Colloids and Surfaces B: Biointerfaces 123 (2014) 419–428

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Pore formation in 1,2-dimyristoyl-sn-glycero-3-phosphocholine/cholesterol mixed bilayers by low concentrations of antimicrobial peptide melittin

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ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form 8 September 2014
Accepted 16 September 2014
Available online 28 September 2014

Keywords:
Antimicrobial peptide
Pore formation
Lipid bilayer
DMPC
Cholesterol
Melittin
Lag time

ABSTRACT

Antimicrobial peptides (AMP) represent a class of compounds to combat antibiotic resistance to microorganisms, neutralize biological warfare agents, and as topical antimicrobial agents. AMP kills microbial cells through insertion and permeabilization of the cytoplasmic membranes. It is important to predict the efficacy of AMP at low concentration to circumvent their toxicity. Leakage of fluorescent dyes (calcein, FD4 and FD20) of different molecular weights entrapped within 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)/cholesterol mixed liposomes by AMP melittin indicated (i) a critical melittin concentration for pore formation and (ii) a lag time for pore formation above this critical concentration. The lag time decreased with an increase in melittin concentration and was in the order FD20 > FD4 > calcein. % α helix of melittin increased when exposed to liposome with this increase being more pronounced at higher concentrations eventually reaching an asymptotic value. The rate of dye leakage following the lag time was found to be larger at higher melittin concentration. A simplified mathematical model for nucleation and growth of pores formed by an aggregate of melittin in lipid bilayer is proposed to predict the variation of rate of dye leakage with melittin concentration which agreed fairly well with the data for calcein and seems to suggest a toroidal mechanism of pore formation with participation of large number of phospholipid heads.

1. Introduction

Antimicrobial peptides (AMP) have the ability to penetrate cell membrane and kill the cell either by cell membrane rupture or through disruption of cell function. They are divided into subgroups on the basis of their amino acid composition and structure [1]. Pore formation by AMP in phospholipid bilayers begins by electrostatic attraction followed by attachment and penetration of the cell membrane and has been subject of several investigations [2–6]. Experimental techniques based on fluorescence [7], oriented circular dichroism [4], NMR spectroscopy [8], X-ray crystallography [4] and quartz crystal microbalance [9] have been employed to monitor the pore formation, secondary conformation of AMP, orientation of AMP and the lipid, thinning of the membrane and mass as well as rigidity of the membrane. Peptides have been shown to bind in two physically distinct states [10]. At low peptide/lipid ratios, α-helical peptides, β-sheet peptides and θ-defensins adsorb and embed into the lipid head group region in a functionally inactive state that stretches the membrane [11,12]. At high peptide/lipid ratios, peptide molecules are orientated perpendicularly and insert into the bilayer [4]. Several studies of pore formation by AMP on model membranes has elucidated different mechanisms of pore formation such as *barrel stave*, *toroidal* or *carpet mechanism* and has helped to identify key factors that control pore formation [3,6,8,12–15].

Since the mechanism of deactivation of microorganisms by AMPs differs from that of antibiotics, they could be very useful for combating drug resistant microbes, for treatment of microbial infections and also for prevention. It is customary to report the effectiveness of AMP as minimum inhibitory concentration. In order to circumvent the problem of toxicity, however, very low doses of these AMPs have to be effective in antimicrobial action. With this in mind, we investigated pore formation at very low concentrations through leakage of fluorescent dyes from liposomes and discovered the existence of a time lag for pore formation by AMP above this critical concentration. Consequently, the effectiveness of AMP at very low concentrations can be quantified by this critical concentration and the time lag at higher concentrations.

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http://dx.doi.org/10.1016/j.colsurfb.2014.09.037
0927-7765/

