Strain-promoted cycloadditions in lipid bilayers triggered by liposome fusion

Coline Jumeaux,[a] Christopher D. Spicer,[a,b,c,] Patrick Charchar,[d] Philip D. Howes,[a],§, Margaret N. Holme,[b] Li Ma,[a,b], Nicholas C. Rose,[c] M. Harunur Rashid,[d],∞ Irene Yarovsky,*[d] and Molly M. Stevens*[a,b]

[a] Dr. C. Jumeaux, Dr. C. D. Spicer, Dr. P. D. Howes,§ Prof. M. M. Stevens
Department of Materials, Department of Bioengineering, and Institute of Biomedical Engineering
Imperial College London
London, SW7 2AZ (United Kingdom)
E-mail: m.stevens@imperial.ac.uk

[b] Dr. C. D. Spicer¶
Department of Medical Biochemistry and Biophysics
Karolinska Institute
Stockholm, 17177 (Sweden)

[c] Dr. C. D. Spicer, Dr. N. C. Rose
Department of Chemistry and York Biomedical Research Institute
University of York
Heslington, YO10 5DD (United Kingdom)

[d] Dr. P. Charchar, Dr. M. H. Rashid, Prof. I. Yarovsky
School of Engineering
RMIT University
Melbourne, Victoria, 3001 (Australia)
E-mail: irene.yarovsky@rmit.edu.au

Present Addresses:
§ Department of Chemistry and Applied Biosciences, ETH Zürich, CH-8093 Zürich, Switzerland.
∞ Department of Mathematics and Physics, North South University, Bashundhara, Dhaka-1229, Bangladesh.
Margaret

Abstract: Due to the variety of roles served by the cell membrane, its composition and structure are complex, making it difficult to study. Bioorthogonal reactions, such as the strain promoted azide–alkyne cycloaddition (SPAAC), are powerful tools for exploring the function of biomolecules in their native environment but have been largely unexplored within the context of lipid bilayers. Here, we developed a new approach to study the SPAAC reaction in liposomal membranes using azide- and strained alkyne-functionalized Förster resonance energy transfer (FRET) dye pairs. This study represents the first characterization of the SPAAC reaction between diffusing molecules inside liposomal membranes. Potential applications of this work include *in situ* bioorthogonal labeling of membrane proteins, improved understanding of membrane dynamics and fluidity, and the generation of new probes for biosensing assays.

Introduction

The attachment of reporter probes to a biological target of interest provides a powerful strategy to study native processes. Bioorthogonal reactions allow site-selective probe conjugation to be achieved within complex cellular or *in vivo* environments. Such reactions must both exhibit high chemo-selectivity in the presence of native biomolecules, and proceed efficiently under ambient biological conditions.[1] In particular, reactions involving azides have found great utility due to the small size, stability under physiological conditions, synthetic accessibility, and absence from native systems.[2] These include the widely used Staudinger-Bertozzi ligation,[3] copper-catalyzed azide-alkyne cycloaddition (CuAAC),[4] and strain-promoted azide-alkyne cycloaddition (SPAAC)[5] reactions.

Bioorthogonal reactions have been most successfully implemented for the tagging of intra- or extracellular components. However, the selective labeling of compounds within lipid membranes is largely unexplored despite the vital role played by lipid bilayers in controlling cell and tissue biology.[6] The lipid bilayer presents a complex and dynamic environment, acting as a barrier to the permeation of polar molecules due to the hydrophobicity of its interior,[7] while also hosting lipophilic or amphiphilic membrane proteins, which are essential to cellular function. The lipid bilayer therefore plays a crucial role in cellular transport, signaling, recognition, and metabolic activity.[8] Intracellular lipid bilayers also enable compartmentalization in the cell by separating chemically distinct environments, and their variation in composition and fluidity allows selective transport of specific molecules from, and into, these compartments. Membrane proteins typically lose activity or undergo denaturation when extracted from lipid bilayers; thus, it is preferable to study their structure and modes of interaction with other biological components *in situ*. This is particularly true given the importance of membrane-spanning regions for the activity and downstream effects of many membrane proteins. The development and investigation of bioorthogonal reactions within the framework of a lipid bilayer is therefore a key challenge.

