The chemical modification of liposome surfaces *via* a copper-mediated [3 + 2] azide–alkyne cycloaddition monitored by a colorimetric assay†

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A generic method for the efficient *in-situ* modification of liposomes is described based on "click" chemistry, and a simple colorimetric assay is developed for monitoring the reaction.

In the search for "high-tech" liposomes as drug carriers,[1] surface modification of lipid vesicles by peptides or proteins has been exploited to target specific cells.[2] The preparation of immunoliposomes as new synthetic peptide-based vaccines appears exceptionally appealing in this context.[3,4] For this, covalent anchorage of (poly)peptides onto phospholipid bilayers has been accomplished by functionalizing the peptide part with a lipidic moiety.[5–7] However, lipidation of peptides lowers their solubility, thereby complicating the efficient incorporation and distribution on the exterior of liposomes. Alternatively, soluble peptides, in their active conformation, have been covalently attached to functionalized lipid anchors already inserted in the membrane of vesicles.[4] Chemical connectivity has been achieved by this approach with variable success using amide[8] or thiol–maleimide coupling[9,10] as well as by imine[11] or hydrazone linkages.[12] In many cases there is a lack of specificity resulting in the uncontrolled formation of the number of covalent bonds between the liposome and the (poly)peptide of interest.

The [3 + 2] cycloaddition[13] between azides and strained[14] or terminal[15] alkynes has recently emerged as a highly useful chemical handle for conjugation in a non copper-mediated[14,16] and copper(I)-catalyzed[15,17] manner, also known as the "click" reaction. The unreactive nature of both azides and alkynes towards functional groups present in biomolecules as well as the thermal and hydrolytical stability of their cycloaddition product established this chemical approach as a powerful means for a wide range of biomolecular applications. For example proteins,[18] enzymes,[19] virus particles[20] and cells,[21] have been selectively modified using this method. Beside chemoselectivity and stability, we also found it particularly intriguing that the copper(I) catalyzed "click" reaction can occur efficiently in aqueous media at room temperature.[15a,22]

Based on these considerations, we investigated the copper-mediated [3 + 2] azide–alkyne cycloaddition as a novel generic chemical tool for the facile *in-situ* surface modification of liposomes. Furthermore, we used fluorescence resonance energy transfer (FRET)[23] to demonstrate that the reaction takes place at the surface and developed a colorimetric assay to follow the reaction in time without the need of any equipment.

A schematic representation of the general approach is shown in Fig. 1. First, small unilamellar lipid vesicles are prepared bearing terminal alkyne groups at their surface. The amount of functional groups can be controlled within a wide range without altering the properties of the liposomes. As a proof of principle an azido- and fluorescent probe modified lysine (**2**) was coupled to the liposome using a catalytic amount of CuBr. When the reaction occurs donor **2** is linked in close proximity to the acceptor (DOPE-LR) present in the lipid bilayer, favouring the energy transfer (FRET). The terminal alkyne functionalities in the internal membrane of the liposomes are screened and do not take part in the reaction, thus the energy transfer occurs only with the acceptor molecules available at the outer surface.

In the study presented here, two solutions of unilamellar liposomes with an average diameter in the range 110–120 nm were prepared in water by sonication of a mixture of a readily prepared terminal alkyne derivative of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE–COC≡CH **1**), DOPC and a commercially available lissamine rhodamine derivative of DOPE (DOPE-LR).‡ Both solutions appeared purple colored due to the presence of the LR. When the vesicle solution was added to 2 equivalents of N_3–Lys(NBD)–NH_2 (**2**) (for details about the straightforward solid phase synthesis see ESI†), the color changed to red–orange. To one solution a catalytic amount of CuBr (0.5 equiv.) was added (vesicle solution **A**). The second solution did not contain any CuBr (negative control **B**). After reaction, which was allowed to occur for 20 h, no dramatic effect on the size of the liposomes (average diameter = 120–130 nm) was observed. Subsequently, samples were dialyzed and the color of the solution **B** turned back to purple, while solution **A** remained orange, indicative of the covalent attachment of the NBD derivative **2** to the outer membrane forming the cycloaddition product **3**. FRET spectra of the reaction mixture before dialysis are shown in Fig. 2. Because of the presence of the NBD derivative **2** both samples appeared red. Interestingly, in the case of vesicle solution **A** the intensity of the LR emission clearly increased due to the energy transfer, demonstrating that the NBD fluorophore is located near the DOPE-LR. This implies that the "click" reaction occurs at the liposome surface. In contrast, only a weak FRET effect was observed in the negative control **B** due to the random presence of non reacted compound **2**.

