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Étude des mécanismes d'extraction lipidique par le peptide mélittine et la protéine BSP1

par

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Résumé

Les peptides et protéines extracteurs de lipides (PEL) se lient aux membranes lipidiques puis en extraient des lipides en formant de plus petits auto-assemblages, un phénomène qui peut aller jusqu'à la fragmentation des membranes. Dans la nature, cette extraction se produit sur une gamme de cellules et entraîne des conséquences variées, comme la modification de la composition de la membrane et la mort de la cellule. Cette thèse se penche sur l'extraction lipidique, ou fragmentation, induite par le peptide mélittine et la protéine *Binder-of-SPerm 1* (BSP1) sur des membranes lipidiques modèles. Pour ce faire, des liposomes de différentes compositions sont préparés et incubés avec la mélittine ou la BSP1. L'association aux membranes est déterminée par la fluorescence intrinsèque des PEL, tandis que l'extraction est caractérisée par une plateforme analytique combinant des tests colorimétriques et des analyses en chromatographie en phase liquide et spectrométrie de masse (LCMS).

La mélittine fait partie des peptides antimicrobiens cationiques, un groupe de PEL très répandu chez les organismes vivants. Ces peptides sont intéressants du point du vue médical étant donné leur mode d'action qui vise directement les lipides des membranes. Plusieurs de ceux-ci agissent sur les membranes des bactéries selon le mécanisme dit « en tapis », par lequel ils s'adsorbent à leur surface, forment des pores et ultimement causent leur fragmentation. Dans cette thèse, la mélittine est utilisée comme peptide modèle afin d'étudier le mécanisme par lequel les peptides antimicrobiens cationiques fragmentent les membranes. Les résultats montrent que la

fragmentation des membranes de phosphatidylcholines (PC) est réduite par une déméthylation graduelle de leur groupement ammonium. L'analyse du matériel fragmenté révèle que les PC sont préférentiellement extraites des membranes, dû à un enrichissement local en PC autour de la mélittine à l'intérieur de la membrane. De plus, un analogue de la mélittine, dont la majorité des résidus cationiques sont neutralisés, est utilisé pour évaluer le rôle du caractère cationique de la mélittine native. La neutralisation augmente l'affinité du peptide pour les membranes neutres et anioniques, réduit la fragmentation des membranes neutres et augmente la fragmentation des membranes anioniques. Malgré les interactions électrostatiques entre le peptide cationique et les lipides anioniques, aucune spécificité lipidique n'est observée dans l'extraction.

La BSP1 est la protéine la plus abondante du liquide séminal bovin et constitue un autre exemple de PEL naturel important. Elle se mélange aux spermatozoïdes lors de l'éjaculation et extrait des lipides de leur membrane, notamment le cholestérol et les phosphatidylcholines. Cette étape cruciale modifie la composition lipidique de la membrane du spermatozoïde, ce qui faciliterait par la suite la fécondation de l'ovule. Cependant, le contact prolongé de la protéine avec les spermatozoïdes endommagerait la semence. Cette thèse cherche donc à approfondir notre compréhension de ce délicat phénomène en étudiant le mécanisme moléculaire par lequel la protéine fragmente les membranes lipidiques. Les résultats des présents travaux permettent de proposer un mécanisme d'extraction lipidique en 3 étapes : 1) L'association à l'interface des

membranes; 2) La relocalisation de l'interface vers le cœur lipidique; 3) La fragmentation des membranes. La BSP1 se lie directement à deux PC à l'interface; une quantité suffisante de PC dans les membranes est nécessaire pour permettre l'association et la fragmentation. Cette liaison spécifique ne mène généralement pas à une extraction lipidique sélective. L'impact des insaturations des chaînes lipidiques, de la présence de lysophosphatidylcholines, de phosphatidyléthanolamine, de cholestérol et de lipides anioniques est également évalué.

Les présentes observations soulignent la complexe relation entre l'affinité d'un PEL pour une membrane et le niveau de fragmentation qu'il induit. L'importance de la relocalisation des PEL de l'interface vers le cœur hydrophobe des membranes pour permettre leur fragmentation est réitérée. Cette fragmentation semble s'accompagner d'une extraction lipidique préférentielle seulement lorsqu'une séparation de phase est induite au niveau de la membrane, nonobstant les interactions spécifiques PEL-lipide. Les prévalences des structures amphiphiles chez certains PEL, ainsi que de la fragmentation en auto-assemblages discoïdaux sont discutées. Finalement, le rôle des interactions électrostatiques entre les peptides antimicrobiens cationiques et les membranes bactériennes anioniques est nuancé : les résidus chargés diminueraient l'association des peptides aux membranes neutres suite à l'augmentation de leur énergie de solvatation.

Mots-clés : Extraction lipidique, Fragmentation, Spécificité lipidique, Mélittine, BSP1, PDC-109, Analogue de mélittine, Membrane modèle, Interactions lipide–protéine, Interactions lipide–peptide, Liposomes, Phospholipides.

Abstract

Lipid-extracting peptides and proteins (LEPs) bind to lipid membranes, extract lipids in the form of smaller auto-assemblies, and ultimately fragment membranes. In nature, this lipid extraction occurs in many different cell systems and causes various consequences, such as a modification of the membrane lipid composition or the cell death. This thesis focuses on the lipid extraction, or fragmentation, induced by the peptide melittin and the protein Binder-of-SPerm 1 (BSP1) on model lipid membranes. To this end, liposomes of different composition are prepared and incubated with melittin or BSP1. The association to membranes is determined by the LEPs intrinsic fluorescence, while the extraction is characterized by a combination of colorimetric phosphorus assays and liquid chromatography-mass spectrometry analyses (LCMS).

Melittin is a cationic antimicrobial peptide, a very common category of LEP found in living organisms. Cationic antimicrobial peptides are interesting to medicine because they directly target membrane lipids. The action of many of these peptides is described by the carpet-like mechanism, by which they adsorb to membrane surface, induce the formation of pores and then cause the fragmentation of the membranes. In this thesis, melittin is used as a model peptide in order to study the mechanism by which cationic antimicrobial peptides fragment lipid membranes. Results show that the phosphocholine (PC) membrane fragmentation is reduced by a gradual demethylation of the ammonium group. Analysis of the fragmented material reveals that PC are preferentially extracted from membranes, due to a local enrichment in PC

near melittin in the membrane. Furthermore, a melittin analogue, for which a majority of its cationic residues were neutralized, is used to investigate the role of the cationic character of native melittin. The neutralization increases the peptide affinity for neutral and anionic membranes, reduces fragmentation of neutral membranes and increases fragmentation of anionic membranes. Despite electrostatic interactions between the cationic peptide and the anionic lipids, no lipid specificity is observed in the extraction.

BSP1 is the most abundant protein of the bovine seminal plasma and constitutes another example of important LEP found in nature. Upon ejaculation, it mixes with spermatozoa and extracts membrane lipids, such as cholesterol and phosphatidylcholines. This crucial process modulates the lipid composition of sperm membranes, which would then facilitate egg fertilization. However, a prolonged contact between the protein and spermatozoa could damage the semen. This thesis is looking to deepen our understanding of this delicate phenomenon by studying the molecular mechanism by which this protein fragments lipid membranes. Results of the present work suggest a 3-step mechanism for the extraction: 1) Association to membrane interface; 2) Relocation towards the lipid core; 3) Fragmentation of membranes. BSP1 binds directly to two interfacial PC; a sufficient quantity of PC in membranes is necessary for protein association and fragmentation. This specific binding generally does not lead to specificity in the lipid extraction. The impact of

unsaturation of the lipid chains, of the presence of lysophosphatidylcholines, of phosphatidylethanolamines, of cholesterol and of anionic lipids is also studied.

The present observations underline the complex relationship between a LEP affinity for membranes and the level of fragmentation it induces. The importance of LEP relocation, from the interface to the hydrophobic core of the membranes, for fragmentation is reiterated. This fragmentation seems to be lipid specific only when a phase separation of the lipids occurs in the membrane, notwithstanding specific LEP-lipid interactions. The prevalence of amphipathic structures in certain LEPs, as well as of the auto-assembled discoidal structures resulting from fragmentation is discussed. Finally, the role of electrostatic interactions between cationic antimicrobial peptides and anionic bacterial membranes is detailed: charged residues lower peptide association to neutral membrane due to an increase of their free energy of solvation.

Keywords: Lipid extraction, Fragmentation, Lipid specificity, melittin, BSP1, PDC-109, Melittin analogue, Model membrane, Lipid-protein interactions, lipid–peptide interaction, liposomes, phospholipids.

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Liste des abréviations et symboles

a_0	Aire de la tête polaire
ABCA1	*ATP-binding cassette transporter*
ACN	Acétonitrile
ADN	Acide désoxyribonucléique
ApoA1	Apolipoprotéine A1
Arg	Arginine
BOP	*(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate*
BSP1 / PDC-109	*Binder-of-SPerm 1*
BSP3	*Binder-of-SPerm 3*
BSP5	*Binder-of-SPerm 5*
CAP	*Cationic Antimicrobial Peptide*
Chol	Cholestérol
Cit-Mel	*Citrullinated Melittin*
Cryo-EM	*Cryo-Electron Microscopy*
D	Coefficient de diffusion
DEPC	1,2-dielaidoyl-*sn*-glycéro-3-phosphocholine
DEPE	1,2-dielaidoyl-*sn*-glycéro-3-phosphoéthanolamine
DMPC	1,2-dimyristoyl-*sn*-glycéro-3-phosphocholine
DMPG	1,2-dimyristoyl-*sn*-glycéro-3-phosphoglycérol
DOPC	1,2-dioleoyl-*sn*-glycéro-3-phosphocholine

DP	Dipalmitoyles
DPMe2PE	1,2-dipalmitoyl-*sn*-glycéro-3-phosphoethanolamine-N,N-diméthyle
DPMePE	1,2-dipalmitoyl-*sn*-glycéro-3-phosphoéthanolamine-N-méthyle
DPPC	1,2-dipalmitoyl-*sn*-glycéro-3-phosphocholine
DPPE	1,2-dipalmitoyl-*sn*-glycéro-3-phosphoéthanolamine
DPPS	1,2-dipalmitoyl-*sn*-glycéro-3-phospho-L-sérine
DPTAP	1,2-dipalmitoyl-3-triméthylammonium-propane
EDTA	*Ethylenediaminetetraacetic acid*
FnII / Fn2	Fibronectine II
HDL	*High-Density Lipoprotein*
HIV	*Human Immunodeficiency Virus*
HPLC	*High-Pressure Liquid Chromatography*
$I_{\lambda free}$	*Intensity at* λ_{free}
$I_{\lambda bound}$	*Intensity at* λ_{bound}
K	Lysine
K_a	Constante d'association
L/M	Rapport lipide/mélittine à l'incubation (mol/mol)
L/P	Rapport lipide/peptide à l'incubation (mol/mol)
ℓ_c	Longueur des chaînes hydrophobes
LCMS	*Liquid Chromatography coupled with Mass Spectrometry*
L_d	Phase liquide désordonnée

LEP	*Lipid-Extracting Peptides and proteins*
L_o	Phase liquide ordonnée
LUV	*Large Unilamellar Vesicle*
Lys	Lysine
Lyso-PC	1-palmitoyl-2-hydroxy-*sn*-glycéro-3-phosphocholine
L_α	Phase fluide
L_β	Phase gel
M/L	Rapport mélittine/lipide à l'incubation (mol/mol)
MALDI-TOF	*Matrix-Assisted Laser Desorption/Ionization coupled with Time-Of-Flight mass spectrometry*
MD	*Molecular Dynamics*
Mel1-20	Mélittine 1-20
Mel1-22	Mélittine 1-22
MLV	*Multilamellar Vesicle*
MOPS	*3-[N-morpholino]propanesulfonic acid*
P/L	Rapport peptide/lipide à l'incubation (mol/mol)
PA	*Phosphatidic Acid*
PC	Phosphatidylcholine
PE	Phosphatidyléthanolamine
PEL	Peptides et protéines Extracteurs de Lipides
PG	Phosphatidylglycérol
PO	Palmitoyle-oléoyle

POPC	1-palmitoyl-2-oleoyl-*sn*-glycéo-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-*sn*-glycéo-3-phosphoéthanolamine
POPS	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-sérine
PS	Phosphatidylsérine
R	Arginine
SM	Sphingomyéline
SMA	Styrène Acide Maléique
TFA	Trifluoroacetic Acid
T_m	Température de fusion
Trp	Tryptophane
X_b	*Fraction of bound melittin*
ΔG	*Change of Gibbs free energy*
ΔH	*Change of enthalpy*
λ	Longueur d'onde
λ_{bound}	λ_{max} *for bound melittin*
λ_{free}	λ_{max} *for free melittin*
μ	Moment amphiphile
υ	Volume de la partie hydrophobe

"Eh, what's up Doc?"

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Chapitre 1 - Introduction

1.1 Mise en contexte: Peptides et protéines extracteurs de lipides

Les peptides et protéines extracteurs de lipides (PEL) se lient aux membranes lipidiques puis en extraient des lipides pour former de plus petits auto-assemblages, un phénomène qui peut aller jusqu'à la fragmentation des membranes. Dans la nature, cette extraction se produit sur une gamme de cellules et entraîne des conséquences variées, comme la modification de la composition de la membrane et la mort de la cellule.

Les peptides antimicrobiens cationiques (CAP) sont un exemple de PEL très répandus chez les organismes vivants [1]. À ce jour, plus de 2500 d'entre eux ont été répertoriés [2], présentant une large gamme de fonctions comme antibactériens, antiviraux, antifongiques, anti-cancers, etc.[3] Ces peptides sont particulièrement intéressants du point de vue médical, étant donné leur mécanisme d'action visant directement les membranes des bactéries. Plusieurs de ceux-ci agissent sur les membranes bactériennes selon le mécanisme dit « en tapis », par lequel ils s'adsorbent à leur surface, forment des pores et arrachent ultimement des portions de membranes [4]. Un de ces peptides antimicrobiens est la mélittine, principal agent actif du venin de l'*Apis mellifera mellifera,* l'abeille domestique européenne. Plus de 8000

articles ayant la mélittine comme sujet ont été publiés à ce jour, faisant de ce peptide l'un des plus étudiés et l'un des plus fréquemment employés comme peptide modèle. L'incubation de la mélittine avec des membranes lipidiques résulte en leur fragmentation et en la formation de petites structures discoïdales [5-7]. Malgré l'imposante littérature sur la mélittine, le mécanisme moléculaire par lequel le peptide forme ces structures et extrait des lipides reste mal compris. Les travaux de la présente thèse se penchent d'abord sur le mécanisme moléculaire par lequel la mélittine fragmente des membranes et en extrait sélectivement certains lipides.

Un autre exemple intéressant de PEL naturel est celui de la *Binder-of-Sperm Protein* 1 (BSP1), la protéine la plus abondante du liquide séminal bovin [8, 9]. Elle se mélange aux spermatozoïdes lors de l'éjaculation [10] et extrait des lipides de leur membrane, notamment le cholestérol et les phosphatidylcholines [11-13]. Cette étape cruciale modifie la composition lipidique de la membrane du spermatozoïde, ce qui faciliterait par la suite la fécondation de l'ovule. Bien que cette action de la BSP1 soit estimée essentielle pour la fertilité, le contact prolongé de la protéine avec les spermatozoïdes endommagerait irréversiblement la semence. Pour l'industrie bovine, la reproduction est largement effectuée par fertilisation *in vitro* : une bonne préservation des semences s'avère donc vitale. L'étude des facteurs qui causent la dégradation des spermatozoïdes est importante pour l'industrie canadienne, qui exporte annuellement des semences bovines pour plus de cent millions de dollars [14]. La compréhension du mécanisme par lequel la BSP1 extrait les lipides des

membranes de spermatozoïdes (et éventuellement les endommage) pourrait permettre de développer de meilleures méthodes de préservation. Le mécanisme d'extraction lipidique par la BSP1 est le second sujet d'étude de cette thèse. Bien que les présents travaux se concentrent sur la mélittine et la BSP1, on retrouve une large gamme de PEL, dans différents domaines, pour lesquels le mécanisme d'extraction n'est pas parfaitement défini.

Un exemple notoire de PEL est celui de l'apolipoprotéine A1 (ApoA1), la protéine principale des lipoprotéines de haute densité (HDL) que l'on retrouve dans le plasma humain et qui contribue au transport inverse du cholestérol [15]. Ce transport inverse correspond au déplacement du cholestérol à partir de certains tissus vers le foie, où il est métabolisé. Le cholestérol, insoluble dans le sang, est d'abord extrait des cellules et des plaques par l'action notamment de l'ApoA1. Malgré le grand intérêt de la médecine moderne pour le taux de cholestérol chez l'humain, le mécanisme moléculaire par lequel les ApoA1 parviennent à extraire les lipides n'est pas complètement élucidé. Il est intéressant de souligner que, comme la mélittine, l'ApoA1 fragmente certaines membranes pour former des particules discoïdales [16].

L'intérêt pour l'extraction de lipides membranaires n'est pas confiné aux PEL. Par exemple, le mécanisme de l'extraction de lipides membranaires par les détergents fut longtemps considéré comme simple et non-spécifique. Il est maintenant démontré que le détergent Triton X-100 extrait préférentiellement certains lipides [17], révélant

ainsi un mécanisme d'extraction plus complexe que ce qui avait été présumé. De même, le copolymère styrène acide maléique (SMA) est un agent synthétique récemment utilisé pour extraire des portions entières de membranes sous forme de nano-disques [18], sans en altérer leur composition ou leur structure. Cette extraction lipidique par le SMA, appliquée sur une cellule par exemple, permet d'isoler une protéine membranaire dans un nano-disque et d'en étudier la structure dans son environnement natif.

L'objectif de cette thèse est d'approfondir la compréhension du phénomène d'extraction lipidique en proposant des mécanismes pour la protéine BSP1 et la mélittine. Plus spécifiquement, la thèse vise à détailler les forces moléculaires qui modulent l'association de ces PEL aux membranes, la fragmentation de ces dernières et la sélectivité lipidique de l'extraction. Pour ce faire, des liposomes de différentes compositions, employés comme membranes modèles, sont préparés afin de dégager les conditions physico-chimiques nécessaires à ces phénomènes. De plus, un analogue de la mélittine est utilisé pour évaluer le rôle de sa région C-terminale cationique. L'association aux membranes est déterminée par la fluorescence des tryptophanes et/ou tyrosines, tandis que l'extraction lipidique est caractérisée par une plateforme analytique combinant des tests colorimétriques et des analyses en chromatographie en phase liquide et spectrométrie de masse (LCMS).

1.2 Propriétés des membranes

La membrane cellulaire est un mélange complexe de lipides et de protéines qui isole l'intérieur de la cellule, le cytoplasme, de l'environnement extérieur [19]. Elle est sélectivement perméable, laissant passer certaines petites molécules comme l'oxygène et le dioxyde de carbone, et bloquant le passage d'autres molécules, notamment les ions, les nutriments et les déchets cellulaires. Des protéines de transport encastrées dans la membrane permettent la diffusion de ces dernières molécules. La base structurale de la membrane consiste en une bicouche lipidique d'environ 5 nm d'épaisseur.

La diversité de la composition lipidique de la membrane cellulaire complexifie grandement l'étude des interactions moléculaires qu'elle entretient avec les PEL. Les liposomes synthétiques, ou vésicules, constitués généralement de phospholipides et de cholestérol, sont fréquemment utilisés comme membranes modèles afin de simplifier le système.

1.2.1 Les auto-assemblages lipidiques comme membranes modèles

En milieu aqueux, les molécules non polaires s'associent selon les interactions hydrophobes afin de minimiser les perturbations au réseau de ponts H formés par les molécules d'eau. Il est entropiquement favorable pour les phospholipides de s'auto-assembler en micelles ou en bicouches pour isoler leur(s) chaîne(s) alkylées de l'eau.

D'un point de vue enthalpique, une telle séparation de phase brise des interactions eau-soluté et crée des interactions soluté-soluté; le signe du ΔH d'agrégation dépend du système en question. Néanmoins, des interactions attractives entre les chaînes alkyles existent toujours à l'intérieur de ce cœur hydrophobe, dues aux forces dispersives de London.

Ainsi, les phospholipides s'auto-assemblent spontanément lors de leur hydratation, généralement en vésicules multi-lamellaires (MLV, de *MultiLamellar Vesicle*). Ces dernières sont composées de plusieurs bicouches emboîtées en « style oignon », et forment une suspension de particules de tailles polydisperses, allant de quelques centaines de nm jusqu'à quelques µm. Le grand avantage que possèdent ces MLV dans le cadre des présents travaux est qu'elles sont plus denses que l'eau et donc centrifugeables, permettant de séparer les fragments extraits des MLV intacts. Il est possible d'extruder les MLV pour obtenir de grandes vésicules unilamellaires (LUV, de *large unilamellar vesicle*), possédant un diamètre de l'ordre de la centaine de nm. Ces dernières sont préférables pour mesurer l'association des PEL aux membranes, puisqu'elles exposent tous les lipides d'un feuillet de toutes les bicouches au milieu extérieur; par contraste, il est difficile d'évaluer la concentration de lipides accessibles aux PEL lorsqu'ils interagissent avec des MLV compte tenu de la lamellarité variable de ces auto-assemblages. Les LUV sont d'ailleurs de mise pour les analyses par fluorescence, car leur petite taille limite la diffusion de la lumière

incidente, un phénomène parfois problématique avec les MLV. La Figure 1-1 illustre les différences morphologiques entre ces deux types de vésicules :

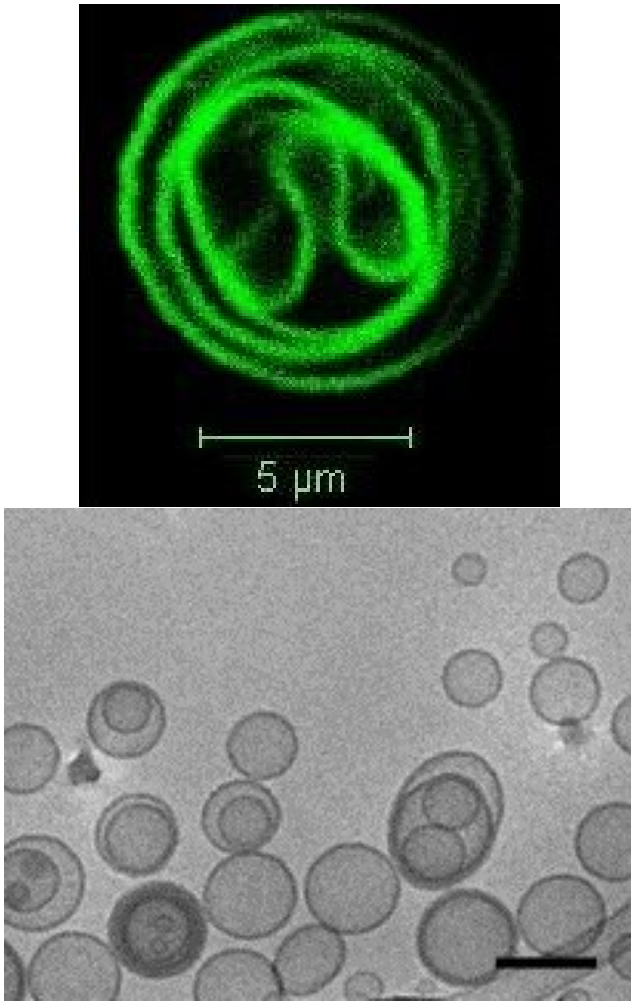


Figure 1-1 : En haut: Image d'une vésicule multilamellaire de 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) marquée au BODIPY obtenue par microscopie de fluorescence. En bas: Image typique de LUV obtenue par cryo-microscopie électronique, la barre correspond à 100 nm [20].

L'image de microscopie de fluorescence illustrée en haut de la Figure 1-1 montre la morphologie d'une MLV d'environ 10 µm de diamètre, constituée de

plusieurs bicouches emboîtées. En bas de la figure se trouve une image de cryo-microscopie électronique de LUV, extrudées à l'aide de filtres de 100 nm. On peut y voir que l'échantillon est principalement constitué de vésicules unilamellaires de tailles similaires bien qu'une fraction des vésicules conserve une certaine multilamellarité.

1.2.2 Les phases lipidiques

Les membranes lipidiques sont caractérisées par différentes phases, définissant entre autres la fluidité des lipides. Les phases les plus importantes pour la compréhension des présents travaux sont la phase fluide (L_α, ou L_d) et la phase gel (L_β). Pour une température sous leur température de fusion respective (T_m), les membranes se trouvent en phase gel, caractérisée par une diffusion latérale relativement lente des lipides ($D \approx 10^{-3}$ $\mu m^2/s$) [21]. En phase gel, les chaînes hydrophobes sont étirées et bien empilées, conduisant à un grand contact entre celles-ci.

En chauffant à une température supérieure à T_m, les membranes passent en phase fluide. L'énergie thermique transférée aux lipides se traduit par un plus grand désordre conformationnel au niveau des chaînes alkyles, qui présentent beaucoup plus d'isomérisation syn/anti (ou gauche/trans, selon les domaines). Cette réorganisation amincit considérablement la membrane (par exemple de 5,5 à 3,6 nm pour la

1,2-dipalmitoyl-*sn*-glycéro-3-phosphocholine (DPPC) [22]). Les membranes fluides sont relativement flexibles et bénéficient d'un plus grand volume libre, ce qui leur permet de plus facilement accommoder l'insertion de corps étrangers comme des peptides/protéines.

Les stérols, comme le cholestérol, constituent une importante portion des lipides retrouvés dans les membranes eucaryotes. Le cholestérol a donc été incorporé dans certaines des membranes modèles préparées dans le cadre de cette thèse. Les 4 cycles formant le cholestérol lui confèrent une structure rigide, peu influencée par la température. Le cholestérol agit comme un stabilisateur de fluidité dans la membrane, puisque sa rigidité diminue le désordre des chaînes en phase fluide, alors qu'il l'augmente en phase gel en perturbant l'empilement régulier des chaînes. La présence de cholestérol dans une membrane introduit donc une phase fluide mais ordonnée, la phase liquide-ordonnée (L_o).

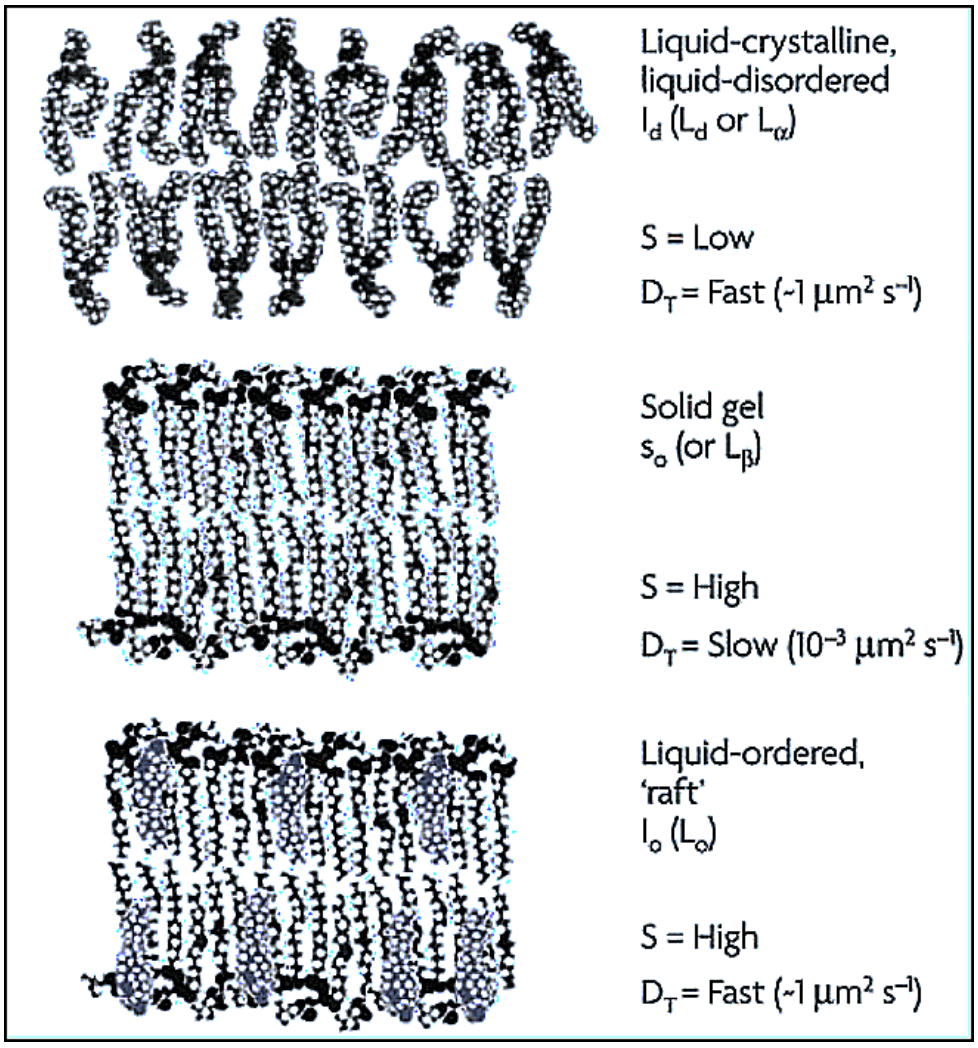


Figure 1-2: Représentation des phases L_β, L_d et L_o [23].

1.2.3 Membranes modèles de mélanges binaires de lipides

Dans le cadre des travaux de cette thèse, de nombreux liposomes sont préparés à partir de mélanges binaires de phospholipides ou de cholestérol; les lipides utilisés sont présentés à la Figure 1-3. Puisque les phospholipides à tête phosphatidylcholine (PC) sont les plus abondants, autant dans les membranes de

spermatozoïdes [24] que dans celles des érythrocytes [25], la PC constitue le phospholipide de base des liposomes formés. Une fois les interactions entre les PEL et des liposomes formés exclusivement de PC établies, on peut alors moduler les propriétés de ces liposomes en leur incorporant un second lipide. Ceci permet d'isoler l'impact de chacun des lipides utilisés sur l'activité du PEL.

