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Liposome functionalization with copper-free "click chemistry"

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ABSTRACT

The modification of liposomal surfaces is of interest for many different applications and a variety of chemistries are available that makes this possible. A major disadvantage of commonly used coupling chemistries (e.g. maleimide–thiol coupling) is the limited control over the site of conjugation in cases where multiple reactive functionalities are present, leading to heterogeneous products and in some cases dysfunctional conjugates. Bioorthogonal coupling approaches such as the well-established copper-catalyzed azide-alkyne cycloaddition (CuAAC) "click" reaction are attractive alternatives as the reaction kinetics are favorable and azide-containing reagents are widely available. In the work described here, we prepared lipids containing a reactive cyclooctyne group and, after incorporation into liposomes, demonstrated successful conjugation of both a small molecule dye (5′-TAMRA-azide) as well as a larger azide-containing model protein based upon a designed ankyrin repeat protein (azido-DARPin). By applying the strain-promoted azido-alkyne cycloaddition (SPAAC) the use of Cu(I) as a catalyst is avoided, an important advantage considering the known deleterious effects associated with copper in cell and protein studies.

We demonstrate complete control over the number of ligands coupled per liposome when using a small molecule azide with conjugation occurring at a reasonable reaction rate. By comparison, the conjugation of a larger azide-modified protein occurs more slowly, however the number of protein ligands coupled was found to be sufficient for liposome targeting to cells. Importantly, these results provide a strong proof of concept for the site-specific conjugation of protein ligands to liposomal surfaces via SPAAC. Unlike conventional approaches, this strategy provides for the homogeneous coupling of proteins bearing a single site-specific azide modification and eliminates the chance of forming dysfunctional ligands on the liposome. Furthermore, the absence of copper in the reaction process should also make this approach much more compatible with cell-based and in vivo applications.

1. Introduction

Liposomes (phospholipid vesicles of 60–200 nm in size) have been widely used as model systems to mimic cell membranes, as nanocompartments to contain complex chemical or enzymatic reactions, and as drug delivery systems for the controlled and targeted delivery of drugs in the human body. Many of these applications require surface modification of the liposomes, i.e. the covalent attachment of functional molecules such as targeting ligands to preformed liposomes. The common strategy is to synthesize a new lipid that contains a reactive group that can react with a complementary reactive group on the ligand. Many different chemistries have been explored for surface modification [1]. Ideally, these reactions should be fast and specific and reaction conditions should be mild enough not to cause damage to the lipid membrane or to the ligand. In cases where the ligand to be coupled is a protein it is crucial that the coupling reaction does not negatively impact the structure and associated activity of the protein. In this regard, the most widely used coupling method is based upon maleimide–thiol coupling, where the liposomes are functionalized with a maleimide group that in turn reacts with a thiol-containing ligand to form a thioether bond. In the case of proteins, both naturally occurring thiol groups (e.g. found on cysteine residues) or introduced by a chemical reaction (e.g. by thiolation of lysines) can be used for coupling [2]. When using peptide or protein ligands, a common disadvantage of using naturally occurring reactive groups can be that the reaction is not site-specific. Thiols, amines, and carboxylic acid groups become more abundant with increasing protein size, and as such generate more possible conjugation sites. This can result in heterogeneous coupling where ligands might be conjugated at different or multiple sites, leading to the need for complicated separation schemes to obtain homogenous products. Even more problematic is the chance that binding affinity might be compromised if the reacting group is too close to the binding site of the protein to be immobilized.

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Site-specific conjugation can be achieved by employing bio-orthogonal coupling reactions such as 'click-chemistry' approaches. In this regard the Cu(I)-catalyzed reaction between an azide and an alkyne is fast and efficient and allows homogeneous and site-specific conjugation because the reactive groups can be introduced at a site of choice. The Cu(I)-catalyzed azide-alkyne [3 + 2] cycloaddition (CuAAC) was first applied in the context of liposomes by the group of Schuber who employed an alkyne-modified lipid to couple an azido-modified mannose ligand [3]. The use of the required copper catalyst however, is a limitation as it is known to be toxic to cells and can interfere with protein activity [4]. An alternative is the Staudinger ligation, in which a phosphine group reacts with the azide to form an amide bond [5]. This was also used for liposome functionalization [6] but the kinetics of this reaction are slow and the phosphine group is prone to oxidation [7]. As an alternative meant to address these limitations, the strain-promoted azido-alkyne cycloaddition (SPAAC) has been developed. In SPAAC ligations a ring-strained alkyne is reactive enough to lead to spontaneous addition with an azide, a process that eliminates the need for a toxic metal catalyst and with faster reaction kinetics [8]. Importantly, the in vivo compatibility and bio-orthogonality of copper-free SPAAC ligations have also been successfully demonstrated inside living organisms [9,10].

In the present work we describe the preparation of two different lipids that contain a reactive bicyclo[6.1.0]nonyne (BCN) cyclooctyne group [11] capable of "clicking" with a variety of azide-containing ligands (Fig. 1). Both small and large (bio)molecules containing azides are readily available and methods to introduce the azide functionality into peptides or proteins are numerous [12]. Azide incorporation can be performed after protein expression using standard labeling approaches [13]. Alternatively, azide-containing amino acids can be specifically introduced during protein biosynthesis, allowing for complete control over the location of the azide [14–17]. We demonstrate here the incorporation of our new BCN-lipids into the bilayer of liposomes followed by the successful coupling of both azide-containing small molecule ligands and recombinantly expressed proteins at the liposomal surface.

