Affinity Induced Surface Functionalization of Liposomes Using Cu-Free Click Chemistry

Bak, Martin; Jølcck, Rasmus Irming; Eliassen, Rasmus; Andresen, Thomas Lars

Published in:
Bioconjugate Chemistry

Link to article, DOI:
[10.1021/acs.bioconjchem.6b00221](https://doi.org/10.1021/acs.bioconjchem.6b00221)

Publication date:
2016

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Bak, M., Jølcck, R. I., Eliassen, R., & Andresen, T. L. (2016). Affinity Induced Surface Functionalization of Liposomes Using Cu-Free Click Chemistry. *Bioconjugate Chemistry*, 27(7), 1673-80.
<https://doi.org/10.1021/acs.bioconjchem.6b00221>

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Affinity Induced Surface Functionalization of Liposomes using Cu-free Click Chemistry

Martin Bak, Rasmus I. Jølck, Rasmus Eliassen and Thomas L. Andresen*

Department of Micro- and Nanotechnology, DTU Nanotech, Centre for Nanomedicine and Theranostics, Technical University of Denmark, Building 423, DK-2800 Kgs. Lyngby, Denmark

* Prof. Thomas L. Andresen, E-mail: Thomas.andresen@nanotech.dtu.dk

ABSTRACT

Functionalization of nanoparticles is a key element for improving specificity of drug delivery systems toward diseased tissue or cells. In the current study we report a highly efficient and chemoselective method for post-functionalization of liposomes with biomacromolecules, which equally well can be used for functionalization of other nanoparticles or solid surfaces. The method exploits a synergistic effect of having both affinity- and covalent anchoring tags on the surface of the liposome. This was achieved by synthesizing a peptide linker system that uses Cu-free strain-promoted click chemistry in combination with histidine affinity tags. The investigation of post-functionalization of PEGylated liposomes was performed with a cyclic RGDfE peptide. By exploring both affinity and covalent tags a $98 \pm 2.0\%$ coupling efficiency was achieved, even a diluted system showed a coupling efficiency of $87 \pm 0.2\%$. The reaction kinetics and overall yield were quantified by HPLC. The results presented here opens new possibilities for constructing complex nanostructures and functionalized surfaces.

INTRODUCTION

Nanoparticle based carrier systems are heavily investigated as therapeutic and diagnostic delivery vectors, and they have the potential to improve clinical practice by offering better diagnosis, treatment and management of human disease.^{1,2} Functionalized nanomaterials for use in drug delivery with targeting ligands on the surface have received considerable attention due to the potential of increasing drug

1 bioavailability at the diseased site by targeting selective or overexpressed receptors on the target cells.³⁻⁵
2 Targeting ligands can broadly be classified as proteins (e.g. antibodies and their fragments), nucleic acids
3 (aptamers) or other receptor ligands (e.g. carbohydrates, peptides, and vitamins). A vast number of
4 bioconjugation methods have been reported for functionalization of liposomes and nanoparticles, all of
5 which have their pros and cons.^{6,7} The method developed in this paper uses liposomes as an exemplifying
6 nanomaterial where both covalent and non-covalent functionalization of nanoparticles is utilized.

7 Post-functionalization of liposomes is typically the method of choice when functionalizing with large and
8 complex ligands such as proteins and antibodies or fragments thereof. This technique ensures the absence of
9 ligand in the interior space of the liposome, thus less ligand is required. Unfortunately, ligand conjugation by
10 post-functionalization can suffer from low yield and large batch-to-batch variation. For some targeting
11 ligands, it is a necessity that they are attached to liposomes by post-functionalization, because larger
12 proteins, e.g. antibodies, are vulnerable to denaturation, hence chemical reactions compatible with the
13 aqueous environment is a prerequisite. Conjugation techniques have until now only been focused on a single
14 attachment point on the liposome surface by either a covalent^{6,8} or non-covalent coupling⁹⁻¹¹. Cu(I)-catalyzed
15 Huisgen 1,3-dipolar cycloaddition (CuAAC) of azides to terminal alkynes has served as a benchmark for a
16 decade, due to the excellent stability of the formed triazole, excellent orthogonality towards other functional
17 groups and easy accessibility of azide and alkyne tags. While the conventional CuAAC reaction^{12,13} is
18 commonly employed in surface derivatization and polymer synthesis¹⁴⁻¹⁶, the use of metal catalyst often
19 limits its utilization in biological settings. Copper ions are cytotoxic¹⁷, can cause DNA degradation^{18,19} and
20 induce protein denaturation²⁰ resulting in loss of the native properties of the attached macromolecule. By
21 substituting terminal alkynes with strain-promoted²¹ or electron deficient alkynes²², triazole formation can be
22 achieved without the addition of catalyst under ambient conditions without disrupting the function of the
23 immobilized macromolecule. This approach has previously been employed for modification of quantum
24 dots²³, liposomes²⁴⁻²⁶, proteins^{27,28} and living cells and organisms²⁹⁻³¹. We have previously showed effective
25 coupling of a somatostatin receptor targeting peptide (TATE) to the lipid moiety via strain-promoted
26 alkyne-azide [3+2] cycloaddition (SPAAC).^{8,32}

1 Recombinant proteins have found widespread use in the growing field of proteomics. Affinity tags are
2 routinely used for fixation of recombinant hybrids to facilitate their purification. In addition to purification
3 and immobilization of biomolecules, they also support a variety of applications such as protein labeling,
4 targeting approaches, 2D crystallization, and biosensing.^{33–36} The parameter-dependent binding affinity of
5 histidine affinity tagged (His-tagged) proteins to nitrilotriacetic acid (NTA) or trisNTA has been explored
6 regarding length of the poly-His-tag, multivalency of NTA moieties, and accessibility of NTA moieties on
7 the surface^{9,37,38}. The aim of these studies was to create a highly efficient receptor system. For example, a
8 His-tag containing six histidine-residues have been shown to have binding affinity in the subnanomolar
9 range, while an increase in histidine-residues resulted in a decreased affinity for binding to the divalent
10 transition metal ions (Ni^{2+} , Zn^{2+} , Co^{2+} or Cu^{2+}).³⁹ This presents a non-covalent approach to ensure a
11 controlled display of macromolecules on the surface of liposomes.^{9,37,40}