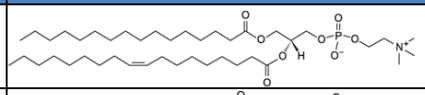
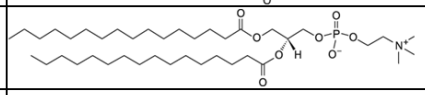
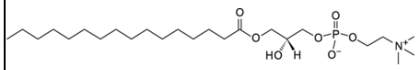
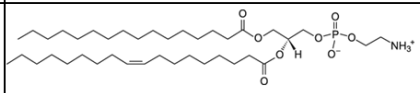
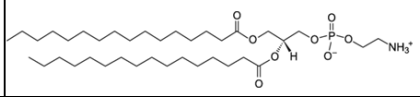
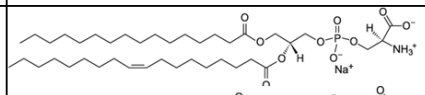
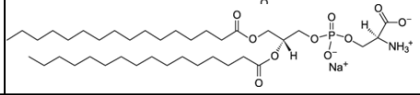
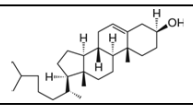
	Nomenclature des lipides UIPAC	Structure	T_m	Caractéristique recherchée
POPC	16:0-18:1 PC 1-palmitoyl-2-oléoyl-sn-glycéro-3-phosphocholine		-2 °C	• Phase fluide (L_α)
DPPC	16:0 PC 1,2-dipalmitoyl-sn-glycéro-3-phosphocholine		41 °C	• Phase gel (L_β)
Lyso-PC	16:0 Lyso PC 1-palmitoyl-2-hydroxy-sn-glycéro-3-phosphocholine		-	• Petit rapport v / $a_0 l_c$
POPE	16:0-18:1 PE 1-palmitoyl-2-oléoyl-sn-glycéro-3-phosphoéthanolamine		25 °C	• Petite tête polaire • Grand rapport v / $a_0 l_c$
DPPE	16:0 PE 1,2-dipalmitoyl-sn-glycéro-3-phosphoéthanolamine		65 °C	• Interactions tête-tête fortes
POPS	16:0-18:1 PS 1-palmitoyl-2-oléoyl-sn-glycéro-3-phospho-L-sérine		14 °C	• Charge négative
DPPS	16:0 PS 1,2-dipalmitoyl-sn-glycéro-3-phospho-L-sérine		54 °C	
Cholestérol			-	• Phase fluide ordonnée (L_o)

Figure 1-3: Liste des structures ainsi que de certaines caractéristiques des lipides utilisés [26].

Pour toutes les espèces de la Figure 1-3, les lipides à chaînes palmitoyle-oléoyle (PO) sont utilisés afin d'obtenir des liposomes en phase fluide à température ambiante, alors que les lipides à chaînes dipalmitoyles (DP) sont utilisés pour obtenir

la phase gel à cette même température. Les phospholipides à tête phosphatidylsérine (PS) sont ajoutés pour donner un caractère anionique aux liposomes. Le potentiel de surface des liposomes introduit la possibilité d'interactions électrostatiques et affecte donc directement l'association des deux PEL étudiés dans les présents travaux. Le cholestérol est utilisé, comme mentionné précédemment, pour obtenir des membranes en phase liquide ordonnée.

La lysophosphatidylcholine (lyso-PC) et les phosphatidyléthanolamines (PE) impactent l'organisation des membranes principalement à cause de leur géométrie. Le paramètre d'empilement d'un lipide $\frac{\nu}{a_0 \ell_c}$, où ν est le volume de la partie hydrophobe, a_0 est l'aire occupée par la tête hydrophile et ℓ_c est la longueur des chaînes, est un bon indicateur de la courbure des monocouches que ce lipide forme en s'auto-assemblant [27]. Les lipides avec un rapport $\frac{\nu}{a_0 \ell_c} < 1$, comme la lyso-PC, s'auto-assemblent en micelles. Les lipides avec un paramètre d'empilement > 1, comme la POPE, favorisent la formation de phases hexagonales inversées. Les lipides avec un paramètre d'empilement voisin de 1 possèdent une forme cylindrique et, en s'auto-assemblant, conduisent généralement à la formation de bicouches. Tous les mélanges de lipides utilisés pour les travaux de cette thèse forment des phases lamellaires aux températures étudiées (Figure 1-3). Cependant, l'inclusion de lipides avec différents rapports $\frac{\nu}{a_0 \ell_c}$ affecte l'empilement des molécules dans la membrane. Cet effet peut avoir un important impact sur l'insertion des PEL et la fragmentation des membranes.

La figure suivante illustre l'effet de l'insertion de la lyso-PC et de la POPE sur la courbure des membranes:

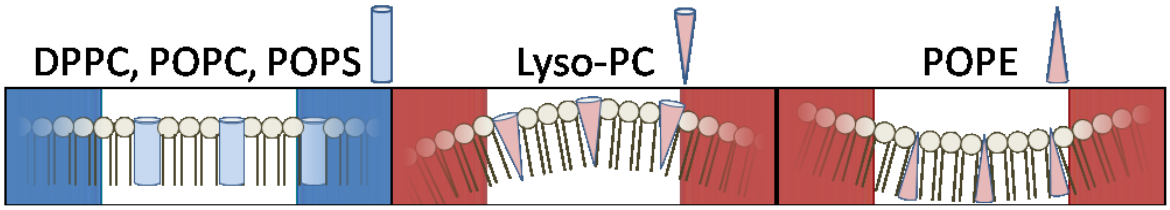


Figure 1-4: Schématisation de l'effet de la lyso-PC et de la POPE sur la courbure des membranes.

La membrane illustrée dans la partie gauche de la Figure 1-4 représente une bicouche plane formée de lipides cylindriques. Comme illustré au centre de la Figure 1-4, les lipides possédant un paramètre d'empilement inférieur à 1 ont plutôt une géométrie conique. Insérés dans une membrane plane, ces derniers augmentent le volume libre des lipides cylindriques et, en quantité suffisante, déstabilisent les membranes et donnent au mélange lipidique une tendance à former des auto-assemblages micellaires.

Au contraire, la géométrie conique (inversée par rapport à la lyso-PC) de la POPE tend à compacter l'empilement des lipides (Figure 1-4, à droite) [28]. Le groupement ammonium primaire $-NH_3^+$ de la tête polaire des PE occupe moins d'espace que l'ammonium quaternaire $-N(CH_3)_3^+$ des PC. De plus, la présence des PE augmente la cohésion membranaire due à leur tendance à former des ponts hydrogène lipide-lipide; les PC tendent plutôt à former des ponts hydrogène avec

l'eau [29]. Ainsi, dans une membrane de PE, il en résulte un empilement plus compact des chaînes lipidiques.

1.3 Extraction lipidique/fragmentation des membranes par les PEL

La fragmentation des membranes, ou extraction lipidique, est au cœur de cette thèse. Elle correspond à la formation de plus petits auto-assemblages par les PEL, à partir de membranes lipidiques. La fragmentation induite par les peptides antimicrobiens cationiques est le premier sujet de ces travaux.

1.3.1 Les peptides antimicrobiens cationiques

Comme mentionné précédemment, certains des peptides antimicrobiens cationiques (CAP), très présents dans la nature [1], agissent comme extracteurs de lipides [30]. Les peptides qui se structurent en hélice-α amphiphile constituent une importante sous-catégorie [3, 31]. Ils sont généralement longs de 10 à 50 résidus, et leurs résidus cationiques ou hydrophobes sont généralement distribués sur des faces opposées de l'hélice-α, lui conférant son caractère amphiphile.

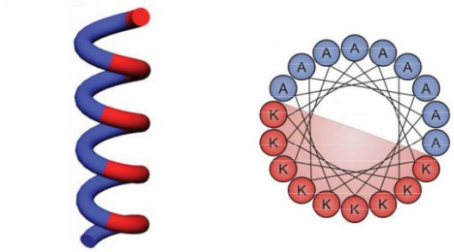


Figure 1-5 : À gauche : Une représentation en hélice-α d'une structure peptidique amphiphile avec une face cationiques (rouge) et une face hydrophobe (bleu). À droite : Projection en roue de l'hélice-α, mettant en évidence le caractère amphiphile de la structure [30].

Plusieurs de ces CAP agissent selon le mécanisme « en tapis » [4]. Selon ce modèle, les peptides se lient à l'interface et recouvrent la membrane en formant un tapis peptidique. Au-delà d'une concentration critique, le modèle prédit que les peptides brisent la membrane et forment des bicelles (nano-disques) ou des micelles.

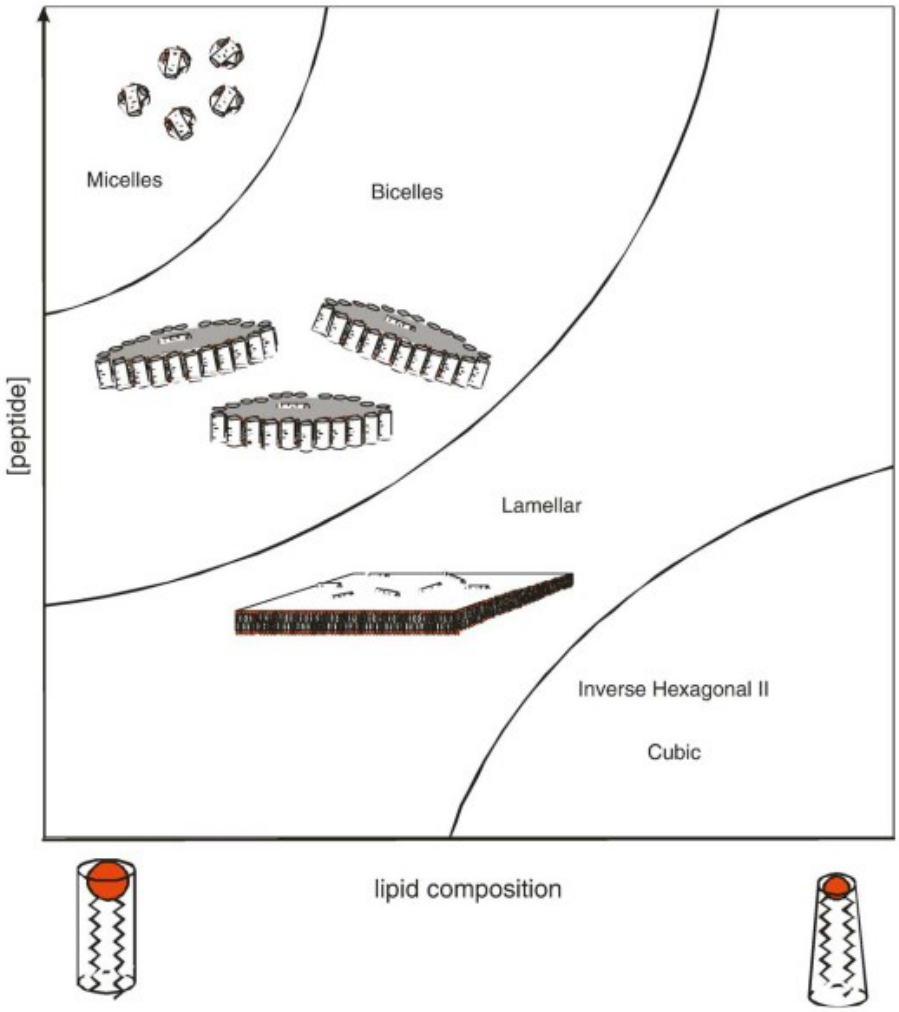


Figure 1-6: Diagramme de phase schématique d'un mélange de phospholipides et de CAP agissant selon le mécanisme en tapis. Différentes phases sont obtenues en fonction du rapport peptide/lipide. Les mélanges formés de lipides cylindriques (à gauche) mènent, à haute concentration en peptide, à la fragmentation des membranes en bicelles et micelles; l'inclusion de lipides coniques dans la bicouche (à droite) inhibe la fragmentation[4].

Il est intéressant de souligner que le modèle prédit que la fragmentation des membranes et la formation de petits auto-assemblages dépend de la composition

lipidique des membranes. Cette extraction se produit pour des bicouches formées de phospholipides cylindriques, comme la POPC (Figure 1-6, à gauche). Par contre, le modèle prévoit que la présence de phospholipides avec une plus petite tête polaire, comme la POPE (Figure 1-6, à droite), inhibe cette extraction lipidique en stabilisant la phase lamellaire. Plusieurs des expériences présentées aux chapitres 2 et 4, caractérisant l'interaction entre des liposomes de PC/PE et des PEL, ont permis d'étudier cet effet.

1.3.2 La mélittine comme peptide modèle

Le peptide mélittine partage les caractéristiques structurelles importantes des CAP présentés plus haut, notamment leur structure en hélice-α amphiphile, en plus de leur caractère cationique (pour une revue, voir [32]). Étant donné sa courte séquence et son faible coût, la mélittine est devenue, au cours des années, l'un des peptides les plus caractérisés et utilisés comme peptide modèle afin d'étudier les interactions peptide-lipide.

La mélittine est le principal agent actif du venin d'abeille. Cette toxine est connue de la médecine depuis des siècles, notamment pour son activité anti-inflammatoire [33]. Plus récemment, la mélittine attira l'attention de la science et de la médecine moderne par son activité anti-bactérienne et anti-virale [32, 34]. Le peptide pourrait également être utilisé comme agent anti-cancer puisque les cellules

cancéreuses présentent un certain nombre de molécules anioniques dans leur feuillet externe; elles seraient préférentiellement ciblées par rapport aux cellules de mammifères, dont le feuillet externe est neutre. Certains y voient donc un potentiel de chimiothérapie croisée, étant donné la capacité de la mélittine à former des pores dans les membranes [35-40]; les pores formés dans les membranes des cellules cancéreuses pourraient faciliter l'action d'un autre agent anti-cancer [33, 41]. De nombreux groupes ont démontré l'activité lytique de la mélittine, qui entraîne la libération du contenu des cellules ou des membranes modèles (pour des revues, voir [32, 42]).

La mélittine est composée de 26 acides aminés (GIGAVLKVLTTGLPALISWIKRKRQQ-NH2) et possède une charge nette de +6 au pH physiologique (les acides aminés cationiques sont soulignés). La région du N-terminal (acides aminés 1 à 20) est amphiphile, alors que sont concentrées 4 des 6 charges près de la région du C-terminal (21 à 26). Cette structure permet à la mélittine d'être très soluble dans l'eau tout en s'associant spontanément aux membranes lipidiques [32]. En solution et à basse concentration, la mélittine est un monomère en pelote statistique. À plus haute concentration, en présence d'une grande concentration en NaCl et/ou à haut pH, la mélittine s'agrège en tétramère sous forme d'une hélice-α (Figure 1-7) [32].

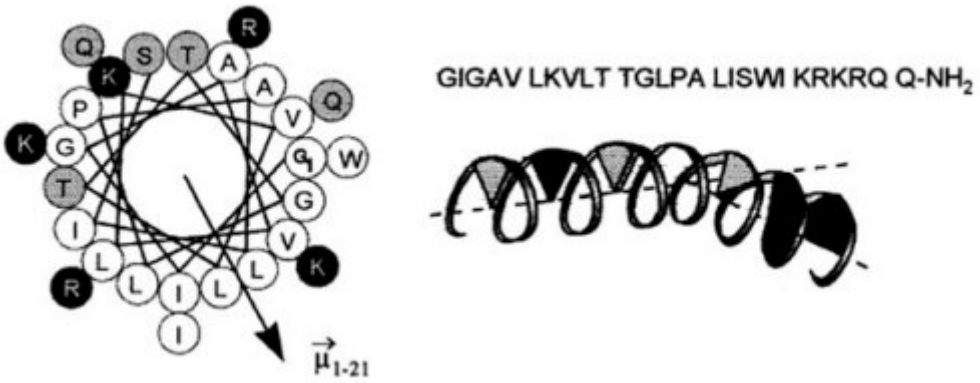


Figure 1-7: Représentation schématique de la structure en hélice-α de la mélittine. Les résidus hydrophobes sont indiqués en blanc, les résidus polaires en gris et les résidus cationiques en noir. Le moment hydrophobe pour le segment 1-21, quantifiant l'amphiphilicité de l'hélice [43], est indiqué par μ_{1-21} [32, 44].

La structure en hélice-α, comprenant les résidus 1 jusqu'à ~20[45, 46], est importante parce que c'est la forme qu'adopte la mélittine associée aux membranes lipidiques [45, 47, 48]. La présence d'une proline en position 14 tord l'hélice qui forme un angle d'environ 120°. Le caractère amphiphile de la mélittine repliée en hélice-α abaisse son énergie d'association à la membrane (Figure 1-8).

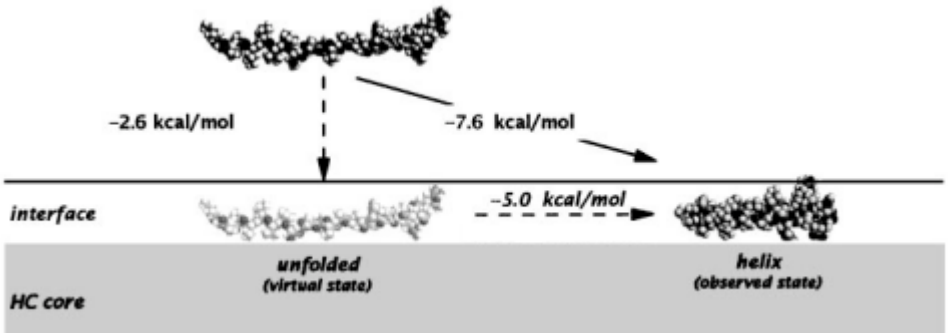


Figure 1-8: Schéma du repliement en hélice-α de la mélittine, qui accompagne sa liaison à l'interface d'une bicouche de POPC [49].

Le ΔG de liaison (ΔG_{bind}) de la mélittine à l'interface d'une membrane de POPC est d'environ -7,6 kcal/mol [49]; l'association est grandement stabilisée (de -5,0 kcal/mol) par le repliement de la mélittine en hélice-α amphiphile. Des simulations ont montré que la stabilisation du système bicouche-mélittine était maximale lorsque le centre de masse de l'hélice-α se trouvait enfoui à environ 0,5 Å de l'interface (Figure 1-9) [50].

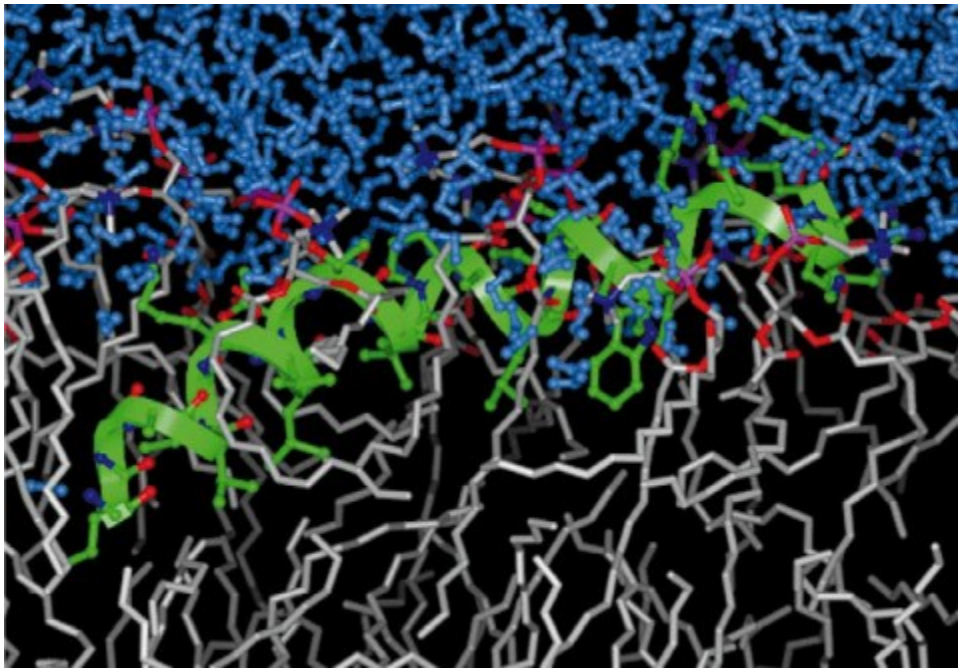


Figure 1-9: Simulation du système mélittine-1,2-dimyristoyl-*sn*-glycéro-3-phosphocholine (DMPC), montrant la position du peptide dans une membrane en phase fluide. La structure secondaire de la mélittine est représentée en vert, les molécules d'eau en bleu clair, les autres oxygènes en rouge, les azotes en bleu foncé, les phosphates en pourpre et les carbones en gris [50]. Les hydrogènes des chaînes alkyles sont omis pour clarté.

La position interfaciale permet de placer la face hydrophobe de l'hélice amphiphile dans la région hydrophobe de la membrane tout en gardant la face hydrophile exposée au milieu aqueux. Il est intéressant de souligner que cette simulation indique que le résidu tryptophane 19 se trouve près du groupement choline d'un phospholipide voisin. Un certain nombre d'études computationnelles et expérimentales se sont penchées sur les interactions entre tryptophanes et les têtes PC ou PE à la surface des membranes [51-56]; les résultats suggèrent qu'une interaction cation-π peut participer à l'association.

À la manière du mécanisme en tapis présenté précédemment pour les CAP, une concentration suffisante de la mélittine à l'interface des membranes induit leur fragmentation [5-7]. De plus, lorsque ces fragments sont refroidis sous la T_m des lipides desquels ils sont composés, ils prennent la forme de disques lipidiques. Pour des membranes de DPPC à température ambiante (T_m=41°C) par exemple, les fragments consistent en une bicouche de lipides dont la région hydrophobe est recouverte par des mélittines (Figure 1-10). Le diamètre de ces disques a été évalué à 25,3 nm et on y retrouve environ 20 lipides par mélittine [5].

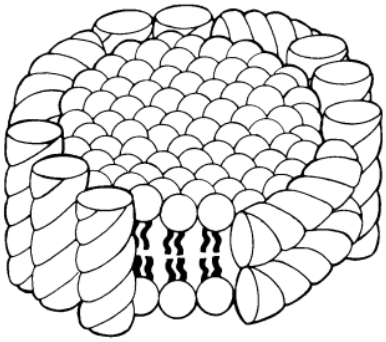


Figure 1-10: Schéma de la structure des disques formés lors de l'interaction entre la DPPC et la mélittine[5, 42]. L'orientation de la mélittine dans les disques n'est pas connue; les deux orientations sont donc possibles.

Il a été démontré que la composition lipidique des membranes module l'extraction lipidique et la formation des disques par la mélittine. D'abord, la longueur des chaînes alkyles est importante; si les chaînes alkyles formées de 16 et 18 carbones sont propices à la formation des disques, les lipides à chaînes de 20 carbones n'en forment pas [57]. Ces derniers forment une bicouche trop épaisse pour que la mélittine puisse bien couvrir la région hydrophobe de la bicouche. De plus, comme mentionné précédemment, les disques sont stables uniquement pour des températures sous la T_m des lipides. Ainsi, la fluidité des lipides joue un rôle dans la fragmentation par la mélittine. Il a d'ailleurs été démontré que la présence d'une quantité suffisante de cholestérol dans des membranes de DPPC inhibe la formation des petits objets [58], la structure volumineuse et rigide du cholestérol étant inadéquate à son insertion dans les disques. Ces travaux ont d'ailleurs démontré que l'extraction lipidique par la mélittine pouvait présenter une spécificité lipidique, puisque les

fragments produits à partir de membranes de DPPC/cholestérol se sont avérés appauvris en cholestérol par rapport aux membranes originales.

Une partie des travaux de cette thèse concerne l'effet de la composition lipidique sur l'extraction par la mélittine. Au chapitre 2, l'impact de la déméthylation du groupement ammonium des lipides à tête PC ($-N(CH_3)_3^+$) sur l'extraction lipidique est étudié. La déméthylation graduelle des groupements ammonium triméthylés (PC) en ammonium diméthylés ($-NH(CH_3)_2^+$), monométhylés ($-NH_2CH_3^+$) ou complètement déméthylés (PE, $-NH_3^+$) induit un empilement plus compact des chaînes lipidiques. Les conséquences de cet empilement plus serré sur l'extraction et la spécificité lipidique sont donc examinées.

Lorsque les CAP interagissent avec des membranes anioniques, d'importantes interactions électrostatiques entrent en jeu. Les travaux du chapitre 3 se concentrent sur le rôle de ces interactions électrostatiques entre le segment C-terminal fortement cationique de la mélittine et les membranes anioniques de DPPC/DPPS. Pour ce faire, l'activité d'un analogue de la mélittine, dont les 4 résidus chargés près du C-terminal (21-Lysine, 22-Arginine, 23-Lysine, 24-Arginine) ont été substitués par des résidus citrullines (Figure 1-11) neutres, a été comparée à l'activité de la mélittine native.

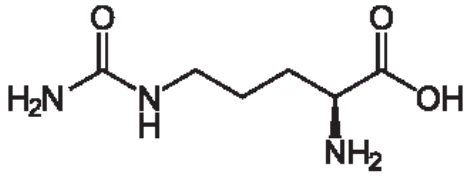


Figure 1-11: Structure de la citrulline.

Les extractions induites sur des liposomes de DPPC/DPPS par la mélittine citrullinée et la mélittine native sont comparées au chapitre 3, permettant de dégager l'impact des résidus cationiques du segment C-terminal sur le pouvoir de fragmentation de la mélittine.

1.4 Système BSP1 et spermatozoïdes

1.4.1 Capacitation du spermatozoïde

Le spermatozoïde est une cellule spécialisée dont le rôle est de livrer du matériel génétique à l'ovule. La tête du spermatozoïde contient le noyau de la cellule tandis que sa queue lui sert de moyen de locomotion, les deux étant liés par le corps.

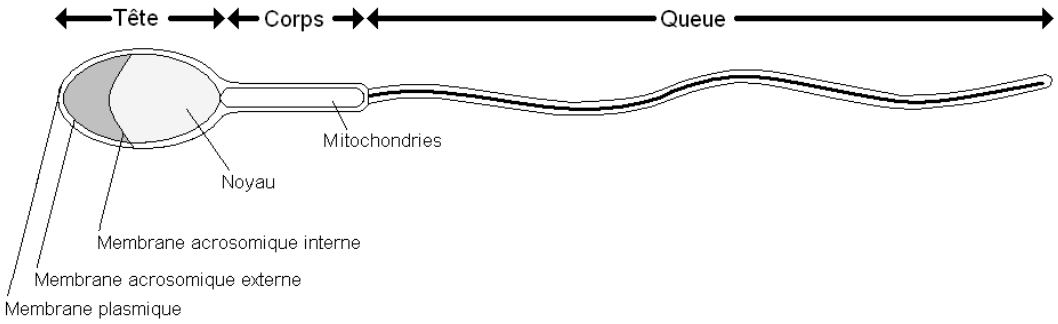


Figure 1-12: Structure schématique du spermatozoïde [59].

Au cours de la vie du spermatozoïde, la composition lipidique de sa membrane est finement ajustée par la nature. Un des facteurs optimisés est le taux de cholestérol contenu dans la membrane, qui rigidifie cette dernière. Un premier contrôle se produit dans l'organe reproducteur mâle, durant la maturation dans l'épididyme. Chez le bélier par exemple, le rapport stérol/phospholipide dans la membrane des spermatozoïdes augmente de 0,26 à 0,44 durant ce passage à travers l'épididyme [60]. La composition lipidique du spermatozoïde bovin à l'éjaculation est donnée au tableau suivant :

Tableau 1-1: Composition lipidique des membranes de spermatozoïdes bovins éjaculés, en mol par 100 phospholipides [24].

Lipide	Proportion (mol%)
Phosphatidylcholines	53
Phosphatidyléthanolamines	12
Sphingomyélines (SM)	9
Lysophosphatidylcholines	1
Phosphatidylsérines	1
Cholestérol	22

Après l'éjaculation, une dernière période de maturation pour le spermatozoïde s'amorce, celle de la capacitation. Il existe de nombreuses définitions de la capacitation. Dans cet ouvrage, elle est définie comme l'ensemble des processus de maturation se produisant après l'éjaculation et qui permettent la réaction d'acrosome, essentielle pour la fertilisation ultérieure de l'oeuf. Les transformations amenées par la capacitation incluent des altérations aux propriétés de la membrane, telles que la composition en protéines membranaires, le potentiel de la membrane, la composition et la répartition asymétrique des lipides dans la bicouche et le pH intracellulaire [61]. La diminution du rapport cholestérol/phospholipide contenu dans la membrane plasmique du spermatozoïde est l'élément de la capacitation le plus important pour les

présents travaux. Cette fluidification de la membrane serait due à l'activité du second sujet de cette thèse, la protéine BSP1 [62-64].

1.4.2 BSP1

La protéine BSP1 (aussi nommée BSP-A1 et PDC-109) fait partie d'un groupe de 3 protéines BSP nommées d'après leur rôle (*Binder of Sperm Protein*) : BSP1 et BSP3, possédant une masse d'environ 13 kDa, et BSP5 avec une masse de 30 kDa [11, 65]. La BSP1 est abondante chez le bovin, représentant jusqu'à 25% de la masse totale des protéines du plasma séminal [8, 9]. Lors de l'éjaculation, les protéines BSP se mélangent et se lient aux spermatozoïdes (Figure 1-13) [10] :

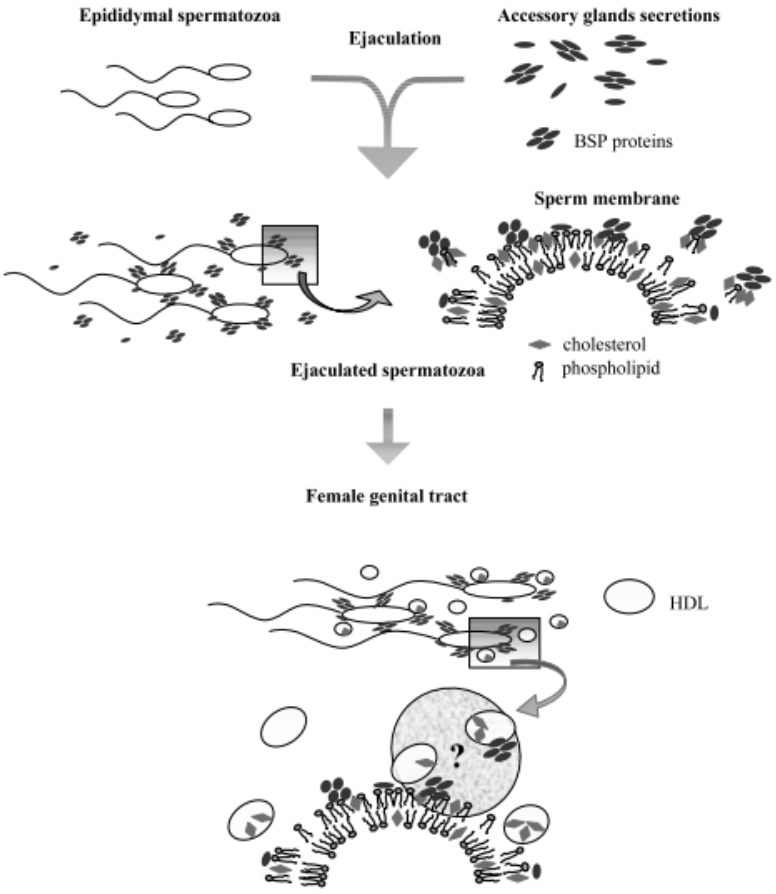


Figure 1-13: Mécanisme de la capacitation des spermatozoïdes bovins par les BSP [11].

