Strain-Promoted Thiol-Mediated Cellular Uptake of Giant Substrates: Liposomes and Polymersomes

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Abstract: *Simple cyclic disulfides under high tension, as small as it gets, mediate the uptake of giant substrates, i.e. liposomes and polymersomes with diameters up to 400 nm, into HeLa Kyoto cells. To place them at the surface of the vesicles, the strained disulfides were attached to the headgroup of cationic amphiphiles. Bell-shaped dose response curves revealed self-activation of the strained amphiphiles by self-assembly into microdomains at low and self-inhibition by micelle formation at high concentrations. Only poor colocalization with endosomes, lysosomes and mitochondria indicate substantial release into the cytosol. Increasing activity with disulfide ring tension, inhibition with Ellman's reagent and inactivity of maleimide and guanidinium controls outline a distinct mode of action that deserves further investigation and invites for use in practice.*

Efficient and reliable delivery into cells remains one of the grand challenges in chemistry and biology.[1-12] Particularly large substrates such as quantum dots, nanoparticles, vesicles, and so on, often end up captured in endosomes without entering into the cytosol. Dynamic covalent disulfide exchange chemistry[13] on cell surfaces is currently emerging as conceptually innovative approach to tackle this challenge.[10,11] Namely, most cells expose thiols on their surface as protection against an oxidative environment (Figure 1b).[1-3] Disulfide exchange[13] with these exofacial thiols covalently attaches transporters to the cell surface; release after uptake is achieved by reduction in the cytosol with glutathione.[10,12] With the guanidinium-rich cell-penetrating poly(disulfide)s (CPDs),[10] this thiol-mediated uptake is coupled with the counterion-mediated uptake and kinetically competing macropinocytosis.[8-10] Counterion-mediated uptake of the classical cell-penetrating peptides[4,8-10] operates with repulsion-driven ion-pairing interactions.[9] However, we found last year that upon application of high ring tension, simple monomeric disulfides can mediate cellular uptake of model fluorophores, also in the absence of positive charges.[11]

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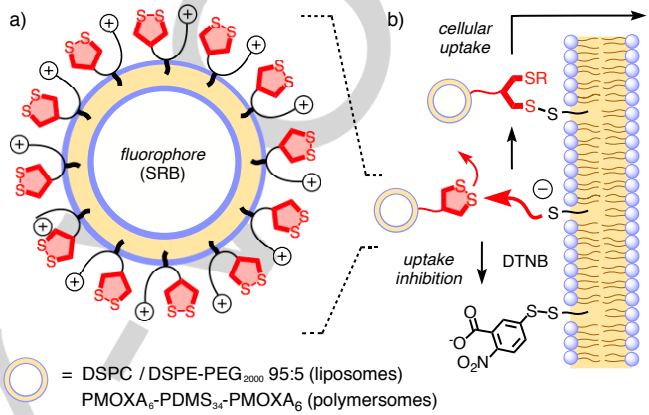

Figure 1. a) For strain-promoted thiol-mediated uptake, cationic amphiphiles **1**-**4** (Figure 2) equipped with cyclic disulfides with increasing ring tension are added to liposomes and polymersomes. b) Dynamic covalent disulfide exchange with thiols on the cell surface initiates uptake, removal of exofacial thiols with Ellman's reagent inhibits uptake.

