Journal of Controlled Release 202 (2015) 14–20

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Liposome functionalization with copper-free "click chemistry"

Erik Oude Blenke [a], Gruson Klaasse [b], Hannes Merten [c], Andreas Plückthun [c], Enrico Mastrobattista [a], Nathaniel I. Martin [b,]*

[a] Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[b] Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute of Pharmaceutical Sciences (UIPS), Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[c] Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

A R T I C L E I N F O

Article history:
Received 25 November 2014
Accepted 23 January 2015
Available online 24 January 2015

Keywords:
Liposomes
Copper-free click chemistry
Strain-promoted azido-alkyne cycloaddition
Site specific conjugation
DARPins

A B S T R A C T

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.

© 2015 Elsevier B.V. All rights reserved.

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.

* Corresponding author.
E-mail address: n.i.martin@uu.nl (N.I. Martin).

http://dx.doi.org/10.1016/j.jconrel.2015.01.027
0168-3659/© 2015 Elsevier B.V. All rights reserved.

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

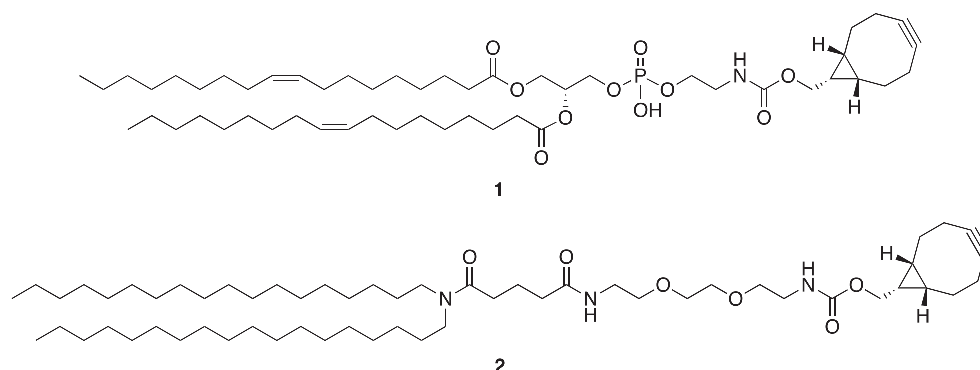

Fig. 1. Structures of BCN-lipid conjugates **1** and **2** prepared for incorporation into liposomes allowing for modification with azides via SPAAC.

Table 1
Size and charge characteristics of the different formulations.

Formulation	Size (μm)	PDI	Zeta potential (mV)
No anchor	0.12 ± 0.03	0.09	−3.5 ± 0.2
Unfunctionalized	0.12 ± 0.03	0.08	−3.1 ± 0.1
10:1 TAMRA-dye (0.1%)	0.12 ± 0.03	0.08	−4.4 ± 0.2
Ac2 DARPin	0.12 ± 0.04	0.1	−16.6 ± 0.6
Off7 DARPin	0.12 ± 0.03	0.09	−17.0 ± 0.4

extruding at least 20 times using a Lipex™ Extruder (Northern Lipids, Burnaby, BC, Canada) through polycarbonate membranes with a final pore size of 100 nm (Nuclepore, Pleasanton, CA, USA). Liposomes were then incubated with 5-TAMRA-PEG3-Azide (Baseclick, Tutzing, Germany) in methanol in a 10:1 ratio of BCN-lipid:azide unless stated otherwise (this 10:1 ratio is corrected for the amount of BCN-lipid facing the inside of the liposome and as such is not available for coupling). The final methanol concentrations added to the liposomes were less than 0.25% (v/v).

2.4. Azido-DARPin conjugation to liposomes

To serve as an azide-containing model protein, a designed ankyrin repeat protein (azido-DARPin) was employed. EpCAM targeting DARPin 'Ac2' and Maltose Binding Protein (MBP) targeting control DARPin 'Off7' were expressed as previously described [18,19] where azido-homoalanine was incorporated at the *N*-terminus instead of the initiator methionine and no cysteine functionality was introduced.

After preparation, liposomes were incubated overnight with azido-DARPin solutions in PBS, in BCN-lipid:azide ratios ranging from 100:1 to 5:1. After incubation, liposomes were ultracentrifuged at 55,000 rpm and resuspended three times, to wash away unconjugated protein. The total amount of phosphate in the resuspended samples was compared to an uncentrifuged sample to calculate the dilution and loss of sample between runs.