Ensuite, selon un mécanisme plus ou moins compris, les BSP induisent un efflux de cholestérol et de phospholipides de la membrane plasmique des spermatozoïdes [11-13]. Cet efflux engendrerait une réorganisation des domaines lipidiques de la membrane qui serait préalable à la réaction d'acrosome se produisant plus tard durant la capacitation [11, 62, 63, 66]. L'efflux serait composé essentiellement de PC, de SM et de cholestérol [12, 13, 67, 68] et fluidifierait la membrane en diminuant le

rapport stérol/phospholipide. Une fois dans l'appareil génital féminin, les BSP seraient complexées par les HDL présentes dans ce nouvel environnement, inhibant leur activité membranaire.

L'association des BSP1 aux membranes de spermatozoïdes est due à une interaction spécifique envers les phospholipides à tête PC. Cette proposition se reflète dans la structure de la BSP1, qui contient deux domaines s'apparentant aux domaines de type II de la fibronectine (FnII) [69]. Ces domaines sont caractérisés par une chaîne d'environ 50-60 acides aminés comprenant 4 cystéines impliquées dans des ponts disulfures [70]. Ces deux domaines forment la presque totalité de la BSP1 (Figure 1-14).

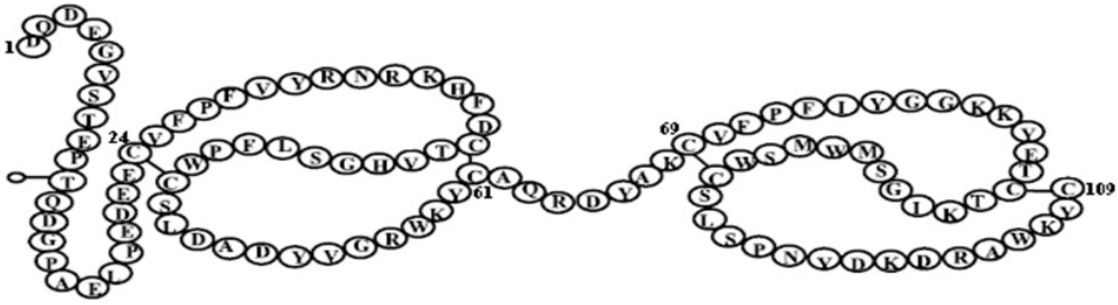


Figure 1-14: Structure primaire de la protéine BSP1 [11].

Les domaines de type FnII sont connus pour permettre à la fibronectine de lier une gamme de substrats incluant le collagène [71], l'ADN et les surfaces des membranes. La BSP1 profite de ces domaines pour se lier spécifiquement aux têtes

PC des lipides des membranes des spermatozoïdes [72-74], via, entre autres, des interactions cation-π et ponts H [69, 75].

1.4.3 Impact pour l'industrie bovine

Le Canada est un important exportateur de semence bovine. En 2014, les exportations représentaient 118 M$, ayant presque que doublées en 5 ans [14]. Un obstacle à l'exportation de semences est la dégradation des spermatozoïdes. Une exposition trop longue au liquide séminal et à la BSP1 endommage les spermatozoïdes, potentiellement dû à la fragmentation des membranes; cette dégradation nuit à leur fertilité. La préservation des semences par l'ajout d'agents protecteurs, nommés diluants, est donc cruciale à cette industrie. Ces diluants sont souvent préparés à base de produits animaliers, ce qui entraîne nécessairement des risques de contaminations. Le mécanisme de préservation associé à ces produits étant toujours méconnu, il est difficile de leur trouver des alternatives. Une meilleure compréhension du phénomène d'efflux lipidique engendré par la BSP1, pourrait par exemple permettre le développement de nouveaux diluants.

Les travaux du chapitre 4 portent sur la fragmentation des liposomes par la BSP1. Différents mélanges lipidiques sont utilisés afin de cerner les conditions menant à l'extraction et à la spécificité lipidique.

1.5 Méthodologie

La section suivante amène quelques précisions sur la méthodologie employée pour les expériences d'association par fluorescence et pour les expériences d'extraction lipidique.

1.5.1 Association aux LUV

L'association PEL-lipides est caractérisée par la fluorescence intrinsèque des PEL, due à la présence d'acides aminés aromatiques dans leur structure respective. Mélittine possède un tryptophane en position 19; BSP1 possède plusieurs tryptophanes, tyrosines et phenylalanines. La position du maximum dans le spectre de fluorescence est sensible à la polarité de l'environnement de ces acides aminés. Ainsi, le transfert d'un PEL du milieu aqueux vers le cœur hydrophobe d'une membrane entraîne un déplacement hypsochromique du maximum de fluorescence.

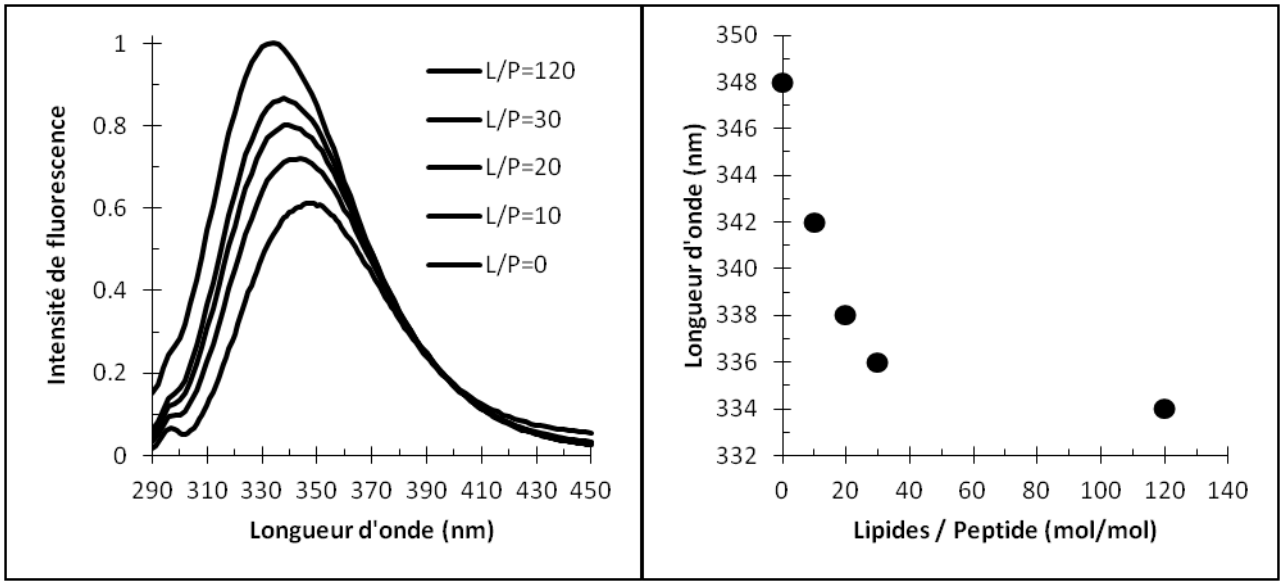


Figure 1-15: À gauche: Déplacement hypsochromique du maximum de la bande de fluorescence de la mélittine, caractéristique du transfert du tryptophane vers un milieu hydrophobe. À droite : Courbe de titrage obtenue après additions successives de lipides à une solution de mélittine. L/P = lipides/peptide (mol/mol).

L'utilisation de liposomes sous forme de LUV plutôt que de MLV est préférable pour la caractérisation de l'association pour deux raisons. Premièrement, pour les peptides/protéines qui ne traversent pas le cœur hydrophobe des liposomes et qui s'associent à leur interface, seuls les lipides du feuillet externe des bicouches sont accessibles. Ainsi, l'unilamellarité des LUV est essentielle aux calculs puisqu'on peut alors estimer la concentration de lipides exposés au milieu comme étant la moitié des lipides totaux; cette approximation n'est pas possible avec les MLV. Deuxièmement, les liposomes diffusent la lumière, ce qui peut induire des artéfacts dans le spectre de fluorescence; l'utilisation de LUV de plus petite taille réduit grandement cet effet.

1.5.2 Protocole d'extraction lipidique/fragmentation

Le principe des expériences d'extraction est résumé à la Figure 1-16 :

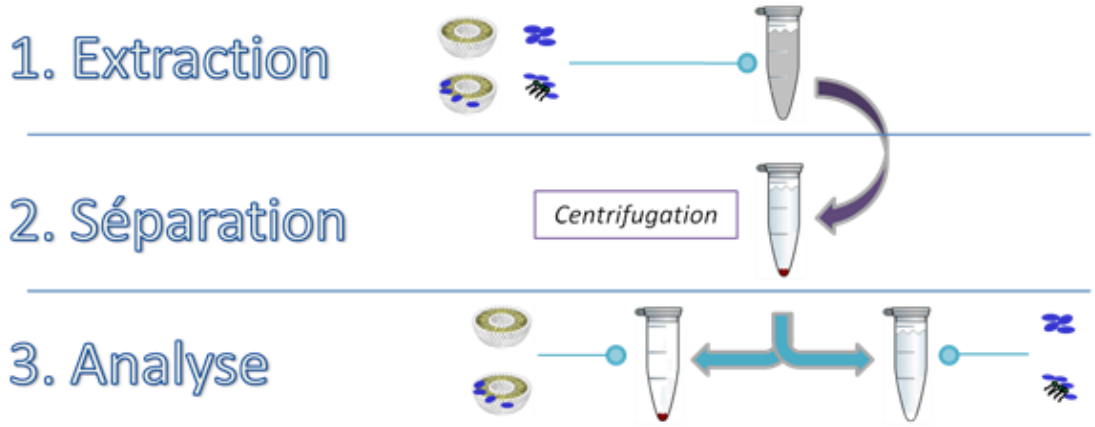


Figure 1-16: Protocole d'extraction en 3 étapes.

1) Les MLV sont tout d'abord incubées avec la BSP1 ou la mélittine au-delà de leur température de transition gel-fluide. Dans le cas de la BSP1, l'incubation est accompagnée de 3 cycles gel-dégel afin de permettre la pleine insertion de la protéine, qui semble autrement incapable de translocation entre les feuillets lipidiques. Cette étape reproduit aussi les conditions de congélation dans lesquels les spermatozoïdes sont conservés. Les échantillons sont ensuite refroidis sous la température de transition fluide-gel des MLV.

2) À cette étape, les lipides sont sous forme de MLV ou de fragments; ces derniers correspondent à de plus petits auto-assemblages, soit des particules PEL-lipides ou de plus petites vésicules. Les protéines/peptides se retrouvent liés aux MLV, dans des petits fragments protéines/peptides-lipides, ou encore sous une forme libre dans le milieu aqueux. Afin de séparer les espèces, les échantillons sont

centrifugés. On considère que tout le matériel lipidique se retrouvant dans le surnageant après centrifugation des MLV correspond aux lipides extraits/fragments. Les surnageants sont retirés et les culots resuspendus.

3) La teneur en lipides ainsi que la composition du surnageant et du culot sont ensuite quantifiés par analyse colorimétrique du phosphore et par chromatographie liquide couplée à la spectrométrie de masse (LCMS) afin de déterminer la proportion de lipides extraits et la sélectivité lipidique de cette extraction.

1.6 Plan de la thèse

Le chapitre 2 présente l'étude de l'impact de la déméthylation du groupement ammonium des PC sur l'association de la mélittine et sur l'extraction et la spécificité lipidiques. Le degré de méthylation est varié en formant des MLV avec des mélanges PC/PE de différentes proportions ou en utilisant de la PE mono- ou di-méthylée. Ces travaux permettent d'approfondir notre compréhension du mécanisme de l'insertion de la mélittine dans les membranes, en étudiant de près l'impact de l'empilement des lipides sur l'association et l'extraction.

Le chapitre 3 décrit l'étude du rôle des interactions électrostatiques sur le pouvoir de fragmentation des membranes anioniques par la mélittine. La mélittine, ainsi qu'un analogue dont les résidus cationiques 21 à 24 sont substitués par des citrullines neutres, interagissent avec des MLV de DPPC/DPPS en différentes

proportions. L'association des deux peptides ainsi que l'extraction qu'ils induisent sont comparées, ce qui permet d'établir l'impact des résidus cationiques du C-terminal de la mélittine sur la fragmentation des membranes anioniques, comme celles des bactéries.

Le chapitre 4 présente l'étude de la fragmentation lipidique de MLV de différentes compositions par la BSP1. L'effet de la fluidité des phases, L_α (ou L_d), L_β ou L_o, sur l'interaction avec la BSP1 y est détaillé. L'interaction de la protéine avec des membranes contenant la lyso-PC ou la POPE est aussi caractérisée afin d'étudier l'impact de l'empilement des chaînes. Finalement, le rôle des têtes polaires des lipides est évalué par la préparation de MLV de POPE (mettant en relief l'impact de la déméthylation du groupement ammonium) et de 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-sérine (POPS) (introduisant une charge négative à l'interface membranaire). Ces travaux ont permis de dégager les conditions chimiques et physiques menant à l'extraction lipidique par la BSP1; un mécanisme d'extraction en 3 étapes est proposé.

Le chapitre 5 conclut la thèse en faisant d'abord un bref résumé des manipulations qui ont été effectuées ainsi que des observations générales qui en ont été tirées. L'importance du motif en hélice-α chez les peptides et protéines extracteurs de lipides est discutée. La prévalence de la formation de bicelles de structures similaires chez différents PEL est ensuite abordée. L'importance des interactions

hydrophobes et des interactions électrostatiques pour la reconnaissance des membranes de procaryotes par les peptides antibactériens est aussi discutée. Finalement, les conclusions générales de la thèse sont présentées.

1.7 Situation des articles à la date du dépot

Chapitre 2: Therrien, A., Lafleur, M., *Melittin-Induced Lipid Extraction Modulated by the Methylation Level of Phosphatidylcholine Headgroups*. Biophysical Journal, **2015**. 110, 400-410.

Les expériences ont été effectuées par Alexandre Therrien. La rédaction a été effectuée par Alexandre Therrien et Michel Lafleur.

Chapitre 3: Therrien, A., Fournier, A., Lafleur, M., *Role of the Cationic C-Terminal Segment of Melittin on Membrane Fragmentation*. Journal of Physical Chemistry B, 2015. **Soumis.**

Alain Fournier a synthétisé l'analogue citrulliné de la mélittine et a rédigé la partie correspondante de la section matériel et méthodes. Le reste des expériences ont été effectuées par Alexandre Therrien. La rédaction a été effectuée par Alexandre Therrien et Michel Lafleur.

Chapitre 4: Therrien, A., Manjunath, P., Lafleur, M., *Chemical and Physical Requirements for Lipid Extraction by Bovine Binder of Sperm BSP1*. Biochimica et Biophysica Acta-Biomembranes, 2013. **1828, 543-551.**

Les expériences ont été effectuées par Alexandre Therrien. La protéine BSP1 a été purifiée dans le laboratoire de Puttaswamy Manjunath par Alexandre Therrien. La rédaction a été effectuée par Alexandre Therrien et Michel Lafleur.

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Chapitre 2 - Melittin-Induced Lipid Extraction Modulated by the Methylation Level of Phosphatidylcholine Headgroups

Alexandre Therrien, Michel Lafleur, *Biophysical Journal*. **2015,** 110, 400-410.

2.1 Abstract

The protein- and peptide-induced lipid extraction from membranes is a critical process for many biological events, including the reverse cholesterol transport and the sperm capacitation. In the present paper, we examine whether such processes could display specificity for some lipid species. Melittin, the main component of dry bee venom, was used as a model amphipathic α-helical peptide. We determined specifically the modulation of melittin-induced lipid extraction from membranes by the change of the methylation level of phospholipid headgroups. Phosphatidylcholine (PC) bilayers were demethylated, either by substitution with phosphatidylethanolamine (PE), or chemically, by using mono, and dimethylated PE. It is shown that demethylation reduces the melittin association to membranes, likely because of the resulting tighter chain packing of the phospholipids, reducing the capacity of membranes to accommodate inserted melittin. This reduced binding of the peptide is accompanied by an inhibition of the lipid extraction caused by melittin. We

demonstrated that melittin extracts selectively PC from PC/PE membranes; this selectivity is proposed to be a consequence of a PE depletion in the surrounding of bound melittin, in order to minimize the disruption of the interphospholipid interactions. The resulting PC-enriched vicinity of melittin would be responsible of the observed formation of PC-enriched lipid/peptide particles resulting from the lipid efflux. These findings reveal that the methylation level of phospholipid headgroups is a simple way to control the specificity of lipid extraction from membranes by peptides/proteins and, therefore, to modulate the lipid composition of membranes.

2.2 Introduction

The protein- and peptide-induced lipid extraction from membranes is a critical process for many biological events. For example, Binder-of-Sperm Protein BSP1, the most abundant protein of the bovine seminal plasma, extracts lipids, more specifically phosphatidylcholines (PC) and cholesterol (Chol), from sperm membranes; this phenomenon is an essential step of the sperm capacitation [1-3]. Apolipoproteins, such as ApoA1, are another species involved in lipid extraction. They extract cholesterol and phospholipids from peripheral tissues to form high-density lipoproteins that are carried towards the liver for catabolism [4]. This phenomenon is pivotal in the reverse cholesterol transport and consequently is intimately related to the risk of coronary heart diseases. The mechanism by which lipids are extracted from cells encompasses a complex series of reactions involving several molecular species [5], but at the end of the process, suitable lipid selectivity must be achieved. These examples illustrate the

complexity of membrane lipid extraction processes. In spite of their importance, the general molecular interactions governing their mechanism and more specifically the basis of their lipid selectivity are still largely unknown.

The present work was carried out to gain insights into membrane lipid extraction processes by studying the lipid efflux induced by melittin from model membranes. Melittin, the main component of dry bee venom [6], is a 26 amino acid peptide that has been widely used as a model peptide for various purposes over the last decades (for a general review, see [7]). Melittin, like ApoA1, acts as a helical amphiphile that can solubilize lipids; this property is due to its amphipathic character coupled with a highly charged C-terminal region (Lys-Arg-Lys-Arg). Melittin also displays an appealing therapeutic potential and reviews discuss its antimicrobial properties [8, 9], its potential for cancer therapy [10, 11], for treatment [12] and prevention of HIV [13], and for treatment of parasitical infections [14], to name a few.

Melittin interacts spontaneously with phospholipid membranes. At low concentrations, it binds to membranes as an amphipathic α-helix [15-17], induces pore formation and causes leakage [18-23]. At higher concentrations, melittin leads to lipid extraction by forming soluble small lipid/peptide bicelles or nanodiscs [24-26]. Melittin-induced lipid extraction was shown to be modulated by the addition of cholesterol [27, 28], phosphatidylethanolamine (PE) [29], negatively charged [15, 30, 31] or positively charged [31] phospholipids in membranes. While these non-PC lipids have been

reported to inhibit the formation of melittin/lipid bicelles, the cause of this inhibition is different for the different lipid species. On one hand, cholesterol, PE, and positively charged phospholipids were reported to simply prevent the association of melittin with bilayers. On the other hand, negatively charged phospholipids enhanced the affinity of melittin to bilayers but anchored the peptide at the bilayer interface, preventing the peptide relocation required for inducing lipid extraction. It is solidly established that melittin-induced lipid extraction is modulated by the bilayer composition but very little is known about the lipid specificity of the extraction. A previous study showed that the particles resulting from melittin-induced extraction from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/Chol bilayers were depleted in cholesterol compared to the sterol content of the original bilayers [27]. This result suggests that melittin does not merely extract membrane lipids as a universal detergent.

The main goal of the present work was to determine the effect of PE headgroups on the lipid extraction induced by melittin and more specifically on its lipid selectivity. Understanding the impact of the methylation of the headgroup, by comparing PE and PC, on the peptide-induced lipid extraction would aid in defining the molecular mechanism of this process. It was already shown that melittin has a reduced affinity for pure 1,2-dielaidoyl-*sn*-glycero-3-phosphoethanolamine (DEPE) bilayers [29]. Moreover, the negative curvature of PE was proposed to inhibit the formation of positively curved toroidal pores by melittin, and therefore to limit the

peptide-induced leakage [32-36]. In order to examine the influence of the methylation level of the phospholipid headgroup on melittin-induced lipid extraction, we characterized the melittin binding to model membranes and the resulting lipid efflux. Model membranes were prepared from DPPC/1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/ 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) mixtures in various proportions, as well as from mono- and di-methyl DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-methyl (DPMePE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N,N-dimethyl ($DPMe_2PE$), respectively). These two series of measurements provide two different ways to modulate the methylation level of the ammonium groups at the bilayer interface. Fluorescence measurements of the tryptophan at the position 19 of melittin were conducted to investigate the melittin binding to bilayers. The extent of the lipid extraction by melittin was analysed using a procedure analogous to the one reported previously [37]. The composition of the lipid/peptide particles resulting from the extraction, and of the remaining bilayers was examined using liquid chromatography coupled with mass spectrometry (LC-MS), in order to reveal any selectivity towards one lipid or another.

2.3 Materials and Methods

2.3.1 Chemicals

Melittin was purified from bee venom (Sigma, St. Louis, Missouri) by ion exchange chromatography on SP-Sephadex C-25 [38]. POPC, POPE, DPPC, DPPE, $DPMe_2PE$ and DPMePE were purchased from Avanti Polar Lipids (Alabaster, Alabama). Ethylenediaminetetraacetic acid (EDTA), NaCl, and 3-[N-morpholino]propanesulfonic acid (MOPS) were obtained from Sigma (St. Louis, Missouri). All chemicals were used as received.

2.3.2 Lipid membrane preparation

First dissolved in a benzene/methanol mixture (90/10 (v/v)), individual lipids were mixed to obtain the desired molar ratio and then lyophilized. The lipid powders were hydrated in a MOPS buffer (50 mM) containing 100 mM NaCl and 100 μM EDTA, pH = 7.4. The samples were submitted to 3 freeze-and-thaw cycles (from the liquid nitrogen temperature to 65 °C) to form multilamellar vesicles (MLVs) used for the extraction experiments. For the fluorescence experiments, the MLVs were extruded at 75 °C using a manual extruder (Northern Lipids, Vancouver, Canada) to obtain 100-nm large unilamellar vesicles (LUVs).

2.3.3 Extraction

Melittin was dissolved in the MOPS buffer and its concentration was determined by its absorbance at 280 nm, using a molar absorptivity coefficient of 5570 M^{-1} cm^{-1}[39]. The lipids and the peptide were mixed in microcentrifuge tubes to obtain the desired molar lipid-to-melittin incubation ratio (L/M), and a fixed phospholipid concentration of 1 mg/ml. The suspensions were then incubated at 70 °C for at least 30 minutes. After the incubation, the samples were centrifuged for 5 minutes at 20 800 g and 1 °C. The centrifugation of control samples (without melittin) showed that more than 95% of lipids were found in the pellets for all the mixtures. The supernatants were isolated and the pellets were resuspended in the MOPS buffer for their analysis. The experiments were carried out in triplicates unless stated otherwise.

2.3.4 Lipid Analysis

The phospholipid content in the supernatants and the pellets was determined by Bartlett's phosphorus assay [40]. The extent of extraction was calculated as

$$Extraction\ \% = \frac{Phospholipid\ content\ in\ supernatant}{Total\ phospholipids}$$ (Eq. 1).

The lipid composition of the supernatants and of the pellets was determined by LC-MS analysis carried out using an Agilent Technologies 1100 series system equipped with a 1100 MSD mass spectrometer. Samples were eluted on a YMC diol

column (4.6 x 150 mm, 5-µm particle size) (Agilent Technologies), maintained at 50 °C. Elution of the phospholipids was achieved over 10 minutes, using an acetonitrile/water (100 mM ammonium acetate) 91/9 mixture at a 0.6-ml/min flow rate. An electrospray ionisation source was used in the positive ionization mode. Nitrogen was used as drying gas at 250 °C and 12 l/min. The nebulizing gas was also nitrogen, held at 241 kPa. The analysis were conducted in the Single Ion Monitoring mode with a dwell time of 290 ms. The extent of extraction was determined for each lipid species, using :

$$Extraction\,\% = \frac{A_s}{A_s + A_p} \quad \text{(Eq. 2)},$$

where A_s and A_p are the lipid peak area from the supernatant and the pellet analysis, respectively.

2.3.5 Fluorescence measurements

The fluorescence spectra were recorded using a Photon Technology International fluorometer, with band widths of 1.0 and 2.0 nm for the excitation and emission monochromator, respectively. The excitation wavelength was set at 283 nm. In order to examine the melittin association to lipid bilayers as a function of the temperature, melittin (0.9 µM) was first mixed with LUVs (L/M = 400) in a sample cell kept at 5 °C. The fluorescence spectra were then acquired at different temperatures, from 5 °C to 90 °C, then back to 5 °C. For each temperature, a 10-

minute equilibration period was introduced prior to the data acquisition. For the binding studies, melittin was first added to a cell, and aliquots of a lipid suspension were added to vary L/M from 0 to 400. After each lipid addition, the fluorescence spectrum of melittin was acquired at 65 °C, then at 20 °C. An equilibration period of at least 20 minutes after the desired temperature was reached was introduced prior to the data acquisition. For the analysis of the supernatants and the pellets resulting from the extraction experiments, the samples were directly transferred to a cell and measured at 20 °C – the pellets were previously resuspended in the MOPS buffer.

2.4 Results

2.4.1 Association to LUVs

The hypsochromic shift of the tryptophan fluorescence that is observed when this amino acid is transferred from water to a more hydrophobic environment is a useful tool to characterize the peptide association to membranes [41]. The binding of melittin to membranes made of DPPC/DPPE was characterized as a function of temperature by plotting the evolution of the tryptophan fluorescence band maximum (λ_{max}) (Figure 2-1).

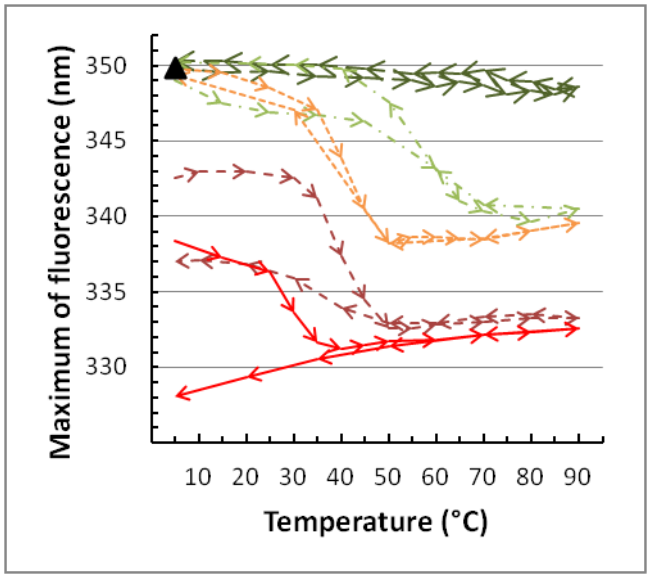

Figure 2-1: Evolution of the tryptophan fluorescence as a function of temperature for melittin in the presence (L/M=400) of DPPE (–→), DPPC/DPPE 25/75 (-·→), DPPC/DPPE 50/50 (---→), DPPC/DPPE 75/25 (- -→) or DPPC (⟶) LUVs, from 5 °C to 90 °C and then back to 5 °C. ▲was obtained for melittin in solution.

The λ_{max} of melittin in the presence of DPPC or DPPC/DPPE 75/25 LUVs, at 5 °C, was observed at 337 and 343 nm, respectively. This hypsochromic shift, compared to 349 nm obtained for free melittin, suggested a lipid-melittin association with these gel-phase membranes. No association was observed with either DPPC/DPPE 50/50, 25/75 or DPPE membranes in the gel phase, as λ_{max} was observed at 349 nm.

The heating of the membranes resulted in a hypsochromic shift for all the DPPC-containing mixtures, indicating an insertion or a deeper insertion of the peptide in the bilayer. For DPPC, this shift occurred between 25 and 35 °C, with λ_{max} decreasing to 331 nm, a value typical for completely membrane-bound melittin. It should be noted that 35 °C corresponds to the pre-transition temperature of DPPC bilayers; it has been shown that melittin added to gel-phase DPPC penetrated the membranes and formed bicelles at the pre-transition temperature [27, 42]. An analogous fluorescence shift was observed for the DPPC/DPPE mixtures. The temperature at which the shift was observed depended on the composition: it was between 30 and 50 °C for DPPC/DPPE 75/25 and 50/50 mixtures whereas it occurred between 45 and 70 °C for DPPC/DPPE 25/75 bilayers. For these 3 mixtures, the hypsochromic shift was thus progressing over a larger temperature span than for DPPC, beginning a few degrees below the apparition of the P_β or L_α phase and ending approximately at a temperature where the lipid bilayers were exclusively in the L_α phase, as inferred from DPPC/DPPE phase diagram [43]; the L_β-to-L_α phase transition was observed over the temperature range of 35-50 °C, 42-57 °C, and 49-62 °C for the DPPC/DPPE (75/25), (50/50), and (25/75) mixtures, respectively. λ_{max} measured at high temperatures was also sensitive to the bilayer composition. It was 333 nm for DPPC/DPPE 75/25 bilayers, 338 nm for the equimolar mixture, and 341 nm for DPPC/DPPE 25/75 bilayers. These results suggested that the DPPE content of the bilayers limits the depth of penetration of melittin and/or limits the proportion of membrane-bound melittin. A free-melittin fluorescence maximum was observed at all

temperatures in the case of pure DPPE membranes (T_m=64 °C), suggesting essentially no insertion of the peptide in these membranes.

The reversibility of the melittin association upon cooling was assessed for the different lipid mixtures. First, λ_{max} decreased to 328 nm upon cooling DPPC membranes to 5 °C, indicating a strong, irreversible association to the gel-phase membrane. For DPPC/DPPE 75/25 bilayers, λ_{max} increased back to about 337 nm upon cooling to 20 °C. This is an indication that a certain proportion of melittin relocated once the bilayers were cooled to the gel phase, either dissociating completely from the membrane or, at least, reorienting the tryptophan towards a more polar environment. For DPPC/DPPE 50/50 and 25/75, the fluorescence shift as a function of lipid phase was practically fully reversible, λ_{max} corresponding to that of free melittin at low temperatures. It is inferred that these mixtures gave rise to a complete dissociation of melittin upon cooling to the gel phase.

