UCL Université catholique de Louvain
Université partenaire de l'Académie universitaire 'Louvain'

Faculté de Pharmacie et des Sciences Biomédicales
Louvain Drug Research Institute

ACYLATED AND UNACYLATED GHRELIN BINDING TO MEMBRANES: TOWARDS A BETTER UNDERSTANDING OF THE UNDERLYING MECHANISMS

Edith Staes

Thèse présentée en vue de l'obtention du grade de
Docteur en Sciences Pharmaceutiques et Biomédicales

Promoteur : Véronique Préat

2010

Défendre sa thèse est l'aboutissement d'un travail de longue haleine qui demande de la persévérance et un caractère bien trempé. Cependant, on ne peut arriver seul à accomplir une telle tâche. C'est grâce aux soutiens psychologiques, financiers et scientifiques que l'on peut y arriver. C'est pourquoi je tiens à remercier ici toutes celles et tous ceux qui m'ont aidé à y parvenir.

Je tiens tout d'abord à remercier ceux que j'aurais pu oublier ci-dessous.

Pour le soutien financier, je remercie le FNRS et la Faculté de Médecine pour les bourses dont j'ai pu personnellement bénéficier. Merci aussi au FSR pour le financement du matériel sans quoi il n'est pas possible d'avancer.

Je tiens à remercier mon promoteur, le professeur Véronique Préat, chef de l'unité de pharmacie galénique à l'UCL, qui m'a suivie tout au long de mon doctorat. Elle m'a laissé libre de m'engager hors des sentiers battus et m'a apporté le soutien nécessaire à réaliser mes choix. Merci aussi de m'avoir appris à aller à l'essentiel, à me recentrer sur mes objectifs.

Je tiens également à remercier les membres de mon comité d'accompagnement, les professeurs Marie-Paule Mingeot, Yves-Jacques Schneider, Jean-Paul Thissen et Rita Vanbever pour leurs questions pertinentes lors de chacune de nos rencontres. Merci également aux membres extérieurs de mon jury de thèse, les professeurs Karl Lohner, Magali Deleu, d'avoir consacré une partie de leur temps pour lire ce manuscrit, le commenter et ainsi que pour les débats constructifs lors de la thèse privée qui ont permis d'améliorer ce travail.
Merci aussi au professeur Bernard Gallez, président du jury, pour avoir dirigé avec brio les débats ainsi qu'à Nathalie Delzenne pour ses remarques constructives lors de l'épreuve de qualification.

Merci tout spécial aussi à Nathalie Dujardin pour m'avoir donné le goût de la recherche et pour m'avoir enseigné les fondements de la recherche expérimentale avec beaucoup de patience et gentillesse.

Merci également à toutes les personnes avec qui j'ai collaboré pour ce travail et qui m'ont aidé malgré leur emploi du temps surchargé. Pierre-Antoine Absil du pôle en ingénierie mathématique à l'UCL pour sa patience et tout le temps qu'il a consacré à la résolution

numérique du coefficient de liaison hydrophobe malgré les nombreux changements successifs que je lui demandé d'apporter! Magali Deleu et Hary Razafindralambo de l'unité de chimie biologique industrielle (CBI) de Gembloux Agro-Bio Tech pour, respectivement, m'avoir initié au titrage calorimétrique isotherme et aidé ponctuellement. Eric Rozet du laboratoire de chimie analytique à l'ULg pour la validation de la méthode HPLC. Laurence Lins du centre de biophysique moléculaire numérique (CBMN) de Gembloux Agro-Bio Tech pour la modélisation moléculaire. Christel Flore, Aurélien Lorin et Laurence Lins de l'unité CBMN pour, respectivement, m'avoir appris à me servir du spectromètre infrarouge, m'avoir conseillé et m'avoir aidé à interpréter mes résultats. Vincent Raussens de l'unité de structure et fonction des membranes biologiques (SFMB) à l'ULB pour m'avoir initié au dichroïsme circulaire, ainsi que pour m'avoir guidé dans les expériences, les calculs et interprétations des résultats. Nancy de l'unité de pharmacologie cellulaire et moléculaire (FACM) à l'UCL pour m'avoir initié à la préparation de liposomes extrudés. Francis Brasseur et Magdalena Swinarska de la Ludwig Institute for Cancer Research (LICR) pour les RT-PCR et l'accès à la, ô combien nécessaire, ultracentrifugeuse. Vincent Stroobant pour ses conseils en matière d'analyse et de lyophilisation des peptides. Jean-François Gohy de l'UCL pour l'expérience de Cryo-TEM. François Verlinden de l'unité de diabétologie à l'UCL pour les marquages de la ghréline à l'iode-125. Bertrand Dodelet de la firme UCB pour les expériences de DSC.

Merci également aux membres des unités CBMN, CBI, SFMB et FACM pour leur formidable accueil et pour la bonne ambiance qui y régnait. Merci aussi pour tous les petits coups de pouce des uns et des autres.

Au sein de notre unité de pharmacie galénique à l'UCL j'ai aussi bénéficié, pour la réalisation de ce travail, de l'aide précieuse de : Virginie Fievez pour la culture des Caco-2/cellules M-like ; Anne des Rieux qui m'a initié à la culture des Caco-2 depuis son labo en Suède et m'a notamment aidé dans l'interprétation des résultats de transport ; Bernard Ucakar et Nathalie Lecouturier pour leur aide dans la réalisation de plusieurs expériences et pour l'organisation logistique du laboratoire ; Jean-Pierre Vandiest qui sait toujours trouver les bons plans pour commander des produits moins chers et sans qui on ne saurait commander nos produits ; Murielle Callier qui m'a entre autres aidé à mettre en forme cette thèse et dans beaucoup de démarches administratives.

Merci à Margarida Jesus, étudiante Erasmus du Portugal, ainsi que Véronique Denis, étudiante en Pharmacie, qui ont travaillé avec ferveur et discernement dans les tous débuts de ce travail.

Je tiens aussi à remercier tous les membres de notre unité, anciens ou présents, pour la formidable ambiance, leurs coups de pouce, leur soutien ainsi que pour les discussions scientifiques mais aussi pour toutes les autres sérieuses et moins sérieuses. Merci pour tout ce que l'on a vécu dans l'unité et en dehors. Merci à : Cynthia, François-Xavier, Anne-Rose, Valérie, Julie, Valentine, Antoine, Frédéric, Louisa, Daniel, Mustapha, Laurence R., Marie-Lise, Benoît, Aline, Nicolas G., Gaëlle R., Farida, Nicolas, Arnaud, Rémy, Fabienne, Laurence P., Régis, Nicolas D., Liévin, Nelson, Benedetta, Ander, Eduardo, Gilson, Nathalie S., Jean-François, Eric, Julie T., Aude, Salomé, Malory et Jean.

Je tiens aussi à remercier tout spécialement mes collègues de bureau et en particulier Catherine Lombry, Gaëlle Vandermeulen, Cláudia Fernandes et Héloïse Ragelle pour leur soutien quotidien. Merci de m'avoir remonté le moral dans les moments de doute et de vous être réjouies avec moi de mes réussites. Merci aussi pour tous les bons moments partagés ensemble, pour avoir écouté mes coups de gueule, mes « Regarde ! » et avoir répondu à mes nombreuses questions et sollicitations. Merci également pour tous nos partages de vie, ils étaient de ceux qui font grandir. Merci enfin pour votre amitié.

Merci aussi à mes amis qui ont suivi d'un œil bienveillant mon parcours. Merci pour tous les partages, les fous rires, … Nos rencontres sont toujours une source de richesse et d'équilibre.

Merci aussi à toute ma famille et belle-famille qui m'ont soutenue d'un point de vue psychologique et matériel dans ce projet fou. Merci tout spécialement à Françoise et Christian pour vous être souvent occupé des filles quand j'en avais besoin. Merci aussi à ma maman pour les corrections d'anglais de mes articles et de ce manuscrit même si les sciences ne sont pas sa tasse de thé. Merci également à mon petit frère Jonathan pour son aide dans les débuts de l'élaboration de la résolution numérique du coefficient de liaison hydrophobe. Merci aussi à Florence pour avoir consacré à plusieurs reprises, avec maman, une partie de ses vacances pour garder les filles pendant que je faisais mes expériences au laboratoire. Merci aussi à mon papa pour m'avoir fait confiance dès mon plus jeune âge et de m'avoir laissé libre de faire

mes propres choix tout en balisant le chemin. Merci également à mamy pour son soutien indéfectible et pour ses nombreuses et fructueuses prières.

Merci aussi à Nolwenn et Gladys, mes filles, vous êtes des rayons de soleil pour moi. Nolwenn tu m'as suivie depuis le début de ce projet puisque j'étais enceinte de plus de 6 mois en commençant cette thèse… puis ta soeur t'a rejointe quatre ans plus tard. Merci d'être là toutes les deux. Votre présence m'a souvent aidé à relativiser les choses. Je suis contente de partager avec vous cette tranche de vie dont j'espère que Nolwenn se souviendra.

Enfin j'aimerais remercier de tout mon cœur mon mari, Geoffroy De Vocht. Merci pour tout l'amour que tu me portes depuis treize ans. Sans toi, je n'aurai pu mener ce projet à bien. Merci d'avoir toujours cru en moi. Ton soutien moral était précieux quand j'avais envie de tout laisser tomber. Merci d'avoir fêté avec moi mes réussites. Merci également pour ton éclairage ingénieux sur moult calculs et problèmes mathématiques ainsi que pour les nombreux dessins. Merci enfin pour les sacrifices financiers que l'on a dû faire pour mener à bien cette entreprise.

ABBREVIATIONS

AA	amino acid
AFM	atomic force microscopy
ATR-FTIR	attenuated total reflection Fourier transform infrared
CD	circular dichroism
CHO	Chinese hamster ovary cells
Chol	cholesterol
DHPC	1,2-dihexanoyl-*sn*-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DMPG	1,2-dimyristoyl-*sn*-glycero-3-phospho-rac-(1-glycerol)
DOPC	1,2-oleoyl-*sn*-glycero-3-phosphocholine
DPC	dodecylphosphocholine
DPH	1,6-diphenylhexatriene
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPS	1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine
DSC	differential scanning calorimetry
EIA	enzyme immunoassay
EM	electron microscopy
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing peptide
GHS	growth hormone secretagogue
GHS-R	growth hormone secretagogue receptor
GHS-R1a	growth hormone secretagogue receptor 1a = ghrelin receptor
GHS-R1b	growth hormone secretagogue receptor 1b
GOAT	ghrelin *O*-acyl transferase
GPCR	G protein coupled receptor
GUV	giant unilamellar vesicles
hAG	human acylated ghrelin
HEK	human embryonic kidney cells
HBSS	Hanks' balanced salt solution

HPLC	high performance liquid chromatography
hUAG	human unacylated ghrelin
IR	infrared (spectroscopy)
ITC	isothermal titration calorimetry
LOQ	limit of quantitation
LUV	large unilamellar vesicles
LUVET	large unilamellar vesicles produced by extrusion
MLV	multilamellar vesicles
NMR	nuclear magnetic resonance
PAMPA	parallel artificial membrane permeability assay
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	phosphatidylserine
RFU	relative fluorescence unit
RNA	ribonucleic acid
RP-HPLC	reverse phase high performance liquid chromatography
RSD	relative standard deviation
SD	standard deviation
SDS	sodium dodecyl sulfate = lauryl sulfate
SEM	standard error of the mean
SM	sphingomyelin
SUV	small unilamellar vesicles
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TM	transmembrane
TMA-DPH	1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene
UV	ultraviolet (spectroscopy)

ABSTRACT

In 1999, Kojima and co-workers isolated ghrelin from stomach extracts as the endogenous ligand of the orphan growth hormone secretagogue receptor 1a (GHS-R1a), now called the ghrelin receptor. Ghrelin is a 28-aa peptide hormone which is *O*-octanoylated at its Ser^3 residue. Ghrelin is a pleiotropic hormone. In particular, besides its growth hormone releasing activity, it plays a key role in energetic homeostasis.

Ghrelin is unique in four ways: (i) it is the only hormone peptide to be modified by a fatty acid on one of its lateral chains; (ii) *O*-octanoylation is a medium chain length fatty acid (C_8) modification whereas other acylated peptides are usually acylated with either much shorter (C_2) or much longer fatty acids (C_{14} or C_{16}); (iii) other acylated peptides are usually *N*- or *S*-linked; (iv) its unacylated form is also endogenous and also seems to play physiological roles, and the charge of both forms is identical.

In the nanomolar range, the acylation of ghrelin is required for efficient binding and activation of the ghrelin receptor that mediates most of its endocrinological activities. However, some other effects of ghrelin have been attributed to the activation of a yet unidentified receptor that could bind acylated and/or unacylated ghrelin. Unacylated ghrelin is also a full agonist of the ghrelin receptor but in the micromolar range. As ghrelin effects seem to be receptor mediated, the interaction of ghrelin with cell membranes is potentially important for all its biological activities. Moreover, membrane lipids and lipidation of peptides have both been shown to modulate the activity of peptides.

Therefore, the aims of this thesis were to determine the importance of this unique post-translational modification of ghrelin as well as of its basic residues (i) in the extent of ghrelin binding to membranes; (ii) on the mechanisms that drive acylated and unacylated ghrelin to the membrane and hence to the ghrelin receptor; (iii) on ghrelin transmembrane and transepithelial transport.

The originality of this work is that we gained some insights into the mechanisms of action of a unique hormone peptide modification, *O*-octanoylation, and the concomitant role of its basic residues, especially at the level of peptide-membrane interactions and their possible biological implications. Moreover, whenever possible, we worked with natural lipids mixtures and this is quite unusual in the field of peptide-lipid interactions.

In this work we have shown differential behaviours of acylated and unacylated ghrelin towards the membrane. We have also shown by PAMPA and intestinal monolayer experiments, that *O*-octanoylation of ghrelin does not seem to help its transport across membranes and epithelia.

After having shown that ghrelin could bind to model plasma membranes (liposomes) as well as to biological membranes and that the acylation significantly enhanced ghrelin binding to negatively charged membranes, we explored the underlying mechanisms of ghrelin binding.
The electrostatic and/or hydrophobic interactions of ghrelin with the membrane were investigated using various liposome and buffer compositions in binding, zeta potential and isothermal titration calorimetry studies. On the basis of the results of these experiments it appears that unacylated ghrelin only seems to interact with the lipids by Coulombic interactions, which are long range electrostatic interactions. Acylated ghrelin, however, is not only driven to the membrane by electrostatic attraction between the positively charged residues of ghrelin and the negatively charged lipids, but acylated ghrelin binding to the membrane is also accompanied by: (i) partitioning into the lipid headgroup and into the lipid backbone regions; (ii) a 120-fold increase in peptide concentration in the membrane. However, as shown by ATR-FTIR, DPH and TMA-DPH fluorescence polarization studies as well as by the use of a binding model, acylated ghrelin did neither deeply penetrate the membrane nor did it perturb its organization.
Conformational studies by circular dichroism and attenuated total reflection Fourier transform infrared as well as *in silico* modelling revealed that both forms of ghrelin mainly adopted the same structure in aqueous, micellar and bilayer environments even though acylated ghrelin structure is slightly more α-helical in a lipid bilayer environment.
Besides, based on our results along with the fact that the ghrelin binding pocket is thought to be located in the transmembrane domains of the ghrelin receptor. I hypothesized that the membrane probably acts as a "catalyst" in acylated ghrelin-ghrelin receptor interactions, as it does for other peptides. Physiologically our findings also imply that unacylated ghrelin is less likely to encounter and thus to bind to the ghrelin receptor than acylated ghrelin. Moreover, ghrelin conformational flexibility may be associated with its various roles in different tissues.

TABLE OF CONTENTS

FOREWORD **1**

CHAPTER I **INTRODUCTION** **3**

CHAPTER II **AIMS OF THE THESIS** **73**

CHAPTER III **VALIDATION OF A METHOD FOR THE QUANTITATION OF GHRELIN AND UNACYLATED GHRELIN BY HPLC** **77**

CHAPTER IV **ACYLATED AND UNACYLATED GHRELIN BINDING TO MEMBRANES AND TO GHRELIN RECEPTOR: TOWARDS A BETTER UNDERSTANDING OF THE UNDERLYING MECHANISMS** **99**

CHAPTER V **DISCUSSION AND PERSPECTIVES** **137**

FOREWORD

Lipids along with genes, proteins and carbohydrates constitute the fundamental material of any living organism. While proteins, genes and carbohydrates are known to have specific biological functions, lipids have long been considered to have non specific functions. However, over the last twenty years, there has been increasing evidence that they also play active roles in cellular functioning. Membrane lipids have been shown to participate in the protein folding, organization, final structure and function of membrane proteins. Some specific membrane lipids even act as second messengers. Moreover, some peripheral proteins are also targeted to the membrane by specific and non specific interactions with the membrane lipids. Besides, the co-translational or post-translational addition of long lipid groups such as myristoyl, palmitoyl, isoprenyl and glycosyl phosphatidylinositol, to peptides and proteins drives them to the membrane and thereby modulates their activity.

In 1999, Kojima and co-workers discovered a hormone peptide that possesses a unique post-translational modification: an *O*-octanoylation at its Ser^3 residue. As this 28 amino acids peptide was the endogenous ligand of the growth hormone secretagogue receptor, they named it ghrelin. Both forms of ghrelin - acylated and unacylated - are endogenous and have biological activities. However, the *O*-octanoylation has been shown to be essential for efficient binding and activation of the receptor that mediates most of its endocrinological effects.

Therefore, the main objective of this thesis was to investigate the role of ghrelin *O*-octanoylation in ghrelin-membrane interaction using model membranes as it could modulate its biological activity.

Chapter I.

INTRODUCTION

TABLE OF CONTENTS

1. GHRELIN 7

1.1. ORIGIN AND DISCOVERY 7

1.2. GHRELIN GENE 7

1.3. GHRELIN STRUCTURE 8

1.4. GHRELIN RECEPTOR FAMILY 13

1.5. GHRELIN BIOLOGY & POTENTIAL CLINICAL RELEVANCE 17

1.6. GHRELIN QUANTIFICATION 20

2. BIOLOGICAL AND MODEL PLASMA MEMBRANES 23

2.1. STRUCTURE AND FUNCTION OF PLASMA MEMBRANES 23

2.2. MEMBRANE MIMICKING MODELS 28

2.3. LIPOSOMES 31

3. PEPTIDE-MEMBRANE INTERACTIONS AND TRANSPORT 39

3.1. PEPTIDE-MEMBRANE INTERACTIONS 39

3.2. ACYLATED PEPTIDES: MEMBRANE INTERACTION & TRANSPORT 52

4. REFERENCES 59

INTRODUCTION

1. GHRELIN

1.1. ORIGIN AND DISCOVERY

In 1976, Bowers and co-workers found that some synthetic enkephalin opiate analogues exhibited some growth hormone (GH) releasing activity *in vitro* [1]. However, these peptide derivatives were not active *in vivo*. In the early 80's, they successfully synthesized potent small peptides that stimulate both *in vitro* and *in vivo* GH release independently of the GH releasing hormone (GHRH) receptors [2,3]. The first of these potent GH releasing peptides (GHRPs) was a hexapeptide called GHRP-6. This peptide was the first compound of a new class of peptide and non-peptide synthetic molecules termed GH secretagogues (GHSs). In 1996, the GHS receptor (GHS-R1a) was identified by expression cloning as the target receptor of these GHSs from the human and swine pituitary and hypothalamus by Howard and co-workers [4]. However, this G protein coupled receptor (GPCR) remained an orphan GPCR for some time as many groups were looking for the endogenous ligand in the brain. In 1999, Kojima and co-workers tested other tissues [5]. To identify rat tissue extracts that could stimulate the GHS-R1a they used a stable CHO cell line expressing rat GHS-R. Several rat tissue extracts were screened: brain, lung, heart, kidney, stomach and intestine. The stomach extracts were unexpectedly exhibiting the highest activity. From these extracts they purified and isolated the endogenous ligand and named it "ghrelin" after its GH releasing activity as "ghre" is the Proto-Indo-European root for growth [5]. Ghrelin is a 28 amino acid (AA) peptide which is unusually modified by a *n*-octanoic acid on the hydroxyl group of its serine 3 residue. Ghrelin was the first known peptide hormone modified by a fatty acid.

1.2. GHRELIN GENE

1.2.1. Ghrelin locus

The human ghrelin gene is localized on the chromosome 3 at location p25–26 [6]. The human ghrelin receptor gene has also been identified on chromosome 3, at position q26–27 [7].

The human ghrelin gene comprises six exons. The 20 bp long exon 0 is non coding [8]. There are several ghrelin gene-derived mRNA transcripts including one that encodes preproghrelin and hence ghrelin; others encode either the C-terminal region of full-length preproghrelin (*C*-ghrelin, which contains the coding region for obestatin) or obestatin only, but there are also natural antisense transcripts termed ghrelin opposite strand transcripts [9]. The ghrelin locus is thus quite complex.

1.2.2. Ghrelin gene-derived products

The main form of gastric human ghrelin mRNA (transcript A) is translated into prepro-ghrelin, a 117 AA polypeptide [5]. Prepro-ghrelin includes a 23 AA signal peptide and a 94 AA polypeptide called pro-ghrelin (1-94). This pro-ghrelin contains the 28 AA des-acyl ghrelin sequence and a 66 AA tail (29-94). This 66 AA tail (29-94) circulates as a full-length peptide (*C*-ghrelin) or is processed into smaller peptides, mainly obestatin, a 23 AA amidated peptide [10]. Both may have physiological importance [11]. Des-Gln-14-ghrelin is another ghrelin gene splicing variant. It is a 27 AA peptide identical to ghrelin except for the deletion of Gln14 [12]. Des-Gln-14-ghrelin can also be acylated and is as potent as ghrelin (in rats) but is present in much lower amounts [12].
Ghrelin post-translational modifications will be discussed in Section 1.3.1.A.

1.3. GHRELIN STRUCTURE

1.3.1. Primary structure

Ghrelin structure is unique as it is the only known hormone peptide to be acylated by a medium chain fatty acid on one of its serine [5]:

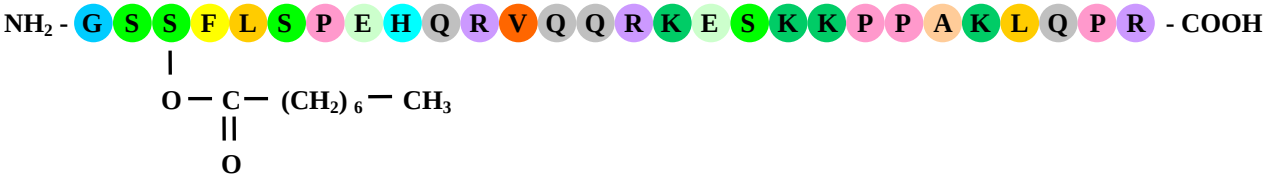


Figure 1 - Human octanoylated ghrelin primary structure.

The structure of mammalian ghrelins is well conserved, especially the first 10 AA that are identical. It has thus been suggested that this NH_2-terminal region is of central importance to the activity of the peptide [13].

A. Ghrelin post-translational isoforms

The major translated form of human ghrelin is a 28 AA des-acyl peptide called unacylated ghrelin or des-acyl ghrelin (1-28). The C-terminal amino acid, Arg^{28}, of unacylated ghrelin (1-28) is sometimes cleaved and leads to the formation of unacylated ghrelin (1-27) [14].

The translated unacylated ghrelin (1-27) and (1-28) forms as well as des-Gln^{14}-ghrelin can be further processed by acylation of their Ser^3 residue. The lipid transferase that mediates this unusual modification has recently been identified and characterized. The ghrelin *O*-acyl transferase (GOAT) is a conserved orphan membrane-bound *O*-acyl transferase that specifically octanoylates the Ser^3 of the ghrelin peptides [15,16].

In humans, ghrelin is predominantly modified by a *n*-octanoyl ($C_{8:0}$) group. However, human ghrelin isolated from the stomach has also been observed to be modified by decanoyl ($C_{10:0}$) and possibly decenoyl ($C_{10:1}$) groups [14].

B. Ghrelin active core

In vitro, both unacylated and acylated ghrelin are full agonists of the GHS-R1a, acylated ghrelin being active in the nanomolar range whereas unacylated ghrelin is only active in the micromolar range [17]. However, *in vivo*, ghrelin acylation has up to now been considered to be essential for potent GHS-R1a activation.

In the early 2000's, two groups investigated many structural aspects of ghrelin that could affect, *in vitro*, its potency to activate the receptor in order to determine the ghrelin active core. They performed many modifications of the peptide such as: the acyl position in the chain, the acyl length and conformation, the serine-acyl bond nature, the third amino acid nature and the peptide length. Their extensive *in vitro* studies highlighted the necessity of (i) a bulky flexible or rigid hydrophobic group in the side chain of residue 3; (ii) the first 4-5 amino acids; (iii) the L-conformation of the third amino acid; (iv) a positive charge at the N-terminus for the full activation of the GHS-R1a, whereas the nature of the bonding structure was not essential [18,19]. In spite of this, *in vivo* subsequent studies revealed that C-terminally truncated analogues of acylated ghrelin other than acylated ghrelin (1-27) [14] were unable to induce a GH release in rats [20,21]. Still Dornonville de la Cour et al. showed that acylated ghrelin (1-18), (1-10) and (1-5) could stimulate gastric emptying in rats but only

at very high concentrations [22] and suggested that the C-terminal portion of ghrelin may increase the potency and stability of the peptide.

1.3.2. Secondary structure

Ghrelin structure studies were up to now mostly concentrated on the primary structure (see Section 1.3.1). Only few studies related to the 3D structure of acylated and unacylated ghrelin have been published so far [23-25]. Circular dichroism (CD) spectroscopy of acylated ghrelin and ^{1}H nuclear magnetic resonance (NMR) spectroscopy of acylated and unacylated ghrelin studies were indicative of a undefined random coil structure in acidic (pH < 2) solution [24]. For acylated ghrelin, energy minimization followed by 10-ns molecular dynamics simulations in water at pH 7 showed an endpoint structure having a short a-helix from Pro^{7} to Glu^{13} and a hairpin structure with Glu^{17} to Lys^{20} in the bending region (**Fig. 2**). The characteristic fold of acylated ghrelin obtained by this molecular dynamics simulation did not significantly change in a subsequent 15-ns molecular dynamics simulation in presence of a DMPC lipid bilayer in water (pH 7) [23,26] (**Fig. 2**). Kukol hypothesized that the difference of time resolution, milliseconds for of ^{1}H NMR and nanoseconds for molecular dynamics simulations as well as pH and hence charge differences, might explain the differences of conformation observed [26].

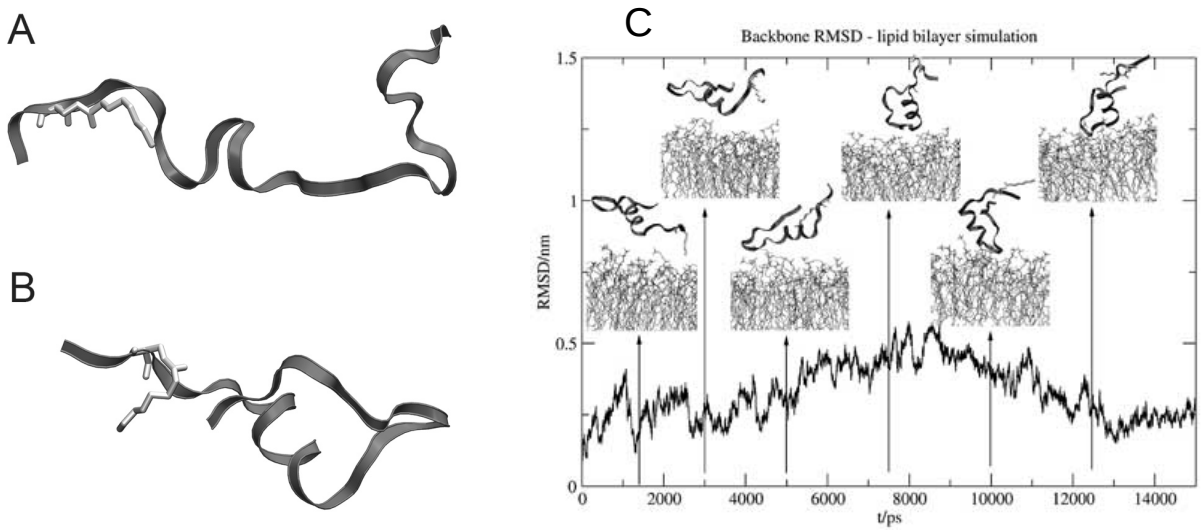


Figure 2 - Peptide backbone structures of ghrelin with the octanoyl side chain of Ser^{3} highlighted. (A) After 2 ns of simulated annealing molecular dynamics from 450 K to 273 K in water. (B) Structure after 10 ns molecular dynamics simulation at 298 K followed by energy minimization in water. (C) Root mean square deviation of the peptide backbone with respect to the starting structure shown in Fig. 2B. Snapshots at different time points are shown. Only a part of the upper bilayer leaflet is shown. Water molecules are not shown. [23,26]

1.3.3. Physico-chemical properties

A. Acido-basic properties

Based upon the pKa values of the N- and C-terminus as well as those of the ionisable lateral residues (Glu, Arg, His and Lys), it is possible to estimate the net charge of a peptide at any pH. As shown on **Fig. 3**, ghrelin is a highly basic peptide and is therefore highly positively charged at physiological pH. Ghrelin calculated isoelectric point is 11.58. It is noteworthy to mention that the net charge profiles of acylated and unacylated ghrelin as a function of pH are identical as the octanoyl is located on a serine and that the hydroxyl group of serine is not ionisable in water.

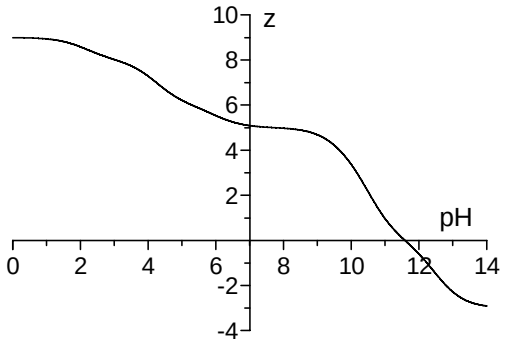


Figure 3 - The net charge (z) of ghrelin as a function of pH. I calculated the net charge as follows:

$$z = \sum_i N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pKa_j}}$$

where N_i and pKa_i are the number and the pKa values [27] of the N-terminus and the side chains of Arg, Lys and His. N_j and pKa_j are the number and pKa values [27] of the C-terminus and the side chain of Glu. pH was varied from 0 to 14 with 0.01 increments.

B. Hydrophilic/hydrophobic properties

The hydrophobicity of a peptide can be calculated on the basis of the hydrophobicity of each residue. There are several hydrophobicity scales, the 2 most commonly used being the Kyte & Doolittle and the Eisenberg scale [28]. The Eisenberg scale, which I have used to calculate ghrelin hydrophobicity, is a normalized consensus scale which shares many features with the other hydrophobicity scales. We can see on **Fig. 4** that most of ghrelin lateral groups are hydrophilic. However, the N-terminal region where the octanoyl is located is more hydrophobic.

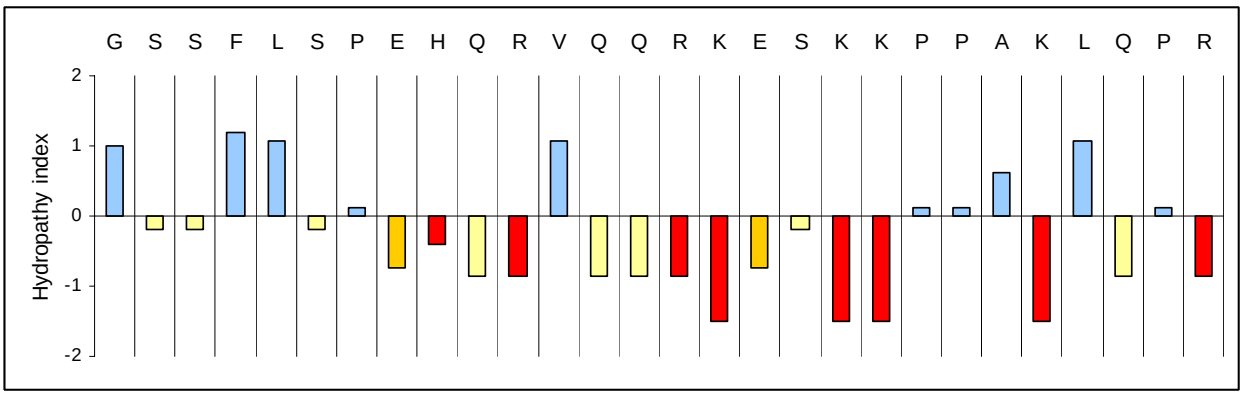


Figure 4 - Hydropathy index of human ghrelin lateral groups according to the normalized consensus scale of Eisenberg [29]. Hydropathy values indicate a tendency of the ghrelin lateral groups to either seek a hydrophobic environment (+ values) or an aqueous environment (- values). The colours of the bars represent the acid-base properties and the polarity of the lateral groups (i) blue: neutral, non polar AA; (ii) yellow: neutral, polar; (iii) orange: acidic, polar; (iv) red: basic, polar. [27]

C. Physico-chemical and biological stability

In rats, the blood half-life of rat ghrelin after intravenous administration is short i.e. ~24 min for unacylated ghrelin and only ~11 minutes for acylated ghrelin [30,31].

Human ghrelin in blood samples is not very stable but its stability can be improved by appropriate handling and storage conditions. Indeed, even though unacylated ghrelin is fairly stable, acylated ghrelin is easily and rapidly converted into unacylated ghrelin ($t_{1/2}$ ~90 min in untreated serum at 37°C) [32]. Butyrylcholinesterases are responsible, at least partially, for acylated ghrelin deoctanoylation in human blood [33,34]. Optimal acylated ghrelin handling and storage stabilities of blood samples are achieved by combination of (1) blood collection on ice; (2) EDTA collection tubes containing proteases and/or esterases inhibitors; (3) 10-fold dilution; (4) acidification to pH 4; (5) centrifugation within 30 minutes [32,35,36].

As regards repeated freezing and thawing, unacylated ghrelin is stable after at least 5 cycles without pre-treatment whereas acylated ghrelin remains relatively stable after acidification to pH 4 (93.8 ± 7.0% of baseline) but not in unacidified samples (14.5 ± 3.3%) [32,36]. While maximal stability of serum samples is achieved at pH 3-4, it is markedly decreased in strongly acidic (pH < 2), neutral and alkaline conditions [32]. Synthetic human ghrelin has been shown to be physically and chemically stable at room temperature for 3 days at pH 5.5 [37].

Synthetic ghrelin counter-ions also influence the stability of rat acylated ghrelin. Whereas trifluoroacetate counter-ions provides a good octanoyl group stability in water, acetate counter-ions lead to deacylation and dehydration of ghrelin at the Ser^3 residue [38].

1.4. GHRELIN RECEPTOR FAMILY

1.4.1. Nomenclature

The GHS-R is a member of the GPCR family. As already mentioned in Section 1.1, the receptor of ghrelin, the GHS-R1a, was discovered before its endogenous agonist. At the time of its cloning, it was an orphan receptor and the only known agonists were the GHS. Therefore, the receptor was named the growth hormone secretagogue receptor (GHS-R). In 2005, the nomenclature of the GHS-R1a was revised by the International Union of Pharmacology (IUPHAR). They published a review that designates the GHS-R1a as the ghrelin receptor, following the IUPHAR convention to name receptors after their endogenous agonist. They also decided that the abbreviation of ghrelin receptor, if necessary, was going to be the GRLN receptor [39]. However, nowadays, the name GHS-R1a is still the most widespread appellation [40,41].

1.4.2. Structure and functions of the GHS-R1a and 1b

At the time of GHS-R cloning, two evolutionary conserved transcript variants were identified: the transcripts 1a and 1b [4]. The transcript 1a (GHS-R1a) encodes for a 366 AA functional protein with the characteristic seven transmembrane (TM) domains of the GPCR, whereas the transcript 1b (GHS-R1b) is formed by an alternative splicing of the GHS-R gene and encodes for a truncated protein of 289 AA that lacks the last two TM domains but possesses an unique 24 AA 'tail'[4]. Besides the ghrelin induced GH releasing effect, the GHS-R1a also mediates food intake and energy expenditure. The constitutive, ligand-independent signalling activity of GHS-R1a is very high, i.e. ~ 50% of the maximum activity [42-44] and it has recently been shown that this high constitutive activity is probably of physiological relevance. Indeed, naturally occurring mutations of the GHS-R1a eliminating the constitutive activity of the receptor but not the ghrelin affinity, potency or efficacy was associated with short stature and obesity [45]. Therefore, there is now a tendency to develop selective inverse agonist instead of antagonists drugs for inhibiting ghrelin receptor function [46-48]. Other ghrelin effects will be discussed in Section 1.5.2.

