Photoactivation Hot Paper

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Temporal Control of Membrane Fusion through Photolabile PEGylation of Liposome Membranes

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Abstract: *Membrane fusion results in the transport and mixing of (bio)molecules across otherwise impermeable barriers. In this communication, we describe the temporal control of targeted liposome–liposome membrane fusion and contents mixing using light as an external trigger. Our method relies on steric shielding and rapid, photoinduced deshielding of complementary fusogenic peptides tethered to opposing liposomal membranes. In an analogous approach, we were also able to demonstrate precise spatiotemporal control of liposome accumulation at cellular membranes in vitro.*

Membrane fusion is a fundamental process of life resulting in the highly regulated transport of (bio)molecules both between and within cells.[1] To achieve fusion, energetic barriers associated with bringing opposing membranes together and subsequent membrane destabilization and merging must be overcome.[2] In vivo, large, often multicomponent, protein fusion complexes have evolved to carry out this task.[3]

The development of synthetic systems that are capable of controlled (non-spontaneous) membrane fusion is a tantalizing prospect, not least for applications in vector (liposomal) based drug and gene delivery in vitro and in vivo. In this context, fusion of drug-loaded vectors with target cellular membranes would result in drug delivery directly to the cell cytoplasm. Crucially, this route to intracellular drug and gene delivery minimizes the degradative loss of encapsulated payloads that is associated with hydro- and proteolytic endocytotic vector uptake.[4]

Given the typical size and complexity of native fusion complexes, significant efforts have been made to develop simplified systems capable of membrane fusion.[5] These can be targeted[6] or non-targeted.[7] Towards this goal, we have previously reported a supramolecular system that is capable of inducing rapid and targeted membrane fusion of distinct liposome populations.[8] Inspired by the native SNARE fusion complex, our targeted fusion system relies on the recognition and binding of complementary coiled-coil-forming peptides (E and K) tethered to opposing liposome membranes (Figure 1). Upon mixing of the E and K liposomes, membrane fusion and leakage-free contents mixing occur spontaneously.

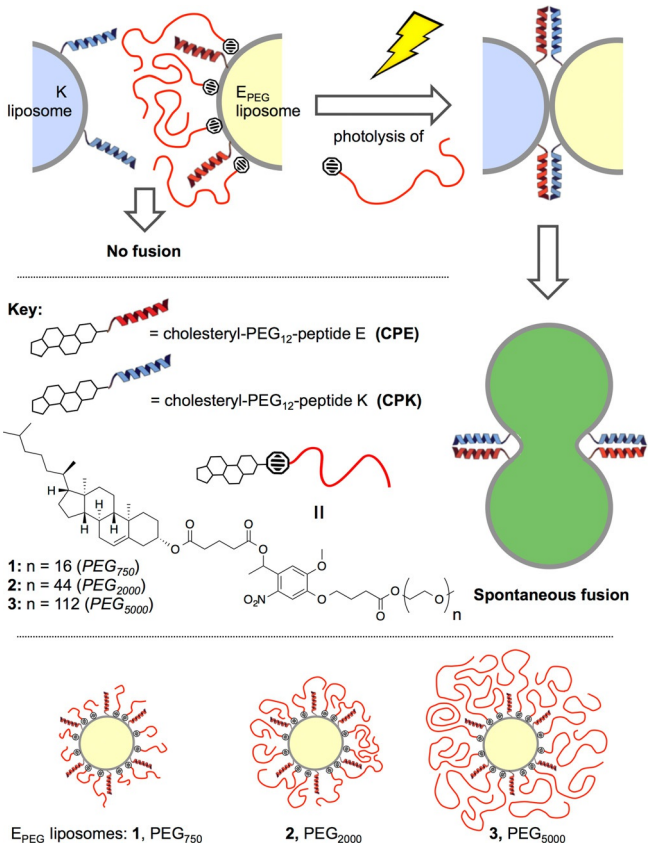

Figure 1. Top: Light-induced temporal control of liposome–liposome fusion through photolabile steric shielding (PEGylation) of fusogenic peptides tethered to opposing liposomal membranes. Bottom: E_{PEG} liposomes sterically shielded with **1**–**3**.

