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Strategies for the Activation and Release of the Membranolytic Peptide Melittin from Liposomes Using Endosomal pH as a Trigger

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Supporting Information

ABSTRACT: Endosomolytic peptides are often coupled to drug delivery systems to enhance endosomal escape, which is crucial for the delivery of macromolecular drugs that are vulnerable to degradation in the endolysosomal pathway. Melittin is a 26 amino acid peptide derived from bee venom that has a very high membranolytic activity. However, such lytic peptides also impose a significant safety risk when applied in vivo as they often have similar activity against red blood cells and other nontarget cell membranes. Our aim is to control the membrane-disrupting capacity of these peptides in time and space by physically constraining them to a nanocarrier surface in such a way that they only become activated when delivered inside acidic endosomes. To this end, a variety of chemical approaches for the coupling of lytic peptides to liposomes via functionalized PEG-lipids were explored, including maleimide−thiol chemistry, click-chemistry, and aldehyde−hydrazide chemistry. The latter enables reversible conjugation via a hydrazone bond, allowing for release of the peptide under endosomal conditions. By carefully choosing the conjugation site and by using a pH activated analog of the melittin peptide, lytic activity toward a model membrane is completely inhibited at physiological pH. At endosomal pH the activity is restored by hydrolysis of the acid-labile hydrazone bond, releasing the peptide in its most active, free form. Furthermore, using an analogue containing a nonhydrolyzable bond as a control, it was shown that the activity observed can be completely attributed to release of the peptide, validating dynamic covalent conjugation as a suitable strategy to maintain safety during circulation.

INTRODUCTION

The major bottleneck of intracellular delivery of therapeutic biomacromolecules using nanoparticulate systems is entrapment in the endosome. It is crucial that the cargo is released into the cytosol before it is degraded in the endolysosomal compartment, a highly acidic and proteolytic environment. A common strategy to enhance endosomal escape is to decorate the nanocarrier with endosomolytic or cell penetrating peptides (CPPs).[1−3] A recurring problem with this approach is that none of these peptides are specific for the endosomal membrane, but interact with all biological membranes including those of red blood cells. In fact, the activity of membrane active peptides is often measured by hemolysis of red blood cells[4] and therefore imposes a significant safety risk if nanocarriers functionalized with such peptides are to be administered into the bloodstream. The application of endosomolytic peptides is therefore a double-edged sword, where more potent peptides are also associated with increased toxicity. Several strategies have been proposed to mask or inactivate the lytic peptide until it has reached the target membrane. For example, Hansen et al. conjugated the cell penetrating peptide TAT to liposomes and constrained the other terminus of the peptide with a UV-cleavable bond, which allowed the construct to be photo-activated.[5] However, UV-radiation may not be the most suitable trigger, as it has limited penetration depth in the human body.[6] Many groups have used the endosomal environment as a trigger, reporting peptides with side-chain modifications that are removed by aminopeptidase and dipeptidyl peptidase[7] or phosphatases.[8] Another attractive strategy is to exploit the difference in pH between the bloodstream (physiological pH, 7.4) and the endosome (5.5−6.5). This could be done by using pH sensitive (variants of) peptides that change conformation at lower pH, usually triggered by protonation of glutamic acid residues in the sequence.[9−11] Alternatively, acid-labile chemistry could be used, such as maleic anhydride to mask the lysines in the peptide, that inhibit the endosomolytic activity, which is then restored when it reaches the acidic environment of the endosome.[12−15] The common goal of these approaches is to maximize the potency of the endosomolytic agent in the endosome, but minimize its activity and thereby toxicity in the bloodstream.

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Table 1. Melittin Peptides Prepared in Present Study

Peptide		Sequence and Structural Modifications
Melittin	1	GIGAVLKVLTTGLPALISWIKRKRQQ
Azido-Melittin	2	$N_3CH_2C(=O)$–NH–GIGAVLKVLTTGLPALISWIKRKRQQ
Acid-Melittin[19]	3	GIGAVLEVLTTGLPALISWIEEEEQQC
Azido-Acid-Melittin	4	$N_3CH_2C(=O)$–NH–GIGAVLEVLTTGLPALISWIEEEEQQ
Hydrazide-Acid-Melittin-Cysteine	5	H_2N–NH–C(=O)–(CH_2)_4–C(=O)–NH–GIGAVLEVLTTGLPALISWIEEEEQQC

In the work presented here, liposomes were functionalized with melittin, a 26 amino acid peptide derived from bee venom known for its high affinity for lipid membranes and potent lytic activity (Table 1).[3] Melittin has previously been used to functionalize cationic polymers for gene delivery[13−17] and has also been used in the clinically evaluated Dynamic Poly-Conjugate (DPC) formulation for siRNA delivery.[18,19] In light of these previous reports we were also interested in developing approaches for coupling lytic peptides like melittin to liposomal surfaces. Our group recently described the use of copper-free "click" chemistry for conjugating other peptides to liposomes.[20] Our preliminary investigations with mellitin indicated that while introduction of an N-terminal azide group did not affect activity, upon conjugation to the liposomal surface a significant reduction in lytic activity was observed. Furthermore, we found that the activity of melittin is also diminished as pH is decreased, an effect which has also been previously reported.[21] We therefore opted to explore strategies wherein the melittin peptide is physically constrained by conjugation to liposomes via acid sensitive linkers so as to facilitate release at the lower pH of the endosome. As a second control mechanism we also selected a variant of the melittin peptide known to exhibit lower lytic activity at physiological pH but an optimal activity at endosomal pH (referred to as acid-melittin, Table 1).[11]

Several different chemical approaches were explored for the in situ coupling of melittin peptides to preformed liposomes and the efficiency of coupling investigated. PEGylated lipids functionalized with cyclooctyne, maleimide, or aldehyde moieties were used for coupling to peptides modified to contain azide, cysteine, or hydrazide groups, respectively. The membrane activity of different constructs was tested in a calcein leakage assay, a model for membrane perturbation and endosomal escape. The results of these investigations demonstrate that the conjugation of an aldehyde-modified lipid to a hydrazide-functionalized acid-melittin peptide via an acid sensitive hydrazine linkage leads to a system wherein lytic activity is abolished at pH 7.4 but restored at pH values below 5.6.