Even though the GHS-R1b mRNA expression is more intense and more widespread than GHS-R1a [49], it has long been thought to be devoid of pharmacological activity but Leung et al. showed that this receptor acts as a dominant-negative mutant of GHS-R1a by the formation

of GHS-R1a/GHS-R1b heterodimers in HEK-293 cells overexpressing GHS-R1a. GHS-R1b may therefore attenuate the activity of GHS-R1a [50].

1.4.3. Representative GHS-R1a ligands

The GHS-R1a ligands are numerous. It is worth mentioning that unlike most 7TM receptors, GHS-R1a is an agonist-prone receptor, i.e. it is relatively easy to discover agonists and relatively difficult to discover antagonists/inverse agonists [51]. In **Table 1**, I listed some of the representative ligands that are active *in vivo*. Some of the agonists are allosteric modulators of ghrelin action. It is to be noted that, *in vitro*, the substance P analogue is a low potency antagonist (micromolar) but a high potency inverse agonist (nanomolar) [42].

Table 1 - Representative *in vivo* active GHS-R1a ligands.

Type of ligand		Ligand	Origin	*In vitro* model	*In vivo* model	Ref
Agonists		Acylated ghrelin	Endogenous peptide	CHO	r, h	[19,52]
		Des-Gln14-acylated ghrelin	Endogenous peptide	CHO	m, r	[12,19,22]
Allosteric modulation	Negative (inhibition)	GHRP-6	Synthetic peptide	COS	r, h	[1,53]
	Neutral	MK-0677	Synthetic small molecule	COS	r, h	[7,53]
	Positive (synergy)	GHRH (1-29) *in vitro* and GHRH *in vivo*	Truncated endogenous peptide	HEK 293 CHO	h	[54,55]
Inverse agonist		[D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu- 11] substance P	Synthetic peptide	COS		[42]
Antagonists		[D-Lys-3]-GHRP-6	Synthetic peptide		m, r	[56,57]
		[D-Arg-1, D-Phe-5, D-Trp-7, 9, Leu- 11] substance P	Synthetic peptide	COS	m	[42,56]
		BIM-28163	Not described (*Ipsen*)	CHO	r	[58]
		L-NOX-B11	RNA-Spiegelmer	CHO	r	[59]

(h) human, (m) mouse, (r) rat

1.4.4. Ghrelin binding pocket and GHS-R1a activation

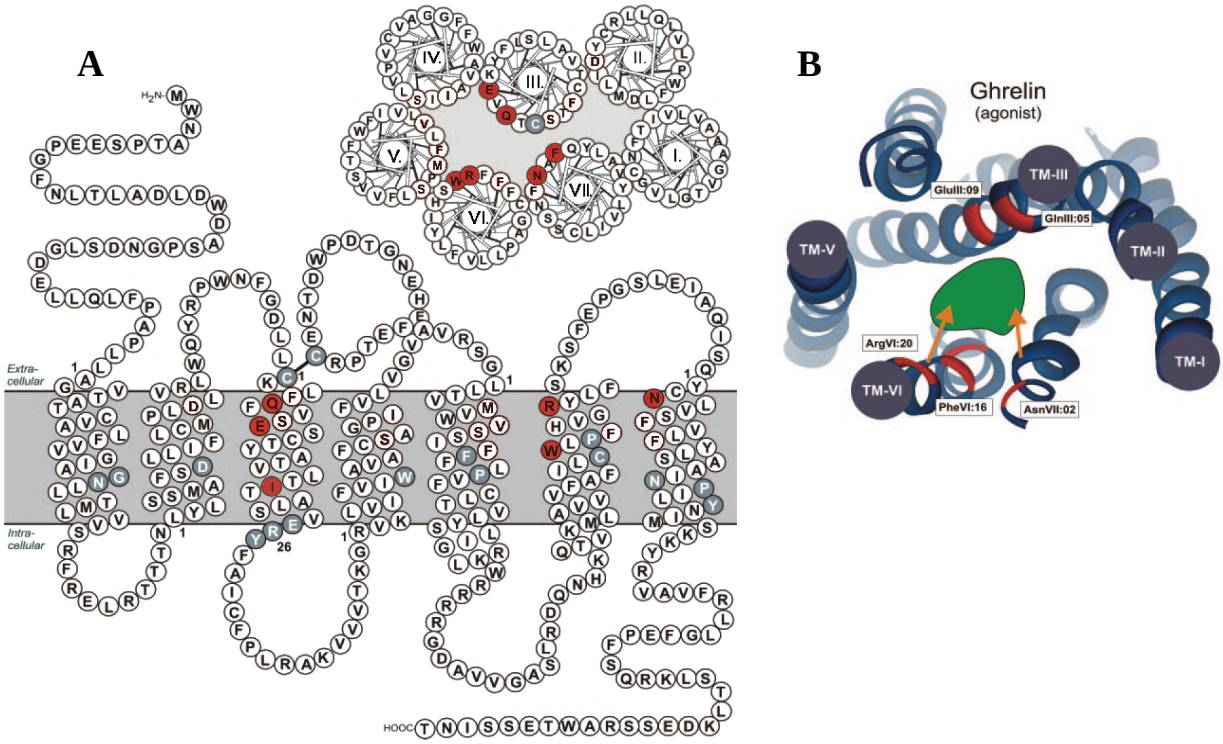


Figure 5 - Structure of the hGHS-R1a and schematic model of the ghrelin binding mode. (A) In the serpentine and helical wheel diagrams of the hGHS-R1a, the mutational hit residues are marked in black on red whereas highly conserved residues are marked with white on grey. The proposed first residue of each TM helix is indicated by "1". (B) Schematic model of the ghrelin binding mode in the main ligand-binding crevice of the ghrelin receptor. Molecular model of the ghrelin receptor - built over the X-ray structure of the inactive form of rhodopsin - as viewed from the extracellular side, where only the seven helical domains are shown in solid ribbon with the identified mutational hits for ghrelin being highlighted in red. The space presumed to be occupied by the agonist ghrelin is indicated in green. Adapted from [43,47].

According to the docking studies performed by Pedretti et al. [60] with a truncated acylated ghrelin (1-4) on a constructed GHS-R1a model, the octanoyl chain is accommodated in an apolar pocket and shows hydrophobic interactions with residues Pro^{192}, Trp^{193}, Pro^{200}, Ala^{204}, and Val^{205}, whereas the ester establishes H bonds with Asn^{188} and Thr^{190}. This apolar pocket is mainly lined by the extracellular loop 2 that partially penetrates the TM segment. They also highlight that the TM-III segment also bears key residues involved in truncated ghrelin recognition [60]. However, even though the naturally occurring mutation of Ala^{204} was found in families with short stature [45,61], this mutation only affects the constitutive but not the ligand induced activity of the receptor [43]. Therefore, it is improbable that Ala^{204} is implicated in ligand recognition and/or receptor activation as suggested by Pedretti et al. Subsequent mutational mapping of the presumed ghrelin binding pocket revealed 3 major (Glu^{124}, Ile^{134}, and Arg^{283}) and 3 minor (Gln^{120}, Trp^{276} and Asn^{305}) binding sites belonging to the TM-III, VI and VII extracellular segments. The role of Phe^{279} in ghrelin binding is not

clearly defined [43,47]. Except for Ile^{134}, these key residues, within the main ligand binding pocket, are located at the opposing faces of the TM-III, TM-VI and TM-VII extracellular ends, which is in accordance with the global toggle-switch model for 7TM receptor activation [47]. According to this model, during receptor activation, the TM-VI and TM-VII perform "vertical" see-saw movements around the central conserved proline, i.e. their extracellular segments bend inward into the main ligand binding pocket and thus toward TM-III, while their intracellular segments will move outwards revealing epitopes that can be recognized by intracellular signalling molecules, G proteins for example [51].

1.4.5. Evidence of other ghrelin receptors

As early as 2002, Baldanzi et al. reported high affinity binding sites for both acylated and unacylated ghrelin, mediating cell protective effects, in cells that did not express GHS-R1a [62]. Since then, there has been increasing evidence for the existence of different GHS-R subtypes (GHS-Rx) that bind acylated and/or unacylated ghrelin. Indeed, several tissues and cell lines that do no express GHS-R1a but display high affinity binding sites for ghrelin have been discovered, even though these receptors have not yet been cloned. Some of the shared binding sites for acylated and unacylated ghrelin ($GHS\text{-}Rx_a$) mediate common effects including cell differentiation and/or cell proliferation of normal tissues, cell survival in pro-apoptotic conditions and stimulation of adipogenesis. In contrast, opposite effects of acylated and unacylated ghrelin on glucose release have been attributed to the binding to distinct receptors, $GHS\text{-}Rx_b$ for unacylated ghrelin. See [11,63-66], for review.

1.4.6. Tissue distribution of GHS-R

As already mentioned, mRNA of GHS-R1b is widely expressed (for details, see [49]).
In the central nervous system, expression of GHS-R1a mRNA is found in the pituitary gland, the hypothalamus and the hippocampus but also in many other brain areas that are linked to ghrelin observed actions. A widespread pattern of expression of the gene encoding GHS-R1a has also been demonstrated by RT-PCR analyses in a variety of peripheral organs, including the pancreas, stomach, small and large intestine, thyroid gland, myocardium, adrenal gland, liver, kidney, spleen, testis, ovaries, adipose tissue and immune cells. This distribution indicates that ghrelin has multiple functions in these organs or tissues [4,13,49,63,65-67].

1.5. GHRELIN BIOLOGY & POTENTIAL CLINICAL RELEVANCE

1.5.1. Tissue distribution

Ghrelin was naturally discovered from stomach extracts [5] as it is the major site of synthesis for circulating ghrelin [68]. In the stomach, the ghrelin-secreting cells are X/A-like cells of the oxyntic mucosa. In these cells, ghrelin is contained in round, compact, electron-dense granules [69]. Ghrelin is present through the intestinal tract from the stomach to the colon with the highest concentration in the stomach fundus [69]. Even though ghrelin is secreted, the acyl modification is not essential for the secretion as unacylated ghrelin circulates in the blood of ghrelin *O*-acyl transferase-null mice [15].

Ghrelin is also produced by the pancreatic islets. In foetus, ghrelin concentration in the pancreas is even more elevated than in the stomach. However, the islet cell type, either the α, β or the newly identified ε cells, that produces pancreatic ghrelin in the pancreatic islets remains controversial [70-72].

Ghrelin has been found to be produced in lower amounts in other organs such as the pituitary gland, hypothalamus, cerebellum, lung, kidney, spleen, eye, placenta, gonads, salivary gland, thyroid, heart, ovary and testis [49,64,68,69,73,74].

Whereas in humans the stomach acylated: unacylated ghrelin ratio is about 3:1 [14], in blood sera this ratio falls down to only 1:20 to 1:5 according to the blood processing method [32,35,75]. Unacylated ghrelin is thus considered to be the main circulating form of ghrelin.

1.5.2. Biological functions

As already mentioned, acylated ghrelin is a pleiotropic hormone that has been discovered due to its GH releasing activity [5]. Besides this significant effect, it has become evident over the years that acylated ghrelin is a key player in the regulation of energetic homeostasis. In particular, acylated ghrelin induces appetite and food intake upon fasting, decreases energy expenditure and promotes fat mass accumulation but also participates in glucose metabolism, among others. Numerous other biological activities have been attributed to acylated ghrelin since its discovery 11 years ago.

Unacylated ghrelin, however, has long been thought to be devoid of biological functions but it now appears that both these forms of ghrelin present similar and opposite actions. **Table 2** is a

non exhaustive list of the acylated and unacylated ghrelin effects and includes the presumed or demonstrated receptors involved.

Table 2 - Effects of acylated and unacylated ghrelin and presumed or demonstrated receptors involved. Adapted from [11,63-66,74,76].

Effect	Acylated ghrelin (AG)		Unacylated ghrelin (UAG)	
	Activity	Receptor type	Activity	Receptor type
Endocrine				
GH	↑ (h)	GHS-R1a	↔ ↓ (mice overexpressing UAG)	
ACTH, PRL, glucocorticoids	↑ (h)	GHS-R1a	↔	
Appetite/body weight				
Appetite	↑ (h)	GHS-R1a	↑↓	
Food intake	↑ (h)	GHS-R1a	↑ ↓ (most available data)	GHS-Rx_b
Body weight	↑ (h)		↓	
Water intake	↓		↔	
Metabolism				
Energy expenditure	↓ (h)	GHS-R1a	nd	
Glucose				
Peripheral insulin sensitivity	↓	GHS-R1a	↑	GHS-Rx_b
Glucose stimulated insulin secretion	↑↓ depending on blood glucose levels	GHS-R1a	↓↑	GHS-Rx_b
Insulin action	↑ (periphery)/ ↓ liver		↔ (periphery)/ ↓ liver	
Glucose production	↑	GHS-R1a	↓	GHS-Rx_b
Lipid content				
Body fat mass	↑			
Adipocytes	↑	GHS-Rx_a	↑↓	GHS-Rx_a
Reproduction				
LH secretion	↓		↓	
Embryogenesis	↓		nd	
Testosterone secretion	↓		nd	
Spermatogenesis	↓		nd	
Gastrointestinal				
Gastric acid secretion	↑↓	GHS-R1a	↔	
Epithelial protection	↑		nd	
Motility	↑ (stomach (h) and colon)	GHS-R1a	↓ (stomach) ↔ (jejunum)	
Cardiovascular				
Arterial blood pressure	↓ but ↔ heart rate (h)		↓	
Endothelium function	↑		nd	
Heart function	↑		↑	
Cardiac cells protection	↑	GHS-Rx_a	↑	GHS-Rx_a

Effect	Acylated ghrelin (AG)		Unacylated ghrelin (UAG)	
	Activity	Receptor type	Activity	Receptor type
Cell proliferation				
Normal cell lines (+differentiation)	↑	GHS-Rx_a	↑	GHS-Rx_a
Tumour cell lines	↑↓	↑ GHS-R1a ↓ GHS-Rx_a	↑↓	↓ GHS-Rx_a
Immune function				
Immune cell production	↑		↔	
Proinflammatory cytokine production	↓ (h)	GHS-R1a	↔	
Neutrophil action	↓		nd	
Bone formation	↑	GHS-R1a ?	↑	
Sleep	↑		↔	
Learning	↑	GHS-R1a	nd	
Memory	↑	GHS-R1a	↔	
Anxiety	↑		↔	
Iris muscle relaxation				
Sphincter	↑		↑	
Dilator	↑		↔	

(↑) stimulation/increased activity, (↓) inhibition/decreased activity, (↔) no effect, (nd) effect not determined, (h) human

1.5.3. Potential therapeutic applications

Due to the wide range of ghrelin biological activities, there are many potential therapeutic applications for ghrelin but also for ghrelin agonists, antagonists and inverse agonists.

Ghrelin and ghrelin agonists have been proposed for the therapy of GH-deficiency states, anorexia, cachexia, metabolic syndrome, gastrointestinal motility disorders, cardiac dysfunction, inflammatory conditions, osteoporosis and Alzheimer's disease, among other things [64,65,77].

Currently, acylated ghrelin most studied and promising therapeutic use lies in the treatment of cachectic patients. Indeed, cachexia is characterized by a loss of weight and muscle mass and frequently associated with elevated inflammatory cytokines, three factors that seem to be positively influenced by ghrelin in cachectic patients and that would improve their quality of life [78,79].

For the treatment of obesity, there was and still is a high interest for drugs that either suppress acylated ghrelin production and action, or have an opposite effect. This led to the development of ghrelin antagonists, anti-ghrelin antibodies, ghrelin-specific RNA spiegelmers

and more recently ghrelin inverse agonists as ghrelin receptor possesses a high constitutive activity [48,80]. The recent discovery of ghrelin *O*-acyl transferase [15,16] will probably also lead to the development of ghrelin *O*-acyl transferase inhibitors to prevent acylated ghrelin production and thus ghrelin orexigenic effects [81].

1.6. GHRELIN QUANTIFICATION

1.6.1. Fluorescence

Ghrelin does not have a high intrinsic fluorescence. Indeed, its amino acid sequence comprises only 1 phenylalanine out of 28 AA and neither tryptophan nor tyrosine. As the phenylalanine (λ_{Exc}: 240-250 nm; $\lambda_{Em\ max}$: 280-282 nm) has a low quantum yield (0.02 to 0.04), it limits its use for fluorescence studies [82-84]. In our laboratory, a 50 µg/ml ghrelin aqueous solution (λ_{Exc}: 240 nm; λ_{Em}: 282 nm) only yielded 4 RFU which is not enough for a quantification use.

Acylated and unacylated ghrelin labelled with fluorescent probes such as rhodamine and fluorescein have been developed by peptide manufacturers (Phoenix Pharmaceuticals and NeoMPS) for imaging purposes. However, on the one hand they are quite expensive, and on the other hand, as they modify the peptide structure, they might also affect their functions.

1.6.2. UV spectroscopy

UV molar extinction coefficients of a protein or of a peptide at a given wavelength λ (ε_λ) is the sum of the molar extinction coefficients of each of its constituents i.e. peptide bond and side chain groups, at that wavelength [85].

Proteins, on the one hand, are usually quantified by UV spectroscopy at 280 nm as they contain tryptophan and tyrosine, and that their side chains highly absorb at that wavelength (i.e. ε_{280} of 5500 and 1210 M^{-1} cm^{-1}, respectively). Phenylalanine, however, does not absorb in this region (ε_{280} = 2 M^{-1} cm^{-1}) [82,83]. As shown in Section 1.3.1, ghrelin sequence lacks tryptophans and tyrosines. It is therefore no wonder that it does not absorb at 280 nm.

Peptides, on the other hand, are often quantified at 210-220 nm. Indeed, peptide bonds and amino acids side chains absorb in this region. Here are the major contributions to the UV absorption at 214 nm by decreasing order and expressed as molar extinction coefficients

(M^{-1} cm^{-1}): tryptophan (29050) > tyrosine ≈ phenylalanine ≈ histidine (5375 to 5125) > proline (2675) > methionine ≈ peptide bond (980 and 923) [85].

Again, ghrelin sequence does not contain any tryptophan that highly absorbs in this UV range. However, it does contain, in addition to the peptide bonds, 1 phenylalanine, 1 histidine and 4 prolines that allows the ghrelin UV quantification in the 210-220 nm range but with a low sensitivity due to its low molar absorption coefficient (ghrelin $\varepsilon_{214} \approx 50000$ M^{-1} cm^{-1}). This value is based on our experimental determination of a ghrelin solution in water (40µg/ml) using a 1 cm path length quartz cuvette, as well as on the value that we calculated on the basis of the amino acid sequence according to Kuipers and Gruppen [85]. The experimentally determined and the calculated molar absorption coefficients of ghrelin at 214 nm were 50984 and 47462 M^{-1} cm^{-1}, respectively.

Therefore, UV detection in combination with a reverse phase high liquid performance chromatography (RP-HPLC) separation system has been widely used in purification processes for the separation of acylated and unacylated human ghrelin [18,86,87]. It has also sometimes been used as a detection method [5,87] although RIA was the typical quantification method in this case [88,89].

1.6.3. Radioactivity

Either ^{125}I or ^{3}H radiolabelled ghrelin is commercially available. ^{125}I radiolabelled ghrelin has the disadvantage to have a short half-life (60 days) and to emit γ-rays. ^{125}I labelling is usually performed by iodination at tyrosine residues but as ghrelin sequence does not comprise any tyrosine, it can either be added to the sequence or replace the Phe in position 4 without change in ghrelin activity [90]. Another solution is to iodinate the histidines of the sequence.

^{3}H ghrelin has a longer half-life (12.4 years) and emits β-rays. As labelling by ^{3}H exchange in tritiated water occurs in basic conditions at some stage, labelling by ^{3}H gas exchange is preferred but requires special facilities.

Besides radioactivity safety concerns, radiolabelled ghrelin has other drawbacks: i) due to the localization of the labelling, direct counts do not give any idea about peptide degradation or acylation state; ii) high cost due to the price of radiolabelled ghrelin and unlabelled ghrelin.

1.6.4. Immunological methods

As concentrations of acylated and unacylated ghrelin in blood [35] or culture media samples are low (pico- nanomolar range), these samples are generally quantified using immunological methods such as RIA [32,75], ELISA [91,92] and EIA [93,94]. Even though the first tests developed did not discriminate acylated from unacylated ghrelin, nowadays most commercially available kits do. Sandwich assays, which allow intact peptide quantification, require monoclonal antibodies against the C-terminal and the N-terminal sequences of ghrelin.

These monoclonal antibodies have been patented and are only available under the form of ELISA or EIA kits and these kits are very expensive (~350 to 800€/ 96-wells plate). As for acylated ghrelin samples both acylated and unacylated ghrelin have to be quantified, the price is a serious drawback.

1.6.5. Mass spectrometry (MS)

An HPLC/MS/MS with online solid-phase-extraction has been developed in 2007 by Rauh et al. [94] to allow the quantification of acylated and unacylated ghrelin from blood and culture media samples. Indeed, the limits of detection of this method are 5.93 pM and 9.24 pM for acylated and unacylated ghrelin, respectively. The limits of quantification are 20.76 pM and 107.88 pM for acylated and unacylated ghrelin, respectively. The method is linear from ~20 pM to ~12 nM for an injection volume of 250 µl. However, this method requires an expensive apparatus and highly qualified staff.

2. BIOLOGICAL AND MODEL PLASMA MEMBRANES

2.1. STRUCTURE AND FUNCTION OF PLASMA MEMBRANES

All eukaryotic cells are surrounded by a plasma membrane. This membrane defines the cell and separates the inside from the outside. The plasma membrane consists of lipids, proteins and carbohydrates and its thickness is about 5–10 nm [95]. The lipids, proteins and carbohydrates proportions and compositions of the biological plasma membranes vary widely with the type of cells as well as with the environmental conditions [95].

On the extracellular side of the plasma membrane, the membrane surface is covered by the glycocalyx formed by the carbohydrates of the glycolipids and the transmembrane glycoproteins. The glycocalyx is involved, among other things, in cell to cell recognition and adhesion. On the intracellular side, the plasma membrane anchors to the cytoskeleton which participates in the cell shape and motility [96].

The organization of the membrane, whose fundamental structure is a lipid bilayer, can be represented (**Fig. 6**) by the fluid mosaic model developed in 1972 by Singer and Nicolson.

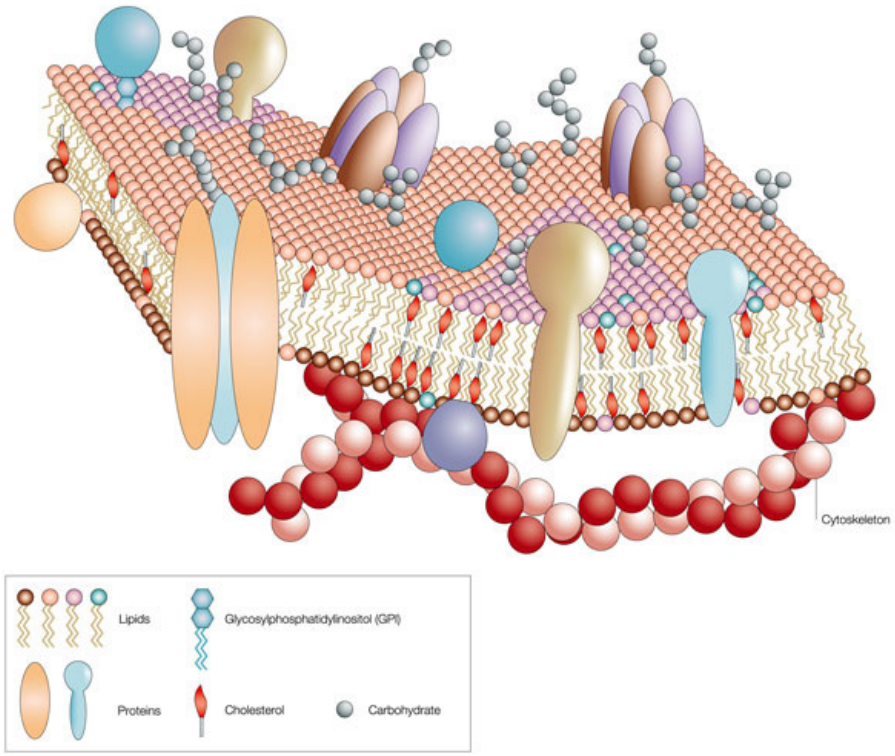


Figure 6 - The fluid-mosaic-model of the cell membrane [95].

The membrane is said to be fluid because a majority of its lipidic and proteic components can move thorough the membrane. The membrane structure is also called a mosaic because the membrane is composed of many different parts such as proteins, glycoproteins, phospholipids, glycolipids and cholesterol [97]. Although this fluid-mosaic model is still widely accepted, some refinements have been brought to the model. These refinements take into account that lipids, proteins and carbohydrates may distribute inhomogeneously and that domains and clusters, such as rafts or calveolae, may form within the membrane [96,98].

2.1.1. ***The lipid bilayer*** [96,99-101]

Lipids represent one of the major components of the mammalian plasma membranes. Lipids form the matrix of the plasma membrane which fundamental structure is a phospholipid bilayer that contains glycolipids and is enriched in cholesterol (**Fig. 7**).

The lipid bilayer acts as a barrier to the diffusion of polar solutes but also plays a role in the distribution, organization and function of membrane proteins [102].

In mammalian cells, except for neural tissues, phospholipids are the major lipid component of cell membranes (40 to 80% of dried weight). Major glycerophospholipids in mammalian plasma membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). Sphingomyelin (SM) and gangliosides are also found in plasma membranes.

The overall plasma membrane lipid and fatty acyl contents vary with the development stage as well as from one tissue to another. It is interesting to note that the differences in phospholipid content between homologous tissues of different species are smaller than between different tissues of the same specie. At the subcellular level, the lipid distribution of organelles and membranes widely differ too. The phospholipid and fatty acyl compositions are adapted to and by each tissue and organelle in order to ensure an appropriate environment and fluidity for its barrier function but also for optimal activity of the proteins associated with the membrane. By varying its local lipid composition, the lipid bilayer can affect a protein function by a change in the hydrophobic matching or mismatching between the protein and the lipid bilayer [102].

As most cells are composed of various types of membranes, nearly all lipid compositions published are overall lipid compositions. However, as the plasma membrane of erythrocytes ghosts is considered to be their only membrane, its composition has been widely studied and is given in **Table 3** as an example.

Table 3 - Lipids of the normal human erythrocyte membrane [103].

	µmol/10^{13} cells	molar % of total lipids
Phospholipids		
PC	1.3	15.3
PE	1.2	14.1
SM	1.0	11.8
PS	0.6	7.0
LysoPC	0.04	0.5
Others	0.07	0.8
Cholesterol	4.0	47.1
Glycolipids	0.21	3.4

Moreover, as shown in **Fig. 7**, the plasma membrane is asymmetric. However, this lipid asymmetry is not absolute as almost every type of lipid is present on both sides of the bilayer, but in different amounts. The outer leaflet consists predominantly of phosphatidylcholine, sphingomyelin and glycolipids, whereas the inner leaflet contains phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. Cholesterol is distributed in both leaflets. By comparison, the glycolipids are exclusively located in the outer leaflet and serve as anchors for some proteins. They are usually associated to membrane microdomains. The outer and inner leaflets thus differ in composition but also in activity and function.

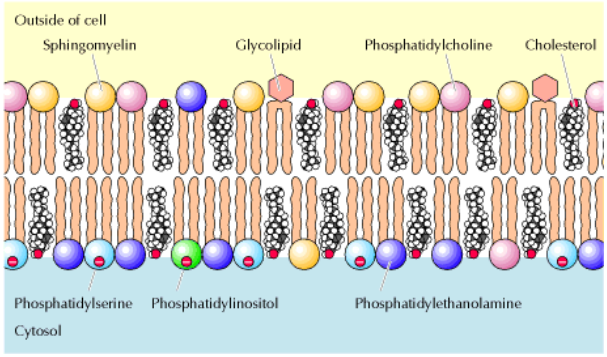


Figure 7 - Major lipid components of the plasma membrane [96,99].

Individual phospholipids can undertake rotational change, rotate and move laterally within a bilayer (**Fig. 8**). However, as it requires the passage of a polar headgroup through the hydrophobic core of the membrane, phospholipids do not spontaneously migrate, or flip-flop from one leaflet to the other. Nevertheless, in some membranes, mostly in endoplasmic

reticulum membranes which is the major site of plasma membrane lipids synthesis, phospholipids can rapidly flip-flop in an ATP-powered process catalyzed by membrane proteins called flippases.

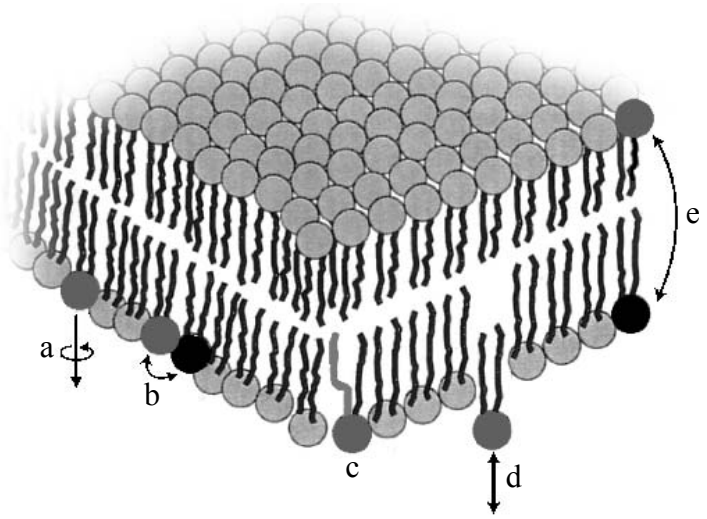


Figure 8 - Mobility of phospholipids in a membrane. (a) Rotation around the molecular axis. (b) Lateral diffusion. (c) Conformational change. (d) Protrusion out of bilayer plane. (e) Flip-flop between lipid monolayers [104].

*2.1.2. **Plasma membrane proteins*** [96,105]

Proteins represent the other major component of the plasma membrane.

Eukaryotic plasma membranes proteins play crucial roles in a variety of essential processes: (i) selective import and export of water, ions, small and large molecules; (ii) binding of signalling molecules leading to cellular response; (iii) mediation of attachment to the extracellular matrix; (iv) stabilization of the cell shape through attachment of the cytoskeleton.

In the fluid mosaic model, proteins are classified according to their location in the membrane as integral, lipid-anchored or peripheral proteins. Integral membrane proteins, also called transmembrane proteins, span the lipid bilayer. These proteins are amphipathic. In all transmembrane proteins examined so far, the transmembrane spanning domains consist of one or more α-helices or of β-barrels. Lipid-anchored proteins are bound covalently to one or more lipid molecules while their polypeptide chains do not enter the phospholipid bilayer. Peripheral membrane proteins, however, are either indirectly associated with the membrane, generally through interactions with integral or lipid-anchored membrane proteins, or directly interact with the lipid headgroups.

Cytoskeletal filaments can be more loosely associated with the cytosolic face of the membrane, usually through one or more peripheral proteins.

In eukaryotic plasma membranes, many membrane proteins are modified by the addition of carbohydrates (glycoproteins) or by the attachment of lipids.

Glycoproteins in the plasma membrane are mainly transmembrane proteins. Their carbohydrates, which are exposed on the cell surface, play an important role as recognition sites in cell-cell interactions.

Some other plasma membrane proteins are modified by the attachment of lipids. Depending on the lipid modification, the protein will be anchored to the cytosolic or to the extracellular face of the plasma membrane. While myristoylation, prenylation and palmitoylation will direct the proteins to the cytosolic face, glycolipids will anchor the protein to the extracellular face of the plasma membrane (**Fig. 9**) [106,107].

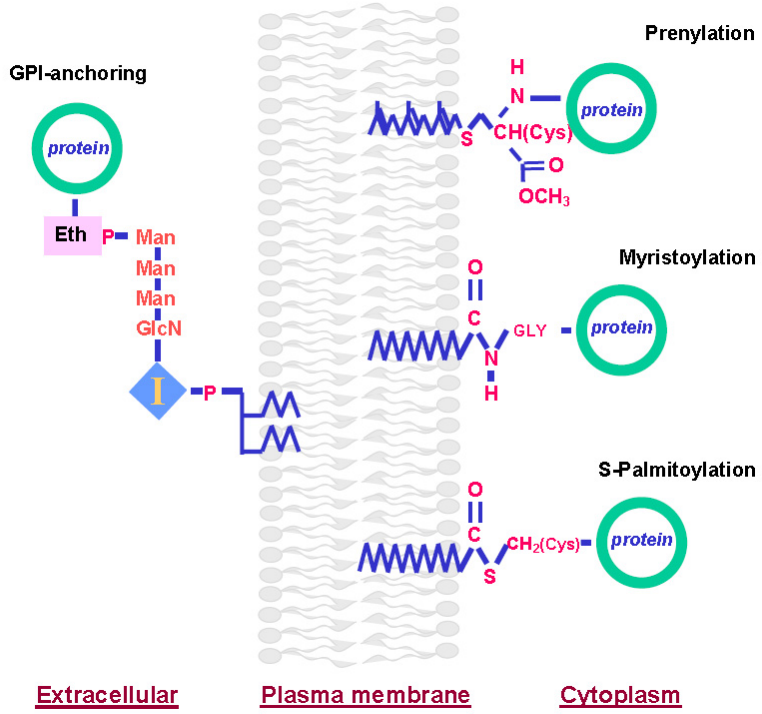


Figure 9 - Major lipid modifications of proteins. Prenylation is an irreversible post-translational modification of the protein by unsaturated fatty acids via the stable thioether bond. Myristoylation is a stable co-translational modification of the N-terminal glycine by the saturated myristic acid. Reversible *S*-palmitoylation occurs at the cysteine residues via the thioester-type bond. The GPI-anchor consists of the phosphatidyl-ethanolamine, trimannoside, glucosamine and inositol phospholipid and serves the attachment of proteins to the outer leaflet of the plasma membrane [106]. Gly-glycine, Cys-cysteine, Eth-ethanolamine, P-phosphate, Man-mannose, GlcN-glucosamine, I-inositol.

2.2. MEMBRANE MIMICKING MODELS

In order to study the interaction of peptides with membrane lipids, several model membranes such as micelles, bicelles, lipid mono- and bilayers are being used (**Fig. 10**).

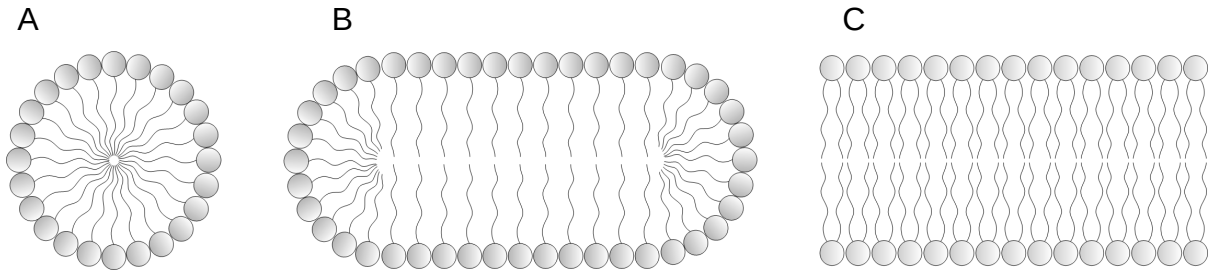


Figure 10 - Membrane mimicking models. (A) Micelles. (B) Bicelles. (C) Lipid bilayers.

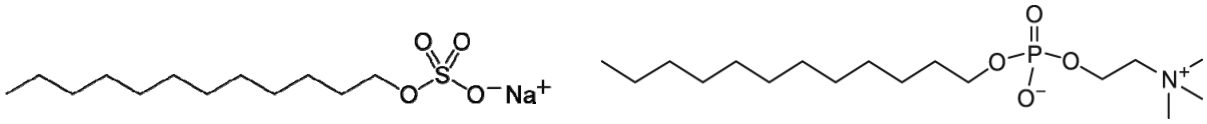
2.2.1. Micelles

Micelles are colloidal aggregates, composed of amphipathic molecules. They form above the critical micellar concentration. The molecules that compose the micelles have a non polar tail which is hydrophobic and a polar headgroup which is hydrophilic.

In a polar environment such as water, micelles composed of one type of unimers can typically adopt two forms: spherical and cylindrical.

Micelles have been and are still extensively employed to mimic a membrane environment in biophysical studies. If we want to study the interaction of a compound with the neutral or the charged part of the membrane, we will choose a zwitterionic detergent, typically dodecylphosphocholine (DPC) and dihexanoylphosphatidylcholine (DHPC), or a negatively charged detergent such as sodium dodecylsulfate (SDS), respectively. Mixed micelles made of DPC and SDS are sometimes used as they are more representative of some membrane environments [108].

As micelles have a monolayer structure with a high curvature, a loose packing of the lipid headgroups and a tight chain entanglement in the centre, they are not the ideal membrane mimicking model, especially for large peptides and proteins [109]. However, their facility of preparation and their small size represent an advantage for circular dichroism studies. For those reasons we utilized SDS and DPC (**Fig. 11**) in our circular dichroism experiments.



sodium dodecylsulfate (SDS) dodecylphosphocholine (DPC)

Figure 11 - SDS and DPC 2D structures [110].

