Bioconjugate Chemistry

Article

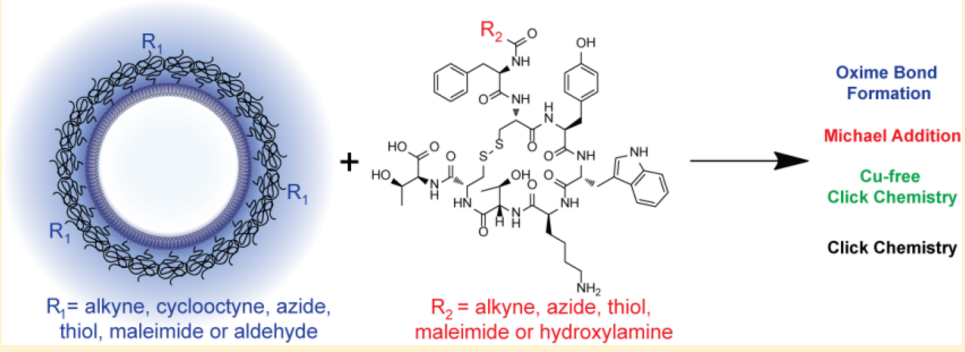
pubs.acs.org/bc

Quantitative Evaluation of Bioorthogonal Chemistries for Surface Functionalization of Nanoparticles

Lise N. Feldborg,[†] Rasmus I. Jølck,[†] and Thomas L. Andresen*

DTU Nanotech, Department of Micro- and Nanotechnology, Technical University of Denmark, Building 423 2800 Lyngby, Denmark

Supporting Information



ABSTRACT: We present here a highly efficient and chemoselective liposome functionalization method based on oxime bond formation between a hydroxylamine and an aldehyde-modified lipid component. We have conducted a systematic and quantitative comparison of this new approach with other state-of-the-art conjugation reactions in the field. Targeted liposomes that recognize overexpressed receptors or antigens on diseased cells have great potential in therapeutic and diagnostic applications. However, chemical modifications of nanoparticle surfaces by postfunctionalization approaches are less effective than in solution and often not high-yielding. In addition, the conjugation efficiency is often challenging to characterize and therefore not addressed in many reports. We present here an investigation of PEGylated liposomes functionalized with a neuroendocrine tumor targeting peptide (TATE), synthesized with a variety of functionalities that have been used for surface conjugation of nanoparticles. The reaction kinetics and overall yield were quantified by HPLC. Reactions were conducted in solution as well as by postfunctionalization of liposomes in order to study the effects of steric hindrance and possible affinity between the peptide and the liposome surface. These studies demonstrate the importance of choosing the correct chemistry in order to obtain a quantitative surface functionalization of liposomes.

INTRODUCTION

Functional nanomaterials have attracted considerable attention due to their highly interesting properties in relation to diagnostic,[1−3] sensor,[4,5] imaging,[6,7] vaccine,[8] and drug delivery applications.[9,10] Within the drug delivery field, liposomes have particularly advantageous properties.[11] However, despite positive results obtained by exploiting the EPR-effect,[12] numerous methods to improve drug bioavailability in the diseased tissue, while maintaining stable liposomes during circulation, is currently being investigated. By coating the liposome surfaces with targeting ligands such as antibodies,[13,14] peptides,[15] proteins,[16] polysaccharides,[17] lectins,[18] and vitamin analogues,[19,20] which are recognized by antigens or receptors selectively, or overexpressed in, e.g., tumor tissue, increased drug accumulation in tumors can be achieved. Numerous bioconjugation methods have been established to surface-modify liposomes with targeting ligands to enhance tissue specificity. To ensure correct orientation of the targeting ligand, bioorthogonal and site-specific surface reactions are required. By the incorporation of unnatural amino acids in peptide or protein sequences,[21] introduction of unnatural functional groups such as maleimides for Michael addition, azides for copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition[22−24] (CuAAC) or Staudinger ligation using triphosphines,[25,26] controlled display of surface ligands can be obtained. Unfortunately, ligand conjugation to the surface of nanoparticles can suffer from low reproducibility and highly varying yields; e.g., we have previously found that it can be very difficult to control the Michael addition of a somatostatin receptor targeting peptide (TATE) to a lipid moiety, even though the maleimide-based Michael addition is one of the most utilized conjugation chemistries for peptides and proteins.[27,28] As a consequence hereof, we have conducted a systematic study on a variety of generally applied conjugation reactions using HPLC as a method to follow and quantify the reactions both in solution and by direct conjugation to the liposomal surface. Reactions conducted directly on liposome surfaces are likely not to show the same reaction kinetics as ones preformed in

Received: September 8, 2012
Revised: October 24, 2012

   dx.doi.org/10.1021/bc3005057 | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

solution, and the yield of functionalization will not necessarily be quantitative and has to be determined case by case.

In this study, we furthermore introduce a novel highly selective and bioorthogonal conjugation reaction relevant for the surface functionalization of liposomes. We show a fast and quantitative coupling of a hydroxylamine functionalized peptide to an aldehyde-modified liposome, a strategy for coating liposomes, which to our knowledge has not previously been reported. In addition, we show a large dependency on the relative position of the conjugation functionalities. A knowledge which we believe is highly useful when designing new functionalized liposomal systems or other functional nanomaterials.

EXPERIMENTAL PROCEDURES

Synthesis of Functionalized TATE Peptides. The functionalized TATE peptides **1a−e** were synthesized manually by using a Wang Resin preloaded with NH_2-Thr(*t*Bu)-OH by standard Fmoc methodology. Each coupling was performed by activating the Fmoc protected amino acid with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) in the presence of 2,4,6-collidine in DMF. Cleavage of the Fmoc group was carried out using piperidine in DMF. Completion of each coupling and deprotection step was monitored by the Kaiser test. Removal of the acetamidomethyl (Acm) protection groups on the two cysteines and simultaneous disulfide bond formation was achieved by addition of $Tl(CF_3COO)_3$ in DMF. The resin containing the cyclized peptide was extensively washed with DMF and subsequently divided into five solid-phase peptide synthesis (SPPS) reaction vials in which the N-terminal was acylated with the desired functionality, cleaved from the solid support, and purified by HPLC. Full experimental details and peptide characterization are available as Supporting Information.