To explore strain-promoted thiol-mediated uptake with giant substrates, biologically significant but as large as possible, liposomes and polymersomes appeared ideal. The usefulness of CPPs on their surface has been probed previously.[4] Following up on early studies with lipoplexes containing maleimide-tagged lipids,[2] pioneering recent reports from Li and Takeoka describe the delivery of liposomes with maleimides on their surface.[3] However, the envisioned conjugate addition of exofacial thiols to maleimides is conceptually different from the topic of this study (see below). For the delivery of liposomes with a few maleimides on their surface (0.3 mol%), conjugate addition of exofacial thiols was found to be overall less important than the fusogenic properties of the pH sensitive lipids used, i.e., activities generally decreased with increasing pH, increased in the presence of serum, and were poorly inhibited by Ellman's reagent (DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); ~20%).[3] Here, we elaborate on strain-promoted[11] thiol-mediated uptake[1] with liposomes[2-5] and polymersomes[6] as representative examples of giant substrates of biological interest (Figure 1a). We find high activity regulated by self-activation in microdomains at lower and self-inhibition by micelle formation at higher concentrations, and a fascinating, most promising, maleimide-independent (inactive),[2,3] guanidinium-independent,[4,8] fluorophilicity- and tension-dependent,[11] thiol-mediated (100% inhibition with DTNB) mode of action.

To decorate the surface of vesicles with disulfides under tension (Figure 1a), cationic amphiphiles **1**-**4** were designed and synthesized, together with controls **5** and **6** (Figure 2a). Details on their synthesis can be found in the SI (Schemes S1-S3).[14] Their ability to mediate the uptake of liposomes was evaluated