In order to further describe the thermal partial reversibility of melittin association to DPPC/DPPE 75/25 membranes, the melittin binding to these membranes was characterized as a function of the lipid/melittin ratio at 20 and at 65 °C; this behavior was compared to that of pure DPPC (Figure 2-2).

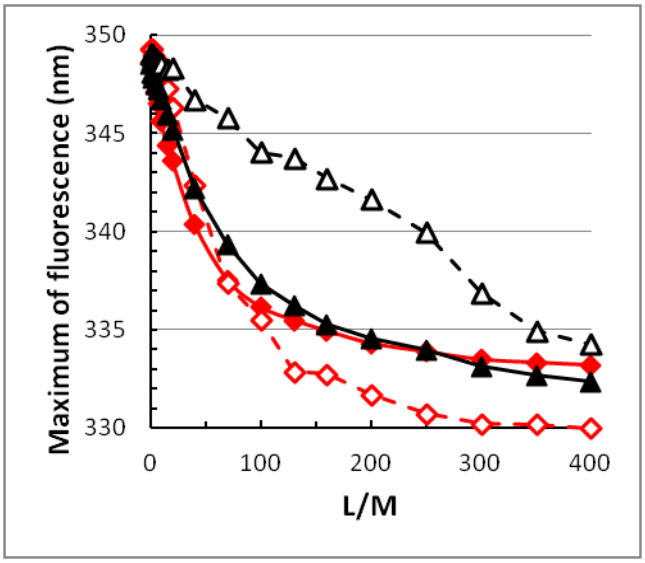

Figure 2-2: Evolution of the tryptophan fluorescence, characteristic of the melittin association with DPPC (diamonds) or DPPC/PE 75/25 (triangles) bilayers, during the incubation at 65 °C (full symbols) and the subsequent cooling to 20 °C (empty symbols) after each addition of lipids.

A melittin solution was titrated with LUVs. After each addition of LUVs, λ_{max} was measured at 65 °C, and then measured again after cooling to 20 °C in order to portray the reversibility of the peptide association. For DPPC bilayers, the shift of λ_{max} obtained as a function of L/M was very similar in the gel and in the fluid phase, the overall shift being slightly larger for the fluid DPPC bilayers. These affinity curves were similar those previously reported [44]. The peptide association to the bilayers was maintained in the gel phase. The association curves obtained for melittin with DPPC/DPPE 75/25 bilayers displayed a different behavior. The shift of λ_{max} observed upon the addition of fluid-phase bilayers was very similar to that obtained with pure DPPC bilayers, indicating a similar melittin/bilayer association. However,

in contrast to DPPC behavior, the fluorescence band was shifted back to higher wavelength upon cooling the bilayers to the gel phase. This finding suggests that the addition of 25% DPPE to DPPC membranes had no significant effect on the peptide insertion in fluid-phase bilayers, but induced the peptide dissociation from gel-phase bilayers upon cooling.

The variation of melittin λ_{max} was also determined in the presence of membranes of $DPMe_2PE$ (T_m=48 °C) and DPMePE (T_m=58 °C) [45] (Figure 2-3), an alternative approach to vary the methylation level of the headgroups.

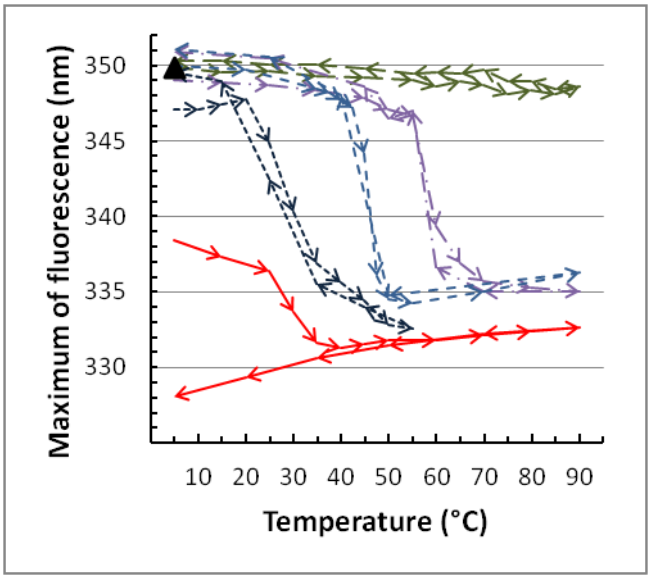

Figure 2-3: Evolution of tryptophan fluorescence as a function of temperature for melittin in the presence (L/M=400) of DPPE (– –), DPMePE (— —), DPMe$_2$PE (– –), POPE (- - -) or DPPC (——) LUVs from 5 °C to 90 °C and then back to 5 °C. ▲was obtained for melittin in solution.

In addition, the melittin association to POPE LUVs (T_m=25 °C) was determined to assess melittin affinity for PE membranes that are less ordered than DPPE LUVs. For the 3 systems, a λ_{max} typical of free melittin was observed at temperatures where the bilayers were in the gel phase. Upon heating, λ_{max} shifted to 330-335 nm, indicating the association of the peptide with the bilayers. The shift was observed between 20 - 50 °C, 43 - 50 °C, and 55 -70 °C for POPE, DPMe$_2$PE, and DPMePE membranes, respectively. It appeared that the peptide insertion was initiated a few degrees prior to the gel-to-fluid phase transition of the phospholipids. Cooling down the membranes resulted in the recovery of free-melittin λ_{max} roughly over the same temperature

intervals, indicating the full reversibility of the melittin association to these membranes.

2.4.2 Lipid extraction by melittin

The influence of the substitution of PC by PE on the lipid extraction was determined using DPPC/DPPE and POPC/POPE membranes. MLVs and melittin (L/M=20) were first incubated above the membrane transition temperature, and then cooled down to 1 °C for centrifugation. It is hypothesized that the remaining MLVs (with bound melittin, if present) would pellet whereas the extracted lipids, forming small self-assemblies with melittin, would remain in suspension. The total lipid extraction was quantified for mixtures with various PE contents (Figure 2-4A).

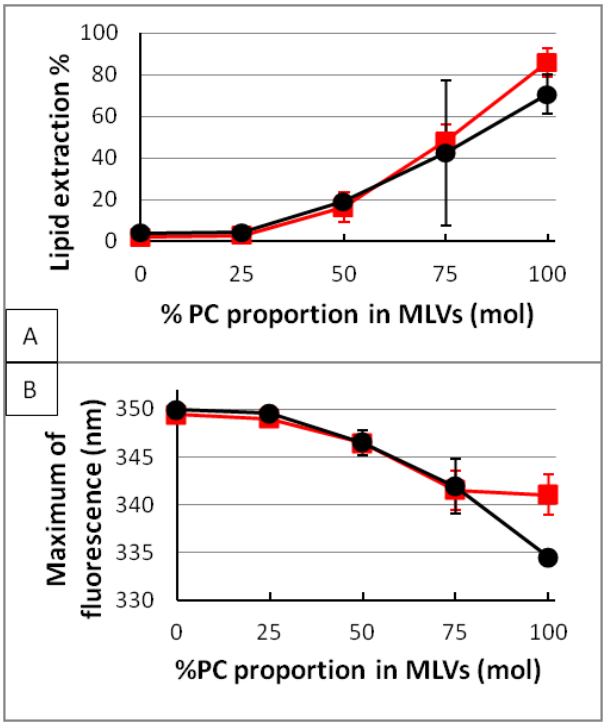

Figure 2-4: Lipid extraction (A) and wavelength of maximum fluorescence in supernatants at 20 °C (B) after the incubation (L/M=20) of melittin with MLVs of DPPC/DPPE (squares) or POPC/POPE (circles).

Melittin extracted 86% of the lipids from pure DPPC membranes; this level is consistent with previous NMR results that showed a similar proportion of lipids involved in an isotropic phase (i.e. bicelles) [31]. Melittin-induced lipid extraction with pure POPC bilayers yielded almost the same proportions, as it was observed with egg-PC using quasi-elastic light scattering [24]. The inclusion of PE in PC membranes inhibited melittin-induced lipid extraction. For both DPPC and POPC, the lipid extraction was reduced by about 50% for membranes with 25% PE compared to those of pure PC. Membranes with more PE offered a greater resistance to melittin-induced extraction, and no lipid could be detected in the supernatant after the incubation of the

membranes with 75% or more PE, for a L/M of 20. This finding is consistent with the previous study indicating that melittin had no impact on the ^{31}P NMR spectrum of gel-phase DEPE at L/M = 20 [29]; our results indicate that the inhibitory effect of PE is progressive and is also observed with DPPE as well as POPE.

The state of melittin in the supernatant after incubation with lipid bilayers and centrifugation was characterized by the position of its fluorescence maximum (Figure 2-4B). These values were consistent with the reported lipid extraction extent (Figure 2-4A). For DPPC and POPC bilayers, the fluorescence maximum appeared between 335 and 341 nm; these values were shifted relative to that observed for free melittin (350 nm). This hypsochromic shift was associated with the contribution of melittin included in the small lipidic complexes where its tryptophan should be in an apolar environment. It should be noted that, at a L/M of 20, there was a considerable proportion of free melittin coexisting with its bound form – see Figure 2-1. Conversely, the fluorescence band of melittin in the supernatant after incubation with DPPE or POPE bilayers was found at 349 nm, a value typical of the peptide free in solution and consistent with the fact that no lipid extraction was observed. The progressive decrease in the lipid extraction with an increasing PE proportion in bilayers was effectively reflected by the progressive shift of the melittin fluorescence band in the supernatant.

Melittin-induced lipid extraction was also modulated by the methylation of the lipid headgroups (Figure 2-5A).

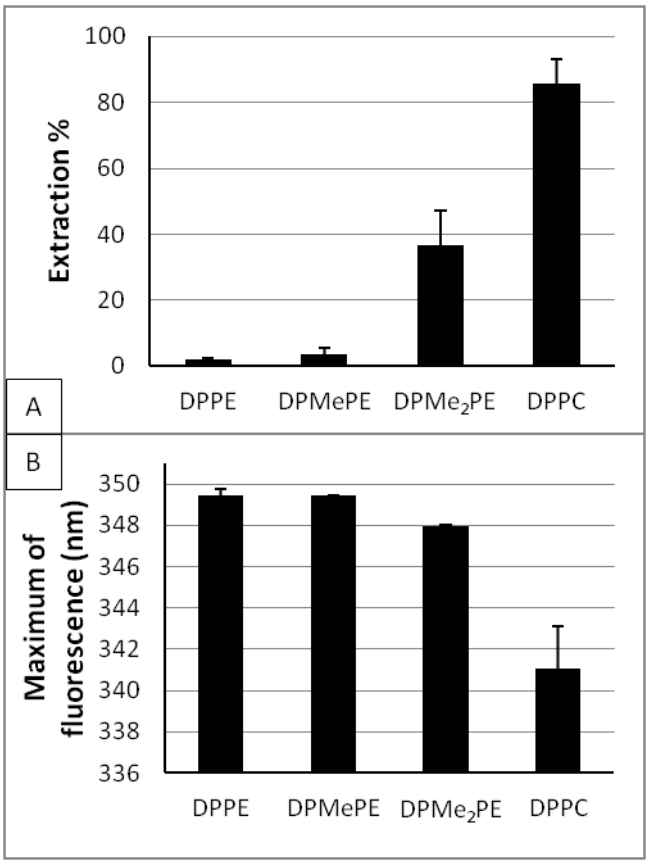

Figure 2-5: A) Quantification of lipid extraction (B) and wavelength of maximum fluorescence in the supernatant at 20 °C after the incubation (L/M=20) of melittin with MLVs of DPPE, methylated DPPE (DPMePE, $DPMe_2PE$) or DPPC.

The removal of one methyl from DPPC ($DPMe_2PE$) reduced the lipid extraction by more than half for a L/M ratio of 20, from 86% with DPPC to 37% with $DPMe_2PE$ bilayers. The removal of a second methyl group on PC headgroup (DPMePE) almost completely inhibited the lipid extraction, similar to what was

observed with DPPE. As in the case of the PC/PE mixtures, the fluorescence maximum of melittin in the supernatant varied in a consistent manner with the lipid extraction level. The position of the band was typical of free melittin in the case of the incubation with DPMePE (which led to no lipid extraction). Melittin fluorescence maximum in the supernatant after the incubation with $DPMe_2PE$ bilayers was shifted to 348 nm, indicative of the co-existence of the lipid-bound, and free melittin states.

The evolution of the phospholipid extraction as a function of the melittin concentration was investigated in more detail for some DPPC/DPPE mixtures (Figure 2-6).

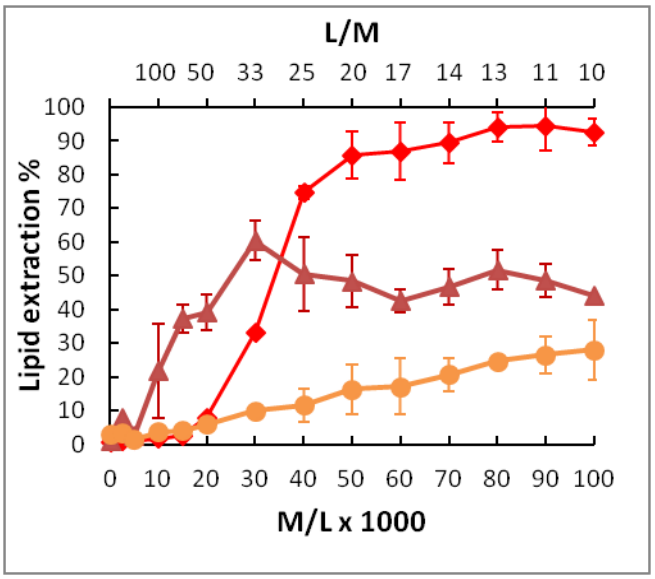

Figure 2-6: Quantification of the lipid extraction after the incubation of melittin with MLVs of DPPC (diamonds), DPPC/DPPE 75/25 (triangles) or DPPC/DPPE 50/50 (circles) at different ratios of incubation. The incubation ratio (the x-axis) is displayed as melittin per 1000 lipids (bottom), as well as L/M (top).

The capacity of melittin to extract lipids from bilayers was dependent on their lipid composition. The lipid extraction increased abruptly with the melittin concentration for DPPC bilayers, from 3% at M/L=15x10^{-3} (L/M = 67) to 86% at M/L=50x10^{-3} (L/M=20). This result is consistent with the evolution of the area of the narrow line, associated with rapidly tumbling bicelles, as a function of melittin content in the ^{2}H-NMR spectra [31]. In general, the presence of DPPE in DPPC membranes decreased their susceptibility to melittin-induced lipid extraction; for M/L=100x10^{-3} (L/M=10), the extent of the lipid extraction decreased from 90% for pure DPPC bilayers to 50% for DPPC/DPPE 75/25 bilayers, and to 30% for DPPC/DPPE 50/50 bilayers. A progressive and monotonous lipid extraction was obtained as a function of the increasing peptide amount for DPPC/DPPE 50/50 bilayers. However, the evolution of the lipid efflux as a function of the melittin proportion displayed a more complex pattern for DPPC/DPPE 72/25 bilayers. The lipid extraction appeared to be more efficient than for DPPC bilayers for low melittin contents, up to a M/L ratio of 30x10^{-3} (L/M=33). For higher melittin contents, the extent of lipid extracted by melittin seemed to reach a plateau; this complex behavior is discussed below.

2.4.3 Lipid selectivity of melittin-induced extraction

We determined the composition of the extracted lipid fraction after the incubation of melittin with PC/PE bilayers in order to identify whether the extraction

was specific for a lipid species. The proportions of extracted PC and PE from bilayers made from mixtures of DPPC/DPPE or POPC/POPE are presented in Figure 2-7.

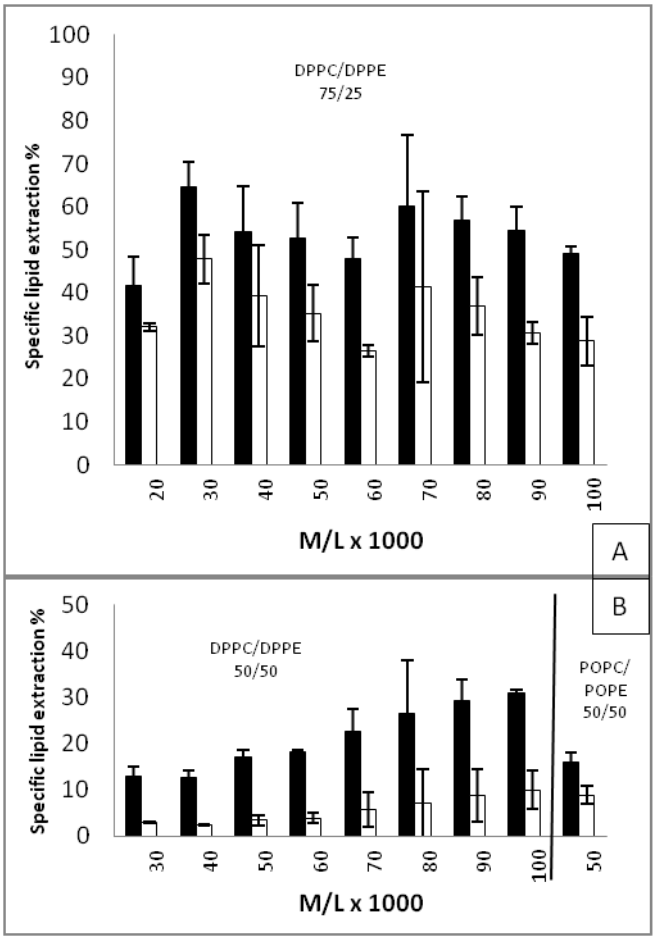

Figure 2-7: Melittin extraction selectivity after the incubation with DPPC/DPPE 75/25 (A) or DPPC/DPPE or POPC/POPE 50/50 (B) MLVs. The black bars represent the PC extraction whereas the white ones represent the PE extraction. The incubation ratio (the x-axis) is displayed as melittin per 1000 lipids.

The results reveal that PC was systematically more extracted than PE from the original bilayers. The extent of melittin-induced extraction was 1.7 - 2.1 times larger for DPPC than for DPPE in DPPC/DPPE 75/25 bilayers, for M/L between $50x10^{-3}$ to

$100x10^{-3}$ (L/M from 20 to 10). The lipid selectivity of the extraction was even more marked for DPPC/DPPE 50/50 membranes, with the proportion of extracted DPPC being 3 to 6 times greater than that of extracted DPPE.

2.5 Discussion

2.5.1 Effect of the demethylation on the association

The binding experiments (Figure 2-1 and Figure 2-3) show that the presence of three methyl groups on the ammonium of the phospholipid headgroup is essential to the association of melittin to gel-phase membranes. The reduction of the methylation level, either by the substitution of PC by PE or by the use of de-methylated PC, led to a reversible fluorescence shift after cooling to the gel phase that was indicative of the dissociation of melittin from the lipid bilayers. The association of melittin with fluid bilayers was considerably favored compared to the gel-phase ones, as is demonstrated by the temperature dependant hypsochromic shift of λ_{max} for the lipid mixtures studied on Figure 2-1 and Figure 2-3. The melittin association with bilayers involves the penetration of the peptide in the apolar core of the bilayers, as was initially suggested by the analysis of the x-ray melittin structure showing its amphipathic α-helix character [46]. The depth of penetration of the 19-Trp residue for melittin bound to 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bilayers was estimated, by the parallax method, to 10.6 Å from the bilayer center [47, 48]. The insertion of melittin into the hydrophobic core of POPC membranes was also

demonstrated by the quenching of the 19-Trp fluorescence upon the addition of brominated lipids [49, 50]. Calculations of the solvation free energy of melittin [51] demonstrated that its binding at the membrane interface was driven by strong hydrophobic interactions, which overcame the energy cost of transferring the polar backbone of the peptide from the high-dielectric aqueous phase to the apolar bilayer core.

The strong intermolecular interactions between PE headgroups are suggested for explaining the reduced interaction of melittin with bilayers with a low interfacial methylation level of the phospholipid ammonium groups. PEs are known to form membranes with a tighter phospholipid packing associated with the small van der Waals volume of their headgroups [52] and their capacity to form intermolecular hydrogen bonds [53]. These features induce the ordering of the lipid chains, as shown by the larger fluid-phase chain order parameters and the derived smaller chain cross-sectional area of POPE compared to POPC [54-56]. The tighter chain packing induced by the decrease of the methylation level of the interfacial ammonium groups is proposed to be thermodynamically unfavorable for melittin insertion in the bilayer. The modulation of the melittin association by the chain packing could also rationalize the enhanced melittin binding observed for fluid-phase membranes, even those with a large proportion of demethylated ammonium group. It has been shown that the melittin binding to DOPC [34] or 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC) [29] membranes in the fluid phase was comparable to that for their equivalent PE

bilayers. The binding was hindered only when DEPE membranes were cooled to the gel phase, like what was observed for POPE in the present work. The absence of effect of substituting PC headgroups by PE on the fluid-phase binding suggests that the tightening effect of PE manifests mostly for membranes with lipid chains that are already motionally restricted. The relatively low cohesion of fluid-phase membranes and the adaptability of the acyl chains to fill potential voids created by the peptide favors, from the energetic point of view, the insertion of melittin in the hydrophobic region of bilayers. However DPPE bilayers appear to display a very low affinity for the peptide even in the fluid phase; these bilayers may be associated with a chain packing that is too ordered even in the fluid phase to accommodate the peptide inside their hydrophobic core. The chain ordering effect of PE is reminiscent of that of cholesterol [57] and it was shown that the presence of cholesterol in PC bilayers reduced melittin affinity for membranes [58]. Such decrease could also be associated with the high energy cost related to the peptide insertion in the ordered hydrophobic core of these membranes.

It is worth mentioning that specific interactions, such as a cation-π interaction between the ammonium group of PC and the indole moiety of tryptophan, may also contribute to the affinity of melittin to PC-rich membranes. MD simulations predicted that melittin binds to DMPC membranes with its 19-Trp near the choline moiety of a neighboring phospholipid. However, at this point, the impact of methylation of the

interfacial ammonium groups on the strength of putative cation-π interactions is not clearly defined [51].

2.5.2 Effect of demethylated headgroups on the lipid extraction

A general two-step mechanism for the lipid extraction by melittin has been proposed [31, 59]. First, melittin inserts at the membrane interface level mainly through hydrophobic interactions. Second, the peptide relocates deeper in the hydrophobic core, disrupting the membrane and extracting fragments of the bilayer. At least three contributions have been proposed as the driving force of this rearrangement. First, it was proposed that this relocation could occur during the fluid to gel phase transition, as the gel phase has a reduced capacity for accommodating the mechanical constraints exerted by inserted melittin [31]; this phenomenon would be the rationale for the bicelle formation observed by cooling after an incubation in the fluid phase [24, 42, 60-62]. Second, the relocation of bound melittin from the interface towards the hydrophobic core of the bilayer could be caused by electrostatic repulsion between bound melittins once the cationic peptide reaches a critical density at the interface. Such a relocation could be related with the change in orientation recently observed in molecular dynamics simulations [63]. In this study, the interpeptide electrostatic repulsion was proposed to contribute to the chemical potential of the peptide and, at a critical peptide concentration, would contribute to drive the peptide from the flat part of bilayers to the membrane interior, leading to the formation of large "pores" that included lipids and melittin molecules. An analogous phenomenon has been proposed

to rationalize the significant increase of the lipid extraction susceptibility of membrane by melittin when the positively charged 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP) was introduced in DPPC membranes; the electrostatic repulsion between DPTAP and melittin makes the interfacial location of melittin unfavorable from a thermodynamics point of view and, therefore, promotes the deeper insertion of melittin in bilayers [31]. Third, it was proposed that the melittin insertion stretches the interface area of the membrane and induces a local thinning of the bilayer [59]. Above a critical peptide concentration, the internal membrane tension caused by the thinning has to be loosened by the relocation of the peptide, leading to the formation of pores and ultimately of bicelles.

The present results demonstrate that the phospholipid headgroup methylation is a determinant factor for the lipid extraction. Phospholipids with demethylated and PE headgroups form membranes that are more resistant to melittin-induced lipid extraction. The extent of the lipid extraction appeared to be dependent on the average number of methyl per ammonium group. For example, the lipid extraction obtained with $DPMe_2PE$ (2 methyls/headgroup) was comparable to that observed for DPPC/DPPE 50/50 (1.5 methyl/headgroup) and DPPC/DPPE 75/25 (2.25 methyls/headgroup) bilayers. This observation suggests that melittin-induced lipid extraction was controlled by the general cohesion properties of the membrane conferred by its level of headgroup methylation, rather than a specific molecular recognition of PC headgroup. We showed that the demethylation of DPPC

(chemically or by the addition of DPPE) reduces melittin association to the membranes in the fluid phase, likely by increasing membrane cohesion. The lower amount of active (bound) melittin naturally contributes to the limited lipid extraction. As an ultimate case, melittin does not bind to DPPE bilayers and, indeed, no lipid extraction was observed. This rationale is reminiscent of the inhibition of melittin-induced lipid extraction caused by the presence of cholesterol in DPPC membranes [27, 28]. It was proposed that cholesterol inhibited lysis by preventing the melittin penetration in the membrane during incubation due to a tighter lipid packing. It should be pointed out, however, that the peptide binding to membranes and the peptide-induced lipid extraction involve different intermolecular interactions and the behavior of one cannot simply be extrapolated to the other. For example, the behavior of DPPC/DPPE 75/25 membranes appeared to be peculiar as, even though less peptide was bound, the observed proportion of extracted lipids was consistently larger for these membranes than for DPPC membranes after incubation at low M/L – smaller than $3x10^{-3}$ or L/M > 333 (Figure 2-6). We propose that DPPC/DPPE 75/25 bilayers have an interfacial cohesion that allows a considerable melittin binding in the fluid phase but the gel-phase formation triggers a relocation of melittin and the formation of small peptide-lipid particles. Figure 2-1 and Figure 2-2 showed that the melittin association to DPPC/DPPE 75/25 bilayers was similar to the DPPC ones in the fluid phase. However, the cooling resulted in a significant level of the melittin dissociation with DPPC/DPPE 75/25 bilayers, likely because of a significantly reduced capability of these gel-phase bilayers to accommodate the peptide at their

interface level. Therefore, the formation of a gel phase in DPPC/DPPE 75/25 bilayers could lead to a destabilization of melittin inserted in the membrane interface; the peptide would then relocate parallel to the bilayer normal and could cause increased lipid extraction. DPPC gel-phase bilayers could, in these conditions, accommodate that limited amount of bound melittin. This phenomenon is reminiscent of the lipid extraction increase observed for DPPC membranes with 10% DPTAP [31]. This cationic lipid induced an overall reduction in the affinity of positively charged melittin for the bilayers. However, its presence increased the extent of the lipid extraction; only small lipid-melittin particles were observed at L/M as high as 100. It was proposed that melittin adsorption to membranes was driven by hydrophobic interactions but electrostatic repulsion between the positively charged bilayer interface and the cationic peptide unfavored the interfacial position and led to melittin redistribution in the bilayers, causing lipid extraction. The cohesion of bilayers containing a limited amount of PE could act similarly to these electrostatic interactions.

2.5.3 Extraction selectivity towards PC

The results clearly show that melittin selectively extracted PC from PC/PE mixed membranes; the proportion of extracted PC was between 2 to 6 times greater than the extracted PE, depending on the composition of the original bilayers. These findings were obtained for DPPC/DPPE as well as POPC/POPE bilayers. It is proposed that this lipid extraction specificity of PC over PE is a consequence of a

putative depletion of PE in the vicinity of bound melittin. As described above, PEs form membranes with a tighter phospholipid packing than PCs, notably because of their capacity to form intermolecular hydrogen bonds. It is expected that the presence of melittin at the membrane interface level leads to a depletion of PE in the peptide vicinity in order to maintain PE-PE contacts. Melittin molecules would subsequently extract a bilayer patch that would be PE-depleted. The suggested PE depletion of the surrounding of membrane-bound melittin is consistent with previous conclusions inferred from the pore formation by the peptide. It was proposed that PE was excluded from melittin-rich domains that led to toroidal pores as the curvature properties of these lipid species were unfavorable to the formation of such curved structures [34]. Such a mechanism could also provide a rationale for the reported PC-specificity in melittin-induced lipid extraction from DPPC/Chol membranes [27]. Cholesterol has an ordering effect on the lipid acyl chains and would be excluded from the surrounding of adsorbed melittin at the bilayer interface. When the local concentration of interfacial melittin reaches a critical value, the relocation of melittin deeper in the bilayer causes the extraction of a bilayer patch that is, in these conditions, depleted in cholesterol relative to the sterol content of the original membrane. The proposed model for rationalizing the lipid specificity of the extraction of bilayer patches by melittin is reminiscent of the mechanism suggested for the action of Triton X-100 on cholesterol-containing membranes [64, 65]. When Triton X-100 was added to vesicles made of POPC, sphingomyelin (SM), and cholesterol, a lipid system that formed homogeneous bilayers, it induced the formation of liquid-

ordered and liquid-disordered domains due to a segregation of SM and Chol. It was found that when a sufficient amount of detergent was added, it would selectively solubilize the liquid-disordered domains, providing POPC-enriched efflux. Therefore, it is suggested that the modulation of lipid mixing properties in bilayers could be a simple way to control the specificity of lipid efflux from membranes.

In this work, it is demonstrated that an amphipathic helical peptide can cause lipid efflux from membranes in a lipid selective manner, based on the methylation level of the headgroup in the present case. Such phenomenon is likely not limited to melittin and may play a pivotal role in other biological events. For example, it has been established that ApoA1 extracts specifically PC over SM and cholesterol from mouse macrophages [66-68] and from fibroblasts [69]. In these cases, the overall lipid efflux mechanism is complex, involving notably the ATP-binding cassette transporter (ABCA1) present in the macrophages. In order to account for the selective PC extraction by Apo-A1, it was pointed out that ABCA1 was primarily found in fluid, SM- and cholesterol-poor regions of the membranes; the lipids transferred from ABCA1 to Apo-A1 lipoparticles originated from the PC-rich vicinity of ABCA1. In that system, the specificity of the lipid efflux was also proposed to be associated with a phase separation in membranes. The specificity regarding PC versus phosphatidylethanolamine (PE) is not clearly established as a PC enrichment was observed in lipoparticles formed with ApoA1 incubated with fibroblast membranes [69] whereas ApoA1 incubated with POPC/POPE MLVs preferentially formed PE-

enriched, saddle-shaped particles [70], a phenomenon to be associated with POPE polymorphic propensities. The rapid development of lipidomics is expected to reveal further specific lipid efflux caused by peptides and proteins, and the understanding of such processes will be required to determine the mechanism of the related biological events.