2.2.2. Bicelles

Bicelles are disk-shaped aggregates composed of bilayer-forming lipids and detergents. Commonly they are composed of a mix of 2 phospholipids: one with two short i.e. C_4-C_6 alkyl chains (usually dihexanoylphosphatidylcholine (DHPC), C_6) and the other with two long i.e. $\geq C_{14}$ alkyl chains (usually dimyristoylphosphatidylcholine (DMPC), C_{14}). It is to be noted that DHPC alone forms micelles, whereas DMPC alone forms liposomes in an aqueous environment. The structure of bicelles is very dependent on the temperature and on the molar ratio of long over short alkyl chains [111]. As these bicelles spontaneously orient the bilayer normal perpendicular to the direction of the applied magnetic field owing to their diamagnetic moment, they are now widely used to study the orientation of soluble and membrane-bound peptides in NMR studies. They can also be employed as a medium for electron spin resonance spectroscopy. Bicelles have the advantage over the micelles to contain a membrane-like bilayer region. Their composition can also be adapted to include lipids that better mimic biological membranes such as cholesterol, dimyristoylphosphatidylglycerol (DMPG), dimyristoylphosphatidylserine (DMPS) and dimyristoylphosphatidylethanolamine (DMPE). However they present the disadvantage to be only stable over a short pH range and are unstable at pH > 7, especially if they contain acidic lipids. For review, see [109,111-113].

2.2.3. Langmuir and Langmuir-Blodgett films

Langmuir lipid films are monolayers formed by the deposition and spreading, on an aqueous subphase, of liquid droplets containing amphiphilic lipids dissolved in a volatile and water-immiscible solvent. In this work, we used Langmuir films in order to obtain the average lipid surface area of our phospholipids using a Langmuir trough technique at a lateral pressure corresponding to the lateral pressure of bilayers i.e. 32 mN/m [114]. It is to be noted that the average phospholipid area is lowered in presence of cholesterol [115,116]. Langmuir film

balance also allows the determination of peptide penetration areas in peptide-lipid interaction studies [117,118].

The Langmuir lipid film can be transferred onto a vertical solid substrate, such as mica, to form a Langmuir-Blodgett film by emersion of the substrate from the aqueous phase.

Each subsequent immersion and following emersion will lead to the deposition of two additional monolayers. In order to get an even number of monolayers, the substrate is positioned horizontally and the last monolayer is deposited by the Langmuir-Schaefer method. The film thickness can thus be accurately controlled. Biomimetic membranes can be created by varying the lipid composition of each monolayer [119]. Langmuir-Blodgett films are often used in atomic force microscopy (AFM) for lipid nanoscale organization studies [120].

2.2.4. Bilayers

Most encountered lipid bilayers systems used in the context of peptide-lipid interactions are freely suspended vesicles like liposomes and substrate-supported bilayers.

A. Black Lipid Membranes [119]

This bilayer spans over a small aperture that separates two aqueous compartments in which reference electrodes are inserted. It is usually formed by the "brushing" or injection of lipid molecules dissolved in an organic solvent over the small hole of a hydrophobic material such as Teflon. Black lipid membranes are principally used for the investigation of the formation of ion channels.

B. Supported bilayers

Substrate-supported bilayers are bilayers that lay onto a solid support that ideally is hydrophilic, smooth and clean [119].

Some of the advantages of supported bilayers are: (i) they increase the robustness and the stability of the lipid bilayer; (ii) they allow the use of surface specific characterization techniques such as AFM [120] and surface plasmon resonance (SPR) [121].

Supported bilayers are also used in spectroscopic studies such as attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) [122].

There are four usual methods to form supported bilayers. The first consists in the Langmuir-Blodgett technique described above. The second is the adsorption and fusion of small

unilamellar vesicles onto the substrate surface. The third is a combination of the first two methods. The last consists in the spreading of a small quantity of lipids dissolved in an organic solvent over the support.

Even though most solid supports are continuous, some are deliberately porous to allow the transport of compounds through them as it is the case in parallel artificial membrane permeability assays (PAMPA). In this system, the lipids are dissolved in dodecane, spread out onto a porous filter and form a bilayer which, together with the filter, separates two aqueous compartments. This system is used to study the passive transfer of drugs and peptides through lipid bilayers [123-125].

C. Vesicles (Liposomes)

In the context of peptide-lipid interactions, liposomes are widely used to study the extent of binding, the electrostatic and hydrophobic interactions but also the peptide conformation, the peptide fusion and membrane destabilization properties, as well as the binding thermodynamics. As liposomes were the predominant model membrane used in this thesis, they will be examined in details in the following section.

2.3. LIPOSOMES

2.3.1. Definition

Liposomes are spherical or oval self-closed vesicles comprising one or multiple lipid bilayers suspended in aqueous solution. The core of the liposomes, if any, is also aqueous. The main lipid bilayer constituents are phospholipids, but cholesterol is also sometimes added.

2.3.2. Classification

Liposomes are mainly classified according to their size and number of lamellae. The major classes of liposomes, as seen in **Table 4**, are the small unilamellar vesicles (SUV), the large unilamellar vesicles (LUV), the multilamellar vesicles (MLV) and the giant unilamellar vesicles (GUV). The size and lamellarity of the liposomes depend upon their method of preparation. The size limits between the different classes of liposomes vary according to the authors. The limit between SUV and LUV varies from 50 to 80 nm [126-128]. This limit can be set at the size at which the packing constraints decrease markedly [126]. As this size limit

depends on the lipid composition, the limit between SUV and LUV also varies with the lipid composition [127].

Table 4 - Major classes of liposomes: classification according to their size and lamellarity [127,128].

Class	Size	Lamellarity
SUV	20-80 nm	Unilamellar
LUV	50-500 nm	Unilamellar
MLV	400 nm – several μm	Multilamellar
GUV	1 μm – several dozens of μm	Unilamellar

2.3.3. Liposomes: lipid classes and structure

A. Phospholipids

As already discussed in Section 2.1.1, phospholipids are the major components of the plasma membrane lipid bilayers. All phospholipids have either a glycerol or a ceramide backbone (**Fig. 12**) with two hydrophobic tails (R_1 and R_2) consisting of fatty acids or alkane chains for glycero- and sphingophospholipids, respectively, as well as a hydrophilic head consisting of a phosphate group and its polar attachment (R_3).

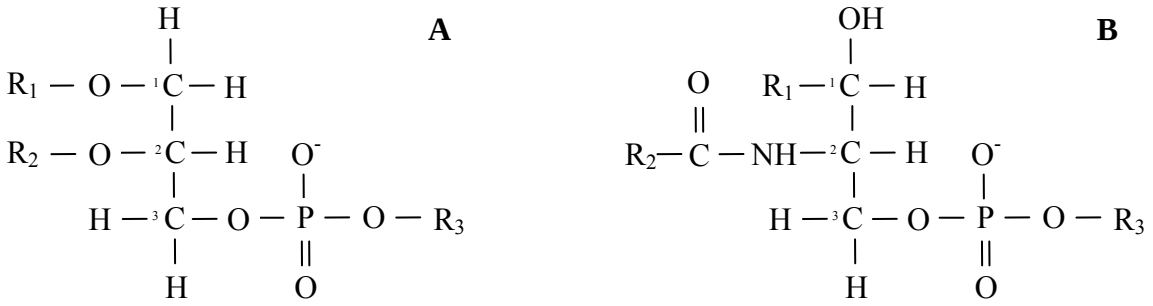


Figure 12 - Phospholipid backbones of (A) glycerophospholipids and (B) sphingophospholipids.

Phospholipids are thus amphiphilic molecules, partly soluble and partly insoluble in water. When they form bilayers, their hydrophilic heads point out towards the aqueous phase whereas their hydrophobic tails associate to form a hydrophobic core (**Fig. 13**).

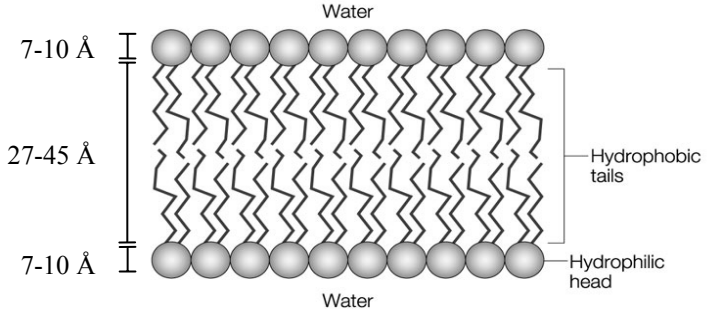


Figure 13 - Phospholipid bilayer structure. Adapted from [95,129-131].

1. Glycerophospholipids [100,127]

Glycerophospholipids are glycerol based phospholipids. Glycerol is a symmetric molecule (**Fig. 12A**) that becomes a chiral molecule centred on carbon-2 when the carbons -1 and -3 are differently substituted. Typically, the glycerol is substituted as follows:

- Carbon-1: saturated fatty acid (C_{16}, C_{18} or C_{20});
- Carbon-2: unsaturated fatty acid (1 to 6 double bonds, usually 18:1, 18:2, 18:3 or 20:4);
- Carbon-3: phosphate group to which a small polar molecule such as choline, ethanolamine, inositol, serine or glycerol is attached.

Some mammalian phospholipids are monoacyl glycerophospholipids such as the lysophosphatidylcholine (LPC). However, the most frequently occurring mammalian phospholipids are diacylglycerophospholipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA) and cardiolipin (CL).

2. Sphingophospholipids

Sphingophospholipids are ceramide based phospholipids. As the ceramide backbone contains one amino and one hydroxyl group, sphingophospholipids are more hydrophilic than their glycerol analogues.

Sphingomyelin is the major sphingophospholipid in mammalian cell membranes and, with respect to the polar group, is an equivalent to PC.

B. Cholesterol

Cholesterol is a sterol present in most eukaryotic membranes at concentrations up to 50 % of the total molar lipid concentration. It cannot form liposomes alone but it can insert between phospholipid chains with its polar hydroxyl group oriented towards and close to the phospholipid headgroups. It can easily flip-flop from one side of a bilayer to the other.

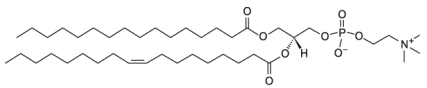
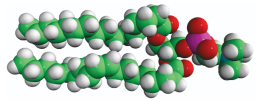
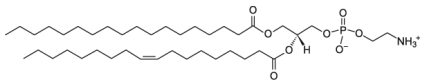
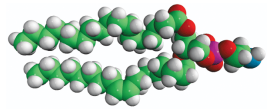
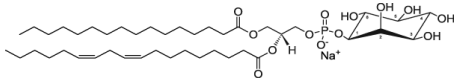
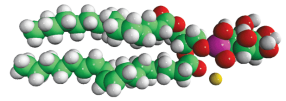
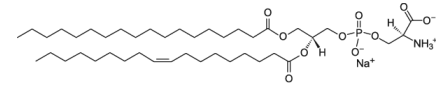
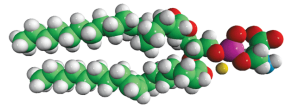
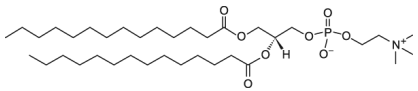
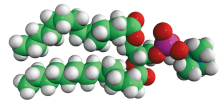
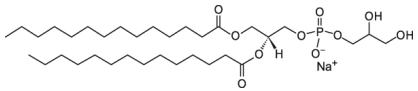
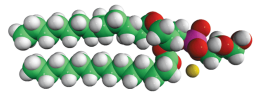
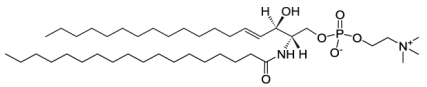
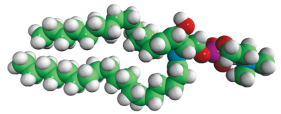
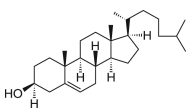
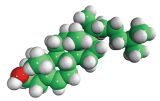
At high concentrations, cholesterol has a dual effect: it orders the liquid crystalline phase by increasing the order and packing density within the membrane, while it disorders the gel phase by decreasing the order parameter. The presence of cholesterol thus creates over a wide range of temperature an intermediate state, the ordered liquid phase, in which the phase transition is suppressed [100,127].

2.3.4. Lipids: structure and physico-chemical properties

A. Overview

Some of the chemical and physical properties of the different natural and synthetic phospholipids that we have used in this thesis, as well as cholesterol are presented in **Table 5**. They include the molecular weight, lipid molecular area, temperature of the phase transition, intrinsic pKa and solubility as well as the 2D and 3D structures.

Table 5 - Structure and physico-chemical properties of the lipids studied [100, 110, 127, 128, 132-136].

Lipids, origin	Mean *MW* (g/mol)	A_L (Å^2)	T_c (°C) gel → l.c.	Intrinsic *pKas*	Solubility		2D structure of predominant species [110]	3D structure of predominant species [110]
					Chloroform	Methanol		
PC, chicken egg yolk	780	60-74	- 15 to -5	phosphate : 1.4-2.0 TMA : 10.5-11.5	soluble	soluble	 3-*sn*-phosphatidylcholine	
PE, chicken egg yolk	744	70	7 to 12	phosphate : 1.4-2.5 amine : 9.0-9.6	soluble	insoluble	 3-*sn*-phosphatidylethanolamine	
PI, wheat germ	856	60-80	not determined	phosphate : 2.0	**insoluble**	soluble	 3-*sn*-phosphatidylinositol	
PS, bovine brain	812.05	60	6 to 14	phosphate : 2.1-2.2 carboxyl : 2.6-4.0 amine : 9.7-9.9	soluble	insoluble	 3-*sn*-phosphatidylserine	
DM**PC**, synthetic	678	54-62	19 to 26	phosphate : 1.4-2.0 TMA : 10.5-11.5	soluble	soluble	 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	
DM**PG**, synthetic	688.85	52-68	21 to 25	phosphate : 2.9	soluble	insoluble	 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol)	
SM, bovine brain	731.1	33-47	28 to 38	phosphate : 1.4-2.0 TMA : 10.5-11.5	soluble	soluble	 Ceramide-1-phosphocholine = sphingomyelin	
Chol	386.6	39-45	not applicable	not applicable	soluble	insoluble	 Cholesterol	

TMA: trimethylammonium; *sn*: stereospecific number; A_L : molecular area per lipid; T_c : phase transition temperature

B. Membrane gel-liquid phase transition [100,104,127]

Among the many aspects of phase behaviour in phospholipid bilayers, gel-liquid phase transition is one of the most important. The gel phase is an ordered phase in which the hydrocarbon chains are fully extended and closely packed, whereas in the liquid crystalline phase the hydrocarbon chains are randomly oriented and fluid. The temperature at which the lipid physical state changes from the gel to the liquid crystalline phase is defined as the phase transition temperature. This temperature mostly depends on the hydrocarbon chain length, saturation state as well as on the nature of the polar headgroup. The amplitude of the change strongly depends on the lipid composition. Membranes composed of lipid mixtures with different chain lengths and/or headgroups do not normally exhibit a phase transition.

Addition of cholesterol is known to increase disorder in the gel phase and to increase the order in the liquid phase and thereby to reduce the intensity of the phase transition. At high cholesterol concentrations (> 30% M/M), it leads to the formation of a new phase, the liquid ordered phase, and the transition is practically eliminated.

2.3.5. Preparation and physico-chemical characterization of liposomes

Among the various methods of liposomes preparation (for review, see [127,128]), we chose to prepare our liposomes by the lipid film hydration method to produce MLV. Subsequent freeze-thawing followed by extrusion were performed in most cases to produce LUV.

A. Thin-lipid film hydration

This is the simplest way to produce liposomes. It was first described by Bangham et al. in 1965 [137]. In this method, lipids are first mixed in a volatile organic solvent. A thin lipid film is then produced by the evaporation of the solvent. Liposomes are formed by the rehydration of this film with an excess of aqueous solution under agitation at a temperature higher than the lipid phase transition temperature T_c. It is to be noted that due to their molecular geometry, i.e. cylindral or inverted truncated cones, and amphiphilicity most natural phospholipids spontaneously form liposomes in aqueous solution [128]. The vesicles produced by this method have a very heterogeneous size distribution, i.e. from SUV to GUV, even though most liposomes formed are MLV. Their diameter can reach tens of μm [127].

B. Extrusion [126,138,139]

In order to facilitate the extrusion of the vesicles generated by the lipid film hydration method, their size is reduced by 5 freeze-thawing cycles. This leads to the formation of smaller MLV [127]. In the extrusion method, these MLV are forced to pass, above T_c, through the pores of 2 stacked polycarbonate membranes with a well defined pore size, from 30 nm to several μm, under high pressure of azote (up to 60 bars). With this method one cannot usually make liposomes smaller than 50-60 nm. The advantages of this method are that it is reliable and produces rather well-characterised liposomes with a narrow size distribution.

The liposome formation using this method is illustrated in **Fig. 14**.

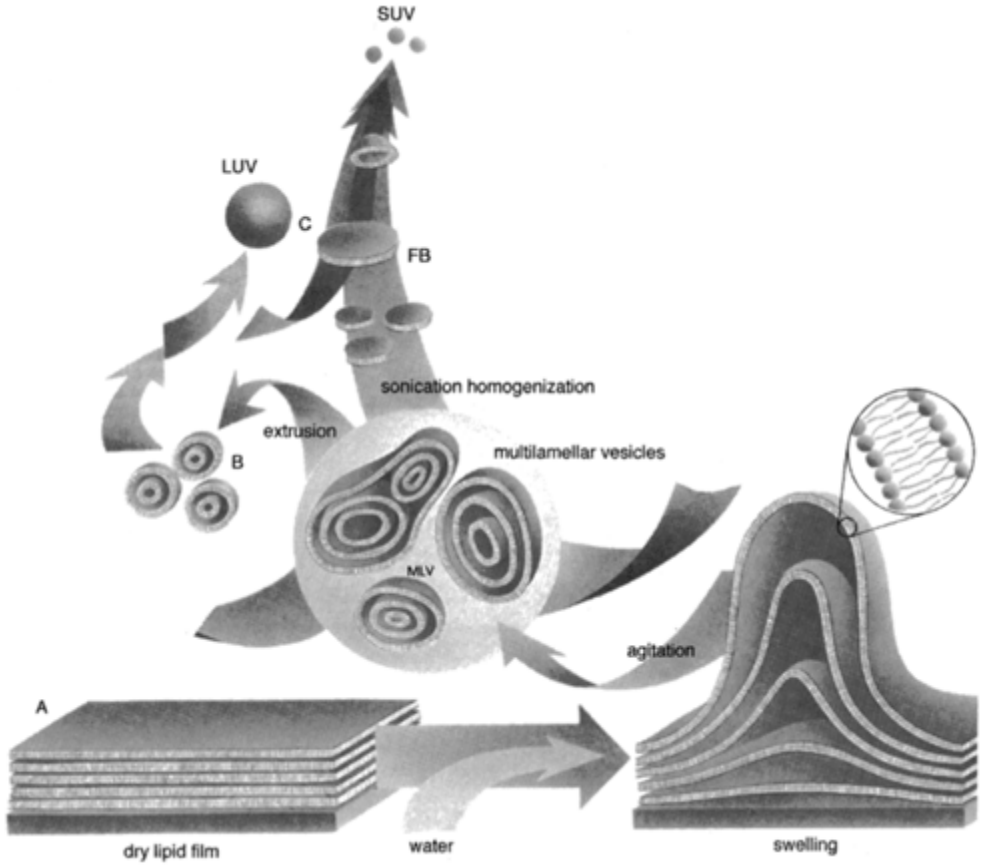


Figure 14 - Growth of tubules upon rehydration of a dry lipid film. Agitation detaches hydrating lipids' mass and particles self-close in large multilamellar vesicles. These can be sonicated, extruded or homogenized to prepare a uniform population of smaller vesicles [140].

C. Liposomes: physico-chemical characterization

After production, liposomes basic characteristics such as their lipid content, size and zeta potential are usually checked but other characteristics such as the lamellarity, the degree of degradation, the permeability or the encapsulation efficiency are also assessed in some cases.

Lipid content

The most widely spread phospholipid quantification method is a phosphorus assay [141]. In the case of cholesterol containing liposomes, it is usually assumed that cholesterol and phospholipids are recovered in a similar way, which is most often the case especially when lipids are mixed in the organic phase [127].

The cholesterol and the different types of phospholipids can be identified and/or quantified by several methods including mass spectrometry, infrared spectroscopy (IR), NMR and refractive index after separation, if necessary, by either capillary electrophoresis, gas chromatography, thin layer chromatography and HPLC (see [142], for review).

Size and lamellarity

Hydrodynamic size and size distribution of liposomes can be assessed by dynamic light scattering; this is the technique that we chose and it has the advantage to be a non-destructive method. They can also be evaluated by size exclusion chromatography, electron microscopy (EM), field-flow fractionation and analytical centrifugation [127,143,144]. If the lamellarity has to be determined, it is usually done by ^{31}P NMR, small angle X-ray scattering, cryo-transmission EM or freeze-fracture EM [144-146].

Oxidation/Hydrolysis [100,127]

Phospholipids are readily subject to hydrolysis in basic or acidic conditions; and to oxidation if they contain unsaturated fatty acid chains under the action of light or in presence of air oxygen. These reactions can lead to a change in the phospholipid structure as well as to their dimerization or splitting into a lysophospholipid and a fatty acid. These induce changes in liposomes properties. In order to limit these phenomena, it is recommended to store the liposomes at 4°C under an inert gas such as nitrogen or argon in dark sealed tubes.

Zeta potential

The zeta potential is usually assessed in order to get an idea of the liposomes surface charge and physical stability in a given solution [143,147]. The zeta potential is the potential difference between the particle slipping plane, which cannot precisely be located, and the bulk solution [148]. It is generally determined from the interpretation of electrokinetic phenomena [148]. In our case, it was calculated on the basis of electrophoretic mobilities measured by the most popular technique i.e. laser Doppler velocimetry, also called electrophoretic light scattering [149].

3. PEPTIDE-MEMBRANE INTERACTIONS AND TRANSPORT

3.1. PEPTIDE-MEMBRANE INTERACTIONS

3.1.1. Overview

Peptides can interact with all membrane components i.e. proteins, cholesterol, phospholipids, glycoproteins and glycolipids. Here, we will focus on peptide-lipid interactions and their consequences on peptide-receptor interactions.

As peptides are involved in many biological processes, they can be classified according to their function. Peptides can simultaneously belong to different families [150]. Regarding their function, they are 3 major classes of peptides:
1) Regulation peptides: hormones (including ghrelin) and neuropeptides;
2) Host defence/attack peptides: antimicrobial peptides and toxins;
3) Carrier peptides: cell penetrating peptides.
As host defence and carrier peptides offer a great therapeutic potential, their mechanisms of action are among the most studied.

Most peptide-lipid interactions in a membrane can lead to (**Fig. 15**):

(i) modification of the membrane elastical/mechanical properties;
(ii) modification of peptide structure;
(iii) receptor binding and a subsequent cellular effect;
(iv) peptide translocation;
(v) drastic destabilisation of membrane (fusion/disruption).

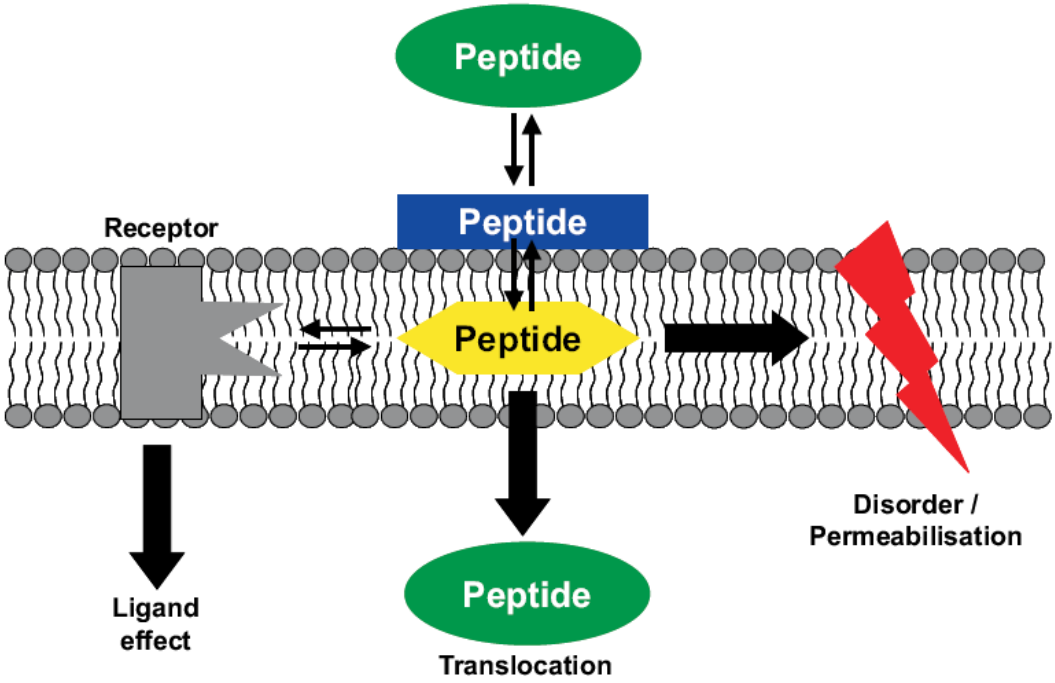


Figure 15 - Outline of peptide-membrane interactions. Peptides in contact with membranes may change conformation, which may facilitate proper binding to a receptor, a physiological effect resulting therefrom. Peptides may also exert local specific effects like disorder and permeabilization (e.g. antimicrobial peptides). Other peptides have the ability to translocate through the membrane, having no apparent effect in the lipid bilayer. [151]

As ghrelin effects seem to be mostly receptor mediated, we will get a closer look at the peptide-lipid-receptor interactions.

3.1.2. Peptide-lipid-receptor interactions

When a peptide approaches its receptor and when this receptor lays in a lipid bilayer, it can either directly interact with the extracellular part of the receptor or it can first encounter the lipid bilayer before reaching its binding site. We will here describe the latter situation with an emphasis on peptide-lipid interactions.

Peptide-lipid interactions are different from chemical reactions in the way that no covalent bonds are formed or broken. Instead, to make it short, we can say that the association of a peptide with a pure lipid membrane corresponds to a physical adsorption process which can be divided into four molecular steps:

(i) Electrostatic adsorption;

(ii) Peptide penetration in the headgroup or in the hydrophobic core region;

(iii) Peptide conformational change;

(iv) Lipid reorganization.

Depending on the peptide and the lipids involved, some or all of these steps will be implicated in the peptide-lipid binding process. When talking about the binding of peptides to lipids, we will refer to these phenomena (see [152] [153], for review). The ultimate step in the peptide-lipid-receptor interaction process will be the encounter and docking of the peptide to the receptor. The first three steps are detailed in the figure below (**Fig. 16**).

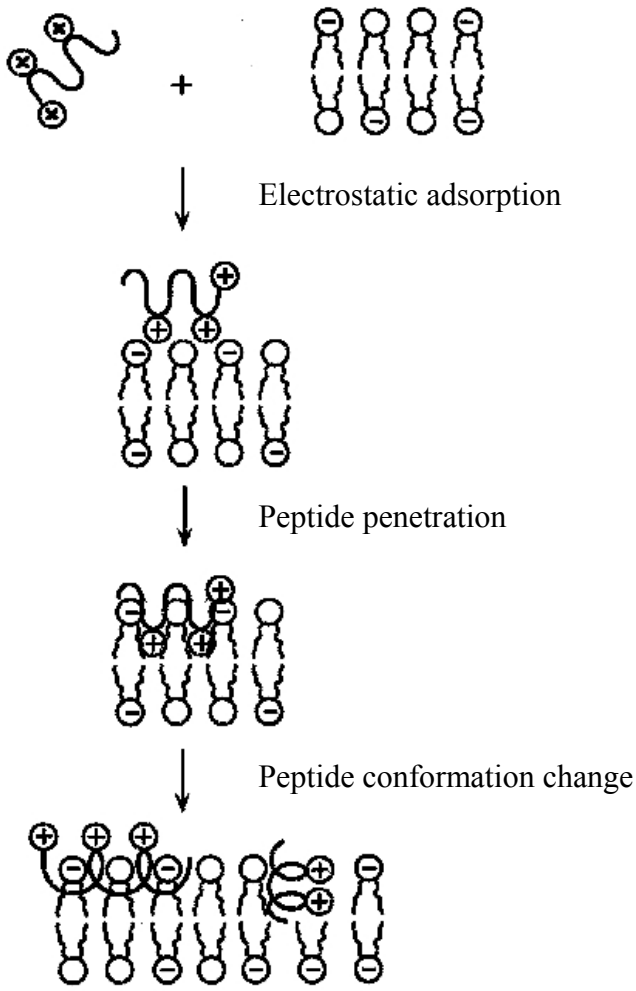


Figure 16 – The first three of the four steps model for peptide binding to lipid membranes: (i) electrostatic adsorption of a cationic peptide to a negatively charged lipid membrane; (ii) penetration into the headgroup region or in the hydrophobic core; (iii) peptide conformational change. Adapted from [152].

A. Electrostatic interactions

As many biological membranes are negatively charged and that most biological peptides carry a net positive charge, the usual first step in the binding process is the electrostatic attraction of the cationic peptide to the membrane surface. Of course, electrostatic attraction is not always a prerequisite for binding.

In the case of charged peptides and/or charged membranes an electrostatic attraction (repulsion) will increase (decrease) the peptide concentration near the membrane surface, compared to the bulk concentration. This will depend on the peptide charge and membrane surface potential. A quantitative treatment of the electrostatic adsorption process can be made using the Gouy-Chapman theory [152-156]. With a non-charged peptide and a neutral membrane, the peptide concentration near the membrane surface is identical to that in bulk solution.

It has been shown that, in some cases, electrostatic interactions between negatively charged membranes and basic peptides play an important role in peptide-lipid interactions [157-159] and that "the negative surface of biological membranes plays a catalytic role in the membrane binding of biologically active cationic species such as anaesthetics, ion channel antagonists, phospholipases, kinases and their substrates" as discussed by Tatulian in Ref [156].

Besides electrostatic interactions, some peptides preferentially or specifically interact with a particular phosphatidyl headgroup regardless of the charge [160,161], e.g. cinnamycin binds to PE but not to PC even though both of them are zwitterionic. In some cases, different phosphatidyl headgroups have the same affinity for a peptide but result in different effects of the peptide, e.g. magainin 2 binds PG and PS in the same way but induces more leakage in PG than in PS liposomes [162].

B. Hydrophobic interactions / peptide penetration

The peptide may remain electrostatically adsorbed in the polar headgroup region or it may insert into the hydrophobic core of the membrane. For some peptides the penetration into the membrane is enthalpy-driven by van der Waals interactions, e.g. PGLa [163] and somatostatin & analogues [164]. For others it is driven by entropic forces, a phenomenon commonly denoted as 'hydrophobic effect' like for *N*-myristoylated hisactophilins [165] but, surprisingly, entropy driven interactions have also been described for a highly basic peptide (Lys_5) and Ca^{2+} interactions with sonicated anionic lipid vesicles [166,167]. The method of preparation of the vesicles might be responsible for the observed effects as suggested by Arseneault and Lafleur for the Ca^{2+} [168]. Some are also entropy and enthalpy driven as it is the case for acylated antimicrobial peptides [169] and talin [118].

Peptide penetration into the hydrophobic region can be affected by [170-173] or affect the membrane fluidity [174-176].

C. Conformational change

When a peptide interacts with the lipids of the membrane, this interaction may be accompanied by a change in the peptide conformation. Peptides that are in random coil conformation in solution can structure in contact of the membrane, e.g. substance P, a neuropeptide, is random coil in solution but adopts a partial α-helical structure in phosphatidylserine vesicles [177]. A structured peptide can also change its conformation upon interaction with the membrane, e.g. Bax-α1, the N-terminal segment of Bax (apoptotic) protein, is in β-sheet conformation in solution but becomes α-helical in membranes [178].

Even though the conformational change of the peptide is most often a transition to a partially α-helical structure [163,177,179-182], the binding to the membrane may induce the formation of β-sheet structures like it is the case for atriopeptin III, an atrial natriuretic peptide [183], or other structures such as 3_{10}-helix, β-turn, hairpin like β-sheet and β-helix [182,184]. The conformational change may also accompany the aggregation of the peptide as it does for the beta-amyloid peptide [185] or the islet amyloid polypeptide [186].

D. Lipid reorganization

Lipid reorganization mostly occurs with host defence, attack and cell penetrating peptides. Cell penetrating peptides that do not enter the cell via endocytic pathways, cross the membrane to reach their final target by a mechanism of action that is still under debate [187]. Several antimicrobial peptides induce, following their interaction with membranes above a threshold concentration, membrane destabilization including pore formation or detergent-like effects (see [188,189], for review). Hormone peptides such as amyloid peptides also provoke membrane perturbation at disease stage, e.g. islet amyloid polypeptide, which is involved in type 2 diabetes, can permeabilize membranes [190]. However, in physiological conditions, most hormone peptides that exert their action via a membrane receptor are unlikely to generate such drastic effects, e.g. insulin and substance P do neither cause vesicle leakage nor fusion [191,192], but they could induce a change in lipid orientation.

E. Receptor binding

A ligand can interact with a membrane-bound receptor in two ways: (i) by direct transfer from the aqueous solution to the receptor; (ii) by binding to the membrane and then diffuse to reach the receptor. As the latest is of particular interest to us, we will get a closer look at it.

In 1986, Sargent and Schwyzer were the first to propose a "membrane catalysis" model in which the membrane lipid phase acts as a mediator for peptide-receptor interactions by speeding up the reactions and giving the right orientation and conformation to the ligand [193]. It relies on evidence that a given receptor only occupies a small fraction of the total cell surface whereas lipids occupy at least half of this surface. Therefore, for any ligand approaching a cell, the probability of contact with the lipid phase is much higher than with one of its receptors. This model is still relevant today even though it has been refined over the years [151,194,195].

The known and foreseen advantages for peptide-receptor binding associated with the peptide-membrane interactions include:

(i) diffusional advantage due to the reduction of dimensionality. Indeed, it restricts the motion of the peptide from a three to a two-dimensional diffusion which is distinctly faster than bulk diffusion [196-198];

(ii) favourable kinetics [193];

(iii) increased local concentration of the peptide in the vicinity of the receptor [196];

(iv) peptide conformation and orientation change and hence pharmacologically active conformation [199-201];

(v) positioning of the peptide at the proper depth for receptor interaction [151,202];

(vi) driving the peptide to the membrane domain in which its receptor is located [203,204].

For some peptides a close relation between their membrane binding ability and their agonist activity has been shown [199,205] e.g. kisspeptin decapeptide analogues [199].

However, peptide binding to the membrane is not always advantageous because when the peptide is bound to the lipids:

(i) less free peptide is available in the aqueous phase and consequently not available anymore for receptor binding if the binding site is in an extracellular loop [164];

(ii) segregation of the peptide in a specific membrane domain could be a drawback if the receptor does not co-localize in this domain;

(iii) they can perturb the membrane structure and therefore induce undesirable side effects [206].

3.1.3. Biophysical techniques for analyzing peptide-lipid interactions

A single technique cannot provide a complete picture of the peptide-lipid interactions. Therefore, it is necessary to use different complementary techniques in order to get a detailed picture of the peptide-lipid interactions. In **Table 6**, I review the commonly used biophysical techniques used to analyse peptide-lipid interactions with their use, advantages and limitations. Even though this covers a wide range of techniques, it does not cover all of them. There are several techniques derived from the presented techniques and some of the techniques are used or can be used in combination. Moreover, it has to be noted that most techniques often require the use of (mathematical) models in order to interpret the results obtained.

Table 6 - Biophysical techniques to study peptide-lipid interactions [150].