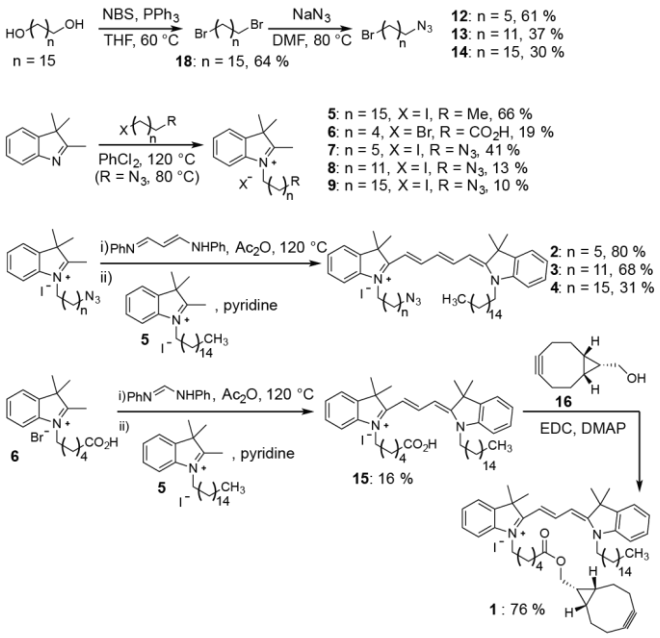
A suitable reaction for covalent conjugation within lipid bilayers must be able to proceed in an apolar, hydrophobic, and densely crowded environment. At the same time, it must proceed with high specificity, while ideally being uncatalyzed and proceeding with fast reaction kinetics. In this respect, the SPAAC reaction between an azide and a strained cyclooctyne has great potential. While the reaction rates of azides with simple cyclooctynes are relatively slow,[9] many derivatives with accelerated reactivities have been reported,[9-10] and the high hydrophobicity of

cyclooctynes results in dramatic rate enhancements of up to 1000-fold being observed in hydrophobic environments.[11] Here, we therefore report the synthesis of novel lipophilic dyes functionalized with SPAAC reactive handles, based on Cy3 and Cy5 cores designed to undergo FRET upon conjugation within lipid bilayers. We use these dyes, Lipo-Cy3-CO and Lipo-Cy5-N_3 respectively, to study bilayer-confined SPAAC by using complementary double-stranded DNA (dsDNA) hybridization to induce liposome–liposome fusion and initiate dye mixing.[12] Through a combination of synthetic design and atomistic simulation, we further show the importance of hydrophobicity on the retention of Lipo-Cy5-N_3 within the membrane, and the fusion-dependent formation of the SPAAC product within the hydrophobic bilayer. This study represents the first example of a bilayer-confined chemical reaction triggered by liposome fusion and offers great potential for novel bioconjugation labeling strategies for membrane components and the use of liposome fusion events for biosensing applications.

Results and Discussion

In order to study SPAAC reactions within a lipid bilayer, we envisaged the use of a FRET pair, which would be brought into proximity only upon covalent conjugation of a suitable cyclooctyne (CO) and azide (N_3). To achieve this goal, we synthesized lipophilic analogues of the cyanine dyes Cy3 and Cy5 bearing complementary SPAAC reactive handles. These novel asymmetric dyes, named Lipo-Cy3-CO (**1**) and Lipo-Cy5-N_3 (**2**-**4**), were synthesized bearing different pendant alkyl chain lengths. The quaternary ammonium indolenine compounds (**5**-**9**) were first obtained by functionalizing 2,3,3-trimethylindolenine with alkyl halide chains of varying lengths and end-functionalities: methyl (**5**), carboxy (**6**) or azide terminated (**7**-**9**) (Scheme 1). These indolenines were found to undergo tautomerism during column chromatography, and so products were isolated from the crude reaction mixture by precipitation as previously reported for similar structures.[13] However, due to the hydrophobic nature of the alkyl chains, a number of the product indolenines were found to be soluble in diethyl ether, requiring the use of *n*-hexane to induce precipitation or separation of the crude products. Although some impurities remained following this process, the products were sufficiently pure for further manipulation. Asymmetric cyanine dyes were then obtained by condensation of two functionalized quaternary ammonium indolenine compounds with a suitable imine linker to produce the Cy3 and Cy5 cores.[13] Lipo-Cy5-N_3 was synthesized from malonaldehyde bis(phenylimine) with three different alkyl azide chain lengths (6-, 12-, and 16-carbon long, **2**, **3**, and **4** respectively), leading to dyes with increased lipophilicity as will be discussed later (Figure S4). In an analogous fashion, the Cy3-based carboxy functionalized dye (**15**) was synthesized utilizing *N,N'*-diphenylformamidine as a linker, and subsequently esterified with alcohol-functionalized bicyclononyne (**16**) to create the functionalized dye Lipo-Cy3-CO (**1**) (Scheme 1).