2.5. Liposome characterization

The total phosphate content of the liposome formulations was determined according to the method of Rouser et al. [20] with sodium biphosphate as a standard after destruction of the phospholipids with perchloric acid (PCA) and heating at 180 °C. The mean particle size and the polydispersity index were measured by dynamic light scattering, using a Malvern CGS-3 multiangle goniometer with He–Ne laser source (λ = 632.8 nm, 22 mW output power) under an angle of 90° (Malvern Instruments, Malvern, UK). The zeta-potential of the liposomes was measured using laser Doppler electrophoresis on a Zetasizer Nano-Z (Malvern Instruments) with samples dispersed in 10 mM Hepes buffer pH 7.4 (no added salts).

2.6. Coupling quantification by UPLC

Quantification of the coupling was done with a Waters Acquity UPLC System (Waters Corporation, Milford, MA, USA) with PDA and FLR detectors on a BEH300 C18 1.7 μm column. The gradient mobile phase at a flow rate of 0.25 ml/min was changed from 100% solvent A (Acetonitrile:H_2O 5:95 with 0.1% PCA) to 100% solvent B (Acetonitrile with 0.1% PCA) in 5 min and ran on solvent B for 2 additional minutes.

UV detection with the PDA detector was done on 210 nm and the FLR detector was used at 545/575 for the 5-TAMRA containing samples. Free ligand had a retention time of 2–3 min whereas conjugated ligand eluted at the end of the chromatogram with the liposomes. Peaks in the FLR channel were integrated and AUCs were used to calculate the ratio of coupled/uncoupled.

2.7. SDS-PAGE and Western blotting

All liposome samples were diluted to a concentration of 2 mM phospholipids as this was found to be the highest amount of lipid that could be loaded without interfering with the electrophoresis of the protein on SDS-PAGE. The DARPin stock solution was diluted to concentrations equaling 100%, 75%, 50%, 40%, 30%, 20% and 10% of the total added amounts of protein in each of the liposome samples (20:1 ratio, 10:1 ratio, 5:1 ratio).

Samples were mixed with reducing loading buffer and run on Bolt® 4–12% Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) and then electrotransferred to a nitrocellulose membrane using the iBlot Dry Blotting system (Life Technologies). The membrane was blocked with 5% BSA in TBS–0.1% Tween 20 (TBS-T) for 1 h at RT and then stained overnight at 4 °C with a mouse anti-polyhistidine tag monoclonal antibody (CLANT227, Cedar Lane Technologies, Burlington, Ontario, Canada) diluted 1:1000 in 5% BSA in TBS-T. After washing with TBS-T the membrane was incubated with goat anti-mouse IgG (H + L) with HRP conjugate (#31430 Pierce Antibodies) diluted 1:2000 in 5% BSA in TBS-T. After washing, protein bands were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and detected using a Gel Doc Imaging system equipped with a XRS camera and Quantity One analysis software (Bio-Rad). Coupling efficiency was determined based on band intensity, as quantified with ImageJ software (v1.48 for Windows PC).

2.8. Flow cytometry

HT29 cells were cultured in Dulbecco's Modified Eagle's Medium with high glucose (Sigma-Aldrich) containing 4.5 g/L D-dextrose and 4 mM L-glutamine supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were regularly tested negative for mycoplasma.

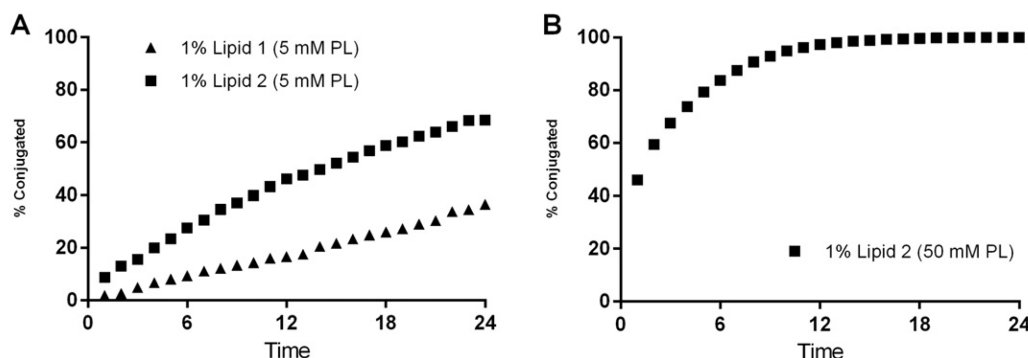

Fig. 2. Reaction kinetics of 5′-TAMRA-azide to the liposomes. A: Initial comparison of both anchors, 1% functional lipid incorporated in 5 mM total phospholipids. B: 1% of Lipid 2 incorporated in 50 mM total phospholipids.