Technique	Principle	Properties studied	Membrane model	Advantages	Limitations	Ref
Atomic force microscopy (AFM)	Scanning with a sharp tip (probe) across a membrane surface while measuring the forces experienced by the tip (z-displacement of the tip is monitored by the reflection of a laser beam focused on the top surface of the cantilever).	3D membrane surface structure and organization with (sub)nanometre resolution	Supported bilayers	No special treatment necessary Can operate in air and liquid Use of a functionalized tip is possible to measure specific interactions or phenomena	Slower scanning speed than an electron microscope	[207-209]
Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)	Absorption by the peptide and the lipids of mid-range infrared radiations that induce molecular vibrations at wavenumbers characteristic of the molecular structure.	Peptide secondary structure Lipid structure Peptide and lipid orientations	Supported bilayers	No external probe necessary Few peptide required (µg) Not affected by light scattering (due to long IR λ) Highly aggregated materials can be investigated Simultaneous study of lipids and peptide structures	TFA interferences SM interference (amide) C=O (buffer) interferences Large amounts of salts from the buffer can interfere Requires D20 flushing to avoid spectra shift due to H20	[122,183, 184,210-212]
Brewster angle microscopy (BAM)	Polarized light is emitted at the Brewster angle and reflected light is recorded. At the Brewster angle polarized light is reflected by the air-oil-water interface but not at the air-water interface.	Visualization of lateral features on surfaces (µm range) Lipid/peptide surface morphology	Monolayers (Langmuir films)	No probe necessary Real-time imaging	Low resolution (µm)	[212-215]
Capillary electrophoresis (CE)	Separation of charged species in a narrow capillary, e.g. silica, according to their electrophoretic mobility under the influence of an electric field.	Binding constant Binding kinetics (rough)	LUV micelles	Rapid	Not ideal for interactions with a rapid dissociation rate Peptide and/or lipids can interact with capillary walls Sensitivity depends on the detection mode	[216-218]
Circular dichroism (CD)	Detection of the wavelength dependence of the variation in ellipticity of the circularly polarized light due to the differential absorbance of the left and right circularly polarized light by the chiral (amide) chromophores of the peptides.	Peptide secondary structure	LUV SUV Micelles Bicelles	Highly sensitive Rapid Ability to monitor structural changes due to the environment No external chromophore is necessary	Does not allow the localization nor the number of similar secondary structures Requires vesicles with size < wavelengths studied	[108,113, 118,219-223]

Technique	Principle	Properties studied	Membrane model	Advantages	Limitations	Ref
Colourimetry	Visible colour change (blue to red) of a polymerized probe (polydiacetylene, PDA) associated to phospholipids in mixed vesicles, induced by phospholipids perturbation and hence rearrangement of PDA side chains.	Membrane disorder effects Peptide penetration depth	"LUV"	Easy to detect Works in the µM-M range Interfacial perturbations induce a stronger colour change Could be used for screening	Peptide penetration depth is not precise	[224-226]
Cryogenic transmission electron microscopy (Cryo-TEM)	Transmission microscopy that uses an electron beam to produce an image of a sample in cryogenic state that has not been previously stained.	Vesicle overall structure upon interaction with peptide Vesicle size Vesicle lamellarity	SUV LUV MLV GUV	Vesicles can be studied in many buffers and in native form High scanning speed (almost real-time) High resolution (~5-20 Å)	Equipment is rare and expensive	[215,223, 227,228]
Differential scanning calorimetry (DSC)	Lipid phase transition is accompanied by absorption or release of heat that is monitored. The phase transition temperature of lipids can change upon peptide interaction.	Phase transitions effects and hence type of peptide-lipid interactions	LUV mostly MLV	Easy sample preparation	Cannot be used with natural lipids mixtures	[215,227, 229,230]
Dynamic light scattering	Time-dependence fluctuations of light scattering intensities due to Brownian motion of the particles.	Vesicle hydrodynamic size Vesicle fusion Vesicle aggregation	SUV LUV MLV	Fast Cheap Non destructive	Sensitive to dust	[37,222, 223,231]
Electrophoretic Light Scattering	Light scattering of moving particles due to an electric field is frequency shifted. This frequency shift is proportional to the particles electrophoretic mobility.	Vesicle zeta potential Peptide adsorption in or close to the vesicles surface	SUV LUV	Rapid Works in the µM range Does not depend on particles size	Dilute dilutions necessary to avoid particle interactions Buffers with a high ionic strength rapidly oxidize electrodes	[148,155, 158,181, 223,232-234]
Ellipsometry	Measurement of the change of polarization (phase and amplitude) of the light reflected by a thin film and thus of the change in film thickness and refractive index.	Amount of peptide adsorbed onto the bilayer Surface structural information	Supported bilayers	Non destructive Contactless Time-resolved	Very expensive equipment Seconds timescale (film thickness must be < λ light)	[215,223]
Equilibrium dialysis	Two compartments (only one which contains the vesicles) are separated by a vertical membrane that only allows peptides to get through. Dialysis is ended when the equilibrium of free peptide between both compartments is reached.	Apparent binding coefficients	LUV	Simple	Sensitivity depends upon the detection method Dialysis membrane can retain the free peptide	[158,234-236]

Technique	Principle	Properties studied	Membrane model	Advantages	Limitations	Ref
Electron spin resonance (ESR) = Electron paramagnetic resonance associated to site-directed spin labelling (EPR)	Absorption of high frequency radio waves by atoms that possess lone pair electrons under a strong magnetic field.	Peptide penetration depth Peptide orientation Peptide dynamics Peptide secondary and tertiary structure	LUV Bicelles	Precision: 1.5 – 3 Å Opaque and highly concentrated samples can be studied	Spin labelling needed as normal PLP and peptide molecules do not contain lone-pair electrons (by introduction of a nitroxide group)	[113,237, 238]
Epifluorescence microscopy	Microscopy of a fluorescent probe (e.g. NBD C_{12}-HPC) that partitions into the disordered phase but not in the ordered phase by steric hindrance.	Membrane lateral organization	Monolayers	Easy to use Sensitive	Necessitates the use of fluorescent lipids Micrometer scale Photobleaching	[239,240]
Filtration	Filtration of peptide-vesicles mixture through a membrane through which only free peptide can pass. Filtration process is accelerated by centrifugation.	Peptide molar partition coefficient into lipids	LUV	Rapid Simple	Membrane can retain the free peptide	[234]
Fluorescence correlation spectroscopy (FCS)	Time-dependence fluctuations of fluorescence intensities due to Brownian motion of the peptide differ if the peptide is free or bound to the vesicles.	Peptide molar partition coefficient into lipids (LP) Lipid mobility (LL) LL: using labelled lipids LP: using labelled peptides	LUV GUV	Works best in the nM range	Not suitable for weak peptide-lipid interactions Peptide or lipid labelling is necessary	[233,241]
Fluorescence enhancement by chelation	Fluorescence of a low fluorescent probe (e.g. Tb^{3+}) drastically increases upon complex formation with a chelate (e.g. DPA) after fusion of vesicles containing either the probe or the chelate.	Vesicle fusion	LUV	Process specific	Necessitates the use of EDTA and Ca^{2+} in the external medium to avoid the complex formation outside the vesicles	[222]
Fluorescence polarization	Orientational anisotropy of some fluorescent probes (e.g. DPH and derivatives) depends upon the probe Brownian motion and thus the probe environment's microviscosity which can be affected upon peptide interaction.	Lipid fluidity near the probe location	SUV LUV	Works in the μM range	Can be affected by light scattering, fluorescence reabsorption or energy transfer	[158,175, 176,209, 242-244]
Fluorescence self-dequenching	Lipid (e.g. R18) or aqueous fluorescent probes (e.g. calcein) that self-quench at high concentrations. Dequenching upon dilution leads to an increase in fluorescence intensity.	Vesicle fusion (R18) Membrane permeabilization Membrane disorder effects	LUV SUV	Simple	Can be affected by the method of mixing the peptides and the lipids (false +) Sensitive to light scattering effects	[209,222, 223,243, 244]

Technique	Principle	Properties studied	Membrane model	Advantages	Limitations	Ref
Fluorescence quenching or dequenching by external quenchers	Aqueous fluorescent probes (e.g. ANTS) are quenched in presence of a quencher (DPX) and dequench (increase in fluorescence intensity) upon release in the solution.	Vesicle fusion Membrane permeabilization Membrane disorder effects	LUV SUV	Simple	Can requench if they associate after their release in solution	[244,245]
Fluorescence quenching of Trp	Trp fluorescence quenching by aqueous (e.g. acrylamide) or hydrophobic (e.g. NBD or doxyl-stearate) quenchers.	Peptide penetration depth Peptide aggregation state within the bilayer	LUV SUV	Precision : 1.5 – 3 Å	Necessitates the presence of a Trp in the peptide sequence and a quencher linked to the lipids or in the aqueous phase	[158,223, 228]
Fluorescence recovery after photobleaching (FRAP)	Lipids mobility monitored by the recovery of lipids fluorescence in a zone that has been photobleached by a laser.	Lipid lateral diffusion	Supported mono- or bilayers Cells GUV	Simple	Labelling of lipids or peptides is necessary	[246,247]
Fluorescence resonance energy transfer (FRET)	Energy transfer intensity between two fluorescent molecules is inversely proportional to the distance between the donor and acceptor probes (e.g. fluorescein and rhodamine for peptides; NBD-PE and Rh-PE for lipids) .	Peptide oligomerization Vesicle fusion	SUV LUV MLV	Working distance: 10-70 Å	For peptide oligomerization: probe choice is limited due to the overlap of the probe spectra	[222,231, 246,248, 249]
Immobilized artificial membrane chromatography (IAMC)	Lipids are grafted onto the silica of a column and peptide retention depends upon its affinity for the lipids vs. the mobile phase.	Partition coefficients	Immobilized phospholipid monolayers	Rapid	Sensitivity linked to peptide detection mode Data interpretation is not straightforward	[124,250, 251]
Intrinsic fluorescence: “Solvatochromism” of Tryptophan	Trp fluorescence wavelength decreases (blue shift) but increases in intensity with the decrease of environment polarity.	Polarity of the peptide environment	LUV	Natural fluorophore in many peptides	Necessitates the presence of a Trp in the peptide sequence Trp fluorescence depends upon its aggregation state	[228,229, 252]
Isothermal titration calorimetry (ITC)	Lipids (or peptide) are injected into peptide (or lipids) while the temperature is kept constant. Release or absorption of heat occurs when lipids and peptide interact.	Binding isotherm Binding coefficients Free energy of binding Binding enthalpy Binding entropy Heat capacity change Lipid affinity	SUV LUV	No need for labelling Works in the µM range	Quantitative analysis of the binding isotherms often necessitates the use of a binding model	[118,152, 153]

Technique	Principle	Properties studied	Membrane model	Advantages	Limitations	Ref
Micropipette aspiration technique	Change in the length of the portion of a vesicle membrane aspirated into a micropipette under controlled suction pressure is monitored by video microscopy and is proportional to the change of the membrane area.	Bilayer area compressibility and bending moduli Bilayer lysis tension	GUV	Easy to obtain qualitative results	Quantitative results require an appropriate model and a long time Friction occurs between the membrane and the pipette	[206,253, 254]
Neutron spectroscopy (diffraction/ reflectometry)	Neutrons interactions with atomic nuclei. Sensitive to nuclear scattering length density.	- Diffraction: Peptide/lipid structures Peptide penetration depth: if in combination with AA deuteration - Reflectometry: Study of buried interfaces Surface structural information and composition normal to the surface	Monolayers Supported mono- or bilayers	Possibility to use aqueous solutions Ability to detect hydrogen atoms positions Possibility to study molecular dynamics Less destructive than RX Good resolution (Å)	Necessitates the use of a nuclear reactor Equipment is rare Large amount of peptide is necessary Long duration (2-6h/sample) Requires an extensive expertise	[177,239, 255,256]
Nuclear magnetic resonance (NMR) (^{1}H, ^{2}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P)	Absorption of high frequency radio waves by nuclei that possess an overall spin under a strong magnetic field.	Complete 3D molecular structure up to ~100 kDa Peptide conformation Peptide insertion depth Lipid phase nature and dynamics Peptide-lipid dynamics	Micelles Bicelles MLV	Possibility to study molecules in physiologically relevant buffers High resolution (atomic)	Poor sensitivity: high µM to low mM range Isotopic labelling required for ^{2}H, (^{13}C) and ^{15}N ^{19}F labelling is not isotopic Requires an extensive expertise	[113,257-259]
Parallel artificial membrane permeability assay (PAMPA)	Transport of peptide between two aqueous compartments separated by a lipid bilayer deposited onto an horizontal hydrophobic porous support in the upper (= acceptor) compartment. Peptides are loaded in the bottom (= donor) compartment.	Peptide passive diffusion through lipid bilayers	Supported bilayers	Simple Could be used for screening	Only sold together with an expensive platform Sensitivity linked to peptide detection mode	[124,125, 260]
Potential-sensitive fluorescence	Variation of the quantum yield of fluorescent probes located at the air/water interface due to probe re-location under the influence of local electric filed or to a change in the probe protonation state.	Surface potential/charge	SUV LUV	Works in the µM range	Necessitates the use of fluorescent lipids like fluoresceinPE Peptides may change the dielectric constant in the vicinity of the fluorescent probe and thus the results	[234,261-263]

Technique	Principle	Properties studied	Membrane model	Advantages	Limitations	Ref
Quartz crystal microbalance (QCM)	A quartz planar resonator covered with gold and lipids is operated at harmonics. Change in quartz resonance frequency is inversely correlated to bilayer mass change.	Lipid mass and structure changes Lipid removal Peptide penetration depth Binding kinetics	Supported bilayers	Not affected by light scattering Works in the ng range	Long preparation time	[207,264, 265]
Surface plasmon resonance (SPR)	Measurement of the change in the refractive index near a surface plasmon sensor (e.g. gold). Refractive index increases if a peptide binds onto the lipid surface.	Binding kinetics Binding coefficients Lipid affinity	Supported monolayers Tethered bilayers Immobilized liposomes	No need for labelling Works in the nM-μM range Coloured, turbid or opaque samples can be studies	BSA sometimes needed to cover non-specific binding sites Lipids form (bilayers or liposomes) on some chips depends upon lipid composition Not suited for peptides <1kDa	[251,259, 266]
Surface pressure area	Lipid film (monolayer with an aqueous subphase) molecular area increases at a given pressure if a peptide absorbs at the interface or into the lipid film.	Peptide penetration Surface thermodynamics Lipid phase transitions Lipid or peptide molecular areas	Monolayers	Can be combined with other techniques (e.g. BAM and epifluorescence microscopy)	Necessitates the use of highly pure buffers Difficult to form stable films at 37°C due to formation of air bubbles	[118,212, 215,240, 267,268]
Ultracentrifugation	Ultracentrifugation of the peptide-vesicles mixture leads to a separation of the free and bound peptide. Bound peptide pellet with the vesicles while the free peptide lays in the supernatant. In presence of a sucrose gradient, the contrary occurs.	Peptide molar partition coefficient into lipids	SUV LUV MLV	Rapid Simple	Sensitivity depends upon detection method	[117,269-274]
X-ray spectroscopy (diffraction/ scattering/ reflection)	X-ray interactions with atomic electrons. Diffracted/scattered/reflected X-ray beams intensities depend on electrons number and angle.	3D electron densities map and hence peptides and lipids 3D structure Bilayer structure Peptide penetration depth and orientation Vesicle lamellarity	Monolayers Supported mono-, bi- and multi-layers SUV LUV MLV	Accurate 3D structure	Destructive Difficult to detect hydrogen atoms positions Large amount of peptide is necessary Peptide penetration depth necessitates use of brominated lipids Requires extensive expertise	[145,230, 239,240, 275]

ANTS: 8-*amino*-1,3,6-napthalene trisulfonate (polyanionic fluorescent probe); NBD : 7-nitrobenz-2-oxa-1,3-diazol-4-yl; DPX: *p*-xylene-bis-pyridinium bromide (cationic quencher); Tb: Terbium; DPA: dipicolinic acid; NBD C_{12}-HPC : 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-2-phosphocholine

We studied ghrelin-lipid interactions by means of different methods described in **Table 6**. The choice of the techniques depended upon the information that they could provide with regard to the question we had to answer, but also upon the technique sensitivity, cost, technical constraints and also on the possibility to gain access to the necessary equipment. Moreover, we often used different techniques for studying a specific aspect of ghrelin-lipid interactions as these are complementary (**Table 7**).

Table 7 - Properties studied and methods used in this thesis.

Properties	**Methods**
Apparent binding coefficient (K_{app})	(Ultra)centrifugation Dialysis
Electrostatic interactions	Binding to liposomes with various lipid compositions at different pH and ionic strengths
Zeta potential	Electrophoretic light scattering
Hydrophobic interactions	
Influence of membrane fluidity	Binding to liposomes above and below T_c
Influence on membrane fluidity	Fluorescence polarization
Hydrophobic partition coefficient	Binding model
Binding thermodynamics	ITC Binding model
Peptide conformation	CD ATR-FTIR Molecular modelization
Orientation of lipids	ATR-FTIR
Passive diffusion through lipids	PAMPA

3.2. ACYLATED PEPTIDES: MEMBRANE INTERACTION & TRANSPORT

3.2.1. Membrane interaction of acylated peptides

A. Naturally acylated peptides and proteins

Eukaryotic peptides and proteins can be co-translationally or post-translationally modified by the addition of an acyl group. The most common acyl chains found in this modified peptides or proteins are acetyl (C_2), myristoyl (C_{14}) and palmitoyl (C_{16}) [107].

The site of acylation on the peptides and proteins typically depends upon the acyl group. While acetylation occurs on Lys side chains, myristoylation generally takes place on N-terminal glycine residues and the palmitoyl group is transferred to the sulfhydryl group of Cys side chains [107]. By transforming an amine into an amide, N^{ϵ}-acetylation and *N*-myristoylation may induce a change in peptide/protein charge depending on the pH. On the contrary, the transformation of the sulfhydryl group into a thioester by *S*-palmitoylation does not change the peptide/protein charge.

Biological consequences of the acylation depend upon the acyl group. On the one hand, acetylation does not seem to play a role in protein-membrane binding but rather seems to modulate protein function in conjunction with other types of post-translational modifications [276]. On the other hand, myristoylation and palmitoylation promote the targeting of the peptides and peripheral membrane proteins to the membranes and thereby modify their function, as for G-protein α subunit [277]. Some proteins are dually acylated either by a myristoyl and a palmitoyl or by two palmitoyls.

1. Myristoylated peptides and proteins

The presence of a myristoyl moiety alone seems to only confer a transient membrane residence, whereas the addition of a second signal such as a basic cluster or a palmitate stabilizes their binding to the membrane [278]. As usually the myristoylate half-life of these proteins is about the same as the polypeptide backbone half-life [277,278], reversibility of myristoylated proteins targeting and/or attachment to the membrane are also regulated by switch mechanisms [278]: a ligand-induced switch e.g. Ca^{2+} for recoverin [279] or an electrostatic switch e.g. phosphorylation of MARCKS [280]. Usually, myristoylated peptides and proteins localize in non-raft domains.

2. Palmitoylated peptides and proteins

Unlike myristoylation, *S*-palmitoylation takes place at different localizations in the sequence: at the N-terminus, C-terminus or other sites in the proteins.

Palmitoylated proteins are more strongly bound to the membrane than myristoylated proteins. Palmitate half-life of palmitoylated proteins, however, is usually much lower than half-life of the polypeptide backbone. This palmitate turn-over is thus a way to regulate the proteins attachment to the membrane and it has been shown, for some proteins, to be mediated by thioesterases [107,278]. Palmitoyl moieties are necessary to target the proteins to raft domains even though palmitoylation is neither always sufficient nor always required. Indeed, on the

one hand some palmitoylated proteins like the transferrin receptor, a non raft marker, do not localize in rafts and, on the other hand, some proteins, like caveolin, still remain detergent-resistant when their palmitoylated cysteines are mutated [277].

B. Non-naturally acylated peptides

A variety of acyl derivatives of natural or purely synthetic peptides have been studied as they are thought to enhance the peptide activity due to an increase in its lipophilicity and thus a potentially increased membrane binding affinity [229].

However, acylated peptides are sometimes less effective than their natural counterpart [281-284]. In this regard, it has to be noted that acylation can modify the physicochemical properties of the peptides not only by enhancing the lipophilicity of the peptide but also by altering the peptide charge, or by disrupting disulfide bonds.

The peptide charge is potentially altered when the peptides are either acylated on the N-terminal amino group [281,285], or on the amino group of charged side chains like Lys [282,283]. Charge alteration can be deleterious to the peptide activity if this charge was necessary to mediate its effect, or necessary to avoid peptide aggregation [281,286] or both. Moreover, it is hard to only isolate the role of the acyl moiety alone in such a case.

The change in lipophilicity [287,288] can also lead to the peptide aggregation [285,289] and increasing the acyl chain length may increase the peptide activity [287,290] but it may also not [281,291]. In fact, there is an optimum chain length for each peptide and each effect. For instance, optimal antimicrobial activity of acylated (C_7, C_{11} and C_{16}) derivatives of magainin-2 was obtained against yeast with the palmitoyl (C_{16}) derivative but maximal activity against fungi was achieved with the undecanoylate (C_{11}) derivative [291].

When acylation induces the disruption of disulfide bonds and if that bond is necessary to the peptide activity, the acylation has to be reversible so that the disulfide bonds can be restored *in vivo* [292].

Acylation of a peptide can also induce a conformational change [291,293] or stabilize the peptide structure [281,289], e.g. myristoylation and/or palmitoylation of eNOS induces a change from a random coil to a β-sheet conformation [293].

One commercial example of a non naturally acylated peptide is insulin detemir, a soluble and long-acting human insulin analogue marketed by Novo Nordisk under the name Levemir™. Insulin detemir lacks Thr^{30} and is myristoylated on Lys^{29} side chain. It possesses a lower molar potency than human insulin [294] and is thus formulated at a 4-fold higher concentration. However, it possesses a protracted action due to self-association, without

precipitation, at the injection site and to albumin binding [294]. These properties contribute to the ability of insulin detemir of maintaining the basal level of insulin with only a once daily administration in patients with diabetes [295].

C. Ghrelin

As already mentioned, ghrelin is the only endogenous hormone peptide to be naturally modified by a middle chain fatty acid. It is the hydroxyl group on Ser^3 that is acylated, most often with a *n*-octanoyl (C_8) moiety; the acyl thus becomes linked to the peptide through an ester linkage.

As ghrelin is also a highly basic peptide at physiological pH and that the acylation does not modify this charge, it will allow us to study the specific role of this middle chain length fatty acid on peptide binding to membranes.

3.2.2. Transepithelial transport of acylated peptides: intestinal model

In order to get through the intestinal epithelium, peptides can follow different pathways: (i) transcellular passive diffusion; (ii) paracellular; (iii) transcytosis (endocytic); (iv) carrier-mediated (**Fig. 17**). The known peptide carriers that transport peptides from the lumen to the interior of the intestinal cells i.e. the peptide transporter 1 (PepT1), the peptide/histidine transporters 1 and 2 (PHT1 and PHT2) and the human oligopeptide intestinal transporter 1 (HPT1), only recognize di- or tripeptides [296]. However, longer peptides have also been shown to be able to cross the intestinal barrier in intact form [297] by mechanisms that are still unclear [298]. Nevertheless, the peptides can also be secreted back into the lumen by efflux proteins such as P-glycoprotein and multidrug resistance associated protein 2 (MRP 2) [299,300]. The detailed transport mechanisms are beyond the scope of this thesis.

Acylation of peptides is considered to be a promising way to increase the delivery of hormone peptides as those are usually highly hydrophilic, rapidly degraded and their transport is limited. Indeed, peptide acylation may enhance the peptide's efficacy through an increase in: (i) its stability i.e. either by decreasing its chemical and/or proteolytic degradation [281,288,291,301] or by prolonging its plasma half-life [283,294,301,302]; (ii) its binding to membranes [303,304]; (iii) its residence time in membranes or in tissues [289,292,302]; (iv) its transport through cell membranes and epithelia [305,306].

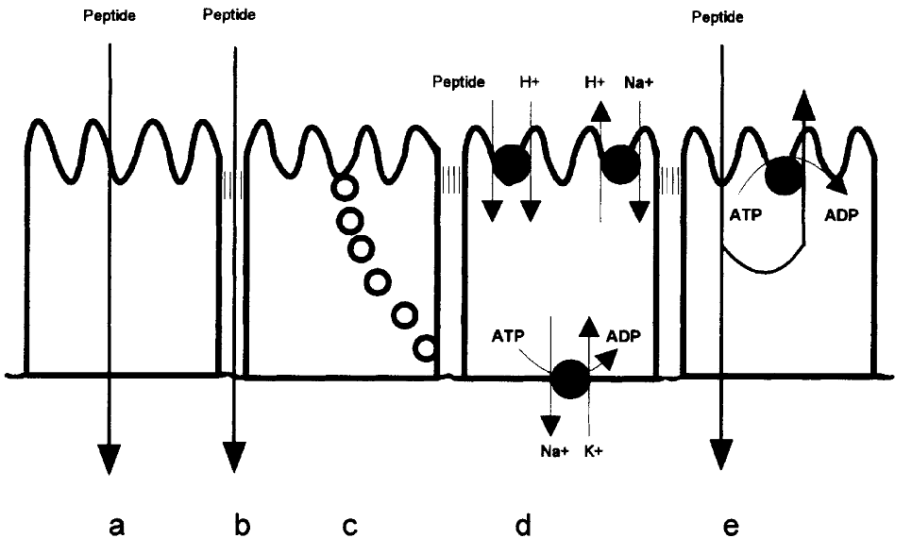


Figure 17 - Basic mechanisms of intestinal peptide absorption: (a) transcellular passive diffusion; (b) paracellular; (c) transcytosis; (d) cellular uptake via the di- and tripeptides carrier (e.g. PepT1); (e) secretion back into the intestinal lumen by efflux proteins (e.g. P-glycoprotein) [298].

A. Passive diffusion – Peptide translocation

As far as I know there is only one study in which the passive diffusion of acylated peptides was investigated by PAMPA. This study reports the passive diffusion of acetylated dipeptides with P_{app} of ~ 2 x 10^{-7} to 2 x 10^{-6} cm/s. However, as they did not study the corresponding unacetylated forms, these values are of little interest [260]. They are few reports of peptides passive diffusion by PAMPA. All studied peptides are no longer than six amino acids and some are cyclic. Reported P_{app} ranged from 5 x 10^{-8} to 10^{-6} cm/s [125,260].

Peptide translocation across lipid bilayers usually requires an acyl modification of at least 14 to 16 carbons, even though it seems that systematic studies on the minimum carbon length required are lacking. This C_{14}-C_{16} lengths roughly correspond to half the bilayer thickness [307-310].

B. Acylated peptides transport through the intestinal epithelia

It has been shown that acylation can enhance the intestinal *in vitro* and/or *in vivo* absorption of short peptides, such as thyrotropin-releasing hormone (3 AA) [288], tetragastrin (4 AA) [305,311] or leu-enkephalin & analogue (5 AA) [284,301], but also of longer peptides, such as insulin (30 AA) [282,312], glucagon-like peptide 1 (30 AA) [283] or Fab antibodies fragments [313]. As for peptide activity and stability, it seems that for each peptide there exists an optimum number of carbons for the fatty acid in order to enhance the peptide

transport i.e. the enhancing effect is not always directly related to the number of carbons of the fatty acid, but is often "bell-shaped" regarding to the lipophilic index [283,284,287,311].

C. Ghrelin

Ghrelin has been shown to be transported through the blood brain barrier. Whereas unacylated ghrelin was transported by an unsaturable mechanism, the transport of acylated ghrelin was saturable [314]. Binding and internalization of acylated ghrelin in a rat cerebral microvessel endothelial cell line has also been investigated and also seemed to be saturable [315].

However, in our case, we were interested in ghrelin transport through the intestinal epithelia. As the blood brain barrier and intestinal barrier differ, the octanoylation of ghrelin may lead to an increase of its transport through the intestinal epithelia. This will thus be investigated along with the passive diffusion of ghrelin through a lipid bilayer.

4. REFERENCES

[1] Bowers C.Y., Growth hormone-releasing peptide (GHRP), Cell Mol. Life Sci., 54 (1998) 1316-1329.

[2] Bowers C.Y., Momany F.A., Reynolds G.A., and Hong A., On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone, Endocrinology, 114 (1984) 1537-1545.

[3] Momany F.A., Bowers C.Y., Reynolds G.A., Hong A., and Newlander K., Conformational energy studies and in vitro and in vivo activity data on growth hormone-releasing peptides, Endocrinology, 114 (1984) 1531-1536.

[4] Howard A.D., Feighner S.D., Cully D.F., Arena J.P., Liberator P.A., Rosenblum C.I., Hamelin M., Hreniuk D.L., Palyha O.C., Anderson J., Paress P.S., Diaz C., Chou M., Liu K.K., McKee K.K., Pong S.S., Chaung L.Y., Elbrecht A., Dashkevicz M., Heavens R., Rigby M., Sirinathsinghji D.J., Dean D.C., Melillo D.G., Patchett A.A., Nargund R., Griffin P.R., DeMartino J.A., Gupta S.K., Schaeffer J.M., Smith R.G., and Van Der Ploeg L.H., A receptor in pituitary and hypothalamus that functions in growth hormone release, Science, 273 (1996) 974-977.

[5] Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H., and Kangawa K., Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature, 402 (1999) 656-660.

[6] Wajnrajch M.P., Ten I.S., Gertner J.M., and Leibel R.L., Genomic organization of the human ghrelin gene, J. Endocr. Genet., 1 (2000) 231-233.

[7] Smith R.G., Van Der Ploeg L.H., Howard A.D., Feighner S.D., Cheng K., Hickey G.J., Wyvratt M.J., Jr., Fisher M.H., Nargund R.P., and Patchett A.A., Peptidomimetic regulation of growth hormone secretion, Endocr. Rev., 18 (1997) 621-645.

[8] Tanaka M., Hayashida Y., Iguchi T., Nakao N., Nakai N., and Nakashima K., Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon, Endocrinology, 142 (2001) 3697-3700.

[9] Seim I., Collet C., Herington A.C., and Chopin L.K., Revised genomic structure of the human ghrelin gene and identification of novel exons, alternative splice variants and natural antisense transcripts, BMC. Genomics, 8 (2007) 298.

[10] Zhang J.V., Ren P.G., Avsian-Kretchmer O., Luo C.W., Rauch R., Klein C., and Hsueh A.J., Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake, Science, 310 (2005) 996-999.

[11] Soares J.B. and Leite-Moreira A.F., Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle, Peptides, 29 (2008) 1255-1270.

[12] Hosoda H., Kojima M., Matsuo H., and Kangawa K., Purification and characterization of rat des-Gln14-Ghrelin, a second endogenous ligand for the growth hormone secretagogue receptor, J. Biol. Chem., 275 (2000) 21995-22000.

[13] Kojima M. and Kangawa K., Ghrelin: structure and function, Physiol. Rev., 85 (2005) 495-522.

[14] Hosoda H., Kojima M., Mizushima T., Shimizu S., and Kangawa K., Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing, J. Biol. Chem., 278 (2003) 64-70.

[15] Gutierrez J.A., Solenberg P.J., Perkins D.R., Willency J.A., Knierman M.D., Jin Z., Witcher D.R., Luo S., Onyia J.E., and Hale J.E., Ghrelin octanoylation mediated by an orphan lipid transferase, Proc. Natl. Acad. Sci. U. S. A, 105 (2008) 6320-6325.

[16] Yang J., Brown M.S., Liang G., Grishin N.V., and Goldstein J.L., Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone, Cell, 132 (2008) 387-396.

[17] Gauna C., van de Zande B., van Kerkwijk A., Themmen A.P., van der Lely A.J., and Delhanty P.J., Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R), Mol. Cell Endocrinol., 274 (2007) 30-34.

[18] Bednarek M.A., Feighner S.D., Pong S.S., McKee K.K., Hreniuk D.L., Silva M.V., Warren V.A., Howard A.D., Van Der Ploeg L.H., and Heck J.V., Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a, J. Med. Chem., 43 (2000) 4370-4376.

[19] Matsumoto M., Hosoda H., Kitajima Y., Morozumi N., Minamitake Y., Tanaka S., Matsuo H., Kojima M., Hayashi Y., and Kangawa K., Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides, Biochem. Biophys. Res. Commun., 287 (2001) 142-146.

[20] Tolle V., Zizzari P., Tomasetto C., Rio M.C., Epelbaum J., and Bluet-Pajot M.T., In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat, Neuroendocrinology, 73 (2001) 54-61.
[21] Torsello A., Ghe' C., Bresciani E., Catapano F., Ghigo E., Deghenghi R., Locatelli V., and Muccioli G., Short ghrelin peptides neither displace ghrelin binding in vitro nor stimulate GH release in vivo, Endocrinology, 143 (2002) 1968-1971.
[22] Dornonville de la Cour C., Lindstrom E., Norlen P., and Hakanson R., Ghrelin stimulates gastric emptying but is without effect on acid secretion and gastric endocrine cells, Regul. Pept., 120 (2004) 23-32.
[23] Beevers A.J. and Kukol A., Conformational flexibility of the peptide hormone ghrelin in solution and lipid membrane bound: a molecular dynamics study, J. Biomol. Struct. Dyn., 23 (2006) 357-364.
[24] Silva Elipe M.V., Bednarek M.A., and Gao Y.D., 1H NMR structural analysis of human ghrelin and its six truncated analogs, Biopolymers, 59 (2001) 489-501.
[25] Dehlin E., Liu J., Yun S.H., Fox E., Snyder S., Gineste C., Willingham L., Geysen M., Gaylinn B.D., and Sando J.J., Regulation of ghrelin structure and membrane binding by phosphorylation, Peptides, 29 (2008) 904-911.
[26] Kukol A., The structure of ghrelin, Vitam. Horm., 77 (2008) 1-12.
[27] Lehninger A.L., Nelson D.L., and Cox M.M., Lehninger principles of biochemistry, W.H. Freeman, New York (2004).
[28] Mullan L.J., Hydropathy--a window on the evasion of water, Brief. Bioinform., 4 (2003) 279-282.
[29] Hu X. and Pan Y., Knowledge discovery in bioinformatics: techniques, methods, and applications, Wiley-Interscience, Hoboken, N.J (2007).
[30] Hosoda H. and Kangawa K., Ghrelin measurement: present and perspectives, in: Ghigo E., Benso A., and Broglio F. (Eds.), Ghrelin, Kluwer Academic, Boston, (2004) 225-236.
[31] Akamizu T., Takaya K., Irako T., Hosoda H., Teramukai S., Matsuyama A., Tada H., Miura K., Shimizu A., Fukushima M., Yokode M., Tanaka K., and Kangawa K., Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects, Eur. J. Endocrinol., 150 (2004) 447-455.
[32] Hosoda H., Doi K., Nagaya N., Okumura H., Nakagawa E., Enomoto M., Ono F., and Kangawa K., Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples, Clin. Chem., 50 (2004) 1077-1080.
[33] De Vriese C., Hacquebard M., Gregoire F., Carpentier Y., and Delporte C., Ghrelin interacts with human plasma lipoproteins, Endocrinology, 148 (2007) 2355-2362.
[34] De Vriese C., Gregoire F., Lema-Kisoka R., Waelbroeck M., Robberecht P., and Delporte C., Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites, Endocrinology, 145 (2004) 4997-5005.
[35] Stengel A., Keire D., Goebel M., Evilevitch L., Wiggins B., Tache Y., and Reeve J.R., Jr., The RAPID method for blood processing yields new insight in plasma concentrations and molecular forms of circulating gut peptides, Endocrinology, 150 (2009) 5113-5118.
[36] Groschl M., Wagner R., Dotsch J., Rascher W., and Rauh M., Preanalytical influences on the measurement of ghrelin, Clin. Chem., 48 (2002) 1114-1116.
[37] Moeller E.H., Holst B., Nielsen L.H., Pedersen P.S., and Ostergaard J., Stability, liposome interaction, and in vivo pharmacology of ghrelin in liposomal suspensions, Int. J. Pharm., 390 (2010) 13-18.
[38] Ishimaru M., Yoshizawa-Kumagaye K., Kubo S., Kitani T., Chino N., Kangawa K., and Kimura T., Stability of the O-octanoyl group of rat ghrelin during chemical synthesis : counter-ion-dependent alteration of an ester bond breakage, Lett. Pept. Sci., 10 (2003) 41-50.
[39] Davenport A.P., Bonner T.I., Foord S.M., Harmar A.J., Neubig R.R., Pin J.P., Spedding M., Kojima M., and Kangawa K., International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function, Pharmacol. Rev., 57 (2005) 541-546.
[40] Ueberberg B., Unger N., Saeger W., Mann K., and Petersenn S., Expression of Ghrelin and its Receptor in Human Tissues, Horm. Metab Res., (2009).
[41] Lau P.N., Chow K.B., Chan C.B., Cheng C.H., and Wise H., The constitutive activity of the ghrelin receptor attenuates apoptosis via a protein kinase C-dependent pathway, Mol. Cell Endocrinol., 299 (2009) 232-239.
[42] Holst B., Cygankiewicz A., Jensen T.H., Ankersen M., and Schwartz T.W., High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist, Mol. Endocrinol., 17 (2003) 2201-2210.
[43] Liu G., Fortin J.P., Beinborn M., and Kopin A.S., Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities, J. Pharmacol. Exp. Ther., 322 (2007) 1036-1043.