■ RESULTS AND DISCUSSION

Peptide Synthesis. The five peptides used in this study (see Table 1) were synthesized following standard Fmoc-based solid-phase peptide synthesis procedure. The introduction of the azido group at the N-terminus in derivatives **2** and **4** was accomplished by coupling commercially available 2-azidoacetic acid in the final step of the synthesis. Similarly, a triply protected hydrazide building block, prepared according to the slightly modified procedure described before,[22] was attached to the N-termini of acid-melittin-cysteine to give the analogue **5**. Both modifications were first activated with HBTU, HOBt, and DIPEA and then added on-resin to the exposed N-terminal amine. Following cleavage using 95% TFA, which simultaneously removed all protecting groups, all peptides were purified by RP-HPLC. The HPLC-MS confirmed the identity and high purity of the desired products.

Surface Modification of Liposomes with Melittin Using Click Chemistry. In earlier work, we reported the surface modification of liposomes using copper-free click chemistry, with a novel lipid containing a bicyclononyne (BCN) cyclooctyne group on a short PEG spacer.[20] This approach allows for the site-specific and bio-orthogonal conjugation of azide-containing ligands. To apply a similar approach in the present study we employed the commercially available DSPE-PEG(2000)-dibenzocyclooctyne (DBCO) lipid containing a much longer spacer and the DBCO click moiety instead of the BCN group.[23] A drawback of the DBCO group is that it is more hydrophobic, which limits the maximum amount of functionalized lipid that can be incorporated. In the liposomes used here, 6% of all phospholipids were PEGylated, but when the DBCO-PEG lipid was used, only 2% of functionalized PEG lipid could be incorporated (with the other 4% unfunctionalized DSPE-PEG). With higher percentages, the liposomes tended to aggregate likely because the hydrophobic DBCO group folds back into the bilayer (resulting in higher average diameters and high polydispersity. see Table S1). However, when conjugating the azido-melittin peptide (compound 4) to these liposomes, coupling was very efficient, reaching complete turnover in almost all cases, as verified by UPLC (Ultra Performance Liquid Chomatography). Thanks to the tryptophan residue, the peptide could be detected with high sensitivity and successful conjugation resulted in a shift of the lipidated peptide to the end of the chromatogram. Size and PDI remained the same after conjugation, indicating that the melittin peptide does not interact with the membrane of the

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liposome itself, when the surface is PEGylated. The zeta potential did change to slightly less negative values as a result of conjugating the peptides (see Table S1).

Activity of Melittin Click Conjugates in Calcein Leakage Assay. The endosomolytic activity of azido-melittin and its conjugates was tested using an established fluorescence-based assay employing liposomes loaded with calcein as model membranes.[10] The concentration of calcein inside the target liposomes is high and quenches the fluorescent signal, but when the membrane is punctured calcein leaks out which can then result in a fluorescent signal. The target liposomes are not PEGylated and are therefore vulnerable to puncture or rupture by endosomolytic peptides, unlike the carrier liposomes used in this experiment, whose surface is protected by PEG-lipids. Calcein release from the target liposomes induced by different concentrations of the peptides examined is plotted as a percentage of maximum leakage, induced by complete lysis with Triton X-100. See Figure 1. The free azido-melittin peptide (**2**)

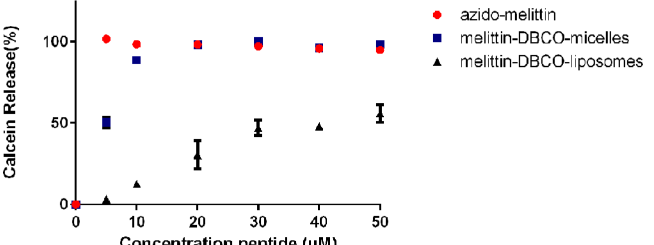

Figure 1. Membrane activity of free azido-melittin and conjugates in the calcein leakage assay. Membrane activity of peptide 2 in free form, conjugated to DSPE-PEG-DBCO as micelles and on the surface of carrier liposomes. Calcein release from target liposomes with self-quenched concentrations of entrapped calcein, was measured after 1 h at pH 7.4. Results are plotted relative to 100% leakage, induced by addition of 0.5% Triton X-100 to the calcein liposomes.

is extremely potent, inducing complete leakage already at the lowest concentration tested (5 μM). The other conditions tested are melittin conjugated to carrier liposomes and to the DSPE-PEG-DBCO lipids alone, which will form micelles in solution. The high potency of the free melittin peptide demonstrates the importance of verifying complete coupling by UPLC. 5 μM of free peptide already induced maximum leakage, so if conjugation to the lipids were incomplete, the results with the micelles or liposomes could be compromised by the unconjugated fraction.