Synthesis of Functionalized DSPE-PEG_{2000} Phospholipids. Functionalized DSPE-PEG_{2000} phospholipids **2a−d** were synthesized in a single step from DSPE-PEG_{2000}-NH_2 by acylation of the amino group with the desired functionality in the presence of either HATU or EDC·HCl. Phospholipid **3** was synthesized in a single step from DSPE-PEG_{2000}-PDP by reduction by dithiothreitol (DTT). Full experimental details and lipid characterization are available as Supporting Information.

Liposome Formulation. Lipids were dissolved in CH_3Cl/MeOH (9:1) and mixed in the ratio 95:4:1 DSPC/DSPE-PEG_{2000}/DSPE-PEG_{2000}-R (R being the different functional groups illustrated in Schemes 2 and 3). The solvent was removed under a stream of nitrogen and the films placed under vacuum overnight to remove remaining traces of organic solvent. The obtained films were hydrated in a Hepes buffer, (10 mM Hepes, pH 6.5, 145 mM NaCl) at 65 °C for 1 h; followed by 10 freeze−thaw cycles and extrusion at 65 °C through a 100 nm polycarbonate filter using an Avanti Polar Lipids mini-extruder. The size distribution of the liposomes was analyzed using dynamic light scattering, as well as their zeta potential using a Brookhaven Zeta PALS analyzer. All sizes were 100−120 nm and all zeta potentials were slightly negative (approximately −5 mV).

Conjugation in Solution. DSPE-PEG_{2000} functionalized lipid (0.2 mM, 0.55 mL, 1.1 equiv.) was dissolved in MeOH and mixed with the corresponding functionalized TATE in MeOH (0.2 mM, 0.50 mL, 1.0 equiv.). The reaction was stirred at room temperature and aliquots (50 μL) were removed at the indicated times and analyzed by analytical HPLC. A linear gradient was used from 100% A (aqueous solution containing 5% MeCN and 0.1% TFA) to 100% B (MeCN containing 0.1% TFA) over 15 min with a flow rate of 1 mL/min and AUC for the free TATE was measured relative to AUC for the coupled product at 280 nm. For the reaction between the maleimide and thiol functionalities Et_3N (50 mM, 6.0 μL, 3.0 equiv.) was added to facilitate the coupling, while for the copper catalyzed click reactions $CuSO_4·5H_2O$ (1.0 M, 2.5 μL, 25 equiv.) and sodium ascorbate (1.0 M, 5.0 μL, 50 equiv.) were added. Before injecting the aliquots from the copper catalyzed click reactions onto the analytical HPLC column 20 μL of a 10% NH_4OH solution was added to ensure the copper ions were in solution and not interfering with the HPLC signals.[29]

Conjugation by Post-Functionalization of Liposomes. Preformed functionalized liposomes ((25 mM, 0.50 mL, 1.1 equiv.) - normalized according to a 45:55 distribution ratio between the inner- and outer liposomal membrane) were mixed with the corresponding TATE peptide (4.2 mM, 15 μL, 1.0 equiv.) dissolved in MeOH. The reactions were shaken (not stirred; to avoid foaming) at room temperature and aliquots (40 μL) removed for analysis by analytical HPLC. A linear gradient was used from 100% A (aqueous solution containing 5% MeCN and 0.1% TFA) to 100% B (MeCN containing 0.1% TFA) over 15 min with a flow rate of 1 mL/min. The AUC for the free TATE was calculated relative to the AUC of the phospholipid coupled product at 280 nm to monitor the conjugation efficiency. For the copper catalyzed click reactions $CuSO_4·5H_2O$ (1.0 M, 1.6 μL, 25 equiv.) and sodium ascorbate (1.0 M, 3.1 μL, 50 equiv.) were added. As for the conjugations done in solution 20 μL of a 10% NH_4OH solution was added to the copper catalyzed click aliquots prior to HPLC analysis.

RESULTS AND DISCUSSION

Synthesis and Characterization. The model peptides **1a−1e** for somatostatin receptor targeting[30] were prepared using a SPPS strategy as shown in Scheme 1.[31] The N-terminal functionalized TATE peptides **1a−e** were synthesized by SPPS on H-Thr(*t*Bu)-OH preloaded Wang Resin. Acylation of the N-terminal of the peptide was achieved by activating the Fmoc-protected amino acid with HATU to ensure high conversion with minimum epimerization. After assembly of the resin immobilized octapeptide, H-D-Phe-Cys(Acm)-Tyr(*t*Bu)-D-Trp-(Boc)-Lys(Boc)-Thr(*t*Bu)-Cys(Acm)-Thr(*t*Bu)-resin, the peptide was treated with $Tl(CF_3COO)_3$, which in a single step removed the two Acm-protection groups present on the cysteine residues and formed the intramolecular disulfide bond. The reaction was followed by cleavage of a small amount of peptide from the resin with TFA and analyzing the crude peptide by MALDI-TOF MS. Complete Acm-removal and disulfide formation were observed after 75 min with no sign of dimerization. After extensive washing of the resin, the N-terminal was acylated with the desired carboxylic acid functionalized linker derivatives and cleaved from the solid support by treatment with a mixture of TFA/H_2O/TIS (95:2.5:2.5) to give the desired functionalized TATE peptides **1a−e**. Final purification of the peptides was accomplished by HPLC and the obtained products **1a−e** characterized by MALDI-TOF MS (Table 1). In order to formulate liposomes exposing the appropriate chemical functionalities at the distal end of the PEG-corona, PEGylated phospholipids with the desired functionalities were synthesized from either DSPE-

dx.doi.org/10.1021/bc3005057 | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

