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Catalyst-Free Conjugation and In Situ Quantification of Nanoparticle Ligand Surface Density Using Fluorogenic Cu-Free Click Chemistry

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Surface functionalized nanoparticles have found widespread applications within the fields of medical diagnostics,[1] drug delivery,[1,2] sensor development,[3] and vaccines.[4] Attachment of highly specific biomolecules such as peptides, proteins, antibodies, or apatamers enables fine-tuning of the nanoparticle constructs resulting in highly functionalized nanoscale materials with high specificity towards, for example, analytes, enzymes, or over-expressed or selectively expressed receptors on diseased cells. Controlling the biomolecule surface density is often a crucial parameter for obtaining the desired properties of the nanomaterial, in particular within the field of drug delivery.[5] To ensure the right composition of the nanoparticle construct, the number of ligands immobilized on the surface must be analyzed. This has previously been done by measuring the fluorescence intensity of probes covalently attached to the targeting ligands.[6] Other methods include phosphorus and amino acid analysis,[7] SDS-PAGE,[8] or protein determination assays.[9] Generally, these techniques are invasive, laborious and time consuming, and are often limited to a semiquantitative determination of the surface density. To rapidly expand the field of highly functionalized nanoparticles there is a crucial need for new methods to efficiently functionalize and analyze nanoparticles. Surface coupling reactions should be fast, efficient, reproducible, mild, and ideally include a reporter functionality that allows direct quantification of coupling efficiency without prior purification. To fulfill these requirements, we have directed our attention towards the pro-fluorophore 3-azidocoumarin.[10] Coumarin derivatives are often used as biological probes, since they are both biocompatible and easy to manipulate synthetically. Introduction of an azido functionality at the 3-position results in efficient quenching of the coumarin fluorescence due to electron donation from the electron-rich α-nitrogen of the azido group into the coumarin backbone.[10] Triazole formation, which can be achieved by the Cu^{I}-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition reaction[11] ("Click" reaction) or by addition of strain-promoted[12] or electron-deficient alkynes,[13] eliminates the quenching through the formation of a triazole ring, thus resulting in a strong fluorescence signal. We envisioned that 3-azidocoumarins could be attached to the desired targeting ligand and function as both a highly specific (orthogonal) conjugation linker molecule and as a quantitative reporter of coupling efficiency in situ. To test this approach we have investigated PEGylated liposomes that have exposed terminal alkynes or cyclooctynes at the distal end of the PEG and shown that post-functionalization with 3-azidocoumarin-modified RGD peptides[14] is highly efficient. Coupling was achieved in high yield by the Cu^{I}-catalyzed approach; however, the direct quantitative determination of the conjugation efficiency was impaired due to quenching of the formed fluorophore by Cu^{II}. By adopting copper-free Click conditions, excellent conjugation efficiency was achieved, which was monitored by the direct and quantitative in-situ read-out that the new method provides.

The new approach utilizes the 3-azido-7-(carboxy methoxy)-chromen-2-one (**3**), which was synthesized in six steps from 2,4-dihydroxybenzaldehyde (**1**) and *N*-acetylglycine (**2**) with slight modifications to previously described methods (Scheme 1).[10,15] Compound **3** was attached to the *N*-terminal of the RGD-peptide after insertion of two glycine spacers, resulting in the peptide **4**. Compound **3** was found to be fully compatible with standard conditions used in Fmoc solid-phase peptide synthesis (SPPS). The two alkyne-modified lipids **6** and **8** were synthesized in a single step from commercially available DSPE-PEG_{2000}-NH_2 by acylation with 4-pentynoic acid (**5**) and **7**, respectively. Compound **7** was synthesized as previously described elsewhere.[16] The model RGD-peptide **4** used in the surface conjugation studies was synthesized by standard Fmoc SPPS methodology using a TentaGel resin with the RinkAmide linker (for a detailed description of the synthesis protocol see the Supporting Information). Visualization of the compounds containing an aromatic azide functionality by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was not possible due to laser-induced photodissociation of the aromatic azides.[17] However, electrospray ionization mass spectrometry (ESI-MS) gave the expected masses. Small unilamellar vesicles (SUVs) with the following compositions; liposome A: DSPC/DSPE-PEG_{2000}/**6** (95:4:1) and liposome B: DSPC/DSPE-PEG_{2000}/**8** (95:4:1) were prepared by the method described by Bangham et al.[18] The liposomes were characterized by dynamic light scattering (DLS) (liposome A: (129±

