PEG-stabilized lipid disks as carriers for amphiphilic antimicrobial peptides

Malin M. Zetterberg\textsuperscript{a}, Karin Reijmar\textsuperscript{a}, Maria Pr"anting\textsuperscript{b}, Åke Engstr"om\textsuperscript{b}, Dan I. Andersson\textsuperscript{b} and Katarina Edwards\textsuperscript{a,c,*}

\textsuperscript{a}Department of Physical and Analytical Chemistry, Uppsala University, Box 579, SE-751 23 Uppsala, Sweden
\textsuperscript{b}Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-751 23 Uppsala, Sweden
\textsuperscript{c}FRIAS, School of Soft Matter Research, University of Freiburg, Freiburg, Germany

* Corresponding author. Phone: +46 18 471 3668 Fax: +46 18 471 3654 E-mail address: Katarina.Edwards@fki.uu.se
Abstract
Antimicrobial peptides hold potential as a possible alternative, or complement, to conventional antibiotics but new, safe and efficient means are needed for formulation and administration of the peptides. In this study we have investigated the utility of a novel type of lipid particles, the polyethylene glycol-stabilized lipid disks, as carriers for the model peptide melittin. The structural integrity of the carrier particle when loaded with the peptide was investigated using cryo-transmission electron microscopy. Liposome leakage upon addition of the peptide-lipid disks was monitored as a means to verify the membrane lytic effect of the formulation. The susceptibility of melittin to tryptic digestion was studied and compared in the absence and presence of lipid disks. Finally, the antibacterial effect of the peptide-lipid disk formulation was compared to that of free melittin after both single and repeated exposure to Escherichia coli. The results show that melittin can redistribute from the disk into a new host membrane and that formulation in the disks does not compromise melittin’s membrane permeabilizing ability. Further, the peptide was found to be fully protected against degradation when bound to the disks. Time-kill experiments revealed that all the antibacterial effect of melittin administered in free form was gone after a single exposure to E. coli. In contrast, the disk formulation showed significant cell-killing effect also upon a second exposure to bacteria, indicating an extended release of peptide from the lipid disks. These results suggest that the lipid disks constitute a new class of promising carriers for peptide antibiotics.