[44] Holst B., Holliday N.D., Bach A., Elling C.E., Cox H.M., and Schwartz T.W., Common structural basis for constitutive activity of the ghrelin receptor family, J. Biol. Chem., 279 (2004) 53806-53817.
[45] Pantel J., Legendre M., Cabrol S., Hilal L., Hajaji Y., Morisset S., Nivot S., Vie-Luton M.P., Grouselle D., de Kerdanet M., Kadiri A., Epelbaum J., Le B.Y., and Amselem S., Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature, J. Clin. Invest, 116 (2006) 760-768.
[46] Holst B., Schwartz T.W., Frimurer T.M., and Rist O., Ghrelin receptor inverse agonist for regulation of feeding behaviours, (2004) [WO/2004/056869].
[47] Holst B., Lang M., Brandt E., Bach A., Howard A., Frimurer T.M., Beck-Sickinger A., and Schwartz T.W., Ghrelin receptor inverse agonists: identification of an active peptide core and its interaction epitopes on the receptor, Mol. Pharmacol., 70 (2006) 936-946.
[48] Holst B., Mokrosinski J., Lang M., Brandt E., Nygaard R., Frimurer T.M., Beck-Sickinger A.G., and Schwartz T.W., Identification of an efficacy switch region in the ghrelin receptor responsible for interchange between agonism and inverse agonism, J. Biol. Chem., 282 (2007) 15799-15811.
[49] Gnanapavan S., Kola B., Bustin S.A., Morris D.G., McGee P., Fairclough P., Bhattacharya S., Carpenter R., Grossman A.B., and Korbonits M., The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans, J. Clin. Endocrinol. Metab., 87 (2002) 2988-2991.
[50] Leung P.K., Chow K.B., Lau P.N., Chu K.M., Chan C.B., Cheng C.H., and Wise H., The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor, Cell Signal., 19 (2007) 1011-1022.
[51] Schwartz T.W., Frimurer T.M., Holst B., Rosenkilde M.M., and Elling C.E., Molecular mechanism of 7TM receptor activation--a global toggle switch model, Annu. Rev. Pharmacol. Toxicol., 46 (2006) 481-519.
[52] Arvat E., Di Vito L., Broglio F., Papotti M., Muccioli G., Dieguez C., Casanueva F.F., Deghenghi R., Camanni F., and Ghigo E., Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans, J. Endocrinol. Invest, 23 (2000) 493-495.
[53] Holst B., Brandt E., Bach A., Heding A., and Schwartz T.W., Nonpeptide and peptide growth hormone secretagogues act both as ghrelin receptor agonist and as positive or negative allosteric modulators of ghrelin signaling, Mol. Endocrinol., 19 (2005) 2400-2411.
[54] Casanueva F.F., Camina J.P., Carreira M.C., Pazos Y., Varga J.L., and Schally A.V., Growth hormone-releasing hormone as an agonist of the ghrelin receptor GHS-R1a, Proc. Natl. Acad. Sci. U. S. A, 105 (2008) 20452-20457.
[55] Hataya Y., Akamizu T., Takaya K., Kanamoto N., Ariyasu H., Saijo M., Moriyama K., Shimatsu A., Kojima M., Kangawa K., and Nakao K., A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans, J. Clin. Endocrinol. Metab., 86 (2001) 4552.
[56] Asakawa A., Inui A., Kaga T., Katsuura G., Fujimiya M., Fujino M.A., and Kasuga M., Antagonism of ghrelin receptor reduces food intake and body weight gain in mice, Gut, 52 (2003) 947-952.
[57] Vlasova M.A., Jarvinen K., and Herzig K.H., Cardiovascular effects of ghrelin antagonist in conscious rats, Regul. Pept., 156 (2009) 72-76.
[58] Zizzari P., Halem H., Taylor J., Dong J.Z., Datta R., Culler M.D., Epelbaum J., and Bluet-Pajot M.T., Endogenous ghrelin regulates episodic growth hormone (GH) secretion by amplifying GH Pulse amplitude: evidence from antagonism of the GH secretagogue-R1a receptor, Endocrinology, 146 (2005) 3836-3842.
[59] Helmling S., Maasch C., Eulberg D., Buchner K., Schroder W., Lange C., Vonhoff S., Wlotzka B., Tschop M.H., Rosewicz S., and Klussmann S., Inhibition of ghrelin action in vitro and in vivo by an RNA-Spiegelmer, Proc. Natl. Acad. Sci. U. S. A, 101 (2004) 13174-13179.
[60] Pedretti A., Villa M., Pallavicini M., Valoti E., and Vistoli G., Construction of human ghrelin receptor (hGHS-R1a) model using a fragmental prediction approach and validation through docking analysis, J. Med. Chem., 49 (2006) 3077-3085.
[61] Wang H.J., Geller F., Dempfle A., Schauble N., Friedel S., Lichtner P., Fontenla-Horro F., Wudy S., Hagemann S., Gortner L., Huse K., Remschmidt H., Bettecken T., Meitinger T., Schafer H., Hebebrand J., and Hinney A., Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and underweight students, and children with short normal stature, J. Clin. Endocrinol. Metab., 89 (2004) 157-162.
[62] Baldanzi G., Filigheddu N., Cutrupi S., Catapano F., Bonissoni S., Fubini A., Malan D., Baj G., Granata R., Broglio F., Papotti M., Surico N., Bussolino F., Isgaard J., Deghenghi R., Sinigaglia F., Prat M., Muccioli G., Ghigo E., and Graziani A., Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT, J. Cell Biol., 159 (2002) 1029-1037.
[63] Camina J.P., Cell biology of the ghrelin receptor, J. Neuroendocrinol., 18 (2006) 65-76.

[64] Leite-Moreira A.F. and Soares J.B., Physiological, pathological and potential therapeutic roles of ghrelin, Drug Discov. Today, 12 (2007) 276-288.
[65] Muccioli G., Baragli A., Granata R., Papotti M., and Ghigo E., Heterogeneity of ghrelin/growth hormone secretagogue receptors. Toward the understanding of the molecular identity of novel ghrelin/GHS receptors, Neuroendocrinology, 86 (2007) 147-164.
[66] Young Cruz C.R. and Smith R.G., The growth hormone secretagogue receptor, Vitam. Horm., 77 (2008) 47-88.
[67] Guan X.M., Yu H., Palyha O.C., McKee K.K., Feighner S.D., Sirinathsinghji D.J., Smith R.G., Van Der Ploeg L.H., and Howard A.D., Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues, Brain Res. Mol. Brain Res., 48 (1997) 23-29.
[68] Ariyasu H., Takaya K., Tagami T., Ogawa Y., Hosoda K., Akamizu T., Suda M., Koh T., Natsui K., Toyooka S., Shirakami G., Usui T., Shimatsu A., Doi K., Hosoda H., Kojima M., Kangawa K., and Nakao K., Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans, J. Clin. Endocrinol. Metab, 86 (2001) 4753-4758.
[69] Date Y., Kojima M., Hosoda H., Sawaguchi A., Mondal M.S., Suganuma T., Matsukura S., Kangawa K., and Nakazato M., Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans, Endocrinology, 141 (2000) 4255-4261.
[70] Hosoda H., Kojima M., and Kangawa K., Biological, physiological, and pharmacological aspects of ghrelin, J. Pharmacol. Sci., 100 (2006) 398-410.
[71] van der Lely A.J., Ghrelin and new metabolic frontiers, Horm. Res., 71 Suppl 1 (2009) 129-133.
[72] Wierup N., Svensson H., Mulder H., and Sundler F., The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas, Regul. Pept., 107 (2002) 63-69.
[73] Ghelardoni S., Carnicelli V., Frascarelli S., Ronca-Testoni S., and Zucchi R., Ghrelin tissue distribution: comparison between gene and protein expression, J. Endocrinol. Invest, 29 (2006) 115-121.
[74] Kojima M. and Kangawa K., Structure and function of ghrelin, Results Probl. Cell Differ., 46 (2008) 89-115.
[75] Yoshimoto A., Mori K., Sugawara A., Mukoyama M., Yahata K., Suganami T., Takaya K., Hosoda H., Kojima M., Kangawa K., and Nakao K., Plasma ghrelin and desacyl ghrelin concentrations in renal failure, J. Am. Soc. Nephrol., 13 (2002) 2748-2752.
[76] De Vriese C. and Delporte C., Ghrelin: a new peptide regulating growth hormone release and food intake, Int. J. Biochem. Cell Biol., 40 (2008) 1420-1424.
[77] Peeters T.L., Potential of ghrelin as a therapeutic approach for gastrointestinal motility disorders, Curr. Opin. Pharmacol., 6 (2006) 553-558.
[78] Akamizu T. and Kangawa K., Translational research on the clinical applications of ghrelin, Endocr. J., 53 (2006) 585-591.
[79] Ashitani J., Matsumoto N., and Nakazato M., Ghrelin and its therapeutic potential for cachectic patients, Peptides, 30 (2009) 1951-1956.
[80] Neary M.T. and Batterham R.L., Gut hormones: implications for the treatment of obesity, Pharmacol. Ther., 124 (2009) 44-56.
[81] Gualillo O., Lago F., and Dieguez C., Introducing GOAT: a target for obesity and anti-diabetic drugs?, Trends Pharmacol. Sci., 29 (2008) 398-401.
[82] Amino acids absorption and fluorescence emission spectra, OMLC, http://omlc.ogi.edu/spectra/PhotochemCAD/html/index.html (Last access date: 2010).
[83] Creighton T.E., Proteins: structures and molecular properties, W.H. Freeman, New York (1993).
[84] Uversky V.N. and Permyakov E.A., Methods in Protein Structure and Stability Analysis: Vibrational Spectroscopy, Nova Science Publishers, New York (2007).
[85] Kuipers B.J. and Gruppen H., Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography-mass spectrometry analysis, J. Agric. Food Chem., 55 (2007) 5445-5451.
[86] Beaumont N.J., Skinner V.O., Tan T.M., Ramesh B.S., Byrne D.J., MacColl G.S., Keen J.N., Bouloux P.M., Mikhailidis D.P., Bruckdorfer K.R., Vanderpump M.P., and Srai K.S., Ghrelin can bind to a species of high density lipoprotein associated with paraoxonase, J. Biol. Chem., 278 (2003) 8877-8880.
[87] Makino T., Matsumoto M., Suzuki Y., Kitajima Y., Yamamoto K., Kuramoto M., Minamitake Y., Kangawa K., and Yabuta M., Semisynthesis of human ghrelin: condensation of a Boc-protected recombinant peptide with a synthetic O-acylated fragment, Biopolymers, 79 (2005) 238-247.

[88] De Vriese C., Gregoire F., De Neef P., Robberecht P., and Delporte C., Ghrelin is produced by the human erythroleukemic HEL cell line and involved in an autocrine pathway leading to cell proliferation, Endocrinology, 146 (2005) 1514-1522.
[89] Pemberton C., Wimalasena P., Yandle T., Soule S., and Richards M., C-terminal pro-ghrelin peptides are present in the human circulation, Biochem. Biophys. Res. Commun., 310 (2003) 567-573.
[90] Muccioli G., Papotti M., Locatelli V., Ghigo E., and Deghenghi R., Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland, J. Endocrinol. Invest, 24 (2001) RC7-RC9.
[91] Akamizu T., Shinomiya T., Irako T., Fukunaga M., Nakai Y., Nakai Y., and Kangawa K., Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay, J. Clin. Endocrinol. Metab., 90 (2005) 6-9.
[92] Paik K.H., Choe Y.H., Park W.H., Oh Y.J., Kim A.H., Chu S.H., Kim S.W., Kwon E.K., Han S.J., Shon W.Y., and Jin D.K., Suppression of acylated ghrelin during oral glucose tolerance test is correlated with whole-body insulin sensitivity in children with Prader-Willi syndrome, J. Clin. Endocrinol. Metab., 91 (2006) 1876-1881.
[93] Broom D.R., Batterham R.L., King J.A., and Stensel D.J., Influence of resistance and aerobic exercise on hunger, circulating levels of acylated ghrelin, and peptide YY in healthy males, Am. J. Physiol. Regul. Integr. Comp. Physiol., 296 (2009) R29-R35.
[94] Rauh M., Groschl M., and Rascher W., Simultaneous Quantification of Ghrelin and Desacyl-Ghrelin by Liquid Chromatography-Tandem Mass Spectrometry in Plasma, Serum, and Cell Supernatants, Clin. Chem., 53 (2007) 902-910.
[95] Pietzsch J., Mind the membrane, *A living frontier - exploring the dynamics of the cell membrane,* Nature Publishing Group, 2008.
[96] Cooper G.M. and Hausman R.E., The cell: a molecular approach, ASM Press, Washington, DC (2007).
[97] Singer S.J. and Nicolson G.L., The fluid mosaic model of the structure of cell membranes, Science, 175 (1972) 720-731.
[98] Heimburg T., Thermal biophysics of membranes, Wiley-VCH, Weinheim (2007).
[99] Yorek M.A., Biological Distribution, in: Cevc G. (Ed.), Phospholipids handbook, Marcel Dekker, Inc, New York, (1993) 745-775.
[100] Cevc G., Phospholipids handbook, Marcel Dekker, Inc, New York (1993).
[101] van Meer G., Voelker D.R., and Feigenson G.W., Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell Biol., 9 (2008) 112-124.
[102] Andersen O.S. and Koeppe R.E., Bilayer thickness and membrane protein function: an energetic perspective, Annu. Rev. Biophys. Biomol. Struct., 36 (2007) 107-130.
[103] Greer J.P. and Wintrobe M.M., Wintrobe's clinical hematology, Lippincott Williams & Wilkins, Philadelphia, Pa (2009).
[104] Mouritsen O.G., Life - As a Matter of Fat, Springer, Berlin (2005).
[105] Lodish H.F., Molecular cell biology, W.H. Freeman, New York (2008).
[106] Papoucheva E., The Functional Role of Palmitoylation of the 5-HT1A Receptor, PhD thesis, University of Göttingen, 2004.
[107] Walsh C.T., Garneau-Tsodikova S., and Gatto G.J., Jr., Protein posttranslational modifications: the chemistry of proteome diversifications, Angew. Chem Int. Ed Engl., 44 (2005) 7342-7372.
[108] Scrima M., Campiglia P., Esposito C., Gomez-Monterrey I., Novellino E., and D'Ursi A.M., Obestatin conformational features: a strategy to unveil obestatin's biological role?, Biochem. Biophys. Res. Commun., 363 (2007) 500-505.
[109] Marcotte I. and Auger M., Bicelles as model membranes for solid- and solution-state NMR studies of membrane peptides and proteins, Concepts in Magnetic Resonance Part A, 24A (2005) 17-37.
[110] Lipids' structure and physico-chemical properties, Avanti Polar Lipids, Inc., http://www.avantilipids.com (Last access date: 2010).
[111] Triba M.N., Warschawski D.E., and Devaux P.F., Reinvestigation by phosphorus NMR of lipid distribution in bicelles, Biophys. J., 88 (2005) 1887-1901.
[112] Arora A. and Tamm L.K., Biophysical approaches to membrane protein structure determination, Curr. Opin. Struct. Biol., 11 (2001) 540-547.
[113] Diller A., Loudet C., Aussenac F., Raffard G., Fournier S., Laguerre M., Grelard A., Opella S.J., Marassi F.M., and Dufourc E.J., Bicelles: A natural 'molecular goniometer' for structural, dynamical and topological studies of molecules in membranes, Biochimie, 91 (2009) 744-751.
[114] Seelig A., Local anesthetics and pressure: a comparison of dibucaine binding to lipid monolayers and bilayers, Biochim. Biophys. Acta, 899 (1987) 196-204.
[115] Chavez A., Pujol M., Alsina M.A., and Cajal Y., Membrane fusion induced by a lipopeptidic epitope from VP3 capside protein of hepatitis A virus, Luminescence., 16 (2001) 135-143.

[116] Kamel A.M., Felmeister A., and Weiner N.D., Surface pressure-surface area characteristics of a series of autoxidation products of cholesterol, J. Lipid Res., 12 (1971) 155-159.
[117] Beschiaschvili G. and Seelig J., Peptide binding to lipid bilayers. Binding isotherms and zeta-potential of a cyclic somatostatin analogue, Biochemistry, 29 (1990) 10995-11000.
[118] Seelig A., Blatter X.L., Frentzel A., and Isenberg G., Phospholipid binding of synthetic talin peptides provides evidence for an intrinsic membrane anchor of talin, J. Biol. Chem., 275 (2000) 17954-17961.
[119] Castellana E.T. and Cremer P.S., Solid supported lipid bilayers: From biophysical studies to sensor design, Surf. Sci. Rep., 61 (2006) 429-444.
[120] El Kirat K., Morandat S., and Dufrene Y.F., Nanoscale analysis of supported lipid bilayers using atomic force microscopy, Biochim. Biophys. Acta, 1798 (2010) 750-765.
[121] Baird C.L., Courtenay E.S., and Myszka D.G., Surface plasmon resonance characterization of drug/liposome interactions, Anal. Biochem., 310 (2002) 93-99.
[122] Goormaghtigh E., Raussens V., and Ruysschaert J.M., Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes, Biochim. Biophys. Acta, 1422 (1999) 105-185.
[123] Sandhya K.V., Gayathri S.D., and Mathew S.T., Liposomal formulations of serratiopeptidase: in vitro studies using PAMPA and Caco-2 models, Mol. Pharm., 5 (2008) 92-97.
[124] Malakoutikhah M., Prades R., Teixido M., and Giralt E., N-methyl phenylalanine-rich peptides as highly versatile blood-brain barrier shuttles, J. Med. Chem., 53 (2010) 2354-2363.
[125] Rezai T., Bock J.E., Zhou M.V., Kalyanaraman C., Lokey R.S., and Jacobson M.P., Conformational flexibility, internal hydrogen bonding, and passive membrane permeability: successful in silico prediction of the relative permeabilities of cyclic peptides, J. Am. Chem. Soc., 128 (2006) 14073-14080.
[126] Hope M.J., Bally M.B., Mayer L.D., Janoff A.S., and Cullis P.R., Generation of Multilamellar and Unilamellar Phospholipid-Vesicles, Chem. Phys. Lipids, 40 (1986) 89-107.
[127] Lasic D.D., Liposomes: from physics to applications, Elsevier, Amsterdam (1993).
[128] Delattre J., Couvreur P., Puisieux F., Philippot J.-R., and Schuber F., Les liposomes: aspects technologiques, biologiques et pharmacologiques, Editions Médicales Internationales, (1993).
[129] Peitzsch R.M., Eisenberg M., Sharp K.A., and McLaughlin S., Calculations of the electrostatic potential adjacent to model phospholipid bilayers, Biophys. J., 68 (1995) 729-738.
[130] Prenner E., Honsek G., Honig D., Mobius D., and Lohner K., Imaging of the domain organization in sphingomyelin and phosphatidylcholine monolayers, Chem Phys. Lipids, 145 (2007) 106-118.
[131] Léonard A., Maillet J.-C., Dufourcq J., and Dufourc E.J., Bilayer thickness from lipid-chain dynamics: Influece of cholesterol and electric charges. A 2H-NMR approach, in: Helm C.M., Lösche M., and Möhwald H. (Eds.), Trends in Colloid and Interface Science VI, Springer, New York, 89 (1992) 315-318.
[132] Phase transition temperatures, LIPIDAT, http://www.lipidat.tcd.ie/ (Last access date: 2010).
[133] Kundu S., Chakraborty H., Sarkar M., and Datta A., Interaction of Oxicam NSAIDs with lipid monolayer: anomalous dependence on drug concentration, Colloids Surf. B Biointerfaces., 70 (2009) 157-161.
[134] Garidel P. and Blume A., 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) monolayers: influence of temperature, pH, ionic strength and binding of alkaline earth cations, Chem. Phys. Lipids, 138 (2005) 50-59.
[135] Castelli F., Sarpietro M.G., Micieli D., Stella B., Rocco F., and Cattel L., Enhancement of gemcitabine affinity for biomembranes by conjugation with squalene: differential scanning calorimetry and Langmuir-Blodgett studies using biomembrane models, J. Colloid Interface Sci., 316 (2007) 43-52.
[136] Rispoli P., Carzino R., Svaldo-Lanero T., Relini A., Cavalleri O., Fasano A., Liuzzi G.M., Carlone G., Riccio P., Gliozzi A., and Rolandi R., A thermodynamic and structural study of myelin basic protein in lipid membrane models, Biophys. J., 93 (2007) 1999-2010.
[137] Bangham A.D., Standish M.M., and Watkins J.C., Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol., 13 (1965) 238-252.
[138] Hope M.J., Bally M.B., Webb G., and Cullis P.R., Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta, 812 (1985) 55-65.
[139] Mayer L.D., Hope M.J., and Cullis P.R., Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta, 858 (1986) 161-168.
[140] Lasic D.D., Liposomes, Recherche, 20 (1989) 904-913.
[141] Bartlett G.R., Phosphorus assay in column chromatography, J. Biol. Chem., 234 (1959) 466-468.
[142] Carrasco-Pancorbo A., Navas-Iglesias N., and Cuadros-Rodriguez L., From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part I: Modern lipid analysis, Trends Analyt. Chem., 28 (2009) 263-278.

[143] Gad S.C., Pharmaceutical Manufacturing Handbook: Production and Processes, John Wiley & Sons, Inc, Hoboken (2008).
[144] Edwards K.A. and Baeumner A.J., Analysis of liposomes, Talanta, 68 (2006) 1432-1441.
[145] Torchilin V.P. and Weissig V., Liposomes: a practical approach, Oxford University Press, Oxford (2003).
[146] Frohlich M., Brecht V., and Peschka-Suss R., Parameters influencing the determination of liposome lamellarity by 31P-NMR, Chem Phys. Lipids, 109 (2001) 103-112.
[147] Hiemenz P.C. and Rajagopalan R., Principles of colloid and surface chemistry, Marcel Dekker, New York (1997).
[148] Delgado A.V., Gonzalez-Caballero F., Hunter R.J., Koopal L.K., and Lyklema J., Measurement and interpretation of electrokinetic phenomena, J. Colloid Interface Sci., 309 (2007) 194-224.
[149] De Meulenaer B., van der Meeren P., and Vanderdeelen J., Electrophoresis of Liposomes, in: Somasundaran P. (Ed.), Encyclopedia of surface and colloid science, Taylor & Francis, New York, (2006) 2372-2384.
[150] Sewald N. and Jakubke H.D., Peptides: chemistry and biology, Wiley-VCH, Weinheim (2009).
[151] Castanho M.A. and Fernandes M.X., Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the "membrane catalysis" model, Eur. Biophys. J., 35 (2006) 92-103.
[152] Seelig J., Titration calorimetry of lipid-peptide interactions, Biochim. Biophys. Acta, 1331 (1997) 103-116.
[153] Seelig J., Thermodynamics of lipid-peptide interactions, Biochim. Biophys. Acta, 1666 (2004) 40-50.
[154] McLaughlin S., Electrostatic Potentials at Membrane-Solution Interfaces, in: Bronner F. and Kleinzeller A. (Eds.), Current Topics in Membranes and Transport, Volume 9, Academic Press, New York, (1977) 71-144.
[155] McLaughlin S., The electrostatic properties of membranes, Annu. Rev. Biophys. Biophys. Chem., 18 (1989) 113-136.
[156] Tatulian S.A., Surface electrostatics of biological membranes and ion binding, in: Sørensen T.S. (Ed.), Surface chemistry and electrochemistry of membranes, M. Dekker, New York, (1999) 872-922.
[157] Backlund B.M., Wikander G., Peeters T.L., and Graslund A., Induction of secondary structure in the peptide hormone motilin by interaction with phospholipid vesicles, Biochim. Biophys. Acta, 1190 (1994) 337-344.
[158] Herbig M.E., Fromm U., Leuenberger J., Krauss U., Beck-Sickinger A.G., and Merkle H.P., Bilayer interaction and localization of cell penetrating peptides with model membranes: a comparative study of a human calcitonin (hCT)-derived peptide with pVEC and pAntp(43-58), Biochim. Biophys. Acta, 1712 (2005) 197-211.
[159] Binder H. and Lindblom G., Charge-dependent translocation of the Trojan peptide penetratin across lipid membranes, Biophys. J., 85 (2003) 982-995.
[160] Sevcsik E., Pabst G., Richter W., Danner S., Amenitsch H., and Lohner K., Interaction of LL-37 with model membrane systems of different complexity: influence of the lipid matrix, Biophys. J., 94 (2008) 4688-4699.
[161] Machaidze G. and Seelig J., Specific binding of cinnamycin (Ro 09-0198) to phosphatidylethanolamine. Comparison between micellar and membrane environments, Biochemistry, 42 (2003) 12570-12576.
[162] Matsuzaki K., Sugishita K., Ishibe N., Ueha M., Nakata S., Miyajima K., and Epand R.M., Relationship of membrane curvature to the formation of pores by magainin 2, Biochemistry, 37 (1998) 11856-11863.
[163] Wieprecht T., Apostolov O., Beyermann M., and Seelig J., Membrane binding and pore formation of the antibacterial peptide PGLa: thermodynamic and mechanistic aspects, Biochemistry, 39 (2000) 442-452.
[164] Seelig J., Nebel S., Ganz P., and Bruns C., Electrostatic and nonpolar peptide-membrane interactions. Lipid binding and functional properties of somatostatin analogues of charge z = +1 to z = +3, Biochemistry, 32 (1993) 9714-9721.
[165] Hanakam F., Gerisch G., Lotz S., Alt T., and Seelig A., Binding of hisactophilin I and II to lipid membranes is controlled by a pH-dependent myristoyl-histidine switch, Biochemistry, 35 (1996) 11036-11044.
[166] Lehrmann R. and Seelig J., Adsorption of Ca2+ and La3+ to bilayer membranes: measurement of the adsorption enthalpy and binding constant with titration calorimetry, Biochim. Biophys. Acta, 1189 (1994) 89-95.
[167] Montich G., Scarlata S., McLaughlin S., Lehrmann R., and Seelig J., Thermodynamic characterization of the association of small basic peptides with membranes containing acidic lipids, Biochim. Biophys. Acta, 1146 (1993) 17-24.

[168] Arseneault M. and Lafleur M., Isothermal titration calorimetric study of calcium association to lipid bilayers: influence of the vesicle preparation and composition, Chem. Phys. Lipids, 142 (2006) 84-93.
[169] Jing W., Hunter H.N., Hagel J., and Vogel H.J., The structure of the antimicrobial peptide Ac-RRWWRF-NH2 bound to micelles and its interactions with phospholipid bilayers, J. Pept. Res., 61 (2003) 219-229.
[170] Tiriveedhi V. and Butko P., A fluorescence spectroscopy study on the interactions of the TAT-PTD peptide with model lipid membranes, Biochemistry, 46 (2007) 3888-3895.
[171] Antharam V.C., Elliott D.W., Mills F.D., Farver R.S., Sternin E., and Long J.R., Penetration depth of surfactant peptide KL4 into membranes is determined by fatty acid saturation, Biophys. J., 96 (2009) 4085-4098.
[172] Zoonens M., Reshetnyak Y.K., and Engelman D.M., Bilayer interactions of pHLIP, a peptide that can deliver drugs and target tumors, Biophys. J., 95 (2008) 225-235.
[173] Lopes S.C., Fedorov A., and Castanho M.A., Lipidic membranes are potential "catalysts" in the ligand activity of the multifunctional pentapeptide neokyotorphin, Chembiochem., 6 (2005) 697-702.
[174] Dufourc E.J., Dufourcq J., Birkbeck T.H., and Freer J.H., Delta-haemolysin from Staphylococcus aureus and model membranes. A solid-state 2H-NMR and 31P-NMR study, Eur. J. Biochem., 187 (1990) 581-587.
[175] Kremer J.J., Sklansky D.J., and Murphy R.M., Profile of changes in lipid bilayer structure caused by beta-amyloid peptide, Biochemistry, 40 (2001) 8563-8571.
[176] Ahn T., Oh D.B., Kim H., and Park C., The phase property of membrane phospholipids is affected by the functionality of signal peptides from the Escherichia coli ribose-binding protein, J. Biol. Chem, 277 (2002) 26157-26162.
[177] Bradshaw J.P., Davies S.M.A., and Hauss T., Interaction of Substance P with Phospholipid Bilayers: A Neutron Diffraction Study, Biophys. J., 75 (1998) 889-895.
[178] Sani M.A., Loudet C., Grobner G., and Dufourc E.J., Pro-apoptotic bax-alpha1 synthesis and evidence for beta-sheet to alpha-helix conformational change as triggered by negatively charged lipid membranes, J. Pept. Sci., 13 (2007) 100-106.
[179] Bechinger B., Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin, J. Membr. Biol., 156 (1997) 197-211.
[180] Dike A. and Cowsik S.M., Membrane-induced structure of scyliorhinin I: a dual NK1/NK2 agonist, Biophys. J., 88 (2005) 3592-3600.
[181] Klocek G., Schulthess T., Shai Y., and Seelig J., Thermodynamics of melittin binding to lipid bilayers. Aggregation and pore formation, Biochemistry, 48 (2009) 2586-2596.
[182] Sankararamakrishnan R., Recognition of GPCRs by peptide ligands and membrane compartments theory: structural studies of endogenous peptide hormones in membrane environment, Biosci. Rep., 26 (2006) 131-158.
[183] Surewicz W.K., Mantsch H.H., Stahl G.L., and Epand R.M., Infrared spectroscopic evidence of conformational transitions of an atrial natriuretic peptide, Proc. Natl. Acad. Sci. U. S. A, 84 (1987) 7028-7030.
[184] Blondelle S.E., Lohner K., and Aguilar M., Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity, Biochim. Biophys. Acta, 1462 (1999) 89-108.
[185] Matsuzaki K., Physicochemical interactions of amyloid beta-peptide with lipid bilayers, Biochim. Biophys. Acta, 1768 (2007) 1935-1942.
[186] Williamson J.A., Loria J.P., and Miranker A.D., Helix stabilization precedes aqueous and bilayer-catalyzed fiber formation in islet amyloid polypeptide, J. Mol. Biol., 393 (2009) 383-396.
[187] Heitz F., Morris M.C., and Divita G., Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics, Br. J. Pharmacol., 157 (2009) 195-206.
[188] Jenssen H., Hamill P., and Hancock R.E., Peptide antimicrobial agents, Clin. Microbiol. Rev., 19 (2006) 491-511.
[189] Khandelia H., Ipsen J.H., and Mouritsen O.G., The impact of peptides on lipid membranes, Biochim. Biophys. Acta, 1778 (2008) 1528-1536.
[190] Knight J.D., Hebda J.A., and Miranker A.D., Conserved and cooperative assembly of membrane-bound alpha-helical states of islet amyloid polypeptide, Biochemistry, 45 (2006) 9496-9508.
[191] Ananthanarayanan V.S. and Orlicky S., Interaction of substance P and its N- and C-terminal fragments with Ca2+: implications for hormone action, Biopolymers, 32 (1992) 1765-1773.
[192] Brimble K.S. and Ananthanarayanan V.S., Induction of Ca2+ transport in liposomes by insulin, Biochim. Biophys. Acta, 1105 (1992) 319-327.
[193] Sargent D.F. and Schwyzer R., Membrane lipid phase as catalyst for peptide-receptor interactions, Proc. Natl. Acad. Sci. U. S. A, 83 (1986) 5774-5778.

[194] Schwyzer R., 100 years lock-and-key concept: are peptide keys shaped and guided to their receptors by the target cell membrane?, Biopolymers, 37 (1995) 5-16.
[195] Vauquelin G. and Packeu A., Ligands, their receptors and ... plasma membranes, Mol. Cell Endocrinol., 311 (2009) 1-10.
[196] Murray D., Arbuzova A., Honig B., and McLaughlin S., The role of Electrostatic and nonpolar Interactions in the Association of Peripheral Proteins with Membranes, in: Simon S.A. and McIntosh T.J. (Eds.), Peptide-lipid interactions, Academic Press, San Diego, California, (2002) 277-307.
[197] Rhodes D.G., Sarmiento J.G., and Herbette L.G., Kinetics of binding of membrane-active drugs to receptor sites. Diffusion-limited rates for a membrane bilayer approach of 1,4-dihydropyridine calcium channel antagonists to their active site, Mol. Pharmacol., 27 (1985) 612-623.
[198] Herbette L.G., Rhodes D.G., and Mason R.P., New approaches to drug design and delivery based on drug-membrane interactions, Drug Des Deliv., 7 (1991) 75-118.
[199] Lee J.Y., Moon J.S., Eu Y.J., Lee C.W., Yang S.T., Lee S.K., Jung H.H., Kim H.H., Rhim H., Seong J.Y., and Kim J.I., Molecular interaction between kisspeptin decapeptide analogs and a lipid membrane, Arch. Biochem. Biophys., 485 (2009) 109-114.
[200] Schwyzer R., Membrane-assisted molecular mechanism of neurokinin receptor subtype selection, EMBO J., 6 (1987) 2255-2259.
[201] Lerch M., Kamimori H., Folkers G., Aguilar M.I., Beck-Sickinger A.G., and Zerbe O., Strongly altered receptor binding properties in PP and NPY chimeras are accompanied by changes in structure and membrane binding, Biochemistry, 44 (2005) 9255-9264.
[202] Lopes S.C., Fedorov A., and Castanho M.A., Chiral recognition of D-kyotorphin by lipidic membranes: relevance toward improved analgesic efficiency, ChemMedChem., 1 (2006) 723-728.
[203] Lopes S.C., Soares C.M., Baptista A.M., Goormaghtigh E., Cabral B.J., and Castanho M.A., Conformational and orientational guidance of the analgesic dipeptide kyotorphin induced by lipidic membranes: putative correlation toward receptor docking, J. Phys. Chem B, 110 (2006) 3385-3394.
[204] Latif R., Ando T., Daniel S., and Davies T.F., Localization and regulation of thyrotropin receptors within lipid rafts, Endocrinology, 144 (2003) 4725-4728.
[205] Biaggi M.H., Riske K.A., and Lamy-Freund M.T., Melanotropic peptides-lipid bilayer interaction. Comparison of the hormone alpha-MSH to a biologically more potent analog, Biophys. Chem, 67 (1997) 139-149.
[206] Ambroggio E.E., Kim D.H., Separovic F., Barrow C.J., Barnham K.J., Bagatolli L.A., and Fidelio G.D., Surface behavior and lipid interaction of Alzheimer beta-amyloid peptide 1-42: a membrane-disrupting peptide, Biophys. J., 88 (2005) 2706-2713.
[207] Mechler A., Praporski S., Atmuri K., Boland M., Separovic F., and Martin L.L., Specific and selective peptide-membrane interactions revealed using quartz crystal microbalance, Biophys. J., 93 (2007) 3907-3916.
[208] Eeman M., Pegado L., Dufrene Y.F., Paquot M., and Deleu M., Influence of environmental conditions on the interfacial organisation of fengycin, a bioactive lipopeptide produced by Bacillus subtilis, J. Colloid Interface Sci., 329 (2009) 253-264.
[209] Domenech O., Dufrene Y.F., Van B.F., Tukens P.M., and Mingeot-Leclercq M.P., Interactions of oritavancin, a new semi-synthetic lipoglycopeptide, with lipids extracted from Staphylococcus aureus, Biochim. Biophys. Acta, 1798 (2010) 1876-1885.
[210] Alegre-Cebollada J., Martinez del P.A., Gavilanes J.G., and Goormaghtigh E., Infrared spectroscopy study on the conformational changes leading to pore formation of the toxin sticholysin II, Biophys. J., 93 (2007) 3191-3201.
[211] Lins L., Brasseur R., Rosseneu M., Yang C.Y., Sparrow D.A., Sparrow J.T., Gotto A.M., Jr., and Ruysschaert J.M., Structure and orientation of apo B-100 peptides into a lipid bilayer, J. Protein Chem., 13 (1994) 77-88.
[212] Arseneault M., Bedard S., Boulet-Audet M., and Pezolet M., Study of the interaction of lactoferricin B with phospholipid monolayers and bilayers, Langmuir, 26 (2010) 3468-3478.
[213] Castano S. and Desbat B., Structure and orientation study of fusion peptide FP23 of gp41 from HIV-1 alone or inserted into various lipid membrane models (mono-, bi- and multibi-layers) by FT-IR spectroscopies and Brewster angle microscopy, Biochim. Biophys. Acta, 1715 (2005) 81-95.
[214] Brewster angle microscopy principle, Optrel GbR, http://www.optrel.de/pdfs/flyer/brewster.pdf (Last access date: 2010).
[215] Deleu M., Paquot M., and Nylander T., Effect of fengycin, a lipopeptide produced by Bacillus subtilis, on model biomembranes, Biophys. J., 94 (2008) 2667-2679.
[216] Ostergaard J. and Moeller E.H., Ghrelin-liposome interactions: characterization of liposomal formulations of an acylated 28-amino acid peptide using CE, Electrophoresis, 31 (2010) 339-345.