Cells were harvested and seeded at 50.000 cells/well in a round-bottom 96-well plate. Rho-labeled liposomes with and without EpCAM targeting DARPin (Ac2) or with MBP-targeting control DARPin (Off7) were added to the wells in triplicate in a final concentration of 7.8125 μm–1 mM total lipid and incubated in the dark at 4 °C.

For the experiment varying the ligand density, Rho-labeled liposomes with targeting DARPin (Ac2) densities of 0.01%–0.2% were added to the wells in triplicate at a fixed final concentration of 0.5 μm or 0.25 μm total lipid and incubated in the dark at 4 °C.

After incubation cells were washed three times with 0.3% BSA in PBS and fixed with a final concentration of 2.5% formalin (from a 10% Neutral Buffered Formalin Solution, Sigma-Aldrich). The mean rhodamine fluorescence intensity was measured with a BD FACSCanto II (Becton & Dickinson, Mountain View, CA, USA) and 10.000 events per well were acquired. Data was analyzed with BD FACSDiva™ software. Histogram overlays were made manually in Photoshop CS6 (Adobe) using the Diva-Fit method [21].

2.9. Calcein leakage

To show that the incorporation of the anchor or that the conditions of the click reaction itself do not damage or cause leakage of the membrane, the leakage of calcein from the liposomes was measured. Liposomes were loaded with 75 mM calcein, which is quenched in this concentration, creating a low baseline signal. When calcein leaks out, it is diluted over the large exterior volume. A further dilution step assures that leaked calcein concentration is in the linear range. Leakage was monitored in the presence and absence of the 5′-TAMRA-azide in 10:1 ratio (BCN-lipid:azide). Liposomes with and without the anchor were also compared, as well as the influence of incubation at 4 °C or at room temperature. Samples were taken at t = 0, t = 1, t = 2, t = 4, t = 8, t = 12 and t = 24 h and calcein fluorescence was measured at 485/520 nm. Leakage was expressed as percentages using the formula

$$\text{Leakage}\ (\%) = \frac{\text{signal}(t) - \text{signal}\ (0)}{\text{signal}(\max) - \text{signal}(0)}$$

where the maximum signal was obtained by the solubilization of the liposomes using 0.5% final concentration of Triton X-100. Samples were diluted before measuring to a concentration where Triton X-100 did not interfere with the fluorescence detection.

3. Results and discussion

3.1. Liposome characterization

Several liposome formulations bearing different surface modifications were examined. Preliminary comparison of the two BCN-lipid conjugates revealed little difference in the ease of incorporation into liposomes. We did, however, observe slightly better azide coupling efficiency with compound **2** and for this reason employed this BCN-lipid in all subsequent experiments. The optimal liposome composition was found to consist of a mixture of DOPC/DOPE/cholesterol in a ratio of 50/25/25 with and without 1% of BCN-lipid **2**. Extrusion through 0.1 μm membranes typically produced vesicles of 0.12 μm in diameter and a polydispersity of <0.1. While the incorporation of 1% of the BCN-lipid did not change the characteristics of the vesicles, the incorporation of 5% of the synthetic lipid did increase the size and polydispersity slightly (data not shown). Measurements of the zeta-potential showed that the liposomes are neutral when unfunctionalized and acquire a negative charge when the DARPins are conjugated. This change was expected as the liposome surfaces here used are not PEGylated and protein conjugation thus has a significant influence on charge. See Table 1.

Table 2
BCN-lipid:azide ratios used for conjugation to the small molecule azide-dye. Corresponding numbers of ligand per liposome are listed in the third row. Numbers in parentheses are theoretical, as conjugation was not complete in these ratios.