Keywords: PEG-stabilized lipid disk, antimicrobial peptide, melittin, E. coli, peptide delivery
1. Introduction
Host defence peptides, also referred to as antimicrobial peptides, constitute part of the innate immune system in virtually all life forms. Since their discovery, host defence peptides have received considerable attention as a potential new class of antibiotics [1-3]. The large interest is mainly due to most peptides broad range of action and the supposedly low risk of resistance development. Administration of peptide drugs is, however, a challenging task. To achieve the desired therapeutic effect sufficiently high peptide concentrations need to be maintained over extended time periods. This often proves difficult, since the half life of peptide drugs typically are short due to enzymatic degradation and rapid renal filtration of the peptides. Several approaches have been attempted to increase peptide half life and stability [2, 3]. Many of the suggested strategies include a chemical modification of the peptide itself by means of, e.g., pegylation [4, 5] or glycosylation [6]. However, altering the chemical structure of the peptide drug risks impairing its therapeutic effect. An alternative route to solving the problem is to formulate the chemically intact peptide in a suitable carrier vehicle. Ideally such a vehicle should, apart from ensuring therapeutic drug concentrations over adequate time periods, also protect the peptide against in vivo and in vitro degradation. The carrier naturally needs to be biocompatible and should preferably have a long circulation time and a high drug loading capacity.
Several systems have been explored in the search of a suitable peptide carrier vehicle. For example there has been a lot of focus on polymeric carrier particles such as polylactic-glycolic acid (PLGA) microparticles as well as on different types of lipid carrier systems [7, 8].
In the present study the potential of a novel vehicle, the polyethylene glycol (PEG)-stabilized lipid disk, as carrier for peptide drugs is evaluated. The PEG-stabilized lipid disk is a nanosized flat circular lipid bilayer surrounded by a highly curved rim [9]. The disks are obtained by mixing lipids that spontaneously form bilayer structures with close to zero curvature with micelle-forming PEG-lipids, i.e., lipids that have a bulky polymer chain covalently attached to their polar head-group. In order to obtain disks the PEG-lipid concentration needs to be above that at which the lipid bilayer becomes saturated with PEG-lipid. In case of PEG-lipids containing PEG of molecular weight 2 and 5 kDa disk formation typically begins at PEG-lipid concentrations corresponding to about 5 mol%, and PEG-lipid concentrations in the range of 15-20 mol% are required to produce pure disk preparations [10, 11]. The lipid and PEG-lipid components of the disk partially segregate in such a way that the PEG-lipids are enriched at the rim. A schematic drawing showing the structure of the disks can be found in Figure 1. The disks, which can be produced from lipid mixtures of various compositions and in sizes from a few tens of nanometres to several hundred nanometres [11, 12], are biocompatible and show good stability against dilution.
We recently presented evidence that a range of linear alpha-helical peptides, including melittin, magainin 2 and alamethicin, display high affinity for the rim of PEG-stabilized lipid disks [13]. Noteworthy, the peptides bind considerably stronger to disks than to liposomes produced from the same lipid components. Furthermore, the maximum loading capacity of the disks is superior to that of liposomes. In the case of melittin, previous studies indicate that the maximum peptide to lipid ratio that can be achieved is about ten times higher in disks than in liposomes [9]. Importantly, under conditions corresponding to maximum binding the disks remain intact and structurally unperturbed whereas liposomes under the same conditions rupture and display severe structural alterations [14]. These findings, together with the biocompatible and stable properties of the disks, lead us to propose PEG-stabilized lipid disks as potential carriers for amphiphilic antimicrobial peptides. In this study we use melittin [15] as a model peptide to further investigate the possibility of using the disks for formulation of antimicrobial peptides. One question we set out to answer is whether the peptides bound to the lipid disk are able to detach from the carrier and redistribute into a target membrane. Equally important is to verify that the peptides remain sufficiently tightly bound to the disk to allow for an extended release effect. Further, by using trypsin as a model enzyme we investigate the hypothesis that the disks may offer protection against proteolysis of the peptides. Finally, we explore the vital question of whether or not the peptide maintains its antibacterial effect after having been formulated in the disk.

2. Materials and Methods

2.1 Materials

Dry powder of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocoline (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG) and N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)5000]} (ceramide-PEG5000) was purchased from Avanti Polar Lipids (Alabaster, AL). Sequencing grade trypsin was purchased from Promega (Madison, WI). Cholesterol, and melittin (purity ≥ 90%, FW 2,846) was purchased from Sigma Aldrich Chemical (Steinheim, Germany). Melittin was dissolved in phosphate buffered saline (PBS\text{a}), 10 mM phosphate, 150 mM NaCl, pH 7.4. The stock solution was divided into 100 µl aliquots and thereafter immediately frozen and kept at -20° C until used. 5(6)-carboxyfluorescein (CF) was from Molecular Probes (Leiden, The Netherlands) and a stock solution of 100 mM was prepared in a salt free phosphate buffer. The solution, isoosmotic with the PBS\text{a}, was pH-adjusted to 7.4. All
chemicals were used without further purification. All experiments were performed in PBS$_a$ buffer unless otherwise stated.