When conjugated to DSPE-PEG micelles, the potency of the peptide is slightly decreased, but maximum leakage is still reached at almost all concentrations. When the peptide is conjugated to the carrier liposome, activity is decreased much further. Even with the highest concentration of melittin conjugated to the liposome only ~50% of the maximum leakage is reached. An explanation for these findings could relate to the orientation in which the peptide is inserted into the target membrane. If the peptide punctures the membrane by insertion of its free N- or C-terminus, conjugation at that terminus would likely interfere with membrane lysis. When using click chemistry, the peptide is conjugated at the N-terminus but the results with the micelles indicate that blocking of this terminus does not hamper the activity. However, when these micelles are anchored to the carrier liposome membrane, the peptide is limited in its movement, which could inhibit the membrane activity, especially if this is dependent on local concentration of the peptide. If that is the case, conjugating more peptides to a liposome may increase the potency, but as shown before, the amount of DBCO lipid that can be incorporated in the liposomes is limited.

To increase the peptide-per-liposome number and achieve higher densities of surface modification with the lytic peptide, maleimide–thiol chemistry was used. When using the DSPE-PEG-maleimide lipid there is no limit to the amount of PEG-lipid that can be substituted with functionalized PEG-lipid. Liposomes with up to 6% of DSPE-PEG-maleimide were stable in size and monodisperse (PDI < 0.1). These liposomes were used to investigate the effect of higher densities of cysteine functionalized peptide **3** on the liposomal surface.

Membrane Activity of Melittin Peptides at Different pHs. Formulation and storage of the liposomes are done at physiological pH and the calcein leakage assay described above was also performed at pH 7.4. However, the pH in the endosome is around 5.5–6.0, low enough to cause conformational changes in the peptide and alter its activity. The calcein leakage assay with free peptide (**1**) was repeated at four different pH values between 5.6 and 7.4 and it was found that, despite its high potency at pH 7.4, the activity of melittin is a lot lower at endosomal pH (see Figure 2). This is a risk, as it could cause toxicity (hemolysis) in the circulation, but is also less potent under endosomal conditions where it is supposed to be active. To tackle both problems, a pH sensitive analog of melittin was used, that has optimized activity at endosomal pH (compound **3**). In "acid-melittin" all basic residues are replaced with glutamic acid, to make the peptide pH responsive.[11] When concentration ranges of free peptides **1**, **3**, and **5** were tested at pH 5.6, 6.2, 6.8, and 7.4 the potencies dramatically changed. At pH 5.6 melittin reached only ~75% of maximum leakage in the highest concentration, while acid-melittin (**3**) was almost as potent as the normal melittin (**1**) was at 7.4. The activity of the pH sensitive variant is only ~25% at physiological pH, addressing the safety issue already in part but should be further decreased in the final design.

For the final design of the conjugate, hydrazide-aldehyde coupling was employed. Therefore, compound **5** with an N-terminal hydrazide was synthesized. This analog behaved similar to compound **3** at different pH values and even performed slightly better, indicating that modification of the N-terminus influences the lytic activity of the peptide, in this case in a positive way (Figure 2).

Click Chemistry vs Maleimide–Thiol Chemistry and Effect of Different Densities. Next, the density of peptides on the surface of the carrier liposomes was investigated. For this, acid-melittin was used (compounds **3** and **4**) because of its favorable behavior at different pHs over normal melittin. Because only 2% of DBCO functionalized lipid could be incorporated in the liposomes, DSPE-PEG(2000)-maleimide lipid was used, to conjugate to a C-terminal cysteine in the acid-melittin peptide. Because the whole fraction of PEGylated lipid could be replaced with PEG-maleimide lipid, densities of 2%, 4%, and 6% were investigated. The total amount of conjugated peptide and of functionalized lipid was kept the same, so complete conjugation resulted in more peptides per liposome when higher densities were used. For conjugation with click-chemistry, compound **4** was used, conjugated at the N-terminus. For conjugation with maleimide–thiol chemistry, compound **3** was used, with a C-terminal cysteine. Therefore, the results of the 2% click chemistry and 2% maleimide–thiol

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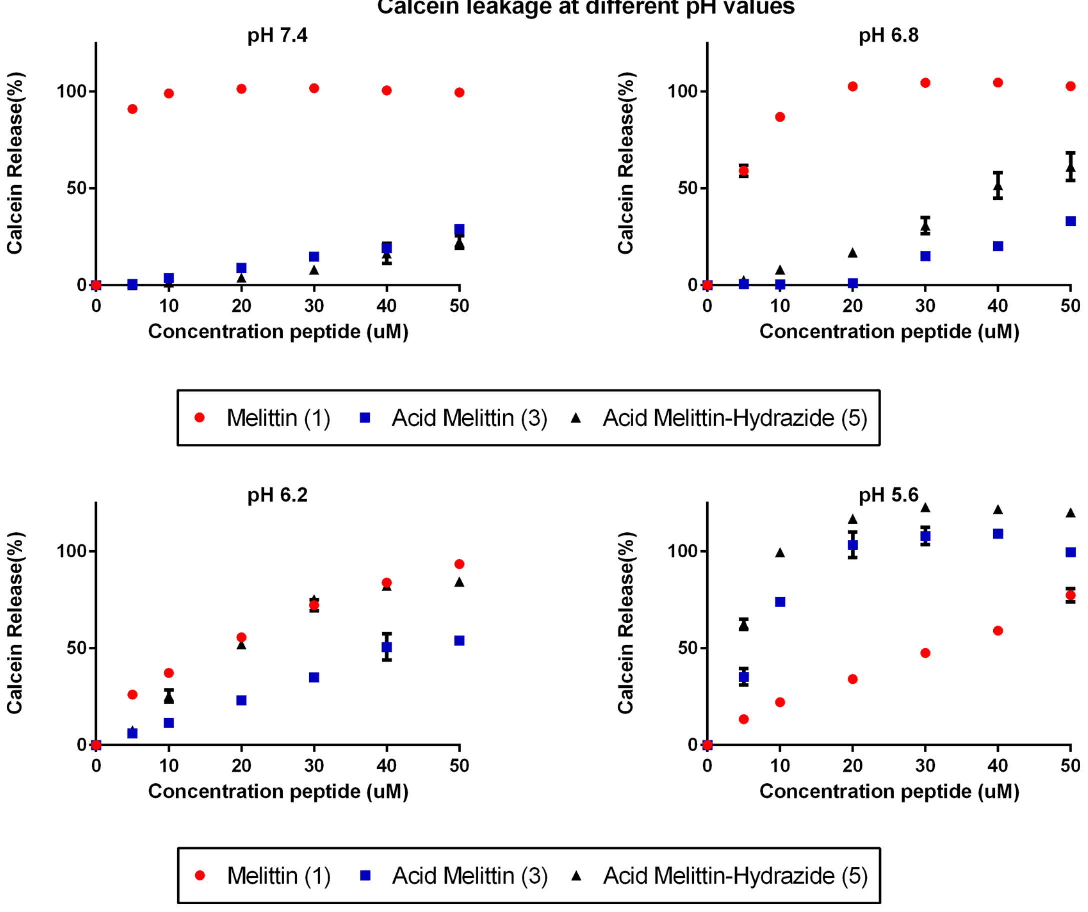