Azide:BCN ratio	0.01	0.02	0.04	0.10	0.20	0.40	1.00	2.00
% azide conjugated	100%	100%	100%	100%	100%	>95%	50%	25%
Ligands conjugated per liposome	5	10	20	50	100	(200)	(500)	(500)

3.2. Coupling efficiency of small molecule azide-dye

To investigate the coupling efficiency of the SPAAC ligation a commercially available 5′-TAMRA-azide dye was coupled the liposomes. Using fluorescence detection, this dye could be readily detected with high accuracy without interference from any of the other components. As expected, the retention time of the TAMRA dye–lipid conjugate increased relative to the azide dye starting material as evidenced by a shifting of the peak to the later end of the UPLC chromatogram. Integration of the peak areas for signals corresponding to the conjugated and unconjugated species were used to calculate the conjugated/unconjugated ratio.

The synthetic BCN-lipids **1** and **2** were compared in a low liposome concentration (5 mM total lipid) to investigate the reaction kinetics of each when coupling to the 5′-TAMRA-azide at 10:1 BCN-lipid:azide ratio. At these concentrations, the conjugation was not complete after 24 h, but the reaction rate when using lipid **2** was significantly faster than for lipid **1** (see Fig. 2A). The difference in reaction rate may be due to a difference in the length of the spacer unit. In BCN-lipid **1**, the reactive cyclooctyne unit is conjugated directly to the phosphate headgroup, whereas lipid **2** contains a longer spacer making the reactive group more mobile and less prone to steric hindrance at the liposomal membrane surface.

Because of the favorable coupling kinetics we chose to continue exclusively with BCN-lipid **2** for further characterization. When the total lipid concentration was increased to 50 mM total lipid, reaction kinetics dramatically increased to ~75% conversion after 4 h and conjugation was essentially complete after overnight incubation (Fig. 2B).

Next, the conjugation efficiency of different BCN-lipid:azide ratios was investigated. At the same lipid concentration, ratios from 100:1 to 1:1 were incubated as well as with a twofold excess of azide (see Table 2). Coupling of all ratios down to 5:1 was complete after overnight incubation. At the ratio of 2.5:1 coupling was more than 95% but in the 1:1 ratio only 50% of the dye molecules were conjugated. This is not explained by the fact that half of the reactive BCN moieties are on the inside of the liposomes, as the ratios mentioned are corrected for this (ratios are relative to the amount of BCN on the outside of the liposomal surface, which is assumed to be 50%). Interestingly, the level of conjugation measured for the sample that was incubated with a 1:2 ratio of BCN:azide, was only 25%. The observation that when working with substoichiometric levels of the BCN-lipid the amount surface conjugated dye cannot be increased by simply adding more of the azide-dye

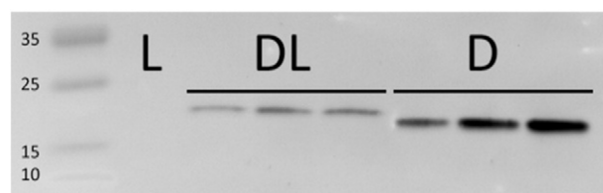

Fig. 3. Western blot of DARPins and conjugated-ADRPin liposome samples. (L) Liposomes without BCN-lipid (DL) DARPin-conjugated liposomes in 20:1, 10:1, 5:1 BCN-lipid:azide ratios (D) unconjugated DARPin samples corresponding to 50% of the amount added to each of the conjugated samples.

Table 3
BCN-lipid:azide ratios used for conjugation to the azido-DARPins.

Azide:BCN ratio	0.05	0.10	0.20
% azide conjugated	23.4%	17.5%	11.4%
Ligands conjugated per liposome	6	9	12

may indicate that a fraction of the BCN groups are not reactive or available for binding. A possible explanation is that part of the liposomes is not unilamellar, although that is generally assumed when membrane extrusion is used. If a liposome has more than one bilayer, the BCN groups in the inner layers are not available for reaction and total available fraction is less than 50%. Another possible reason for incomplete availability of the BCN moiety may be due to its relative hydrophobicity, causing it to fold back into the bilayer as has been previously described in polymer vesicles [22].

A final explanation for the incomplete coupling when using equimolar ratio is that the surface of the liposome becomes occupied at a certain point after which no additional dye molecules can be conjugated. This may explain why conjugation of the 1:1 sample did not go to completion while half of the BCN groups remain unreacted.

Working with the assumption that a liposome consists of 100.000 lipid molecules [23], this results in the theoretical number of molecules per liposome as shown in Table 2. Between ratios of 1:5 and 1:100 conjugation of small molecule azide is complete, so the exact number of conjugated molecules can be controlled by the feed concentration. It obviously depends on the application which density of surface modification is desired, but in the case of targeting ligands our data (see 3.4) as well as previous reports show that numbers well below 100 are sufficient to achieve cell association [24].