2.2 Preparation of liposomes and lipid disks
The PEG-stabilized lipid disks were composed of POPC/cholesterol/ceramide-PEG$_{5000}$ (35:40:25 mol%) and the liposomes were composed of POPC/POPG (90:10 mol%). Lipid films were prepared by dissolving the desired lipids in chloroform and thereafter removing the solvent under a gentle stream of nitrogen gas. The films were subsequently dried in vacuum for at least 15 hours. The dry lipid films were then rehydrated in PBS$_a$ or, in the case of CF-filled liposomes, the salt free CF buffer. Liposomes were prepared by subjecting the hydrated films to five freeze thaw cycles. The liposome dispersions were then extruded 20 times through a polycarbonate filter with a pore size of 100 nm (Avestin, Ottawa, Canada) using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Liposome preparations were stored at 4 °C for 24 hours before use. Untrapped CF was removed by gel-filtration on a PD-10 desalting column (GE Healthcare, Uppsala, Sweden). Lipid disks were prepared by sonication of the hydrated lipid films for 45 minutes in an ice-bath using a Soniprep 150 sonicator (MSE, London, England). Metal debris from the sonicator tip was removed by centrifugation.

2.3 Melittin to lipid ratio
Melittin and lipid disks were mixed and used 24 hours after mixing. In all experiments the free melittin concentration was kept as low as possible while still allowing maximum melittin loading on the disks. Binding isotherm data collected by Lundquist et al. [9] was used to determine the optimal melittin to lipid ratio. The peptide to lipid ratio in the disks was 0.041 giving a free melittin concentration of 1.3 µM. The lipid concentration was held at 824 µM in all melittin-lipid disk mixtures unless otherwise stated. This lipid concentration gave a total melittin concentration of 35.1 µM (100 µg/ml).

2.4 Cryo-TEM
The cryogenic transmission electron microscopy (Cryo-TEM) investigations were performed using a Zeiss EM 902A Transmission Electron Microscope (Carl Zeiss NTS, Oberkochen, Germany). All observations were made in zero loss bright-field mode and at an accelerating voltage of 80kV. Digital images were recorded under the low dose conditions with a BioVision Pro-SM Slow scan CCD camera (Proscan GmbH, Scheuring, Germany and analysis software (Soft Imaging System GmbH, Münster, Germany). An underfocus of 1-2 µm was used to enhance the image contrast. In short, the Cryo-TEM specimens were prepared by depositing a small drop (~1 µl) of the sample on a copper grid covered with a carbon reinforced holey polymer film. Thin sample films (10-500 nm) were prepared by blotting the grid with a filter paper. All sample preparations were performed in a custom-built climate chamber at 25 °C and >99 % relative humidity. After blotting the grid was immediately plunged into liquid ethane kept just above its freezing point. Samples were kept below -165 °C and protected from atmospheric conditions during both transfer from the preparation chamber to the microscope and during examination. A detailed description of the technique can be found in [16].
2.5 Liposome leakage
Melittin induced leakage from liposomes was monitored at 25 °C using a SPEX Fluorolog 1650 0.22-m double spectrometer (SPEX Industries, Edison, NJ). Disk-peptide mixtures were added to liposomes encapsulating a self-quenching fluorescent dye and the increase in fluorescence was monitored over time. The spontaneous leakage from the liposomes, as well as the leakage obtained when adding a disk-free solution with melittin concentration corresponding to that of the unbound melittin in the disk-peptide mixture, was also measured. The excitation and emission wavelengths were set at 495 and 520 nm respectively. The degree of leakage L(t), expressed as the percentage of total leakage, was monitored and calculated according to

\[ L(t) = 100 \times \left( \frac{I(t) - I_0}{I_{tot} - I_0} \right) \]

where \( I(t) \) is the time dependent fluorescence intensity, \( I_0 \) is the background intensity and \( I_{tot} \) is the maximum intensity obtained by lysing the liposomes with 2.5 mM Triton-X100. All experiments were performed in triplicates and corrected for the spontaneous leakage.