In nature, however, membrane fusion is highly regulated in both time and space, ensuring correct biological function. Likewise, if simplified fusion systems are to be applied to drug and gene delivery systems, the ability to control when and where fusion occurs will be essential in ensuring clinically relevant therapeutic indices. However, control of membrane fusion by using simplified fusion systems in either time and/or space has yet to be demonstrated. Herein, we demonstrate precise temporal control of membrane fusion in model (liposome–liposome) systems for the first time. This was achieved through steric shielding and rapid, photoinduced deshielding of complementary and fusogenic liposome populations (Figure 1).

Polyethylene glycol (PEG) was chosen as a steric "shield" given its widespread use in improving the pharmacokinetics and dynamics of biomolecules, nanoparticles, and lipo-

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somes.[9] We have previously shown that 2 mol% PEGylation of both liposomal membranes in our simplified fusion system effectively shut down membrane fusion through steric shielding of the liposome-tethered peptides E and K.[10]

The syntheses of the photolabile cholesterol-*ortho*-nitrobenzyl-PEG constructs **1**–**3** are outlined in the Supporting Information. Upon UV irradiation (365 nm, 3–5 mW cm^{-2}) in H_2O/MeCN/*t*BuOH (1:1:1), complete photolysis was achieved within 20 minutes as shown by UV/Vis spectroscopy (see Figure 2 for **2**). The appearance of three clear isosbestic points confirmed the clean photoconversion of **2** into its

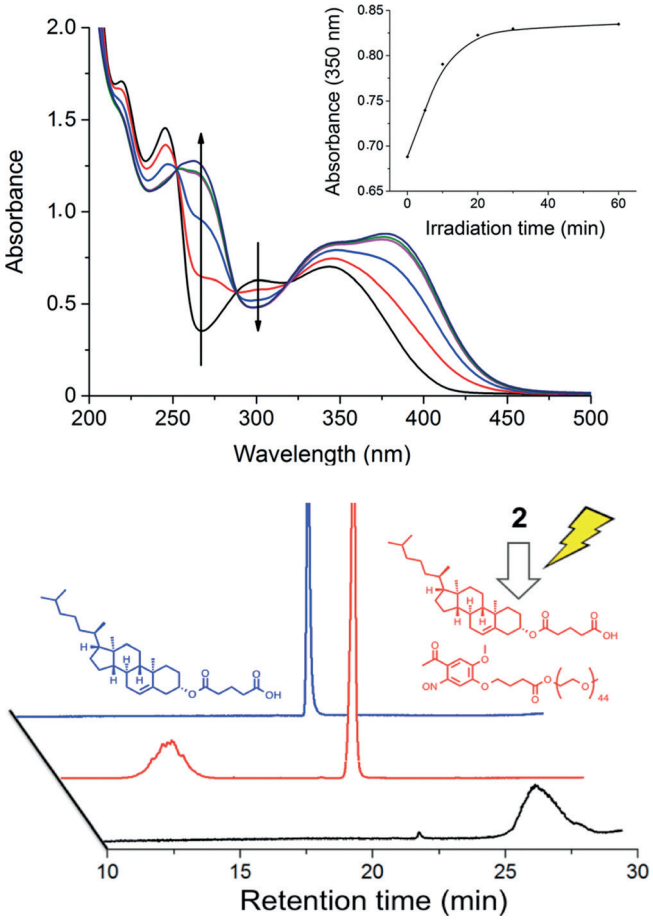

Figure 2. Top: Time evolution of the UV/Vis spectra of a solution of **2** (200 μM; H_2O/MeCN/*t*BuOH, 1:1:1) during photolysis (365 nm, 3–5 mW cm^{-2}). Inset: Time evolution of the absorbance at 350 nm. Bottom: HPLC-ELSD analysis of **2** (200 μM in PBS) before (black) and after (red) UV irradiation (30 min, 365 nm, 3–5 mW cm^{-2}). The corresponding trace of cholesteryl hemisuccinate (200 μM in PBS), an expected photoproduct, is shown in blue.

photoproducts. Comparable spectra and rates of photolysis were found for **1** and **3** under identical irradiation conditions (Supporting Information, Figure S9). HPLC-ELSD analysis (ELSD = evaporative light-scattering detection) of the photolysis products following irradiation of **2** in both H_2O/MeCN/*t*BuOH (1:1:1; Figures S10b and S11) and PBS (Figure 2, bottom) confirmed the conversion of **2** into cholesteryl hemisuccinate as expected. Conversion of **1** and **3** into their expected photoproducts was similarly observed (Figure S10).