3.3. Conjugation of an azide-containing DARPin to liposomal surfaces

The site-specific introduction of azido-amino acids into recombinant proteins allows for full control over the position of the conjugation site. As previously described, azido-homoalanine was introduced at the *N*-terminus of an anti-EpCAM DARPin, where it replaces the initiator methionine, providing a single, orthogonal conjugation site that does not interfere with the binding domain of the DARPin [19]. Liposomes containing BCN-lipid **2** were incubated with the azido-DARPin at ratios of 20:1, 10:1, 5:1 BCN-lipid:azide. For optimal electrophoresis the samples were diluted and protein was detected using a polyhistidine antibody after Western blotting, alongside free protein dilutions as a calibration standard. A liposome sample without the BCN-lipid was included to show that there is no non-specific binding to the liposomes and that the washing steps are sufficient to wash away all unconjugated protein. Free DARPin samples were loaded in concentrations corresponding to 50% of the amount that was added to the samples for conjugation. Band intensities in Fig. 3 already indicate that conjugation is less than that amount.

For more accurate quantification, the three samples mentioned above were loaded together with protein dilutions equaling 100%, 75%, 50%, 40%, 30%, 20% and 10% of total added protein. Bands were quantified and conjugation ratios were calculated as shown in Table 3. (Quantitative blots are included in Supplementary materials Figs. S1–3.) Compared with coupling of the small molecule dye, coupling of the protein was less efficient (based on molar quantities) and did not reach completion, even though BCN is always in molar excess, and the reaction time was sufficient to reach a plateau. This may be due to the large size of the protein leading to possible steric hindrance. Higher levels of protein conjugation are achievable by increasing the amount of azido-protein added, but the efficiency of the process (percentage of total protein coupled) is then lowered.

By increasing the feed ratio of azide, more protein could be coupled, but the amount coupled relative to the amount that was added is much lower, resulting in the low conjugation percentages.

3.4. Cell association of DARPin functionalized liposomes

To show the functionality of the DARPin modified liposomes, binding studies were undertaken using EpCAM expressing HT-29 cells. Using the SPAAC approach the liposomes were functionalized with DARPin Ac2 which is known to bind EpCAM with low nanomolar affinity [18] or with DARPin Off7, which binds Maltose Binding Protein (used as off-target control). Non-functionalized liposomes were also used as secondary control. Experiments were carried out at 4 °C to block internalization mechanisms so any cell association is considered surface binding and not internalization.

When lipid concentrations were varied, i.e. more liposomes were added, increased cell binding was seen with increasing amounts of those liposomes functionalized with Ac2. The liposomes that were functionalized with the off-targeting DARPin did not show any cell association and neither did the unfunctionalized liposomes, indicating that the cell association was DARPin specific. See Fig. 4A.

In a second experiment, liposome concentrations were kept constant, while the density of surface modification was varied. BCN-lipid: azide ratios from 100:1 to 5:1 were used (keeping the azide concentration constant), corresponding to a theoretical maximal conjugation of 5 to 100 ligands per liposome (ligand densities from 0.01% to 0.2% BCN-lipid to total lipid). Cell association was found to be independent of ligand density, suggesting that the lower ligand loading is sufficient to achieve cell binding in vitro. The number of total liposomes did have an effect, showing more total cell association in the higher liposome concentration, but again showing no influence of the degree of surface modification (see Fig. 4B).

It has been previously demonstrated that for cell targeting, there is no need for extremely high degrees of surface modification [24] and