Interestingly, it was recently found that styrene-maleic acid copolymers solubilized membranes into nanodisks, a process that is reminiscent of the bicelle formation by melittin. The organization of these self-assemblies is actually similar, with a small discoidal lipid bilayer whose edges are coated by the amphiphilic molecules [24-26, 71]. The proposed mechanism for the nanodisk formation by the copolymer is also similar to the one associated with the bicellization process induced by melittin: the copolymer first binds the interface, inserts into the membrane, and then extracts a piece of the lipid bilayer to form nanodisks [71]. The copolymer displayed a reduced ability to form nanodiscs with PE-containing membranes compared to DOPC ones, like melittin. However no selectivity in the lipid extraction was observed, the extent of PE and PC extraction being the same. This discrepancy with the bicellization process caused by melittin indicates that some interactions impacting the lipid extraction induced by molecular species such as peptides, proteins, detergent and polymers, have yet to be understood. The present work highlights that molecular details such as the methylation level of the lipid headgroup

are sufficient to tune the susceptibility of membranes to lipid extraction and to lead to a selective extraction.

Author Contributions

Michel Lafleur and Alexandre Therrien designed the project, analyzed data, and wrote the manuscript. Alexandre Therrien performed experiments.

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Chapitre 3 - Role of the Cationic C-Terminal Segment of Melittin on Membrane Fragmentation

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3.1 Abstract

The widespread distribution of cationic antimicrobial peptides capable of membrane fragmentation in animal and plants underlines their importance to living organisms. In the present work, we determined the impact of the electrostatic interactions associated with the cationic C-terminal segment of melittin, a 26-aminoacid peptide from bee venom (net charge +6), on its binding to model membranes and on the resulting fragmentation. In order to detail the role played by the C-terminal charges, we prepared a melittin analogue for which the 4 cationic amino acids in positions 21 to 24 were substituted with the polar residue citrulline, providing a peptide with the same length and amphiphilicity but with a lower net charge (+2). We compared the peptide bilayer affinity and the membrane fragmentation for bilayers prepared from dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) mixtures. It is shown that neutralization of the C-terminal considerably increased melittin affinity for

zwitterionic membranes. The unfavorable contribution associated with transferring the cationic C-terminal in a less polar environment was reduced, leaving the hydrophobic interactions, which drive the peptide insertion in bilayers, with limited counterbalancing interactions. The presence of negatively charged lipids (DPPS) in bilayers increased melittin binding by introducing attractive electrostatic interactions, the augmentation being, as expected, greater for native melittin than for its citrullinated analogue. The membrane fragmentation power of the peptide was shown to be controlled by electrostatic interactions and could be modulated by the charge carried by both the membrane and the lytic peptide. The analysis of the lipid composition of the extracted fragments from DPPC/DPPS bilayers revealed no lipid specificity. It is proposed that extended phase separations are more susceptible to lead to the extraction of a lipid species in a specific manner than a specific lipid-peptide affinity. The present work on the lipid extraction by melittin and citrullinated melittin with model membranes emphasizes the complex relation between the affinity, the lipid extraction/membrane fragmentation, and the lipid specificity.

3.2 Introduction

The widespread distribution of cationic antimicrobial peptides (CAPs) in animal and plants underlines their importance to living organisms. To this day, more than 2500 naturally occurring CAPs have been catalogued. They present a wide range of activity: antibacterial, antiviral, antifungal, and anticancer, to name a few [1, 2].

Notably, the interest they generate in modern medicine comes from their straightforward mode of action: in general, CAPs directly target and disrupt vital membrane features, allowing CAPs to preserve their potency despite some microbial mutations.

A characteristic feature of CAP structures is the coexistence of an amphipathic character, and positively charged amino acids. Upon binding to membranes, they often organize as α-helixes with hydrophobic and hydrophilic residues on opposite faces, a feature referred to as secondary amphipathic character. Designers of synthetic CAPs found that it was possible to modify CAP activity towards microbial and mammalian cells independently by varying the hydrophobicity [3-5], the number and the position of the positive charges in the amphiphilic structure [6-8]. Therefore, fine-tuning the primary structure of CAPs can optimize cell selectivity. It is believed that the cationic charge of CAPs plays a fundamental role in the targeting of bacterial cells [9-11] as these typically possess negatively charged membranes [12]. For example, the increase of the net positive charge of synthetic CAPs was found to improve their antimicrobial activity while maintaining low toxicity towards mammalian cells [6, 8]. It appeared, however, that there was a positive-charge threshold as further increase of the CAP net positive charge led to a higher activity toward erythrocyte membranes [7], a phenomenon that was associated with the putative attraction of these highly charged CAPs by the negative membrane potential inside the cell [13].

In the present work, we determined the impact of electrostatic interactions associated with the cationic C-terminal segment of melittin on bilayer affinity and on the resulting lipid extraction. Melittin, the main component of dry bee venom, is a 26-amino-acid CAP [14]. It has been used as a model peptide for various purposes over the last decades (for a general review, see [15]), and has shown therapeutic potential with antimicrobial [16, 17], antiparasitical [18], and anticancer [19, 20] activity, as well as for treatment [21] and prevention [22] of HIV. Like many CAPs, melittin binds to membranes as an amphipathic α-helix [23-25]. Its primary sequence, GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2 [14], includes a highly hydrophilic C-terminal segment with 4 positive charges (K^{21}-R^{22}-K^{23}-R^{24}). The 1-20 segment folds as an amphipathic α-helix upon the binding of the peptide to bilayers. When interacting with membranes, melittin induces leakage [26-31] and it leads, at higher concentrations, to membrane fragmentation and the formation of small lipid/peptide bicelles, or nano-disks [32-34].

The cationic character of melittin was shown to be essential to its activity on membranes. It is well documented that the presence of negatively charged lipids in membranes, such as phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidic acid (PA), significantly increases the melittin affinity due to attractive electrostatic interactions [35-39]. However, it was observed that melittin-induced bicellization of membranes was strongly hindered by the presence of anionic lipids in

membranes [23, 35, 37, 40, 41]. This observation was proposed to be due to an electrostatic anchoring of the peptide to the interface, preventing the relocation of the peptide deeper in the hydrophobic core, an essential step for the membrane fragmentation. This inhibition of the lipid extraction was found to be proportional to the interfacial negative charge density of the bilayer, and independent of the nature of the anionic lipid [35].

Melittin analogues with modified or omitted residues have been used to determine the role of the cationic C-terminal part. At this point, there is no coherent conclusion regarding the impact of the electrostatic interactions on bilayer binding. For instance, the properties of melittin-21Q, a truncated analogue of melittin in which residues 21 to 25 (K^{21}-R^{22}-K^{23}-R^{24}-Q^{25}) are omitted, were investigated. Its binding to zwitterionic 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers as well as to anionic 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) bilayers was measured by Surface Plasmon Resonance (SPR) [42]. Melittin-21Q exhibited a reduced binding to DMPC and to DMPG bilayers compared to native melittin, suggesting a contribution of the cationic C-terminal portion favorable to membrane affinity. However, another SPR study [43] concluded that, despite the different kinetics, the extent of binding to DMPC and to DMPC/DMPG (4/1 mol/mol) bilayers was similar for native melittin and melittin-21Q. The different conclusions of the two studies were at least partly attributed to the fact that the former investigation was conducted using hybrid bilayers on long-chain alkane-coated (HPA) chips, preventing a deep

insertion of the peptide in the supported bilayers. Another investigation, using tryptophan fluorescence, revealed that melittin and its C-terminal truncated versions 1-22 (Mel1-22), and 1-20 (Mel1-20), all showed an increased affinity for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (POPG) 70/30 large unilamellar vesicles (LUVs)compared POPC LUVs [44] (See Supplementary Material). These findings highlighted the role of attractive electrostatic interactions. However, it was also reported that Mel1-22 and Mel1-20 showed a larger affinity for POPC bilayers than melittin, hinting for a detrimental role of the cationic C-terminal in the association with neutral membranes. Similarly, the disordering effect of membrane apolar core by melittin fragments, as assessed by Attenuated Total Reflectance - infrared (ATR-IR) spectroscopy, suggested that the hydrophobic 1-15 fragment of melittin inserted well into 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and POPC membranes, while the binding of the hydrophilic 16-26 fragment was limited [45]. The results relative to the impact of the C-terminal residues on cell lysis do not provide a consistent description either. Mel1-22 was shown to induce less leakage of encapsulated fluorescein from DPPC LUVs than melittin [46]. This conclusion was in agreement with the findings resulting from the comparison of melittin cytolytic activity with that of Mel1-22 or Mel1-20, evaluated by ^{51}Cr release assays from human lymphoblast cells; this study also found that the truncated analogues were less active [47]. However, it was shown that the removal of any of the 21 to 26 (KRKRQQ) residues to form 25-residue analogues of melittin (net charge +5 or +6) had no effect on lytic power; the induced

leakage of cell material from erythrocytes and bacterial cells was similar between analogues and native melittin [48].

In the present paper, we detail the role played by the electrostatic interactions involving the C-terminal charges of melittin in the lipid extraction induced upon interacting with membranes. We compared the bilayer affinity and the extent of lipid extraction of melittin bearing either its native cationic C-terminal segment or a neutral hydrophilic segment. The neutral segment was prepared by substituting the 4 basic residues of melittin (21K-22R-23K-24R) by citrullines, a polar but neutral amino acid [49]. Citrullination of the peptide isolated the effect of the charges since it preserved the solubility, the length and the number of peptide bonds of the native peptide. We characterized the interactions with membranes prepared from DPPC and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS) in various proportion in order to modulate the negative charge of the bilayer interface. In addition, we examined whether melittin-driven lipid extraction was specific for anionic lipids, considering the attractive interactions between the two species. It has been recently shown that melittin could preferentially extract DPPC molecules when interacting with DPPC/1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) membranes [50]. It was proposed that the surrounding of bound melittin was depleted in DPPE, as the stronger inter-PE interactions were unfavorable to the peptide insertion in the bilayer. The resulting enrichment in DPPC of the peptide environment was proposed to be at the origin of the enhanced DPPC extraction relative to DPPE.

3.3 Materials and Methods

3.3.1 Chemicals

Melittin was purified from bee venom (Sigma, St. Louis, MO, USA) by ion exchange chromatography on SP-Sephadex C-25 [51]. Solid phase synthesis of citrullinated melittin (Cit-Mel) was carried out on methylbenzhydrylamine resin, using commercial Boc-amino acid residues (Chem-Impex, Wood Dale, IL, USA) and BOP as the coupling reagent (Matrix Innovation, Quebec City, QC, CAN). Acidolytic Boc removal was obtained by treating the protected peptide-resin with trifluoroacetic acid (TFA)/methylene chloride (45%). After a final Boc deprotection step, following a cleavage with hydrofluoric acid (Matheson, Edmonton, AB, CAN) containing *m*-cresol (10%) as a scavenger, a crude peptide preparation was isolated and washed with ethylether. The crude material was purified by reverse-phase HPLC using an acetonitrile (ACN) gradient in aqueous TFA (0.1%). Pure fractions corresponding to the expected mass of Cit-Mel, as established by MALDI-TOF mass spectrometry, were pooled, evaporated to remove ACN, and lyophilized. The mellitin analogue was kept at -80 °C before use. DPPC and DPPS were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Ethylenediaminetetraacetic acid (EDTA), NaCl, and 3-[N-morpholino]propanesulfonic acid (MOPS) were obtained from Sigma (St. Louis, MO, USA). All chemicals were used as received.

3.3.2 Lipid membrane preparation

First dissolved in a benzene/methanol mixture (90/10 (v/v)), individual lipids were mixed to obtain the desired molar ratio and then lyophilized. The lipid powders were hydrated in a MOPS buffer (50 mM) containing 100 mM NaCl and 100 μM EDTA, and adjusted to pH7.4. The samples were submitted to 3 freeze-and-thaw cycles (from liquid nitrogen temperature to 65 °C) to form the multilamellar vesicle (MLV) suspensions used for the extraction experiments. For the fluorescence experiments, the MLVs were extruded at 75 °C using a manual extruder (Northern Lipids, Vancouver, BC, CAN) to obtain 100-nm LUVs.

3.3.3 Fluorescence measurements

Tryptophan fluorescence spectra were recorded to assess the binding of melittin to lipid bilayers. For these binding studies, LUV aliquots were added to a melittin solution (14 μM in the MOPS buffer; its concentration was determined from its absorbance at 280 nm, using a molar absorptivity coefficient of 5 570 M^{-1} cm^{-1}[52]). After each addition, the fluorescence spectrum of melittin was acquired at 65 °C; this temperature was selected to ensure that all the investigated lipid systems were in the fluid phase. The wavelengths at maximum for free (λ_{free}) and bound (λ_{bound}) melittin were determined from the spectra recorded from samples with a lipid to peptide incubation ratio (L/P) of 0, and 400, respectively. The fraction of bound melittin, X_b,

was then calculated from the ratio of the fluorescence intensity at λ_{free} ($I_{\lambda free}$) and at λ_{bound} ($I_{\lambda bound}$) using

$$X_b = \frac{R-R_0}{R_{400}-R_0},$$

where R is $I_{\lambda bound}/I_{\lambda free}$, R_0 is $I_{\lambda bound}/I_{\lambda free}$ at L/P=0, and R_{400} is $I_{\lambda bound}/I_{\lambda free}$ at L/P=400. The association constants of melittin to the membranes (K_a) were calculated as the slope of the fitted lines describing the variations of $X_b/(1-X_b)$ as a function of $[lipid]_{external}$, according to:

$$K_a = \frac{[peptide]_{bound}}{[peptide]_{free} \times [lipids]_{external}}.$$

The data points for which most of melittin was bound ($X_b \geq 0.8$) deviated from the model and were discarded for the determination of K_a. $[lipids]_{external}$ took into account only the lipid external leaflet (i.e. the total lipid concentration/2) as the association was assumed to occur essentially at the bilayer interface. The molar Gibbs free energy of binding (ΔG_{bind}) was calculated using:

$$\Delta G_{bind} = \mathrm{RT} \ln\left(\frac{c^\circ}{K_a}\right),$$

where R is the ideal gas constant, T is the temperature, and c°=1 M is the standard reference concentration. Fluorescence measurements were carried using a Photon Technology International fluorometer. The excitation wavelength was set at 270 nm and the bandwidths for the excitation and emission monochromators were set at 1.0 and 2.0 nm, respectively. In these conditions, no significant light diffusion caused by the liposomes was observed.

3.3.4 Lipid Extraction

Lipid extraction induced by melittin was determined using an approach previously described [50]. Briefly, a melittin solution and a MLV suspension prepared in the MOPS buffer were mixed in a microcentrifuge tube to obtain the desired L/M and a fixed phospholipid concentration of 1 mg/mL. The suspensions were then incubated for at least 30 min at 65 °C, a temperature above the membrane gel-to-fluid phase transition temperature (T_m = 41 °C for DPPC and 54°C for DPPS). After the incubation, the samples were centrifuged for 5 min at 20 800 g and 1 °C. It was assumed that the extracted lipids, existing as small melittin-lipid assemblies, stay in the supernatant while the remaining MLVs (possibly with bound melittin) pellet. Centrifugation of control samples (without melittin) showed that more than 85% of lipids were found in the pellets for all phospholipid mixtures. The supernatants were isolated and the pellets were resuspended in the MOPS buffer for their analysis. The phospholipid contents in the supernatants and in the pellets were determined by a Bartlett's phosphorus assay [53]. The extent of extraction was calculated using

$$\text{Extraction } \% = \frac{phospholipids\ in\ supernatant}{total\ phospholipids}.$$

The lipid composition of the supernatants and of the pellets were determined by HPLC-MS analysis, using an Agilent Technologies 1100 series system equipped with a 1100 MSD mass spectrometer. Samples were eluted on a YMC diol column (4.6 x 150 mm, 5-µm particle size) (Agilent Technologies), maintained at 50 °C. Elution of the phospholipids was achieved in 7 min, using acetonitrile/aqueous ammonium

acetate solution (100 mM) (85/15) at 0.6 mL/min. The ESI source was used in the positive ionization mode. Nitrogen was used as drying gas at 250 °C and 12 L/min. Nebulizing gas was also nitrogen, held at 241 kPa. The extent of extraction was determined for each lipid species, using

$$Extraction\ \% = \frac{A_S}{A_S + A_p},$$

where A_s and A_p are the lipid peak area from the supernatant and the pellet analysis, respectively. Experiments were carried out in triplicates unless stated otherwise.

3.4 Results

3.4.1 Association to LUVs

First, the impact of the C-terminal charges of melittin on bilayer affinity was determined. The hypsochromic shift of the tryptophan fluorescence band was used to characterize the transfer of this amino acid from water (in the case of free melittin) to a more hydrophobic environment (associated with membranes) [54]. The binding of melittin and Cit-Mel to DPPC zwitterionic bilayers and to DPPC/DPPS anionic membranes was characterized (Figure 3-1). The binding experiments were carried out at 65 °C, a temperature at which membranes were in the fluid phase. The effect of citrullination on the melittin association to pure DPPC membranes is presented in Figure 3-1A. Free melittin displayed a fluorescence maximum at 348 nm; the band was progressively shifted upon the addition of DPPC LUVs towards 335 nm, a value

characteristic of the bound state [38] (see Supplementary Material). From these fluorescence data, the proportion of membrane-bound melittin was estimated as described in the Materials and Methods section.

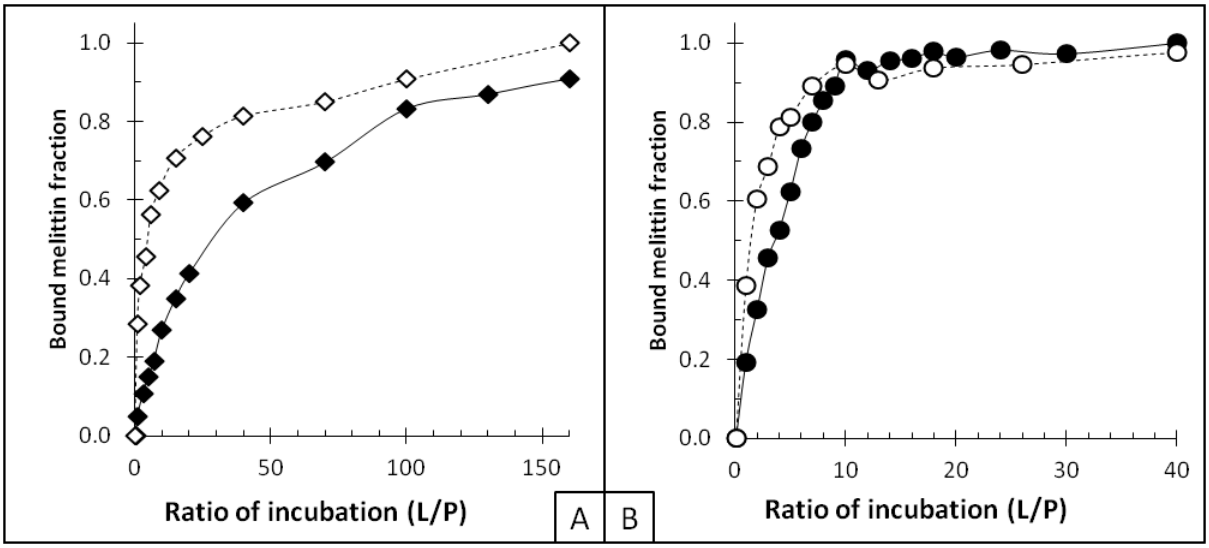

Figure 3-1: Evolution of tryptophan fluorescence of melittin (full symbols) and of citrullinated melittin (empty symbols), characteristic of melittin association with DPPC (A) or DPPC/DPPS 70/30 (B) bilayers at 65°C.

The L/P at which 50% of melittin was bound (L/P_{50}) was evaluated at 30 for DPPC. K_a and ΔG_{bind} were also inferred from these data (Table 3-1). The ΔG_{bind} value for the association of melittin to DPPC membranes was -23.8 kJ/mol, which is similar to the values that have been reported for melittin binding to fluid POPC LUVs (varying between -21.3 to -24.7 kJ/mol) [55, 56]. The titration of Cit-Mel with DPPC LUVs showed a similar hypsochromic shift (from 344 nm to 336 nm; see Supplementary Material) but occurring at lower L/Ps, with L/P_{50}=4. This result indicated that ~7

times less lipids were required to bind half the peptides. In addition, ΔG_{bind} was -28.0 kJ/mol, indicating a stronger association to DPPC than native melittin.

Table 3-1: Association parameters obtained from binding data.

Peptide	Membrane	L/P at 50% binding	K_a (M^{-1})	ΔG_{bind} (kJ/mol)
Melittin	DPPC	30	$4.8x10^3$	-23.8
	DPPC/DPPS 70/30	3.6	$5.1x10^4$	-30.5
Citrullinated	DPPC	4.0	$2.1x10^4$	-28.0
	DPPC/DPPS 70/30	1.3	$1.2x10^5$	-32.6

The peptide affinity was also determined for negatively charged membranes formed by DPPC/DPPS 70/30 (mol/mol) mixture (Fig. 3-1B). The titration curves indicated that melittin had a stronger affinity for negatively charged bilayers than for pure DPPC membranes; in the case of DPPC/DPPS 70/30 bilayers, the L/P_{50} was shifted to 3.6 and ΔG_{bind} was -30.5 kJ/mol. A consistent increased melittin affinity had already been reported for anionic membranes of PS, of phosphatidylglycerol (PG), or containing deprotonated palmitic acid (PA^-) [38, 55, 57]. This enhanced attraction was proposed to be due to electrostatic interactions. The binding experiments with Cit-Mel also revealed an increased affinity for DPPC/DPPS membranes compared to pure DPPC bilayers, with L/P_{50} reaching 1.3, and a ΔG_{bind} of -32.6 kJ/mol. It should be noted that these results indicated that the affinity of Cit-Mel for DPPC/DPPS

bilayers was stronger than that of melittin, even though 4 of the 6 positively charged amino acids of the latter have been substituted with citrullines.

3.4.2 Lipid extraction by melittin and citrullinated melittin

The impact of electrostatic interactions on melittin-induced lipid extraction was characterized by modulating both the bilayer charge, using DPPC/DPPS membranes in different proportions, and the peptide charge, by comparing melittin and Cit-Mel. Figure 3-2 indicates that the addition of anionic DPPS to neutral DPPC MLVs modulated lipid extraction induced by melittin and Cit-Mel in opposite ways.

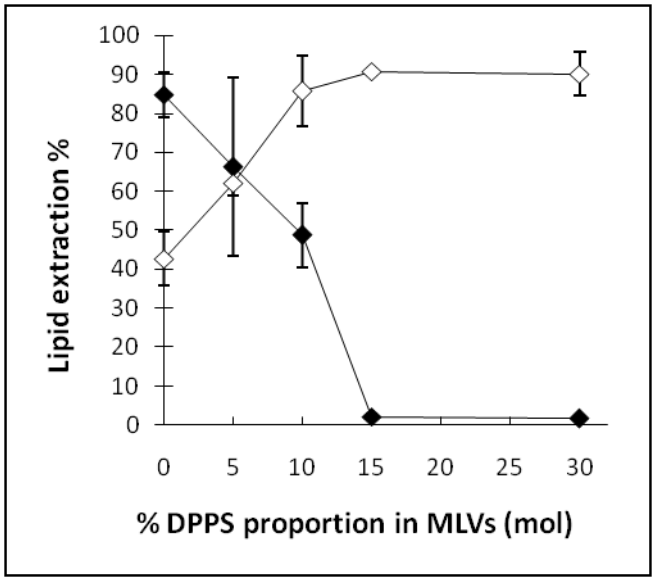

Figure 3-2 : Lipid extraction after incubation (L/P=20) of melittin (full diamonds) or melittin-cit (empty diamonds) with DPPC/DPPS MLVs with different proportions of anionic lipids.

At L/P=20, DPPC bilayers were almost completely destructed after an incubation with melittin as the lipid extraction reached 85%; this extent is in agreement with previous results [35, 50]. The insertion of DPPS in membranes progressively inhibited the lipid extraction, a phenomenon previously demonstrated for other negatively charged phospholipids [35]. Membranes with 15 DPPS % or more were completely resistant to the lipid extraction by melittin at L/P=20; this inhibition is consistent with previous ^{2}H-NMR results obtained with DPPC bilayers containing 10 (mol)% DPPG or DMPS [35]. The lipid extraction from pure DPPC bilayers by Cit-Mel was ~40 %, about half of the level observed for melittin. Conversely to the inhibiting effect on melittin-induced extraction, the addition of DPPS appeared to enhance the ability of Cit-Mel to extract lipids; the addition of 5 and 10 % DPPS increased the extent of the lipid extraction by about 20% and 40% respectively, mirroring the pattern observed with melittin. The lipid extraction reached a plateau at about ~90% for membranes with 10% DPPS or more.

The lipid extraction as a function of the melittin concentration was detailed for DPPC and DPPC/DPPS 90/10 membranes (Fig. 3-3) to better distinguish the activity of both peptides towards neutral and anionic liposomes. Melittin started to cause a lipid extraction from DPPC membranes when P/L reached 15×10^{-3} (L/P=67). The lipid extraction increased abruptly with an increased melittin concentration, from 6% at P/L=15×10^{-3} (L/P=67) to 91% at P/L=50×10^{-3} (L/P=20). These results are in agreement with previous studies using the same centrifugation approach [50] as well as

those using ^{2}H-NMR measurements to assess the formation of extracted small lipid/peptide particles [35]. The curve obtained with DPPC/DPPS 90/10 bilayers illustrates the resistance of anionic bilayers to the lipid extraction by melittin. The lipid extraction was observed for P/L proportions greater than 30×10^{-3} (L/P=33); it increased from 5% at P/L=30×10^{-3} to 80% at P/L=70×10^{-3}.

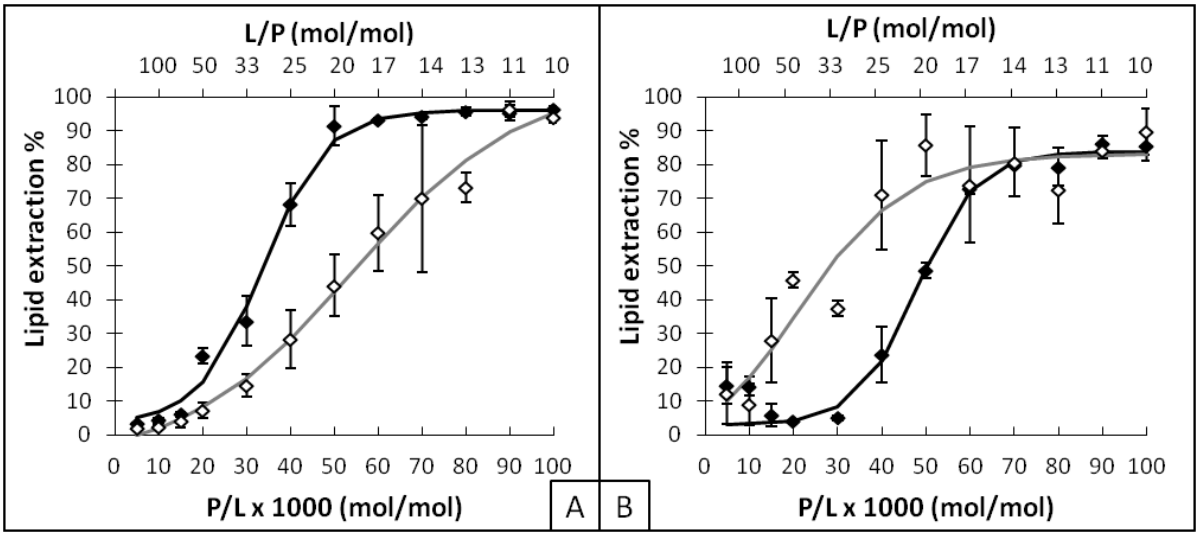

Figure 3-3: Quantification of lipid extraction after incubation of melittin (full symbols) or citrullinated melittin (empty symbols) with MLVs of DPPC (A) or DPPC/DPPS 90/10 (B) at different ratios of incubation. The incubation ratio is displayed as peptide per 1000 lipids, P/L, or L/P.

The lipid extraction from pure DPPC bilayers by Cit-Mel reflected the reduced activity of this peptide compared to melittin. The lipid extraction was observed when P/L reached 20×10^{-3}: 9% lipid extraction was observed. It increased with the peptide concentration, reaching >95% at P/L=90×10^{-3}. The results obtained with DPPC/DPPS 90/10 bilayers showed the enabling effect of DPPS on the lipid extraction by Cit-Mel,

as a P/L as low as 15×10^{-3} led to 28% lipid extraction. The maximal bilayer disruption was already obtained for a P/L of 50×10^{-3}. It should be noted that DPPC/DPPS 90/10 bilayers were more difficult to pellet by centrifugation in the absence of peptide, likely because of the electrostatic repulsion between the anionic membranes (Figure 3-3B). This was reflected by a small extent of lipid extraction reported for very low peptide contents (P/L≤10) that were, in fact, corresponding to the amount of lipid obtained for the blank. Upon the addition of more melittin (10≤P/L≤30), the MLVs were pelleted more efficiently, leading to an estimated lipid extraction closer to 0%. This observation is in agreement with the proposed bridging of adjacent anionic bilayers by melittin that would force the expulsion of some interlamellar water [23, 57].

3.4.3 Absence of lipid selectivity in melittin-induced extraction

The composition of the extracted lipid fraction after the incubation of DPPC/DPPS membranes with the peptides was determined in order to identify whether the extraction was specific for one of the two lipid species (Fig. 3-4). The 90/10 DPPC/DPPS mixture was chosen for these experiments since the resulting membranes led to intermediate levels of lipid extraction; these were required to obtain sufficient DPPS for reliable quantitation and to get remaining MLVs to allow specific lipid extraction. The overall lipid extraction could be inferred from the weighted extraction average of the two lipids. As shown on Fig. 3-2, the lipid extraction augmented as P/L increased. The higher lipid extraction extent observed

for Cit-Mel at P/Ls between 15×10^{-3} and 60×10^{-3} demonstrated again its increased activity towards DPPS-containing membranes. The results show that both DPPC and DPPS were extracted by the peptides to a similar extent. Therefore, melittin and Cit-Mel extracted lipids from DPPC/DPPS 90/10 bilayers in a non-selective manner. No significant difference between the DPPS content in the initial membrane composition and in the extracted lipid fractions was observed for membranes with up to 30% of DPPS for P/L of 50×10^{-3} (L/P=20) (Supplementary Material).