12 We envisioned that liposomes exposing affinity tags in addition to chemically reactive functionalities would
13 aid in the attraction and orientation of the macromolecules thus affecting the kinetic profile and the coupling
14 efficiency of surface conjugation reactions. To test the hypothesis we have investigated PEGylated
15 liposomes with NTA-chelates, strained cyclooctynes and a combination of both at the liposome interface and
16 shown that post-functionalization of His-tagged cyclic RGDfE exposing an azide functionality in close
17 proximity to the His-tag can be induced using this strategy. We believe this general concept seen in Figure 1,
18 exemplified by affinity induced surface functionalization of liposomes, will be highly beneficial when
19 designing new functionalized nanomaterials, especially in highly diluted environments.

1

2 **Figure 1.** Linker system (4) for post-functionalization of liposomes. Red box: Targeting moiety, c(RGDfE). Blue box:
3 Azide and strained cyclooctyne for SPAAC coupling. Green box: His₃-tag and nitrilotriacetic acid (NTA) for formation
4 of hexa-coordinated nickel complex.

1

2 **RESULTS AND DISCUSSION**3 **Scheme 1.** Synthesis of the c(RGDfE) functionalized peptide linker (**3** and **4**)

4

5

6 **Synthesis and Characterization.** The c(RGDfE) functionalized linker peptides **3** and **4** were prepared using
 7 a SPPS strategy as shown in Scheme 1. Intramolecular cyclization was achieved using an orthogonal
 8 protection group scheme.⁴¹ Peptides **3** and **4** were cleaved from the solid support using TFA:TIPS:H₂O and
 9 subsequently purified by HPLC. Laser-induced reduction of the azido functionality to the corresponding
 10 amine was observed during MALDI-TOF MS analysis as previously observed by Budyka *et al.*⁴². The intact
 11 azido functionality was confirmed by FT-IR analysis at 2112 cm⁻¹. A His₆-tag was successfully synthesized

1 and isolated, but a large degree of aggregation was observed for this molecule in aqueous solutions. This
2 would presumably create complications in the later liposome study. A His₃-tag was made, which was
3 sufficient to create the coupling to the chelate of NTA-Ni²⁺ ($K_D = 2.23 \times 10^{-6}$ M)³⁷ and limit the aggregation.
4 This was also in correspondence with a previous study by Knecht *et al.*^{37, 10}
5 In order to formulate liposomes exposing the appropriate chemical functionalities at the distal end of the
6 PEG-layer, the desired functionalized PEGylated phospholipids were synthesized in solution from either
7 DSPE-PEG₂₀₀₀-NH₂ or DSPE-PEG₂₀₀₀-NHS. The phospholipid with the strained cyclooctyne at the distal end
8 of the polymer was synthesized by acylating DSPE-PEG₂₀₀₀-NH₂ with 1-fluorocyclooct-2-ynecarboxylic
9 acid.³² 1-Fluorocyclooct-2-ynecarboxylic acid was synthesized as described elsewhere.⁴³ DSPE-PEG₂₀₀₀-
10 NTA was synthesized in a single acylation step between DSPE-PEG₂₀₀₀-NHS and NTA.
11 Three different compositions of liposomes were prepared in order to investigate the effect on surface
12 conjugation efficiency induced by having an affinity tag present on the liposomal surface. A concentrated
13 TRIS/HCl buffer was used to minimize non-specific electrostatic interactions when using the negatively
14 charged Ni-NTA complex.⁴⁴ Formed liposomes were characterized by DLS and ζ -potential (Table 1), and
15 stored at 5 °C until use. Post-functionalization investigations were carried out by adding the required
16 components for the individual reactions (e.g. Ni²⁺ for the His-tag affinity coupling), followed by addition of
17 **4** in CH₃OH. Liposome integrity was tested in different concentrations in the final solvent mixture by DLS to
18 ensure their stability in the given reaction environment. The CH₃OH concentration of 1.5-3 % was not found
19 to affect the stability of the liposomes. All reactions were monitored for 72 h by HPLC using UV/VIS-
20 detection. The conjugation efficiency was calculated based on the AUC for **4** and the DSPE-PEG₂₀₀₀-
21 c(RGDfE) conjugate. Triplicates of all reactions were carried out to ensure reproducibility and elucidate the
22 variance of the post-functionalization reactions at the liposomal surfaces.

23 **Table 1.** DLS and Zeta-potential measurements of the liposome formulations

Entry #	DLS	Zeta-potential
1	115.2 ± 0.9 nm	-4.02 ± 2.85 mV
2	115.8 ± 1.7 nm	-9.68 ± 2.56 mV
3	112.1 ± 0.7 nm	-2.22 ± 3.06 mV

24

1 **Coupling of 4 to preformed liposomes by SPAAC.** The efficiency of the individual conjugation techniques
2 was first addressed. Coupling of 4 to preformed liposomes via SPAAC reaction was investigated using the
3 DSPE-PEG₂₀₀₀-cyclooctyne (5) and azide functionalized 4. The combined data acquisition gave the
4 conclusive result that the SPAAC chemistry proceeds in good yield, with a mean conversion of 83±1.5 %
5 after 72 h at rt (Figure 2) in good correlation with previous work by Feldborg *et al.*³². Presence of Ni²⁺ during
6 the SPAAC reaction did not alter the reaction kinetics or conversion degree (data not shown).