Scheme 1. Synthesis of the TATE-Derivatives 1a−1e

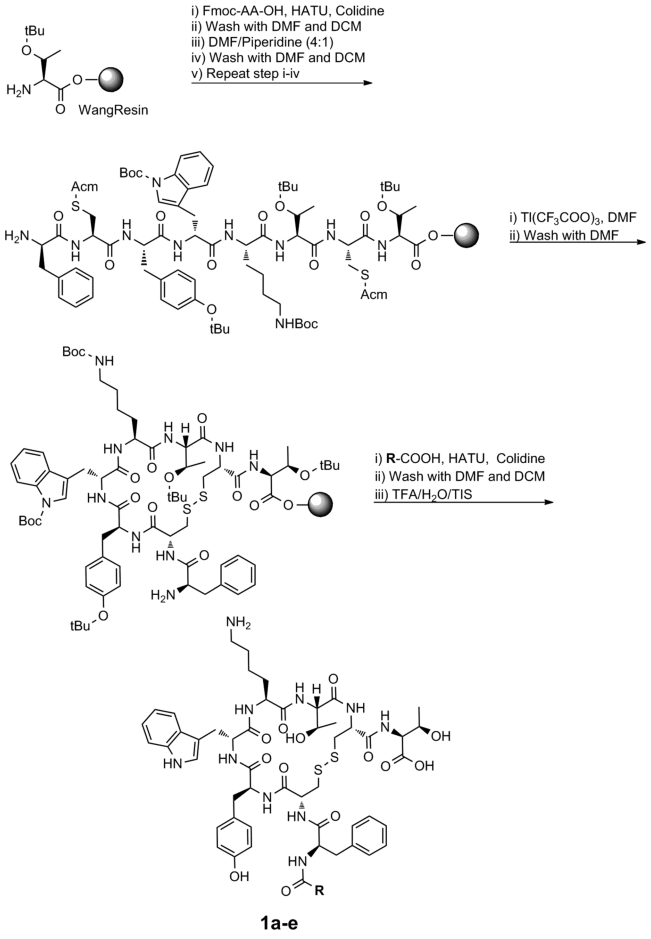
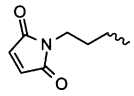
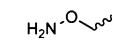
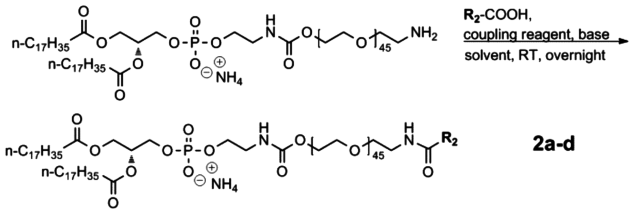


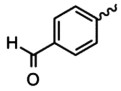
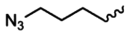
Table 1. Chemical Structure of R-COOH Used for Synthesis of TATE Derivatives 1a−1e and Corresponding Characterization by MALDI TOF MS

Compound #	R_1	MS $(M+Na)^+$ (cald.)	MS $(M+Na)^+$ (found)
1a		1236.35	1236.39
1b		1159.33	1159.57
1c		1196.33	1196.60
1d		1129.31	1129.47
1e		1144.25	1144.41

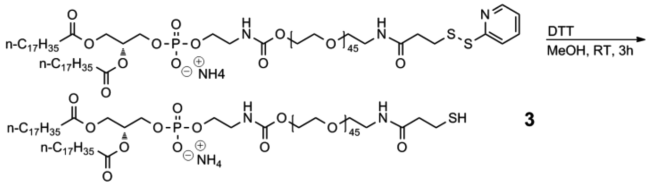
PEG_{2000}-NH_2 or DSPE-PEG_{2000}-PDP as illustrated in Schemes 2 and 3. PEGylated phospholipids exposing a terminal alkyne, a cyclic alkyne, an aldehyde, or an azide functionality at the distal end of the polymer (**2a−d**) were synthesized by acylating DSPE-PEG_{2000}-NH_2 with 4-pentynoic acid, 1-fluorocyclooct-2-ynecarboxylic acid, 4-carboxybenzaldehyde, or 5-azidopentanoic acid, respectively. 1-Fluorocyclooct-2-ynecarboxylic acid was synthesized as described elsewhere.[32] DSPE-PEG_{2000}-SH (**3**) was synthesized in a single step from DSPE-PEG_{2000}-PDP by reduction of the pyridyldithiopropionate (PDP) group by DTT applying standard conditions[33] which resulted in the desired

Scheme 2. Synthesis of the DSPE-PEG_{2000}-NH_2 Modified Phospholipids 2a−d



Compound #	R_2	Coupling reagent	Base	Solvent
2a		EDC·HCl	DMAP	CH_2Cl_2
2b		HATU	Et_3N	DMF
2c		HATU	Et_3N	DMF
2d		HATU	Et_3N	DMF

Scheme 3. Synthesis of the DSPE-PEG_{2000}-SH (3) Functionalized Phospholipid



free thiol (Scheme 3). The DSPE-PEG_{2000}-maleimide (**4**) functionalized phospholipid was purchased from Avanti Polar Lipids, Inc., Alabama.

