Membrane Recruitment

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Recruitment of SH-Containing Peptides to Lipid and Biological Membranes through the Use of a Palmitic Acid Functionalized with a Maleimide Group**

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Abstract: *This study presents a novel and easily applicable approach to recruit sulfhydryl-containing biomolecules to membranes by using a palmitic acid which is functionalized with a maleimide group. Notably, this strategy can also be employed with preformed (biological) membranes. The applicability of the assay is demonstrated by characterizing the binding of a Rhodamine-labeled peptide to lipid and cellular membranes using methods of fluorescence spectroscopy, lifetime measurement, and microscopy. Our approach offers new possibilities for preparing biologically active liposomes and manipulating living cells.*

The recruitment of soluble molecules to membranes is of particular interest from biological, medical, and pharmacological perspectives. Various biological processes are determined by the association of proteins or peptides to membranes and often this binding is a prerequisite for subsequent membrane-related processes and/or interactions. Such a membrane association can be realized by a number of mechanism(s),[1] including 1) specific interaction with membrane lipids,[2] 2) conformational changes and membrane penetration of soluble proteins,[3] 3) binding by means of electrostatic interactions,[1b,4] and 4) decoration of the soluble protein with a lipid anchor (e.g. fatty acyl chain).[5] In order to study these processes at the molecular level and apply them, it is important to develop systems that mimic the membrane binding of the protein/peptide under investigation.

In addition, many applications have been developed using lipid vesicles (liposomes) that are decorated with biologically active molecules in order to produce, for example, immunogenic or anticancer drug and carrier systems.[6] In this context, different strategies have been introduced for the covalent attachement of proteins/peptides to vesicles.[6a,7] In general, a functionalized membrane anchor is used which reacts with the respective biomolecule by distinct chemical reaction(s). This can be achieved by first incorporating the anchor into the membrane and adding the respective protein/peptide to the modified membrane or by first reacting membrane anchor and protein/peptide and subsequently adding the complex to the membrane. The structure of the membrane anchor crucially determines its ability to be incorporated into the membranes. For example, phospholipid-derived anchors having two long fatty acyl chains can be efficiently incorporated into lipid membranes only during their preparation.[6a,8] However, it is difficult to incorporate these molecules into preformed artificial and, more interestingly, biological membranes due to their very low water solubility; these molecules spontaneously form stable membrane structures in aqueous buffer which complicates their transfer into a host membrane. It is a challenge to find membrane anchors that are partly water soluble such that they can also be incorporated into preformed membranes.

In the present study we have revived the use of fatty acids as a reactive membrane anchor by applying a palmitic acid functionalized with a maleimide moiety (PAEM) (Figure 1), which facilitates the ready recruitment of sulfhydryl (SH)-containing peptides to membranes. Several studies have already applied fatty acids for recruiting peptides to membranes and triggering biological effects.[9] However, the linkage of the fatty acid to the appropriate biomolecule was typically elaborate and time consuming. For example, a fatty acid was coupled to a lysine residue of a peptide which was bound to the resin after solid-phase synthesis.[9d] We show here that PAEM mediates the effective and rapid binding of a Rhodamine-labeled peptide (Rhodamine-βA-βA-FWDTN-βA-C (RLP, see the Supporting Information) to lipid vesicles and to biological membranes. Notably, by this approach it should be principally possible to bind every soluble molecule/

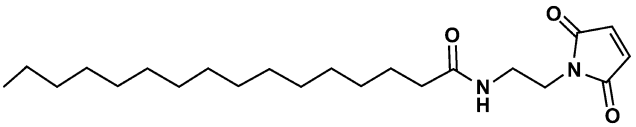

Figure 1. Structure of *N*-(2-(*N*-palmitoylaminoethyl)maleimide (PAEM).

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construct containing an accessible SH group to the surface of (preformed) membranes.

All details of the experiments including the synthesis of PAEM and the experimental conditions are given in the Supporting information. The reaction between PAEM and RLP in aqueous buffer was followed by mass spectrometry. We found that already 15 min after mixing the two substances a significant proportion of the RLP–PAEM complex had formed (see Supporting Information, Figure S1).

To demonstrate that PAEM is able to recruit RLP to lipid membranes, large unilamellar vesicles (LUVs) consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 0.5 mol% of the fluorescent phospholipid 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphocholine (C_{12}-NBD-PC) were prepared. It is possible to follow the association of RLP to the membrane surface by measuring the Förster resonance energy transfer (FRET) between the two fluorophores. FRET was determined by recording the fluorescence intensities of the vesicle suspension in the absence of RLP and in the presence of peptide or peptide along with PAEM. The results indicate that PAEM mediates the binding of RLP to LUV membranes (see the Supporting Information, Figure S2).