Figure 2. Membrane activity of free peptides in the calcein leakage assay at different pH values. Membrane activity of free peptides in the calcein leakage assay at different pH values ranging from physiological pH (7.4) to endosomal pH (5.6). Activity of melittin decreases when pH is lowered while the acid-optimized analog acid-melittin increases in potency at endosomal pH.

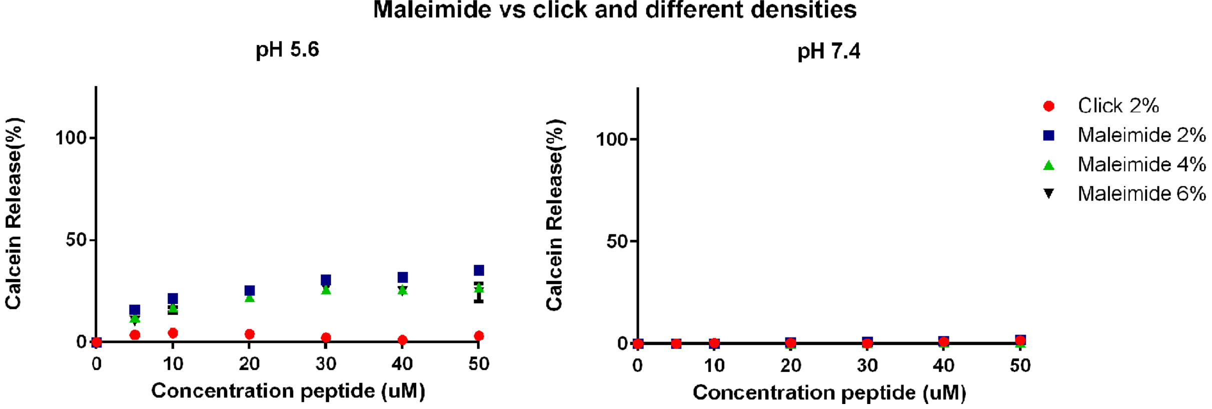

Figure 3. Liposome formulations with acid-melittin coupled with click chemistry or maleimide−thiol chemistry at different densities. When coupling **4** with click chemistry, activity in the calcein leakage assay is completely abolished. C-terminal conjugation of **3** to the carrier liposomes preserves membrane activity but does only reach a maximum of ~40%. The density of the peptide on the surface of the carrier liposomes does not drastically alter the outcome.

conjugation could be used to compare the influence of N- versus C-terminal conjugation on the endosomolytic effect (see Figure 3).

Strikingly, the conjugation of **4** to liposomes via the N-terminus completely abolished its effect in the calcein leakage assay, also at pH 5.6. This again demonstrates that the N-terminus is important for the lytic activity. Conjugation at the C-terminus of **3** did result in leakage of ~40%, but the density of peptides on the surface of the liposome did not have a very big effect. The observation that C-terminal conjugation to a carrier system results in higher activity is in line with the report of Boeckle et al. where melittin was coupled to polymeric PEI nanoparticles. Also there, it was reported that conjugation of the peptide decreases the activity, regardless of the terminus.[16] It is likely that the mechanism of membrane penetration is different when the peptide is free or conjugated. For example, it

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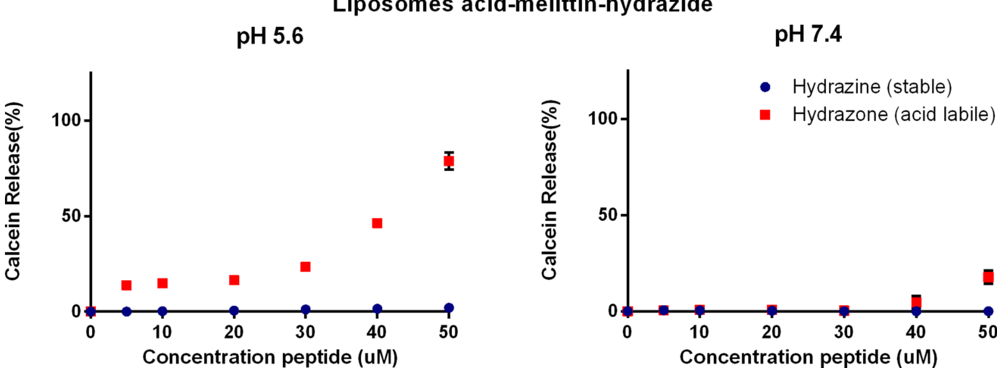