In order to evaluate the efficiency of the different coupling strategies and make a valid comparison, a standardized protocol was used to minimize the influence of other parameters. Functionalized liposomes composed of DSPC/DSPE-PEG_{2000}/DSPE-PEG_{2000}-R (95:4:1) (R = alkyne (**2a**), cycloalkyne (**2b**), aldehyde (**2c**), azide (**2d**), thiol (**3**), or maleimide (**4**)) were prepared by the method described by Bangham and co-workers.[34] The lipid components were dissolved in a mixture of $CHCl_3$/MeOH (9:1), and the solvent was removed to form a transparent lipid film. The lipid film was hydrated in HEPES-buffer (pH 6.5) at 65 °C and extruded through 100 nm polycarbonate filters to form liposomes in the 100 nm range. The liposomes were characterized by dynamic light scattering (DLS), and the ζ-potentials were measured to ensure that liposomes were formed. All liposomes had a diameter of approximately 100 nm and slightly negative ζ-potential due to the presence of the DSPE-PEG_{2000} lipids. Postfunctionalization was carried out by adding the required components for the individual reactions (e.g., $CuSO_4 \cdot 5H_2O$ and sodium ascorbate for the CuAAC reaction), followed by addition of the required functionalized TATE peptide **1a−e** (1 equiv peptide to 1.1 equiv of the functionalized lipid component) in MeOH. Postfunctionalization reactions were carried out at room temperature and carefully mixed using a plate shaker.

dx.doi.org/10.1021/bc3005057 | Bioconjugate Chem. XXXX, XXX, XXX−XXX

For the conjugation reactions in solution, functionalized phospholipids (1.1 equiv) and functionalized TATE peptides (1 equiv) were dissolved in methanol, mixed with the required additives to facilitate reaction, as described in the Experimental Procedures, and stirred at room temperature. Triplicates of all reactions were carried out to ensure reproducibility and elucidate the heterogeneity of liposomal postfunctionalization. The amount of functionalized TATE added in the two conjugation approaches was carefully controlled by measuring the concentration of the peptide stock solution by UV−vis, thus ensuring exactly the same peptide concentration in all reactions. To ensure the same total concentration of phospholipids in all reactions, phosphorus content measurement was carried out by ICP-AES. All reactions were monitored for 24 h by HPLC using UV-detection (λ = 280 nm), and up to 72 h in selected cases, by removing small aliquots from the reaction vial, and the conjugation efficiency was calculated based on the area under the curve (AUC) for the free peptide and the DSPE-PEG_{2000}-TATE conjugate, respectively (Figure 1). MALDI-TOF MS was used to verify that an excess of the functionalized phospholipids reagent existed in all reactions that did not go to completion within 72 h.

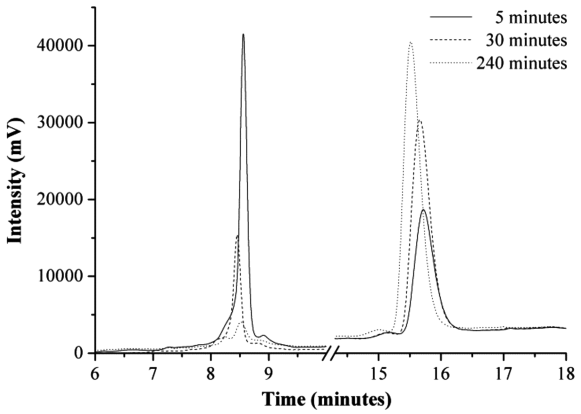


Figure 1. Example of the monitoring of a conjugation reaction by HPLC over time at 280 nm. The decreasing peak at 8.5 min belongs to the free TATE-maleimide (**1a**), while the peak increasing at 15.6 min is the product from the Michael addition between TATE-maleimide (**1a**) and DSPE-PEG_{2000}-SH (**3**).

Conjugation of TATE and DSPE-PEG_{2000} by Oxime Formation. This method, which has not previously been reported for the surface functionalization of liposomes, is chemoselective and bioorthogonal to functional groups in most natural occurring ligands and could serve as an excellent alternative to existing liposome postfunctionalization methods.[35] TATE-hydroxylamine (**1e**) and DSPE-PEG_{2000}-benzaldehyde (**2c**) were conjugated, both in solution and on the liposome surface, by simply mixing the two components without any additives. Oxime bond formation in solution was found to be quantitative within one hour, whereas eight hours of incubation was needed once performed directly on the liposome surface (Figure 2). Both procedures resulted in complete conversion to the phospholipid coupled TATE within 24 h under mild conditions.

Oxime bond formation is known to be accelerated by the presence of aniline,[36] acetic acid,[37] or Lewis acids.[38] In order to verify this, acetic acid (0.1% v/v) was added to a liposome batch containing TATE-hydroxylamine (**1e**). Under these

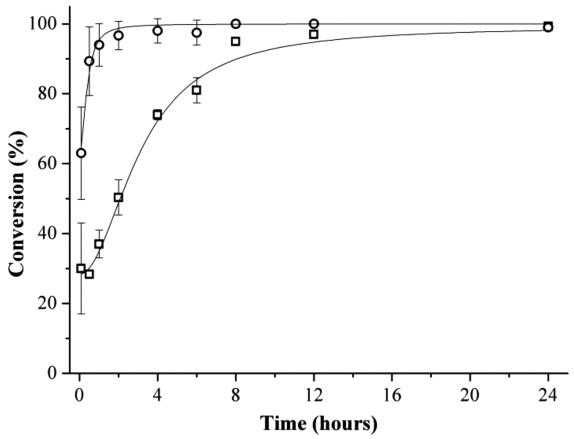


Figure 2. Conjugation of DSPE-PEG_{2000} and TATE by oxime bond formation. Reactions carried out in solution and directly on the liposome surface. TATE-hydroxylamine (**1e**) + DSPE-PEG_{2000}-benzaldehyde (**2c**) in solution (○), TATE-hydroxylamine (**1e**) + DSPE-PEG_{2000}-benzaldehyde (**2c**) on liposomes (□). All values are means ± SEM (n = 3).

conditions, the conjugation yield reached 78% within 5 min. Without the presence of the catalyst, 30% conversion was observed within the same time frame. The oxime bond formation is a reversible process; however, HPLC and MALDI TOF MS did not show any reversible formation of the starting materials within one week in aqueous medium, which supports the reporting of oxime bonds to be more hydrolytically stable than, e.g., its analogous hydrazine bond at physiologically relevant pH.[39] The position of these two functional groups is not interchangeable, as placing the aldehyde functionality on TATE will induce the risk of intramolecular imine formation.