7
8 **Figure 2.** Coupling efficiencies of 4 to DSPE-PEG2000-cyclooctyne (5) by SPAAC chemistry at the liposome surface
9 (Liposome 1). All values are means ± SEM (n = 3).

10

11 **Complexation of 4 to preformed liposomes by His₃-tag.** Complex-formation to preformed liposomes via
12 His-tag was investigated using the DSPE-PEG₂₀₀₀-NTA (6) and His₃-tag functionalized 4. Reactions were
13 carried out using two different amount of Ni²⁺ (1 or 10 equiv.) to investigate how the Ni²⁺-concentration
14 affected the complex formation with 4. No notable difference in using 1 or 10 equiv. of Ni²⁺ was observed,
15 hence, 1 equiv. was used onwards. The dissociation constants of four Ni-NTA complexes have been taken
16 into consideration to verify the affinity based complex formation between the liposome and targeting ligand
17 (Ni²⁺-His-tag complex: $K_D \approx 10^{-6}$ M, Ni²⁺-Imidazole: $K_D = 9.8 \times 10^{-4}$ M, Ni²⁺-NTA: $K_D = 1.8 \times 10^{-11}$ M and
18 Ni²⁺-EDTA complex: $K_D = 4.0 \times 10^{-19}$ M)⁴⁵⁻⁴⁸. Firstly, the reversibility of the complex was tested by
19 competitive complexation to determine the stability of the complex. Trans-complexation by addition of
20 acetic acid resulted in recovery of 4 as analyzed by HPLC. Furthermore, in order to confirm the NTA-Ni²⁺-

1 His-tag complex formation, imidazole was added to the Ni-NTA complex prior to addition of **4**, thus
 2 hindering the coupling of the His-tag. Because the K_D of the NTA- Ni^{2+} -Imidazole complex is larger than K_D
 3 for the NTA- Ni^{2+} -His₃-tag complex, the His₃-tag will still associate to the Ni-NTA complex but to a less
 4 extend than previously observed. Hindering the Ni-NTA complexation with imidazole resulted in only
 5 approximately 10% association of **4** after 42 h. In comparison, the triplicate data showed a mean complex
 6 efficiency of $72 \pm 2.0\%$ after 72 h (Figure 3). This implies that imidazole is able to slow down the
 7 coordination degree significantly by competitive binding to the NTA- Ni^{2+} -moiety on the liposomal surface.
 8 Combined, the obtained data confirms that NTA-tags efficiently associate with His-tag ligands on the
 9 liposome surface and that the complexation is reversible as shown by trans-complexation.

10

11 **Figure 3.** Complexation efficiencies of **4** to DSPE-PEG2000-NTA- Ni^{2+} (**6**) by a complex-formation via the His₃-tag at
 12 the liposome surface (Liposome 2). All values are means $\pm$ SEM (n = 3).

13

14 **Affinity induced covalent coupling of **4** to liposomes.** The liposome composition with both NTA (1 mol%)
 15 and cyclooctyne (1 mol%) moieties was incubated with Ni^{2+} (1 equiv.) and was subsequently added **4**. The
 16 progression of the affinity induced covalent coupling of **4** was monitored by HPLC. The complexation
 17 (NTA- Ni^{2+} -His₃-tag) was disrupted by addition of acetic acid to aliquots after 72 h, however, no change in
 18 AUC for the coupled product was observed. This confirmed that **4** is covalently attached to the liposome
 19 surface by triazole formation. Comparison of the conjugation studies of the SPAAC coupling versus the
 20 SPAAC reaction via the affinity His₃-tag shows that the His₃-tag is able to increase the coupling efficiency

(Figure 4). The mean conjugation of **4** by a SPAAC coupling was $83 \pm 1.5\%$ after 72 h whereas the pure affinity based association of His₃-tag was $72 \pm 2.0\%$. Interestingly, the fact is that the mean conjugation of the combined system was significantly better by $98 \pm 2.0\%$ after 72 h, which can be explained by the higher local concentration of the targeting ligand at the liposomal surface due to the presence of the affinity tag. The combined data acquisition supports that the His₃-tag having a promoting effect on the SPAAC-coupling on the surface of preformed liposomes (Figure 4).

In order to validate this observation and further investigate the difference between the SPAAC-coupling versus the His₃-tag induced SPAAC-coupling surface functionalization of liposomes exposing both NTA (1 mol%) and cyclooctyne (1 mol%) moieties with **4** in highly diluted conditions was investigated.

Figure 4. Coupling efficiencies of **4** to the liposome surface by SPAAC chemistry via affinity NTA-Ni²⁺-His₃-complex at the liposome surface (Liposome 3). All values are means $\pm$ SEM (n = 3).

Dilution test of conjugating **4 to preformed liposomes.** Next we investigated the ligand conjugation under diluted conditions (diluted 5 fold) to investigate the effects of the affinity-based conjugation to the functionalized liposome surface. The reaction kinetics for the covalent coupling will decrease in the dilute system and the affinity complexation effect will presumably be more pronounced. The reaction kinetics decreased as expected in comparison to the study done under more concentrated conditions (Figure 5). The

liposome formulation with the SPAAC-moiety showed a coupling efficiency of $58 \pm 2.2\%$ after 72 h, whereas the combined SPAAC and His₃-tag system showed a coupling efficiency of $87 \pm 0.2\%$ after 72 h. The increased difference in coupling efficiency from 15 – 29 % between concentrated and dilute conditions, respectively, shows the potential of using affinity-based complexation followed by covalent conjugation for functionalizing liposomes.

