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Liposomes

Orthogonal Click Conjugation to the Liposomal Surface Reveals the Stability of the Lipid Anchorage as Crucial for Targeting

Thomas Fritz,[a] Matthias Voigt,[a] Matthias Worm,[b] Inka Negwer,[a, c] Sophie S. Müller,[b, d] Kathrin Kettenbach,[e] Tobias L. Ross,[f] Frank Roesch,[e] Kaloian Koynov,[c] Holger Frey,[b] and Mark Helm*[a]

Abstract: Synthetic access to multiple surface decorations are a bottleneck in the development of liposomes for receptor mediated targeting. This opens a complex multiparameter space, exploration of which is severely limited in terms of sample numbers and turnaround times. Here, we unlock this technological barrier by a combination of a milligram-scale liposome formulation using dual centrifugation and orthogonal click chemistry on the liposomal surface. Application of these techniques to conceptually new amphiphilic compounds, which feature norbornene and alkyne groups at the apex of sterically stabilizing, hyperbranched polyglycerol moieties, revealed a particular influence of the membrane anchor of functional amphiphiles. Folic acid residues clicked to cholesterol-based amphiphiles were inefficient in folate-mediated cell targeting, while dialkyl-anchored amphiphiles remained stable in the liposomal membrane and imparted efficient targeting properties. These findings are of specific importance considering the popularity of cholesterol as a lipophilic anchor.

Targeted nanoparticular drug delivery is one of the most promising current fields in pharmaceutical sciences. Liposomes are among the established nanoparticular delivery systems, being in clinical use for over two decades, and yet the targeting of liposomes is a topic still at the cutting edge of drug delivery research.[1] A large and growing body of literature has investigated the decoration of liposomes with antibodies, small molecules, or peptides to obtain targeting functionalities.[2] The arguably best-established targeting ligand is folic acid, which was first reported to mediate specific binding of folate-functionalized liposomes to the surface of cells expressing the folate receptor.[3] Surprisingly, liposomal targeting, which typically relies on conjugates of folic acid with DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine)[3] or cholesterol,[4] is not well understood in terms of the mechanistic aspects of the cellular uptake. This assessment is based on the fact that most older studies use quantification of radiolabeled cargo or lipids as a readout for uptake rather than the direct observation for example, by microscopy.[4,5] In contrast, the report from Chau and co-workers[6] that systematically investigated the uptake of folate-decorated particles by microscopy is quite recent.

Modern liposomes are typically stabilized by so-called "stealth" polymers that are covalently bound to the hydrophilic head groups of the lipids. These systems can strongly increase the half life time in the blood stream by lowering the uptake by the reticuloendothelial system.[7] While the gold-standard polymer in this context is PEG, recent reports showed comparable circulation times and a reduced serum aggregation by making use of branched poly(glycerol) (PG).[8,9] PG is also a promising candidate for the attachment of multiple functional moieties, which was lately reported to increase the liposomal concentrations in the target tissue and the specificity.[10,11] Indeed, the development of liposomal surfaces is in large part driven by the exploration of such new amphiphilic compounds. However, starting from conventional liposomes with only cholesterol (Ch) and egg phosphatidyl choline (EPC) as components, the creation of liposomes featuring a stealth layer and at least two different surface modification entities opens a multidimensional parameter space to be investigated. The limiting factors with conventional methods[12–14] are material and time consumption, which leaves many case reports at the proof-of-concept stage without in-depth understanding of new experimental amphiphiles. To address this problem, we employed dual centrifugation as recently developed by Massing et al.[15] that we have refined to a stage, in which the formulation of liposomes can be conducted on a 1 milligram scale and with eight samples in parallel.

[a] *T. Fritz, M. Voigt, I. Negwer, Prof. M. Helm*
Institute of Pharmacy and Biochemistry, Johannes Gutenberg-University Mainz, Staudingerweg 5, 55128 Mainz (Germany)
E-mail: m.helm@uni-mainz.de

[b] *M. Worm, Dr. S. S. Müller, Prof. H. Frey*
Institute of Organic Chemistry, Johannes Gutenberg-University Mainz
Duesbergweg 10–14, 55128 Mainz (Germany)

[c] *I. Negwer, Dr. K. Koynov*
Max Planck Institute for Polymer Research
Ackermannweg 10, 55128 Mainz (Germany)

[d] *Dr. S. S. Müller*
Graduate School MAINZ, Johannes Gutenberg-University Mainz
Staudingerweg 9, 55128 Mainz (Germany)

[e] *K. Kettenbach, Prof. F. Roesch*
Institute of Nuclear Chemistry, Johannes Gutenberg-University Mainz
Fritz Strassmann Weg 2, 55128 Mainz (Germany)

[f] *Prof. T. L. Ross*
Department of Nuclear Medicine, Hannover Medical School
Carl-Neuberg-Str.1, 30625 Hannover (Germany)

Supporting information and ORCID(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/chem.201602758.