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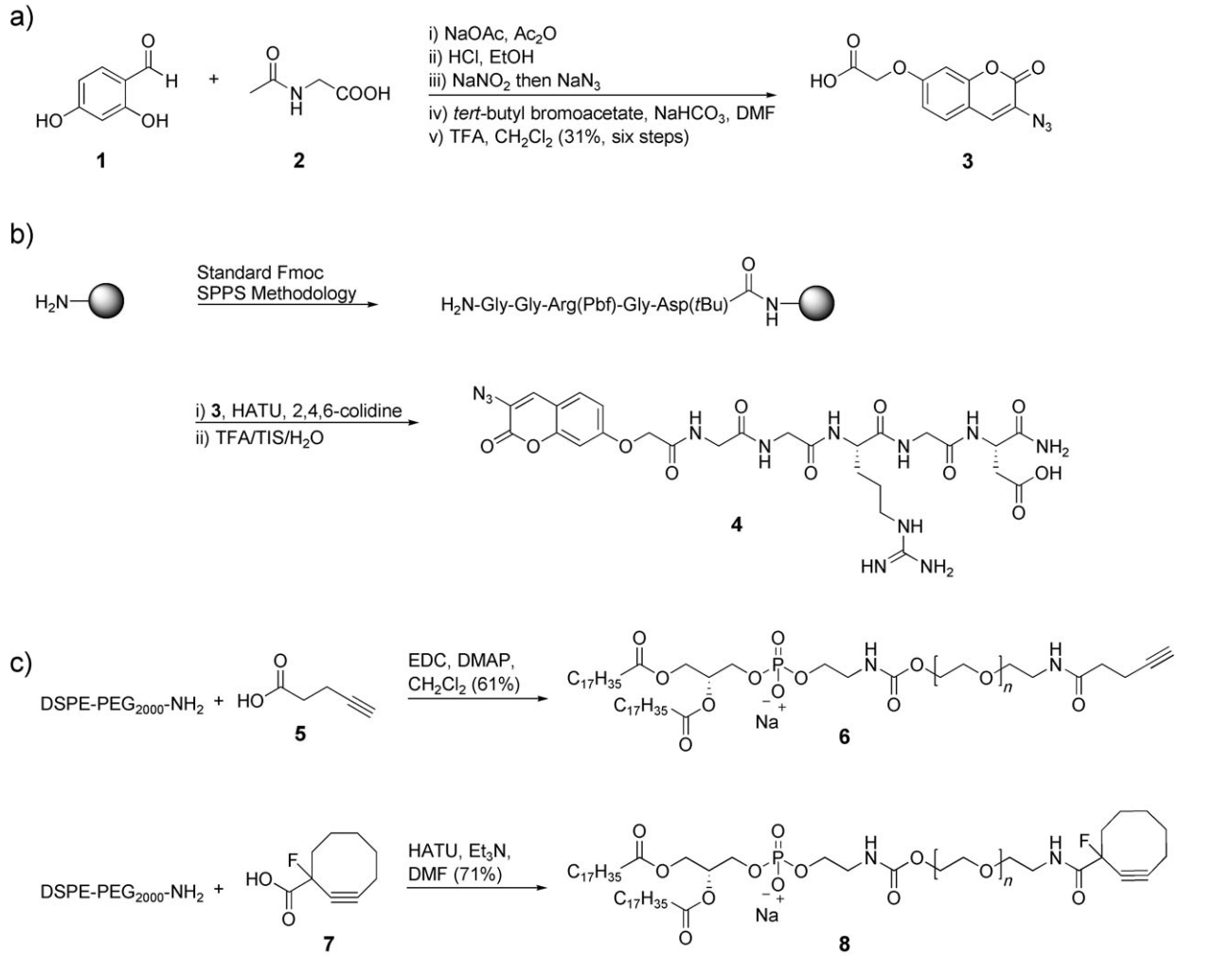

Scheme 1. a) Synthesis of 3-azido-7-(carboxymethoxy)-chromen-2-one (**3**); b) synthesis of 3-azidocoumarin-functionalized RGD-peptide **4**; c) synthesis of the alkyne-functionalized PEGylated phospholipids **6** and **8**. Fmoc = 9-Fluorenylmethoxycabonyl; HATU = *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate: TFA = trifluoroacetic acid; TIS = triisopropylsilane; EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMAP = 4-Dimethylaminopyridine; DSPE-PEG_{2000}-NH_2 = 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000].