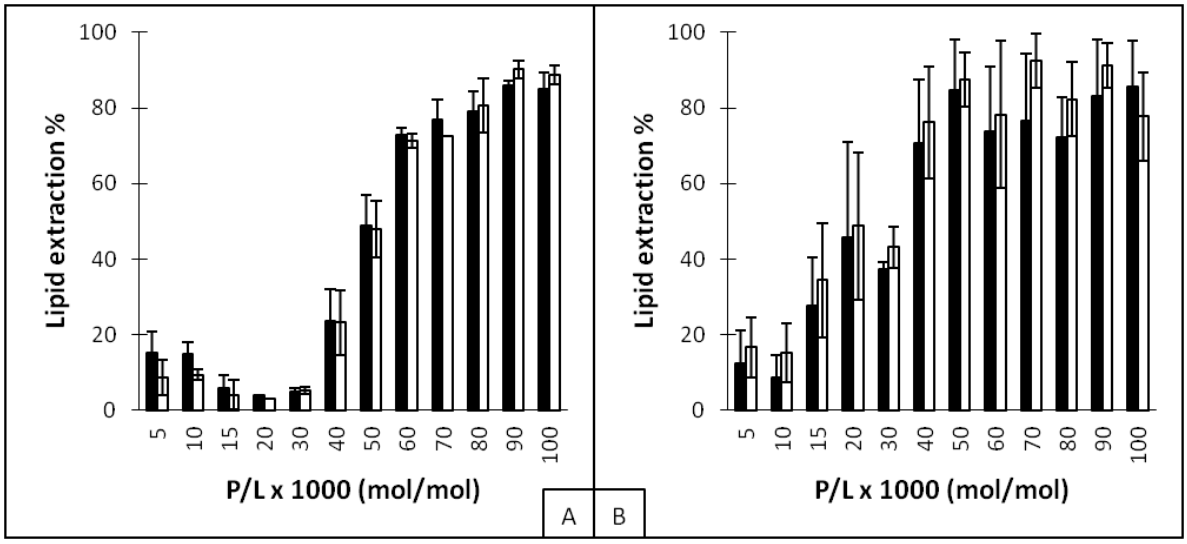

Figure 3-4: Melittin (A) and citrullinated melittin (B) extraction selectivity after incubation with DPPC/DPPS 90/10 MLVs. Black bars represent PC extraction, white bars represent PS extraction. The incubation ratio is displayed as peptide per 1000 lipids, P/L, or L/P.

3.5 Discussion

3.5.1 The electrostatic effect on the melittin association

It is generally accepted that the positive character of CAPs is pivotal for the peptide association with negatively charged membranes. The binding of CAPs to lipid bilayers is increased by the presence of anionic lipids; the more negative surface potential of bacterial membranes compared to eukaryotic cell is assumed to be a key feature for providing cell specificity [9-11, 58]. The stronger affinity of melittin for anionic than for neutral membranes had already been demonstrated [37, 38, 57] and is corroborated by the present results (Fig. 3-1). This preference for anionic membranes is likely due to attractive electrostatic interactions associated with the cationic residues of the peptide. However, the present findings suggest that the cationic C-terminal grants cell binding specificity to melittin not only by providing attractive electrostatic interactions with anionic membranes, but also by reducing its association with zwitterionic bilayers. The binding experiments (Table 3-1) showed that the substitution with citrullines of the C-terminal cationic amino acids increased melittin affinity for zwitterionic DPPC membranes, rising ΔG_{bind} from -23.8 to -28.0kJ/mol. This result is consistent with a previous study showing that the affinity of truncated amidated Mel1-22 (net charge +4) or Mel1-20 (net charge +2) for POPC membranes was increased compared to native melittin ([44], Supplementary Material). In that study, half-association was reached at L/P_{50}=5 and L/P_{50}=2 for melittin 1-22 and 1-20, compared to L/P_{50}=19 for melittin. Considering these observations, it is concluded

that the cationic C-terminal K^{21}-R^{22}-K^{23}-R^{24} segment has an adverse effect on the association of melittin to neutral membranes. The melittin binding to membranes is a process involving substantial hydrophobic interactions. A continuum mean-field model describing the melittin transfer from the polar aqueous medium to the membrane apolar core isolated the contributions of the hydrophobic and of the electrostatic interactions to the ΔG of association [59]. It was found that the favorable hydrophobic interactions were dominating, overcoming the energy required for peptide desolvation. The thermodynamics of the melittin association to POPC LUVs was also determined experimentally by Isothermal Titration Calorimetry (ITC), and concluded that ΔG_{bind} was the combination of a favorable entropic contribution, driven by hydrophobic interactions, and an unfavorable enthalpic contribution [55]. The increased affinity of Cit-Mel for zwitterionic membranes could originate from at least two consequences resulting from the substitution of the charged residues with citrullines. First, it is possible that neutralization of the cationic C-terminal segment lowered the free energy of peptide desolvation mentioned above, and effectively decreased the free energy of the peptide association. ΔG associated with the water-to-interface transfer of citrulline is not reported but such a value is typically lower for neutral residues like glutamine (2.4 kJ/mol) or asparagine (1.8 kJ/mol) than for cationic arginine (3.4 kJ/mol) and lysine (4.2 kJ/mol) [60]. Such contributions would be consistent with the more negative ΔG of the bilayer association found for Cit-Mel compared to that for melittin. Second, the positive membrane surface potential associated with the presence of bound melittin has been proposed to create a melittin

concentration gradient, from the bulk to the interface, as a response for intermelittin electrostatic repulsion [36, 56]. The reduced melittin concentration close to the bilayer interface was proposed to reduce the apparent binding of melittin [56]. Such an interpeptide repulsion was also proposed to affect the binding of cecropins and may be a general feature of the association of CAPs to membranes [61]. In the case of Cit-Mel, the neutralization of the C-terminal charges would considerably decrease the impact of this phenomenon and would increase the apparent binding constant, as observed here. The present results therefore reveal that the C-terminal charges of melittin reduce the affinity of the peptide for neutral membranes. These conclusions are in contradiction with those inferred from the SPR binding studies concluding that truncated amidated melittin-21Q exhibits a lower affinity than melittin for DMPC membranes [42] or that both peptides bind to the same extent to DMPC bilayers [43]. These SPR experiments were conducted on supported bilayers and the nature of the interactions with the peptides, particularly regarding the peptide insertion, is probably different.

As a matter of fact, the association of Mel and Cit-Mel with negatively charged bilayers included attractive electrostatic interactions. Melittin (charge +6) and Cit-Mel (charge +2) both displayed a greater association with DPPC/DPPS bilayers than with DPPC ones (Fig. 3-1); this behavior is likely due to attractive electrostatic interactions between the negative surface charge of the membranes and the cationic peptides, as previously demonstrated for other anionic membranes [38, 57].

The ΔG_{bind} (Table 3-1) of melittin increased by 28% when 30 (mol)% DPPS were included in DPPC bilayers, whereas the augmentation was 16% for Cit-Mel; these differences highlight the fact that the extent of increase was dependent on the peptide charge. However, despite the fact that negatively charged membranes led to larger attractive electrostatic interactions, it appeared that hydrophobic interactions remained a prevailing contribution as Cit-Mel still displayed greater affinity for DPPC/DPPS bilayers than native melittin. It must be noted that truncated Mel1-22 and Mel1-20 (net charge +4 and +2) displayed also a greater association with POPC/POPG 70/30 membranes than melittin ([44], Supplementary Material). The present findings suggest that the C-terminal charges of melittin actually play two roles in membrane association. They improve the association to anionic membranes by attractive electrostatic interactions. In addition, they reduce the peptide affinity for neutral membranes by counterbalancing the favorable hydrophobic interactions associated with the penetration of apolar segments of the peptide in the bilayer core with an unfavorable desolvation energy of the cationic C-terminal. These combined contributions are proposed to play a central role in the cell selectivity of melittin towards bacterial cell membranes compared to mammalian cell membranes [62, 63]. This fine modulation of affinity should be investigated for other CAPs in order to establish whether this phenomenon is a general feature.

3.5.2 Electrostatic effect on lipid extraction

Proposed mechanisms for the lipid extraction by melittin generally include two steps: first, the binding of the peptide to the bilayer interface, and second, its relocation in a transmembrane position that would cause the fragmentation of a part of the bilayer if the peptide amount is sufficient [35, 64]. The details of the mechanism of membrane fragmentation occurring upon the relocation of the peptide are not well identified, but at least 3 phenomena have been suggested to trigger this relocation. First, the relocation is observed when the peptide reaches a critical/limit interfacial concentration. The insertion of melittin in bilayers causes their thinning [64, 65], leading to a membrane tension. Hence, as more peptide molecules are inserted at the bilayer interface level, the tension is increased. Eventually, a critical peptide concentration is reached, and melittin then relocates from the interface to a transmembrane position. This change is proposed to lead to the creation of membrane defects and leaks and, at high peptide concentrations, it would cause the membrane fragmentation. The second trigger is the fluid-to-gel phase transition. Cooling the membrane to the more ordered gel phase reduces its capacity to accommodate melittin at the interface level and, as a consequence, induces the redistribution of the peptide towards the apolar core [33, 66-69]. Third, melittin relocation from the interface can also be triggered by electrostatic repulsions at the interfacial level. For example, the presence of positively charged 1,2-dipalmitoyl-3-(trimethylammonium)propane (DPTAP) in DPPC membranes has been shown to promote melittin-induced lipid extraction [35]. Accordingly, it was proposed that the enhanced activity of melittin was due to the electrostatic repulsion

between melittin and DPTAP, thus causing a disfavored interfacial location of the peptide and a deeper insertion in the membrane, and thereby resulting in bilayer fragmentation. Similarly, intermelittin electrostatic repulsion was also proposed as a trigger for the change in peptide location [70].

The present results show that the substitution of the 4 positively charged C-terminal K^{21}-R^{22}-K^{23}-R^{24} residues with the neutral polar amino acid citrulline decreased the lipid extraction potential of melittin from neutral DPPC membranes. This inhibition highlights the fact that there is no direct relationship between the extent of lipid extraction by a peptide and its affinity for a particular membrane since Cit-Mel showed a greater association than melittin for DPPC membranes. Therefore, the reported reduction of lipid extraction potential upon citrullination of melittin is not due to a smaller number of bound (active) melittin but results from a reduced ability of the peptide to cause membrane fragmentation. It was mentioned above that the electrostatic repulsion between melittin bound at a bilayer interface could lead to the peptide relocation in a transmembrane conformation and, consequently, to the bilayer fragmentation. The fact that Cit-Mel possesses a decreased net charge should reduce the interpeptide repulsion. It is then possible that the critical number of bound peptide molecules leading to relocation is higher for Cit-Mel than for native melittin.

Negatively charged phospholipids are known to inhibit melittin-induced lipid extraction [23, 35, 37, 40, 41]. The attractive electrostatic interactions between melittin and

the lipid head groups have been shown to prevent the relocation of the peptide by anchoring it to the interface, thereby significantly reducing the lipid extraction. However, the present results indicate that Cit-Mel is more efficient for the fragmentation of anionic membranes compared to native melittin (Fig. 3-2 and 3-3B). Like melittin, Cit-Mel affinity for bilayers was increased by the presence of DPPS (Fig. 3-1B and Table 3-1). Therefore, for any given L/P, the proportion of bound peptide was larger for DPPC/DPPS than for DPPC bilayers. However, in the conditions used for the fragmentation (lipid/peptide ratios > 20), most Cit-Mel peptides were membrane-bound, even when the LUVs were prepared exclusively from DPPC. The observed increase in Cit-Mel-induced fragmentation caused by the presence of anionic lipids cannot be rationalized on the basis of the electrostatic interactions discussed above. In fact, the anchoring of the peptide at the bilayer interface via negatively charged lipids in membranes, and the resulting inhibition of the membrane fragmentation, should be less significant for Cit-Mel. Hence, the observed increase of fragmentation must imply particular phenomena. It is possible that a positive intrinsic curvature of the bilayer, provided by electrostatic repulsion between anionic phospholipids at the head group level, lowers the energy of penetration of Cit-Mel, as proposed for surfactins [71]. Combined with the absence of anchoring effect, this would rationalize the increased activity of Cit-Mel on anionic membranes compared to native melittin. Consequently, these results demonstrate that the extent of the membrane fragmentation can be regulated by the electrostatic

interactions, which are modulated by the charges carried by both membranes and the lytic peptides.

3.5.3 Lipid extraction specificity

The present work reveals the absence of specificity in the lipid extraction from anionic membranes induced by melittin: melittin extracted the same proportions of DPPC and of DPPS from membranes made of binary mixtures of these lipids. The same absence of specificity was observed for Cit-Mel. This absence of specificity was somehow unexpected given the strong electrostatic attraction between melittin and DPPS. Recently, it was shown that melittin-induced lipid extraction from PC/PE bilayers was specific. For example, the PC/PE ratio increased from 1/1 in the original membranes to up to 6/1 in the small particles resulting from the membrane fragmentation [50]. It was then proposed that this specificity was due to a local DPPC enrichment near the bound melittin molecules, a consequence of the stronger lipid-lipid interactions of PE relative to those of PC. Melittin would extract lipids from this PC-enriched environment, leading to PC-enriched fragments. Incubation of melittin with DPPC/cholesterol bilayers also led to PC-specific lipid extraction [72] and a similar mechanism was proposed. The absence of specificity in the lipid extraction by melittin with DPPC/DPPS bilayers suggests that there was no local enrichment of the anionic lipid species in the environment of the adsorbed peptide. Actually, two studies using either Raman spectroscopy [73] or ^{2}H-NMR [41] have reported the absence of lipid phase separation upon melittin insertion in DPPC/DPPG or DMPC/DMPS

bilayers. A putative homogeneous lipid distribution in melittin surroundings in anionic membranes would be consistent with the absence of lipid selectivity in the fragmentation process reported here.

Our current knowledge of the lipid selectivity in membrane fragmentation by CAPs is very limited. The present work on the bilayer activity of melittin and citrullinated melittin emphasizes the complex relation between affinity, lipid extraction/membrane fragmentation and lipid specificity. It is shown that an augmented affinity for membranes via neutralization of cationic C-terminal can lead either to an increased (on anionic membranes) or a decreased (on zwitterionic membranes) lipid extraction activity. It is also shown that lipids inducing a stronger binding via electrostatic interactions do not necessarily lead to lipid specificity in the extraction process. Other CAPs have been found to extract lipids from phospholipid membranes, including magainin [74], δ-lysin [75-78], and aurein [79]; the formation of bilayer fragments at high peptide concentrations has been proposed as a general consequence of the carpet mechanism [75]. Indeed, the incubation of phospholipid vesicles with one of these peptides gave rise to a narrow signal in ^{31}P-NMR, indicating that, like melittin, they extracted phospholipids from bilayers to form small fast-tumbling particles. Furthermore, it was proposed that magainin [74] and δ-lysin [76] formed bicelles out of PC bilayers, as does melittin. Interestingly, magainin was shown to be unable to fragment 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) anionic bilayers [74]. Also, following a study using three histidine-containing

amphipathic helical CAPs, it was proposed that their penetration in anionic bilayers was reduced due to anchoring electrostatic interactions at the interface [80]. These reports, including the present work, suggest that a partial neutralization of the cationic residues of CAPs would modulate their membrane fragmentation capacity. Therefore, the study of a wider selection of CAPs needs to be carried out in order to precisely establish the role played by electrostatics in the mechanism of CAP-induced bilayer fragmentation.

3.6 Acknowledgments

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3.8 Supplementary material

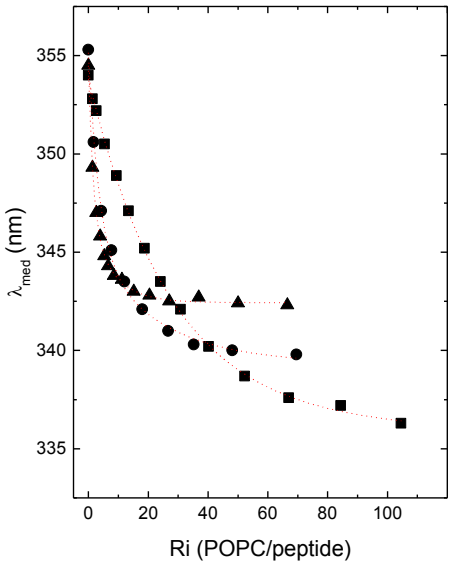

Figure 3-5: Affinity of the peptides for POPC vesicles, as reported by the fluorescence shift of Trp[19]. Melittin (■), Mel_{1-22} (●) and Mel_{1-20} (▲). (From ref. 44).

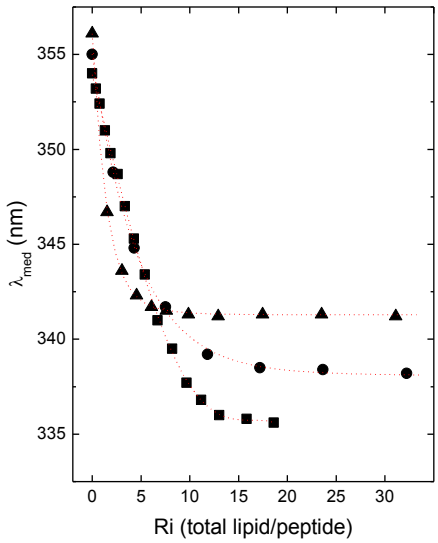

Figure 3-6: Affinity of the peptides for POPC:POPG (70:30) vesicles, as reported by the fluorescence shift of Trp[19]. Melittin (■), Mel_{1-22} (●) and Mel_{1-20} (▲). (From ref. 44).

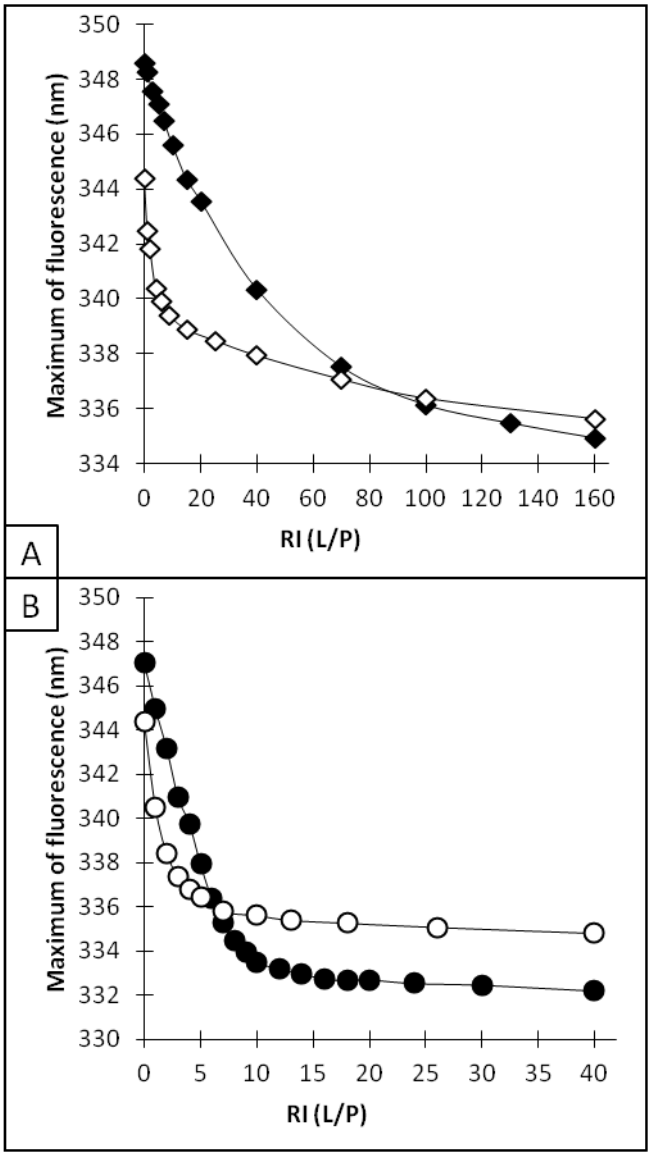

Figure 3-7: Evolution of tryptophan fluorescence of melittin (full symbols) and of citrullinated melittin (empty symbols), characteristic of melittin association with DPPC (A) or DPPC/DPPS 70/30 (B) bilayers at 65°C.

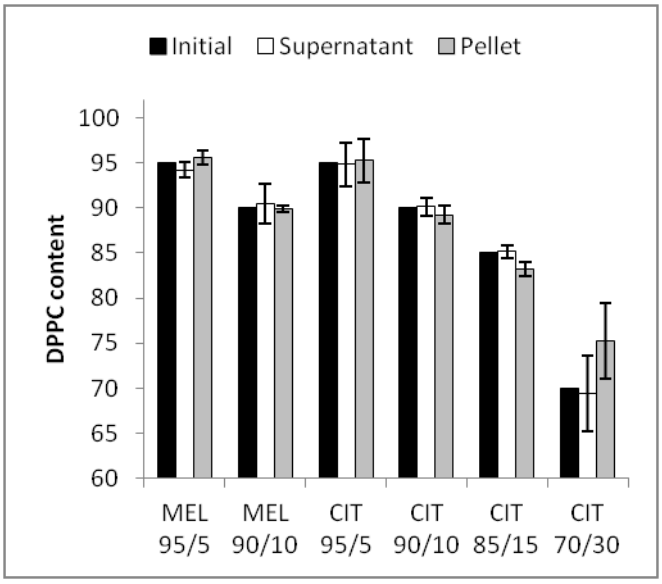

Figure 3-8: DPPC content as % of total lipids in lipid extraction experiments before and after incubation and centrifugation with melittin or Cit-Mel at P/L=50x10^{-3}. The initial DPPC/DPPS ratios of the liposomes are indicated under the x-axis.

Chapitre 4 - Chemical and Physical Requirements for Lipid Extraction by Bovine Binder of Sperm BSP1

Alexandre Therrien, Puttaswamy Manjunath, Michel Lafleur, *Biochimica et Biophysica Acta-Biomembranes.* **2013,** 1828, 543-551.

4.1 Abstract

The bovine seminal plasma contains phosphocholine-binding proteins, which associate to sperm membranes upon ejaculation. These binder-of-sperm (BSP) proteins then induce a phospholipid and cholesterol efflux from these membranes. In this work, we determined physical and chemical parameters controlling this efflux by characterizing the lipid extraction induced by BSP1, the most abundant of BSP protein in bull seminal plasma, from model membranes with different composition. The model membranes were formed from binary mixtures of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) with 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) or cholesterol. The modulation of BSP1-induced lipid extraction from membranes by their chemical composition and their physical properties brings us to propose a 3-step extraction mechanism. First, the protein associates with membranes via specific binding to phosphocholine groups.

Second, BSP1 penetrates in the membrane, essentially in the external lipid leaflet. Third, BSP1 molecules solubilize a lipid patch coming essentially from the outer lipid leaflet, without any lipid specificity, to ultimately form small lipid/protein auto-assemblies. The stoichiometry of these complexes corresponds to 10–15 lipids per protein. It is also shown that fluid-phase membranes are more prone to BSP1-induced lipid extraction than gel-phase ones. The inhibition of the lipid extraction in this case appears to be related to the inhibition of the protein penetration in the membrane (step 2) and not to the protein association with PC head groups (step 1). These findings contribute to our understanding of the mechanism by which BSP1 modify the lipid composition of sperm membranes, a key event in sperm capacitation.

4.2 Introduction

The bovine seminal plasma contains proteins that bind to sperm membranes upon ejaculation [1-3]. These bovine Binder-of-SPerm (BSP) proteins have been shown to be necessary for sperm capacitation [4]. They consist of 3 different proteins: BSP1 and BSP3, with molar weight around 13 kDa, and BSP5, with a 30-kDa mass [4-6]. BSP1, the most abundant of them, accounts for 25 (w/w)% of all the proteins in bull seminal plasma [7, 8]. It has been shown that, after binding to sperm, BSP1 induces a cholesterol and phospholipid efflux from their plasma membranes [4, 8-10]. This action leads to a modification of the membrane lipid composition, a phenomenon that is believed to play a key role in sperm maturation. Specific tuning of the

cholesterol/phospholipid ratio of the sperm membrane is an essential step to the acrosomal reaction [11-13], likely involving a reorganization of membrane lipid domains or lipid rafts [4, 11, 12, 14]. Moreover, BSP1 has recently been shown to function as a molecular chaperone[15, 16].

Several studies have led to the conclusion that BSP1 binds to spermatozoa via a specific interaction with phosphatidylcholines (PC) in sperm plasma membranes. The 109-amino acid sequence of BSP1 includes two fibronectin type II (Fn2) domains that are responsible for binding the choline head groups [17-21]. A protein structure that includes one choline group per Fn2 domain has been determined by X-ray crystallography [20, 22]. The structure reveals that the interactions between the ammonium moiety of the choline group and the π electrons of the indole ring of Trp-47 or Trp-106, and an H-bond between the oxygen of the choline phosphate group and the hydroxyl group of nearby tyrosines (Tyr30 and Tyr54, or Tyr75 and Tyr100) account for the choline binding specificity of the protein. As reviewed elsewhere [23], the specific affinity of BSP1 for PC has been confirmed from several approaches including Electron Spin Resonance (ESR) [24], protein intrinsic fluorescence experiments [25, 26], Surface Plasmon Resonance (SPR) [27], ^{31}P NMR [28] and Isothermal Titration Calorimetry (ITC) [29, 30]. These experiments, carried out with model lipid membranes, established the protein binding to PC membranes and indicated a reduced affinity of BSP1 for membranes containing phosphatidylethanolamine (PE), phosphatidylglycerol (PG) or phosphatidylserine (PS) [24, 25, 27]. It was also reported

that the binding capacity of these model PC membranes was 1 protein for about 10-12 lipids [24-26, 29].

Bovine sperm membranes contain cholesterol; the cholesterol to phospholipid molar ratio is estimated to 0.38 [31]. The impact of cholesterol on the binding of BSP1 to membranes is still controversial. Some studies reported no direct interaction between BSP1 and membrane-inserted cholesterol [17, 32, 33], whereas a recent publication identified specific cholesterol recognition amino acid consensus (CRAC) regions in BSP1, suggesting a direct intermolecular association of the protein with cholesterol [34]. It was reported that the presence of cholesterol in phospholipid membranes increases the affinity of BSP1 [35], decreases the binding capacity and the binding molar energy of the protein to PC membranes [29], or has no effect on BSP1 binding to PC membranes [26]. Cholesterol was shown to hinder BSP1-induced leakage from PC vesicles [26].

Once bound, BSP1 induces lipid efflux from sperm membranes. It was found that the extracted lipids are essentially PC [9, 36], sphingomyelin (SM) [36], and cholesterol [37]; phospholipids bearing different head groups, such as PE and PS, were not significantly extracted from sperm membranes [36]. It has been shown that BSP1 was more efficient to extract PC from the external leaflet of sperm membranes relative to the inner-leaflet PC. It was then suggested that this extraction preference towards the outer leaflet lipids was one of the factors explaining why BSP1 would not

considerably extract PE and PS from sperm membranes as these are mainly found in the inner leaflet [36].

The lipid extraction induced by BSP1 is not limited to sperm membranes but has also been reported for other cells. For example, BSP1 similarly produces lipid extraction from fibroblasts cells; the lipids are extracted with a molar ratio of 20 choline phospholipids and 24 cholesterols per protein [32]. Having previously shown that BSP1 exhibits no direct interaction with cholesterol and that free choline in solution inhibits the extraction, the authors speculated that cholesterol and phospholipids are concomitantly extracted from the membrane once BSP1 is bound to choline binding sites [38]. BSP1 also extracts lipids from human erythrocytes. It was found that mainly PC and SM were extracted by the protein but the efflux contained some PS and a very limited amount of PE [36].

The study of lipid extraction by BSP1 on model systems can provide a better understanding of the physico-chemical features that modulate this phenomenon. However, even though model membranes have been widely used to characterize the affinity of BSP1 for membranes, the potential of this approach, up to now, has not been effectively exploited for studying lipid extraction. In the sparse literature on the topic, it was shown that BSP1 can induce lipid extraction from PC membranes [24, 36]. With an incubation lipid-to-protein molar ratio of 20, 10% of the lipids were extracted from egg yolk-PC multilamellar vesicles (MLVs) [36]. Recently, it was

reported that BSP1 could solubilize 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (DMPG) MLVs, a phenomenon that may be associated with an ultimate form of lipid extraction. It was also shown that the presence of 25 mol % cholesterol in DMPC membranes did not affect BSP1-induced membrane solubilization [24] whereas 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) MLVs were found resistant to this solubilization [30].

The goal of this work was to determine the chemical and physical membrane parameters controlling lipid extraction induced by BSP1 in order to gain insights into the mechanism of this vital phenomenon. The BSP1-induced lipid extraction was characterized for a variety of membrane model systems composed of different binary mixtures for which the chemical composition, as well as the physical properties, was modulated. Since choline phosphoglycerides are the most abundant lipid species in bull sperm membranes [31], 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was used as the core of these model membranes. Various proportions of POPC were substituted by other species to examine the impact of different features. In order to determine the influence of lipid phase propensities on BSP1-lipid interactions, micelle-forming 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Lyso-PC) or gel-phase inducing 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) has been included in POPC membranes. The specific requirement of the choline head group has been investigated by substituting POPC by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-

phosphoethanolamine (POPE), the negatively charged 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) or cholesterol. Phosphatidylserine is the most abundant anionic phospholipid in sperm membranes [39].

The lipid extraction was produced by mixing model membranes with BSP1 and then subjecting them to freeze-and-thaw cycles, in a similar fashion to other previous studies [24]. This procedure was essential to expose the protein to all of the lipids within the MLVs, since BSP1 has shown limited ability to translocate membranes and reach their inner leaflet [36]. This approach also provided a convenient way to isolate the extracted lipids since, after centrifugation, they were found in the supernatant associated with BSP1 whereas the remaining MLVs were pelleted. The freeze-and-thaw cycles were indeed an integral part of the lipid extraction processes. Therefore this study examines the extractive properties of the protein within a procedure that includes freeze-and-thaw cycles. Nevertheless, this *in vitro* lipid extraction is believed to be influenced and be representative of the same lipid–protein intermolecular interactions that are controlling the *in vivo* process. The ability of the protein to extract lipids and the extraction selectivity were determined for these various systems.