In our recently reported copper-catalyzed azide–alkyne cycloaddition (CuAAC)-mediated fluorescence functionalization of liposomes containing novel experimental amphiphiles based on poly(glycerol),[16] one intriguing result was a rapid, cell-line independent fluorescent staining of cellular membranes. Lack of a concomitant cellular uptake of the liposomal cargo suggested a rapid translocation of the cholesterol-based amphiphiles from the liposomal to the cellular membrane. Since such unusual behavior has strong implications for the use of cholesterol-derived compounds (**1**) in liposomal applications in vivo, we decided to establish structure–function relationships between the lipophilic substructure of the amphiphile and the membrane stability of functional surface features on liposomes.

Herein, we show how the combined application of a particular combination of orthogonally clickable amphiphiles and small scale dual centrifugation formulation allows for the fast and efficient preparation of a series of liposomes as sketched in Figure 1. Investigation of the amphiphile mobility revealed that the nature of the lipophilic anchor is crucial for the efficient folate targeting of liposomes, because it ensures durable anchorage of stealth amphiphiles in lipid vesicles.

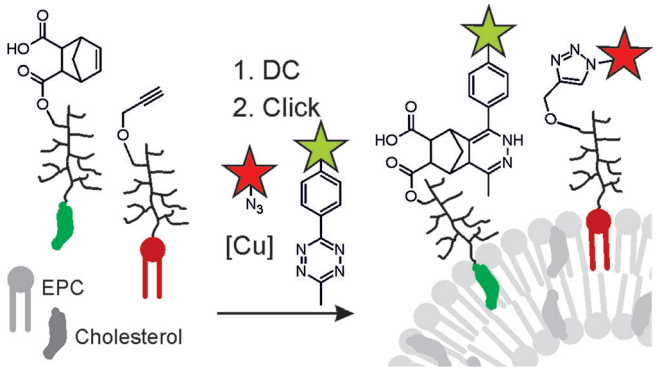

Figure 1. Scheme of the post-preparational liposome functionalization in an orthogonal reaction with two different fluorophores.

We designed and synthesized a panel of amphiphiles based on a 1,2-bis-*n*-hexadecyl-substituted glycerol (BHG) macroinitiator **2** as shown in Figure 2, which was employed in oxyanionic ring-opening polymerizations as reported earlier,[17] yielding hyperbranched poly(glycerol) (*hb*PG) amphiphiles. Further on, these amphiphiles were subjected to end-group modifications, either introducing terminal alkyne groups **3** utilizing propargyl bromide, or norbornene residues **4** by esterification with dicarboxy-norbornene anhydride. Systematic variation of these building blocks yielded four amphiphiles **5–8** listed with corresponding depictions in Figure 2 and structurally detailed in the Supporting Information. Our panel contained compounds carrying permutations of clickable groups for orthogonal surface functionalizations, to independently track the whereabouts of these compounds, for example between liposomal and cellular membranes. While conventional conjugation chemistry is typically utilized for the attachment of targeting ligands to liposomes, a few groups (including us) have reported the use of click chemistry by means of CuAAC.[16,18,19] Furthermore, the norbornene moiety in Figure 2 is known as a suitable substrate for the inverse-electron demand Diels–Alder cycloaddition (IEDDA), which has so far not been applied to liposomes.[20] Similar to strain-promoted azide–alkyne cycloadditions (SPAAC), IEDDA is a route to overcome the potentially toxic copper catalysis.[21]

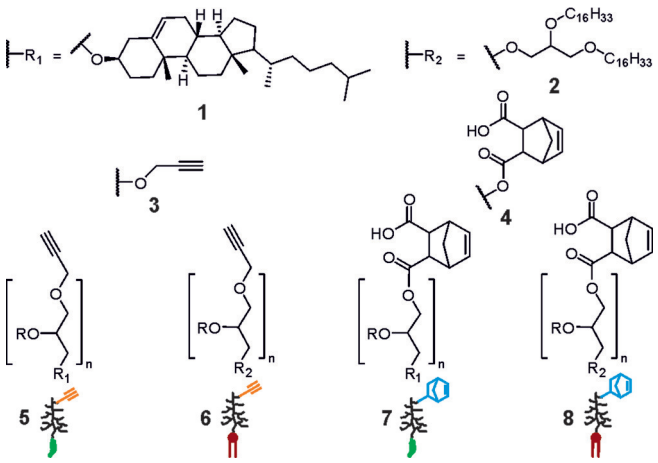

Figure 2. Building blocks of polymeric amphiphiles.