Figure 5. Comparison of the diluted coupling efficiency study: Coupling of DSPE-PEG2000-cyclooctyne (**5**) and the peptide linker (**4**) to the liposome surface by SPAAC chemistry (○). Coupling efficiencies of **4** to the liposome surface by SPAAC chemistry via affinity NTA-Ni²⁺-His₃-complex at the liposome surface (■). All values are means $\pm$ SEM (n = 3).

Table 2. Overview of the yields in the liposomal post-functionalization study determined by analytical HPLC

Entry #	R ₁	Yields after 72 h
1	SPAAC	83 $\pm$ 1.5%
2	His ₃ -Ni ²⁺ -NTA-tag	72 $\pm$ 2.0%
3	SPAAC via His ₃ -Ni ²⁺ -NTA-tag	98 $\pm$ 2.0%
4	Diluted SPAAC	58 $\pm$ 2.2%
5	Diluted SPAAC via His ₃ -Ni ²⁺ -NTA-tag	87 $\pm$ 0.2%

CONCLUSION

In conclusion, a conceptual new approach has been described for effectively post-functionalizing liposomes. It has been shown that it is possible to induce a covalent coupling via a synergetic effect with an affinity tag, see Table 2. The His₃-tag in the peptide linker system, **4**, is able to improve the reaction kinetics of the

SPAAC chemistry. This presumably happens through an up-concentration at the interface of the liposome. The conjugation technique is bioorthogonal and chemoselective to the functional groups in most relevant ligands such as proteins and aptamers. The mean conjugation efficiency of **4** by a SPAAC coupling was $83 \pm 1.5\%$ after 72 h and $72 \pm 2.0\%$ for the His₃-tag affinity complex-formation. The combined system was significantly faster and gave a total conjugation efficiency of $98 \pm 2.0\%$ after 72 h. This is a 15% improvement relative to the SPAAC coupling reaction, but also provides a method that would not require post-conjugation purification of unbound ligand. This is presumably due to favorable interactions between the liposome surface and the His₃-tag, which results in up-concentration of **4** at the liposome surface and thereby increased coupling efficiency. The symbiotic effect of the dual coupling setup was further verified in a dilution test where the difference in conjugation efficiency increased by 29%. The findings described are useful for future post-functionalization designs not only for liposomes but also for other nanoparticles. This linker system has the potential to improve the post-functionalization of large and sterically hindered target molecules, such as antibodies or fragments hereof.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma-Aldrich Inc. (Broendby, Denmark) unless otherwise stated. 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-(polyethylene glycol)₂₀₀₀ (DSPE-PEG₂₀₀₀), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)₂₀₀₀] (DSPE-PEG₂₀₀₀-NH₂), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[succinyl(polyethylene glycol)₂₀₀₀] (ammonium salt) (DSPE-PEG₂₀₀₀-NHS) were purchased from Avanti Polar Lipids Inc. (Alabama, USA). *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU), Tentagel[®] Rink Amide Resin, 2-chlorotrityl resin and all Fmoc protected amino acids used for the solid phase peptide synthesis were purchased from GL Biochem (Shanghai, China). All chemicals and reagents were of analytical grade and used without further purification.

1 **Instrumentation**

2 Reactions were monitored by thin layer chromatography (TLC); visualization was carried out by UV-light
3 exposure (254 nm) and KMnO₄-stain. Chromatography refers to open column chromatography on silica gel.
4 Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Gilson
5 HPLC (Gilson Valvemate, UV/VIS-155, 321 Pump, 234 Auto injector) or Waters LC/MS (2695 HPLC
6 system, 2998 UV/VIS, 3100 ZQ single quadrupole MS with ESI) by employing an XBridge™ C₁₈ 5 μm
7 (4.6×150 mm), XBridge™ C₁₈ 3.5 μm (2.1×50 mm) or a Waters XTerra® C₈ 5 μm (4.6×150 mm) column.
8 Semi-preparative HPLC was performed on a Waters Semi-preparative HPLC equipped with a Waters 600
9 Pump & Controller and a Waters 2489 UV/VIS Detector using a Knauer Eurosphere 100-5 C₁₈ (20×250 mm)
10 column or a Waters XTerra® C₈ 5 μm (19×150 mm) column. HPLC Eluent A consisted of a 5 % CH₃CN
11 aqueous solution with 0.1 % trifluoroacetic acid (TFA); HPLC Eluent B consisted of 0.1 % TFA in CH₃CN.
12 Preparative HPLC analysis was monitored using UV/VIS detection at 220/280 nm. NMR spectra were
13 recorded on a Varian Mercury 400 MHz Spectrometer. ¹H and ¹³C NMR were recorded at 400- and 100
14 MHz, respectively. Chemical shifts (δ) are reported in parts per million (ppm) relative to the solvent's signal
15 peak. Mass spectra were recorded on a Bruker Reflex IV MALDI-TOF Spectrometer using 2,5-
16 dihydroxybenzoic acid (DHB) spiked with sodium trifluoroacetate in CH₃OH as matrix. FT-IR was recorded
17 neat on a Perkin Elmer Instruments Spectra One FT-IR Spectrometer. Dynamic light scattering (DLS) and ζ-
18 potential measurements were performed on a Brookhaven Instruments Corporation ZetaPALS ζ-potential
19 analyzer. Phosphorous analysis was measured by ICP-MS performed on a Dionex™ ICS-5000⁺ system
20 (Thermo Scientific™, Dreieich, Germany).