Figure 4. Liposome formulations with hydrazide-acid-melittin coupled via a stable or acid labile bond. Stable conjugation of the hydrazide-acid-melittin to the carrier liposomes does not result in any leakage at all, neither at pH 5.6 where the peptide is most active, demonstrating again that conjugation to the N-terminus completely inactivates the peptide. The acid labile hydrazone bond is hydrolyzed at pH 5.6, releasing the peptide in its free form, inducing leakage at endosomal pH.

has been hypothesized that melittin peptides form multimers that create pores, which is less likely to happen when they are anchored to a liposome. Alternatively, they insert one of their termini in the hydrophobic core of the membrane, which can be prevented by conjugation to that terminus. Finally, it is speculated that these mechanism are concentration dependent and therefore also influenced by the mobility of the peptides that is obviously lower when anchored. The mechanisms of action of these lytic peptides and the factors that influence them have been extensively studied and described in the following references.[3,16,24−27]

At physiological pH, there is no membrane activity in any formulation, which is advantageous given the fact that the peptides are also lytic toward erythrocyte membranes in circulation, but the activity at endosomal pH is also significantly lower than that of the free peptide. For optimal performance and safety, the difference in lytic activity between pH 5.6 and 7.4 is preferred to be as big as possible. Considering the results of the calcein leakage assay after conjugation and at different pH values, this goal can be reached by having the acid-melittin peptide conjugated at pH 7.4 but free in solution at the endosomal pH of 5.6. To achieve this, an acid-labile bond was used for conjugation.

Conjugation of Acid-Melittin to Liposomes via an Acid-Labile Hydrazone Bond. To conjugate the peptide via a bond that is released at endosomal pH, a hydrazide was introduced at the N-terminus of compound **5**. For conjugation of this peptide analog to the liposomes, a fourth type of PEG lipid was used, DSPE-PEG(2000)-aldehyde. The conjugation of a hydrazide to an aldehyde results in an acid-labile hydrazone bond. This bond can be reduced with hydride, resulting in an irreversible hydrazine bond. The hydrazine conjugation was used as a control, to distinguish between the contribution of pH on the activity of the peptide at different pHs and of the effect of covalent conjugation. In other words, any effect between different pHs with the stable conjugate is caused by peptide conformation, whereas the difference between the stable and unstable conjugate per pH is caused by hydrolysis of the bond.

However, conjugation of the peptide to preformed liposomes as done in the previous experiments was not possible with this type of chemistry. Possibly, the conjugation efficiency was too low, or the product was hydrolyzed again, because of the equilibrium that is formed between the hydrazone form and free aldehyde.[28] However, also when this reaction was performed in the presence of hydride to immediately reduce the bond, no conjugates could be detected by UPLC, indicating that the bond is never formed in the first place, when such relatively low concentrations are used. Therefore, the reaction was performed in DMSO and the formed micelles were inserted into the membrane of the PEGylated carrier liposomes, as described before.[29] The small percentages of DMSO present in the final sample did not interfere with the calcein leakage assay (see Table S3 in Supporting Information).

The resulting formulations of liposomes were tested in the calcein leakage assay as shown in Figure 4. In both pH conditions, the reduced stable bond completely inhibited the lytic activity of the peptide. This is in line with the stable N-terminal conjugation with click chemistry as shown in Figure 3. The acid-labile hydrazone bond is hydrolyzed after 1 h at pH 5.6 and releases the peptide to induce calcein leakage up to 80%. The effect of concentration appears to be biphasic, indicating that a certain threshold of free peptide has to be reached to catalyze efficient membrane puncture. This suggests there is indeed a concentration dependent mechanism of action, as discussed before. The finding that the calcein release at pH 5.6 is not complete when the labile bond is used suggests that not all the bonds are hydrolyzed in the time frame of 1 h, because Figure 2 shows that the free acid melittin peptides already induce complete leakage at the lower concentrations. Furthermore, the fact that the hydrazone formulation is not completely inactive in the highest concentration of peptide at pH 7.4 indicates that the bond is not completely stable at physiological pH and that some hydrolysis may still occur.

■ DISCUSSION AND CONCLUSION

In the work presented here, three types of chemistry were explored to conjugate peptides to preformed liposomes. The gold standard is maleimide−thiol chemistry, where maleimide-functionalized PEG lipids are used to couple cysteine residues of peptides. The main disadvantages of this approach are that cysteines may form disulfide bridges in solution (and already before conjugation) and that maleimides are not stable in aqueous solution, which prohibits storage of the liposomes for longer times until coupling. Furthermore, in more complex ligands like small proteins or antibodies, present cysteines usually have a different function or are already used in disulfide bridges, so they are not available for coupling.