1.7) nm, liposome B: (118.9 ± 0.7) nm) and the zeta potential was measured (liposome A: (−14.68 ± 0.50) mV, liposome B: (−12.22 ± 0.81) mV).

Initially, the Cu^I-catalyzed approach to functionalize liposomes was investigated. Site-specific conjugation of the 3-azidocoumarin-functionalized RGD-peptide **4** to the terminal alkyne-functionalized liposome A was carried out in the presence of **4** (0.125 mM), $CuSO_4 \cdot 5H_2O$ (1.25 mM), and sodium ascorbate (25 mM) (Figure 1). The same conditions without addition of $CuSO_4$ were used as a negative control. The reaction mixtures were protected from sunlight, gently stirred, and the degree of conversion was monitored by measuring the increase in fluorescence intensity (excitation 342 nm, emission 421 nm, Figure 2a) as a function of time. At each time point, 50 μL of the reaction solution was added to a cuvette containing HEPES buffer (1200 μL) and measured in a spectrofluorometer. By correlating the observed fluorescence intensity to a standard curve based on the core probe **9** in HEPES buffer (Figure 2b), the coupling efficiency could be directly quantified without prior purification. The fluorescence of **9** showed a linear correlation in the concentration range from 0.1–4.0 μM ($R^2 = 0.99$). The obtained conversion plot illustrated in Figure 2c was normalized according to a 60:40 distribution ratio of the functionalized lipid **6** due to the curvature-induced asymmetry of lipid bilayers in 100 nm liposomes.[19] As clearly evident from the conversion plot illustrated in Figure 2c, triazole formation occurs rapidly within the first hour, whereas the negative control (no copper added) remained non-fluorescent over the entire time-span of the experiment due to lack of triazole formation. However, a quenching effect was observed shortly after reaching full conversion. This phenomenon has not previously been described by other authors using coumarin as a analytical tool in protein conjugation chemistry,[15] even though the nature of the problem seems general as described below. Initially, our experiments were carried out by measuring the fluorescence in HEPES buffer giving the plot shown in Figure 2c (triangles). The data indicated