21 **Synthesis**

22 **DSPE-PEG₂₀₀₀-1-fluorocyclooct-2-yne (5).** DSPE-PEG₂₀₀₀-1-fluorocyclooct-2-yne (DSPE-PEG₂₀₀₀-
23 cyclooctyne) was synthesized as previously described by Jølk *et al.*²⁴ and isolated as a white powder.
24 MALDI-TOF MS (m/z): Calc. mass [M+H]⁺: 2967.9 ± n×44.0; found mass [M+H]⁺: 2968.3 ± n×44.0. ¹H-
25 NMR (400MHz, CDCl₃): δ 6.83 (bs, 1H), 5.93 (bs, 1H), 5.22-5.16 (m, 1H) , 4.35-4.01 (m, 10H),

1 3.95-3.89 (m, 2H), 3.62 (bs, 164H), 3.48-3.36 (m, 6H), 2.32-2.23 (m, 6H), 1.71-1.49 (m, 6H), 1.23 (bs, 56H),
2 0.85 (t, J= 6.6 Hz, 6H). FT-IR: $\nu(\text{cm}^{-1})$ 3445.3, 2918.2, 2851.4, 1741.6, 1648.9, 1104.6.

3 **DSPE-PEG₂₀₀₀-NTA (6).** *N_ωN_α*-Bis(carboxymethyl)-L-lysine (9.1 mg, 34.6 μmol) and triethylamine (48 μL ,
4 0.35 mmol) in anhydrous dimethylformamide (1.0 mL) were added to a flame dried flask containing DSPE-
5 PEG₂₀₀₀-NHS (20.7 mg, 6.9 μmol) in anhydrous CH_2Cl_2 (1.0 mL) under argon atmosphere and with reaction
6 molecular sieves (4 Å). The reaction was stirred at rt for 15 h after which the solvent was removed *in vacuo*
7 and the product purified by semi-preparative HPLC employing a Waters XTerra[®] C₈ 5 μm (10x150 mm)
8 column. Eluent: (A) 5 % CH_3CN + 0.1 % TFA in H_2O , (B) 0.1 % TFA in CH_3CN . Gradient profile: Linear
9 gradient from 20 % B to 100 % B over 20 min. Flow rate: 7 mL/min. UV/VIS monitoring at 220 and 280
10 nm. Column temperature: 40 °C. DSPE-PEG₂₀₀₀-NTA was isolated as a broad homogenous peak with
11 retention time of 10.8 min. The solvent was removed *in vacuo* and the product lyophilized from a mixture of
12 H_2O and CH_3CN to give a white fluffy powder (9.1 mg, 40 %, purity > 98 %). MALDI-TOF MS (*m/z*): Calc.
13 mass $[\text{M}+\text{H}]^+$: $3212.9 \pm n \times 44.0$; found mass $[\text{M}+\text{H}]^+$: $3213.1 \pm n \times 44.0$. ¹H-NMR (400 MHz, CDCl_3): δ 5.17
14 (m, 1H), 4.07 (m, 2H), 4.01-3.69 (m, 4H), 3.54 (m, 180H), 3.51-3.46 (m, 4H), 3.42 (t, 2H), 3.36 (t, 1H),
15 3.17-3.09 (m, 4H), 2.27-2.19 (m, 4H), 2.14-2.09 (m, 4H), 1.80 (m, 2H), 1.66 (f, 2H), 1.61-1.30 (m, 8H), 1.20
16 (m, 58H), 0.81 (t, 6H). FT-IR: $\nu(\text{cm}^{-1})$ 3337.3, 2918.5, 2852.1, 1696.9, 1106.2.

17

18 **c(RGDfE)-EG₂-K(EG₂-N₃)-EG₂-His₃ (4).** The peptide linker was synthesized by solid-phase peptide
19 synthesis (SPPS) on a Tentagel[®] resin (loading 0.25 mmol/g) with a Rink amide linker by standard Fmoc
20 methodology. Each coupling was performed using 4.0 equiv. Fmoc protected amino acid, 3.95 equiv. HATU
21 and 8 equiv. 2,4,6-collidine in DMF. Deprotection of the Fmoc group was carried out using 20 % piperidine
22 in DMF for 2x5 min. Completion of each coupling and deprotection step was monitored by the Kaiser test.
23 The first step was synthesis of Fmoc-EG₂-K(Alloc)-EG₂-(H(Trt))₃-resin. Removal of Alloc protection group
24 on the lysine residue was achieved by washing the resin with dry CH_2Cl_2 (5x30 sec) under N₂-atmosphere. A
25 solution of PhSiH_3 (24 equiv.) in dry CH_2Cl_2 was mixed with the resin. A dispersion of $\text{Pd}(\text{PPh}_3)_4$ (0.5
26 equiv.) in dry CH_2Cl_2 was added and stirred for 10 min with N₂-atmosphere. The peptide was washed with

1 CH₂Cl₂ (8×30 sec) and the process was repeated.⁴⁹ N₃-EG₂-COOH was coupled to the deprotected lysine side
2 chain as described above. The couplings were continued at the N-terminal end with the linear peptide
3 sequence Fmoc-R(Pbf)-G-D(^tBu)-f-E-OAll. Allyl deprotection on the glutamic acid residue was achieved
4 using the same procedure like for deprotection of the lysine residue, but repeated twice.⁴⁹ The intramolecular
5 cyclization was achieved by deprotecting Fmoc followed by standard HATU coupling procedure for 16 h.
6 The resin was cleaved with TFA:triisopropylsilane(TIPS):H₂O (95:2.5:2.5) for 2 h. Final purification was
7 achieved by semi-preparative HPLC by employing a Waters XTerra[®] C₈ 5 μm (19×150 mm) column. Eluent:
8 (A) 5 % CH₃CN + 0.1 % TFA in H₂O, (B) 0.1 % TFA in CH₃CN. Gradient profile: Linear gradient from 0 %
9 B to 100 % B over 20 min. Flow rate: 17 mL/min. Solvent for injection: H₂O/acetic acid (4:1). The solvent
10 was removed *in vacuo* and the product lyophilized from a mixture of H₂O and CH₃CN to give a white fluffy
11 powder (**4**) (41.8 mg, 24 %, purity > 97 %). MALDI-TOF MS (m/z): Calc. mass [M+H]⁺: 1604.7, found
12 mass [M+H]⁺: 1604.6. FT-IR: ν(cm⁻¹) 3022; 2926; 2868; 2112; 1668; 1201; 1127; 1107; 698.