Based on the observation that a color shift occurred after dialysis, we developed a colorimetric assay to follow the reaction in time (Fig. 3). Samples were taken from the reaction at several time intervals and dialyzed. Already after 30 min a color change was

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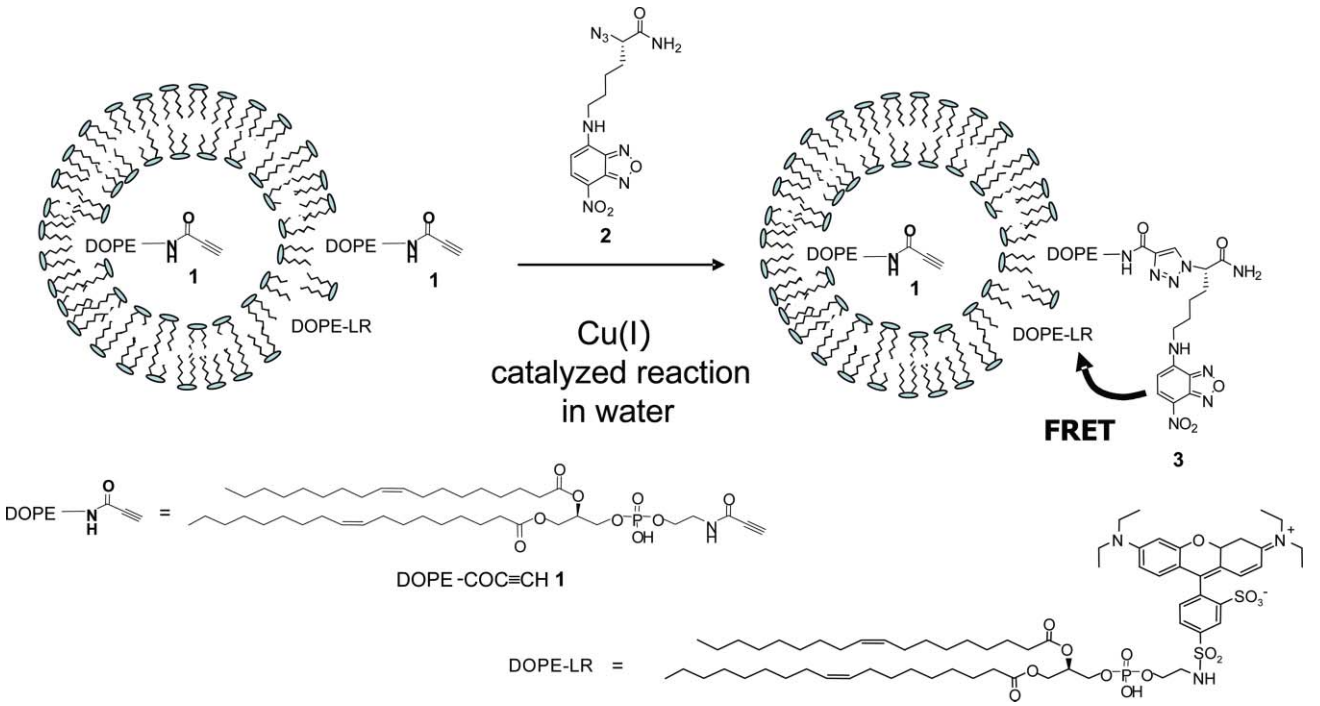

Fig. 1 Schematic representation of the general click-FRET approach.

observed in the case of vesicle solution **A** (Fig. 3, row A). In contrast, the vesicle solution **B** did not show any color change in time (Fig. 3, row B). The relevance of this easy test is based on its practical use, allowing fast monitoring of the reaction, simply by observing the color change with the naked eye. In contrast, the detection of the lipid-peptide conjugate **3** by LC-MS was rather time consuming and laborious (see ESI†). The rate of formation of product **3** was followed by measuring UV absorbance at 472 nm as a function of time (Fig. 4). In the case of vesicle solution **A** the intensity increased fast and leveled off after approximately 4 h indicative of an efficient reaction. While the absence of the Cu(I) catalyst (vesicle solution **B**) resulted in no reaction at the surface of liposomes. Finally, HPLC analysis performed after the reaction revealed that 57% of the DOPE–COC≡CH (**1**) reacted implying that only the outer layer of the liposome membrane was accessible and reacted to form product **3**.