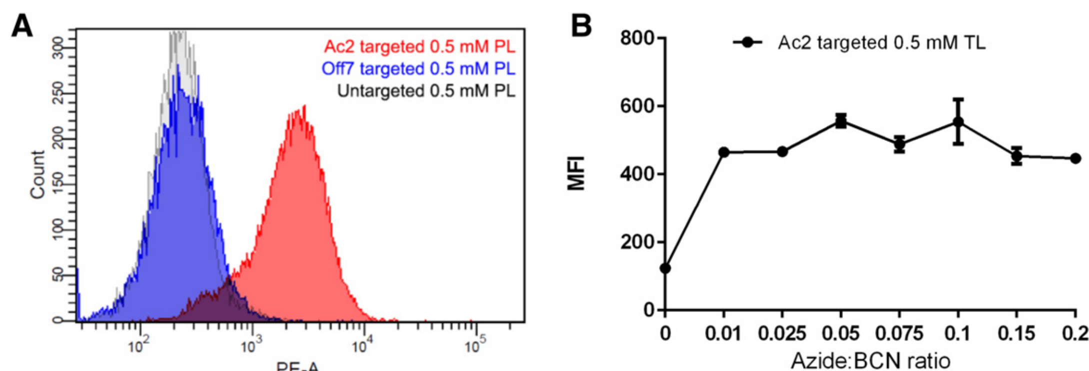

Fig. 4. Binding of Rho-PE labeled DARPin-modified liposomes to HT-29 cells. A: Ac2 targeted liposomes bind to the EpCAM expressing HT-29 cells whereas Off7 targeted and untargeted liposomes do not. B: When the number of ligands is varied, no increased binding is seen.

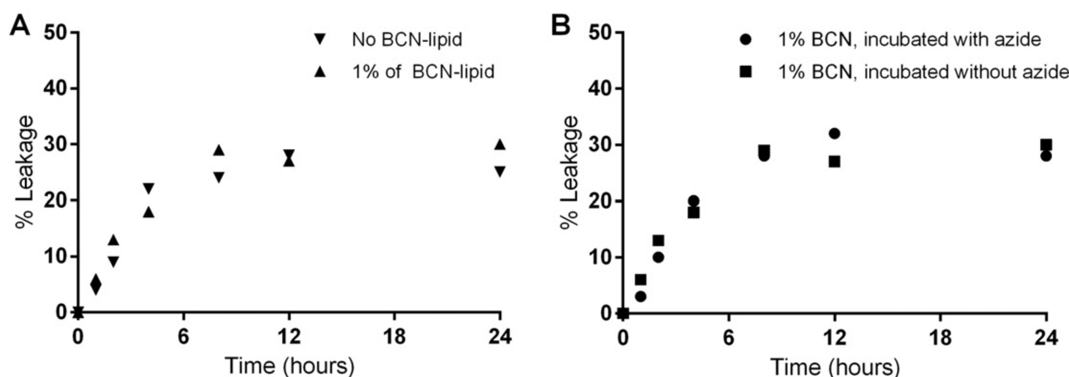

Fig. 5. Leakage of calcein from liposomes under coupling conditions. A: Liposomes with and without 1% of BCN-lipid show similar leakage profiles. B: Liposomes with 1% of BCN-lipid, incubated with and without azide. The click reaction did not have an influence on the amount of leaked calcein.

certainly not when the binding affinity of the ligand is as high as that of the DARPin. On the contrary, increasing ligand density on a liposomal surface can result in an avidity effect, wherein the discriminative capacity for cells expressing high levels of target versus low expressing cells is lost [24]. It was recently shown that increasing the avidity of a nanoparticulate system led to nanomolar affinities, also for cell types that were considered 'low expressers' of the target antigen [25]. The receptor density–avidity relationship is a nonlinear one and most probably is also very much influenced by receptor and ligand type. In the experiments we here describe all the conditions explored were found to result in strong cell association in vitro.

3.5. Leakage assay

To assess the effect of the coupling reaction on the stability of the liposomes, a calcein leakage assay was performed. While no effect on size or polydispersity was observed after incubation and conjugation of the ligands (see Table 1), the leakage assay was performed to show that there is no (temporary) destabilization or rupture of the liposomal membrane during conjugation. The liposome composition used here is inherently leaky due to the use of unsaturated lipids. Despite the presence of cholesterol a quick release was observed in the first hours after the removal of the calcein from the exterior volume. Fig. 5A shows the leakage rates of azide-treated liposomes with and without BCN-lipid **2** and Fig. 5B shows the leakage of liposomes containing the BCN-lipid that were incubated with or without the azide. No increased leakage was seen after incorporation of the BCN-lipid or during the click reaction. Leakage rates were expressed relative to the maximum leakage that was measured after solubilization of the liposomes with Triton X-100. Leakage reached a plateau of approximately 30% and could not be decreased by incubation at 4 °C as compared to RT (see Supplementary materials Fig. S4).