13

14 **Liposome Formulation**

15 Liposomes exposing 1 mol% of the reactive cyclooctyne (**5**), the NTA affinity tag (**6**) or both were prepared
16 with an overall PEGylation density of 5 mol%. The functionalized liposomes composed of DSPC/DSPE-
17 PEG₂₀₀₀/DSPE-PEG₂₀₀₀-R (95:4:1) (R = cyclooctyne (**5**), NTA (**6**)) and DSPC/DSPE-PEG₂₀₀₀/DSPE-
18 PEG₂₀₀₀-cyclooctyne (**5**)/DSPE-PEG₂₀₀₀-NTA (**6**) (95:3:1:1) were prepared by the method described by
19 Bangham *et al.*⁵⁰. Lipids were dissolved in CHCl₃:CH₃OH (9:1) and mixed in the desired ratios. The solvent
20 was removed under a stream of nitrogen, and the lipid films placed under vacuum overnight to remove
21 remaining traces of organic solvent. The obtained films were hydrated in tris(hydroxymethyl)-aminomethane
22 (TRIS)/HCl buffer (100 mM, pH 8) at 65 °C for 1 h and vortexed every 10 min followed by 10 freeze-thaw
23 cycles to form multilamellar liposomes. Unilamellar liposomes of approximately 100 nm in size were
24 prepared by extrusion (Avanti[®] Mini-Extruder) of the multilamellar liposome suspension 21 times through a
25 100 nm polycarbonate filter (Nuclepore[®]) at 65 °C. The resulting unilamellar liposomes were stored at 5 °C
26 until usage. The Ni-NTA-complex was initiated by addition of NiCl₂ (1 or 10 equiv. in H₂O), followed by 2
27 h incubation.³⁴

1 **Liposome Characterization**

2 The hydrodynamic diameter of the formed liposomes was analyzed by DLS and their ζ -potentials measured
3 using a Brookhaven Zeta PALS analyzer. Buffer: Sterile TRIS/HCl buffer (100 mM, pH 8). Dilution factor:
4 500. Number of runs: 10 runs per analysis. All liposomes were in the 110 nm range and all ζ -potentials were
5 slightly negative. The lipid concentration after extrusion was determined by measuring the phosphor content
6 by ICP-MS and the overall liposome concentration adjusted accordingly.

7 **Post-functionalization of Liposomes**

8 Preformed functionalized liposomes (25 mM, 500 μ L or 5 mM, 1000 μ L) were mixed with **4** (15 μ L in
9 CH₃OH, 0.5 equiv.) giving an overall ligand concentration of 67 μ M and 13 μ M. The reactions were shaken
10 at rt and aliquots (40 μ L or 100 μ L) were removed at specific time intervals for analysis by analytical HPLC.
11 Sample aliquots were stored at -80 °C to quench the functionalization prior to HPLC analysis. The coupling
12 efficiency was monitored by analytical HPLC employing a Waters XTerra[®] C₈ 5 μ m (4.6 x 150 mm) column
13 or Waters XBridge[™] C₁₈ 3.5 μ m (2.1 x 50 mm). Eluent: (A) 5 % CH₃CN + 0.1 % TFA in H₂O, (B) 0.1 %
14 TFA in CH₃CN. Gradient profile: Linear gradient from 0 % B to 100 % B over 20 min. Flow rate: 1.0
15 mL/min. The coupling efficiency was calculated based on the area under the curve (AUC) for **4** and the
16 DSPE-PEG₂₀₀₀ conjugated product by using UV/VIS-detection at 220nm.

17

18 **AUTHOR INFORMATION**

19 **Corresponding Author**

20 *E-mail: Thomas.andresen@nanotech.dtu.dk

21 **Notes**

22 The authors declare no competing financial interest.

23

1 **ACKNOWLEDGEMENT**

2 The authors acknowledge financial support provided by the Technical University of Denmark (DTU) and the
3 Lundbeck Foundation.

4

5 **ABBREVIATIONS**

6 AUC, Area Under the Curve; DLS, Dynamic Light Scattering; DSPC, 1,2-distearoyl-*sn*-glycero-3-
7 phosphocholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; EPR, Enhanced Permeability and
8 Retention; HPLC, High-Performance Liquid Chromatography; ICP-MS, Inductively Coupled Plasma Mass
9 Spectrometry; MALDI-TOF MS, Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass
10 Spectrometry; NTA, Nitrilotriacetic Acid; SPAAC, Strain Promoted Azide-Alkyne [3+2] Cycloaddition;
11 SPPS, Solid Phase Peptide Synthesis; TATE, [Tyr³,Thr⁸]-octreotate.