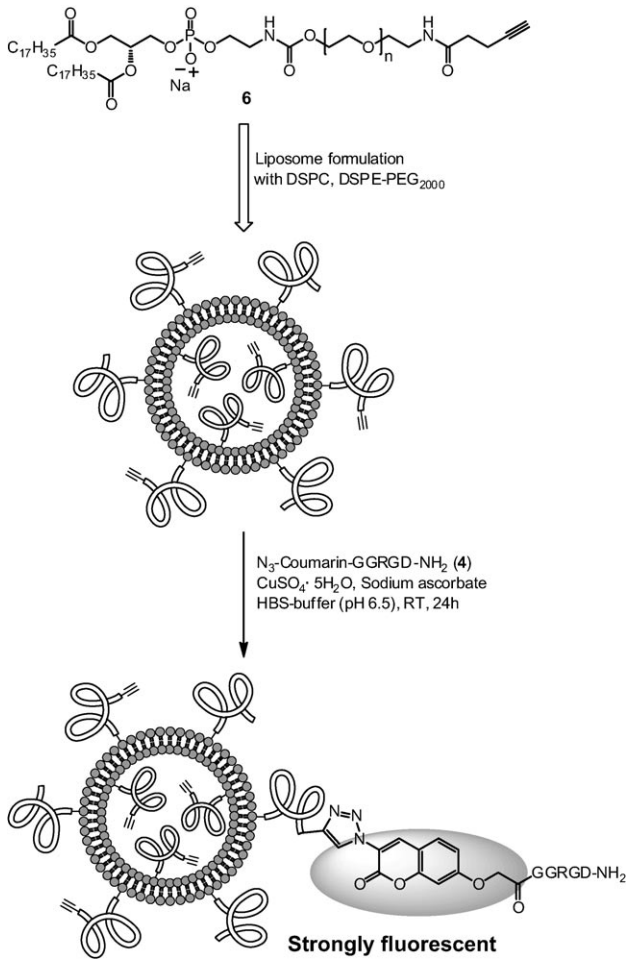

Figure 1. Liposomes consisting of DSPC/DSPE-PEG_{2000}/**6** were formulated and incubated with **4** in the presence of $CuSO_4$ and sodium ascorbate, and the increase of fluorescence intensity (Ex. 342 nm, Em. 421 nm) was monitored as a function of time.

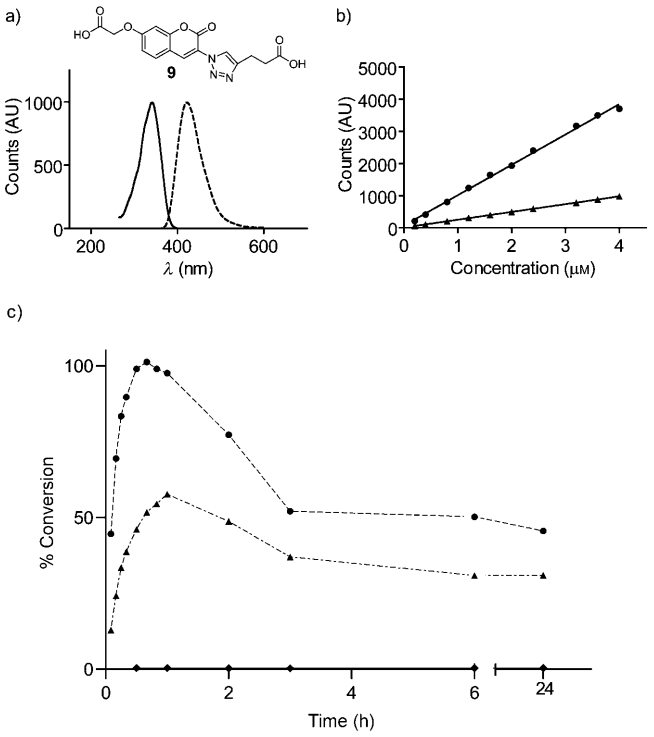

Figure 2. a) Excitation and emission scan of **9**. $\lambda_{Ex.\ max} = 342$ nm, $\lambda_{Em.\ max} = 421$ nm. b) Standard curves based on **9** in either $CHCl_3$/MeOH/H_2O (16:8:1) (●) or HEPES buffer (▲). c) Conversion plot of the Cu^I-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition reaction between the alkyne-modified liposome (liposome A; DSPC/DSPE-PEG_{2000}/**6** (95:4:1)) and **4**. Fluorescence intensity measured either in $CHCl_3$/MeOH/H_2O (16:8:1) (●) or HEPES buffer (▲) and converted to degree of conversion by using the standard curves based on **9**. Negative control performed without addition of copper (◆).

to us that self-quenching was occurring on the liposome surface due to a high local concentration of the coumarin probe. To test this, we disrupted the liposome by solubilization of the lipids in organic solvent. This was achieved by adding 50 μL of the reaction mixture to a solution of $CHCl_3$/MeOH (2:1) (1200 μL), thereby forming a homogenous solution with maximum intermolecular distance between the lipids. Disruption of the liposomes was confirmed by DLS, which showed no presence of colloids in the solution. As clearly illustrated in Figure 2c, a parallel shift along the *y* axis was observed when measuring the fluorescence in organic solvent compared to aqueous buffer, and we observed that the reaction proceeded to completion within one hour (Figure 2c, circles). However, as the time-dependent decrease in fluorescence signal remained, self-quenching was not the only effect. We speculated that there were a number of conditions that could influence the experiments. The fluorescence signal of **9** was found to be independent of pH (Figure 3a), whereas an increase in temperature caused a decrease in signal intensity (Figure 3b). However, since all measurements were performed at 30 °C this could not have caused the observed decrease. In addition to the temperature dependency, Cu^{II} was found to efficiently quench the fluorescence intensity of **9** (Figure 3c) and since most of the copper in the solution is Cu^I that was found not to quench the fluorescence, there is a time delay before this effect becomes dominant. This phenomenon, which recently has been described for 7-aminocoumarins[20] as well as other fluorophores,[21] was found to cause the observed drop in intensity. The plateau reached after 3 h represents the scenario where the formed probes at the liposomes interface are fully complexed with Cu^{II}. To regain the maximum fluorescence intensity, the Cu^{II}-chelator ethylenediaminetetraacetic acid (EDTA) ($\log K = 18.76$)[22] was added to the solution, but it did not result in complete recovery of the fluorescence intensity (Figure 3c). These findings encouraged us to develop a method in which the reaction could be carried out without the copper catalyst.