4. Conclusion

To date, modification of liposomes via "click chemistry" has relied upon either the Staudinger ligation [6] or the copper (I) catalyzed alkyne/azide cycloaddition (CuAAC) [26,27]. We here described what is, to our knowledge, the first demonstration of liposome functionalization by using a strain-promoted alkyne/azide cycloaddition (SPAAC) wherein the cyclooctyne moiety is incorporated into the liposomal membrane. The inverse approach, employing azide-modified lipids in the liposome has been previously reported, however, this then requires modification of the ligand to introduce a cyclooctyne group [28]. The SPAAC approach eliminates the need of the copper catalyst, which can be toxic in many liposome applications. When a small molecule azide is used as the 'ligand' reasonable reaction rates are achieved and the degree of surface modification can be fully controlled. A growing number of azide-modified compounds are commercially available and methods to incorporate azides in peptides and proteins are also increasing. In this work we show the application of site-specific conjugation by conjugating an azide-modified DARPin to a liposome and the feasibility of this approach for cell targeting. Site-specific conjugation is an advantage over other conjugation methods and SPAAC is an attractive alternative for copper-catalyzed CuAAC.

Acknowledgments

The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement no. 115363 resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution. Financial support also provided by Utrecht University and the Netherlands Organization for Scientific Research (VIDI grant to NIM 700.59.427).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.01.027.

References

[1] R.I. Jølck, L.N. Feldborg, S. Andersen, S.M. Moghimi, T.L. Andresen, Engineering liposomes and nanoparticles for biological targeting, Adv. Biochem. Eng. Biotechnol. 125 (2011) 251–280. http://dx.doi.org/10.1007/10_2010_92.
[2] M. Fleiner, P. Benzinger, T. Fichert, U. Massing, Studies on protein — liposome coupling using novel thiol-reactive coupling lipids: influence of spacer length and polarity, Bioconjug. Chem. 12 (2001) 470–475. http://dx.doi.org/10.1021/bc000101m.
[3] F. Said Hassane, B. Frisch, F. Schuber, Targeted liposomes: convenient coupling of ligands to preformed vesicles using "click chemistry", Bioconjug. Chem. 17 (2006) 849–854. http://dx.doi.org/10.1021/bc050308l.
[4] B.C. Bundy, J.R. Swartz, Site-specific incorporation of p-propargyloxyphenylalanine in a cell-free environment for direct protein–protein click conjugation, Bioconjug. Chem. 21 (2010) 255–263. http://dx.doi.org/10.1021/bc9002844.
[5] E. Saxon, Cell surface engineering by a modified Staudinger reaction, Science 80 (287) (2000) 2007–2010. http://dx.doi.org/10.1126/science.287.5460.2007.
[6] H. Zhang, Y. Ma, X.-L. Sun, Chemically-selective surface glyco-functionalization of liposomes through Staudinger ligation, Chem. Commun. (Camb.) (2009) 3032–3034. http://dx.doi.org/10.1039/b822420j.
[7] F.L. Lin, H.M. Hoyt, H. van Halbeek, R.G. Bergman, C.R. Bertozzi, Mechanistic investigation of the staudinger ligation, J. Am. Chem. Soc. 127 (2005) 2686–2695. http://dx.doi.org/10.1021/ja044461m.
[8] N.J. Agard, J.A. Prescher, C.R. Bertozzi, A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems, J. Am. Chem. Soc. 126 (2004) 15046–15047. http://dx.doi.org/10.1021/ja044996f.
[9] P.V. Chang, J.A. Prescher, E.M. Sletten, J.M. Baskin, I.A. Miller, N.J. Agard, et al., Copper-free click chemistry in living animals, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 1821–1826. http://dx.doi.org/10.1073/pnas.0911116107.
[10] S.T. Laughlin, C.R. Bertozzi, In vivo imaging of *Caenorhabditis elegans* glycans, ACS Chem. Biol. 4 (2009) 1068–1072. http://dx.doi.org/10.1021/cb900254y.
[11] J. Dommerholt, S. Schmidt, R. Temming, L.J. a Hendriks, F.P.J.T. Rutjes, J.C.M. Van Hest, et al., Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells, Angew. Chem. Int. Ed. 49 (2010) 9422–9425. http://dx.doi.org/10.1002/anie.201003761.