The amphiphiles were then combined with cholesterol and EPC and formulated to liposomes by dual centrifugation, yielding a vesicular phospholipid gel (VPG), from which liposomes were obtained after resuspension. In keeping with the literature, an optimization series afforded a substitution of 5 mol% of lipids with experimental amphiphiles (data not shown). Thus, the formulated nanoparticular suspensions with the various amphiphiles yielded sizes in the range of 130–250 nm in diameter with narrow size distributions ($\mu 1 < 0.3$) as determined by dynamic light scattering (DLS), qualifying them for potential use in drug delivery applications (Figure S1 in the Supporting Information). Characteristic encapsulation efficiencies (EE) for DC-formulations were found to be roughly 50%.

Introduction of the single amphiphiles **5–8** in the formulations yielded liposomes that were functionalizable either by CuAAC or IEDDA chemistry. Liposomes bearing terminal alkyne and norbornene residues were derivatized with fluorophores, either with Atto 488 tetrazine or with Alexa Fluor 594 azide under conditions that allowed simultaneous and orthogonal click conjugation in a one-pot reaction. Since only single-digit milligram amounts of the lipids were used, typical characterization in terms of NMR or IR spectroscopy had to be forgone in favor of fluorescence-based quantitation of the reacted surface features.[16] The reaction mixtures were subjected to size exclusion chromatography and the fluorescence of the eluting fractions was quantified to determine yields (Figure S2). As expected, we observed that liposomes carrying only terminal alkyne residues did not react with Atto 488 tetrazine, and inversely, tetrazine carrying liposomes did not react with Alexa Fluor 594 azide (data not shown). The number of fluorophores was estimated from the size of the liposomes and the encapsulation efficiency to yield approximately 200–300 fluorophores per lip-

osome. These numbers were in general agreement with measurements by fluorescence correlation spectroscopy (FCS) (Figure S2), which also confirmed the absence of residual dye azides or tetrazines after the workup.

When eukaryotic cells were incubated with liposomes containing a cholesterol-anchored, Alexa Fluor 594-labeled amphiphile, rapid staining of the cellular membranes was observed within 30 min, as evident in the confocal laser scanning microscopy images (CLSM) in Figure S3 A in the Supporting Information. To understand this finding that essentially recapitulates our previously reported observation,[16] we put forward the hypothesis that the fast staining behavior might be conferred by either of the three principal structural elements of the amphiphiles, that are, the dye, the *hb*PG-body, or the lipid membrane anchoring part, in the present case a cholesterol moiety. The abovementioned panel of amphiphiles (Figure 2) was, thus, employed to generate permutations of these three elements. Since equally fast membrane staining was also observed with liposomes carrying cholesterol-anchored Atto 488 tetrazine attached by IEDDA (Figure S3 B), the dye structure and attachment chemistry could be ruled out. Next, we investigated the polymer architecture and lipid anchoring, by exchanging *hb*PG versus linear PEG (compounds **9** and **10** in the Supporting Information) on one hand, and cholesterol versus BHG on the other hand. In a direct comparison of the membrane staining of the four resulting compounds, cells were incubated with liposomes containing the structurally altered amphiphiles **5**, **6**, **9**, and **10**. Rapid membrane staining was observed only for cholesterol-anchored compounds, irrespective of the architecture of the polymer (Figure S4).

We next extended these studies to liposomes containing multiple surface functionalities. When cells were simultaneously treated with different permutations of Atto 488 and Alexa 594 that were lipid-anchored to either cholesterol or dialkyl-moieties by either type of click chemistry, the cell membranes faithfully developed a fluorescence emission corresponding only to dyes conjugated to cholesterol. When both dyes were anchored to cholesterol, the membrane was stained both in green and red, but when both dyes were attached to dialkyl-based amphiphiles, membrane staining was negligible (Figure 3 A). Evidently, the anchorage of cholesterol-containing amphiphiles shown in Figure 2 appears to be too instable, promoting rapid exchange to other lipid phases. In contrast, amphiphiles anchored by a dialkyl chain are more stably integrated in the liposomal bilayer.