Liposome B, which contains exposed cyclooctynes at the outer PEG-layer, was incubated with **4** but without addition of either $CuSO_4$ or sodium ascorbate (Figure 4). The liposomes and the peptide were simply mixed and the fluorescence intensity was monitored as a function of time using the $CHCl_3$/MeOH solution as described above. The observed intensity (excitation 337 nm, emission 402 nm, Fig-

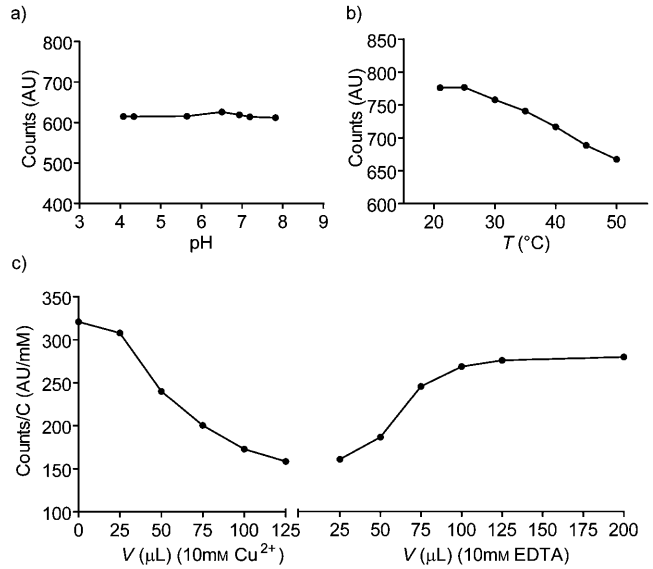

Figure 3. a) The influence of pH on the fluorescence intensity of **9**. b) The influence of temperature on the fluorescence intensity of **9**. c) Quenching of the fluorescence intensity of **9** by addition of $CuSO_4$ followed by recovery of the fluorescence intensity by addition of EDTA.