[217] Haunschmidt M., Ortner K., Hainz K., Bradt E., Sternbauer L., Buchberger W., and Klampfl C.W., Investigations on the migration behavior of insulin and related synthetic analogues in CZE, MEKC and MEEKC employing different surfactants, Electrophoresis, 31 (2010) 1560-1564.
[218] Bilek G., Kremser L., Blaas D., and Kenndler E., Analysis of liposomes by capillary electrophoresis and their use as carrier in electrokinetic chromatography, J. Chromatogr. B Analyt Technol. Biomed. Life Sci., 841 (2006) 38-51.
[219] Zhong L. and Johnson W.C., Jr., Environment affects amino acid preference for secondary structure, Proc. Natl. Acad. Sci. U. S. A, 89 (1992) 4462-4465.
[220] Chou H.T., Wen H.W., Kuo T.Y., Lin C.C., and Chen W.J., Interaction of cationic antimicrobial peptides with phospholipid vesicles and their antibacterial activity, Peptides, (2010).
[221] Li H., Li F., Sun H., and Qian Z.M., Membrane-inserted conformation of transmembrane domain 4 of divalent-metal transporter, Biochem. J., 372 (2003) 757-766.
[222] Kashiwada A., Matsuda K., Mizuno T., and Tanaka T., Construction of a pH-responsive artificial membrane fusion system by using designed coiled-coil polypeptides, Chemistry, 14 (2008) 7343-7350.
[223] Stromstedt A.A., Wessman P., Ringstad L., Edwards K., and Malmsten M., Effect of lipid headgroup composition on the interaction between melittin and lipid bilayers, J. Colloid Interface Sci., 311 (2007) 59-69.
[224] Kolusheva S., Shahal T., and Jelinek R., Peptide-membrane interactions studied by a new phospholipid/polydiacetylene colorimetric vesicle assay, Biochemistry, 39 (2000) 15851-15859.
[225] Porat Y., Kolusheva S., Jelinek R., and Gazit E., The human islet amyloid polypeptide forms transient membrane-active prefibrillar assemblies, Biochemistry, 42 (2003) 10971-10977.
[226] Kolusheva S., Lecht S., Derazon Y., Jelinek R., and Lazarovici P., Pardaxin, a fish toxin peptide interaction with a biomimetic phospholipid/polydiacetylene membrane assay, Peptides, 29 (2008) 1620-1625.
[227] Ye G., Gupta A., DeLuca R., Parang K., and Bothun G.D., Bilayer disruption and liposome restructuring by a homologous series of small Arg-rich synthetic peptides, Colloids Surf. B Biointerfaces, 76 (2010) 76-81.
[228] Agirre A., Flach C., Goni F.M., Mendelsohn R., Valpuesta J.M., Wu F., and Nieva J.L., Interactions of the HIV-1 fusion peptide with large unilamellar vesicles and monolayers. A cryo-TEM and spectroscopic study, Biochim. Biophys. Acta, 1467 (2000) 153-164.
[229] Pedersen T.B., Sabra M.C., Frokjaer S., Mouritsen O.G., and Jorgensen K., Association of acylated cationic decapeptides with dipalmitoylphosphatidylserine-dipalmitoylphosphatidylcholine lipid membranes, Chem Phys. Lipids, 113 (2001) 83-95.
[230] Lohner K. and Prenner E.J., Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems, Biochim. Biophys. Acta, 1462 (1999) 141-156.
[231] Wiessner J.H., Mar H., Baskin D.G., and Hwang K.J., Peptide-carrier interaction: induction of liposome fusion and aggregation by insulin, J. Pharm. Sci., 75 (1986) 259-263.
[232] Bordi F., Cametti C., Sennato S., and Diociaiuti M., Direct evidence of multicompartment aggregates in polyelectrolyte-charged liposome complexes, Biophys. J., 91 (2006) 1513-1520.
[233] Rusu L., Gambhir A., McLaughlin S., and Radler J., Fluorescence correlation spectroscopy studies of Peptide and protein binding to phospholipid vesicles, Biophys. J., 87 (2004) 1044-1053.
[234] Kim J., Mosior M., Chung L.A., Wu H., and McLaughlin S., Binding of peptides with basic residues to membranes containing acidic phospholipids, Biophys. J., 60 (1991) 135-148.
[235] Schmidt M.C., Rothen-Rutishauser B., Rist B., Beck-Sickinger A., Wunderli-Allenspach H., Rubas W., Sadee W., and Merkle H.P., Translocation of human calcitonin in respiratory nasal epithelium is associated with self-assembly in lipid membrane, Biochemistry, 37 (1998) 16582-16590.
[236] Peitzsch R.M. and McLaughlin S., Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins, Biochemistry, 32 (1993) 10436-10443.
[237] Cafiso D.S., Peptide-Membrane Interactions Determined Using Site-Directed Spin Labeling, in: Simon S.A. and McIntosh T.J. (Eds.), Peptide-lipid interactions, Academic Press, San Diego, Calif, (2002) 3-29.
[238] Fanucci G.E. and Cafiso D.S., Recent advances and applications of site-directed spin labeling, Curr. Opin. Struct. Biol., 16 (2006) 644-653.
[239] Dietrich U., Kruger P., Gutberlet T., and Kas J.A., Interaction of the MARCKS peptide with PIP2 in phospholipid monolayers, Biochim. Biophys. Acta, 1788 (2009) 1474-1481.
[240] Gidalevitz D., Ishitsuka Y., Muresan A.S., Konovalov O., Waring A.J., Lehrer R.I., and Lee K.Y., Interaction of antimicrobial peptide protegrin with biomembranes, Proc. Natl. Acad. Sci. U. S. A, 100 (2003) 6302-6307.

[241] Smith-Dupont K.B., Guo L., and Gai F., Diffusion as a probe of the heterogeneity of antimicrobial peptide-membrane interactions, Biochemistry, 49 (2010) 4672-4678.
[242] Mateo C.R., Protein-lipid interactions: new approaches and emerging concepts, Springer, Berlin (2006).
[243] Mingeot-Leclercq M.P., Lins L., Bensliman M., Van Bambeke F., Van Der Smissen P., Peuvot J., Schanck A., and Brasseur R., Membrane destabilization induced by beta-amyloid peptide 29-42: importance of the amino-terminus, Chem. Phys. Lipids, 120 (2002) 57-74.
[244] Lorin A., Lins L., Stroobant V., Brasseur R., and Charloteaux B., The minimal fusion peptide of simian immunodeficiency virus corresponds to the 11 first residues of gp32, J. Pept. Sci., 14 (2008) 423-428.
[245] Yandek L.E., Pokorny A., Floren A., Knoelke K., Langel U., and Almeida P.F., Mechanism of the cell-penetrating peptide transportan 10 permeation of lipid bilayers, Biophys. J., 92 (2007) 2434-2444.
[246] Trugnan G., Fontanges P., Delautier D., and it-Slimane T., [FRAP, FLIP, FRET, BRET, FLIM, PRIM...new techniques for a colourful life], Med. Sci. (Paris), 20 (2004) 1027-1034.
[247] Sponne I., Fifre A., Drouet B., Klein C., Koziel V., Pincon-Raymond M., Olivier J.L., Chambaz J., and Pillot T., Apoptotic neuronal cell death induced by the non-fibrillar amyloid-beta peptide proceeds through an early reactive oxygen species-dependent cytoskeleton perturbation, J. Biol. Chem, 278 (2003) 3437-3445.
[248] You M., Li E., Wimley W.C., and Hristova K., Forster resonance energy transfer in liposomes: measurements of transmembrane helix dimerization in the native bilayer environment, Anal. Biochem., 340 (2005) 154-164.
[249] Lorin A., Flore C., Thomas A., and Brasseur R., Les liposomes : description, fabrication et applications, Biotechnol. Agron. Soc. Environ., 8 (2004) 163-176.
[250] Davies-Tuck M., Lee T.H., Apffel A., and Aguilar M.I., Hydrophobic and electrostatic forces control the retention of membrane peptides and proteins with an immobilised phosphatidic acid column, J. Chromatogr. A, 1156 (2007) 167-173.
[251] Lee T.H., Mozsolits H., and Aguilar M.I., Measurement of the affinity of melittin for zwitterionic and anionic membranes using immobilized lipid biosensors, J. Pept. Res., 58 (2001) 464-476.
[252] Powers J.P., Rozek A., and Hancock R.E., Structure-activity relationships for the beta-hairpin cationic antimicrobial peptide polyphemusin I, Biochim. Biophys. Acta, 1698 (2004) 239-250.
[253] Kim D.H. and Frangos J.A., Effects of amyloid beta-peptides on the lysis tension of lipid bilayer vesicles containing oxysterols, Biophys. J., 95 (2008) 620-628.
[254] Sun Y., Hung W.C., Chen F.Y., Lee C.C., and Huang H.W., Interaction of tea catechin (-)-epigallocatechin gallate with lipid bilayers, Biophys. J., 96 (2009) 1026-1035.
[255] Deleu M., Wathelet B., Brasseur R., and Paquot M., Aperçu des techniques d'analyse conformationnelle des macromolécules biologiques, Biotechnol. Agron. Soc. Environ., 2 (1998) 234-247.
[256] Penfold J., Richardson R.M., Zarbakhsh A., Webster J.R.P., Bucknall D.G., Rennie A.R., Jones R.A.L., Cosgrove T., Thomas R.K., Higgins J.S., Fletcher P.D.I., Dickinson E., Roser S.J., Mclure I.A., Hillman A.R., Richards R.W., Staples E.J., Burgess A.N., Simister E.A., and White J.W., Recent advances in the study of chemical surfaces and interfaces by specular neutron reflection, J. Chem. Soc. - Faraday Trans., 93 (1997) 3899-3917.
[257] Grage S.L., Wang J., Cross T.A., and Ulrich A.S., Solid-state 19F-NMR analysis of 19F-labeled tryptophan in gramicidin A in oriented membranes, Biophys. J., 83 (2002) 3336-3350.
[258] Gawrisch K. and Koenig B.W., Lipid-Peptide Interaction Investigated by NMR, in: Simon S.A. and McIntosh T.J. (Eds.), Peptide-lipid interactions, Academic Press, San Diego, Calif, (2002) 163-190.
[259] Gehman J.D., Luc F., Hall K., Lee T.H., Boland M.P., Pukala T.L., Bowie J.H., Aguilar M.I., and Separovic F., Effect of antimicrobial peptides from Australian tree frogs on anionic phospholipid membranes, Biochemistry, 47 (2008) 8557-8565.
[260] Ano R., Kimura Y., Shima M., Matsuno R., Ueno T., and Akamatsu M., Relationships between structure and high-throughput screening permeability of peptide derivatives and related compounds with artificial membranes: application to prediction of Caco-2 cell permeability, Bioorg. Med. Chem., 12 (2004) 257-264.
[261] Wall J., Golding C.A., Van V.M., and O'Shea P., The use of fluoresceinphosphatidylethanolamine (FPE) as a real-time probe for peptide-membrane interactions, Mol. Membr. Biol., 12 (1995) 183-192.
[262] Matos P.M., Goncalves S., and Santos N.C., Interaction of peptides with biomembranes assessed by potential-sensitive fluorescent probes, J. Pept. Sci., 14 (2008) 407-415.
[263] Przybylo M., Borowik T., and Langner M., Fluorescence Techniques for Determination of the Membrane Potentials in High Throughput Screening, J. Fluoresc., (2010).
[264] Nielsen S.B. and Otzen D.E., Impact of the antimicrobial peptide Novicidin on membrane structure and integrity, J. Colloid Interface Sci., (2010).

[265] Kotarek J.A. and Moss M.A., Impact of phospholipid bilayer saturation on amyloid-beta protein aggregation intermediate growth: a quartz crystal microbalance analysis, Anal. Biochem., 399 (2010) 30-38.
[266] Besenicar M., Macek P., Lakey J.H., and Anderluh G., Surface plasmon resonance in protein-membrane interactions, Chem Phys. Lipids, 141 (2006) 169-178.
[267] Maget-Dana R., The monolayer technique: a potent tool for studying the interfacial properties of antimicrobial and membrane-lytic peptides and their interactions with lipid membranes, Biochim. Biophys. Acta, 1462 (1999) 109-140.
[268] Eeman M., Berquand A., Dufrene Y.F., Paquot M., Dufour S., and Deleu M., Penetration of surfactin into phospholipid monolayers: nanoscale interfacial organization, Langmuir, 22 (2006) 11337-11345.
[269] Buser C.A. and McLaughlin S., Ultracentrifugation technique for measuring the binding of peptides and proteins to sucrose-loaded phospholipid vesicles, Methods Mol. Biol., 84 (1998) 267-281.
[270] Frkanec R., Travas D., Krstanovic M., Spoljar B.H., Ljevakovic D., Vranesic B., Frkanec L., and Tomasic J., Entrapment of peptidoglycans and adamantyltripeptides into liposomes: an HPLC assay for determination of encapsulation efficiency, J. Liposome Res., 13 (2003) 279-294.
[271] Gobbi M., Re F., Canovi M., Beeg M., Gregori M., Sesana S., Sonnino S., Brogioli D., Musicanti C., Gasco P., Salmona M., and Masserini M.E., Lipid-based nanoparticles with high binding affinity for amyloid-beta1-42 peptide, Biomaterials, 31 (2010) 6519-6529.
[272] Matsubara M., Titani K., and Taniguchi H., Interaction of calmodulin-binding domain peptides of nitric oxide synthase with membrane phospholipids: regulation by protein phosphorylation and Ca(2+)-calmodulin, Biochemistry, 35 (1996) 14651-14658.
[273] Terzi E., Holzemann G., and Seelig J., Self-association of beta-amyloid peptide (1-40) in solution and binding to lipid membranes, J. Mol. Biol., 252 (1995) 633-642.
[274] Taniguchi H. and Manenti S., Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids, J. Biol. Chem, 268 (1993) 9960-9963.
[275] Neville F., Ivankin A., Konovalov O., and Gidalevitz D., A comparative study on the interactions of SMAP-29 with lipid monolayers, Biochim. Biophys. Acta, 1798 (2010) 851-860.
[276] Yang X.J. and Seto E., Lysine acetylation: codified crosstalk with other posttranslational modifications, Mol. Cell, 31 (2008) 449-461.
[277] Levental I., Grzybek M., and Simons K., Greasing their way: lipid modifications determine protein association with membrane rafts, Biochemistry, 49 (2010) 6305-6316.
[278] Resh M.D., Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, Biochim. Biophys. Acta, 1451 (1999) 1-16.
[279] Senin I.I., Churumova V.A., Philippov P.P., and Koch K.W., Membrane binding of the neuronal calcium sensor recoverin - modulatory role of the charged carboxy-terminus, BMC. Biochem., 8 (2007) 24.
[280] McLaughlin S. and Aderem A., The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions, Trends Biochem. Sci., 20 (1995) 272-276.
[281] Radzishevsky I.S., Rotem S., Zaknoon F., Gaidukov L., Dagan A., and Mor A., Effects of acyl versus aminoacyl conjugation on the properties of antimicrobial peptides, Antimicrob. Agents Chemother., 49 (2005) 2412-2420.
[282] Hashimoto M., Takada K., Kiso Y., and Muranishi S., Synthesis of palmitoyl derivatives of insulin and their biological activities, Pharm. Res., 6 (1989) 171-176.
[283] Knudsen L.B., Nielsen P.F., Huusfeldt P.O., Johansen N.L., Madsen K., Pedersen F.Z., Thogersen H., Wilken M., and Agerso H., Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration, J. Med. Chem, 43 (2000) 1664-1669.
[284] Uchiyama T., Kotani A., Tatsumi H., Kishida T., Okamoto A., Okada N., Murakami M., Fujita T., Fujiwara Y., Kiso Y., Muranishi S., and Yamamoto A., Development of novel lipophilic derivatives of DADLE (leucine enkephalin analogue): intestinal permeability characateristics of DADLE derivatives in rats, Pharm. Res., 17 (2000) 1461-1467.
[285] Sankaram M.B., Membrane interaction of small N-myristoylated peptides: implications for membrane anchoring and protein-protein association, Biophys. J., 67 (1994) 105-112.
[286] Shalev D.E., Rotem S., Fish A., and Mor A., Consequences of N-acylation on structure and membrane binding properties of dermaseptin derivative K4-S4-(1-13), J. Biol. Chem, 281 (2006) 9432-9438.
[287] Wang J., Wu D., and Shen W.C., Structure-activity relationship of reversibly lipidized peptides: studies of fatty acid-desmopressin conjugates, Pharm. Res., 19 (2002) 609-614.
[288] Tanaka K., Fujita T., Yamamoto Y., Murakami M., Yamamoto A., and Muranishi S., Enhancement of intestinal transport of thyrotropin-releasing hormone via a carrier-mediated transport system by chemical modification with lauric acid, Biochim. Biophys. Acta, 1283 (1996) 119-126.

[289] Cheng W. and Lim L.Y., Comparison of reversible and nonreversible aqueous-soluble lipidized conjugates of salmon calcitonin, Mol. Pharm., 5 (2008) 610-621.
[290] Greathouse D., Vostrikov V., McClellan N., Chipollini J., Lay J., Liyanage R., and Ladd T., Lipid interactions of acylated tryptophan-methylated lactoferricin peptides by solid-state NMR, J. Pept. Sci., 14 (2008) 1103-1110.
[291] Avrahami D. and Shai Y., Conjugation of a magainin analogue with lipophilic acids controls hydrophobicity, solution assembly, and cell selectivity, Biochemistry, 41 (2002) 2254-2263.
[292] Wang J., Shen D., and Shen W.C., Preparation, purification, and characterization of a reversibly lipidized desmopressin with potentiated anti-diuretic activity, Pharm. Res., 16 (1999) 1674-1679.
[293] Yelamos B., Roncal F., Albar J.P., Rodriguez-Crespo I., and Gavilanes F., Influence of acylation of a Peptide corresponding to the amino-terminal region of endothelial nitric oxide synthase on the interaction with model membranes, Biochemistry, 45 (2006) 1263-1270.
[294] Kurtzhals P., Havelund S., Jonassen I., Kiehr B., Larsen U.D., Ribel U., and Markussen J., Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo, Biochem. J., 312 (Pt 3) (1995) 725-731.
[295] Raslova K., An update on the treatment of type 1 and type 2 diabetes mellitus: focus on insulin detemir, a long-acting human insulin analog, Vasc. Health Risk Manag., 6 (2010) 399-410.
[296] Herrera-Ruiz D. and Knipp G.T., Current perspectives on established and putative mammalian oligopeptide transporters, J. Pharm. Sci., 92 (2003) 691-714.
[297] des Rieux A., Fievez V., Momtaz M., Detrembleur C., Alonso-Sande M., Van Gelder J., Cauvin A., Schneider Y.J., and Préat V., Helodermin-loaded nanoparticles: characterization and transport across an in vitro model of the follicle-associated epithelium, J. Control. Release, 118 (2007) 294-302.
[298] Fricker G. and Drewe J., Current concepts in intestinal peptide absorption, J. Pept. Sci., 2 (1996) 195-211.
[299] Zhou S.F., Wang L.L., Di Y.M., Xue C.C., Duan W., Li C.G., and Li Y., Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development, Curr. Med. Chem, 15 (2008) 1981-2039.
[300] Oswald S., Grube M., Siegmund W., and Kroemer H.K., Transporter-mediated uptake into cellular compartments, Xenobiotica, 37 (2007) 1171-1195.
[301] Wang J., Hogenkamp D.J., Tran M., Li W.Y., Yoshimura R.F., Johnstone T.B., Shen W.C., and Gee K.W., Reversible lipidization for the oral delivery of leu-enkephalin, J. Drug Target, 14 (2006) 127-136.
[302] Yuan L., Wang J., and Shen W.C., Reversible lipidization of somatostatin analogues for the liver targeting, Eur. J. Pharm. Biopharm., 70 (2008) 615-620.
[303] DiNitto J.P., Cronin T.C., and Lambright D.G., Membrane recognition and targeting by lipid-binding domains, Sci. STKE., 2003 (2003) re16.
[304] Zhang W., Crocker E., McLaughlin S., and Smith S.O., Binding of peptides with basic and aromatic residues to bilayer membranes: phenylalanine in the myristoylated alanine-rich C kinase substrate effector domain penetrates into the hydrophobic core of the bilayer, J. Biol. Chem., 278 (2003) 21459-21466.
[305] Fujita T., Kawahara I., Quan Y., Hattori K., Takenaka K., Muranishi S., and Yamamoto A., Permeability characteristics of tetragastrins across intestinal membranes using the Caco-2 monolayer system: comparison between acylation and application of protease inhibitors, Pharm. Res., 15 (1998) 1387-1392.
[306] Yamamoto A., [Improvement of intestinal absorption of peptide and protein drugs by chemical modification with fatty acids], Nippon Rinsho, 56 (1998) 601-607.
[307] Chopineau J., Robert S., Fenart L., Cecchelli R., Lagoutte B., Paitier S., Dehouck M.P., and Domurado D., Monoacylation of ribonuclease A enables its transport across an in vitro model of the blood-brain barrier, J. Control. Release, 56 (1998) 231-237.
[308] Gallina A. and Milanesi G., Trans-membrane translocation of a myristylated protein amino terminus, Biochem. Biophys. Res. Commun., 195 (1993) 637-642.
[309] Harishchandran A. and Nagaraj R., Interaction of a pseudosubstrate peptide of protein kinase C and its myristoylated form with lipid vesicles: only the myristoylated form translocates into the lipid bilayer, Biochim. Biophys. Acta, 1713 (2005) 73-82.
[310] Takada R., Satomi Y., Kurata T., Ueno N., Norioka S., Kondoh H., Takao T., and Takada S., Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion, Dev. Cell, 11 (2006) 791-801.

[311] Yodoya E., Uemura K., Tenma T., Fujita T., Murakami M., Yamamoto A., and Muranishi S., Enhanced permeability of tetragastrin across the rat intestinal membrane and its reduced degradation by acylation with various fatty acids, J. Pharmacol. Exp. Ther., 271 (1994) 1509-1513.
[312] Asada H., Douen T., Waki M., Adachi S., Fujita T., Yamamoto A., and Muranishi S., Absorption characteristics of chemically modified-insulin derivatives with various fatty acids in the small and large intestine, J. Pharm. Sci., 84 (1995) 682-687.
[313] Chekhonin V.P., Ryabukhin I.A., Zhirkov Y.A., Kashparov I.A., and Dmitriyeva T.B., Transport of hydrophobized fragments of antibodies through the blood-brain barrier, Neuroreport, 7 (1995) 129-132.
[314] Banks W.A., Tschop M., Robinson S.M., and Heiman M.L., Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure, J. Pharmacol. Exp. Ther., 302 (2002) 822-827.
[315] Pan W., Tu H., and Kastin A.J., Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin, Peptides, 27 (2006) 911-916.

Chapter II.

AIMS OF THE THESIS

AIMS OF THE THESIS

Membrane lipids and lipidation of peptides have both been shown to modulate the activity of peptides. Moreover, ghrelin *O*-octanoylation is necessary to ensure an efficient binding and activation of the ghrelin receptor but the unacylated form also seems to play physiological roles. However, little was known about the role of the octanoyl moiety in ghrelin membrane binding and transport. The objectives of this thesis were thus to determine the importance of this unique post-translational modification, as well as of its basic residues (i) in the extent of ghrelin binding to membranes; (ii) on the mechanisms that drive acylated and unacylated ghrelin to the membrane and hence to the ghrelin receptor; (iii) on ghrelin transmembrane and transepithelial transport.

The preliminary step to ghrelin-membrane interactions studies was to find an affordable and reproducible method that allowed the quantification and differentiation of both forms of ghrelin i.e. acylated and unacylated ghrelin. As ghrelin is devoid of fluorescent amino acids, that radioactive labelling was neither very stable nor reproducible and that immunoassays were much too expensive, we optimized and validated a RP-HPLC/UV method to separate and quantify acylated and unacylated ghrelin in aqueous solutions.

After showing that the octanoyl significantly enhanced the binding of ghrelin to model (liposomes) and biological (erythrocytes) membranes by (ultra)centrifugation studies, we investigated the underlying mechanisms driving ghrelin to the membrane. The molecular steps than can accompany peptide-lipid interactions are: (i) electrostatic attraction / membrane adsorption of the peptide; (ii) peptide penetration into the headgroup or hydrophobic core region; (iii) peptide conformational change; (iv) lipid reorganization. We investigated the occurrence of each of these steps as it depends on the lipid type and the peptide.
Firstly, the electrostatic interactions were studied by binding and zeta potential studies with liposomes of various compositions and at different pH and ionic strengths.
Next, we considered the hydrophobic interactions, including the effect of the membrane fluidity. The hydrophobic penetration coefficient of acylated ghrelin was determined by means of a mathematical binding model as quantitative analysis was not straightforward due to the electrostatic interactions. We also established the thermodynamics of ghrelin binding to

membranes on the basis of this binding model together with isothermal titration calorimetry experiments.

Then, the ghrelin conformation in aqueous solutions as well as in hydrophobic environments and membrane mimicking models was investigated by circular dichroism, ATR-FTIR and molecular modelization.

Finally, the influence on the lipid organization was assessed by, on the one hand, the effect of ghrelin on the membrane fluidity by fluorescence polarization studies and, on the other hand, the effect on lipids orientation by ATR-FTIR.

Based on the ghrelin-membrane interactions results, we discussed the potential "catalytic" role of the membrane in the interactions of ghrelin with the ghrelin receptor.

Besides, we also looked at the influence of the octanoyl group on ghrelin translocation by PAMPA and on ghrelin transport through model epithelia.

The conclusions on the role of the *O*-octanoylation on peptide-membrane interactions are discussed with a critical approach regarding to our experimental results and the existing literature. The future directions are also given.

Chapter III.

VALIDATION OF A METHOD FOR THE QUANTITATION OF GHRELIN AND UNACYLATED GHRELIN BY HPLC

VALIDATION OF A METHOD FOR THE QUANTITATION OF GHRELIN AND UNACYLATED GHRELIN BY HPLC

Adapted from Staes E., Rozet E., Ucakar B., Hubert Ph., Préat V.
Journal of Pharmaceutical and Biomedical Analysis, 51: 633-639 (2010)

ABSTRACT

An HPLC/UV method was first optimized for the separation and quantitation of human acylated and unacylated (or des-acyl) ghrelin from aqueous solutions. This method was validated by an original approach using accuracy profiles based on tolerance intervals for the total error measurement. The concentration range that achieved adequate accuracy extended from 1.85 to 59.30 μM and 1.93 to 61.60 μM for acylated and unacylated ghrelin, respectively. Then, optimal temperature, pH and buffer for sample storage were determined. Unacylated ghrelin was found to be stable in all conditions tested. At 37 °C acylated ghrelin was stable at pH 4 but unstable at pH 7.4, the main degradation product was unacylated ghrelin. Finally, this validated HPLC/UV method was used to evaluate the binding of acylated and unacylated ghrelin to liposomes.

TABLE OF CONTENTS

1. INTRODUCTION 83

2. EXPERIMENTAL PROCEDURES 85

2.1. CHEMICALS 85

2.2. QUANTITATION OF GHRELIN BY RP-HPLC 85

2.3. VALIDATION OF THE HPLC/UV METHOD 86

2.4. PREPARATION OF STABILITY SAMPLES 86

2.5. GHRELIN BINDING TO LIPOSOMES 86

3. RESULTS AND DISCUSSION 88

3.1. HPLC METHOD 88

3.2. VALIDATION OF THE HPLC METHOD 88

3.2.1. SELECTIVITY 89

3.2.2. TRUENESS AND PRECISION 89

3.2.3. ACCURACY, LIMITS OF QUANTITATION AND RANGE 91

3.2.4. RESULTS LINEARITY 92

3.3. STABILITY OF GHRELIN IN AQUEOUS SOLUTIONS 93

3.4. GHRELIN BINDING TO LIPOSOMES 94

4. CONCLUSIONS 96

5. REFERENCES 97

1. INTRODUCTION

Peptides offer a great therapeutic interest. Nevertheless, their administration is often limited not only due to their high molecular weight, but also owing to their hydrophilicity and their metabolic instability [1]. In 1999, Kojima et al. [2] isolated and characterized an unusual endogenous peptide of 28 amino acids, which was modified by a *n*-octanoic acid on the hydroxyl group of its serine 3 residue and called it ghrelin. The physiological roles of ghrelin are numerous, among which growth hormone (GH) secretion, stimulation of food intake and gastrointestinal motility, glucose and energy homeostasis, cardiovascular protective effects, cell proliferation and differentiation as well as immune regulation [3]. Ghrelin is mainly produced by the stomach in endocrine X/A-like cells and by the pancreas. Another naturally occurring variant of ghrelin is unacylated ghrelin also called des-acyl ghrelin. This form is the most abundant in plasma [3]. The acylation of ghrelin is required for the binding and the activation of the GHS-R1a that mediates most of its endocrinological activities [2]. However, some other biological activities have been attributed to the binding of acylated and/or unacylated ghrelin to a yet unidentified receptor [4]. Acylated and unacylated ghrelin present similar and opposite actions (see [3,4] for review). As both these peptides offer a great therapeutic potential, their physiological and pathological roles have been widely investigated. Since ghrelin is a pleiotropic hormone, it has several potential clinical applications, among which the diagnostic of GH deficiency, but also the treatment of hypomotility syndrome, cachexia-anorexia syndrome and aging [3,5]. If we want to formulate ghrelin as a drug, the stability of the formulation is essential. Indeed, the octanoyl-serine ester bond is both chemically and enzymatically unstable. Therefore, deoctanoylation is likely to occur during manufacturing, storage, handling and *in vitro* testing. Ghrelin storage stability has been assessed but only in blood samples [6]. As ghrelin is degraded by esterases [7,8], it has been recommended to collect blood samples in EDTA-aprotinin tubes and then to acidify samples to a final pH of 3-4 to avoid deacylation of ghrelin [6]. Even though the ester bond is meant to be more stable in solution devoid of enzymes, it is important to know whether ghrelin stability is affected in water or in commonly used buffers for *in vitro* tests or parenteral formulations. Selection of optimal conditions, including temperature, pH and buffer is therefore required.

Acylated and unacylated human ghrelin are generally quantitated by immunological methods such as RIA [6,9], ELISA [10,11], EIA [12,13]. An HPLC/MS/MS method has also been

developed [13]. However, all these methods are expensive, time consuming and quantitate samples in the pico- or nanomolar range. Therefore, dosing micromolar samples with these techniques require high dilutions and are thus subject to errors. Even though RP-HPLC has been widely used in purification processes [14-16] to separate acylated from unacylated human ghrelin, UV was sometimes used as a detection method [2,16] but RIA was typically used as the quantification method [17,18]. Here we provide the first validated RP-HPLC/UV method to separate and reliably quantify both forms of human ghrelin in the micromolar range.

The objectives of this paper were first to adapt the described HPLC/UV method for rat ghrelin [2,19,20] in order to separate and quantify human acylated (hAG) and unacylated (hUAG) ghrelin in the micromolar range adapted to ghrelin injectable formulations and *in vitro* tests [21,22]. The stability and storage conditions of the acylated and unacylated ghrelin in media that are commonly used for formulations or *in vitro* studies were also evaluated. Secondly, a thorough validation of this HPLC/UV method was performed to provide guarantees about the accuracy and thus the reliability of the results. Finally, this validated HPLC/UV method was used to quantify the ghrelin binding to liposomes to illustrate its applicability.

2. EXPERIMENTAL PROCEDURES

2.1. CHEMICALS

Synthetic human acylated ghrelin (hAG) and human unacylated ghrelin (hUAG) were purchased from NeoMPS (Strasbourg, France). Peptide HPLC purity was > 97%, according to the manufacturer.

Acetonitrile, HPLC grade for far UV, was purchased from Acros Organics (Thermo Fisher Scientific Inc., Waltham, MA, USA). Trifluoroacetic acid (TFA), HPLC grade, was obtained from Fisher Scientific (Thermo Fisher Scientific Inc.). Hanks' balanced salt solution (HBSS) and phosphate-buffered saline pH 7.4 (PBS) were purchased from Invitrogen (Paisley, United Kingdom). Bovine brain sphingomyelin (SM) and cholesterol (Chol) were obtained from Sigma-Aldrich (St. Louis, MO, USA), whereas phosphatidylcholine (PC) (Grade 1, from egg yolk), phosphatidylinositol (PI) (Grade 1) and phosphatidylethanolamine (PE) (Grade 1) were obtained from Lipid Products (Redhill Surrey, United Kingdom). All other reagents were of ACS or analytical grade.

2.2. QUANTITATION OF GHRELIN BY RP-HPLC

The concentrations of human acylated and unacylated ghrelin were determined by an HPLC method adapted from Date et al. [20]. The HPLC Waters system (Waters Associates Inc., Milford, MA) consisted of a refrigerated autosampler (717plus), a binary pump (1525), a column heater and a dual wavelength absorbance UV detector (2487). 50 µl of samples were injected in a TSK-gel ODS 120 column (150 mm x 4.6 mm I.D.; 5 µm particle size: Tosoh Bioscience, Stuttgart, Germany) protected by a Hypersil Gold guard column (10 mm x 4 mm I.D: 5 µm particle size: Thermo Fisher Scientific), both thermostatised at 37 °C. The mobile phase A was water with 0.1% (v/v) trifluoroacetic acid, and B was acetonitrile with 0.1% trifluoroacetic acid. The samples were eluted with a linear gradient of A and B, from 12% B (0 min) to 52% B (32 min). The flow rate was 1 ml/min. The acylated and unacylated ghrelin UV absorbances were recorded at 210 nm.

2.3. VALIDATION OF THE HPLC/UV METHOD

In each experiment the stocks of acylated and unacylated ghrelin were reconstituted at 1 mg/ml with water or with 12.5 mM sodium citrate 14 mM NaCl pH 6 from pre-weighted 100 µg or 1 mg lyophilized peptide (NeoMPS). A calibration curve was established for each analyte from the reconstituted stock by twofold serial dilutions (7 dilutions). The concentrations of the calibration curve ranged from 0.93 to 118.67 µM and 0.96 to 123.28 µM for human acylated and unacylated ghrelin, respectively. The validation standards, prepared from similarly reconstituted stock solutions, were set at three concentrations ranging from 1.85 to 59.30 µM and from 1.93 to 61.60 µM, for human acylated and unacylated ghrelin, respectively. The number of replicates per concentration level and the number of runs were all ≥ 3. Enoval software v2.0 (Arlenda, Liège, Belgium) was used to compute accuracy profiles and validation results.

2.4. PREPARATION OF STABILITY SAMPLES

Lyophilized human acylated and human unacylated ghrelin were reconstituted in water or in buffer at a final concentration of 200 µg/ml. Buffers tested were: (i) 12.5 mM sodium citrate 14 mM NaCl pH 6 (I_c = 0.06 M); (ii) 12.5 mM sodium citrate 46.2 mM NaCl pH 4 (I_c = 0.06 M); (iii) HBSS pH 6; (iv) HBSS pH 7.4 and (v) PBS pH 7.4. Peptide stability was evaluated at 37 °C for 4 h in all solutions. Stability of the peptides was also assessed at 4 °C in PBS pH 7.4 and in 12.5 mM sodium citrate 14 mM NaCl pH 6. 200 µg/ml stock solutions of each buffer were used as controls. Controls were immediately frozen and stored at -80 °C upon preparation whereas samples were incubated prior to freezing and storage in the same conditions. Samples and controls were thawed at room temperature 5 to 10 min before injection into the HPLC system.

2.5. GHRELIN BINDING TO LIPOSOMES

The binding of human acylated and unacylated ghrelin to large unilamellar liposomes made of Chol/PC/SM/PI/PE (5.5:4.0:1.7:3.0:2.3, M/M) in 12.5 mM sodium citrate 14 mM NaCl pH 6 buffer was assessed. These liposomes were prepared by extrusion under nitrogen through two 100 nm pore superposed Nucleopore® Track-Etched polycarbonate membranes (Whatman, Maidstone, UK) using a Thermobarrel extruder (Lipex Biomembranes Inc., Vancouver,

Canada), as previously described [23]. The phospholipids concentration was determined by phosphorus assay [24] and the total lipid concentration was calculated assuming similar recovery of phospholipids and cholesterol. Liposomes were stored under nitrogen at 4 °C and used within 3 days. The *z*-average diameter of these liposomes was 107 ± 5 nm as measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). The ghrelin binding to liposomes was assessed at lipid/peptide molar ratios from 13:1 to 40:1 by ultracentrifugation. The liposomes/peptide (either acylated or unacylated ghrelin at 100 µg/ml final concentration) mixture (160 µl) was incubated for 3 h at 37 °C and then ultracentrifuged at 4 °C (250,000 × *g*) for 1 h. In controls, liposomes were replaced by the equivalent volume of buffer. The supernatants that contained the free peptide were then collected for HPLC/UV quantitation. The binding percentage was calculated as follows: ((control concentration - supernatant concentration) / control concentration) × 100.

3. RESULTS AND DISCUSSION

3.1. HPLC METHOD

As shown in **Fig. 1** the HPLC method developed allowed the separation of acylated and unacylated ghrelin. Retention times were 11.4 min and 21.0 min for unacylated and acylated ghrelin, respectively.