To better quantify the binding of RLP to lipid membranes, FRET efficiencies were determined from the measurements of the NBD fluorescence lifetimes of DOPC/C_{12}-NBD-PC-LUVs. PAEM was added either after or during LUV preparation. In the second case, a significant FRET efficiency of 56% was measured upon addition of RLP to vesicles, whereas FRET efficiency decreased to 12% when the peptide was pre-incubated with DL-dithiothreitol (DTT) (Figure 2). DTT reacts with the sulfhydryl (SH) groups of RLP, preventing the reaction between PAEM and RLP. Sequential addition of RLP and DTT to DOPC/C_{12}-NBD-PC/PAEM vesicles kept FRET efficiency high. DTT is not able to break the bond between PAEM and RLP.

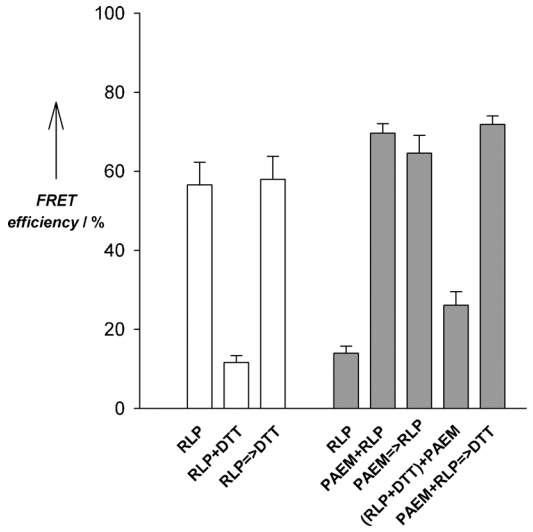

Figure 2. FRET efficiency between NBD of C_{12}-NBD-PC and Rhodamine of RLP. PAEM (4 μM) was added to 200 μM DOPC/C_{12}-NBD-PC (1 mol%) LUVs either during (white columns) or after LUV preparation (gray columns). The substances (8 μM RLP, 140 μM DTT) were added as indicated (+, addition as mixture after pre-incubation; =>, sequential addition). The fluorescence lifetime of NBD was measured at 25 °C and the FRET efficiencies were determined as described in the Supporting information. The data represent the mean ± standard deviation (seven measurements each of three vesicle preparations).

When PAEM was incorporated into LUVs after vesicle preparation and RLP was added subsequently, a large FRET efficiency of about 65% was measured. When PAEM with RLP was incubated in a small volume of buffer solution and this mixture was added to the vesicles, a similar high FRET efficiency was observed. Subsequent addition of DTT to this solution had no influence on this efficiency. However, a very low FRET efficiency was measured for 1) vesicles mixed with RLP in the absence of PAEM and 2) vesicles first mixed with PAEM and, subsequently with RLP pre-incubated with DTT. The results indicate that PAEM mediates a recruitment of RLP to LUV membranes which depends on the reaction between the maleimide moiety of PAEM and the thiol group of RLP.

When PAEM and the peptide are added to preformed membranes, solely the outer membrane leaflet is functionalized, whereas the addition of PAEM during vesicle preparation causes its localization on both leaflets such that half of the PAEM molecules are accessible for reaction with RLP. Note that in both protocols the same amount of PAEM was added. The difference in PAEM localization is quantitatively reflected by the FRET efficiencies measured on LUV membranes. The FRET efficiency in vesicles containing PAEM on both membrane leaflets was lower than that determined for vesicles where PAEM had been added to the preformed LUVs and thereby localized solely in the outer leaflet. This explanation presumes that the trans-bilayer movement of PAEM is very low in contrast to that of nonmodified palmitic acid which is very rapid with halflives of seconds or less.[10] Additional experiments were performed in order to investigate the influence of the RLP/PAEM ratio on PAEM-mediated binding of RLP to LUVs (see the Supporting Information, Figure S3). We found that the FRET efficiency becomes saturated at constant PAEM and increasing RLP concentration.

The binding of RLP to giant unilamellar vesicles (GUVs) was followed by fluorescence microscopy. For better visualization of the membranes, GUVs were additionally labeled with the membrane-embedded fluorophore C_{12}-NBD-PC. Again, two strategies were followed to recruit RLP to the membrane: the addition of PAEM either before or after GUV formation. For the latter, a significant Rhodamine fluorescence at the GUV membrane was observed when RLP was pre-incubated with PAEM, whereas GUVs showed no labeling when the peptide was 1) added without PAEM or 2) pre-incubated with DTT and PAEM (Figure 3). A similar dependence of peptide binding on the presence of PAEM was found when PAEM was incorporated into the GUV membranes during their formation. Upon addition of RLP to these vesicles, the membranes showed a clear Rhodamine fluorescence, whereas pre-incubation of the peptide with DTT prevented the labeling (see the Supporting Information, Figure S4). These results show that PAEM mediates the recruitment of RLP to GUV membranes via the reaction with the thiol group.