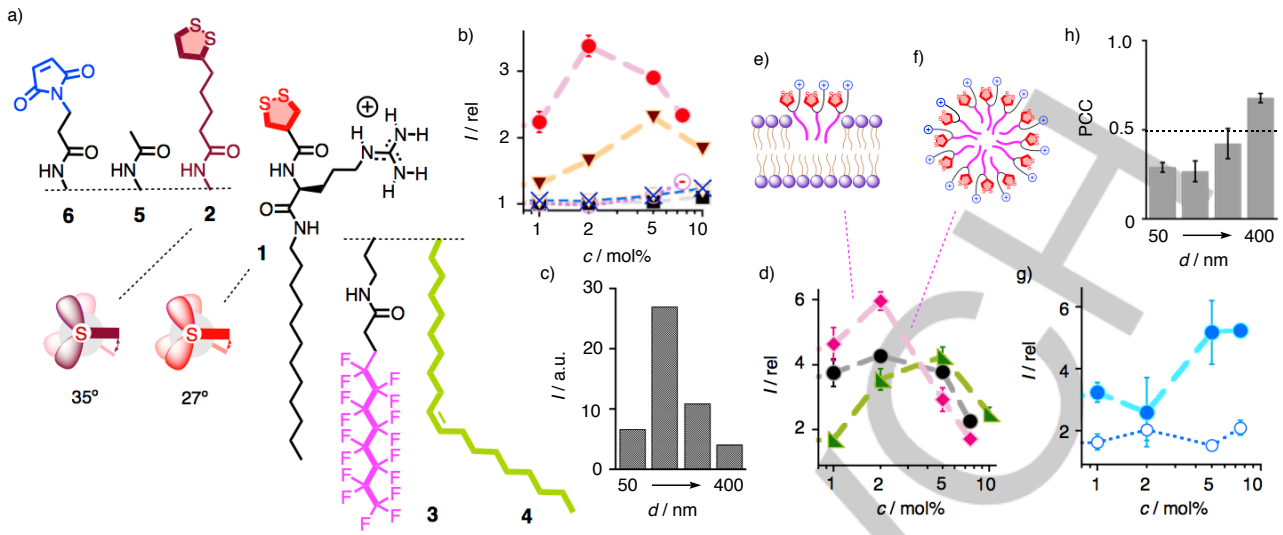

Figure 2. a) Structure of transporters and controls for strain-promoted thiol-mediated uptake of red-fluorescent liposomes (DSPC/DSPE-PEG_{2000} 95:5, 30 mM SRB, 5 mM HEPES, 115 mM NaCl (outside: 172 mM), pH 7.4) and polymersomes ($PMOXA_6$-$PDMS_{34-39}$-$PMOXA_6$) into HeLa Kyoto cells (4 h, 37 °C, Leibovitz medium). b) The dependence of liposome uptake efficiency (diameter d = 200 nm) on the concentration of transporters **1** (●, ○), **2** (▼, ▽), **5** (□) and **6** (×) without (●, △, □, ×) and with (○, ▽) preincubation with DTNB (1.2 mM, 30 min). Data (average ± standard deviation from three experiments) are normalized against those obtained with liposomes alone. c) The dependence of uptake efficiency mediated by **1** (5 mol%, constant lipid concentration) on diameter of liposomes (d = 50, 100, 200 and 400 nm). Enhancements relative to respective liposomes without **1** are compared (Figure S11). d) The dependence of liposome uptake (d = 100 nm) on the concentration of **1** (●), **3** (◆) and **4** (◣), with schematic illustration of self-activation in microdomains (e) and self-inactivation in micelles (f). g) Dependence of uptake efficiency of polymersomes (d = 160 nm) composed of $PMOXA_6$-$PDMS_{39}$-$PMOXA_6$ (●) and $PMOXA_6$-$PDMS_{34}$-$PMOXA_6$ (○) on the concentration of **1**. h) Colocalization of liposomes (5 mol% **1**) of d = 50, 100, 200 and 400 nm diameter with mitochondria (MitoTracker Green).

with established systems composed of solid-ordered DSPC (1,2-distearoyl-*sn*-phosphatidylcholine) membranes that are surrounded by protective PEG (poly(ethylene glycol)) tails and loaded with red-fluorescent sulforhodamine B (SRB, Figure 1a).[5] Amphiphiles **1**-**6** were simply added to the preformed vesicles. The temperature of addition, below or above the T_m = 55 °C of DSPC membranes, did not change the properties of the resulting systems.

The uptake of the red-fluorescent liposomes into HeLa Kyoto cells was measured after incubation for 4 h in Leibovitz medium at 37 °C, either by flow cytometry or by confocal laser scanning microscopy (CLSM), following previously reported procedures.[10,11] Without strained disulfides on their surface, the liposomes did not enter the cells (Figure 3a). Cellular uptake in the presence of transporter **1** was characterized by a bell-shaped dose-response curve with maximal activity at 2 mol% (Figures 2b●, 3b). In transporter **2**, the cyclic disulfides relax by 8°, from a CSSC dihedral angle of 27° in **1** to 35° in **2** (Figure 2a).[11] This reduction in ring tension caused a significant loss in uptake activity, together with a shift in maximal activity from 2 mol% for **1** to 5 mol% for **2** (Figures 2b▼). Preincubation of the cells with Ellman's reagent, DTNB fully inhibited uptake at all concentrations of **1** and **2** (Figures 2b, ○ and ▽). Both results, i.e., increasing activity with tension and Ellman inhibition, were important because they supported that dynamic covalent disulfide exchange on cell surfaces accounts for strain-promoted uptake of giant substrates (Figure 1b). Inactivity of control amphiphiles **5** without disulfides confirmed that guanidinium cations alone are unable to mediate liposomal uptake (Figures 2b■). This negative control was important because it excluded significant contributions from mechanisms related to CPPs[4,8,9] and thus supports that the main role of the cations in transporters **1** and **2** is to provide the amphiphilic structure needed to deliver and position the disulfides at the membrane surface (Figure 1a).

Strain-promoted uptake mediated by **1** depended strongly on the size of the liposomes (Figures 2c, S10, S11). Namely, independent of the dose of **1**, the maximal enhancement of uptake efficiency was observed with liposomal diameter d = 100 nm (Figure 2c). Considering that at constant lipid concentration, diameters shortened by half produce 4-times more vesicles with 8-times smaller volume, the intrinsic fluorescence intensity at, e.g., 50 nm should be half of that at 100 nm. However, these considerations should not influence the results in Figure 2c because uptake efficiency is measured by comparing fluorescence intensities with and without disulfides at a given diameter, using automated microscopy (Figure S11). Based on these results, studies were continued with 100 nm liposomes.