2. Materials and method

2.1. Chemicals

Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and L-α-phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-PE) were from Avanti Polar Lipids (Alabaster, AL, USA). (1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate was from SynAffix BV, (Oss, The Netherlands) dioctadecylamine, cholesterol, calcein and Triton X-100 were from Sigma-Aldrich (St. Louis, MO, USA). 5-Carboxytetramethylrhodamine-PEG3-Azide (5-TAMRA-PEG3-Azide) was from Baseclick GmbH (Tutzing, Germany).

2.2. Synthesis of lipid-BCN conjugates ***1*** and ***2***

DOPE-BCN conjugate (1): (2*R*)-3-(((2-(((((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)amino)ethoxy)(hydroxy)phosphoryl)oxy)propane-1,2-diyl dioleate.

(1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-ylmethyl succinimidyl carbonate (50 mg, 0.17 mmol) was dissolved in 6 ml dry CH_2Cl_2 which was then added to a solution of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (127 mg, 0.17 mmol) in 4 ml dry CH_2Cl_2. NEt_3 (78 μl, 0.55 mmol) was added and the solution was left stirring overnight. The product was purified directly by column chromatography (95:5 CH_2Cl_2/MeOH) to yield the product as a colorless oil (114 mg, 73%). Analytical data: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.35 (bs, 2H), 5.82 (s, 1H), 5.31 (m, 3H), 5.18 (m, 1H), 4.36 (dd, 2H), 4.11 (m, 3H), 3.92 (m, 4H), 3.57 (m, 1H), 3.38 (d, 2H), 3.07 (q, 6H), 2.62 (s, 4H), 2.23 (m, 8H), 1.97 (q, 6H), 1.26 (m, 38H, should be 40H), 0.84 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.57, 130.00, 129.62, 129.59, 98.74, 62.68, 34.19, 34.01, 31.89, 29.75, 29.53, 29.31, 29.24, 29.22, 29.18, 29.15, 27.22, 24.90, 24.83, 22.67, 21.41, 20.15, 14.11; HRMS (ESI) calcd for $C_{52}H_{89}NO_{10}P$ $[M-H]^-$ 918.6224 found 918.6198.

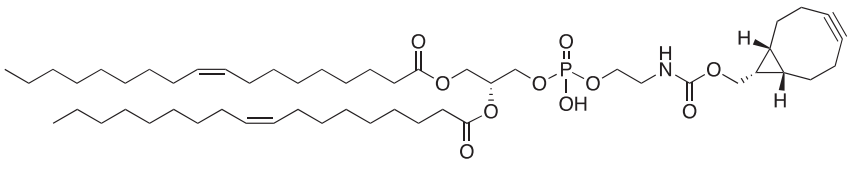
Dioctadecylamine-BCN conjugate (2): ((1*R*,8*S*,9*s*)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (15-octadecyl-10,14-dioxo-3,6-dioxa-9,15-diazatritriacontyl)carbamate.

BCN-POE_3-NH-C(O)$CH_2CH_2CH_2$C(O)OSu (100 mg, 0.186 mmol) was dissolved in 6 ml dry CH_2Cl_2 which was then added to a suspension of dioctadecylamine (100 mg, 0.192 mmol) in 4 ml dry CH_2Cl_2. Et_3N (78 μl, 0.55 mmol) was added and over 2 h the solution became homogenous. The solution was left to stir after which the product was purified directly by column chromatography (95:5 EtOAc:MeOH) resulted in a colorless oil (152 mg, 87%).

Analytical data: R_f 0.25 (95:5 EtOAc/MeOH); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.38 (s, 1H), 5.40 (s, 1H), 4.16 (d, 2H), 3.61 (bs, 4H), 3.56 (q, 5H), 3.41 (m, 5H), 3.27 (t, 2H), 3.19 (t, 2H) 2.37 (t, 2H), 2.25 (m, 8H), 1.95 (t, 2H), 1.26 (bs, 80H, should be 72), 0.88 (m, 8H); ^{13}C NMR (75 MHz, D_2O) δ 170.27, 169.30, 154.24, 96.18, 67.62, 67.52, 67.30, 60.09, 45.39, 43.36, 38.17, 36.53, 33.18, 29.32, 27.10, 27.06, 26.98, 26.86, 26.79, 26.76, 26.54, 26.46, 25.23, 24.50, 24.33, 20.09, 20.05, 19.00, 18.82, 17.52, 15.20, 11.51; HRMS (ESI) calcd for $C_{58}H_{108}N_3O_6$ $[M + H]^+$ 942.8232 found 942.8257.

2.3. Liposome preparation

Lipid stock solutions were prepared in ethanol and combined in a round bottom flask in a DOPC/DOPE/cholesterol/BCN-lipid ratio of 49/25/25/1. Labeled liposomes for flow cytometry were made by adding 0.2 mol% of Rho-PE. After removal of organic solvent with a rotary vacuum pump the resulting lipid film was flushed with nitrogen. Liposomes were formed by hydration of the lipid film with Hepes buffered saline (HBS; 10 mM Hepes, 145 mM NaCl, pH 7.4) to a final concentration of 50 mM total lipid (TL). The size of the liposomes was reduced by



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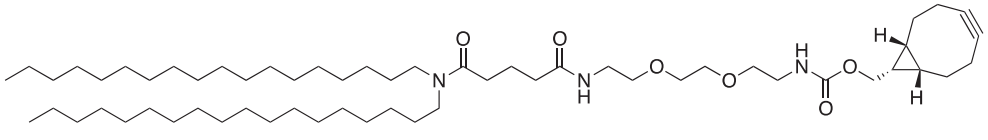

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Fig. 1. Structures of BCN-lipid conjugates **1** and **2** prepared for incorporation into liposomes allowing for modification with azides via SPAAC.