Conjugation of TATE and DSPE-PEG_{2000} by CuAAC. An often applied procedure for nanoparticle functionalization is the CuAAC reaction—commonly referred to as the Click Reaction.[22−24] Conjugation of TATE and DSPE-PEG_{2000} by the CuAAC reaction was carried out using sodium ascorbate to generate Cu^{I} from $CuSO_4 \cdot 5H_2O$ in situ. Reactions were carried out with the azide present on the TATE peptide, the alkyne at the PEG terminal of DSPE-PEG_{2000}, and vice versa. The relative position of the functional moieties was found to have no significant effect on the reaction efficacy under the solution conditions, as both orientations result in approximately 98% yield after 12 h (Figure 3). A different result was obtained when monitoring the postfunctionalization reaction on the liposome surface. The cycloaddition between the azide-functionalized TATE (**1c**) and the alkyne-modified phospholipid (**2a**) resulted in about 75% yield after 2 h, while the opposite position having the azide functionality present on the liposomes and the alkyne at the N-terminal of the peptides leveled out at 43% (Figure 3).

Functionalization of azide-modified liposomes by the CuAAC reaction has not previously been reported elsewhere to our knowledge. Functionalization of liposomes by the CuAAC reaction has consistently been carried out with alkyne-modified liposomes and azide-functionalized ligands; thus, these reports have not observed the clear negative trend observed here with the azide-functionalized liposomes. Furthermore, removing the copper fully from the final product to avoid copper induced cytotoxicity can be challenging, which

dx.doi.org/10.1021/bc3005057 | Bioconjugate Chem. XXXX, XXX, XXX−XXX

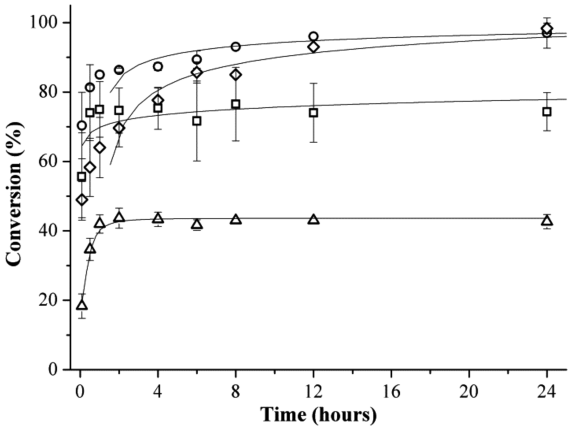


Figure 3. Conjugation of DSPE-PEG$_{2000}$ and TATE by the CuAAC reaction. Reactions carried out in solution and directly on the liposome surface. TATE-azide (**1c**) + DSPE-PEG$_{2000}$-alkyne (**2a**) in solution (○), TATE-azide (**1c**) + DSPE-PEG$_{2000}$-alkyne (**2a**) on liposomes (□), TATE-alkyne (**1d**) + DSPE-PEG$_{2000}$-azide (**2d**) in solution (◊), TATE-alkyne (**1d**) + DSPE-PEG$_{2000}$-azide (**2d**) on liposomes (Δ). All values are means ± SEM (n = 3).

to some degree reduces the usability of this reaction for biological purposes.

Conjugation of TATE and DSPE-PEG$_{2000}$ by Cu-Free Click Chemistry. A method to avoid addition of toxic copper salts as catalyst in the triazole formation is to use strained or electron-deficient alkynes.[40,41] This strategy was studied using the DSPE-PEG$_{2000}$-cyclooctyne (**2b**) and azide-functionalized TATE (**1c**). In solution, the conjugation was remarkably slow, yet highly reliable and reproducible with no form of decomposition or side product formation. As the reaction was terminated after 50 days, a product yield of 86% was obtained without reaching a plateau. Applying the post-functionalization protocol resulted in a remarkably faster initial reaction (Figure 4), and a level of 84% yield was reached after 72 h.

Interestingly, triazole formation was significantly faster when performed on liposome surfaces compared to the reaction in solution. This is presumably due to favorable interactions between the liposome surface and TATE-azide (**1c**). As TATE-azide (**1c**) is not highly soluble in the buffer, it prefers to bind to the PEG layer, bringing it closer to the reaction sites on the surface, which leads to a faster reaction. Choosing a more reactive cyclooctyne could not only increase the reaction rate in solution, but also increase the risk of side product formation.[42,43] However, a relatively long synthetic procedure to obtain the cyclooctyne moiety limits the use of this type of copper-free click reaction. Furthermore, it has been shown that thiols can add spontaneously to cyclooctynes limiting the bioorthogonality, as some degree of nonspecific reactions can occur.[44]

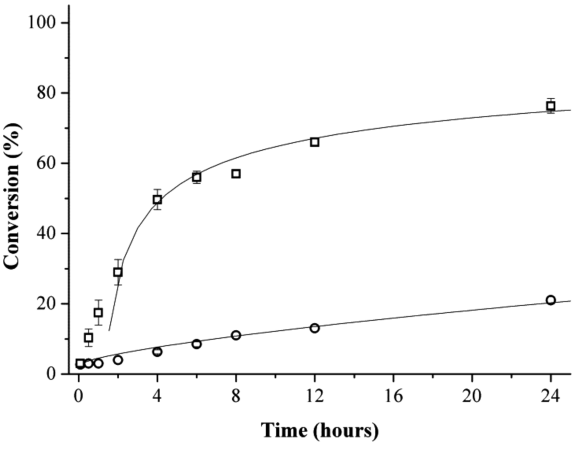


Figure 4. Conjugation of DSPE-PEG$_{2000}$ and TATE by strain-promoted Click reaction. Reactions were carried out in solution and directly on the liposome surface. TATE-azide (**1c**) + DSPE-PEG$_{2000}$-cyclooctyne (**2b**) in solution (○), TATE-azide (**1c**) + DSPE-PEG$_{2000}$-cyclooctyne (**2b**) on liposomes (□). All values are means ± SEM (n = 3).