The origin of the bell-shaped dose-response curves was clarified with transporters **3** and **4**. Compared to homolog **1**, (Figure 2d●), the unsaturated tail in transporter **4** shifted maximal activity to higher concentrations (Figure 2d◣). Most importantly, the fluorinated tail in transporter **3** caused higher activity at lower concentrations (Figures 2d◆, 3c, 3d) but lower activity at higher concentrations (Figure 2d◆). Consistent with previous findings on an otherwise unrelated topic,[15] these important trends confirmed that micelle formation accounts for self-inactivation of the transporters at higher concentrations (Figure 2f). The increased activity of fluorophile **3** at low concentrations indicated that self-activation by fluorophilic self-assembly[9,16] into microdomains in the outer leaflet of the membrane matters for function, presumably by increasing the local effective concentration of strained disulfides (Figure 2e).

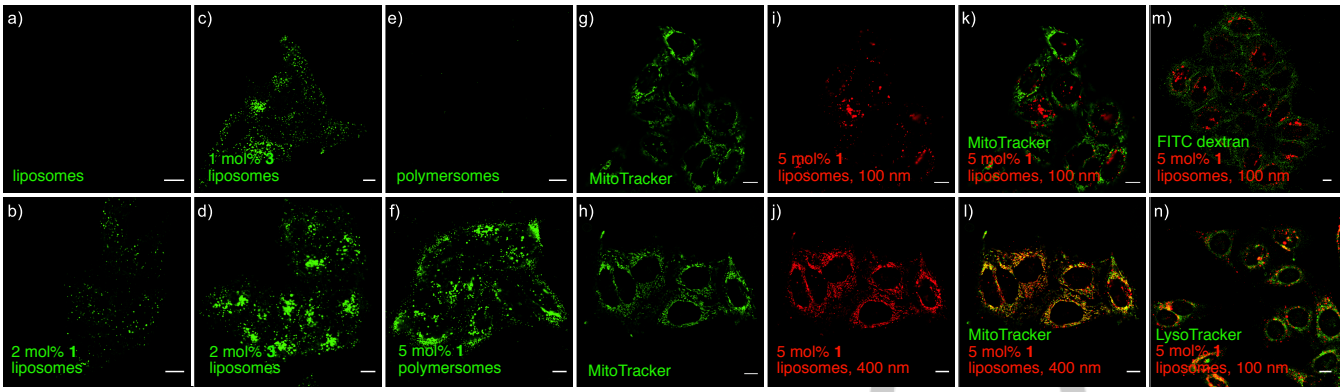

Figure 3. Representative CLSM images of HeLa Kyoto cells after 4 h of incubation with (a-b) liposomes (DSPC/DSPE-PEG_{2000} 95:5, 30 mM SRB, 100 nm) without (a) and with 2 mol% **1** (b), (c-d) liposomes with 1 mol% **3** (c) and 2 mol% **3** (d), (e-f) polymersomes ($PMOXA_6$-$PDMS_{39}$-$PMOXA_6$, 30 mM SRB, 160 nm) without (e) and with 5 mol% **1** (f), (g-l) liposomes with MitoTracker Green (g, h), liposomes, 5 mol% **1**, 100 nm (i) and 400 nm (j) diameter, and merged images (k, l), and (m-n) liposomes, 5 mol% **1**, 100 nm, with FITC Dextran 40 kDa (m, 50 µM in dish, green; LUVs, red) and LysoTracker Green (n, 100 nM in dish, green; LUVs, red). Scale bar: 10 µm