The quantum yield (QY) and the fluorescence absorption and emission spectra of the synthesized dyes closely matched those of the commercially available lipophilic cyanine dyes DiI and DiD (non SPAAC-active analogues of Lipo-Cy3-CO and Lipo-Cy5-N_3) (Figure S1, Table S1). Introduction of the azide and



Scheme 1. Synthesis of Lipo-Cy3-CO (**1**) and Lipo-Cy5-N_3 (**2**-**4**) dyes, bearing cyclooctyne and azide functional groups respectively.

cyclooctyne groups did not therefore significantly alter the spectroscopic properties of the cyanine core. To test whether Lipo-Cy3-CO and Lipo-Cy5-N_3 were able to undergo SPAAC, they were incubated together in dichloromethane (DCM) and the fluorescence spectra measured following excitation at 530 nm. A strong FRET response indicated efficient formation of the cycloaddation product, in contrast to control mixtures of the functionalized dyes with DiI or DiD, which gave negligible FRET signal (Figure S2, Table S2).

Next, we investigated whether our dyes were able to undergo SPAAC within a lipid bilayer. To do so, we made use of our previously reported double stranded DNA (dsDNA)-mediated liposome fusion assay (Figure 1a).[12] The synthesized dyes (**1** & **2**, **3**, **4**) were individually integrated into the membranes of separate liposome populations composed of DOPC:DOPE:cholesterol (50:25:25). This lipid composition has previously been shown to be optimal for liposome fusion assays as it provides fast kinetics of lipid fusion when the liposomes are brought together by a coupling mechanism.[14] The liposome surfaces were functionalized with complementary double-stranded DNA, as detailed later. Upon DNA-triggered fusion, mixing of the membranes would be expected to bring the complementary reactive dyes into close contact and initiate SPAAC. However, in initial experiments with unfunctionalized liposomes (no DNA-triggered fusion) we observed an unexpected FRET signal when liposomes containing the Lipo-Cy5-N_3-C_{12} (**3**) acceptor were mixed with liposomes containing either Lipo-Cy3-CO (**1**) or DiI donors (Figure S3a). By contrast, liposome mixtures containing DiI and DiD did not generate a FRET response, while a mixture of Lipo-Cy3-CO (**1**) donor and DiD acceptor liposomes showed a relatively low response (Figure S3a). We reasoned that the FRET signal arising from the use of Lipo-Cy5-N_3-C_{12} (**3**) containing liposomes could be due to the migration of **3** between liposomes during random collision events, as well as by diffusion of the amphiphilic dyes through the aqueous media.[15] Such

effects have been previously reported for amphiphilic cargoes, such as phospholipids.[16]