2.6 Proteolytic assay
Peptide digestion upon incubation with trypsin was monitored for free and disk associated melittin. To ensure the effect of the enzyme both in the presence and absence of disks, degradation of a hydrophilic peptide KGVDA QGTLs KIFKL GGRDS RSGSP M was monitored. Digestion was performed by incubating melittin or melittin-lipid disk mixtures with trypsin (enzyme to substrate ration 1/100) in a temperature controlled chamber held at 25 °C for 2.5, 5, 10, 20 and 40 minutes. The peptide concentration was 57 µg/ml in all experiments and, in the experiments with lipid disks, the lipid concentration was 456 µM. The digestion was terminated by lowering the pH to 2.3 by addition of 10% acetic acid. Samples were then analysed by MALDI-Tof using an Ultraflex Tof/Tof system (Bruker Germany). The instrument was calibrated using Peptide Calibration standard II from Bruker and the matrix used was α-Cyano-4-hydroxycinnamic acid. Samples were analysed after dilution with 0.1 % trifluoroacetic acid.

2.7 Bacterial killing
*Escherichia coli* MG1655 (λ, F') were used for all bacterial killing experiments. Dilutions were performed in phosphate buffered saline (PBSb), 13mM phosphate 137 mM NaCl pH 7.4, and cells were plated on Luria Bertani agar (high salt, Sigma-Aldrich Chemical, Steinheim, Germany) for viable counts. BBL Trypticase soy broth (TSB) was purchased from Becton, Dickinson and Company, Sparks, USA.

2.7.1 Bacterial time-kill kinetics
Bacteria were grown overnight in 10 mM sodium phosphate buffer supplemented with 0.1% TSB and diluted to approximately 4 × 10^6 colony forming units (cfu)/ml in the same medium. 90 µl bacterial solution was then mixed with 10 µl PBSb (positive control 1), 10 µl lipid disks (positive control 2), 10 µl melittin solution (final concentration 5 µg/ml (1.76µM)) and 10 µl melittin mixed with lipid disks (final melittin concentration 5 µg/ml) in microcentrifuge tubes. The tubes were incubated with continuous shaking at 37
°C. Samples of 10 µl (resulting in a detection limit of 100 cfu/ml) were removed from the tubes after 0, 20, 60, 120 and 240 minutes to determine the number of viable cells. At each time point, percentage of viable cells remaining of the starting inoculum was determined. Survival at each time point is given as the geometric mean (±SEM) of at least 4 separate experiments. An approximate value of 0.001% was used in calculations for values below the detection limit of the assay.

2.7.2 Prolonged effect
Bacteria were grown overnight and diluted as described above. Then 135 µl bacterial solution was added to three different microcentrifuge tubes and mixed with 15 µl PBSb (positive control), 15 µl melittin solution and 15 µl melittin-lipid disk formulation, respectively. Final melittin concentration in the two latter tubes was 10 µg/ml (3.51 µM). Viable counts were determined before and after incubation for 15 min at 37 °C in a shaking incubator. Subsequently, the tubes were centrifuged for 10 min at 16060 × g to remove cell debris and remaining viable bacteria. The supernatants (110 µl/tube) were transferred to clean tubes to which new bacteria were added in a small volume, resulting in a bacterial concentration of approximately 4 × 10^6 cfu/ml. The bacteria were again incubated at 37 °C and the viable count determined at 0, 20, 60, 120 and 240 min. The experiment was carried out four times and survival at each time point was calculated as described above.

3. Results & Discussion
3.1 Cryo-TEM
Cryo-TEM enables direct visualisation of micelles, liposomes and related lipid structures in the nanosize range [16]. The technique was used to determine the particle size and shape in the disk preparations. Micrographs of the pure disk preparation revealed disk-shaped structures with a radius of approximately 20 – 100 nm. Cryo-TEM investigations revealed no structural change in the disk sample upon incubation with melittin (Fig. 2). Note that due to the poor contrast of the polymer, the PEG chains are invisible to the electron beam and can therefore not be detected in the micrographs.
Figure 2
Cryo-TEM images of disks composed of POPC/cholesterol/ceramide-PEG$_{5000}$ (35:40:25 mol%) mixed with melittin (peptide to lipid ratio 0.041). The arrow and arrowhead indicate disks observed edge-on and face-on, respectively. Scale bar = 100 nm.