12

1 REFERENCES

- 2 (1) Malam, Y., Loizidou, M., and Seifalian, A. M. (2009) Liposomes and nanoparticles: Nanosized vehicles
3 for drug delivery in cancer. *Trends Pharmacol. Sci.* 30, 592–9.
- 4 (2) Kaasgaard, T., and Andresen, T. L. (2010) Liposomal cancer therapy: exploiting tumor characteristics.
5 *Expert Opin. Drug Deliv.* 7, 225–43.
- 6 (3) Sapra, P., and Allen, T. M. (2003) Ligand-targeted liposomal anticancer drugs. *Prog. Lipid Res.* 42, 439–
7 462.
- 8 (4) Peer, D., Karp, J. M., Hong, S., Farokhzad, O. C., Margalit, R., and Langer, R. (2007) Nanocarriers as an
9 emerging platform for cancer therapy. *Nat. Nanotechnol.* 2, 751–60.
- 10 (5) Maeda, H., Nakamura, H., and Fang, J. (2013) The EPR effect for macromolecular drug delivery to solid
11 tumors: Improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging in vivo.
12 *Adv. Drug Deliv. Rev.* 65, 71–9.
- 13 (6) Jøllek, R. I., Feldborg, L. N., Andersen, S., Moghimi, S. M., and Andresen, T. L. (2010) Engineering
14 Liposomes and Nanoparticles for Biological Targeting, in *Biofunctionalization of Polymers and their*
15 *Applications*, pp 251–281.
- 16 (7) Shieh, P., and Bertozzi, C. R. (2014) Design strategies for bioorthogonal smart probes. *Org. Biomol.*
17 *Chem.* 12, 9307–9320.
- 18 (8) Lallana, E., Sousa-Herves, A., Fernandez-Trillo, F., Riguera, R., and Fernandez-Megia, E. (2012) Click
19 chemistry for drug delivery nanosystems. *Pharm. Res.* 29, 1–34.
- 20 (9) Chikh, G. G., Li, W. M., Schutze-Redelmeier, M.-P., Meunier, J.-C., and Bally, M. B. (2002) Attaching
21 histidine-tagged peptides and proteins to lipid-based carriers through use of metal-ion-chelating lipids.
22 *Biochim. Biophys. Acta* 1567, 204–12.
- 23 (10) Terpe, K. (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to
24 commercial systems. *Appl. Microbiol. Biotechnol.* 60, 523–33.
- 25 (11) Algar, W. R., Prasuhn, D. E., Stewart, M. H., Jennings, T. L., Blanco-Canosa, J. B., Dawson, P. E., and
26 Medintz, I. L. (2011) The controlled display of biomolecules on nanoparticles: A challenge suited to
27 bioorthogonal chemistry. *Bioconjug. Chem.* 22, 825–58.
- 28 (12) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-Triazoles
29 by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem.*
30 67, 3057–64.
- 31 (13) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen
32 cycloaddition process: Copper(I)-catalyzed regioselective “ligation” of azides and terminal alkynes. *Angew.*
33 *Chem. Int. Ed. Engl.* 41, 2596–9.
- 34 (14) Fournier, D., Hoogenboom, R., and Schubert, U. S. (2007) Clicking polymers: a straightforward
35 approach to novel macromolecular architectures. *Chem. Soc. Rev.* 36, 1369–80.
- 36 (15) Hein, J. E., and Fokin, V. V. (2010) Copper-catalyzed azide-alkyne cycloaddition (CuAAC) and
37 beyond: new reactivity of copper(I) acetylides. *Chem. Soc. Rev.* 39, 1302–15.
- 38 (16) Mamidyala, S. K., and Finn, M. G. (2010) In situ click chemistry: probing the binding landscapes of
39 biological molecules. *Chem. Soc. Rev.* 39, 1252–61.
- 40 (17) Gaetke, L. (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology* 189, 147–163.
- 41 (18) Burrows, C. J., and Muller, J. G. (1998) Oxidative Nucleobase Modifications Leading to Strand
42 Scission. *Chem. Rev.* 98, 1109–1152.

- 1 (19) Gierlich, J., Burley, G. A., Gramlich, P. M. E., Hammond, D. M., and Carell, T. (2006) Click chemistry
2 as a reliable method for the high-density postsynthetic functionalization of alkyne-modified DNA. *Org. Lett.*
3 8, 3639–42.
- 4 (20) Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003)
5 Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *J. Am. Chem. Soc.* 125, 3192–3.
- 6 (21) Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004) A strain-promoted [3 + 2] azide-alkyne
7 cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc.* 126, 15046–7.
- 8 (22) Li, Z., Seo, T. S., and Ju, J. (2004) 1,3-Dipolar cycloaddition of azides with electron-deficient alkynes
9 under mild condition in water. *Tetrahedron Lett.* 45, 3143–3146.
- 10 (23) Bernardin, A., Cazet, A., Guyon, L., Delannoy, P., Vinet, F., Bonnaffé, D., and Texier, I. (2010)
11 Copper-free click chemistry for highly luminescent quantum dot conjugates: application to in vivo metabolic
12 imaging. *Bioconjug. Chem.* 21, 583–8.
- 13 (24) Jølleck, R. I., Sun, H., Berg, R. H., and Andresen, T. L. (2011) Catalyst-free conjugation and in situ
14 quantification of nanoparticle ligand surface density using fluorogenic Cu-free Click chemistry. *Chem. - A*
15 *Eur. J.* 17, 3326–3331.
- 16 (25) Bostic, H. E., Smith, M. D., Poloukhine, A. A., Popik, V. V., and Best, M. D. (2012) Membrane
17 labeling and immobilization via copper-free click chemistry. *Chem. Commun.* 48, 1431–1433.
- 18 (26) Oude Blenke, E., Klaasse, G., Merten, H., Plückthun, A., Mastrobattista, E., and Martin, N. I. (2015)
19 Liposome functionalization with copper-free “click chemistry.” *J. Control. Release* 202, 14–20.
- 20 (27) Fernández-Suárez, M., Baruah, H., Martínez-Hernández, L., Xie, K. T., Baskin, J. M., Bertozzi, C. R.,
21 and Ting, A. Y. (2007) Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule
22 probes. *Nat. Biotechnol.* 25, 1483–7.
- 23 (28) Link, A. J., Vink, M. K. S., Agard, N. J., Prescher, J. A., Bertozzi, C. R., and Tirrell, D. A. (2006)
24 Discovery of aminoacyl-tRNA synthetase activity through cell-surface display of noncanonical amino acids.
25 *Proc. Natl. Acad. Sci. U. S. A.* 103, 10180–5.
- 26 (29) Laughlin, S. T., Baskin, J. M., Amacher, S. L., and Bertozzi, C. R. (2008) In vivo imaging of
27 membrane-associated glycans in developing zebrafish. *Science* 320, 664–7.
- 28 (30) Poloukhine, A. A., Mbua, N. E., Wolfert, M. A., Boons, G.-J., and Popik, V. V. (2009) Selective
29 labeling of living cells by a photo-triggered click reaction. *J. Am. Chem. Soc.* 131, 15769–76.
- 30 (31) Chang, P. V., Prescher, J. A., Sletten, E. M., Baskin, J. M., Miller, I. A., Agard, N. J., Lo, A., and
31 Bertozzi, C. R. (2010) Copper-free click chemistry in living animals. *Proc. Natl. Acad. Sci. U. S. A.* 107,
32 1821–6.
- 33 (32) Feldborg, L. N., Jølleck, R. I., and Andresen, T. L. (2012) Quantitative evaluation of bioorthogonal
34 chemistries for surface functionalization of nanoparticles. *Bioconjug. Chem.* 23, 2444–50.
- 35 (33) Rüger, R., Müller, D., Fahr, A., and Kontermann, R. E. (2005) Generation of immunoliposomes using
36 recombinant single-chain Fv fragments bound to Ni-NTA-liposomes. *J. Drug Target.* 13, 399–406.
- 37 (34) Dietrich, C., Schmitt, L., and Tampé, R. (1995) Molecular organization of histidine-tagged
38 biomolecules at self-assembled lipid interfaces using a novel class of chelator lipids. *Proc. Natl. Acad. Sci.*
39 *U. S. A.* 92, 9014–8.
- 40 (35) Rüger, R., Müller, D., Fahr, A., and Kontermann, R. E. (2006) In vitro characterization of binding and
41 stability of single-chain Fv Ni-NTA-liposomes. *J. Drug Target.* 14, 576–82.
- 42 (36) Dong, X.-Y., Feng, X.-D., and Sun, Y. (2010) His-tagged protein purification by metal-chelate affinity
43 extraction with nickel-chelate reverse micelles. *Biotechnol. Prog.* 26, 1088–94.