As an alternative, copper-free click chemistry was used, with a DBCO-functionalized PEG lipid and an azide that was

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introduced at the N-terminus of the peptide. This worked very efficiently and much faster than with the BCN-lipid previously described by our lab, demonstrating again that this is an attractive option, especially if bioorthogonal conjugation is required.[20] However, in the case of melittin, it turned out that the activity in the calcein leakage assay decreased when the peptide was conjugated, in particular, to the N-terminus. If desired, an azide could also be incorporated at the C-terminus (or anywhere else in the peptide) with a different technique, for example, by introducing an unnatural amino acid such as azido-homoalanine during synthesis.[30] Instead, this finding was used to our advantage as a means to inactivate the peptide when circulating in the bloodstream, simply by conjugating it to the liposomal surface. As a second safety mechanism against hemolysis and interaction with nontarget cells, the acid sensitive variant of melittin was used,[11] and after conjugation, this resulted in complete elimination of the lytic activity at physiological pH.

To take full advantage of the maximized activity of acid-melittin at *endosomal pH*, the coupling strategy was changed to release the peptide in its free form at low pH. For this, a PEG-aldehyde lipid was used and two strategies were explored to conjugate this to the N-terminus of the peptide with an acid-labile imine bond. The first one was to directly couple the aldehyde to the N-terminal amine; however, this did not result in any conjugation at all (data not shown). The second strategy employed the addition of a hydrazide to the N-terminus of the peptide during synthesis. The aldehyde and hydrazide form a hydrazone bond, which is more stable than the imine bond. Due to the simultaneous hydrolysis of the formed bond, yields of such reactions are very low and again no conjugation could be detected when the reaction was performed in aqueous environment. To solve this, the reaction was done in organic solvent and the formed DSPE-PEG-peptide micelles were inserted into preformed liposomes according to a previously published procedure known as "post-insertion".[29] As a control, the hydrazone bond was reduced with sodium borohydride, to form a stable hydrazine bond that is not reversible in acidic environment. Liposomes functionalized with hydrazine coupled peptides did not induce any leakage at all in the calcein assay, nor at the pH of 5.6 where acid-melittin has the maximum activity. The formulation in which the peptide is reversibly conjugated, the lytic activity is restored at endosomal pH, and the absence of any effect in the irreversible conjugate proves that the leakage must be caused by the release of the free peptide. However, the submaximal leakage induced even at the highest concentration of peptide indicates that not all of the peptides are released, as free hydrazide-acid-melittin (**5**) already induced complete leakage at concentrations of 10−20 uM. Complete release may be achieved at lower pH or after longer incubation times at pH 5.6, but the residence time in the endosome is likely not longer than the 1 h time frame that was used here.[31,32]

However, this work undoubtedly demonstrates that the activity of the peptide can be completely killed by conjugating acid-melittin to the N-terminus and that it can be restored by releasing the peptide in its free form using endosomal pH as a spatiotemporal trigger. The effect of N- versus C-terminal conjugation was demonstrated here when comparing click-chemistry to maleimide−thiol chemistry in Figure 3 because the azide and cysteine were placed on the N- and C- terminus, respectively, for practical reasons. Because the peptide is more active in its free form also when compared to the C-terminus conjugated form, we employed the strategy to completely release it in the endosome and used N-terminal conjugation for safety in the bloodstream. The effect of the conjugation side of melittin was already reported by Boeckle et al.[16] and it was explained by the hypothesis that the N-terminus of the peptide is inserted into the membrane to exert its function.[25,26]

To summarize, this work shows the proof-of-concept that an endosomolytic peptide can be reversibly conjugated to a carrier liposome, using endosomal pH as the trigger for release. Calcein leakage from model liposomes was used as read-out both for toxicity (safety) and endosomolytic activity. As a second safety mechanism to avoid hemolysis and activity toward nontarget membranes, the pH-sensitive variant of melittin was used, resulting in an inactive formulation at physiological pH. Triggered by a conformational change of the acid-melittin at pH 5.6 and hydrolysis of the hydrazone bond, activity was restored. This effect can be completely attributed to release of the peptide, as the formulation with a noncleavable bond did not show any activity, even at pH 5.6.

Drug delivery systems equipped with an endosomal escape mechanism as described here are typically used for the delivery of biomacromolecules, such as peptides/proteins and nucleic acids. This may require a liposome with slightly different composition (for example, containing some cationic lipid to complex nucleic acid cargo) but both the in situ coupling of peptides to preformed liposomes as well as the post-insertion of peptide-micelles in a preformed liposome can be applied to any lipid composition.[29] Additionally, before reaching the endosome, the liposomes need to be internalized via endocytosis, which could be achieved by active targeting with a targeting ligand. The coupling chemistries described here could all be used to couple targeting ligands as well, so to maintain specificity, one technique could be used to couple the endosomolytic peptide and another could conveniently be used to couple the targeting peptide. It is important to note that peptides conjugated to the outside of a liposome are susceptible to enzymatic degradation and do not remain intact very long in the bloodstream. This problem is usually not addressed in the scientific literature because it is not a technical challenge to, e.g., make the peptides out of (D) amino acids instead of (L). But interestingly, apart from C- versus N-terminal conjugation Boeckle et al. also reported an increase in membrane activity of the peptide when it was composed of all (D) amino acids. It is not clear whether this influences the conformation of the peptide which makes it more potent or that it is a result of increased stability, but it is another opportune effect.[16] Alternatively, drug delivery systems can be functionalized with peptoids or peptidomimetics to enhance enzymatic resistance.[33]

Altogether, this work demonstrates the feasibility of using a two-pronged safety mechanism to render a membrane active peptide inactive while circulating in the blood, but at the same time maximizing the potency of the peptide within the acidic environment of the endolysosomal pathway.