As a next step, liposomes containing 1 mol% CPE or CPK, referred to as the E and K liposomes, respectively, were formulated by lipid film hydration and sonication. Following previously published procedures,[11] in situ modification of the E liposomes with **1**–**3** yielded E_{PEG} liposomes whose outer membrane leaflet contained between 0 and 10 mol% of **1**–**3**. In all cases, the liposomes, both before and after in situ modification, were about 100 nm in diameter as shown by dynamic light scattering analysis (polydispersity index < 0.2; Figure S12).

For the photolabile PEG constructs **2** and **3**, lipid-mixing experiments between K and E_{PEG} liposomes revealed that the degree of lipid mixing was inversely correlated to the degree of membrane PEGylation (Figure S14). In both cases, 4 mol% PEGylation of the E liposomes alone was sufficient to completely nullify lipid mixing between the E and K liposomal membranes. For the shorter (PEG_{750}) construct, **1**, the degree of PEGylation had no influence on the rate or extent of lipid mixing between the E_{PEG} and K liposomes (Figure 3). This was confirmed by circular dichroism (CD)

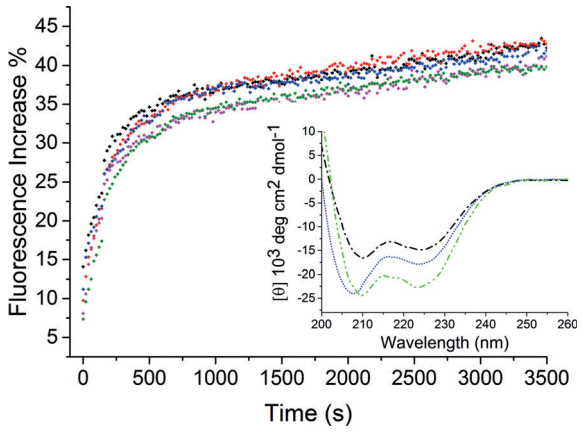

Figure 3. Lipid mixing of E_{PEG} and K liposomes with various amounts of **1** presented on the E liposome membrane: 0 mol% (black), 2 mol% (red), 4 mol% (blue), 8 mol% (violet), and 10 mol% (green). For the lipid-mixing measurements, donor (DOPE-NBD) and acceptor (DOPE-LR) fluorescent dyes were incorporated into the E_{PEG} liposomal membrane, and the donor emission was monitored. Upon mixing of the E_{PEG} and K liposomes, membrane merging resulted in a decrease in Förster resonance energy transfer between donor and acceptor and an increase in donor fluorescence emission. Inset: CD spectra of K liposomes (black), E_{PEG} liposomes modified with 4 mol% of **1** (blue), and a mixed solution of the K and E_{PEG} liposomes (green).

measurements, which, for mixed K and E_{PEG} liposomes (4 mol% **1**), showed a significant increase in helical content (Table S13), indicative of the formation of the expected heterodimeric coiled-coil complex between peptides E and K (Figure 3). It should be noted that when the peptides E and K are tethered to the liposome membrane, they are themselves about 50% folded. This is consistent with previous reports on the conformation of these peptides when tethered to a liposome membrane.[8a,c,d]

The inability of the shorter PEG construct, **1**, to sterically shield the interaction between peptides E and K reflects

a critical length requirement for the steric shield, which is determined by the molecular size of peptides E and K.[12]

To assess how liposome–liposome fusion was influenced by UV light irradiation, E_{PEG} liposomes containing 4 mol% of **2** and **3** were irradiated for increasing periods of time prior to the addition of K liposomes. As expected, the lipid-mixing efficiencies are directly correlated to the pre-irradiation times (Figure 4). Complete lipid mixing, as compared to E and

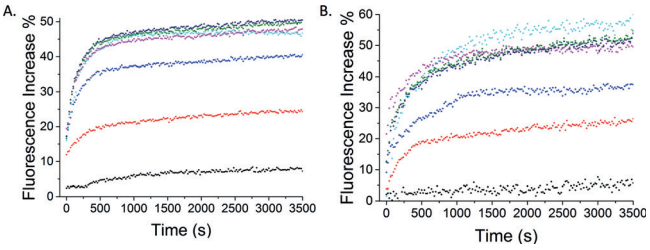

Figure 4. Lipid mixing of the E_{PEG} (4 mol%) and K liposomes. The E_{PEG} liposomes were irradiated for increasing periods of time prior to mixing with the K liposomes. A) E_{PEG} (4 mol% **2**). B) E_{PEG} (4 mol% **3**). Irradiation times: 0 (black), 5 (red), 10 (light blue), 20 (pink), 30 (green), and 60 min (dark blue). The turquoise line corresponds to 0 mol% of **2** or **3**; in these cases, the liposomes were not irradiated.