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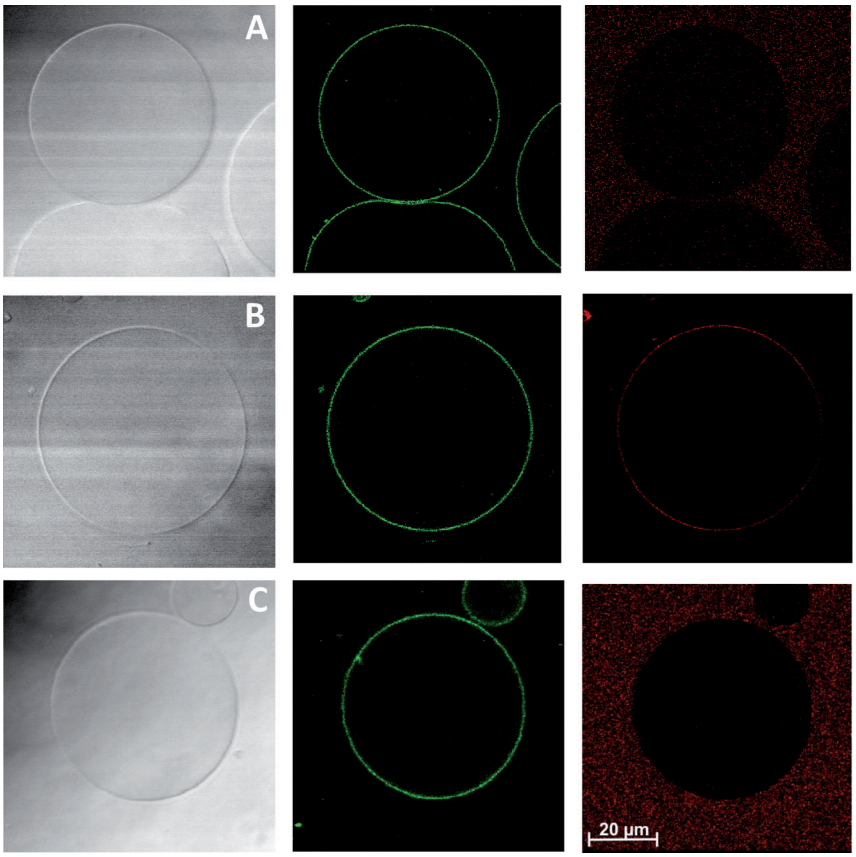

Figure 3. Binding of RLP to GUV membranes. 50 µM GUVs consisting of DOPC and C_{12}-NBD-PC (0.5 mol%) were mixed with A) 1 µM RLP, B) 1 µM RLP pre-incubated with 1 µM PAEM, and C) 1 µM RLP pre-incubated with 50 µM DTT and 1 µM PAEM and observed by differential interference contrast microscopy (left column) and by confocal scanning laser microscopy in the green channel (middle column) and in the red channel (right column) at 25 °C. The scale bar (20 µm) applies to all images.

PAEM also triggers the binding of RLP to a biological membrane. The peptide was added in the absence or in the presence of PAEM to macrophages and the staining of the cells was followed by fluorescence microscopy (Figure 4). Upon addition of RLP in the presence of PAEM, most of the cell membranes showed a significant Rhodamine fluorescence indicating a PAEM-mediated binding of the peptide to the plasma membrane. Under these conditions, also some bright extracellular labeling was observed which might be caused by RLP/PAEM micelles and/or RLP aggregates adhered to the membrane surface. No homogenous staining of the plasma membranes could be observed when RLP alone or RLP with DTT and PAEM was added to the cells. Only some extracellular fluorescent particles could be observed which may reflect membrane-attached aggregates of RLP.