Consolidation of these findings outside living cells came from analysis of the membrane exchange among liposomes by fluorescence cross-correlation spectroscopy (FCCS). Two liposomal populations with either Atto 488 or SulfoCy5 labeling showed strong cross-correlation directly after mixing of the liposomes with labelled polymeric amphiphiles based on cholesterol (Figure 4). This indicates a fast exchange of the cholesterol-anchored amphiphiles among liposomes. In contrast, the amplitude of the cross-correlation function G(0) is significantly lower for a mixture of BHG-based labelled amphiphiles, showing that dialkyl-derived amphiphiles are more stably anchored in lipid bilayers in general.

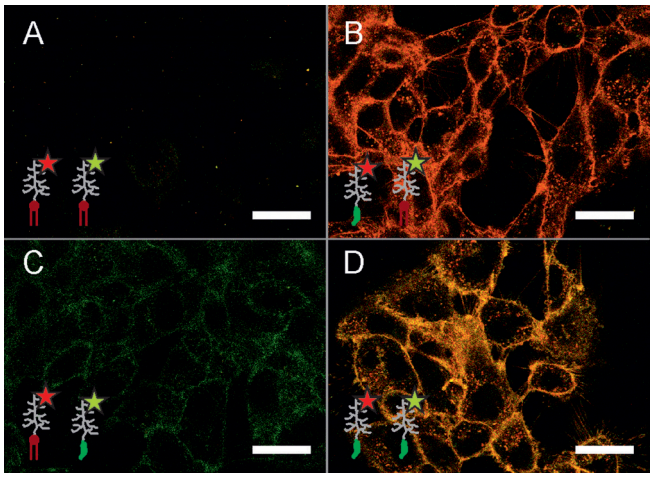

Figure 3. RBE4 cells after incubation with orthogonally labelled liposomes. In contrast to BHG (1,2-bis-*n*-hexadecyl-substituted glycerol)-based lipids (red anchor depictions, red and green stars are red and green fluorophores, respectively), only Ch (cholesterol)-based lipids (green depictions in B, C, and D) integrate into the cellular membrane (scale bar: 25 μm).

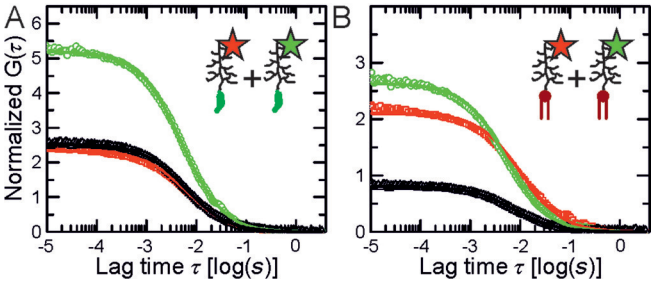

Figure 4. FCCS (fluorescence cross-correlation spectroscopy) data illustrating the strong cross-correlation of Ch-based amphiphiles (A) and weak cross-correlation of BHG-based amphiphiles (B). Green (Atto 488) and red (SulfoCy5) traces indicate the two fluorophore-specific autocorrelations, black indicates cross-correlations.

To determine the biological relevance of these findings, we investigated in how far the differential anchoring of cholesterol versus dialkyl chains affected the targeting properties of the functionalized liposomes. Folic acid, which had already been applied on liposomes in a variety of settings,[3,5] was chosen as a well-established ligand for directed targeting. In order to accommodate folic acid azide as yet another functionality in addition to the two fluorescent dyes, we now resorted to the synthesis of a folic acid conjugate by click chemistry before the formulation. To promote the steric accessibility of the targeting moiety on the outer liposomal polymer shell, we used folate conjugates of linear PEG (**9** and **10**) in a comparative analysis. KB cells overexpressing the folate receptor were incubated with liposomes loaded with the red fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) as a drug-model cargo, and observed by CLSM. Figure 5 shows an overlay of the green fluorescence from the Atto 488-labeled dialkyl-anchored amphiphile and the red signal from the drug-model cargo, as well as the transmission channel for an outline of the cell features. Figure 5 A clearly shows the effi-