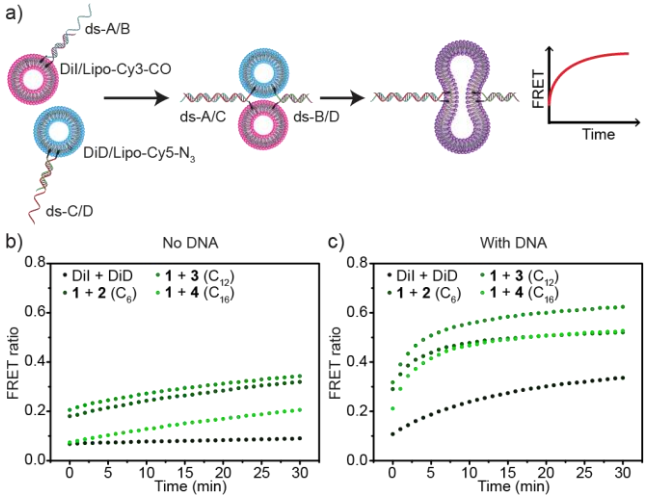


Figure 1. a) Schematic of the liposome fusion assay. Liposomes containing either a FRET donor or acceptor dye undergo fusion, mediated by zipper-like DNA hybridization between complementary sequences A/C and B/D,[14] resulting in a FRET increase following SPAAC.[12] b) Influence of Lipo-Cy5-N_3 alkyl chain length on FRET ratio as a result of non-specific dye transfer in the absence of complementary DNA. c) FRET ratio induced by SPAAC upon DNA mediated liposome fusion. Donor in Lipo-Cy5-N_3 (**2**, **3**, **4**) assays is Lipo-Cy3-CO (**1**).

To probe this hypothesis, an excess of liposomes containing no dye (unlabeled liposomes) was added to a 1:1 mixture of liposomes containing either Lipo-Cy3-CO (**1**) or Lipo-Cy5-N_3-C_{12} (**3**). In this scenario, if the exchange of dye between liposomes was non-specific, rather than induced by cycloaddition, the probability of Lipo-Cy5-N_3-C_{12} (**3**) transfer to unlabeled liposomes would be greater than the probability of transfer to Lipo-Cy3-CO (**1**) containing liposomes. As predicted, the addition of unlabeled liposomes resulted in an almost complete elimination of a FRET response upon mixing of liposomes containing Lipo-Cy3-CO (**1**) and Lipo-Cy5-N_3-C_{12} (**3**), or DiI and Lipo-Cy5-N_3-C_{12} (**3**) (Figure S3). This result strongly supports our hypothesis that non-specific transfer of Lipo-Cy5-N_3-C_{12} (**3**) between liposomes was responsible for the observed background FRET signal.

To improve membrane retention of the azide dye, we studied the influence of alkyl chain length and lipophilicity on migration for three different alkyl chain lengths, C_6 (**2**), C_{12} (**3**) and C_{16} (**4**). In all cases, the Lipo-Cy5-N_3 dyes underwent non-specific transfer into Lipo-Cy3-CO containing liposomes, causing an increase in FRET signal; however, the rate of transfer was greatly diminished for dye **4** (Figure 1b). Lipo-Cy5-N_3-C_{16} (**4**) is more lipophilic than the corresponding C_6 and C_{12} dyes (**2**, **3**) (Figure S4), leading to a stronger retention within the lipid bilayer and a corresponding decrease in the rate of transfer.

Atomistic molecular dynamics (MD) simulations were employed to explore the influence of Lipo-Cy5-N_3 alkyl chain length on dye–bilayer interactions and bilayer properties. Three experimentally consistent bilayer models with a DOPC:DOPE:cholesterol (50:25:25 mass ratio) composition and: a 2 mol% insertion of Lipo-Cy5-N_3-C_6 (**2**); Lipo-Cy5-N_3-C_{16} (**4**); or no dye (unlabeled) were constructed and simulated for 720 ns each in explicit water and physiological salt (150 mM NaCl).