3.2 Liposome leakage
Melittin is a well-characterized haemolytic peptide (26 amino acids). The peptide spontaneously forms an α-helix upon insertion in a lipid membrane and is well known to induce leakage from liposomes [15]. The ability of disk-associated melittin to detach and redistribute into another lipid membrane was investigated by monitoring liposome leakage after addition of peptide-lipid disk mixtures. In order to mimic the negatively charged surface of a bacterial membrane, the liposomes employed in the experiment contained 10 mol% negatively charged lipids. The leakage data presented in Figure 3 shows that the peptide was released from the disks and, moreover, that the released peptides were able to induce leakage from the liposomes. Addition of melittin at a concentration corresponding to that of the free, non-bound, melittin in the lipid disk mixture induced a liposome leakage of 11% 30 minutes after peptide addition. In contrast, the melittin-lipid disk mixture caused a CF leakage that amounted to 70% 30 minutes after peptide addition. Thus, the leakage induced when adding the melittin-lipid disk mixture to the CF encapsulating liposomes was higher than what would be expected if only due to the fraction of free peptide (1.3 μM). In fact, the observed leakage corresponded to that obtained upon addition of a 15 μM melittin solution (data not shown).
3.3 Proteolytic assay
Rapid enzymatic degradation of peptides in solution is one of the major problems that must be surmounted in order to make peptides promising as drug candidates. One solution to the problem might be to formulate the peptide drugs in a carrier particle, such as the PEG-stabilized lipid disk. In order to evaluate the protective properties of the disks, the susceptibility of melittin to degradation by trypsin was investigated in the presence and absence of lipid disks. Trypsin is a highly specific enzyme that selectively cleaves peptide bonds of lysine (K) and arginine (R). Since the melittin sequence contains three lysine and two arginine residues, trypsin was chosen as a suitable enzyme to study melittin stability. Mass spectra of the enzymatically digested melittin are shown in Figure 4 and the peptide sequences are given in Table 1. As expected, the tryptophan residue became oxidized and in the spectra all peaks from melittin and its digestion products are accompanied by an additional peak from a fragment that is 32 m/z heavier than the unoxidized one.
Figure 4
Mass spectra showing the digestion of melittin after incubation with trypsin for 2.5, 5, 10, 20 and 40 minutes. (a) free melittin, (b) melittin-lipid disk mixture. The peptides labelled $a_1$-$g_1$ are listed in table 1.
Table 1
Tryptic digestion products of melittin. Trypsin cleaves the peptide at lysine (K) and arginine (R) residues (indicated in bold). Listed in the right column are the labels used in figure 4.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
<th>Mw</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIGAV LKVLT TGLPA LISWI KRKRQ Q-COH</td>
<td>1-26</td>
<td>2847.49</td>
<td>a</td>
</tr>
<tr>
<td>GIGAV LKVLT TGLPA LISWI KRKR</td>
<td>1-24</td>
<td>2590.63</td>
<td>b</td>
</tr>
<tr>
<td>GIGAV LKVLT TGLPA LISWI KRK</td>
<td>1-23</td>
<td>2434.53</td>
<td>c</td>
</tr>
<tr>
<td>GIGAV LKVLT TGLPA LISWI KR</td>
<td>1-22</td>
<td>2306.43</td>
<td>d</td>
</tr>
<tr>
<td>VLT TGLPA LISWI KRKRQ Q-COH</td>
<td>8-26</td>
<td>2208.33</td>
<td>e</td>
</tr>
<tr>
<td>VLT TGLPA LISWI KRKR</td>
<td>8-24</td>
<td>1952.21</td>
<td>f</td>
</tr>
<tr>
<td>VLT TGLPA LISWI KR</td>
<td>8-22</td>
<td>1668.02</td>
<td>g</td>
</tr>
</tbody>
</table>