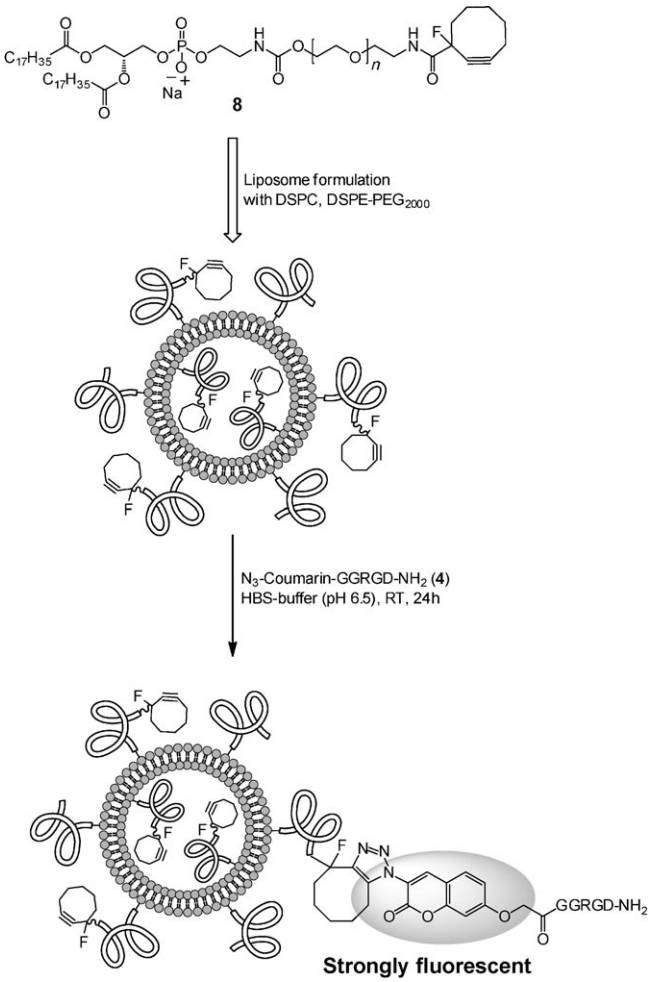

Figure 4. Liposomes consisting of DSPC/DSPE-PEG_{2000}/**8** were formulated and incubated with **4** and the increase of fluorescence intensity (Ex. 337 nm, Em. 402 nm) was monitored as a function of time.

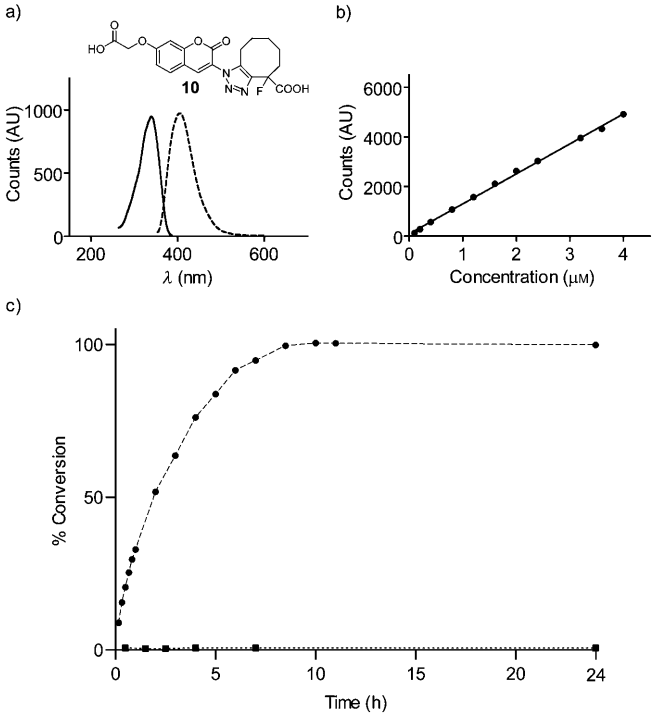

Figure 5. a) Excitation (full line) and emission scan (dashed) of **10**. $\lambda_{Ex.\ max} = 337$ nm, $\lambda_{Em.\ max} = 402$ nm. b) Standard curve based on **10** solubilized in $CHCl_3$/MeOH/H_2O (16:8:1). c) Conversion plot of the Cu-free strain-promoted azide/alkyne Huisgen 1,3-dipolar cycloaddition reaction between the alkyne-modified liposome (liposome B; DSPC/DSPE-PEG_{2000}/**8** (95:4:1)) and **4** (●). Negative control performed without addition of liposome B (■).

ure 5a) was correlated to a standard curve based on the two core-probe **10** (Figure 5b), which was isolated as a 1:1 mixture of possible regioisomers. The two regioisomers were found to have the same fluorescence spectral properties. As illustrated in Figure 5a, a blue shift was observed for **10** compared to the previously used probe **9**. The fluorescence of **10** was found to have a linear correlation in the concentration range from 0.1–4.0 μM ($R^2 = 0.99$). By adopting the Cu-free approach and disruption of the liposomes prior to the fluorescence measurements, a smooth conversion plot was achieved. The strain-promoted Click reaction proceeded slower than the Cu^I-catalyzed counterpart; however, quantitative conversion was achieved after approximately 8.5 h. The employed reaction conditions were found not to alter the size or the surface charge of the liposomes. Furthermore, the negative control without addition of liposome B remained non-fluorescent during the entire time span of the experiment.