EXPERIMENTAL SECTION

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), egg-phosphatidylcholine (EPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were a gift from Lipoid GmbH (Ludwigshafen, Germany). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-maleimide) and 1,2-distearoyl-*sn*-glyc-

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ero-3-phosphoethanolamine-*N*-[dibenzocyclooctyl-(polyethylene glycol)-2000] (DSPE-PEG2000-DBCO) were from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[aldehyde(polyethylene glycol)-2000] (DSPE-PEG2000-aldehyde) was from Nanocs (Bio-Connect Services BV, Huissen, Netherlands). Calcein, cholesterol, and Triton X-100 were from Sigma-Aldrich (St. Louis, MO, USA).

All the amino acid derivatives were purchased from GL Biochem (Shanghai) Ltd. 2-Azidoacetic acid was provided by Sigma-Aldrich (St. Louis, MO, USA). Hydrazide building block was synthesized following the procedure described previously,[22] with slight modifications.

HPLC was carried out on Shimadzu (analytical) and Applied Biosystems (preparative) chromatographs equipped with a UV detector (λ = 214 nm). The following solvent system was used: (A) acetonitrile in 0.1% aqueous TFA (5:95, v/v) and (B) acetonitrile in 0.1% aqueous TFA (95:5, v/v). Preparative HPLC was carried out using Dr. Maisch C18 column (10 μm, 22 × 250 mm) in a linear gradient from 40% to 70% of (B) for 60 min, except for analogue **4**, where the applied gradient was from 50% to 80% of (B) for 60 min. All purifications were performed at a flow rate of 12 mL/min. The chromatograms of the pure peptides and mass spectra were recorded on an HPLC-MS system (Shimadzu - Finnigan LCQ Deca XP Max) using Dr. Maisch C_{18} column (5 μm, 4.6 × 250 mm).

Peptide Synthesis. All peptides were obtained by the solid-phase method on a Symphony Multiple Peptide Synthesizer (Protein Technologies Inc., USA), i.e., by stepwise coupling of Fmoc-amino acids to the growing peptide chain on rink amide resin (TentaGel S RAM, Rapp Polymere GmbH, capacity 0.22 mmol/g). The amino acid side chain protecting groups were Trt for Cys and Gln, OtBu for Glu, Boc for Lys and Trp, Pbf for Arg, and tBu for Ser and Thr. Fully protected peptide resins were synthesized according to the standard procedures[28] involving (i) deprotection steps using 20% solution of piperidine in DMF, 5 and 15 min, and (ii) couplings in DMF using HBTU, HOBt in the presence of DIPEA. The amino acids were coupled twice at a 3-fold excess. After second coupling remaining free amino groups were terminated with 0.5 M acetic anhydride solution in DMF. N-terminal azide or hydrazide building block (analogues **2**, **4**, **5**) were attached manually in the final coupling step using the same procedure as that for Fmoc-amino acids. After completion of the synthesis the protected peptidyl resins were treated with cleavage cocktail, TFA:H_2O:EDT:TIPS, and stirred under N_2 for 2 h or in the case of analogues **1** and **2** for 3 h. Then the peptides were precipitated with cold diethyl ether and centrifuged to afford crude products. The resulting materials were dissolved, frozen, and lyophilized. The crude peptides were purified by RP-HPLC as described before. HPLC-MS was used to confirm the identity of the compounds and their purity.

Liposome Preparation. All carrier liposomes used in this work were composed of DPPC:Cholesterol:DSPE-PEG2000 in a ratio of 1.88:1:0.12 (equaling 6% of surface PEGylation). For conjugation of peptides, a fraction or the total amount of the DSPE-PEG2000 was replaced with DBCO- or maleimide-functionalized PEG-lipid. For post-insertion of the peptide-**5** micelles, liposomes of DPPC:Cholesterol:DSPE-PEG2000 in a ratio of 1.92:1:0.08 were used. For all preparations, lipids were added to a round-bottom flask and dissolved in chloroform. The organic solvent was then evaporated using a rotary evaporator until a dry lipid film was formed. This lipid film was further flushed with a stream of nitrogen to remove all residual chloroform. The lipid film was hydrated in PBS 7.4 (B. Braun Melsungen AG, Melsungen, Germany) and the formed liposome dispersion was extruded through 100 nm pore-sized filters (Nuclepore, Pleasanton, CA, USA) using a Lipex Extruder (Northern Lipids, Burnaby, BC, Canada) for 10−12 times. The liposomes for the calcein leakage assay were composed of EPC:Cholesterol in a ratio of 2:1 and the lipid film was hydrated with a solution of 75 mM calcein and extruded as described above. After extrusion, calcein liposomes were transferred to a 10K MWCO Slide-A-Lyzer G2 Dialysis Cassette (Life Technologies) and dialyzed against a ~300-fold volume of HEPES (10 mM) buffered saline (HBS) at 4 °C. After intervals of 8 h or more, dialysis fluid was replaced with fresh HBS until no calcein could be detected in it fluorometrically (see below). The hydrodynamic diameter and polydispersity index of all liposomes were measured by dynamic light scattering, using a Malvern CGS-3 multiangle goniometer with He−Ne laser source (λ = 632.8 nm, 22 mW output power) under an angle of 90° (Malvern Instruments, Malvern, UK). The zeta-potential of the liposomes was measured using laser Doppler electrophoresis on a Zetasizer Nano-Z (Malvern Instruments) with samples dispersed in 10 mM Hepes buffer pH 7.4 (with no additional salts added).