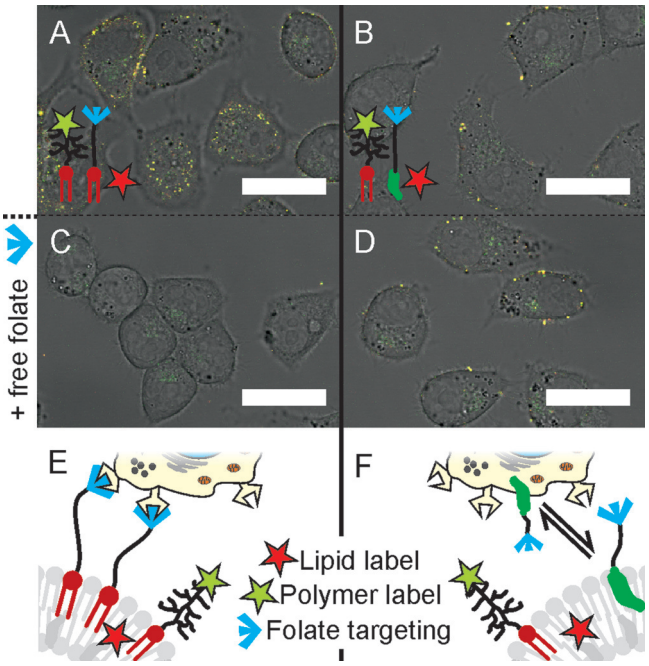

Figure 5. Incubation of KB cells results in specific FR (folate receptor)-mediated binding with colocalized fluorescence on the surface (A) and the ability to chase (C) the binding with free folic acid for BHG-based folic acid conjugates, while the cholesterol-based derivatives show less surface adhesion (B) and stronger unspecific binding (D, F). Cholesterol-based species are presumably exchanging with the cellular membranes (E, F).

cient targeting of liposomes containing dialkyl-anchored folate to the cells, which was confirmed to be folate dependent by a chase control experiment shown in Figure 5C.

Clearly, an excess of folate in the medium visibly outcompeted the liposomal binding. In contrast, no efficient targeting could be obtained with liposomes containing a cholesterol-anchored folate. The few signals observed in Figure 5B were revealed as unspecific binding by the corresponding control experiment, in which an excess of competing folate did not reduce those signals. Based on all the above findings, which demonstrate that liposomes shed cholesterol-based amphiphiles, we propose an interpretation as depicted in Figure 4E and F. We posit that the cholesterol-anchored folate compound rapidly interchanges between liposomes and cellular membranes, which prevents efficient targeting. In contrast, the stable folate anchoring can sustain folate-dependent binding of liposomes to the corresponding receptor on the cellular surface, effectively mediating successful targeting. A comparison of both chase experiments (Figure 5C and D), even suggests an increased unspecific binding of the liposomes formulated with the cholesterol-anchored folate, possibly as a consequence of its shedding. This interpretation, however, will have to be verified by further experiments.

Our systematic characterization of new amphiphilic prototype compounds involved the repeated preparation of liposomes of approximately 50 different compositions, which, by virtue of limited supply, typically had to be conducted at the 1–5 mg scale. These numbers illustrate quite well how dual centrifugation unlocks a technological barrier, since other formulation methods require significantly larger amounts of experimental compounds and time. In contrast to post-insertion approaches that often rely on potentially incompatible, elevated temperatures,[22,23] we prepared liposomal nanoparticles that allow for a post-preparational, orthogonal surface derivatization. While this concept has been described only in a single work to date[24] for the use of SPAAC, we add CuAAC and IEDDA reactions to this repertoire, thereby only relying biocompatible conjugation sites.

Having achieved the above technological prerequisites led us to the discovery of differential strength of the membrane anchoring. In principle, the propensity of cholesterol and cholesterol-PEG conjugates to integrate into existing membranes, for example, by post-insertion of liposomes[23] is known in the field. On the other hand, literature conveys a notion of the tendency to exchange between lipid phases,[4] while plain cholesterol exhibits only slow desorption kinetics.[25] As detailed experimental investigations remain elusive, the influence of the lipid anchor is bound to attract renewed interest.[26] Importantly, no mechanistic studies are available that combine the pertinent conclusions and point to the disadvantage of using such compounds in liposomes, as discovered here. On the other hand, there are known and potential advantages of using cholesterol-PEG conjugates, which are actually quite popular.[27] In addition to their efficient use in post-insertional applications, a reversal in terms of shedding may allow tuning of the circulation time in the bloodstream as demonstrated earlier.[28] As a closing remark, we raise the issue of the stability of ligand anchoring, and the field may profit by considering and tuning lipophilicity of the anchoring moiety in relation to the hydrophilicity of the stealth-type polymeric part of the amphiphiles. This may potentially include even more stably anchored compounds featuring, for example, longer dialkyl chains.

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