The average number of peptides exposed at the outer liposome membrane (surface density) can be calculated by using Equations (1)–(3). By assuming that all liposomes are unilamellar and using an averaged lipid surface area, the number of lipid molecules in a single liposome can be calculated as described in Equation (1),

$$N_{tot} = \frac{[4\pi(\frac{d}{2})^2 + 4\pi(\frac{d}{2} - h)^2]}{a} \quad (1)$$

where N_{tot} is the number of lipids in a single liposome, d is the diameter of the liposome (118.9 nm), h is the thickness of the bilayer (4 nm), and a is the lipid head group area (PC lipids 0.70 nm^2).[23] The number of liposome particles per milliliter of liposome solution can be calculated as described in Equation (2),

$$N_{lipo} = \frac{M_{lipid} \times N_A}{N_{tot} \times 1000} \quad (2)$$

where N_{lipo} is the number of liposomes per milliliter and N_A is the Avogadro number. The absolute number of formed fluorophores can be calculated by correlating the observed fluorescence intensity to the standard curve based on **10** (Figure 5b). By dividing the number of formed fluorophores with the number of liposome particles the average surface density can be calculated as described in Equation (3),

$$\text{surface density} = \frac{c_f \times V \times N_A}{N_{lipo}} \quad (3)$$

where c_f is the concentration of the formed fluorophore and V is the reaction volume. By using Equations (1)–(3) the strain-promoted Click reaction (liposome B) resulted in an average surface density of 712 peptides per liposome.

From an overall perspective, this method is superior to other post-functionalization methods, as it proceeds without addition of a catalyst, under mild conditions, and includes a reporter functionality by which the conversion efficiency can be monitored directly without prior purification. Furthermore, avoiding addition of Cu is highly desirable for liposomal drug-delivery purposes since Cu is considered toxic and has been reported to promote oxidation of unsaturated phospholipids.[24] In addition, a shorter reaction time for the strain-promoted Click reaction may be achieved by employing more reactive cycloalkynes.[25]

In conclusion, we have developed a unique method to post-functionalize liposomes and directly quantify the achieved ligand density on the liposome surface. Our results have shown that 3-azidocoumarins are a valuable class of linker molecules that can be utilized to give a quantitative in-situ read out of conjugation efficiency. The Cu^{I}-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition reaction resulted in efficient conjugation; however, quantitative measurements of the coupling efficiency were not possible due to quenching by Cu^{II}. This limitation was solved by adopting Cu-free conditions by using electron-deficient strain-promoted alkynes. This approach resulted in a quantitative conversion, which could be monitored by fluorescence spectroscopy without prior workup. This method is the first to combine both functionalization and quantification of the conjugation efficiency on the surface of liposomes in one single step, and it is equally suited for other nanoparticle constructs (e.g. polymer micelles and polymersomes), including systems that cannot be dissolved by addition of organic solvents as described herein, if the fluorophore surface density is kept below the self-quenching concentration.

Experimental Section

The synthesis of the compounds **3**, **4**, **6**–**10**, their characterization (^{1}H NMR, ^{13}C NMR, IR, MALDI-TOF MS, ESI-MS, HPLC), liposome preparation, and protocols for surface functionalization are available in the Supporting Information.

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Keywords: click chemistry · coumarin · liposomes · nanoparticles · surface functionalization