K liposome fusion in the absence of any steric shielding, was achieved after 30 minutes of pre-irradiation of E_{PEG} liposomes. This result was mirrored by analogous contents-mixing experiments (Figure S15).

To validate that membrane fusion is governed through the interaction of peptides E and K, mixed populations of E_{PEG} and K liposomes were subjected to CD analysis both before and after irradiation (Figure 5). In both cases, following

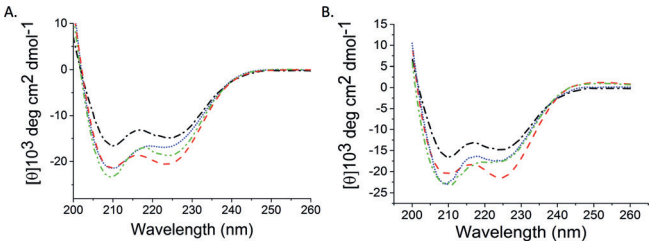

Figure 5. CD spectra of K liposomes (black), 4 mol% E_{PEG} liposomes (blue), and mixed solutions of the K and E_{PEG} liposomes before (green) and after (red) irradiation. A) E_{PEG} (4 mol% **2**). B) E_{PEG} (4 mol% **3**).

irradiation, an increase in helical content together with a shift towards an equal ratio of mean residue molar ellipticities at 208 and 222 nm, respectively, confirmed the formation of the expected E/K heterodimeric coiled-coil complex (see Table S13 for quantitative CD analysis).

To monitor the photolysis and subsequent liposome–liposome fusion in situ, populations of E_{PEG} liposomes (containing 4 mol% **2**) and K liposomes were premixed, and contents mixing was monitored before and during continuous UV irradiation (Figure 6; for experimental details, see the Supporting Information). In the absence of UV light, we

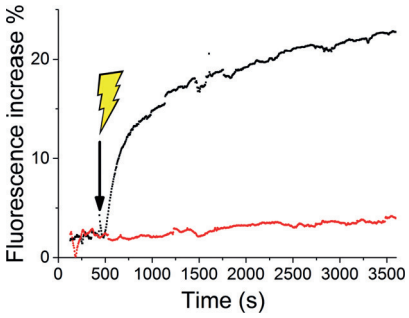

Figure 6. Contents mixing of a 1:1 mixture of E_{PEG} liposomes (4 mol% **2**) and K liposomes upon (black) and in the absence of (red) UV irradiation. The black arrow indicates the point at which UV irradiation was turned on. For contents-mixing measurements, a self-quenching concentration of a fluorescent dye (sulforhodamine B, 10 mM) was encapsulated within the E_{PEG} liposomes. Upon mixing and fusion with empty K liposomes, dilution of the dye resulted in fluorescence dequenching.

observed no contents mixing between the liposomes; however, immediately upon UV irradiation, liposome–liposome fusion commences. This result demonstrates the direct dependence of liposome–liposome fusion on photolysis of the PEG shield from the E_{PEG} liposomal membrane, enabling precise temporal control of the fusion process. This result was mirrored by the analogous experiment with E_{PEG} liposomes containing 4 mol% of **3** (Figure S17). The influence of continuous UV irradiation on the rate of fusion between E and K liposomes in the absence of any PEGylation was found to be insignificant (Figure S16).