Nomenclature

$g_{formation}(n_a, n_b)$	free energy of formation of pore consisting of n_a peptides and n_b phospholipids per peptide
$d_{pore}(n_a, n_b)$	diameter of pore consisting of n_a peptides and n_b phospholipids per peptide
λ	line tension of hydrophilic head of lipid molecule
γ	interfacial tension of phospholipid bilayer
d_h	diameter of phospholipid head
κ	Debye Huckel parameter
l	length of lipid tail
k	Boltzmann constant
T	temperature
ξ	flexibility parameter of peptide molecule
q	net charge of peptide molecule
ϕ	electrostatic potential
σ^*	surface charge density
t_{pore}	thickness of the bilayer
ε_r	dielectric constant of aqueous medium inside the pore
ε_0	permittivity of vacuum
q_{ph}	net charge of phospholipid head
g_{bend}	bending free energy of phospholipid heads
$\Delta G_{pep,ads}$	free energy of adsorption of peptide onto phospholipid membrane
$\Delta G_{pore}(n_a, n_b)$	free energy of pore consisting of n_a peptides and n_b phospholipids per peptide with respect to adsorbed peptides
$\Delta G_{sol}(n_a, n_b)$	free energy of pore consisting of n_a peptides and n_b phospholipids per peptide with respect to peptides in solution
X	binding constant of peptide onto phospholipids
σ_s	surface free energy change per unit length for pore formation
s	supersaturation
c_p	bulk peptide concentration
c_{ps}	critical peptide concentration for pore formation
n_c	number of peptides in a pore of critical size
ΔG^*	free energy of pore of critical pore size
J	rate of nucleation of pores per unit volume
ρ_{eq} (1)	surface concentration of adsorbed peptides on phospholipid surface
ρ_{eq} (n_c)	surface concentration of pores of critical size
$\dot{n}$	rate of growth of pores
t_g	time of growth of pores
$N(n, t)$	number of pores of size n at time t
c_b	bulk concentration of fluorescent dye
ϕ	volume fraction of liposomes
d_{lip}	liposome diameter
D	diffusion coefficient of dye through pores
D_s	surface diffusion coefficient of peptides on liposome surface
F	fluorescence intensity of dye
$F_{\max}$	maximum fluorescent intensity of dye
t_{lag}	time lag for fluorescence

2. Materials and methods

2.1. Materials

DMPC and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), dihexadecyl hydrogen phosphate, calcein, fluorescein isothiocyanate-labeled dextrans with average molecular masses of 4 and 20 kDa (FD4 and FD20) and melittin (purity: 91.8%, HPLC) from honey bee venom were purchased from Sigma–Aldrich (St. Louis, MO). Sephadex G-75 (GE Healthcare) was used to separate free calcein from liposomes. The composition of the bilayer is chosen to mimick the cell membrane.

2.2. Preparation of liposomes

Liposome of composition DMPC, cholesterol, and dihexadecyl hydrogen phosphate in a molar ratio of 5:4:1 and with entrapped dye (calcein, FD4 or FD20) was prepared using protocol described elsewhere [16–19]. 100 μl of lipid stock solution (DMPC, cholesterol, and dihexadecyl hydrogen phosphate in a molar ratio of 5:4:1 [16] in chloroform) was dried under N_2 to form a thin film in a glass vial. Three fluorescent reporter molecules, calcein [17], FD4 (molecular weight 4000) and FD20 (molecular weight 20,000) [18] were trapped inside the lumen of the liposomes. Dye entrapment was accomplished by adding 1 ml of calcein (50 mM), FD4 or FD20 (10 mg/ml) in 0.02 M phosphate buffer at pH 6.0 to the lipid film before liposome formation. The suspension was vortexed for several minutes and then allowed to rest for 30 min to form large unilamellar, dye-encapsulating vesicles. The vesicle suspension was then forced through a 1000 nm polycarbonate filter (Avanti Polar Lipids) for clacein, FD4 and FD20 using a miniextruder (Avanti Polar Lipids, Inc.) with two gastight Hamilton syringes [16] fifteen times to form unilamellar liposomes. The diameter of the liposomes was measured by laser light scattering (Zetasizer nano series, Malvern Instruments, Worcestershire, UK). Free dye was removed by size-exclusion chromatography using a Sephadex G-75 column for calcein[19], FD4 and FD20. To avoid photobleaching, both liposomes were stored in a dark environment at −4 °C until use.

2.3. Fluoroscence measurement

The fluorescence of the released dye was measured with a spectrofluorometer (Flex Station II, Molecular Device) at an excitation wavelength of 488 nm and an emission wavelength of 518.5 nm. All experiments were conducted at 25 °C which is above the phase transition temperature for DMPC–cholesterol mixtures of cholesterol concentration above 12.5 mol% [20].

2.4. Circular dichroism (CD)

CD spectra were recorded at room temperature using a Jasco 810 spectropolarimeter (Jasco Spectroscopic Co., Hachioji, Japan) with a quartz cell of 0.2 cm path-length. Data were collected every 0.2 nm with 2 nm bandwidth, at a scan speed of 100 nm/min. Molar ellipiticity (deg cm^{-1} $dmol^{-1}$) is expressed on a mean residue concentration basis in the far-UV. Spectra were analyzed for secondary structure content by using the program *Contin* [21].

2.5. Confocal laser-scanning microscopy (CLSM)

CLSM images were taken using LSM710 (Carl Zeiss, Göttingen, Germany) with an argon ion laser (488 nm). Liposome with encapsulated calcein was mixed with different concentration of melittin. The poly-L-lysine slide was used to fix the sample. The fluorescence intensity was measured as a function of time. Images were then recorded digitally in a 512 × 512 pixel format.

3. Results

The liposomes were monodispersed with a narrow distribution around the mean size of 551 nm. The liposome concentration in the collected fraction from size exclusion chromatographic column is inferred from the measurement of count rate that is obtained from