Polymersomes are analogs to liposomes, but formed by self-assembly of bolaamphiphilic block copolymers.[7] Their mono- or bilayer membrane is overall thicker and less ordered than lipid bilayer membranes, and both thickness and order can be varied significantly with the nature of the polymers used. Compatibility with strain-promoted thiol-mediated uptake was tested with polymersomes composed of ABA-block copolymers.[7] $PMOXA_6$-$PDMS_{39}$-$PMOXA_6$ and $PMOXA_6$-$PDMS_{34}$-$PMOXA_6$ at a constant diameter of ~160 nm (PMOXA: poly(2-methyl-2-oxazoline)s, PDMS: poly(dimethylsiloxane)s). Like the liposomes, they were loaded with red-fluorescent SRB. In the absence of strained disulfides, cellular uptake of polymersomes was not detectable under these conditions (Figure 3e). In the presence of transporter **1**, cellular uptake of polymersomes increased with the thickness of their membrane (Figures 2g, 3f). Even thinner $PMOXA_3$-$PDMS_{22}$-$PMOXA_3$ polymersomes were not delivered by 5 mol% transporter **1** (Figure S8b). The origins of this thickness dependence remain unclear, although decreasing stability leading to more fluorophore leakage with thinner membranes would explain the found decrease in fluorescence with more fragile polymersomes well.

Colocalization experiments were performed with liposomes containing 5 mol% of transporter **1**. Colocalization with endosomes, was probed with FITC-Dextran 40 kDa. The obtained CLSM images were analysed following routine procedures to extract Pearson's correlation coefficient (PCC, Figure 3m). The found PCC = 0.26 ± 0.06 was far below the PCC > 0.5 threshold for correlation.[17] Colocalization with lysosome (Figure 3n, PCC = 0.41 ± 0.05) and mitochondria (Figure 3k, PCC = 0.27 ± 0.06) was similarly poor (Figure S9). More significant colocalization with mitochondria was found by increasing diameter of the liposomes, ending up in with a PCC = 0.68 ± 0.03 for d = 400 nm (Figures 2i, 3l). However, d = 400 nm liposomes are the only ones with marginal uptake activity also in the absence of strained disulfides on their surface, accumulation in mitochondria is thus possibly unrelated with strain-promoted thiol-mediated uptake. In agreement with the literature,[3] poor colocalization with endosomes, lysosomes and mitochondria at highest impact of strain-promoted thiol-mediated uptake, i.e., liposomes with d = 100 nm, supported substantial delivery to the cytosol.

Control transporters **6** with maleimides in place of strained disulfides were inactive (Figure 2b✕). This finding demonstrated that strain-promoted thiol-mediated uptake is different and, at least under our conditions, much more powerful than eventual uptake mediated by maleimides.[2,3] Different also from CPP-like counterion-mediated uptake,[8,9] the question concerning the mechanism of strain-promoted thiol-mediated delivery gains in significance. Proteomics screens support that the transferrin receptor can be involved,[18] participation of other partners and mixed mechanisms are likely. Mechanistic studies along these lines, concerning also the nature and fate of the vesicles after uptake, are ongoing and will be reported in due course.

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Keywords: Cellular uptake • liposomes • polymersomes • ring tension • disulfides • maleimides

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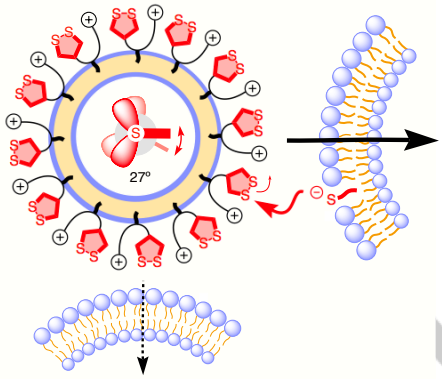
COMMUNICATION

Giant Substrates, High Tension: Realized with liposomes and polymersomes, strain-promoted thiol-mediated cellular uptake of giant substrates is characterized by increasing activity with disulfide ring tension, inactivity of cationic charges and maleimides, inhibition by Ellmans reagent, self-activation in microdomains and self-inhibition by micelle formation.



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