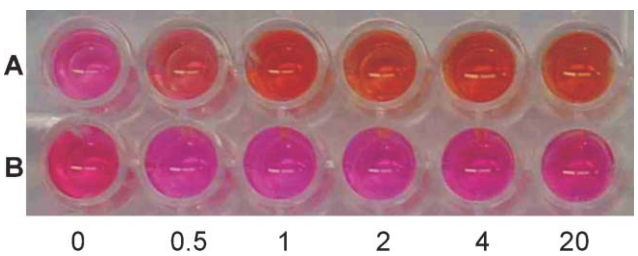

Fig. 3 Colorimetric test for (row A) the vesicle solution **A** and (row B) vesicle solution **B**, which did not contain CuBr. Samples were taken after 0, 0.5, 1, 2, 4 and 20 h and dialyzed.

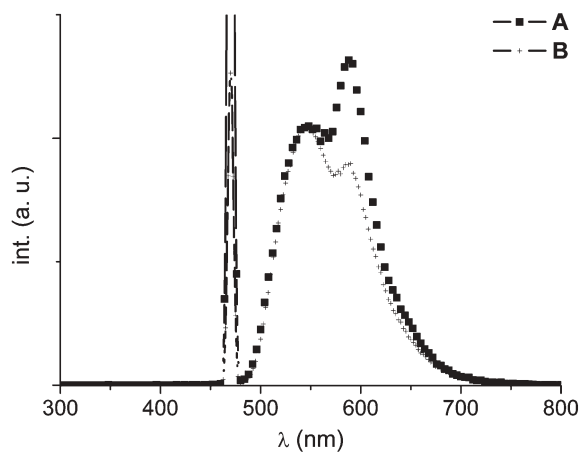

Fig. 2 FRET (exciting λ = 470 nm) spectra for vesicle solution **A** and negative control solution **B**, which did not contain CuBr. Samples were measured after 20 h reaction before dialysis.

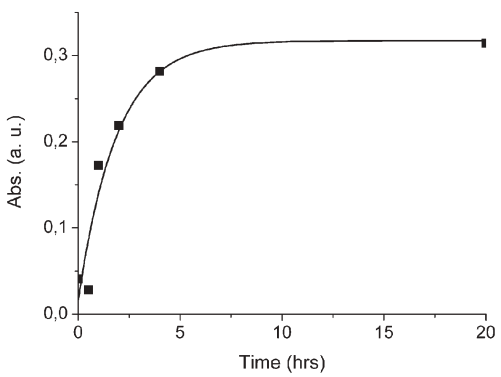

Fig. 4 UV absorbance of NBD at 472 nm as function of time for vesicle solution **A**. Absorbance values were corrected using the total lipid concentration obtained performing a phosphorous test.[24]

In conclusion, we have shown that liposome surface can be efficiently modified using copper-mediated [3 + 2] azide–alkyne cycloaddition. The reaction proceeds at room temperature and is finished within 4 h. FRET studies prove that the chemical modification truly occurs at the surface of the liposomes and a colorimetric assay was developed allowing the monitoring of the product formation in time by exploiting the visible color change upon reaction. The generic nature of our approach allows any azido-functionalized peptide[25] to be conjugated to the outer membrane of liposomes in a straightforward manner, using a similar synthetic protocol as shown for compound **2**. Additionally, NBD can be easily introduced as well[26] in order to follow the reaction using the colorimetric assay. The versatility and scope of the "click" chemistry approach for surface modification of vesicles described here is currently under investigation in our laboratory.

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Notes and references

‡ *General protocol*: A lipid mixture consisting of 50 mol% of DOPE–COC≡CH (**1**), 1 mol% of DOPE-LR and 49 mol% of DOPC was homogeneously dissolved in $CHCl_3$ (2 ml). The solvent was evaporated under a flow of N_2 and then under vacuum for 10 min. Next, the lipid film was hydrated for 1 h at room temperature with milli-Q water (total lipid concentration 1 mM). The samples were vortexed resulting in a turbid suspension and sonicated for 1 h until a clear solution appeared.[27] The size of the particles was measured by Photon Correlation Spectroscopy (PCS). Simultaneously, 2 equivalents (relative to DOPE–COC≡CH **1**) of crude N_3–Lys(NBD)–NH_2 **2** was weighed in a vial and dissolved in 1 : 1 v/v MeOH–$CHCl_3$ mixture followed by evaporation of the solvents to form a thin film. The vesicle solution (solution **A**) was added to the vial containing **2** and CuBr (0.5 equivalent). Negative control solution **B** did not contain CuBr. The solutions were incubated at 40 °C for 10 min and then under gentile swirling at RT. Samples (300 μl) were taken at several time intervals (t = 0, 0.5, 1, 2, 4 and 20 h) and dialyzed against water after which FRET (exciting λ = 470 nm) and UV (λ = 472 nm) spectra were measured. Finally, all solutions were lyophilized, the formation of the product was confirmed by LC-MS and the efficiency of the reaction determined by HPLC. The total lipid concentration was determined according the lipid manufacturer guidelines.[24]

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