[1] a) M. A. Phillips, M. L. Gran, N. A. Peppas, *Nano Today* **2010**, *5*, 143–159; b) S. Azarmi, W. H. Roa, R. Löbenberg, *Adv. Drug Delivery Rev.* **2008**, *60*, 863–875.
[2] a) M. E. Davis, Z. Chen, D. M. Shin, *Nat. Rev. Drug Discovery* **2008**, *7*, 771–782; b) V. Torchilin, *Expert Opin. Drug Delivery* **2008**, *5*, 1003–1025; c) R. A. Petros, J. M. DeSimone, *Nat. Rev. Drug Discovery* **2010**, *9*, 615–627.
[3] a) V. Pavlov, Y. Xiao, B. Shlyahovsky, I. Willner, *J. Am. Chem. Soc.* **2004**, *126*, 11768–11769; b) J. Liu, Y. Lu, *Nat. Protoc.* **2006**, *1*, 246–252.
[4] A. Muhs, D. T. Hickman, M. Pihlgren, N. Chuard, V. Giriens, C. Meerschman, I. van der Auwera, F. van Leuven, M. Sugawara, M. Weingertner, B. Bechinger, R. Greferath, N. Kolonko, L. Nagel-Steger, D. Riesner, R. O. Brady, A. Pfeifer, C. Nicolau, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 9810–9815.
[5] T. L. Andresen, S. S. Jensen, K. Jørgensen, *Prog. Lipid Res.* **2005**, *44*, 68–97.
[6] J. Lu, M. Shi, M. S. Shoichet, *Bioconjugate Chem.* **2009**, *20*, 87–94.
[7] S. Zalipsky, B. Puntambekar, P. Boulikas, C. M. Engbers, M. C. Woodle, *Bioconjugate Chem.* **1995**, *6*, 705–708.
[8] a) S. W. A. Reulen, W. W. T. Brusselaars, S. Langereis, W. J. M. Mulder, M. Breurken, M. Merkx, *Bioconjugate Chem.* **2007**, *18*, 590–596; b) B. Gamier, A. Bouter, C. Gounou, K. G. Petry, A. R. Brisson, *Bioconjugate Chem.* **2009**, *20*, 2114–2122.
[9] G. Bendas, D. Rothe, G. L. Scherphof, J. A. A. M. Kamps, *Biochim. Biophys. Acta Biomembr.* **2003**, *1609*, 63–70.
[10] K. Sivakumar, S. Xie, B. M. Cash, S. Long, H. N. Bamhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603–4606.
[11] a) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599; c) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596–2599.
[12] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
[13] Z. Li, T. S. Seo, J. Ju, *Tetrahedron Lett.* **2004**, *45*, 3143–3146.
[14] a) G. Huang, Z. Zhou, R. Srinivasan, M. S. Penn, K. Kottke-Marchant, R. E. Marchant, A. S. Gupta, *Biomaterials* **2008**, *29*, 1676–1685; b) L. Auzzas, F. Zanardi, L. Battistini, P. Burreddu, P. Carta, G. Rassu, C. Curti, G. Casiraghi, *Curr. Med. Chem.* **2010**, *17*, 1255–

1299; c) R. I. Jølck, L. N. Feldborg, S. Andersen S. M. Moghimi, T. L. Andresen, *Adv. Biochem. Eng./Biotechnol.* **2010**, DOI: 10.1007/10 2010 92.
[15] A. J. Dirks, J. J. L. M. Cornelissen, R. J. M. Nolte, *Bioconjugate Chem.* **2009**, *20*, 1129–1138.
[16] M. K. Schultz, S. G. Parameswarappa, F. G. Pigge, *Org. Lett.* **2010**, *12*, 2398–2401.
[17] M. F. Budyka, *Russ. Chem. Rev.* **2008**, ***77***, 709–723.
[18] A. D. Bangham, R. D. Horne, *J. Mol. Biol.* **1964**, *8*, 660–668.
[19] M. Rovira-Bru, D. H. Thompson, I. Szleifer, *Biophys. J.* **2002**, *83*, 2419–2439.
[20] H. S. Jung, P. S. Kwon, J. W. Lee, J. I. Kim, C. S. Hong, J. W. Kim, S. Yan, J. Y. Lee, J. H. Lee, T. Joo, J. S. Kim, *J. Am. Chem. Soc.* **2009**, *131*, 2008–2012.
[21] E. Espada-Bellido, D. M. Galinda-Riano, M. Garcia-Vargas, R. Narayanaswamy, *Appl. Spectrosc.* **2010**, *64*, 727–932.
[22] A. M. Crouch, L. E. Khotseng, M. Polhuis, D. R. Williams, *Anal. Chim. Acta* **2001**, *448*, 231–237.
[23] S. M. Gruner, M. W. Tate, G. L. Kirk, P. T. C. So, D. C. Turner, D. T. Keane, C. P. S. Tilcock, P. R. Cullis, *Biochemistry* **1988**, *27*, 2853–2866.
[24] a) C. Lee, J. Barnett, P. D. Reaven, *J. Lipid Res.* **1998**, *39*, 1239–1247; b) S. Gal, I. Pinchuk, D. Lichtenberg, *Chem. Phys. Lipids* **2003**, *126*, 95–110.
[25] J. A. Codelli, J. M. Baskin, N. J. Agard, C. R. Bertozzi, *J. Am. Chem. Soc.* **2008**, *130*, 11486–11493.

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