Peptide Conjugation. For coupling of **2**, the peptide was dissolved in PBS to a concentration of 500 μM and added to the liposomes with a total DPPC concentration of 20 mM and containing 1%, 2%, or 3% PEG-DBCO lipid, in the ratios as noted in Table S1. These samples where then incubated overnight on a roller bench. The liposomes used in the leakage assay of Figure 1 had a concentration of 100 mM DPPC and contained 2% PEG-DBCO. **2** was added to those liposomes to a final concentration of 500 uM. To make micelles of DSPE-PEG-DBCO and **2**, both were dissolved in PBS and added together in a ratio of 2:1 with a final peptide concentration of 500 uM.

For coupling of **3** and **4**, stock solutions of peptide were made in DMSO in a concentration of 2.5 mM. These were added to liposomes containing PEG-DBCO or PEG-maleimide in a ratio of 4:1 (peptide to functionalized PEG). Considering that half of the functionalized PEG lipids is facing to the inside of the liposome, this results in an effective concentration of 2:1. The ratio was kept the same, so different densities of coupled peptide required different concentrations of liposomes. Liposomes containing 2% of PEG-DBCO or PEG-maleimide lipid were hydrated to a final concentration of 60 mM DPPC. Liposomes containing 4% and 6% of PEG-maleimide were hydrated to 30 mM and 20 mM DPPC respectively. The final concentration of DMSO in the liposomes was 10% and the final concentration of acid-melittin was 250 μM. Coupling efficiency was measured with UPLC (see below) and DLS measurements were done again after conjugation. No aggregation of the liposomes was seen, unless otherwise stated (see Table 1).

Coupling of **5** to preformed liposomes containing 6% of PEG-aldehyde was attempted in aqueous solution but this did not lead to any measurable conjugation (nor in the presence of 20 mM sodium borohydride (Sigma-Aldrich)). Therefore, micelles were made in DMSO in an approximate ratio of 3:1 peptide to aldehyde in a concentration of 1.25 mM peptide. After overnight incubation, the sample was split and to one sodium borohydride was added to a final concentration of 20 mM and to the other 20 mM of TCEP (Sigma-Aldrich) to reduce the C-terminal cysteine. Two batches of liposomes

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containing 4% DSPE-PEG were made, hydrated in PBS pH 7.4 and PBS set to pH 5.6 with a total concentration of DPPC of 50 mM. These batches were split and 20 mM sodium borohydride and 20 mM TCEP were added to each of them (four samples total). The micelles with hydride and TCEP were added to the corresponding liposome samples and incubated at 40 °C for 30 min. The final concentration of DMSO in the liposomes was 20% and the final concentration of acid-melittin was 250 μM.

Coupling Quantification by UPLC. Coupling of the peptides was verified on a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) with PDA and FLR detectors on a BEH300 C18 1.7 μm column. The mobile phase was changed in a gradient from 100% solvent A (acetonitrile:water 5:95 with 0.1% TFA) to 100% solvent B (acetonitrile with 0.1% TFA) in 5 min and run on solvent B for 2 additional minutes at a flow rate of 0.25 mL/min. UV detection with the PDA detector was carried out at 210 and 280 nm and the FLR detector was used at 295/350 to detect the tryptophan residue in the peptides. Free peptides had a retention time around 3−4 min whereas the lipid conjugated peptide eluted at the end of the chromatogram. Conjugation was confirmed by the complete disappearance of the free peptide peak. The lowest amount that was injected was 0.301625 μM of peptide and this was still detectable. With the typical concentration of 250 μM of peptide added, the absence of the free peptide peak means less than 0.01% of free peptide was present.

Calcein Leakage Assay. Membrane activity of free peptides and the peptides conjugated to liposomes was measured in a calcein leakage assay. EPC liposomes containing 75 mM of calcein were used as model membranes that could be punctured or lysed by the peptides/liposomes. Leakage causes dilution of the self-quenched calcein to the external volume of the liposomes, increasing the fluorescent signal which was measured using a Jasco FP8300 plate reader (Jasco, Tokyo, Japan) at 495/520 excitation/emission wavelengths. Leakage was expressed as a percentage of the maximum release (max), induced by lysis of the target liposomes with Triton X-100 (0.5% final concentration). To determine 0% leakage, calcein liposomes were incubated in buffer and release was calculated using the following formula

$$\text{Calcein Release (\%)} = \frac{\text{Fluorescence (Sample)} - \text{Fluorescence (0)}}{\text{Fluorescence (max)} - \text{Fluorescence (0)}} \times 100$$

For free peptide samples and for all melittin samples the ratio of sample to target liposomes was 1:9, and therefore dilutions at 10× the assay concentration were made in PBS pH 7.4 (Braun) or set to pH 6.8, 6.2, or 5.6 with hydrochloric acid. For acid-melittin samples, the ratio of sample to target liposomes was 1:4, and therefore dilutions at 5× the assay concentration were made in the appropriate buffers. Samples were incubated at room temperature for 1 h and then measured in the platereader.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00677.

Additional tables with data and results (PDF)

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Notes

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■ ABBREVIATIONS

Boc, *tert*-butyloxycarbonyl group; DIPEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; EDT, 1,2-ethanedithiol; HBTU, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)-uronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high pressure liquid chromatography; MS, mass spectrometry; OtBu, *tert*-butyl ester group; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group; tBu, *tert*-butyl group; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; Trt, trityl group; CPP, cell penetrating peptide; DPC, Dynamic PolyConjugate; PEG, poly(ethylene glycol); RP-HPLC, reverse phase-high pressure liquid chromatography; HPLC-MS, high pressure liquid chromatography−mass spectrometry; UPLC, ultra performance liquid chromatography; PDI, polydispersity index; BCN, bicyclononyne; DBCO, dibenzocyclooctyne

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