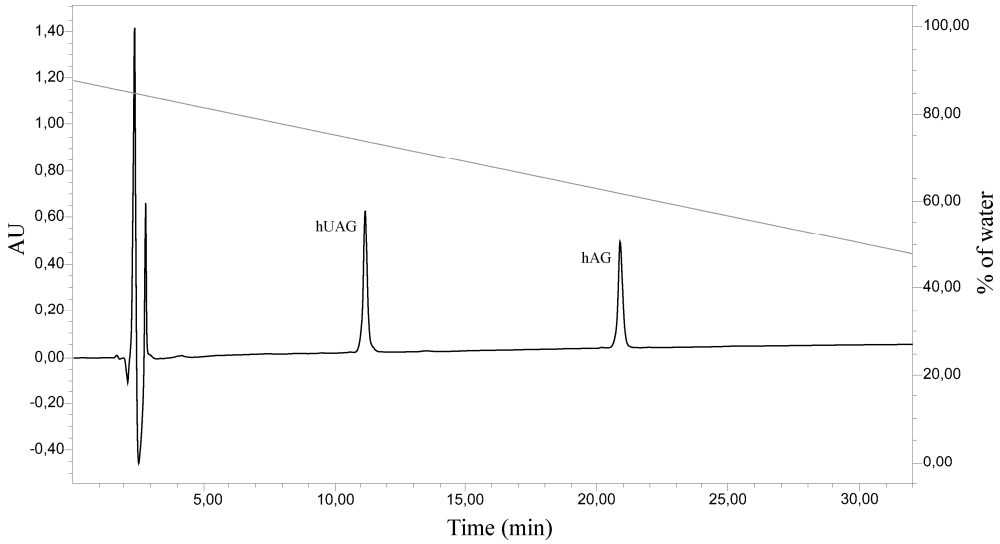


Figure 1 - HPLC chromatogram of 33.9 µM of human acylated ghrelin (hAG) and 35.5 µM of human unacylated ghrelin (hUAG) in 12.5 mM sodium citrate 14 mM NaCl pH 6 detected at 210 nm.

3.2. VALIDATION OF THE HPLC METHOD

An original approach using accuracy profiles based on tolerance intervals for the total error measurement, including both bias and standard deviation for intermediate precision, was applied to demonstrate the method validity [25-27]. It allows to evaluate the calibration curve, trueness, precision, accuracy, linearity, dosing range, limits of quantitation and of detection [28,29]. The method is declared as valid within the range for which the accuracy profile computed by means of β-expectation tolerance intervals is fully included inside the accuracy acceptance limits set at ± 30% [30]. The β-expectation tolerance interval that describes a region where, on average, a proportion β of future measurements will fall, was fixed at 80% [31]. In this way, the guarantee that each further measurement of unknown samples will be included within the acceptance limits is fixed.

3.2.1. Selectivity

The selectivity of the developed method is acceptable since no interfering peak was observed at the retention times of the well separated acylated and unacylated ghrelin for all the buffers tested (**Figs. 1 and 2**).

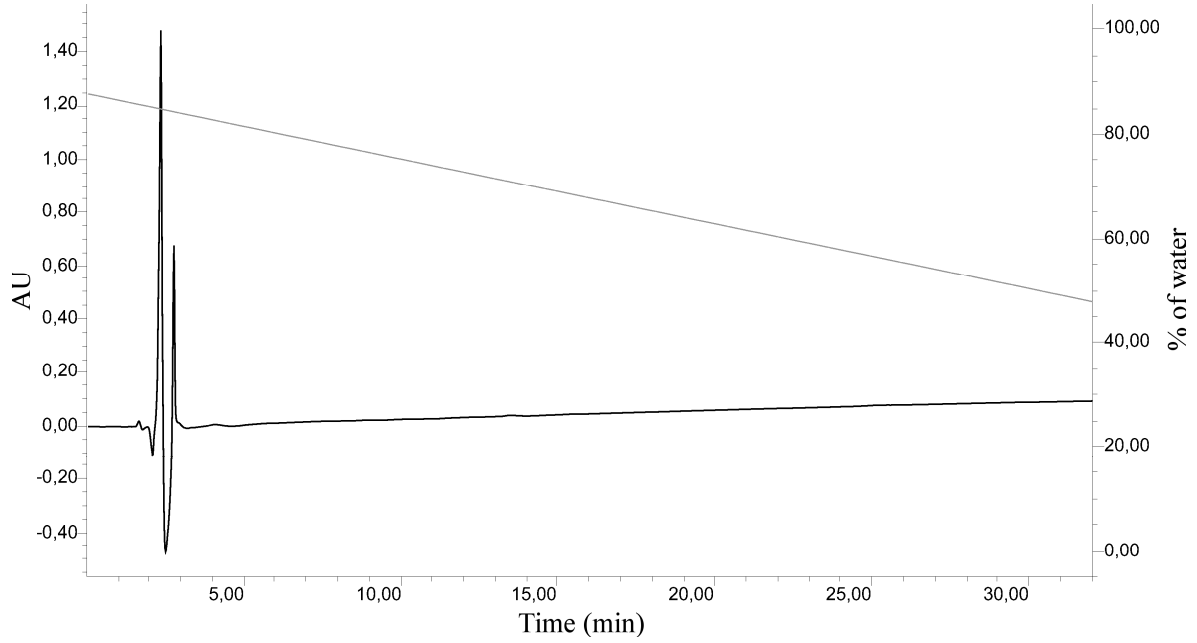


Figure 2 - HPLC chromatogram of 12.5 mM sodium citrate 14 mM NaCl pH 6 detected at 210 nm.

3.2.2. Trueness and precision

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [28,29,32]. It gives information on systematic error. **Tables 1 and 2** report trueness expressed as absolute bias and relative bias for the different level of validation standards for both analytes. The maximum relative biases were 7% and 4% for human acylated and unacylated ghrelin, respectively.

Precision is the closeness of agreement among measurements from multiple sampling of a homogeneous sample under the recommended conditions [28,29,32]. It gives information on random errors and it can be evaluated at two levels: repeatability and intermediate precision. Results are presented in **Tables 1 and 2**. Relative standard deviation for repeatability and intermediate precision seemed relatively high, with maximum values of about 14% and 6% for acylated and unacylated ghrelin, respectively.

Table 1 - Validation results for the human acylated ghrelin using a weighted (1/X) linear regression as calibration curve.

Trueness		
True Concentration (µM)	Bias (µM)	Relative bias (%)
1.85	0.12	6.7
7.42	-0.42	-5.6
59.30	1.69	2.9
Precision		
True Concentration (µM)	Repeatability (RSD %)	Intermediate precision (RSD %)
1.85	9.9	14.3
7.42	5.2	13.9
59.30	2.6	6.1
Accuracy		
True Concentration (µM)	Lower relative tolerance interval (%)	Upper relative tolerance interval (%)
1.85	-14.6	28.0
7.42	-29.3	18.0
59.30	-5.4	11.1

Table 2 - Validation results for the human unacylated ghrelin (hUAG) using a weighted (1/X) linear regression as calibration curve.

Trueness		
True Concentration (µM)	Bias (µM)	Relative bias (%)
1.93	0.02	1.2
7.71	0.30	3.9
61.60	0.98	1.6
Precision		
True Concentration (µM)	Repeatability (RSD %)	Intermediate precision (RSD %)
1.93	4.0	5.9
7.71	4.3	4.6
61.60	2.7	4.1
Accuracy		
True Concentration (µM)	Lower relative tolerance interval (%)	Upper relative tolerance interval (%)
1.93	-8.3	10.6
7.71	-2.4	10.2
61.60	-3.9	7.1

3.2.3. Accuracy, limits of quantitation and range

Accuracy refers to the closeness of agreement between the test result and the accepted reference value, namely the conventionally true value [28,29,32]. The accuracy takes into account the total error, i.e. systematic and random errors, related to the test result (**Tables 1 and 2**). It is assessed from the accuracy profile illustrated in **Figs. 3A and 3B** for acylated and unacylated ghrelin, respectively. The method is considered as valid in the range for which the accuracy profile is within the accuracy acceptance limits set at ± 30%. This approach gives the guarantee that each further measurement of unknown samples will be included within the tolerance limits with a probability of 80%.

The lower limit of quantitation (LOQ) is the smallest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy [28,29,32]. The definition can also be applicable to the upper LOQ, which is the highest quantity of the targeted substance in the sample that can be assayed under experimental conditions with well defined accuracy. The limits of quantitation are obtained by calculating the smallest and highest concentration beyond which the accuracy limits or β-expectation limits step outside the acceptance limits. Experimentally, the lower LOQs were evaluated at 1.85 and 1.93 µM for acylated and unacylated ghrelin, respectively and the upper LOQs at 59.30 and 61.60 µM for acylated and unacylated ghrelin, respectively. The concentration range extends then to the interval between the lower and upper limits where the procedure achieves adequate accuracy.

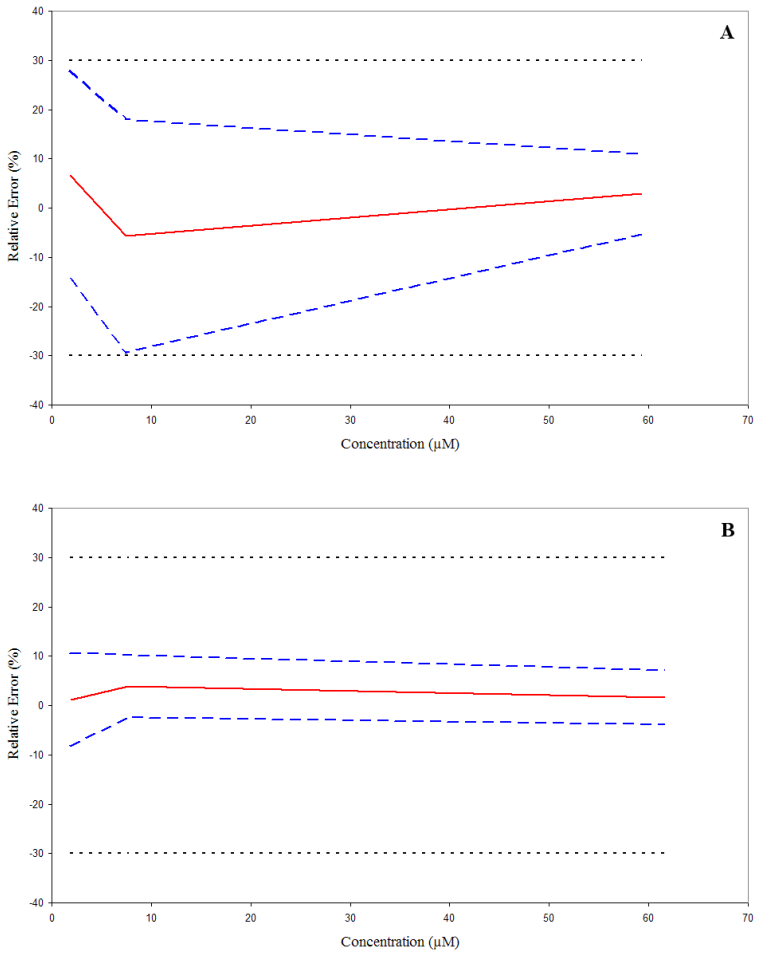


Figure 3 - Accuracy profiles obtained for the validation of the HPLC/UV analytical method for the quantitation of human acylated ghrelin (A) and human unacylated ghrelin (B) by considering a weighted (1/X) linear regression as calibration curves; plain line: relative bias, dashed lines: β-expectation tolerance limits (β=80%), dotted lines: ± 30% acceptance limit.

3.2.4. Results linearity

In order to demonstrate results linearity [28,29], a regression line was fitted on the calculated concentrations of the validation standards as a function of the introduced concentrations by applying a linear regression model. The equations obtained for each ghrelin with their coefficient of determination are presented in **Table 3**. The slopes values obtained for the two analytes were between 0.85 and 1.15. The linearity of the method is demonstrated using the β-expectation tolerance interval approach. Indeed, as illustrated in **Fig. 4** the upper and lower β-expectation tolerance limits were included inside the absolute acceptance limits irrespective of the concentration levels for the two analytes studied.

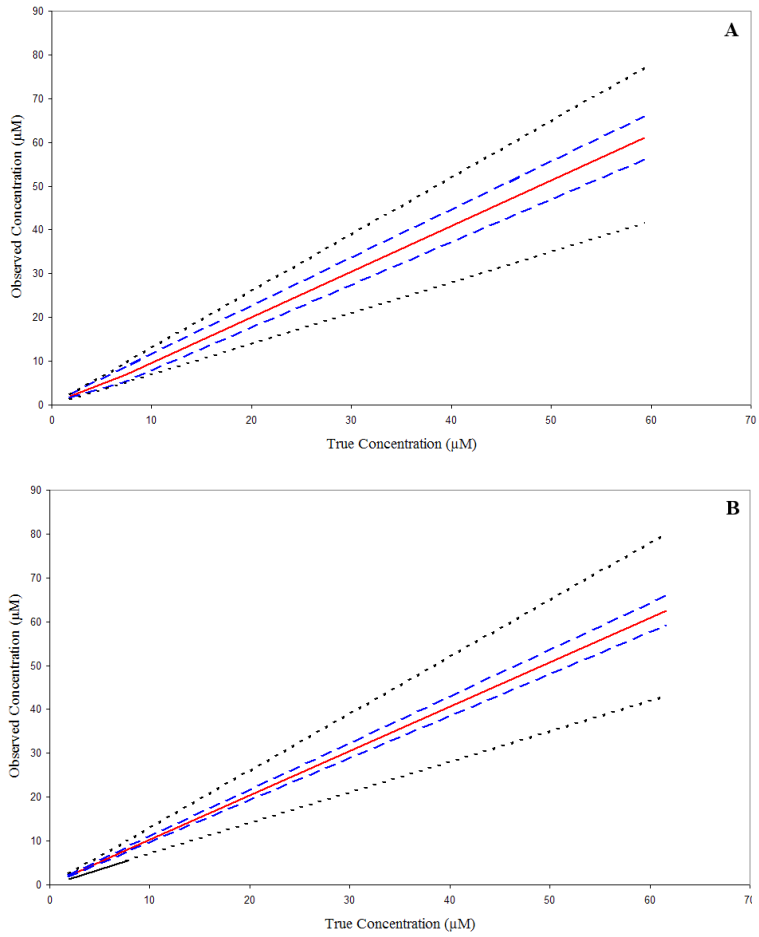


Figure 4 - Linearity obtained for the validation of the HPLC/UV analytical method for the quantitation of human acylated ghrelin (A) and human unacylated ghrelin (B) by considering a weighted (1/X) linear regression as calibration curves; plain line: relative bias, dashed lines: β-expectation tolerance limits (β=80%), dotted lines: ± 30% acceptance limit expressed in µM.

Table 3 - Evaluation of the results linearity for human acylated ghrelin (hAG) and human unacylated ghrelin (hUAG).

	Equation	R^2
hAG	y = 1.0326x-0.2792	0.999
hUAG	y = 1.0147x-0.0854	0.999

3.3. STABILITY OF GHRELIN IN AQUEOUS SOLUTIONS

To determine the optimal storage conditions for ghrelin samples, the stabilities of acylated ghrelin and unacylated ghrelin were tested in different types of buffers, at different temperatures and different times of storage. The stability of each analyte was determined as a percentage of the stock solutions concentrations (= controls). As acylated ghrelin stock solutions (~202 µg/ml = 100% hAG) already contained some unacylated ghrelin (~8 µg/ml = 100% hUAG), the degradation of acylated ghrelin into unacylated ghrelin implies that

unacylated ghrelin mean percentage of controls exceeds 100%. As shown in **Table 4** unacylated ghrelin was stable under all conditions tested while the stability of acylated ghrelin was impaired at 37 °C at pH 7.4. The main degradation product of acylated ghrelin was unacylated ghrelin, probably due to the saponification of the ester bound in basic conditions. The higher stability of acylated ghrelin at acidic pH is also consistent with the optimal storage conditions of ghrelin, i.e. pH 4, that were determined for blood samples by Hosoda et al. [6]. Consequently, ghrelin samples can be kept at 4 °C for at least 24 h before analysis. If formulated at pH 7.4, acylated ghrelin should be kept at 4 °C or used and analysed immediately.

Table 4 - Effect of temperature and pH on human acylated (hAG) and unacylated ghrelin (hUAG) stability.

Medium	pH	Temp (°C)	Time (h)	Mean (SD) percentage of controls[a]		
				hAG		**hUAG**
				hAG	hUAG	
H2O	~4.7[b]	37	4	99.7 (2.0)	99.2 (2.7)	101.3 (2.4)
Citrate buffer	4	37	4	98.9 (2.0)	100.5 (2.4)	100.3 (0.2)
Citrate buffer	6	37	4	96.2 (1.3)	109.7 (0.5)*	99.3 (1.5)
	6	4	4	99.3 (0.6)	103.4 (7.8)	99.5 (1.6)
HBSS	6	37	4	98.5 (3.3)	103.0 (4.9)	99.4 (0.9)
HBSS	7.4	37	4	91.9 (2.2)£	178.5 (7.7)**	99.2 (1.8)
PBS	7.4	37	4	95.1 (1.8)£	126.2 (1.7)***	98.6 (0.2)
PBS	7.4	37	24	85.3 (1.0)***	269.9 (9.9)***	98.7 (1.3)
PBS	7.4	4	24	98.2 (0.7)	104.5 (3.1)	100.0 (1.6)

No statistical difference unless otherwise stated.
[a] hUAG control concentrations were ~202 μg/ml, whereas hAG controls contained ~198 μg/ml hAG and ~8 μg/ml hUAG.
[b] as determined using a pH-meter.
£ $p<0.05$ (statistical analysis by unpaired *t-test* within each condition tested vs. control).
* $p<0.01$ (statistical analysis by unpaired *t-test* within each condition tested vs. control).
** $p<0.0005$ (statistical analysis by unpaired *t-test* within each condition tested vs. control).
*** $p<0.0001$ (statistical analysis by unpaired *t-test* within each condition tested vs. control).

3.4. GHRELIN BINDING TO LIPOSOMES

To check if acylation could affect the binding of ghrelin to lipid membranes, the validated method was used to assess the binding of acylated and unacylated ghrelin to negatively charged liposomes made of Chol/SM/PC/PE/PI (5.5:4.0:1.7:3.0:2.3, M/M) at different

lipid/peptide molar ratios. Indeed, peptide-membrane interactions are mainly due to electrostatic and/or hydrophobic interactions [33,34]. As shown in **Fig. 5**, the binding of acylated ghrelin to Chol/SM/PC/PE/PI liposomes was higher than the binding of unacylated ghrelin at all the lipid/peptide ratios. It thus indicates that acylation promotes the interaction of the peptide with the lipid membrane and hence that hydrophobic interactions seem to play a role in ghrelin-membrane binding as both peptides carry the same net positive charge. The binding of acylated ghrelin increased with the lipid content.

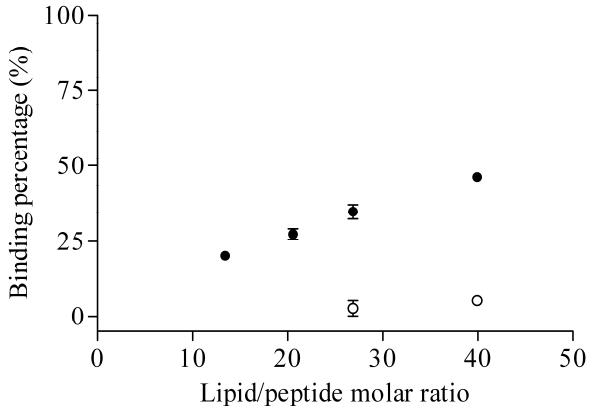


Figure 5 - Binding of human acylated ghrelin (hAG) (●) and human unacylated ghrelin (hUAG) (○) to Chol/PC/SM/PI/PE (5.5:4.0:1.7:3.0:2.3, M/M) liposomes at increasing lipid/peptide molar ratios, T = 37 °C (n = 3). Circles represent the mean values ± SD.

4. CONCLUSIONS

The described HPLC/UV method allows the separation and the quantitation of the human acylated and unacylated ghrelin. An original validation approach using accuracy profiles based on β-expectation tolerance intervals for the total measurement error demonstrated the reliability of the results generated. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the lower limit of quantitation and the range over which the method can be considered as valid. The described method is therefore reliable for its intended use, the accurate quantitation of human acylated and unacylated ghrelin in the micromolar concentration range. In addition, this method was used to assess the stability of acylated and unacylated ghrelin in various media. Unacylated ghrelin was found to be stable in all conditions tested. Acylated ghrelin solutions, however, required preparation or storage at acidic pH and/or at low temperature to avoid deacylation. Finally, this validated HPLC/UV method was successfully applied to evaluate the binding of acylated and unacylated ghrelin to liposomes.

5. REFERENCES

[1] Banga A.K., Therapeutic peptides and proteins formulation, processing, and delivery systems, CRC/Taylor & Francis, Boca Raton, FL (2006).

[2] Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H., and Kangawa K., Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature, 402 (1999) 656-660.

[3] Kojima M. and Kangawa K., Ghrelin: structure and function, Physiol. Rev., 85 (2005) 495-522.

[4] Soares J.B. and Leite-Moreira A.F., Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle, Peptides, 29 (2008) 1255-1270.

[5] Akamizu T. and Kangawa K., Translational research on the clinical applications of ghrelin, Endocr. J., 53 (2006) 585-591.

[6] Hosoda H., Doi K., Nagaya N., Okumura H., Nakagawa E., Enomoto M., Ono F., and Kangawa K., Optimum collection and storage conditions for ghrelin measurements: octanoyl modification of ghrelin is rapidly hydrolyzed to desacyl ghrelin in blood samples, Clin. Chem., 50 (2004) 1077-1080.

[7] De Vriese C., Gregoire F., Lema-Kisoka R., Waelbroeck M., Robberecht P., and Delporte C., Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites, Endocrinology, 145 (2004) 4997-5005.

[8] De Vriese C., Hacquebard M., Gregoire F., Carpentier Y., and Delporte C., Ghrelin interacts with human plasma lipoproteins, Endocrinology, 148 (2007) 2355-2362.

[9] Yoshimoto A., Mori K., Sugawara A., Mukoyama M., Yahata K., Suganami T., Takaya K., Hosoda H., Kojima M., Kangawa K., and Nakao K., Plasma ghrelin and desacyl ghrelin concentrations in renal failure, J. Am. Soc. Nephrol., 13 (2002) 2748-2752.

[10] Akamizu T., Shinomiya T., Irako T., Fukunaga M., Nakai Y., Nakai Y., and Kangawa K., Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay, J. Clin. Endocrinol. Metab., 90 (2005) 6-9.

[11] Paik K.H., Choe Y.H., Park W.H., Oh Y.J., Kim A.H., Chu S.H., Kim S.W., Kwon E.K., Han S.J., Shon W.Y., and Jin D.K., Suppression of acylated ghrelin during oral glucose tolerance test is correlated with whole-body insulin sensitivity in children with Prader-Willi syndrome, J. Clin. Endocrinol. Metab., 91 (2006) 1876-1881.

[12] Broom D.R., Batterham R.L., King J.A., and Stensel D.J., Influence of resistance and aerobic exercise on hunger, circulating levels of acylated ghrelin, and peptide YY in healthy males, Am. J. Physiol. Regul. Integr. Comp. Physiol., 296 (2009) R29-R35.

[13] Rauh M., Groschl M., and Rascher W., Simultaneous Quantification of Ghrelin and Desacyl-Ghrelin by Liquid Chromatography-Tandem Mass Spectrometry in Plasma, Serum, and Cell Supernatants, Clin. Chem., 53 (2007) 902-910.

[14] Beaumont N.J., Skinner V.O., Tan T.M., Ramesh B.S., Byrne D.J., MacColl G.S., Keen J.N., Bouloux P.M., Mikhailidis D.P., Bruckdorfer K.R., Vanderpump M.P., and Srai K.S., Ghrelin can bind to a species of high density lipoprotein associated with paraoxonase, J. Biol. Chem., 278 (2003) 8877-8880.

[15] Bednarek M.A., Feighner S.D., Pong S.S., McKee K.K., Hreniuk D.L., Silva M.V., Warren V.A., Howard A.D., Van Der Ploeg L.H., and Heck J.V., Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a, J. Med. Chem., 43 (2000) 4370-4376.

[16] Makino T., Matsumoto M., Suzuki Y., Kitajima Y., Yamamoto K., Kuramoto M., Minamitake Y., Kangawa K., and Yabuta M., Semisynthesis of human ghrelin: condensation of a Boc-protected recombinant peptide with a synthetic O-acylated fragment, Biopolymers, 79 (2005) 238-247.

[17] De Vriese C., Gregoire F., De Neef P., Robberecht P., and Delporte C., Ghrelin is produced by the human erythroleukemic HEL cell line and involved in an autocrine pathway leading to cell proliferation, Endocrinology, 146 (2005) 1514-1522.

[18] Pemberton C., Wimalasena P., Yandle T., Soule S., and Richards M., C-terminal pro-ghrelin peptides are present in the human circulation, Biochem. Biophys. Res. Commun., 310 (2003) 567-573.

[19] Ishimaru M., Yoshizawa-Kumagaye K., Kubo S., Kitani T., Chino N., Kangawa K., and Kimura T., Stability of the O-octanoyl group of rat ghrelin during chemical synthesis : counter-ion-dependent alteration of an ester bond breakage, Lett. Pept. Sci., 10 (2003) 41-50.

[20] Date Y., Kojima M., Hosoda H., Sawaguchi A., Mondal M.S., Suganuma T., Matsukura S., Kangawa K., and Nakazato M., Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans, Endocrinology, 141 (2000) 4255-4261.

[21] Enomoto M., Nagaya N., Uematsu M., Okumura H., Nakagawa E., Ono F., Hosoda H., Oya H., Kojima M., Kanmatsuse K., and Kangawa K., Cardiovascular and hormonal effects of subcutaneous administration of ghrelin, a novel growth hormone-releasing peptide, in healthy humans, Clin. Sci. (Lond), 105 (2003) 431-435.
[22] Bossola M., Tazza L., and Luciani G., Mechanisms and treatment of anorexia in end-stage renal disease patients on hemodialysis, J. Ren. Nutr., 19 (2009) 2-9.
[23] Mathot F., Schanck A., Van Bambeke F., Arien A., Noppe M., Brewster M., and Préat V., Passive diffusion of polymeric surfactants across lipid bilayers, J. Control. Release, 120 (2007) 79-87.
[24] Bartlett G.R., Phosphorus assay in column chromatography, J. Biol. Chem., 234 (1959) 466-468.
[25] Hubert P., Nguyen-Huu J.J., Boulanger B., Chapuzet E., Chiap P., Cohen N., Compagnon P.A., Dewe W., Feinberg M., Lallier M., Laurentie M., Mercier N., Muzard G., Nivet C., and Valat L., Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal. Part I, J. Pharm. Biomed. Anal., 36 (2004) 579-586.
[26] Hubert P., Nguyen-Huu J.J., Boulanger B., Chapuzet E., Cohen N., Compagnon P.A., Dewe W., Feinberg M., Laurentie M., Mercier N., Muzard G., Valat L., and Rozet E., Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal. Part III, J. Pharm. Biomed. Anal., 45 (2007) 82-96.
[27] Hubert P., Nguyen-Huu J.J., Boulanger B., Chapuzet E., Chiap P., Cohen N., Compagnon P.A., Dewe W., Feinberg M., Lallier M., Laurentie M., Mercier N., Muzard G., Nivet C., Valat L., and Rozet E., Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal. Part II, J. Pharm. Biomed. Anal., 45 (2007) 70-81.
[28] International Conference on Harmonization (ICH) of technical requirements for registration of Pharmaceutical for human use, topic Q2(R1): Validation of analytical procedures: text and methodology, Geneva (2005).
[29] Rozet E., Ceccato A., Hubert C., Ziemons E., Oprean R., Rudaz S., Boulanger B., and Hubert P., Analysis of recent pharmaceutical regulatory documents on analytical method validation, J. Chromatogr. A, 1158 (2007) 111-125.
[30] Viswanathan C.T., Bansal S., Booth B., DeStefano A.J., Rose M.J., Sailstad J., Shah V.P., Skelly J.P., Swann P.G., and Weiner R., Workshop/conference report - Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays, Aaps J., 9 (2007) E30-E42.
[31] Boulanger B., Dewe W., Gilbert A., Govaerts B., and Maumy-Bertrand M., Risk management for analytical methods based on the total error concept: conciliating the objectives of the pre-study and in-study validation phases, Chemom. Intell. Lab. Syst., 86 (2007) 198-207.
[32] ISO 5725 Application of the statistics-accuracy (trueness and precision) of the results and methods of measurement, Part 1 to 6, International Organization for Standardization (ISO) (1994).
[33] Castanho M.A. and Fernandes M.X., Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the "membrane catalysis" model, Eur. Biophys. J., 35 (2006) 92-103.
[34] Seelig J., Thermodynamics of lipid-peptide interactions, Biochim. Biophys. Acta, 1666 (2004) 40-50.

Chapter IV.

ACYLATED AND UNACYLATED GHRELIN BINDING TO MEMBRANES AND TO GHRELIN RECEPTOR: TOWARDS A BETTER UNDERSTANDING OF THE UNDERLYING MECHANISMS

ACYLATED AND UNACYLATED GHRELIN BINDING TO MEMBRANES AND TO GHRELIN RECEPTOR: TOWARDS A BETTER UNDERSTANDING OF THE UNDERLYING MECHANISMS

Adapted from Staes E., Absil P.A., Lins L., Brasseur R., Deleu M., Lecouturier N., Fievez V., des Rieux A., Mingeot-Leclercq M.P., Raussens V., Préat V.

Biochimica et Biophysica Acta – Biomembranes, 1798: 2102-2113 (2010)

ABSTRACT

The *O*-octanoylation of human ghrelin is a natural post-translational modification that enhances its binding to model membranes and could potentially play a central role in ghrelin biological activities. Here, we aimed to clarify the mechanisms that drive ghrelin to the membrane and hence to its receptor that mediates most of its endocrinological effects. As the acylation enhances ghrelin lipophilicity and that ghrelin contains many basic residues, we examined the electrostatic attraction and/or hydrophobic interactions with membranes. Using various liposomes and buffer conditions in binding, zeta potential and isothermal titration calorimetry studies, we found that whereas acylated and unacylated ghrelin were both electrostatically attracted towards the membrane, only acylated ghrelin penetrated into the headgroup and the lipid backbone regions of negatively charged membranes. The *O*-acylation induced a 120-fold increase in ghrelin local concentration in the membrane. However, acylated ghrelin did neither deeply penetrate the membrane nor did it perturb its organization. Conformational studies by circular dichroism and attenuated total reflection Fourier transform infrared as well as *in silico* modelling revealed that both forms of ghrelin mainly adopted the same structure in aqueous, micellar and bilayer environments even though acylated ghrelin structure is slightly more α-helical in a lipid bilayer environment. Altogether our results suggest that membrane acts as a "catalyst" in acylated ghrelin binding to the ghrelin receptor and hence could explain why acylated and unacylated ghrelin are both full agonists of this receptor but in the nanomolar and micromolar range, respectively.

TABLE OF CONTENTS

1. INTRODUCTION 105
2. MATERIAL AND METHODS 107
2.1. MATERIAL 107
2.2. BUFFERS 107
2.3. PREPARATION OF LIPOSOMES 107
2.4. BINDING TO LIPOSOMES BY ULTRACENTRIFUGATION 108
2.5. BINDING TO ERYTHROCYTES BY CENTRIFUGATION 108
2.6. FLUORESCENCE POLARIZATION 109
2.7. ZETA POTENTIAL DETERMINATION 109
2.8. INFRARED SPECTROSCOPY MEASUREMENT 109
2.9. CIRCULAR DICHROISM 110
2.10. PEPLOOK METHOD (BOLTZMANN-STOCHASTIC METHOD) 110
2.11. ISOTHERMAL TITRATION CALORIMETRY 111
2.12. BINDING MODEL 111
2.13. PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAY (PAMPA) 114
2.14. TRANSPORT EXPERIMENTS 115
2.15. STATISTICAL ANALYSIS 115
3. RESULTS 116
3.1. BINDING OF GHRELIN TO MODEL AND BIOLOGICAL MEMBRANES 116
3.2. ELECTROSTATIC ADSORPTION STUDIES 117
3.3. LIPID-PEPTIDE INTERACTIONS: EFFECT OF AND ON MEMBRANE FLUIDITY 120
3.4. HYDROPHOBIC INTERACTIONS 122
3.5. PEPTIDE CONFORMATION AND LIPID ORIENTATION STUDIES 125
3.6. TRANSMEMBRANE AND CELLULAR TRANSPORT STUDIES 127
4. DISCUSSION 129
4.1. ELECTROSTATIC-HYDROPHOBIC INTERACTIONS 129
4.2. PEPTIDE CONFORMATION 130
4.3. CELLULAR TRANSPORT 131
4.4. GHRELIN ACYLATION VS. RECEPTOR ASSOCIATION 131
5. REFERENCES 133

1. INTRODUCTION

O-acylation of medium chain length fatty acids is a novel type of natural peptide modification which was discovered a decade ago by Kojima and co-workers [1]. In 1999, ghrelin was isolated from stomach extracts as the endogenous ligand of the orphan growth hormone secretagogue receptor 1a (GHS-R1a), now called the ghrelin receptor. Ghrelin is a 28-aa peptide hormone which possesses a unique post-translational modification: an *O*-acylation at its Ser^3 residue. The main acylated forms of ghrelin are modified with an *n*-octanoic acid and to a lesser extent with decanoyl and possibly decenoyl fatty acids [1,2]. The enzyme that catalyzes the acylation of ghrelin is a membrane-bound *O*-acyl transferase called ghrelin *O*-acyltransferase (GOAT) [3,4]. Ghrelin has been discovered thanks to its growth hormone releasing activity [1]. Since then, numerous biological activities have been attributed to ghrelin. The orexigenic effects are the ones that generated the highest interest among endocrinologists. Yet, ghrelin also affects energy and glucose homeostasis, cell proliferation and differentiation, gastrointestinal, cardiovascular, pulmonary and immune functions as well as bone physiology. Another naturally occurring variant of ghrelin is unacylated ghrelin also called des-acyl ghrelin. This form is the most abundant in the plasma. Even though unacylated ghrelin has long been thought to be devoid of physiological activities, it now appears that acylated and unacylated ghrelin present similar and opposite actions (see [5-7] for review). In the nanomolar range, the acylation of ghrelin is required for efficient binding and activation of the ghrelin receptor that mediates most of its endocrinological activities [1]. However, some other effects of ghrelin have been attributed to the activation of a yet unidentified receptor that could bind acylated and/or unacylated ghrelin [7]. Unacylated ghrelin is also a full agonist of the ghrelin receptor but in the micromolar range [8]. As ghrelin effects seem to be receptor mediated, the interaction of ghrelin with cell membranes is potentially important for all its biological activities.

In 1986, Sargent and Schwyzer were the first to propose a “membrane catalysis” model in which the membrane lipid phase acts as a mediator for peptide-receptor interactions by speeding up the reactions [9]. It relies on evidence, that for any ligand approaching a cell, the probability of contact with the lipid phase is much higher than with one of its receptors. This model is still relevant today even though it has been refined over the years [10,11]. The known and foreseen advantages for peptide-receptor binding associated with the peptide-membranes interactions include favourable kinetics, increased local concentration of the

peptide in the vicinity of the receptor, peptide conformation and orientation change, as well as positioning of the peptide at the proper depth for receptor interaction and in some cases diffusional advantage due to the reduction of dimensionality. These peptide-membrane-receptor interactions can be divided into four molecular steps: (i) electrostatic attraction / membrane adsorption of the peptide; (ii) peptide penetration into the headgroup region or in the hydrophobic backbone; (iii) conformational change of the peptide; (iv) peptide-receptor docking [10,12,13]. When basic peptides such as ghrelin interact with acidic lipids, their interactions can either be purely electrostatic like for polylysines or a mix of hydrophobic and electrostatic interactions which occurs for most peptides [12,13].

As we already mentioned, there has been a lot of interest for ghrelin regarding to its therapeutical potential. However, besides the octanoyl role in binding and activation of the ghrelin receptor, little is known about the other roles of this unusual moiety [14]. The acylation of ghrelin increases its lipophilicity [15]. Moreover, as we have previously shown, it also increases its binding extent to model membranes [16]. We can therefore hypothesize that this modification could enhance the binding of ghrelin to biological membranes and hence to its receptor as well as its transmembrane and transepithelial transport [17,18].

The aims of this paper are thus focused on the roles of the octanoyl moiety and the basic residues of ghrelin in the extent of ghrelin binding to membranes and on the underlying mechanisms of ghrelin-lipid interactions in order to give new insights about the differential binding of acylated and unacylated ghrelin to the ghrelin receptor, as well as its role on ghrelin transmembrane and transepithelial transport.

2. MATERIAL AND METHODS

2.1. MATERIAL

Synthetic human acylated ghrelin (hAG) and human unacylated ghrelin (hUAG) were purchased from NeoMPS (Strasbourg, France). Peptide HPLC purity was >97%, according to the manufacturer. Bovine brain sphingomyelin (SM), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) , 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) (DMPG), cholesterol (Chol), phosphatidylserine (PS), trifluoroethanol (TFE) and furosemide were obtained from Sigma-Aldrich, St. Louis, MO, USA. Egg yolk phosphatidylcholine (PC) (Grade 1), wheat germ phosphatidylinositol (PI) (Grade 1) and egg yolk phosphatidylethanolamine (PE) (Grade 1) were obtained from Lipid Products, Redhill Surrey, UK. Acetonitrile, HPLC grade for far UV, was purchased from Acros organics. Trifluoroacetic acid (TFA), HPLC grade was purchased from Fisher Scientific. All other reagents were of ACS or analytical grade. Peptide quantities are always expressed as the net peptide content.