Finally, applying this method to a biological context, we were able to demonstrate precise spatiotemporal control of liposome accumulation at prefunctionalized cellular membranes (Figure 7). To achieve this, we adapted previously reported methods,[13] first incorporating lipopeptide K into the membranes of cells, then incubating the cells with E_{PEG} liposomes (4 mol% **2**). The membrane-associated fluorescent probe DOPE-LR (1 mol%) was added to the E_{PEG} liposome membrane composition for visualization. Remarkably, after photolysis of the steric shield from the E_{PEG} liposomes, the interaction between the peptides E and K, which are displayed by the liposome and the cell surface, respectively, is both specific and strong enough to enable the well-defined, light-templated accumulation of liposomes at the cell surface. We have yet to confirm whether this interaction leads to full fusion of the liposome and target plasma membrane. However, even if this process results in docking alone, we can expect these liposomes, now localized at the cellular membrane, to be internalized over time, most likely by an endocytotic pathway.[14] Subsequent liposome degradation and endosomal escape would result in the intracellular release of liposome-encapsulated contents.

In summary, we have successfully synthesized the photolabile cholesterol-*ortho*-nitrobenzyl-PEG constructs **1**–**3** and incorporated these into E liposomal membranes. We have illustrated the need for a minimum PEG length ($\geq$ 2 K) to effectively shield the interaction between the fusogenic peptides E and K. Rapid photoinduced deshielding of the

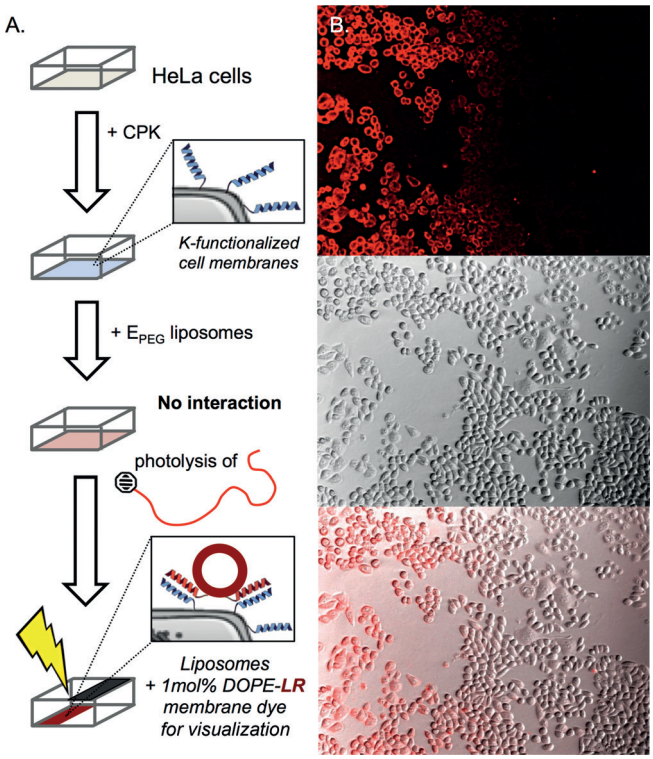

Figure 7. A) Light-directed spatiotemporal control of liposome accumulation at prefunctionalized HeLa cell membranes in vitro. B) Top: Fluorescence image (10× magnification) of E_{PEG} liposomes (containing 1 mol% of the DOPE-LR fluorescent probe) docked at prefunctionalized HeLa cell membranes following localized UV irradiation. Middle: Bright-field image (10× magnification). Bottom: Merge. Experimental details: 1) A CPK solution (5 μM) was incubated with cells for 15 min followed by washing. 2) An E_{PEG} liposome solution (250 μM, containing 1 mol% CPE and 1 mol% DOPE-LR fluorescent probe) was incubated with cells for 15 min. 3) Localized irradiation (10 min, 10 mW cm^{-2}) and further incubation for 15 min. 4) Wash and image.

E_{PEG} liposomal membranes resulted in spontaneous, and temporally controlled, fusion between distinct liposome populations in situ. Applying this approach to a biological context, we demonstrated the light-directed spatiotemporal control of liposome accumulation at prefunctionalized cellular membranes in vitro. It should be noted that no phototoxicity, which could arise from the use of UV-A (365 nm) light, was observed in cell experiments. In any event, potential issues of phototoxicity can largely be alleviated through the use of longer-wavelength, two-photon excitation sources, to which *ortho*-nitrobenzyl moieties are also photosensitive.[15] Likewise, whereas UV-A light suffers from poor tissue penetration, the use of two-photon excitation sources enables light activation at tissue depths of up to 1 cm. In conclusion, the general method described here holds significant promise towards non-invasive, user-defined, vector-based drug and gene delivery both in vitro and in vivo.

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