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PEG-stabilized lipid disks as carriers for amphiphilic antimicrobial peptides

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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.

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 could potentially impair 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

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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 Fig. 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 disks 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-PEG_{5000}) 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_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.

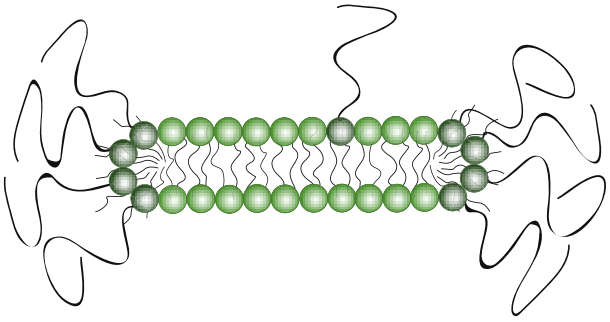

Fig. 1. A schematic illustration of the cross section of a polyethylene glycol (PEG)-stabilized lipid disk with the PEG-ylated lipids covering the disk rim.

The solution, isoosmotic with the PBS_a, was pH-adjusted to 7.4. All chemicals were used without further purification. All experiments were performed in PBS_a 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 h. 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 h 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 min 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 h 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 80 kV. 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