Conjugation of TATE and DSPE-PEG$_{2000}$ by Michael Addition. The final approach tested to conjugate TATE and DSPE-PEG$_{2000}$ was using the Michael addition procedure. In general, the Michael addition proceeded rapidly within the first four hours until reaching a plateau, and reactions carried out in solution were slightly faster compared to the liposomal counterpart. The orientation of the maleimide and the thiol was found to have a decisive impact on the conjugation efficiency. Michael addition in solution between DSPE-PEG$_{2000}$-SH (**3**) and TATE-maleimide (**1a**) resulted in 97% conversion, whereas when performed on the liposome surface, 92% conversion was observed. The opposite orientation of functionalities expressed in the form of DSPE-PEG$_{2000}$-maleimide (**4**) and TATE-thiol (**1b**) only resulted in 53% product formation when performed in solution and 40% for the postfunctionalization of liposomes (Figure 5), which was surprising. The origin for this observation is not clear, however; multiple factors could have caused this drop in conversion. Hydrolysis of the maleimide moiety to the nonreactive maleamic acid at pH > 8.5 has been reported in the literature,[45] but at pH 6.5 where our reactions are carried out, this should be minimized.[46,47] This was confirmed by MALDI-TOF MS where intact DSPE-PEG$_{2000}$-maleimide as well as unreacted TATE-SH was observed. However, a minor amount of methoxy-substituted maleimide was also observed by MALDI-TOF MS indicating that the oxa-Michael addition is a competing side

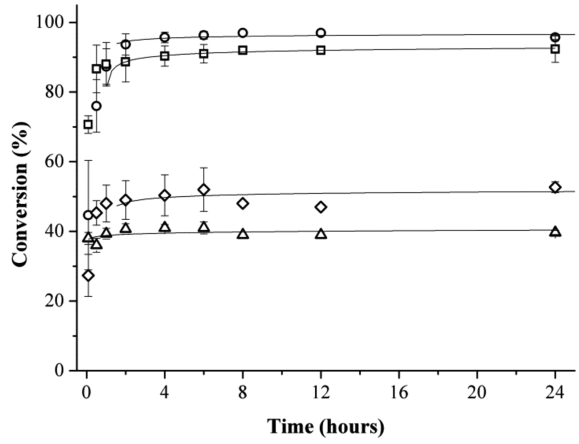


Figure 5. Conjugation of DSPE-PEG$_{2000}$ and TATE by Michael addition. Reactions carried out in solution and directly on the liposome surface. TATE-maleimide (**1a**) + DSPE-PEG$_{2000}$-SH (**3**) in solution (○), TATE-maleimide (**1a**) + DSPE-PEG$_{2000}$-SH (**3**) on liposomes (□), TATE-thiol (**1b**) + DSPE-PEG$_{2000}$-maleimide (**4**) in solution (◊), TATE-thiol (**1b**) + DSPE-PEG$_{2000}$-maleimide (**4**) on liposomes (Δ). All values are means ± SEM (n = 3).

dx.doi.org/10.1021/bc3005057 | *Bioconjugate Chem.* XXXX, XXX, XXX−XXX

reaction.[48] As poor conversion was observed for the reaction carried out in solution as well as on the surface, we speculated that the TATE-thiol (**1b**) peptide was the limiting factor in this reaction. TATE-thiol (**1b**) is highly prone to oxidation resulting in nonreactive TATE-dimers; however, dimerization was not observed by HPLC or by MALDI-TOF MS. As a positive control of the reactivity of the commercial DSPE-PEG_{2000}-maleimide (**4**), a test reaction using 2-mercaptoethanol was conducted. Using this small and highly reactive thiol, complete product formation was observed within two hours as confirmed by MALDI-TOF MS, confirming excellent reactivity of DSPE-PEG_{2000}-maleimide (**4**). Hence, the reason for the low coupling efficiency between DSPE-PEG_{2000}-maleimide (**4**) and TATE-SH (**1b**) is not clear.

CONCLUSION

In conclusion, a novel highly effective method to functionalize liposomes by postfunctionalization as well as under solution conditions has been introduced in the form of an oxime bond formation between an aldehyde and a hydroxylamine. This conjugation is chemoselective and bioorthogonal to functional groups in most naturally occurring relevant ligands, and based on our investigations, it could serve as an excellent alternative to existing liposome postfunctionalization methods.

In addition, a systematic study has been conducted to elucidate the optimal postfunctionalization chemistry with focus on the relative position of the reactive functionalities. In general, the reactions carried out directly on the surface of the functionalized liposomes were slower than the solution-phase counterpart, except for the strain-promoted Click reaction, which surprisingly showed the opposite trend. Despite of possible heterogeneities of liposomal formulations, surface conjugation reactions were, in general, highly reproducible. The relative position of the reactive functionalities was found to have a significant impact on the degree of conversion for the Michael addition and the CuAAC reaction. The Michael addition was found to be most effective when the nucleophilic thiol was present at the distal end of the PEG polymer compared to the N-terminal of TATE. A similar observation was observed for the CuAAC reaction. The relative positions of the azide and the alkyne did not influence the degree of conversion in solution; however, once performed on the liposome surface it had a decisive impact. Having the alkyne functionality present at the liposome surface resulted in 75% conversion, whereas the azide functionalized liposomes only resulted in 43% product formation.

The findings described above will be useful for future postfunctionalization designs and clearly illustrate that care should be taken to select the most appropriate chemistry for the desired chemical manipulation. Furthermore, it is also evident that postfunctionalized nanoparticles in general should always be analyzed in order to ensure the desired composition of the nanoparticle constructs.