4.3 Materials and Methods

4.3.1 Chemicals

BSP1 was purified according to previously established protocols [1]. POPC, lyso-PC, POPE, DPPC, and POPS were purchased from Avanti Polar Lipids. POPC and lyso-PC were also obtained from Northern Lipids (Vancouver, Canada), and Sigma (St-Louis, Missouri), respectively. Cholesterol, ethylenediaminetetraacetic acid (EDTA), NaCl, and 3-[N-Morpholino]propanesulfonic acid (MOPS) were obtained from Sigma. All chemicals were used as received.

4.3.2 Extraction

First dissolved in a benzene/methanol mixture (90/10 (v/v)), individual lipids were mixed to obtain the desired molar ratio and then lyophilized. The lipid powders were hydrated in a MOPS buffer (50 mM) containing 100 mM NaCl and 100 μM EDTA. They were submitted to 3 freeze-and-thaw cycles (from liquid nitrogen temperature to 50 °C) to form MLV suspensions. BSP1 was separately dissolved in the same buffer and quantified by absorbance using a molar absorptivity coefficient of 36,000 M^{-1} cm^{-1} at 280 nm [40]. The lipids and the protein were mixed in microcentrifuge tubes to obtain 1 mg/ml of each species(providing a lipid/protein molar ratio between 17 and 20, depending on the nature of the lipids), unless stated otherwise. The suspensions were then frozen-and-thawed 3 times in order to favor the

homogeneity of the samples. These cycles were carried out from liquid nitrogen (until the sample was frozen) to a temperature at which the lipid membranes were in the fluid state (namely room temperature or 50 °C) for a 20-minute period. In the case of DPPC-containing samples, a series was thawed at room temperature while a parallel series was heated to 50 °C. After incubation, samples were centrifuged for 5 minutes at 20 800 g and 4 °C. Centrifugation of control samples (without BSP1) showed that more than 95% of lipids were found in the pellets, except for some POPC/Lyso-PC mixtures as discussed below. Supernatants were isolated and pellets were resuspended in MOPS buffer. Experiments were carried out in triplicates.

4.3.3 Lipid Analysis

The phospholipid contents in the supernatants and in the pellets were determined by Bartlett phosphorus assay [41]. The cholesterol content in the samples was quantified by HPLC-MS, using the method and the conditions specified in the extraction selectivity section. The extraction was then calculated as

$$\textbf{Extraction } \% = \frac{\boldsymbol{lipids\ in\ supernatant}}{\boldsymbol{total\ lipids}}.$$

4.3.4 Extraction selectivity

The lipid composition of the supernatants as well as of the pellets was determined by HPLC-MS analysis carried out using an Agilent Technologies 1100 series system including a 1100 MSD mass spectrometer. Samples were eluted on a Zorbax column (2.1 x 30 mm, 3.5 µm particle size) (Agilent Technologies), maintained at 50 °C. The following elution gradient was used: from methanol/water (0.1% formic acid) 90/10 to 100/0 over 1 minute, then methanol for 5.5 minutes except in the case of Lyso-PC-containing samples where the initial conditions were 80/20 methanol/water (0.1% formic acid). An ESI source was used in the positive ionization mode. Nitrogen was used as drying gas at 250 °C and 12 l/min. Nebulizing gas was also nitrogen, held at 35 psig. Analysis was conducted in SIM mode with a dwell time of 290 ms. Extraction selectivity, or preferential extraction of POPC over other lipids, is reported using the "POPC enrichment factor", calculated as:

$$\boldsymbol{POPC\ enrichment\ factor} = \frac{\boldsymbol{Extraction\ \%\ of POPC}}{\boldsymbol{Extraction\ \%\ of\ the\ other\ lipidic\ species}}.$$

For each lipid species, $\boldsymbol{Extraction\ \%} = \frac{\boldsymbol{A_s}}{\boldsymbol{A_p + A_s}}$, where A_p and A_s are the lipid peak area from the pellet and the supernatant analysis, respectively.

4.3.5 Protein analysis

BSP1 was first quantified using fluorescence. Fluorescence intensities (λexcitation = 283 nm) at the maximum of the spectra of the supernatant and of the

pellet were directly compared to evaluate the protein partitioning. The binding of the protein was characterized by:

$$\textit{Proportions of liposome-bound protein} = \frac{I_p}{I_p + I_s},$$

where I_p and I_s are the fluorescence intensity from the pellet and the supernatant respectively. These values do not take into account any fluorescence amplification due to BSP1-lipid binding [25]; this approximation has however a very limited impact because, for most systems, BSP1 was found almost exclusively in the supernatants. The measurements were recorded using a PTI fluorometer (band widths for the excitation and emission monochromator were 1.8 and 3.6 nm, respectively). The sampling cell was kept at 20 °C. Controls with the protein in the absence of lipid incubated according to the same protocol as for the samples (i.e. frozen then thawed at room temperature or at 50 °C) led to no pellet after centrifugation and showed no change in the protein fluorescence spectra, indicating that the incubation conditions did not induce significant changes of the protein structure.

The composition of the extracted materials is given by:

$$\textbf{Lipid/protein molar ratio in efflux} = \frac{\textbf{supernatant lipids (mol)}}{\textbf{supernatant protein (mol)}},$$

where "S*upernatant lipids"* were assayed using the Bartlett assay, as stated above, whereas "S*upernatant protein"* was determined as (1 - "*Proportion of liposome-bound protein"*) multiplied by the total amount (mol) of BSP1.

4.4 Results

4.4.1 Extraction

The lipid extraction by BSP1 was first quantified for different protein concentrations with pure POPC or POPC/POPE (50/50 (mol)) liposomes (Figure 4-1).

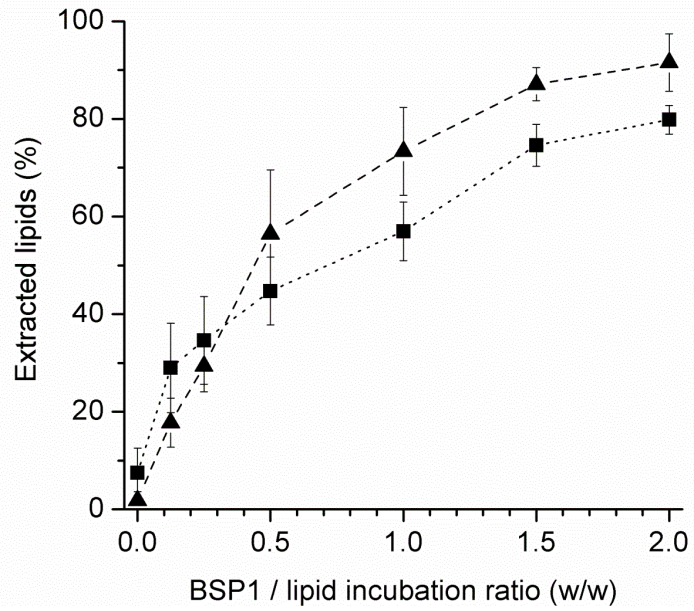

Figure 4-1 : Quantification of lipid extraction after incubation of BSP1 with MLVs of pure POPC (■) or POPC/POPE (50/50 mol) (▲). BSP1/lipid mass ratios of 0.5, 1.0 and 2.0 correspond to lipid/protein molar ratios of about 36, 18 and 9, respectively. Each point is the mean value of 3 experiments; error bars represent standard deviations.

As expected, BSP1 could extract lipids from POPC model membranes and the fraction of extracted lipids increased with BSP1 concentration. When the BSP1/lipid (w/w) ratio reached 2, between 80 and 90% of liposomes were disrupted. For a ratio of 1 (lipid/protein molar ratio of 18), about 60% of the lipids were extracted by the protein. This protein content was used in subsequent experiments for examining the influence of lipid composition on the lipid extraction. The results showed a similar behavior for POPC/POPE MLVs except that, for a protein/lipid weight ratio of 0.5 (lipid/protein molar ratio of ~ 36) and above, BSP1 extracted about 20% more lipids from POPC/POPE MLVs compared to pure POPC MLVs.

4.4.2 Effect of the phase

The effect of the lipid phase propensities on the lipid extraction was characterized by preparing MLVs with binary mixtures of phosphatidylcholines (Figure 4-2).

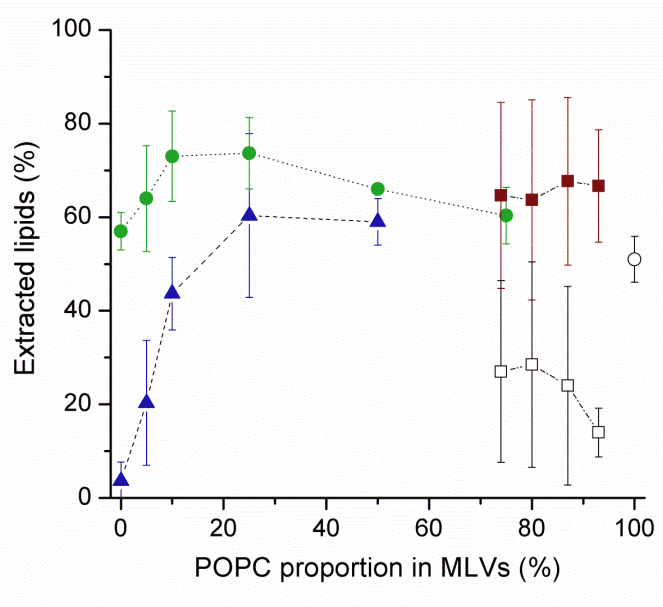

Figure 4-2 : Quantification of lipid extraction after incubation of BSP1 with MLVs of POPC/Lyso-PC (■), POPC/DPPC incubated at 50 °C (●) or at room temperature (▲) or pure POPC (○). Controls of POPC/Lyso-PC MLVs without BSP1 are also shown (□). The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 19 depending on the lipid mixture. Each point is the mean value of 3 experiments; error bars represent standard deviations.

A proportion of micelle-forming Lyso-PC or of gel-phase inducing DPPC was included in POPC model membranes. It is reported that the micelle-forming Lyso-PC is practically completely inserted in POPC membranes for proportions lower than 35 mol% [42]. However, our centrifugation conditions could not pellet all the lipids in the control samples (i.e. without BSP1), suggesting that a fraction of these lipids formed micelles. Between 10 to 30% of the lipids were found in the supernatant after

centrifugation as indicated in Figure 4-2. Therefore, the lipid extraction reported for LysoPC-containing mixtures may actually be a combination of the lipid micelles that could not be pelleted (but that may interact with BSP1) and BSP1-extracted lipids. The presence of up to 25 mol % of Lyso-PC in POPC MLVs did not appear to influence significantly the lipid extraction induced by BSP1. For these MLVs, about 70% of lipids were extracted.

POPC/DPPC membranes exist in the fluid or gel phase, depending on lipid composition and temperature. The temperature of the main gel-to-liquid crystalline phase transition is 41 and -2 °C for pure DPPC and pure POPC respectively. At room temperature, the phase diagram [43] predicts exclusively gel-phase membranes below 25% POPC, fluid-phase membranes above 60% POPC, and phase coexistence in between these proportions. For all POPC/DPPC MLVs, including those formed exclusively of DPPC, BSP1 achieved a lipid extraction equivalent or slightly greater compared to that obtained with pure POPC MLVs, when the samples were incubated at 50 °C, i.e. when the membranes, irrespectively of their composition, were in the fluid phase. The situation was however different when the protein-lipid mixture was incubated at room temperature. Under these conditions, pure DPPC MLVs were in the gel phase and, in this case, no lipid extraction was observed. When the POPC proportion increased from 0 up to 25 (mol) % in the POPC/DPPC MLVs, the lipid extraction also increased. It became similar to the one obtained for samples incubated

at 50 °C when the POPC proportion exceeded about 25%, a composition for which there is a fluid-gel phase coexistence [43].

The partitioning of BSP1 between the aqueous phase and the remaining (pelleted) MLVs was determined. For the POPC/Lyso-PC and the POPC/DPPC MLVs that were incubated with the protein in conditions leading to fluid membranes, BSP1 was exclusively present in the supernatant (Figure 4-3).

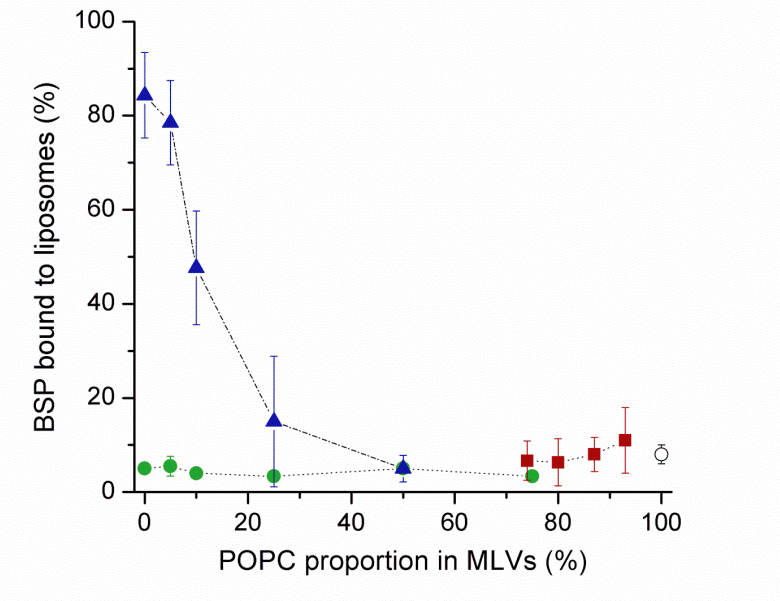

Figure 4-3: Quantification of BSP1 bound to MLVs of POPC/Lyso-PC (■), POPC/DPPC incubated at 50 °C (●) or at room temperature (▲) or pure POPC (○). The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 19 depending on the lipid mixture. Each point is the mean value of 3 experiments; error bars represent standard deviations.

In contrast, the protein was mainly found in the pellet with DPPC liposomes when the samples were incubated at room temperature. It appeared that the protein could bind to DPPC MLVs in gel phase but could not extract lipids to form small self-assemblies that remain in the supernatant after centrifugation. Under these conditions (room temperature incubation), the proportion of MLV-bound BSP1 decreased with the increased proportion of POPC in DPPC MLVs, in parallel with the increased extraction reported in Figure 4-2. When POPC content exceeded 25%, where the formed MLVs were partially or completely in a fluid phase [43], essentially no protein was found in the pellet, as observed for these systems when they were incubated at 50 °C. These results established that the inhibition of the lipid extraction observed for DPPC-rich MLVs incubated at room temperature is associated with the lipid gel phase.

The composition of the self-assemblies found in the supernatant, resulting from the BSP1-induced lipid extraction, could be inferred from these analyses. It was calculated that the lipid/protein molar ratio was about 10 for pure POPC MLVs if one assumes that all the proteins and all the lipids in the supernatant are included in the mixed self-assemblies resulting from the lipid extraction (Figure 4-4).

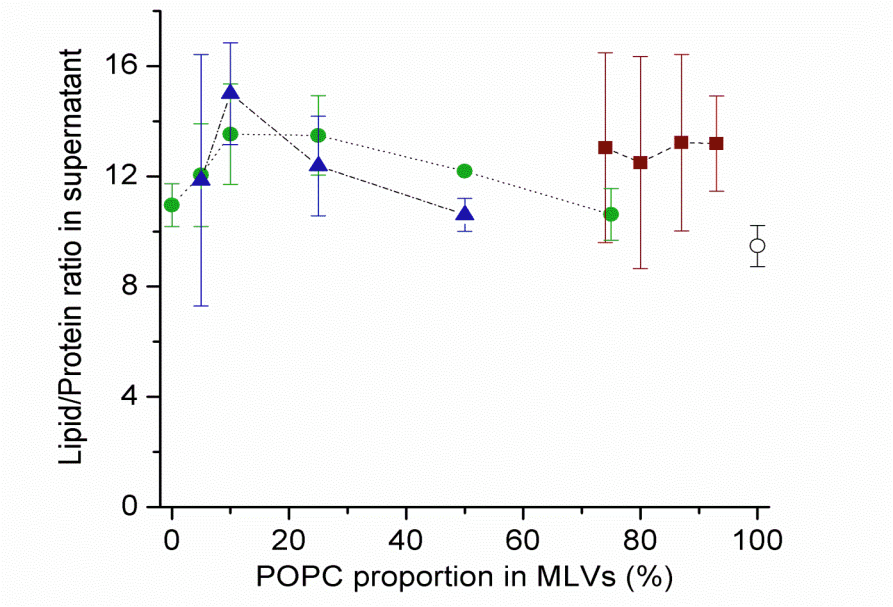

Figure 4-4 : Lipid/BSP1 molar ratio in the supernatant after BSP1 incubation with MLVs of POPC/Lyso-PC (■), POPC/DPPC incubated at 50 °C (●) or at room temperature (▲) or pure POPC (○). The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 19 depending on the lipid mixture. Each point is the mean value of 3 experiments; error bars represent standard deviations.

BSP1 incubation with POPC/Lyso-PC mixtures gave rise to lipid/protein ratios of about 13. In that case, it is probable that some of the supernatant lipids were in micellar form and not necessarily bound to proteins. POPC/DPPC liposomes, incubated in the presence of BSP1 at 50 °C and thus in the fluid phase, led to extracted lipid/BSP1 ratios between 10 and 15. The lipid/protein self-assemblies found in the supernatant after incubation at room temperature of mixtures containing between 5% and 25% DPPC with BSP1 were characterized by a phospholipid/BSP1

ratio between 12 and 15, these values being similar to those observed when the mixtures were incubated at 50 °C. It should be noted that very little lipids and protein were found in the supernatant in the case of pure DPPC MLVs incubated with BSP1 and, as a consequence, the lipid/protein ratio value in the supernatant could not be determined.

We analyzed the lipid composition of the BSP1-extracted phase found in the supernatant. The variations of the lipid composition relative to that of the initial liposomes are expressed in terms of POPC enrichment in the extracted fraction. It was found that BSP1 did not show any extraction selectivity for mixtures of POPC with either Lyso-PC or DPPC when the samples were incubated in the fluid phase (Figure 4-5).

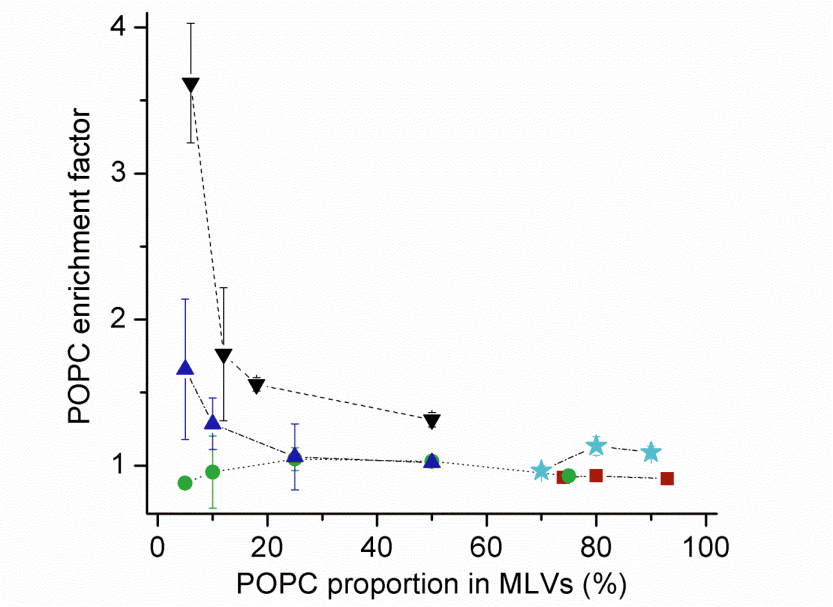

Figure 4-5: BSP1 extraction selectivity for POPC after incubation with POPC/Lyso-PC (■), POPC/DPPC at 50 °C (●) or at room temperature (▲), POPC/POPE (▼), or POPC/Chol (*) MLVs. POPC enrichment factor is given as the POPC extraction % divided by the second species extraction %. The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 20 depending on the lipid mixture. Points below 60% POPC are mean values of 3 experiments, points above 60% POPC are mean values of 2 experiments; error bars represent standard deviations.

Despite the different phase propensities of these phosphatidylcholines, the extracted lipids were representative of the initial liposome composition. In the case of POPC/DPPC MLVs incubated with BSP1 at room temperature, it appeared that a slightly greater proportion of POPC was extracted by the protein than that of DPPC,

for mixtures with 90-95% DPPC. This finding reinforces the hypothesis that BSP1 is less efficient to induce extraction from gel-phase membranes.

The position of the maximum of the intrinsic fluorescence band of BSP1 is sensitive to the polarity of the environment of the fluorescent amino acid side chains and, as a consequence, varies upon the binding of the protein to a membrane. For example, it was shown to vary for the free and the POPC-bound BSP1 from 342 to 333 nm [25]. The position of the fluorescence maximum of BSP1 in the supernatant was measured to assess its binding to a lipid phase. Our measurements indicate that the emission maximum of BSP1 in the supernatant after incubation with the lipid mixtures was ~331 nm for most systems (Figure 4-6).

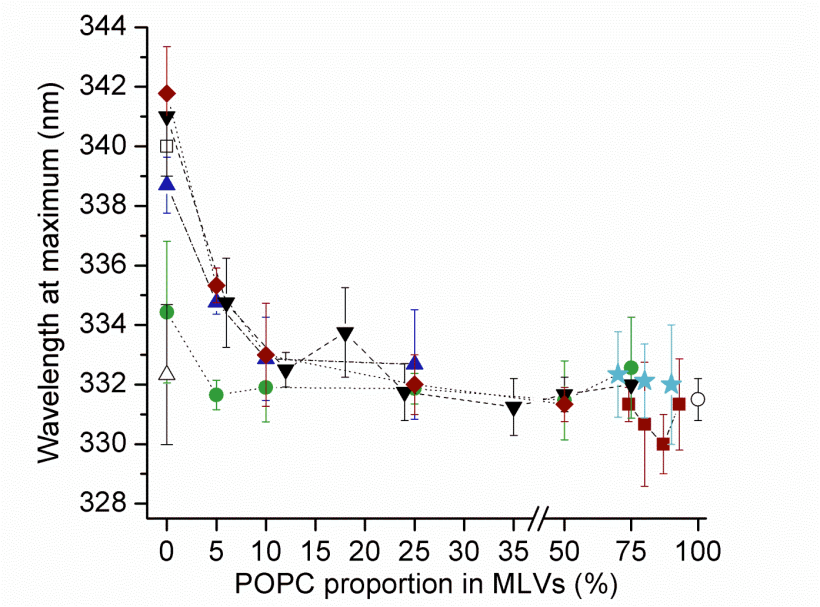

Figure 4-6: Wavelength of maximum BSP1 fluorescence in supernatant after incubation with MLVs of POPC/Lyso-PC (■), POPC/DPPC at 50 °C (●) or at room temperature (▲), POPC/POPE (▼), POPC/Chol (*), POPS (♦), or pure POPC (○). Protein fluorescence in the pellet is shown for DPPC incubated at room temperature (△). BSP1 solution has been measured as a control (□). The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 20 depending on the lipid mixture. Each point is the mean value of 3 experiments; error bars represent standard deviations.

These results indicated a similar binding of the proteins to the various lipid self-assemblies. In the case of the protein incubated with pure DPPC MLVs, the fluorescence maximum appeared at a slightly higher value, 334 nm. It is possible that the structure of the BSP1/DPPC complexes is somewhat different as all the lipid chains are saturated and this could lead to different environments of the tryptophans

and tyrosines. It should be noted that a small shift towards longer wavelengths was observed when BSP1 was incubated at room temperature with POPC/DPPC MLVs containing 90% of DPPC or more. It was shown that, with incubation at room temperature, there was practically no lipid extraction with DPPC MLVs: less than 20% (see Figure 4-2), and that most of the protein was associated with the pelleted MLVs (see Figure 4-3). The emission maximum of the protein in the supernatant was 340 nm, a value in agreement with the conclusion that BSP1 is free in solution in the supernatant. The BSP1 fluorescence maximum measured for the resuspended pellet was 332 nm (see Figure 4-6), confirming that the protein was bound to the remaining MLVs and was dragged with them in the pellet during the centrifugation. When the protein was incubated at room temperature with POPC/DPPC (5/95) MLVs, the protein fluorescence maximum was 334 nm. At this point, it is not clear whether this value slightly different to those observed for the other complexes is associated with complexes with a slightly different structure or if it is due to the co-existence of the lipid-bound and free forms of BSP1 in the supernatant.

4.4.3 Effect of the phosphocholine head group

The choline group requirement for the lipid extraction was investigated (Figure 4-7).

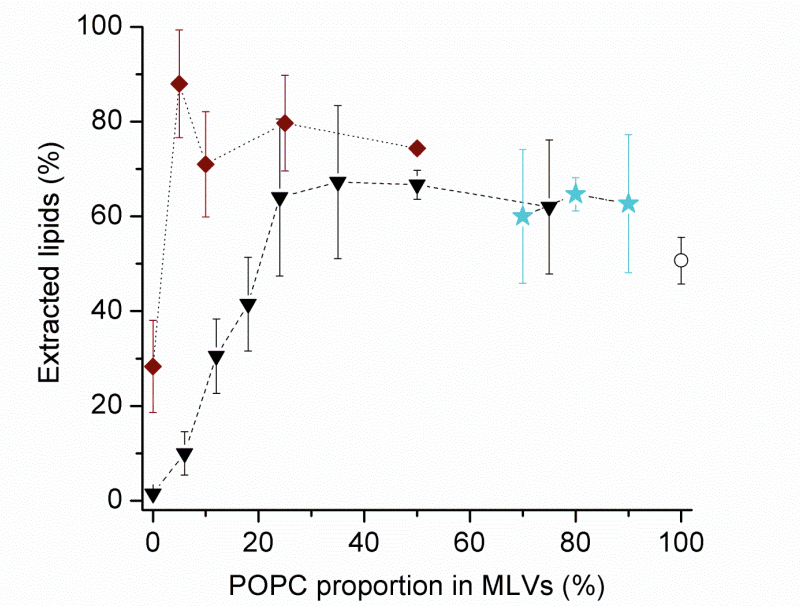

Figure 4-7: Quantification of lipid extraction after incubation of BSP1 with MLVs of POPC/POPE (▼), POPC/POPS (♦), POPC/Chol (*), or pure POPC (○). The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 20 depending on the lipid mixture. Each point is the mean value of 3 experiments; error bars represent standard deviations.

POPE-, POPS- and cholesterol-containing liposomes with different proportion of choline (POPC) were incubated with BSP1. Substituting POPC by POPE, up to 75%, slightly increased the lipid extraction (from 50 to ~65% extraction). For POPE proportion greater than 75%, the lipid extraction was inhibited; it decreased progressively with increasing POPE content to reach 0% for pure POPE MLVs. Practically all the protein was in the supernatant for all the POPC/POPE system (data not shown). Consequently, it was found that the lipid/protein molar ratio in the

supernatant was around 12 for POPC/POPE MLVs containing 75% POPE or less (Figure 4-8).

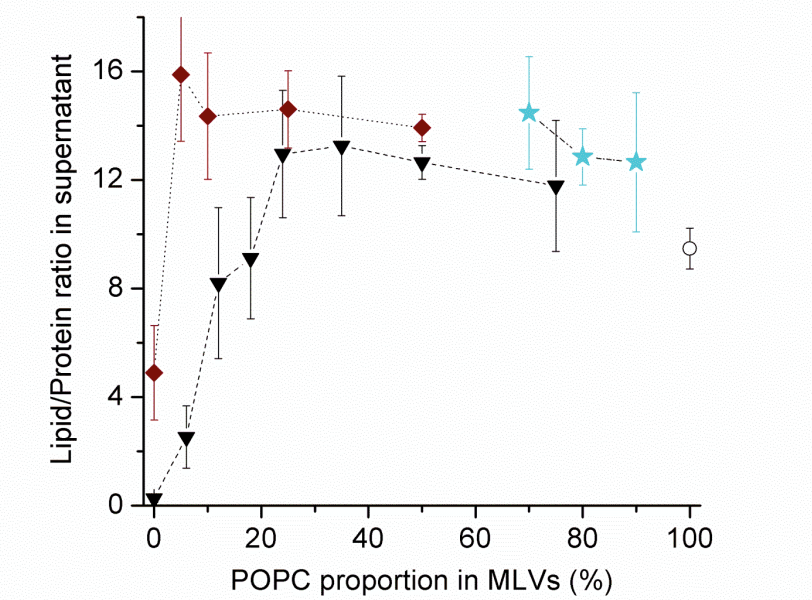

Figure 4-8: Lipid/BSP1 molar ratio in the supernatant after BSP1 incubation with MLVs of POPC/POPE (▼), POPC/POPS (♦), POPC/Chol (*), or pure POPC (○). The lipid/BSP1 incubation mass ratio was 1, corresponding to a molar ratio between 17 and 20 depending on the lipid mixture. Each point is the mean value of 3 experiments; error bars represent standard deviations.

For these systems, the position of the fluorescence of BSP1 in the supernatant after incubation with the MLVs corresponded to the lipid-bound state of the protein (Figure 4-6). Therefore we conclude that the lipid extraction was not modified by the presence of 75% POPE or less in POPC/POPE MLVs. When the mixture contained more than 75% POPE, the lipid/protein ratio of the self-assemblies found in the

supernatant decreased (Figure 4-8) in a concomitant manner with the lipid extraction; this observation results from the fact that the protein was found almost exclusively in the supernatant. Increasing the POPE proportion above 75% in POPC/POPE MLVs also led to a bathochromic shift of the fluorescence of the protein in the supernatant. The position of the fluorescence maximum obtained for pure POPE MLVs was 341 nm, the value obtained for free protein in solution. This observation is consistent with the absence of lipid extraction. Lipid analysis revealed that POPC and POPE were extracted with a similar efficiency when BSP1 induced the lipid extraction from POPC/POPE MLVs containing less than 25 (mol)% POPE. When the proportion was larger, specificity for POPC over POPE was observed (Figure 4-5). Under these conditions, the extraction was POPC-enriched compared to the initial liposome composition. The extreme value was found when BSP1 was incubated with POPC/POPE MLVs containing only 5% POPC: it induced a small lipid extraction that included almost 15% POPC, corresponding to an enrichment factor of about 3. POPC/POPS liposomes with 95% POPS or less sustained a slightly greater lipid extraction than the one observed with pure POPC, with extraction levels ranging from 70 to 90% (Figure 4-7). Extraction from these POPS-containing liposomes yielded to high lipid/protein molar ratios, between 14 and 16 (Figure 4-8). The fluorescence maxima obtained for BSP1 incubated with POPC/POPS MLVs corresponded to those obtained for lipid-bound proteins (Figure 4-6). These results indicate that BSP1 proceeded to lipid extraction in a fashion similar to that observed with pure POPC MLVs except that slightly more lipids per protein appeared to be extracted. Pure

POPS MLVs led to only 25% of extracted lipids, leading to a lipid/protein ratio of 5 for the self-assemblies found in the supernatant. This value is considerably smaller than those measured for the POPC/POPS mixtures (Figure 4-7). Even though 25% lipids were extracted, the fluorescence maximum measured for BSP1 in the supernatant was 343 nm, a value corresponding to the one measured for the free BSP1.