The assay presented allows a rapid and effective recruitment of SH-containing molecules to membranes, especially also to biological membranes. Principally, PAEM can be incorporated into membranes by two protocols. First, PAEM can be added to model membranes (LUVs, GUVs) during their preparation and the peptide can then be bound to the membrane-embedded fatty acid derivative. Second, PAEM and the respective mole-

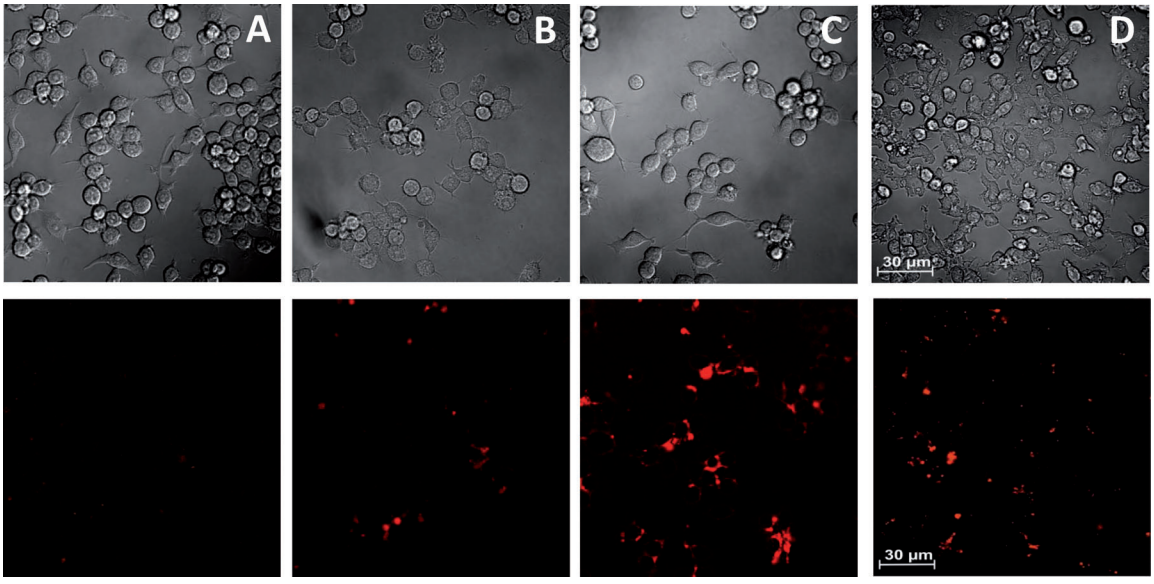

Figure 4. Binding of RLP to macrophages. The cells were incubated with A) buffer, B) 10 µM RLP, C) 10 µM RLP pre-incubated with 3 µM PAEM, and D) 10 µM RLP pre-incubated with 50 µM DTT and 3 µM PAEM for 5 min on ice and washed with buffer. Subsequently, the cells were observed by differential interference contrast microscopy (top row) and confocal laser scanning microscopy in the red channel (bottom row) at 25 °C. The scale bar (30 µm) applies to all images.

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cule are pre-incubated in buffer triggering their reaction and, subsequently, the complex is given to the respective membrane. In this way, the biomolecules can be recruited also to preformed membranes allowing the use for biological membranes. The method takes advantage of the behavior of fatty acids in aqueous media, which are organized as monomers (below the critical micellar concentration, cmc) or as micelles and monomers (above the cmc). Under our conditions the PAEM–RLP complex (as well as PAEM) does not form micelles and is present as monomers (see the Supporting Information). The transfer of these monomers from the buffer into the membrane results in the rapid and efficient incorporation of the complex into the membrane.

The PAEM-mediated binding of RLP to the membranes depends on the reaction between the maleimide moiety and the sulfhydryl group since blocking the SH groups by preincubation with DTT prevented peptide binding 1) to membranes as seen from the low FRET efficiencies (see Figure 2) and 2) to GUVs and macrophages (Figure 3 and Figure 4). We note that if this method is to be used in biological systems the putative reaction of PAEM and of the SH-bearing peptide/molecule with thiol groups of cellular proteins has to be considered.[11]

In future experiments the structure of the fatty acid will be modified with regard to length and saturation degree. These modifications will influence the aqueous solubility of the complex (affecting its membrane incorporation) and/or its membrane arrangement (affecting for example, its lateral distribution). In addition to peptide labeling, it is also possible to label the fatty acid derivative with a fluorophore by using an appropriately labeled fatty acid for PAEM synthesis. In this way, it will be possible to follow both the peptide and the fatty acyl chain, for example, during intracellular trafficking. Biological cells continuously take up part of their plasma membrane by endocytic processes, allowing the intracellular uptake of the membrane-anchored peptide and the triggering of respective biological effect(s).

A particular advantage of our approach is the close analogy to the physiological palmitoylation process.[12] It has been shown that the de- and re-palmitoylation of proteins plays an important physiological role since it determines protein localization and function.[13] Similar to the cellular palmitoylation machinery, we utilize cysteines to modify biomolecules with a fatty acid anchor. Therefore, we believe that this approach can be also used to mimic the biological palmitoylation, but potentially also other lipid modifications such as myristoylation, cholesteroylation, and farnesylation in in vitro approaches with purified proteins and peptides in different membrane model systems.

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