ASSOCIATED CONTENT

Supporting Information

Detailed description of the synthetic approach to compounds **1a–e**, **2a–d**, **3** and their characterization (^{1}H NMR, FT-IR, HPLC, and MALDI-TOF MS). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

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

Author Contributions

†Authors contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The Danish Strategic Research Council (NABIIT) grant 2106-07-0033 and 2106-08-0081 is gratefully acknowledged for funding.

ABBREVIATIONS

EPR, enhanced permeability and retention effect; CuAAC, copper catalyzed azide–alkyne cycloaddition; TATE, [Tyr^3,Thr^8]-octreotate; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; HPLC, high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; DLS, dynamic light scattering; ICP-AES, inductively coupled plasma atomic emission spectroscopy.

REFERENCES

(1) Petersen, A. L., Hansen, A. E., Gabizon, A., and Andresen, T. L. (2012) Liposome imaging agents in personalized medicine. *Adv. Drug Delivery Rev. 64*, 1417–1435.

(2) Phillips, M. A., Gran, M. L., and Peppas, N. A. (2010) Targeted nanodelivery of drugs and diagnostics. *Nano Today 5*, 143–159.

(3) Petersen, A. L., Binderup, T., Jølck, R. I., Rasmussen, P., Henriksen, J. R., Pfeifer, A. K., Kjaer, A., and Andresen, T. L. (2012) Positron emission tomography evaluation of somatostatin receptor targeted 64Cu-TATE-liposomes in a human neuroendocrine carcinoma mouse model. *J. Controlled Release 160*, 254–263.

(4) Pavlov, V., Xiao, Y., Shlyahovsky, B., and Willner, I. (2004) Aptamer-functionalized Au nanoparticles for the amplified optical detection of thrombin. *J. Am. Chem. Soc. 126*, 11768–11769.

(5) Liu, J., and Lu, Y. (2006) Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes. *Nat. Protoc. 1*, 246–252.

(6) Taylor, K. M. L., Kim, J. S., Rieter, W. J., An, H., Lin, W., and Lin, W. (2008) Mesoporous silica nanospheres as highly efficient MRI contrast agents. *J. Am. Chem. Soc. 130*, 2154–2155.

(7) Sun, I. C., Eun, D. K., Koo, H., Ko, C. Y., Kim, H. S., Yi, D. K., Choi, K., Kwon, I. C., Kim, K., and Ahn, C. H. (2011) Tumor-targeting gold particles for dual computed tomography/optical cancer imaging. *Angew. Chem., Int. Ed. 50*, 9348–9351.

(8) Muhs, A., Hickman, D. T., Pihlgren, M., Chuard, N., Giriens, V., Meerschman, C., van der Auwera, I., van Leuven, F., Sugawara, M., Weingertner, M. C., Bechinger, B., Greferath, R., Kolonko, N., Nagel-Steger, L., Reisner, D., Brady, R. O., Pfeifer, A., and Nicolau, C. (2007) Liposomal vaccines with conformation-specific amyloid peptide antigens define immune response and efficacy in APP transgenic mice. *Proc. Natl. Acad. Sci. U.S.A. 104*, 9810–9815.

(9) Davis, M. E., Chen, Z. G., and Shin, D. M. (2008) Nanoparticle therapeutics: An emerging treatment modality for cancer. *Nat. Rev. Drug Discovery 7*, 771–782.

(10) Petros, R. A., and DeSimone, J. M. (2010) Strategies in the design of nanoparticles for therapeutic applications. *Nat. Rev. Drug Discovery 9*, 615–627.

(11) Gregoriadis, G., Wills, E. J., Swain, C. P., and Tavill, A. S. (1974) Drug-carrier potential of liposomes in cancer chemotherapy. *Lancet 1*, 1313–1316.