The addition of cholesterol (up to 30%) in POPC MLVs had little effect on the BSP1-induced lipid extraction. The proportion of extracted lipids (Figure 4-7), and the lipid/protein ratio in the supernatant (Figure 4-8) for POPC/Chol system were similar to those obtained with pure POPC MLVs. The composition of the extracted lipid fraction was similar to the composition of the original liposome composition (Figure 4-5) so cholesterol was extracted as efficiently as POPC by the protein. Finally, the maximum of the protein fluorescence in the supernatant corresponded to the one obtained for BSP1 incubated with pure POPC (Figure 4-6), indicating BSP1 was bound to lipids.

4.5 Discussion

The present results show that BSP1 induces lipid extraction from model membranes except in the cases where the MLVs have a very low PC content (Figure

4-5). We used a lipid/protein mass ratio of 1 for incubations, for which PC-MLVs are partially solubilized, a finding in agreement with previous results [24]. This lipid extraction is reminiscent of the action of BSP1 on sperm membranes [9, 36, 37], a phenomenon that has also been observed for other cell membranes including fibroblast [32, 38] and red blood cells [36]. In incubation conditions similar to the ones used in the present study, Tannert and coworkers quantitated the lipid extraction from lipid-labeled sperm membranes and red blood cells and showed that ~30-40 % of the labeled lipids were extracted [36]. Assuming that non-labeled lipids were extracted as well, this leads to a stoichiometry of about 8 extracted lipids per protein. These figures match the 50 % extraction and 9 lipids per protein reported here for POPC MLVs. These numbers do not appear, however, to describe BSP1-induced lipid extraction in a universal manner as, for example, the lipid extraction by BSP1 from fibroblasts yields a different extraction stoichiometry, with 20 PC and 24 cholesterol being extracted per protein [32]. In our case, it should be noted that extraction does not increase linearly with BSP1/Lipid ratio (see Figure 4-1). For diluted BSP1 (incubation ratio of less than 1), we obtained increasing extraction stoichiometry, up to 40 lipids per protein in extraction when a 0.13 mass ratio was used for incubation.

Few models rationalizing BSP1-induced lipid extraction have been proposed [25, 26, 32]. The molecular details of the mechanism leading to BSP1-induced lipid extraction are however not well understood. The findings reported here, combined with the literature, suggest the existence of a 3-step mechanism. First,

BSP1 binds to membranes via specific interactions between two lipid choline head groups and the two Fn2 domains of BSP1. Second, the apolar region of BSP1 penetrates into the outer leaflet of the membrane, a step driven by hydrophobic interactions. Finally, a certain number of BSP1 molecules extract a fragment mainly originating from the outer leaflet of the membrane, without lipid specificity, leading to the formation of lipid/BSP1 complexes. The information associated with each step is discussed below.

4.5.1 BSP1 binding to membrane

BSP1 binding to membranes via specific interactions between its Fn2 domains and the lipid choline head group is displayed in the present work by the complete absence of protein binding to membranes exclusively formed by POPE (Figure 4-7). This is in agreement with an early binding analysis by Desnoyers et al [17] and the analysis of Anbazhagan and coworkers who showed that BSP1 could not solubilize DMPE membranes [30]. Interestingly, the lipid extraction from POPC/POPE MLVs when POPE content is more than 75% suggests the extraction of about 2 POPC per BSP1. It is possible to calculate the proportion of PC in the extracted fraction from the initial PC proportion of PC in the MLVs and the corresponding enrichment factor. If one considers that one BSP1 extracts 12 lipids, as indicated on Figure 4-8, the number of PC molecules extracted per BSP1 can be inferred. For the 3 POPC/POPE mixtures containing less than 25 (mol)%PC, such calculations lead to an average of

2.5 extracted PC/BSP1, a value close to the expected 2. The specific binding of the protein to PC head groups is reinforced by the BSP1 x-ray structure showing the insertion of phosphocholine groups in the two Fn2 domain grooves [20], and with the more significant PC immobilization observed in the ESR experiments [24].

Despite the specific requirement of PC for BSP1 binding, our results indicate an interaction between BSP1 and POPS as a sizable extraction (30%) was measured from pure POPS MLVs. In addition, the capacity of BSP1 to induce lipid extraction is completely recovered, if not slightly increased, when POPS MLVs included as low as 5% POPC. It should be noted that the position of the fluorescence band of BSP1 found in the supernatant after the incubation of POPC/POPS MLVs with 5 and 15% POPC with BSP1 is slightly higher than that measured for POPC-bound BSP1 (Figure 4-6), suggesting different tryptophan and/or tyrosine environments and likely different type of structures of the resulting self-assemblies. This result is in agreement with a previous study [25] showing that BSP1 binding to PC/PS mixed membranes induced also a more limited change of the fluorescence intensity compared to its binding to pure PC membranes. The serine head is negatively charged and putative electrostatic interactions with the positively charged residues of BSP1 could contribute to the binding. In fact, it has been shown that BSP1 displays an affinity for negatively charged PS and PG, even though this affinity is not as high as for PC [24, 27, 30].

4.5.2 BSP1 penetration in membrane

After the BSP1 binding to the membranes, it is proposed that the protein inserts into the outer leaflet of the MLV. BSP1 has an amphipathic character due to the presence of hydrophobic cavities [44]. The shift of the position of the tryptophan fluorescence band for the membrane-bound protein is indicative of a more apolar environment. Previous studies have shown that the binding with soluble phosphocholine (only the head group moiety) does not increase intrinsic BSP1 fluorescence nor does it induce a blue shift as much as binding with membranes [25, 45]. Previous cryo-electron microscopy (cryo-EM) and fluorescence microscopy investigations reported drastic changes in vesicle morphology when liposomes are incubated with BSP1, including the formation of protrusions, bead necklace self-assemblies, and small vesicles [26, 46]. These structures, on the basis of the elastic model of vesicle shape [47-49], would be compatible with an increased relative area of the external leaflet, a phenomenon that was proposed to be associated with the protein insertion.

In the present work, we observed that POPC-BSP1 incubation without freeze-and-thaws induced only a limited extraction (~10%, data not shown). This is in agreement with a previous study that also reported that only 10% of the lipids were extracted from PC MLVs after the incubation with the protein in the absence of freeze-and-thaw cycles [24, 36]. These findings are compatible with the penetration of

BSP1 in the membrane limited to the external leaflet. Without freeze-and-thaw cycles during incubation, only the outer leaflet of the outermost bilayer of an MLV would be exposed to the protein. The freeze-and-thaw cycles promote a homogeneous distribution of the proteins in the sample, ensuring a contact of the proteins with all the lipids [50, 51]. The capacity of BSP1 to translocate membranes and reach the inner leaflet is not established but the present results suggest that it may be limited. This conclusion is also consistent with the more efficient lipid extraction from the outer leaflet compared to that of the inner leaflet [36]. We are aware that the freeze-and-thaw procedure is a rather disturbing step and that it differs considerably from the *in vivo* conditions of the BSP1 action. We have observed, for example, that these cycles promoted the extent of the lipid extraction compared to plain incubation and to hydrating solid POPC with the buffer already containing BSP1 (data not shown). However, the freeze-and-thaw procedure, required for isolating lipids extracted by BSP1 from the remaining MLV lipids, clearly did not rule the extractions because large differences were observed between different systems; the chemical difference between phosphocholine and phosphoethanolamine and the physical difference between fluid- and gel-phase bilayers in the case of DPPC led to considerable differences in lipid extraction (actually from all to nothing). Therefore, we conclude that the lipid extractions resulting from this procedure that includes freeze-and-thaw cycles and those occurring in vivo should essentially result from the same molecular interactions and thus the lipid fractions extracted by BSP1 in our protocol depict in a reasonable manner the main features affecting the extraction process.

4.5.3 BSP1-induced lipid extraction

In the third step of the extraction mechanism, it is proposed that BSP1 with lipids mainly from the external leaflet undergo a rearrangement (that could include oligomerization of the protein), leading to the formation of lipid/protein assemblies that leave the membrane. The complex stoichiometry would be, according to the present results, between 9 and 16 lipids per BSP1. This value is in good agreement with those obtained from lipid extraction from sperm membranes and erythrocytes [36]. The present results indicate that this extraction is rather unspecific with regards to the lipids, provided the presence of two PC per BSP1 to ensure the protein anchoring. We did not observe a lipid selectivity regarding the extraction between POPC, Lyso-PC, DPPC, POPE, POPS and cholesterol. Our results show that, except when membranes do not contain enough PC to ensure BSP1 binding, PS and PE are extracted by BSP1 as efficiently as PC, the extraction being representative of the liposome composition. This is valid even for membranes where the proportions of PC were rather limited (30 (mol)%). Such mechanism supports the hypothesis that the large proportion of PC obtained in lipid extraction from sperm membranes is essentially due to the fact that the protein extracts mainly lipids from the outer leaflet, a leaflet rich in PC [36]. The lipid extraction from the outer leaflet of the membranes must eventually lead to lipid redistribution in the membrane in order to maintain the bilayer structure. Such phenomenon would take place when there is a considerable lipid extraction. This situation could actually take place when there is an extended contact between BSP1

and sperm cells. This could be the origin of the detrimental interaction of BSP1 on sperm survival when ejaculates are collected for artificial inseminations [4].

The present results reveal the binding of BSP1 to DPPC MLVs (Figure 4-3) but also the incapacity for the protein to extract lipids from these membranes when the incubation was carried out at room temperature (Figure 4-2), when pure DPPC MLVs were in gel phase. In this case, either the penetration in the membrane or the solubilization of a membrane patch was hindered by a tight packing of the lipid chains. As shown on Figure 4-2, incubation of DPPC MLVs with BSP1 at 50 °C, which brought these membranes in the fluid phase, induced an extraction identical to that observed for POPC. It is concluded that, despite the BSP1 binding to gel-phase DPPC MLVs, as assessed by the protein intrinsic fluorescence, the extraction process can only take place with fluid-phase membranes. It must be noted that BSP1 induced a small extraction from gel-phase DPPC MLVs containing 5 or 10% POPC (Figure 4-2). Even though the phase diagram of this phospholipid mixture indicates that these membranes should be in the gel phase at room temperature [43], it appeared that the presence of a small amount of POPC was sufficient to induce some lipid extraction. The extraction was however enriched in POPC, reinforcing the idea that fluid phase promotes lipid extraction. The favored POPC extraction led to a reduced POPC content in the remaining DPPC MLVs and this consequence may have impeded further phospholipid extraction.

The general solubilization of membrane patch by BSP1 provides a rationale for the extraction of cholesterol without evoking a direct interaction between BSP1 and cholesterol; phospholipids and cholesterol would be concomitantly extracted, as previously hypothesized [38]. This conclusion is in agreement with a previous study indicating that the solubilization of DMPC membranes by BSP1 was almost unaffected by the presence of 25 mol % cholesterol [24]. The determination of the protein affinity for membranes obtained from the intrinsic fluorescence of the protein [26] and ITC measurements [30] showed that cholesterol had little effect on BSP1 affinity to PC. It should be noted however that some previous reports concluded that cholesterol addition to PC membranes inhibited BSP1 activity on liposomes. For example, it was shown that 30 mol% cholesterol in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) small unilamellar vesicles (SUVs) limited BSP1-induced leakage of the entrapped fluorophore from the vesicles [26]. An ITC study concluded that the presence of cholesterol in POPC bilayers reduced drastically the capacity of the membranes to bind BSP1 [29]. A SPR study showed that the presence of cholesterol in the supported bilayers inhibited the lipid extraction induced by BSP1 [27]. These results indicate that BSP1 interactions can be modulated by cholesterol and this influence is dependent on factors that remain to be identified. BSP1 binding, the membrane permeability perturbation and the lipid extraction may not be affected by the presence of cholesterol in the membrane in the same fashion. For example, it has been reported that addition of 30% cholesterol to POPC bilayers prevented BSP1-induced leakage of the liposome content, but had no effect on protein binding to the

bilayers [51]. In addition, SUVs, MLVs and supported bilayers may not interact in an identical way with BSP1. It was actually previously suggested that the size of liposomes (and thus lipid plane curvature), which is different for these 3 lipid self-assemblies, impacts on BSP1-membrane interactions [24].

4.5.4 Lipid/BSP1 auto-assemblies

The knowledge regarding the structure of lipid/BSP1 complexes resulting from the membrane lipid extraction is very limited. Their stoichiometry corresponds to about 10 to 15 lipids per BSP1. They cannot be pelleted by centrifugation. The structures resulting from the lipid extraction from fibroblasts were proposed to have a diameter of about 80 nm, as assessed by gel filtration chromatography [32]. Spherical membrane fragments of about 10-nm diameter resulting from the interaction of BSP1 with DOPC SUVs were observed by transmission electron microscopy [26]. Cryo-electron microscopy and fluorescence microscopy reported the presence of various structures after the incubation of POPC MLVs with BSP1, including tubes, small vesicles and spiral-like threads [46]. The later nanostructures would likely not be pelleted under our centrifugation conditions and were proposed to correspond to the lipid/protein complexes resulting of the lipid extraction. Additional investigations are required to determine the molecular details of these complexes.

In conclusion, the finding of the present study proposes a three-step mechanism for the BSP1-induced lipid extraction from sperm membranes. It appears that the presence of PC is essential for the protein binding but, once BSP1 is associated with the membrane, the resulting lipid extraction shows practically no specificity. The putative impact of this non lipid-specific membrane perturbation caused by BSP1 on sperm capacitation as well as on sperm preservation should be examined.

4.6 Acknowledgements

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Chapitre 5 - Conclusion

Les résultats de cette thèse ont permis d'approfondir la compréhension du phénomène de l'extraction lipidique par des PEL. Une plateforme de caractérisation de l'extraction lipidique a été mise en place. Elle inclut un protocole d'incubation des PEL et des MLV, suivi de leur centrifugation, permettant de séparer rapidement et facilement les lipides extraits des membranes. La quantification des lipides après incubation et centrifugation par l'analyse du phosphore et LC-MS mène à la caractérisation de l'extraction lipidique par les PEL. L'analyse de la nature des lipides extraits par LC-MS révèle que dans certaines conditions, l'extraction est spécifique envers les PC. L'observation de la spécificité lipidique est un élément clé pour l'élaboration de mécanismes d'extraction plus fins, puisqu'elle fournit de l'information sur la composition lipidique dans l'environnement immédiat des PEL associés aux membranes. La caractérisation de l'association pour les systèmes PEL-lipides, effectuée par la fluorescence intrinsèque des PEL, permet, en plus, de dégager certains facteurs moléculaires influençant l'affinité entre peptides/protéines et membranes lipidiques.

5.1 Mécanisme d'extraction lipidique en 3 étapes

Les présents travaux contribuent à établir un mécanisme décrivant l'extraction lipidique par les PEL en 3 étapes: 1) l'association des PEL aux membranes; 2) la

relocalisation des PEL dans le coeur lipidique; 3) l'extraction lipidique. La Figure 5-1 résume comment différentes contributions et phénomènes interviennent à différentes étapes du processus de fragmentation.

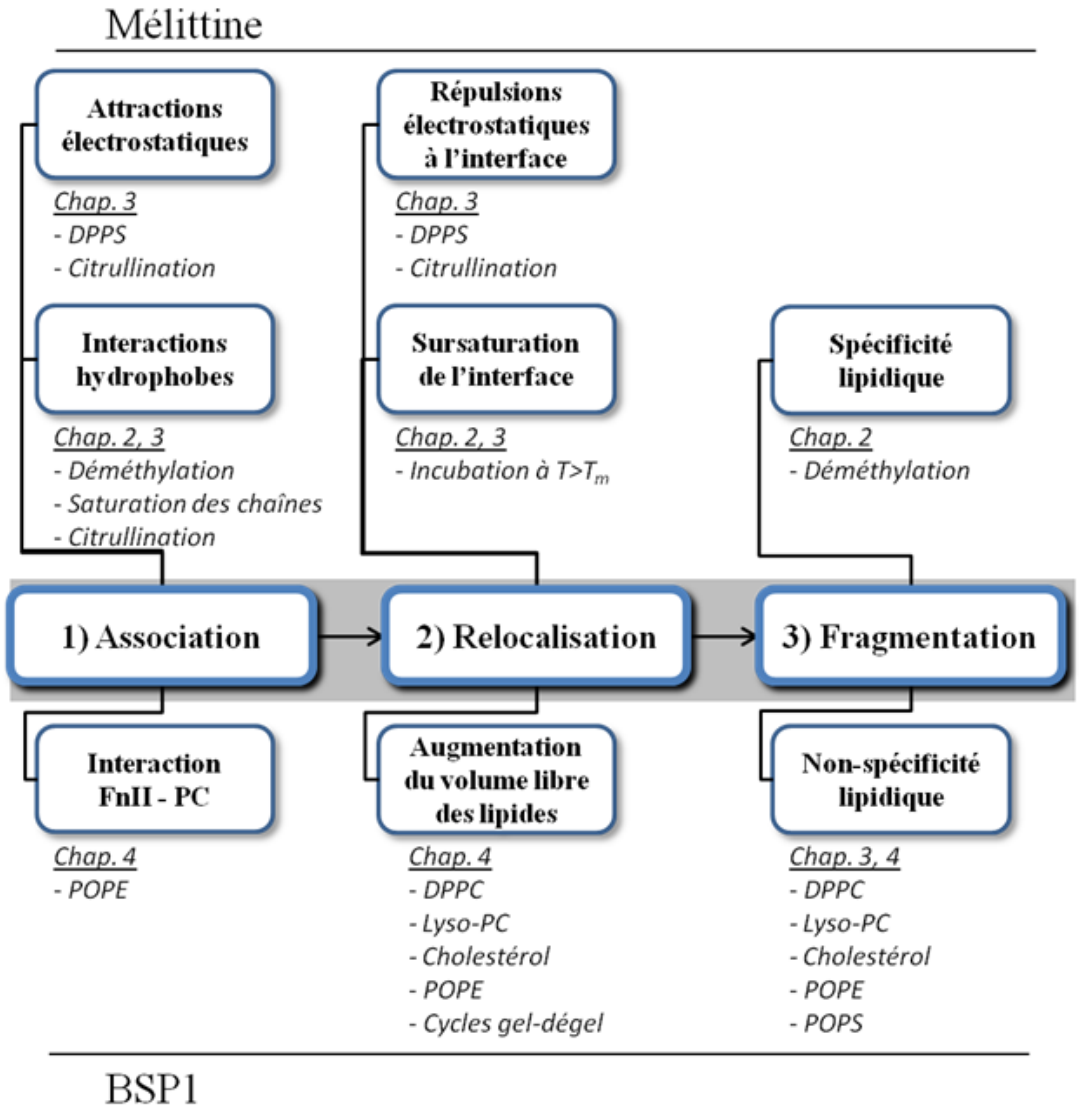

Figure 5-1: Résumé des travaux de cette thèse qui ont permis de détailler les étapes du mécanisme de l'extraction lipidique par les PEL.

Bien que les mécanismes d'extraction pour les deux PEL étudiés puissent être résumés par les 3 étapes mentionnées plus haut, ces mécanismes diffèrent et les facteurs influençant ces étapes varient d'un peptide/protéine à l'autre. Dans le cas de la mélittine, l'association à l'interface est largement due aux forces hydrophobes. Les forces hydrophobes dominent même lorsque la mélittine, cationique, s'associe à des membranes de DPPC/DPPS 70/30 anioniques; la plus grande constante d'affinité mesurée pour la mélittine citrullinée le démontre (chapitre 3). L'association de la mélittine est inhibée par l'augmentation de la cohésion de la membrane; un empilement plus compact des chaînes dû à des interactions attractives entre les têtes polaires ou à la présence de chaînes saturées réduit l'affinité (chapitre 2). Un phénomène complètement différent est observé pour l'association de la BSP1, qui est due à l'interaction spécifique de ses deux domaines de type FnII et les lipides à tête choline (chapitre 4). Plus précisément, les mesures d'extraction sur les liposomes de PC/PE très riches en PE démontrent que chaque BSP1 nécessite 2 PC pour se lier.

Comme l'association, la relocalisation vers le cœur hydrophobe est une étape essentielle de l'extraction. Elle est influencée par différents facteurs, selon le PEL étudié. Pour la mélittine, la relocalisation est provoquée par le refroidissement sous la T_m des lipides durant l'incubation. En phase $L_{\beta,}$ l'interface accommode moins de peptides qu'en phase L_{α}, ce qui pousse les PEL hors de l'interface, vers le cœur lipidique (chapitres 2 et 3). La répulsion électrostatique entre les mélittines à l'interface est aussi un facteur qui contribue à la relocalisation; la diminution de la

charge effective de la mélittine à l'interface par citrullination des résidus cationiques réduit la relocalisation et donc la fragmentation (chapitre 3). Finalement, la présence de lipides anioniques dans les membranes inhibe la relocalisation de la mélittine, qui se trouve alors ancrée à l'interface à cause des interactions électrostatiques attractives; la citrullination des résidus cationiques réduit l'ancrage et favorise la fragmentation (chapitre 3). La protéine BSP1, d'une masse environ 6 fois plus grande que la mélittine, nécessite un plus grand stimulus pour pénétrer les membranes. La congélation des liposomes fait augmenter leur volume et brise les bicouches. Les défauts ainsi formés permettent la relocalisation de la BSP1 vers le cœur lipidique (chapitre 4). Il est intéressant de souligner que la relocalisation ne semble pas se produire après le gel des liposomes lorsque la BSP1 est incubée avec des liposomes de DPPC en phase L_{β}, bien que l'association à l'interface soit complète. Il se pourrait que la BSP1 s'insère moins profondément dans les membranes en phase $L_{\beta,}$ ce qui préviendrait la relocalisation de la protéine lors des cycles gel-dégel.

Un des points les plus originaux de la thèse est la caractérisation de la spécificité lipidique lors de la fragmentation. Les résultats montrent que selon la composition des membranes, l'extraction peut ou ne pas se produire préférentiellement envers une espèce lipidique. Les deux interactions spécifiques PEL-lipides étudiées, soit mélittine-PS et BSP-PC, ne mènent pas à une extraction sélective (chapitres 3 et 4). Une sélectivité est plutôt observée lorsqu'une séparation de phase se produit sur un domaine plus grand de la membrane. L'insertion de la

mélittine dans les membranes de PC/PE crée un enrichissement en PC dans une région autour du peptide, ce qui mène ensuite à l'extraction préférentielle des PC (chapitre 2).

Un certain degré d'amphiphilicité semble être essentiel à l'activité des PEL. Presque tous les peptides antimicrobiens présentent une structure amphiphile, leur permettant de s'associer aux membranes [1]. Selon les données de l'*Antimicrobial Peptide Database,* environ 14% des peptides antimicrobiens possèdent une structure hélicoïdale [2]. La distribution des résidus polaires et hydrophobes sur des faces opposées des hélices leur confère une grande affinité pour l'interface des membranes lipidiques [3], où leurs deux faces sont se retrouvent dans un environnement favorable. Cette structure est cohérente avec le mécanisme d'action en tapis, par lequel les peptides recouvrent l'interface des membranes. La présence de la structure en hélice-α amphiphile chez l'Apo-A1 démontre que ce motif est également important pour des catégories de PEL autres que les peptides antimicrobiens. Il serait intéressant de vérifier si le mécanisme proposé pour la mélittine s'applique pour d'autres peptides s'arrangeant en hélice-α amphiphile. Ces travaux pourraient potentiellement permettre de dégager des règles plus générales s'appliquant également aux plus grosses protéines comme l'Apo-A1.

Une particularité intéressante de l'extraction lipidique est la prévalence de la formation de nano-disques. Les modèles proposés pour les particules discoïdales formées sont remarquablement similaires (Figure 5-2).

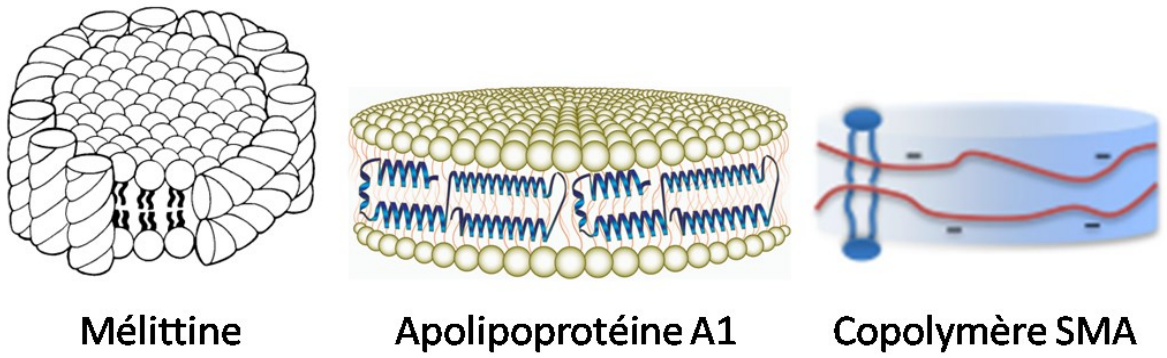

Figure 5-2: Structures schématiques des objets formés par la mélittine [4], l'Apo-A1 [5] et le copolymère SMA [6] après incubation avec des membranes lipidiques.

Dans les cas présentés à la Figure 5-2, les particules sont formées d'une bicouche lipidique plane formant un disque d'environ 20-30 nm de diamètre pour la mélittine et de 9 nm pour les deux autres [6-8]. La région des chaînes lipidiques est recouverte par la partie hydrophobe du peptide/protéine/polymère amphiphile, l'isolant du milieu aqueux. La compréhension du mécanisme de leur formation est liée à la fragmentation des membranes. À ce jour, ce mécanisme n'a pas été complètement élucidé. Peu de chercheurs se sont intéressés à la composition lipidique de ces disques, mais la mince littérature à ce sujet rapporte tout de même quelques faits intéressants. D'abord, il a été démontré que le cholestérol est difficilement accommodé dans les bicelles formées par la mélittine [9]. La stabilité des disques serait en cause : puisque les bicelles se forment seulement pour des lipides en phase gel

avec la mélittine, il a été proposé que le cholestérol déstabilise les disques en désordonnant les lipides. D'autre part, les disques formés par les Apo-A1 prennent une forme différente lorsqu'ils accommodent les lipides à tête PE [8]. La forme conique des lipides à tête PE est inadaptée pour leur inclusion dans un disque plan; ils tendent plutôt à former des particules plus petites (8 nm de diamètre) en forme de selle. Hormis ces deux exemples, la littérature sur les PEL porte peu d'attention à l'étude des objets formés par l'extraction lipidique. Une meilleure connaissance de la structure de ces objets accroitrait la compréhension générale du mécanisme de l'extraction lipidique. L'étude de l'impact de la composition lipidique sur la taille, la forme et la stabilité des petits auto-assemblages est une avenue peu explorée, potentiellement riche en découvertes intéressantes.

La fragmentation par les PEL est fortement modulée par la composition lipidique des membranes. Les observations sur le taux de méthylation des têtes polaires (chapitres 2 et 4) suggèrent que la cohésion du coeur hydrophobe des membranes pourrait être importante dans le contrôle de la susceptibilité à la fragmentation. Cette cohésion peut être modulée par le cholestérol, dont l'effet dramatique sur l'activité de la mélittine a été démontré [9-11]. Ce dernier élément est important puisque le cholestérol est abondant dans les membranes des cellules animales. Peu d'attention est pourtant portée à l'effet de la cohésion du coeur hydrophobe des membranes, modulant principalement les interactions hydrophobes, sur l'activité sélective des peptides antimicrobiens envers les bactéries [1, 12-14]; la

sélectivité est plutôt attribuée aux interactions électrostatiques entre peptides cationiques et membranes bactériennes anioniques. Il a été proposé au chapitre 3 que le rôle du caractère cationique de la mélittine serait plutôt de réduire l'association aux membranes neutres, comme celles des cellules animales. Ainsi, dans une optique de design de nouveaux peptides antimicrobiens, il semble important de tenir compte, en plus des attractions électrostatiques, des interactions hydrophobes qui pourraient jouer un rôle dans la discrimination entre les bactéries et les cellules. Les différences de composition lipidique entre membranes de cellules bactériennes et membranes de cellules animales sont grandes; la composition des membranes procaryotes varie d'ailleurs entre les souches de bactéries [15]. Ainsi, l'exploration systématique de l'effet de la composition lipidique des membranes sur l'action des peptides antimicrobiens, notamment l'impact de la balance PC/PE et du cholestérol, permettrait d'approfondir notre compréhension du mécanisme de l'extraction lipidique par ses peptides. De telles avancées pourraient, par exemple, permettre ensuite le développement de peptides plus spécifiques et actifs envers certaines membranes bactériennes.

5.2 Conclusions générales et travaux futurs

L'extraction lipidique est présente dans de nombreux processus biologiques et biotechnologiques. L'étude de l'action de PEL modèles comme la mélittine permet de comprendre les aspects fondamentaux de la fragmentation. Les présents travaux démontrent quelques unes des propriétés moléculaires des membranes qui sont

déterminantes pour l'extraction lipidique par les PEL. Bien qu'un cadre commun de fragmentation membranaire soit proposé, des différences mécanistiques significatives ont cependant été observées entre les deux peptides/protéines étudiés. Il est donc incorrect d'extrapoler le détail du mécanisme d'action d'un PEL à un autre. Ainsi, il serait intéressant d'étudier le mécanisme de fragmentation de différents PEL comportant des caractéristiques structurales similaires à celles de la mélittine et de la BSP1, comme la structure en hélice-α et les domaines de type FnII, afin de détailler davantage les liens entre la structure d'un PEL et son action.

Le concept du mécanisme d'extraction lipidique en est un fondamental qui rejoint plusieurs domaines; dans cette optique, l'intérêt limité qui est porté à l'étude des facteurs menant et résultant à/de la fragmentation des membranes est surprenant. La plateforme développée pour la caractérisation de l'extraction lipidique est un outil permettant d'étudier les interactions entre les membranes et les PEL qui s'y insèrent; cette approche pourrait être au cœur de découvertes importantes concernant les peptides antimicrobiens, le transport du cholestérol, la préservation des semences, etc.

5.3 Références

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