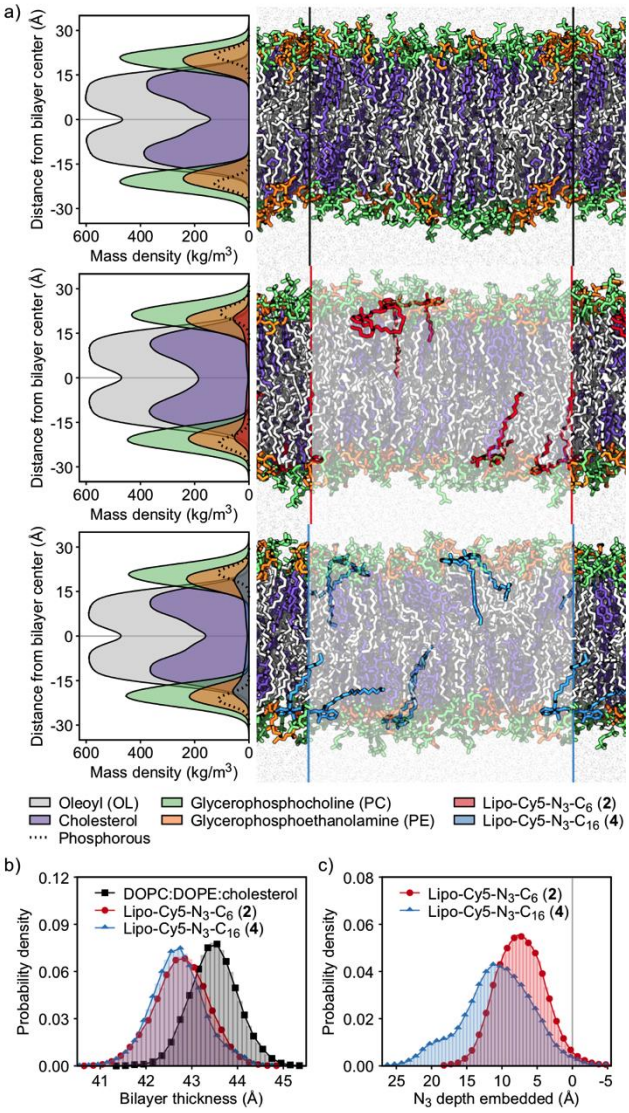


Figure 2. a) Mass density profiles (left) and representative structures (right) obtained from molecular dynamics (MD) simulations of an unlabeled DOPC:DOPE:cholesterol bilayer (top), and bilayers implanted with Lipo-Cy5-N_3-C_6 (**2**) (red, middle) and Lipo-Cy5-N_3-C_{16} (**4**) (blue, bottom). Solvent is not included in the density plots and only lightly shown in the images. MD obtained probability distributions for: (b) bilayer thickness and (c) Lipo-Cy5-N_3 azide embedding depth relative to the lipid/solvent interface (a depth of zero indicates N_3 is at the interface, whereas positive and negative distances are within the bilayer bulk or solvent, respectively).

MD simulations showed that the Lipo-Cy5-N_3 (2 mol%) bilayers remained stable and did not significantly restructure on the timescale of several hundred nanoseconds (Figure 2a). Compared to the non-defected (unlabeled) bilayer, compositional inclusion of dyes resulted in a vertical compression and lateral expansion of the membranes (ca. 2% in both directions), as signified by a decrease in the average bilayer thickness (Figure 2b, Figure S5), and an increase in the average area per bilayer molecule (Figure S6). Inspecting the dyes' bilayer integration more closely, in all cases the bulky cyanine headgroup remained near the solvent interface throughout the simulations (Figure S7, Table S3), sterically unable to embed into the lipid layer despite

being relatively hydrophobic. In contrast, the dyes' azide group could traverse from deeply inserted in the lipid layer to solvent exposed (Figure 2c, Figure S8), subject to the conformational extension and contortion of the pendant alkyl chain that the N_3 moiety flanked. While an increased chain length intuitively suggests that the N_3 group could access a deeper bilayer penetration depth, interestingly we found that the lipophilic nature of the C_{16} chain of **4** stabilized alkyl–alkyl interactions and encouraged localization of the azide moiety close to the internal interface between the two bilayer leaflets. This is emphasized in Figure 2c by the shoulder that emerges (> 16 Å) in the probability distribution of the N_3 embedding depth (also see Table S3). Although this finding cannot be directly correlated with the experimental results, it suggests that Lipo-Cy5-N_3-C_{16} (**4**) has an increased retention capacity within the lipid bilayer and therefore during random liposome collision events fewer dye transfer events would take place, in line with the observed decrease in experimental FRET signal (Figure 1b). The simulations also demonstrated that while both dyes (**2** and **4**) displayed similar lateral mobility characteristics within the membrane environment, the shorter C_6 dye disrupted lipid/cholesterol mobility more significantly than the C_{16} dye (Figure S9).