(12) Maeda, H., Wu, J., Sawa, T., Matsumura, Y., and Hori, K. (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics. A review. *J. Controlled Release 65*, 271−284.
(13) Koning, G. A., Gorter, A., Scherphof, G. L., and Kamps, J. A. (1999) Antiproliferative effect of immunoliposomes containing 5-fluorodeoxyuridine dipalmitate on colon cancer cells. *Br. J. Cancer 80*, 1718−1725.
(14) Koning, G. A., Morselt, H. W., Gorter, A., Allen, T. M., Zalipsky, S., Scherphof, G. L., and Kamps, J. A. (2003) Interaction of differently designed immunoliposomes with colon cancer cells and Kupffer cells. An in vitro comparison. *Pharm. Res. 20*, 1249−1257.
(15) Nardin, A., Lefebvre, M. L., Labroquère, K., Faure, O., and Abastado., J. P. (2006) Liposomal muramyl tripeptide phosphatidylethanolamine: Targeting and activating macrophages for adjuvant treatment of osteosarcoma. *Curr. Cancer Drug Targets 6*, 123−133.
(16) Chiu, S. J., Liu, S., Perrotti, D., Marcucci, G., and Lee, R. J. (2006) Efficient delivery of a Bcl-2-specific antisense oligodeoxyribonucleotide (G3139) via transferrin receptor-targeted liposomes. *J. Controlled Release 112*, 199−207.
(17) Zhu, J., Xue, J., Guo, Z., Zhang, L., and Marchant, R. E. (2007) Biomimetic glycoliposomes as nanocarries for targeting P-selectin on activated platelets. *Bioconjugate Chem. 18*, 1366−1369.
(18) Matsui, M., Shimizu, Y., Kodera, Y., Kondo, E., Ikehara, Y., and Nakanishi, H. (2010) Targeted delivery of oligomannose-coated liposome to the omental micrometastasis by peritoneal macrophages from patients with gastric cancer. *Cancer Sci. 101*, 1670−1677.
(19) Lee, R. J., and Low, P. S. (1994) Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J. Biol. Chem. 269*, 3198−3204.
(20) Sudimack, J., and Lee, R. J. (2000) Targeted drug delivery via the folate receptor. *Adv. Drug Delivery Rev. 41*, 147−162.
(21) O'Donnell, M. J., Zhou, C., and Scott, W. L. (1996) Solid-phase unnatural peptide synthesis. *J. Am. Chem. Soc. 118*, 6070−6071.
(22) Tornøe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J. Org. Chem. 67*, 3057−3064.
(23) Meldal, M., and Tornøe, C. W. (2008) Cu-catalyzed azide-alkyne cycloaddition. *Chem. Rev. 108*, 2952−3015.
(24) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew. Chem., Int. Ed. 41*, 2596−2599.
(25) Staudinger, H., and Meyer, J. (1919) New organic compounds of phosphorus. III. phosphinemethylene derivatives and phosphinimines. *Helv. Chim. Acta 2*, 635−646.
(26) Saxon, E., and Bertozzi, C. R. (2000) Cell surface engineering by a modified Staudinger reaction. *Science 287*, 2007−2010.
(27) Reubi, J. C., and Maecke, H. R. (2008) Peptide-based probes for cancer imaging. *J. Nucl. Med. 49*, 1735−1738.
(28) Jølck, R. I., Feldborg, L. N., Andersen, S., Moghimi, S. M., and Andresen, T. L. (2011) Engineering liposomes and nanoparticles for biological targeting. *Adv. Biochem. Eng. Biotechnol. 125*, 251−280.
(29) Sharpless, W. D., Wu, P., Hansen, T. V., and Lindberg, J. G. (2005) Just click it: Undergraduate procedures for the copper(I)-catalyzed formation of 1,2,3-triazoles from azides and terminal acetylenes. *J. Chem. Educ. 82*, 1833−1836.
(30) Bauer, W., Briner, U., Doepfner, W., Haller, R., Huguenin, R., Marbach, P., Petcher, T. J., and Pless, J. (1982) SMS 201−995: A very potent and selective octapeptide analog of somatostatin with prolonged action. *Life Sci. 31*, 1133−1140.
(31) Merrifield, R. B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc. 85*, 2149−2154.
(32) Schultz, M. K., Parameswarappa, S. G., and Pigge, F. C. (2010) Synthesis of a DOTA-biotin conjugate for radionuclide chelation via Cu-free click chemistry. *Org. Lett. 12*, 2398−2401.
(33) Cleland, W. W. (1964) Dithiothreitol, a new protective reagent for SH groups. *Biochemistry 3*, 480−482.
(34) Bangham, A. D., and Horne, R. W. (1964) Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol. 8*, 660−668.
(35) Richard, A., and Bourel-Bonnet, L. (2005) Internalization of a peptide into multilamellar vesicles assisted by the formation of an alpha-oxo oxime bond. *Chem.—Eur. J. 11*, 7315−7321.
(36) Dirksen, A., and Dawson, P. E. (2008) Rapid oxime and hydrazone ligations with aromatic aldehydes for biomolecular labeling. *Bioconjugate Chem. 19*, 2543−2548.
(37) Gustafsson, M., Olsson, R., and Andersson, C.-M. (2001) General combinatorial synthesis of tertiary amines on solid support. A novel conditional release strategy based on traceless linking at nitrogen. *Tetrahedron Lett. 42*, 133−136.
(38) Sridhar, M., Narsaiah, C., Raveendra, J., Reddy, G. K., Reddy, M., Reddy, M. K. K., and Ramanaiah, B. C. (2011) Efficient microwave-assisted synthesis of oximes from acetohydroxamic acid and carbonyl compounds using $BF_3{\cdot}OEt_2$ as the catalyst. *Tetrahedron Lett. 52*, 4701−4704.
(39) Rose, K., Zeng, W., Regamey, P. O., Chernushevich, I. V., Standing, K. G., and Gaertner, H. F. (1996) Natural peptides as building blocks for the synthesis of large protein-like molecules with hydrazone and oxime linkages. *Bioconjugate Chem. 7*, 552−556.
(40) Agard, N. J., Prescher, J. A., and Bertozzi, C. R. (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc. 126*, 15046−15047.
(41) Li, Z., Seo, T. S., and Ju, J. (2004) 1,3-dipolar cycloaddition of azides with electron-deficient alkynes under mild condition in water. *Tetrahedron Lett. 45*, 3143−3146.
(42) Chenoweth, K., Chenoweth, D., and Goddard, W. A., III. (2009) Cyclooctyne-based reagents for uncatalyzed click chemistry: A computational survey. *Org. Biomol. Chem. 7*, 5255−5258.
(43) Codelli, J. A., Baskin, J. M., Agard, N. J., and Bertozzi, C. R. (2008) Second-generation difluorinated cyclooctynes for copper-free click chemistry. *J. Am. Chem. Soc. 130*, 11486−11493.
(44) Fairbanks, B. D., Sims, E. A., Anseth, K. S., and Bowman, C. N. (2010) Reaction rates and mechanisms for radical, photoinitated addition of thiols to alkynes, and implications for thiol-yne photopolymerizations and click reactions. *Macromolecules 43*, 4113−4119.
(45) Gregory, J. D. (1955) The stability of N-ethylmaleimide and its reaction with sulfhydryl groups. *J. Am. Chem. Soc. 77*, 3922−3923.
(46) Barradas, R. G., Fletcher, S., and Porter, J. D. (1976) The hydrolysis of maleimide in alkaline solution. *Can. J. Chem. 54*, 1400−1404.
(47) Matsui, S., and Aida, H. (1978) Hydrolysis of some N-alkylmaleimides. *J. Chem. Soc., Perkin Trans. 2*, 1277−1280.
(48) Finnegan, R. A., and Mueller, W. H. (1965) Base-catalyzed addition and solvolysis reactions of N-phenyl-maleimide in methanol. *J. Pharm. Sci. 54*, 1257−1260.