Analysis of the mass spectra belonging to pure melittin (Fig. 4a) shows that the peptide was rapidly digested by trypsin, and after ten minutes of incubation with the enzyme there was no intact melittin left. The fast degradation of melittin was prevented, however, when the peptide was associated to lipid disks. Even after 40 minutes of incubation with trypsin no trace of digestion products were observed in the mass spectra (Fig. 4b). It could be speculated that the protection from degradation was an effect of the disks inhibiting the enzyme rather than the peptide being protected when bound to the disks. To test this possibility the hydrophilic peptide KGDSA QGTLS KIFKL GGRDS RSGSP M was incubated with trypsin in the presence and absence of disks. The mass spectra in Figure 5a and 5b show that the enzymatic activity was unaffected by the presence of the lipid disks. This indicates that the effect observed in Figure 4 is a result of the peptide being protected in the disks.
Figure 5

(a) Intens. [a.u.] vs. m/z for different time points.

(b) Intens. [a.u.] vs. m/z for different time points.
Mass spectra showing the digestion of the peptide KGVDAGQGTLSEQIFKLGGRDSRSGSPMG after incubation with trypsin for 2.5, 5, 10, 20 and 40 minutes. (a) free peptide, (b) peptide-lipid disk mixture. The peptides labelled a₂-d₂ are listed in Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
<th>Mw</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>KGVDAGQGTLSEQIFKLGGRDSRSGSPMG</td>
<td>1-26</td>
<td>2593.07</td>
<td>a₂</td>
</tr>
<tr>
<td>KGVDAGQGTLSEQIFKLGGRDSRSGSPMG</td>
<td>1-21</td>
<td>2333.24</td>
<td>b₂</td>
</tr>
<tr>
<td>KGVDAGQGTLSEQIFKLGGRDSRSGSPMG</td>
<td>1-18</td>
<td>1975.08</td>
<td>c₂</td>
</tr>
<tr>
<td>IFKLGGRDSRSGSPMG</td>
<td>12-26</td>
<td>1907.83</td>
<td>d₂</td>
</tr>
</tbody>
</table>

Previous studies of melittin in phosphatidylcholine bilayers showed that although the peptide was associated with the membrane, all peptide cleavage sites were still accessible for trypsin cleavage [17, 18]. This indicates that the observed protection of the peptide in the lipid disks is not merely due to the fact that the peptide is embedded in the lipid bilayer. Conceivably, the PEG chains covering the disk rim sterically hinders the enzyme from interaction with the peptide. It is plausible that also other proteases will be hindered by the polymer. Further studies are needed, however, to prove this hypothesis right.

### 3.4 Activity of melittin-disk formulation against bacteria

#### 3.4.1 Bactericidal activity

One possible field of application for peptide drugs would be as antibacterials, either to replace existing antibiotics rendered ineffective by bacterial resistance development or as a complement to existing treatments. Melittin is bactericidal and acts by disrupting the bacterial membrane. An important aspect of the present work was to establish if melittin retains bactericidal activity when administered together with the lipid disks, and therefore killing assays with *E. coli* were performed. Bacteria were subjected to melittin and to melittin-lipid disk formulations, and the number of viable bacterial cells was monitored over time. As can be seen in Figure 6, free melittin at 5 µg/ml efficiently killed the bacteria and the number of cells decreased to below 0.1% of the starting inoculum within 20 minutes (>3 log reduction). The melittin-lipid disk formulation also killed the bacteria and >3 log reduction in the number of viable cells was achieved within 60 min of incubation (Fig. 6). Although initial killing was slightly slower than for free melittin, similar reduction in the number of bacterial cells was reached in one to two hours. The lipid disks in themselves had no inhibitory effect on the bacteria, as control cultures with and without lipid disks grew equally well. These results demonstrate that melittin is bactericidal when administered together with the disks, and that the lipid disks are relevant as peptide delivery system.
3.4.2 Extended activity of melittin-lipid disk formulation