Next, the formation of a SPAAC product within the lipid membrane was studied following DNA self-assembly triggered liposome–liposome fusion. Liposomes containing either a donor (DiI or Lipo-Cy3-CO (**1**)) or an acceptor dye (DiD or Lipo-Cy5-N_3 (**2**-**4**)) were surface-functionalized with 100 copies of self-inserting, cholesterol-functionalized dsDNA per liposome, ds-A/B or ds-C/D (DNA sequences in Table S4).[12, 14b] In this system, liposome fusion is promoted by the hybridization of ds-A/B and ds-C/D in a zipper-like fashion, and fusion induces mixing of the fluid lipid components (Figure 1a). An intensified FRET signal therefore results as the complementary dyes are brought into close proximity and SPAAC is enabled. The kinetics of FRET evolution during DNA-triggered fusion was measured for 30 min (Figure 1c). When liposomes were loaded with DiI and DiD a gradual increase in FRET ratio was observed, due to freely diffusing dyes being brought into transient proximity within the fused membranes, enabling some level of energy transfer. Consequently, the ability to generate a FRET response in this system is limited by the concentration of the dyes within the membrane and is therefore ultimately controlled by dye diffusion. In contrast, when utilizing SPAAC functionalized dyes the FRET ratio was up to two times higher. At first, we believed this increase to be indicative of covalent conjugation within the lipid membrane, ensuring that the FRET dye pair was maintained within close proximity, and leading to effective energy transfer. However, similar intensified FRET signals were also observed for control experiments using mixtures of DiI and Lipo-Cy5-N_3 liposomes (Table S5), potentially indicating that the increased FRET signal was caused by the intrinsic properties of the SPAAC-functionalized dyes (e.g. diffusion, solubility, critical aggregation concentration). To distinguish between these two possibilities and to quantify the efficiency of the SPAAC reaction in lipid bilayers, it was therefore important to delineate the FRET occurring in response to the proximity of freely diffusing dyes or non-covalent interactions, from those that were covalently conjugated.

Post-fusion liposomes were therefore lysed at various time points through the addition of ethanol (Figure S10),[17] leading to rapid dilution of the dyes, and ensuring that the resultant FRET signal emanated exclusively from covalently conjugated dye products. To prevent donor–acceptor dye SPAAC conjugation occurring after liposome disruption, a large excess of an azide-PEG (**17**) was present during lysis to react with residual Lipo-Cy3-CO (**1**).

To elucidate contributions that arise from semi-transient donor–acceptor dye confinement versus covalent dye conjugation, FRET spectra for different dye combinations were measured on intact liposomes following DNA-mediated fusion (30 min) and then again after liposome disruption with ethanol (Table S5, Figure S11). While a significant FRET was observed for all dye combinations in fused liposomes, a strong FRET signal was only retained after lysis for mixtures of Lipo-Cy3-CO/Lipo-Cy5-N_3 liposomes, demonstrating that SPAAC had successfully taken place to form the cycloaddition product **19** within the lipid bilayer. Formation of the SPAAC product was further confirmed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Figure S12).

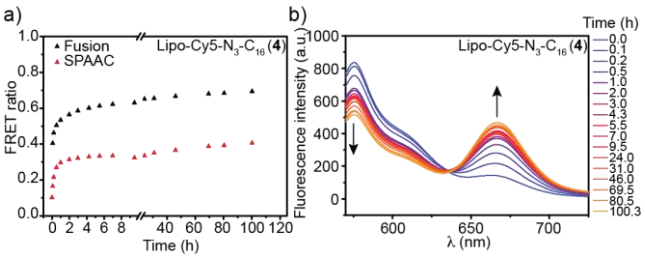


Figure 3. Kinetics of the SPAAC reaction between Lipo-Cy5-N_3-C_{16} (**4**) and Lipo-Cy3-CO (**1**) loaded liposomes. a) Kinetic evaluation of FRET ratio to assess liposome fusion (black symbols) and chemical conjugation after lysis (red symbols). b) FRET spectra evolution over time, post liposome disruption with ethanol.