- 1 (37) Knecht, S., Ricklin, D., Eberle, A. N., and Ernst, B. (2009) Oligohis-tags: mechanisms of binding to
2 Ni²⁺-NTA surfaces. *J. Mol. Recognit.* 22, 270–9.
- 3 (38) Lata, S., Reichel, A., Brock, R., Tampé, R., and Piehler, J. (2005) High-affinity adaptors for switchable
4 recognition of histidine-tagged proteins. *J. Am. Chem. Soc.* 127, 10205–15.
- 5 (39) Tanner, P., Ezhevskaya, M., Nehring, R., Van Doorslaer, S., Meier, W., and Palivan, C. (2012) Specific
6 His6-tag attachment to metal-functionalized polymersomes relies on molecular recognition. *J. Phys. Chem. B*
7 116, 10113–24.
- 8 (40) Platt, V., Huang, Z., Cao, L., Tiffany, M., Riviere, K., and Szoka, F. C. (2010) Influence of multivalent
9 nitrilotriacetic acid lipid-ligand affinity on the circulation half-life in mice of a liposome-attached His6-
10 protein. *Bioconjug. Chem.* 21, 892–902.
- 11 (41) McCusker, C. F., Kocienski, P. J., Boyle, F. T., and Schätzlein, A. G. (2002) Solid-phase synthesis of
12 c(RGDfK) derivatives: on-resin cyclisation and lysine functionalisation. *Bioorg. Med. Chem. Lett.* 12, 547–9.
- 13 (42) Budyka, M. F. (2008) Photodissociation of aromatic azides. *Russ. Chem. Rev.* 77, 709–723.
- 14 (43) Schultz, M. K., Parameswarappa, S. G., and Pigge, F. C. (2010) Synthesis of a DOTA-biotin conjugate
15 for radionuclide chelation via Cu-free click chemistry. *Org. Lett.* 12, 2398–2401.
- 16 (44) Pack, D. W., and Arnold, F. H. (1997) Langmuir monolayer characterization of metal chelating lipids
17 for protein targeting to membranes. *Chem. Phys. Lipids* 86, 135–52.
- 18 (45) Nieba, L., Nieba-Axmann, S. E., Persson, A., Hämäläinen, M., Edebratt, F., Hansson, A., Lidholm, J.,
19 Magnusson, K., Karlsson, Å. F., and Plückthun, A. (1997) BIACORE analysis of histidine-tagged proteins
20 using a chelating NTA sensor chip. *Anal. Biochem.* 252, 217–228.
- 21 (46) Smith, R. M., Martell, A. E., and Motekaitis, R. J. (2004) NIST Standard Reference Database 46 1–20.
- 22 (47) Hochuli, E., Döbeli, H., and Schacher, A. (1987) New metal chelate adsorbent selective for proteins and
23 peptides containing neighbouring histidine residues. *J. Chromatogr. A* 411, 177–184.
- 24 (48) Crowe, J., Döbeli, H., Gentz, R., Hochuli, E., Stüber, D., and Henco, K. (1994) 6xHis-Ni-NTA
25 chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol. Biol.*
26 31, 371–87.
- 27 (49) Thieriet, N., Giralt, E., Guib, F., and Albericio, F. (1997) Use of Alloc-amino Acids in Solid-Phase
28 Peptide Synthesis . Tandem Deprotection-Coupling Reactions Using Neutral Conditions. *Tetrahedron Lett.*
29 38, 7275–7278.
- 30 (50) Bangham, A. D., and Horne, R. W. (1964) Negative staining of phospholipids and their structural
31 modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* 8, 660–668.

32

1 Table of Contents Artwork

2

3

4

5