A potential problem with positively charged antimicrobial peptides is high affinity for negatively charged bacterial membranes combined with a low turnover rate. Once associated with the bacteria, the peptides may not be released and available for attack of another cell. Although binding to bacterial membranes is desirable, a considerable amount of peptide may be consumed at first contact with bacteria whether these are viable or not. This would leave no free active peptide to kill bacteria that survive the first exposure and thus the bacteria might start proliferating again. An interesting question is if the peptide-lipid disk formulation can extend peptide activity due to a continuous release of peptides from the disks while still allowing efficient bacterial killing. To test this, we used a two-step killing assay.

After an initial 15 minutes exposure of bacteria to melittin or melittin-lipid disk formulation, remaining bacteria, cell debris and any associated melittin was removed by centrifugation. Subsequently, new bacteria were added to the supernatant and the number of viable cells was determined at different time points (Figure 7). In the first exposure, melittin, given in free form, reduced the number of bacterial cells to less than 0.01% of the starting inoculum. However, very little activity remained in the supernatant and when new bacteria were added, most cells survived exposure.
Figure 7
Prolonged effect of melittin-lipid disk formulation. *E. coli* cells were subjected to 10 µg/mL melittin or melittin-lipid disk formulation for 15 min, after which remaining cells were removed by centrifugation and new bacteria were added to the supernatant (indicated by an arrow and a vertical dashed line). The number of viable cells was determined at different time points and survival was calculated as a percentage of the starting inoculum. Filled diamonds, positive control *E. coli*; open squares, *E. coli* + melittin-lipid disks; open circles, *E. coli* + melittin.

We know from the killing experiments (described above) that melittin at 5 µg/ml is enough to efficiently reduce the amount of cells to low numbers within 20 minutes. It appears that the majority of peptides binds to the bacteria added in the first exposure and are removed simultaneously during the centrifugation step. This is in contrast to what was observed for the melittin-lipid disk mixture. When administered together with lipid disks, melittin caused the number of bacterial cells to decrease to below 1% in the first 15 minutes. After centrifugation a substantial amount of melittin was still present in the supernatant, and upon addition of fresh bacteria the number of viable cells decreased to about 0.1% in 120 min (a 3 log reduction in number of cells). In other words, it appears that the lipid disks extend the bactericidal activity by releasing peptide to the media at a slow enough rate to prevent the consumption of all peptide during the first exposure. It is conceivable that the release rate can be adjusted and optimized by altering the lipid composition of the disks. A slower release of melittin can for instance be expected upon inclusion of negatively charged lipids in the disk.

4. Conclusions
Results of this study show that the antibacterial effect of melittin is retained upon formulation in the disks. Additionally, when bound to the disk the peptide is protected against digestion by trypsin. Rather than being due to specific interactions between melittin and the disk, this is likely an effect of the PEG-chains sterically hindering the enzyme from coming into close contact with the peptide. Therefore, it can be speculated that also other peptide drugs would be protected against digestion, provided that they bind to the disk rim. It is also shown that the release of melittin from the formulation is extended, since the antibacterial effect of the peptide-lipid disk mixture is maintained when the formulation is repeatedly exposed to bacteria. The slow release of peptide from the disks presumably enables a more even drug concentration to be maintained in the media over time. Thus, the risk of exhaustion of peptide upon an initial interaction with bacteria is reduced. The encouraging data of this study motivate further studies focused on the application of PEG-stabilized lipid disks as carriers for peptide drugs. In particular, subsequent studies would involve tests of melittin and other peptides against a broader spectrum of both Gram-positive and Gram-negative bacteria.

5. Acknowledgements
Dr. Jonny Eriksson is gratefully acknowledged for skilful technical assistance with the cryo-TEM analysis. Financial support was received from the Swedish Research Council
(DIA and KE), the Swedish Cancer Society (KE), Vinnova (DIA) and The Knut and Alice Wallenberg foundation (MS instrument).

6. References