Finally, we studied the kinetics of the SPAAC reaction within the lipid bilayer (Figure 3, Figure S13). Equal volumes of dsDNA modified, Lipo-Cy3-CO (**1**) and Lipo-Cy5-N_3 (**4**) containing liposomes were mixed, and aliquots from the mixture were removed at various time intervals. FRET spectra were measured both prior to and post liposome disruption as described above, enabling the kinetics of both liposome fusion and chemical conjugation to be followed. The kinetics of SPAAC closely followed the evolution of the liposome fusion kinetics, albeit with a lowered FRET ratio. This reduction can be rationalized through the interaction of multiple donor and acceptor dyes together within the confined space of the lipid membrane, increasing FRET efficiency.[18] After liposome disruption, the FRET signal is limited to interactions between a single chemically conjugated donor–acceptor pair. Little difference was observed in SPAAC reaction kinetics between Lipo-Cy5-N_3 dyes with different alkyl chains lengths (**2**-**4**) (Figure S13), which could suggest that the reaction rate under these conditions is not limited by the diffusion of the dyes within the lipid bilayer, but rather by liposome fusion events, although further investigations are required to probe this effect. Finally, we investigated whether decreasing the concentration of dyes inside the liposomes could enable us to follow the kinetics of the SPAAC reaction directly, without resorting to the irreversible ethanol step (Figure S14). We postulated that decreasing the dye-to-lipids ratio would also decrease the random collision FRET. When the dye concentration was decreased from 2 mol% to 0.5 mol% and 0.1 mol%, the FRET signal arising from random collision events also decreased, and the best signal-to-noise ratio was obtained with a dye concentration of 0.5 mol% (Figure S14).

Conclusion

In conclusion, we have presented the synthesis of novel cyclooctyne- and azide-functionalized lipophilic dyes, Lipo-Cy3-CO (**1**) and Lipo-Cy5-N_3 (**2**-**4**), and demonstrated that they successfully undergo SPAAC within the complex hydrophobic environment of a lipid bilayer. This study represents the first example of a liposome fusion-triggered chemical reaction, and sheds light on the features of SPAAC in lipid bilayers. Our results highlight the importance of rational molecular design to maximize the retention of amphiphilic species within liposomal membranes, and to ensure mixing of reactive pairs only upon liposome fusion. The work presented here demonstrates the potential uses of small lipophilic molecules within liposome bilayers for novel bioconjugation strategies and opens up powerful possibilities for the study of native cell membranes and membrane-embedded biomolecules. Importantly, the FRET signal generated by the Lipo-Cy3-CO and Lipo-Cy5-N_3 dyes in this work was significantly greater than the commercially available dyes DiI and DiD, which have previously found use in liposome fusion based biosensing assays.[12, 19] SPAAC-enhanced FRET generation therefore also offers the potential for greatly improved liposome-fusion biosensing assays for the detection of disease, through a reduced limit of detection and increased sensitivity. We are currently undertaking further explorations to this extent.

Acknowledgements

C.J. and M.M.S. gratefully acknowledge financial support from the Rosetrees Trust. M.M.S. acknowledges support from the Engineering and Physical Sciences Research Council (EPSRC) through the Interdisciplinary Research Centre (IRC) "Early Warning Sensing Systems for Infectious Diseases" (EP/K031953/1) and from the grant "Bio-functionalised Nanomaterials for Ultrasensitive Biosensing" (EP/K020641/1). I.Y. and M.M.S. acknowledge support from the Australian Research Council Discovery grants DP140101888 and DP170100511. P.C., M.H.R. and I.Y. acknowledge generous allocation of high-performance computing resources by the Australian computational infrastructure facilities NCI, iVEC and Melbourne Bioinformatics (grant e87). M.M.S. acknowledges support from the EPSRC programme grant “Advanced Functional Materials” (EP/M020398/1).