[12] A.J. de Graaf, M. Kooijman, W.E. Hennink, E. Mastrobattista, Nonnatural amino acids for site-specific protein conjugation, Bioconjug. Chem. 20 (2009) 1281–1295. http://dx.doi.org/10.1021/bc800294a.
[13] S.F.M. van Dongen, R.L.M. Teeuwen, M. Nallani, S.S. van Berkel, J.J.L.M. Cornelissen, R.J.M. Nolte, et al., Single-step azide introduction in proteins via an aqueous diazo transfer, Bioconjug. Chem. 20 (2009) 20–23. http://dx.doi.org/10.1021/bc8004304.
[14] Y. Ryu, P.G. Schultz, Efficient Incorporation of Unnatural Amino Acids Into Proteins in *Escherichia coli*, 3 (2006) 263–265. http://dx.doi.org/10.1038/NMETH864.
[15] A. Deiters, T.A. Cropp, D. Summerer, M. Mukherji, P.G. Schultz, Site-specific PEGylation of proteins containing unnatural amino acids, Bioorg. Med. Chem. Lett. 14 (2004) 5743–5745. http://dx.doi.org/10.1016/j.bmcl.2004.09.059.
[16] M. Fernández-Suárez, H. Baruah, L. Martínez-Hernández, K.T. Xie, J.M. Baskin, C.R. Bertozzi, et al., Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes, Nat. Biotechnol. 25 (2007) 1483–1487. http://dx.doi.org/10.1038/nbt1355.
[17] K.L. Kiick, E. Saxon, D.A. Tirrell, C.R. Bertozzi, Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 19–24 http://dx.doi.org/10.1073/pnas.012583299.
[18] N. Stefan, P. Martin-Killias, S. Wyss-Stoeckle, A. Honegger, U. Zangemeister-Wittke, A. Plückthun, DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display and engineered for multivalency, J. Mol. Biol. 413 (2011) 826–843. http://dx.doi.org/10.1016/j.jmb.2011.09.016.
[19] M. Simon, U. Zangemeister-Wittke, A. Plückthun, Facile double-functionalization of designed ankyrin repeat proteins using click and thiol chemistries, Bioconjug. Chem. 23 (2012) 279–286. http://dx.doi.org/10.1021/bc200591x.
[20] G. Rouser, S. Fleischer, A. Yamamoto, Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, Lipids 5 (1970) 494–496. http://dx.doi.org/10.1007/BF02531316.
[21] K. Weber, B. Fehse, Diva-Fit: a step-by-step manual for generating high-resolution graphs and histogram overlays of flow cytometry data obtained with FACSDiva software, Cell. Ther. Transplant. 1 (2009) 1–4. http://dx.doi.org/10.3205/ctt-2009-en-000045.01.
[22] S.a. Meeuwissen, M.F. Debets, J.C.M. van Hest, Copper-free click chemistry on polymersomes: pre- vs. post-self-assembly functionalisation, Polym. Chem. 3 (2012) 1783. http://dx.doi.org/10.1039/c2py00466f.
[23] D.A. Marsh, CRC Handbook of Lipid Bilayers, CRC Press, Boca Raton, FL, 1990.
[24] D. Kirpotin, J.W. Park, K. Hong, S. Zalipsky, W.L. Li, P. Carter, et al., Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro, Biochemistry 36 (1997) 66–75. http://dx.doi.org/10.1021/bi962148u.
[25] J.E. Silpe, M. Sumit, T.P. Thomas, B. Huang, A. Kotlyar, M.A. van Dongen, et al., Avidity modulation of folate-targeted multivalent dendrimers for evaluating biophysical models of cancer targeting nanoparticles, ACS Chem. Biol. 8 (2013) 2063–2071. http://dx.doi.org/10.1021/cb400258d.
[26] R. Tarallo, A. Accardo, A. Falanga, D. Guarnieri, G. Vitiello, P. Netti, et al., Clickable functionalization of liposomes with the gH625 peptide from Herpes simplex virus type I for intracellular drug delivery, Chemistry 17 (2011) 12659–12668. http://dx.doi.org/10.1002/chem.201101425.
[27] S. Cavalli, A.R. Tipton, M. Overhand, A. Kros, The chemical modification of liposome surfaces via a copper-mediated [3 + 2] azide-alkyne cycloaddition monitored by a colorimetric assay, Chem. Commun. (Camb.) (2006) 3193–3195. http://dx.doi.org/10.1039/b606930d.
[28] H.E. Bostic, M.D. Smith, A.A. Poloukhtine, V.V. Popik, M.D. Best, Membrane labeling and immobilization via copper-free click chemistry, Chem. Commun. (Camb.) 48 (2012) 1431–1433. http://dx.doi.org/10.1039/c1cc14415d.