We thank Dr Yiyang Lin for help with Matrix-Assisted Laser Desorption/Ionization (MALDI) experiments.

Keywords: SPAAC • liposomes • bioconjugation • molecular dynamics • FRET

[1] a) J. C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272-1279; b) C. D. Spicer, B. G. Davis, *Nat. Commun.* **2014**, *5*, 4740.

[2] L. Shah, S. T. Laughlin, I. S. Carrico, *J. Am. Chem. Soc.* **2016**, *138*, 5186-5189.

[3] a) E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007-2010; b) E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141-2143.

[4] Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192-3193.

[5] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046-15047.

[6] A. B. Neef, C. Schultz, *Angew. Chem.* **2009**, *121*, 1526-1529; A. B. Neef, C. Schultz, *Angew. Chem. Int. Ed.* **2009**, *48*, 1498-1500.

[7] W. K. Subczynski, A. Wisniewska, J. J. Yin, J. S. Hyde, A. Kusumi, *Biochemistry* **1994**, *33*, 7670-7681.

[8] a) I. G. Denisov, S. G. Sligar, *Nat. Struct. Mol. Biol.* **2016**, *23*, 481-486; b) M. S. Khan, N. S. Dosoky, J. D. Williams, *Int. J. Mol. Sci.* **2013**, *14*, 21561-21597.

[9] M. F. Debets, S. S. van Berkel, J. Dommerholt, A. T. Dirks, F. P. Rutjes, F. L. van Delft, *Acc. Chem. Res.* **2011**, *44*, 805-815.

[10] a) J. A. Codelli, J. M. Baskin, N. J. Agard, C. R. Bertozzi, *J. Am. Chem. Soc.* **2008**, *130*, 11486-11493; b) M. F. Debets, S. S. van Berkel, S. Schoffelen, F. P. Rutjes, J. C. van Hest, F. L. van Delft, *Chem. Commun.* **2010**, *46*, 97-99; c) J. C. Jewett, E. M. Sletten, C. R. Bertozzi, *J. Am. Chem. Soc.* **2010**, *132*, 3688-3690.

[11] a) H. Tian, T. P. Sakmar, T. Huber, *ChemBioChem* **2015**, *16*, 1314-1322; b) G. I. Anderton, A. S. Bangerter, T. C. Davis, Z. Feng, A. J. Furtak, J. O. Larsen, T. L. Scroggin, J. M. Heemstra, *Bioconjug. Chem.* **2015**, *26*, 1687-1691.

[12] C. Jumeaux, E. Kim, P. D. Howes, H. Kim, R. Chandrawati, M. M. Stevens, *Nanoscale Adv.* **2019**, *1*, 532-536.

[13] M. V. Kvach, A. V. Ustinov, I. A. Stepanova, A. D. Malakhov, M. V. Skorobogatyi, V. V. Shmanai, V. A. Korshun, *Eur. J. Org. Chem.* **2008**, *2008*, 2107-2117.

[14] a) G. Stengel, L. Simonsson, R. A. Campbell, F. Hook, *J. Phys. Chem. B* **2008**, *112*, 8264-8274; b) G. Stengel, R. Zahn, F. Hook, *J. Am. Chem. Soc.* **2007**, *129*, 9584-9585.

[15] L. R. McLean, M. C. Phillips, *Biochemistry* **1981**, *20*, 2893-2900.

[16] a) F. J. Martin, R. C. MacDonald, *Biochemistry* **1976**, *15*, 321-327; b) S. Loew, A. Fahr, S. May, *J. Drug Deliv.* **2011**, *2011*, 376548.

[17] J. Zhou, Q. X. Wang, C. Y. Zhang, *J. Am. Chem. Soc.* **2013**, *135*, 2056-2059.

[18] A. I. Fabian, T. Rente, J. Szollosi, L. Matyus, A. Jenei, *ChemPhysChem* **2010**, *11*, 3713-3721.

[19] C. Jumeaux, O. Wahlsten, S. Block, E. Kim, R. Chandrawati, P. D. Howes, F. Hook, M. M. Stevens, *ChemBioChem* **2018**, *19*, 434-438.