2.2. BUFFERS

As the ester bond of acylated ghrelin is chemically unstable and converts into unacylated ghrelin at basic pH, even at pH 7.4 [16], most experiments were conducted at pH 6. Unless otherwise stated, experiments were performed in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer (I_c = 0.06 M), referred as citrate buffer hereafter. In absence of buffering the peptide solution pH is about 4.5. Two other buffers, 12.5 mM Na-citrate 46.2 mM NaCl pH 4 buffer (I_c = 0.06 M) and 12.5 mM Na-citrate 103.2 mM NaCl pH 6 buffer (I_c = 0.15 M), were used for the pH and ionic strength influence studies, respectively.

2.3. PREPARATION OF LIPOSOMES

We prepared large unilamellar vesicles by extrusion (LUVET) using a Thermobarrel extruder (Lipex Biomembranes Inc., Vancouver, Canada) as previously described [19,20]. The *z*-average diameters of the liposomes extruded on either 100 nm ($LUVET_{100}$) or 200 nm ($LUVET_{200}$) pore size filters, as determined using a Zetasizer Nano ZS (Malvern Instruments

Ltd., Worcestershire, UK), were 100 ± 2 nm and 135 ± 12 nm, respectively. The phospholipid concentrations were determined by phosphorus assay [21]. When liposomes contained cholesterol, the total lipid concentration was calculated assuming similar recovery of phospholipids and cholesterol. Liposomes were stored under nitrogen at 4 °C and used within 5 days.

2.4. BINDING TO LIPOSOMES BY ULTRACENTRIFUGATION

Human acylated and unacylated ghrelin were used to assess the binding to $LUVET_{100}$ or $LUVET_{200}$ at lipid/peptide molar ratios of 12:1 to 375:1 by ultracentrifugation. Liposomes were either made of Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) [22], DMPC/PI (10.3:1, M/M), DMPC/PI (5.15:1, M/M), DMPC/DMPG (5.15:1, M/M) or DMPC alone. Peptide concentration was 100 or 200 µg/ml. The liposomes/peptide (either acylated or unacylated ghrelin) mixture (~160 µl) was incubated for 3 h at 4 or 37 °C and then ultracentrifuged at 4 °C (250,000 × *g*) for 1 h. In controls, liposomes were replaced by the equivalent volume of buffer. 100 µl of the supernatants, that contained the free peptides, were then collected. Controls and supernatants were quantified by HPLC/UV as previously described [16]. The binding percentage was calculated as follows: ((control concentration - supernatant concentration) / control concentration) × 100. Acylated and unacylated ghrelin binding rates at 37 °C to Chol/PC/SM/PI/PE $LUVET_{200}$ at a lipid/peptide molar ratio of 375:1, as determined by ultracentrifugation (98.7 ± 1.0% and 47.7 ± 2.1%, respectively), were confirmed by equilibrium dialysis of ^{125}I-human Tyr^4-acylated ghrelin and ^{125}I-human Tyr^4-unacylated ghrelin (Eurogentec, Herstal, Belgium), 96.8 ± 1.9% and 46.9 ± 3.4%, respectively, $p>0.05$ (unpaired t-test).

2.5. BINDING TO ERYTHROCYTES BY CENTRIFUGATION

Blood samples were collected in citrate tubes from 6 healthy volunteers who gave informed consent (3 male and 3 female). Erythrocytes were isolated by centrifugation (10 min, 330 *g*, 20 °C) and then washed 3 times with PBS. After addition of acylated ghrelin or unacylated ghrelin (200 µg/ml) to the erythrocytes (45% V/V), the erythrocytes were incubated for 3 h at 4 °C and then centrifuged at 4 °C (330 × *g*) for 10 min. Thereafter the supernatants were collected and quantified by UV after an RP-HPLC separation, as previously described [16].

2.6. FLUORESCENCE POLARIZATION

The effect on membrane fluidity was studied by measuring the degree of fluorescence polarization of 1,6-diphenylhexatriene (DPH) and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Invitrogen, Carlsbad, CA, USA) in Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{200}$ at 4, 37 and 55 °C according to the method of Shinitzky and Barenholz [23]. Samples preparation was adapted from [24]. Lipid/peptide molar ratio was 240:1, whereas lipid to DPH and TMA-DPH molar ratios were 315:1 and 477:1, respectively. Fluorescence polarization measurements and determination of the degree of polarization were performed as previously described [20].

2.7. ZETA POTENTIAL DETERMINATION

The vesicles electrophoretic mobility was determined using a Zetasizer Nano ZS. For the electrophoretic mobility measurements at constant peptide concentration (200 µg/ml) the samples were prepared as follows: DMPC or Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{200}$ were added to the peptide solution (or buffer for controls) at a lipid/peptide molar ratio of either 240:1 or 60:1, with a final mixture volume of about 160 µl. After 3h incubation at 37 °C, 1 ml of citrate buffer was added prior to the sample measurement. For electrophoretic mobility measurements at different peptide concentrations, the peptide (1 mg/ml stock concentration) was added to 1 ml of 61.5 µM Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{100}$ and incubated for 1 h at 37 °C and then measured at the same temperature. The zeta potentials were calculated using Oshima's analytical expression of Henry's equation described in Section 2.12.

2.8. INFRARED SPECTROSCOPY MEASUREMENT

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was used to evaluate any secondary structure change of acylated and unacylated ghrelin in presence of DMPC/DMPG (5.15:1, M/M) $LUVET_{200}$ regarding to their structure in solution. Before sample preparation, the trifluoroacetate (TFA) counterions, which strongly associate with the peptides, were replaced by chloride counterions by three successive lyophilisations against HCl 10 mM [25]. These were followed by 2 successive lyophilisations in water in order to remove the excess of HCl. This allowed the elimination of a strong C=O stretching absorption

band due to TFA near 1673 cm^{-1} [26]. Spectra were recorded on a Bruker Equinox 55 at room temperature as previously described [27]. About 265 µl of either peptide alone (200 µg/ml) or of a lipid/peptide mixture (at a 50:1 lipid/peptide molar ratio), in NaCl 3 mM was spread out on the plate. Samples were flushed with N_2 saturated with D_2O for 4 h. The secondary structure changes were monitored by analysis of the amide I vibrational bands (1600– 1700 cm^{-1}) as described in [28]. The effects of the peptides on the orientation of the phospholipids acyl chains were assessed as previously described [29]. In brief, we monitored the transition dipole moment of the $\gamma_w(CH_2)$ peak at 1202 cm^{-1} as it lies along the lipid hydrocarbon chains. The angle between the germanium crystal and the dipole was calculated from the dichroic ratio R_{ATR}. The dichroic ratio (R_{ATR}) was calculated by determining the ratio of the peak absorbance as follows: $R_{ATR} = A(90°)/A(0°)$.

2.9. CIRCULAR DICHROISM

Far-UV circular dichroism (CD) spectra (185–260 nm) of the peptides were recorded on Jasco-710 spectropolarimeter. A quartz cell with a path length of 0.05 or 0.02 cm was used. All measurements were made at room temperature (22 °C). The parameters used were as follows: bandwidth, 1.0 nm; step resolution, 0.5 nm; scan speed, 50 nm/min; response time, 2 s. Each spectrum was obtained from an average of 4 scans. The contribution of the buffer was subtracted. Peptide concentration was 200 µg/ml.

2.10. PEPLOOK METHOD (BOLTZMANN-STOCHASTIC METHOD)

In order to explore conformational possibilities of the peptide in a hydrophobic environment we used the Boltzmann-Stochastic *in silico* method, PepLook [30]. This method requires several successive steps of calculation. At each step, a random population of 10,000 conformations of ghrelin (acylated or not) is generated and the energy of all conformations is calculated using the force field described elsewhere [30]. The first step uses a set of 64 pairs of Φ/Ψ of angles with equal probability. In the next steps, the probabilities of Φ/Ψ values per residue vary according to whether they had previously contributed to exclusively poor or, exclusively good structural solutions for the peptide, respectively. The calculation was iterated up to when the probability of all Φ/Ψ angles remains constant. Then, the 99 models of lower energy were further minimized using a Simplex method [31,32] with a precision of 5

degrees and a maximum of 1000 steps. The structure with the best energy (called the "prime") was considered.

2.11. ISOTHERMAL TITRATION CALORIMETRY

The heat flows resulting from the binding of acylated and unacylated ghrelin to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{100}$ were measured by high-sensitivity isothermal titration calorimetry (ITC) using a Microcal VP-ITC (Microcal, Northampton, MA) with a reaction cell volume of 1.4565 ml [33] and Milli-Q water as a reference. Solutions were degassed for 10 min under vacuum prior to use. Peptide-into-lipid and lipid-into-peptide titrations were performed under constant stirring (305 rpm). After an initial delay of 1800 s and a 2 µl initial injection, 5 µl (in 5 s) or 10 µl (in 7.1 s) of either peptide (200 µM) or lipids (12.2 to 48.4 mM) were injected from the 300 µl syringe into the sample cell containing either the lipids (12.1 to 18.2 mM) or the peptide (10 to 100 µM). The interval between each injection was 4 min. The heats of dilution were determined in control experiments by injection of either the peptides or the lipids into the buffer and were subtracted from the heats determined in the corresponding peptide-into-lipid or lipid-into-peptide experiment. All experiments were performed at 37 °C in duplicate.

2.12. BINDING MODEL

The binding of ghrelin to lipids can be described by its overall or apparent binding coefficient, K_{app}, that includes both the electrostatic attraction and the chemical partition equilibriums. This apparent binding coefficient is linked to the degree of peptide binding, X_b^i, and to the bulk molar concentration of the peptide, C_f^i, as follows:

$$K_{app} = X_b^i / C_f^i \qquad (1)$$

where X_b^i is the number of moles of peptides bound, $n_{pep,b}^i$, per mole of lipids that the peptides can theoretically reach, i.e. 50% of the total lipids in the case of LUVET if the peptide does not cross the bilayer [34]. *i* represents the *i*th external lipid/peptide molar ratio L_{ext}/P.

However, as the peptide chemical (hydrophobic) partition is of high interest to us, we used another binding model described by Seelig et al. [35,36]. In this model, the chemical partition

coefficient K_p is not assumed anymore to be linked to the bulk concentration of the peptide but rather to its concentration near the membrane surface, C_M^i, as follows:

$$K_p = X_b^i / C_M^i \qquad (2)$$

The X_b^i have been calculated here from the binding experiments by ultracentrifugation of ghrelin – Chol/PC/SM/PI/PE LUVET mixtures at L_{ext}/P molar ratios at which the membrane is not yet saturated by the peptide i.e. at $L_{ext}/P \geq 10$, as follows:

$$X_b^i = n_{pep,b}^i / \gamma\, n_L^i = (C_0^i - C_f^i)/\gamma\, C_L^i \qquad (3)$$

where n_L^i is the total number of moles of lipids, C_0^i is the total or control molar peptide concentration, C_f^i is the free molar peptide concentration, γ is the proportion of external lipids (50%) and C_L^i is the total molar lipid concentration.

The peptide membrane concentration, C_M^i, is correlated to the free peptide concentration, C_f^i, as well as to the peptide effective charge, z_{eff}, and to the membrane surface potential, ψ_0^i, according to the Boltzmann relation [37] as follows:

$$C_M^i = C_f^i \exp\left(-z_{eff}\, F\, \psi_0^i / R\, T\right) \qquad (4)$$

where F is the Faraday constant (C/mol), R is the gas constant (J/K mol) and T is the temperature (K).

Unfortunately, direct measurement of ψ_0^i cannot be performed. However, ψ_0^i is related to the zeta potential, ζ^i, and to the surface charge density, σ^i, the first being related to the electrophoretic mobility, u_e^i, and the latter being connected to X_b^i.

We first calculated ζ^i, from the measured u_e^i, of the ghrelin-Chol/PC/SM/PI/PE LUVET mixtures at different L_{ext}/P molar ratios, using the Oshima's analytical expression of Henry's equation [38]:

$$\zeta^i = \frac{3\,\eta}{2\,\varepsilon_r\,\varepsilon_0\, f(\kappa R_s)}\, u_e^i \qquad (5)$$

with

$$\kappa = \sqrt{\frac{2000\, e^2\, N_A\, I}{\varepsilon_r\,\varepsilon_0\, k_B\, T}} \qquad (5a)$$

and

$$f(\kappa R_s) = 1 + \frac{1}{2}\left[1 + \left(\frac{2.5}{\kappa R_s\left[1 + 2\exp(-\kappa R_s)\right]}\right)\right] \qquad (5b)$$

where ζ^i and u_e^i are respectively expressed in (V) and (m^2 V^{-1} s^{-1}), η is the dispersant viscosity (Pa.s), ε_r is the relative permittivity of water (74.4 at 37 °C), ε_0 is the vacuum electric permittivity (F/m), κ is the reciprocal of the Debye screening length (m^{-1}), R_s is the radius of the liposomes (m), e is the elementary charge (C), N_A is the Avogadro's number (mol^{-1}), I is the buffer ionic strength (M) and k_B is the Boltzmann constant (J/K).

From the ζ^i we could then calculate the ψ_0^i using the Debye-Hückel approximation for spherical double layers [39,40]:

$$\psi_0^i = \frac{\zeta^i}{\frac{R_s}{R_s + x}\exp(-\kappa x)} \qquad (6)$$

where x is the distance of the hydrodynamic plane of shear from the liposomes surface (m).

In our model we consider the sodium binding to the membrane as described by Seelig et al. [35]. However, we neglected the peptide penetration area into the lipid bilayer. The surface charge density σ^i was therefore related to X_b^i as follows:

$$\sigma_i = \sigma_0 + \sigma_k = -\frac{e\, X_{PI}\left(1 - X_{Na}^i\right)}{A_L} + \frac{e\, z_{eff}\, X_b^i}{A_L} \quad \text{Eq. (7)}$$

with

$$X_{Na}^i = \frac{K_{Na}\, C_{M,Na}^i}{1 + K_{Na}\, C_{M,Na}^i} \qquad (7a)$$

where σ_0 is the surface charge density in absence of the peptide (C/m^2), σ_k is the increment of charge density due to the addition of peptide to the liposomes (C/m^2), X_{Na}^i is the molar fraction of PI with a Na^+ bound and $C_{M,Na}^i$ is the Na^+ membrane concentration (M). The molar fraction of PI is X_{PI} = 0.164. The mean lipid molecular area, A_L = 5.26 × 10^{-19} m^2 , was determined using a Langmuir film balance by air/water surface compression isotherms at 37 °C of a Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) monolayer at a lateral pressure of 32 mN/m which corresponds to the monolayer-bilayer equivalent pressure [41]. K_{Na} is the Na^+ binding coefficient (M^{-1}). This value was calculated, for each plane of shear distance x, from the electrophoretic mobilities of the liposomes in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer in absence of the peptide, where X_b^i = 0, and hence by the combination of the Eqs. (7) and (7a). For x = 2 Å, K_{Na} = 2.35 M^{-1}, this value is in good agreement with the previously reported value for PI containing liposomes [42].

The surface charge density σ^i, creates a surface potential ψ_0^i which can be calculated using the Grahame equation based on the Gouy-Chapman theory [37]:

$$\sigma_i = \text{sign}\left(\psi_0^i\right)\left\{2000\,\varepsilon_r\,\varepsilon_0\,\mathrm{R}\,T\sum_j C_{j,\text{eq}}\left[\exp\left(\frac{-z_j\,\mathrm{F}\,\psi_0^i}{\mathrm{R}\,T}\right)-1\right]\right\}^{1/2} \quad \text{Eq. (8)}$$

where $C_{j,\text{eq}}$ is the molar concentration of the *j*th electrolyte in the bulk aqueous phase and z_j is the signed valency of this *j*th electrolyte.

The experimental values of u_e^i and X_b^i were obtained with Chol/PC/SM/PI/PE LUVET and ghrelin in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer as described above. The unknowns in the above equations that are needed to determine K_p from u_e^i and X_b^i are the surface potential ψ_0^i, the plane of shear distance x and the peptide effective charge z_{eff}. As no analytical solution exists, we developed a numerical solution. For each plane of shear distance x, from 0 to 7.5 Å with a 0.5 Å step, we computed a solution and the best fit to the experimental data was found for x = 2 Å, as found in many other systems [43-45]. First, we determined the K_{Na} as described above and hence X_{Na}^i. As there are 2 independent ways to calculate σ^i from the experimental data, i.e. either from the u_e^i by the combination of Eqs. (5), (6) and (8), or from the X_b^i by the combination of Eqs. (3) and (7), Eqs. (7) and (8) were thus equalized to obtain z_{eff} by a one-parameter linear regression. On the basis of this z_{eff} value we calculated C_M^i using Eq. (4) and hence K_p by linear regression of $K_p\,C_M^i = X_b^i$.

2.13. PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAY (PAMPA)

The commercial PAMPA "sandwich" from pION Inc. (P/N 110163, Woburn, MA, USA) is composed of a receiver 96-well filter plate deposited on a donor 96-well microtiter plate. On the filter (hydrophobic PVDF, 125 µm thick, 0.2 µm pores) we deposited 4 µl of either a 20% (w/v) *n*-dodecane solution of a lecithin mixture (pION, P/N 110669) containing excess negative charges, or a 2% w/v DOPC *n*-dodecane solution (*p*ION P/N 110615). The donor and receiver chambers were both filled with 200 µl of 12.5 mM Na-citrate 103.2 mM NaCl pH 6 buffer. The donor chamber also contained 200 µg/ml of peptide. Incubation lasted for 18 h at 25 °C, after which the receiver chamber was quantified. Acylated and unacylated ghrelin were analyzed using specific enzyme immunoassays (EIA) kits (A05106 and A05119, SPI-bio, Montigny le Bretonneux, France). Carbamazepine and furosemide at 25 µg/ml (2.5%

DMSO) were used as controls and quantified by UV following a RP-HPLC separation (carbamazepine P_{app} was 2.76 ± 0.11 and 2.74 ± 0.05 × 10^{-6} cm/s with DOPC and lecithin, respectively, whereas furosemide P_{app} was 0.45 ± 0.02 × 10^{-6} cm/s with lecithin and not detectable in the acceptor well with DOPC and thus its P_{app} was <0.19 × 10^{-6} cm/s).

2.14. TRANSPORT EXPERIMENTS

Mono-cultures of Caco-2 cells and co-cultures of Caco-2 and Raji cells were grown on/under inverted 12-wells inserts as previously described by des Rieux et al. [46,47]. 400 µl of acylated or unacylated ghrelin in HBSS pH 6 (100 µg/ml) were added to cell monolayer apical compartments of mono- and co-cultures. Basolateral compartments were filled with 1200 µl of HBSS pH 7.4. Cell monolayers were incubated for 105 min at 37 °C. Then, 30 µl of 1% TFA was added to acidify the basolateral compartments of acylated ghrelin samples (final pH ~5.7). Basolateral solutions were sampled and stored at -80 °C before quantification. Amounts of acylated and unacylated ghrelin transported were measured in duplicates using the specific EIA kits described above. For acylated ghrelin samples, both acylated and unacylated ghrelin were dosed. The *in vitro* model functionality was assessed by the transport of control nanoparticles [47].

2.15. STATISTICAL ANALYSIS

Unless otherwise stated, all average results are represented as mean ± SD. Differences between 2 groups were analyzed by unpaired t-test with Welch's correction, whereas differences between three or more groups were analyzed by one-way ANOVA combined with the Tukey's test. Differences from 0 were analyzed with one sample t-test. A probability level < 0.05 was considered significant.

3. RESULTS

3.1. BINDING OF GHRELIN TO MODEL AND BIOLOGICAL MEMBRANES

To determine if acylated and unacylated ghrelin bind to membranes, we assessed the binding extent of these peptides to model plasma membranes (liposomes) or to biological membranes (erythrocytes) by (ultra)centrifugation. As displayed in **Fig. 1A** both acylated (hAG) and unacylated (hUAG) ghrelin bound to liposomes and to erythrocytes. The extent of binding of ghrelin to both liposomes and erythrocytes was enhanced by its acylation from ~30 to ~90% (**Fig. 1A**). Interestingly, acylated and unacylated ghrelin bound to erythrocytes to the same extent than to the Chol/PC/SM/PI/PE liposomes, which mimic the plasma membrane [22,48-50], at a lipid/peptide molar ratio of 240:1. Ghrelin has been shown to circulate in the bloodstream associated with lipoproteins [51,52] in the plasma compartment. However, to our knowledge, we were the first to investigate the fate of ghrelin in the erythrocytes fraction. The acylation of ghrelin enhanced its binding extent to erythrocytes but also to human serum albumin (49.2 ± 2.8% for hAG and 18.8 ± 1.1% for hUAG). Knowing that (i) the erythrocytes represent 45% of the blood volume, (ii) albumin has a pseudo-esterase activity [53] and (iii) other studies highlighted the implication of esterases in the deoctanoylation of ghrelin [54], it could partly explain why des-acyl ghrelin is the major form of ghrelin in the plasma [55]. This would, however, require further investigation.

The binding extent of acylated and unacylated ghrelin to Chol/PC/SM/PI/PE liposomes also depended on the lipid/peptide molar ratio. The binding extent increase was steeper for acylated ghrelin and, in our conditions, reached a 100% plateau that was not observed for unacylated ghrelin (**Fig. 1B**). At all lipid/peptide molar ratios, the binding extent of acylated ghrelin to the Chol/PC/SM/PI/PE liposomes was significantly higher ($p<0.001$, except at the lipid/peptide molar ratio of 12:1 where $p<0.01$) than that of unacylated ghrelin (e.g. 77.0 ± 0.7% vs. 12.0 ± 0.8%, for hAG and hUAG, respectively, at a lipid/peptide molar ratio of 60:1). The size of the liposomes extruded either through 100 or 200 nm pores, did not influence the binding of the peptides (**Fig. 1C**) [16]. The apparent binding coefficients determined from these results will be calculated and analyzed together with the chemical partition coefficients (see Section 3.4.).

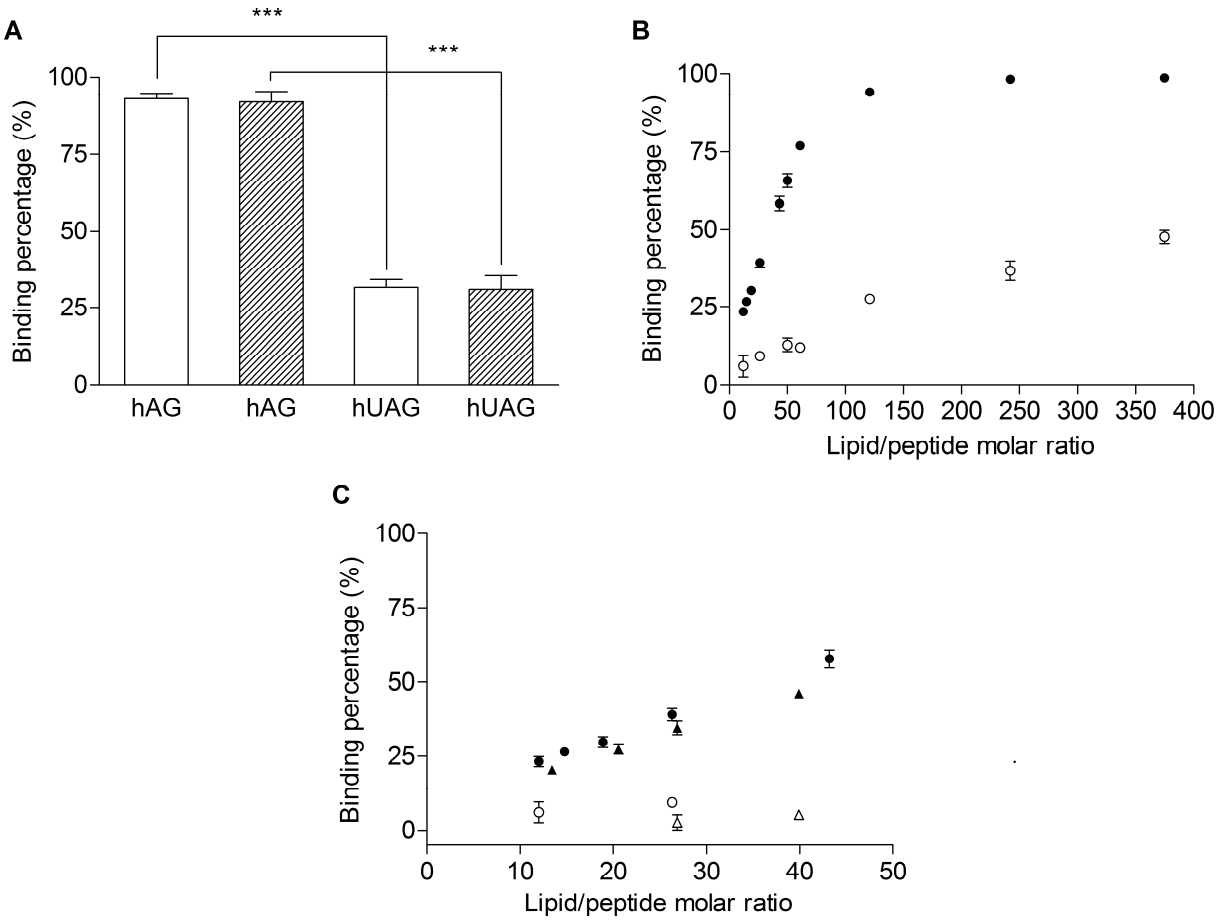


Figure 1 - Acylation influence on ghrelin binding to model and biological membranes. (A) Binding of acylated ghrelin (hAG) and unacylated ghrelin (hUAG) at 200 µg/ml to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{200}$ (empty bars) in Na-citrate 12.5 mM NaCl 14 mM pH 6 buffer at a lipid/peptide molar ratio of 240:1 or to erythrocytes (45%, V/V) (hatched bars) in PBS pH 7.4. T = 4 °C (n = 3-6). Bars represent the mean values ± SD. *** p<0.0001. (B) and (C) Binding of hAG (black symbols) and hUAG (open symbols) to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{200}$ (circles) and $LUVET_{100}$ (triangles) in Na-citrate 12.5 mM NaCl 14 mM pH 6 buffer at increasing lipid/peptide molar ratios, T = 37 °C (n = 3-6). Circles represent the mean values ± SD.

3.2. ELECTROSTATIC ADSORPTION STUDIES

As acylated and unacylated ghrelin are highly charged peptides at pH 6 and 7.4 (theoretical charges of ~5.5 and ~5.0, respectively), we first investigated the possible electrostatic interactions between ghrelin and liposomes. For that purpose, we used liposomes of different compositions. By comparison with the binding to negatively charged liposomes (**Fig. 2A**), acylated ghrelin only slightly bound to zwitterionic liposomes made of DMPC (7.7 ± 1.3%) whereas unacylated ghrelin did not (**Fig. 2A**). As the presence of an acidic phospholipid seemed to be important for the binding of ghrelin and as some peptides specifically bind to a specific headgroup [56,57], we investigated whether acylated and unacylated ghrelin could bind to different acidic lipids such as PI, PG and PS. The binding of acylated and unacylated

ghrelin to the liposomes was essentially charge dependent as both bound to negatively charged liposomes whatever the acidic phosphatidyl head carrying the negative charge: PI, DMPG (**Fig. 2A**) or PS (data not shown). Moreover, when doubling the number of acidic phospholipids in the liposomes composition (**Fig. 2B**), there was an increase in the bound peptide concentration. This concentration increase was larger for unacylated ghrelin (3.8-fold) than for acylated ghrelin (1.3-fold) (**Fig. 2B**). The acylation of ghrelin induced a highly significant increase (e.g. from 16.8 ± 6.2% to 66.1 ± 7.4% at the neutral lipids to PI molar ratio of 5.15:1) in the binding extent of ghrelin to all negatively charged liposomes (**Figs 2A and 2B**).

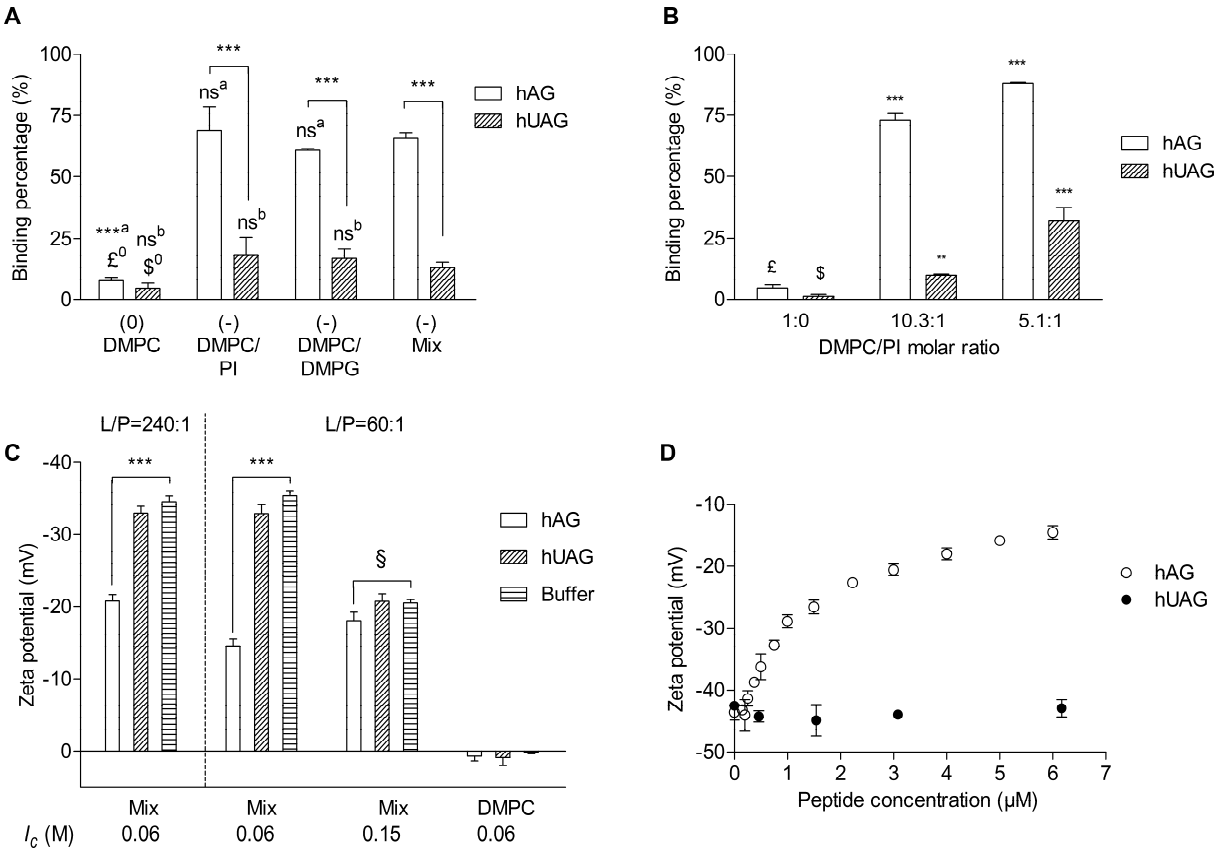


Figure 2 - Electrostatic adsorption of ghrelin to liposomes. Studies conducted at 37°C with acylated ghrelin (hAG) and unacylated ghrelin (hUAG) in Na-citrate 12.5 mM NaCl 14 mM pH 6 (I_c = 0.06 M) buffer (n = 3-6), unless otherwise stated. Mix = Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M). (A) Binding of hAG and hUAG to $LUVET_{200}$ with different charges at a lipid/peptide molar ratio of 50:1. DMPC/PI and DMPC/DMPG molar ratios of 5.15 to 1. $\$^0$ p>0.05, £^{0} p<0.05 compared to 0; ns^a p>0.05, ***^{a}p<0.0001 compared to Mix hAG; ns^b p>0.05 compared to Mix hUAG. (B) Binding of hAG and hUAG to DMPC/PI $LUVET_{200}$ with increasing charges at a lipid/peptide molar ratio of 200:1. For DMPC, \$ p>0.05, £ p<0.05 compared to 0; ***p<0.0001 compared to all other groups; **p<0.001 compared to hUAG 1:0. (C) Zeta potential of Mix $LUVET_{200}$ after addition of peptide (200 µg/ml) or buffer at a lipid/peptide molar ratio of either 240:1 or 60:1. Mix (I_c = 0.15 M) buffer = Na-citrate 12.5 mM NaCl 103.2 mM pH 6. § p<0.05; ***p<0.0001. Only significant differences with the buffer within each condition tested are indicated. (D) Zeta potential of Mix $LUVET_{100}$ as a function of hAG or hUAG concentrations. Lipid total concentration was 60.5 µM. Peptide concentrations are the total peptide concentrations. V = 1 ml.

Zeta potential (ζ) measurements revealed that, in any case, unacylated ghrelin did not affect the ζ of the liposomes (**Figs. 2C and 2D**). However, even though acylated ghrelin did not affect the ζ of neutral liposomes (DMPC), the ζ of negatively charged liposomes made of Chol/SM/PC/PI/PE became less negative in presence of acylated ghrelin, e.g. from –34 ± 1 mV to –21 ± 1 mV at a lipid/peptide molar ratio of 240:1 in 12.5 mM Na-citrate 14 mM NaCl buffer (**Figs. 2C and 2D**). The ζ of DMPC/PI (5.15:1; M/M) liposomes at lipid/peptide molar ratios of 200:1 and 50:1 were similar to those of Chol/SM/PC/PI/PE liposomes shown in **Fig. 2C** in presence of acylated and unacylated ghrelin (data not shown).

The electrostatic forces which influence the binding of the peptides were also investigated by examining the pH (4 and 6) and ionic strength effects (0.06 and 0.15). At pH 4, the theoretical charge (~7.3) of ghrelin is increased compared with pH 6 (~5.5) whereas the liposomes charge is not much affected as the intrinsic phosphate pK_a of phospholipids is ranging from 1.2 to 2.5 [58]. Acylated ghrelin binding to Chol/PC/SM/PI/PE liposomes was unaffected by lowering the pH whereas it decreased with the increase in ionic strength (**Fig. 3**). Unacylated ghrelin binding to the liposomes increased while lowering the pH and was suppressed at higher ionic strength (**Fig. 3**). Why was the binding of acylated ghrelin at pH 4 unaffected? There are several possible explanations, among which: (i) all the acylated ghrelin electrostatic binding sites of the liposomes are already saturated at pH 6; (ii) the hydrophobic interaction forces are stronger than the electrostatic interactions forces. In the case of increased ionic strength, more screening ions are present and the distance that separates the plane of shear and the vesicle surface diminishes. This might explain why there was less peptide bound and a lower increase of the zeta potential for acylated ghrelin (**Fig. 2C**) due to a reduced amount of peptide inside that plane. Indeed, as there are more screening ions in the case of increased ionic strength, the membrane negative surface potential is reduced which facilitates the adsorption of anions and hinders the binding of cations [58]. Based on these hypotheses, hydrophobic interactions have been investigated (see Section 3.4.).

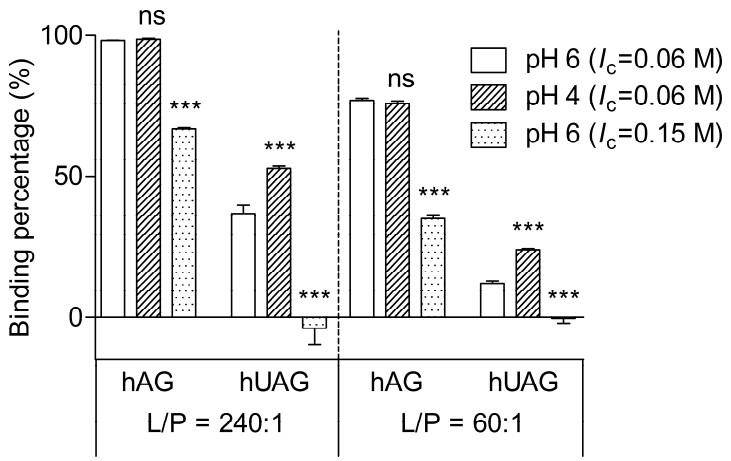


Figure 3 - Ionic strength and pH influence on ghrelin binding to liposomes. Acylated ghrelin (hAG) and unacylated ghrelin (hUAG) binding to Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.4, M/M) $LUVET_{200}$ at T = 37 °C (n = 3-6). pH 4 (I_c = 0.06 M) = 12.5 mM Na-citrate 46.2 mM NaCl pH 4 buffer; pH 6 (I_c = 0.06 M) = 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer; pH 6 (I_c = 0.15 M) = 12.5 mM Na-citrate 103.2 mM NaCl pH 6 buffer. ns $p>0.05$, ***$p<0.0001$ vs. pH 6 (I_c = 0.06 M) for each peptide and each lipid/peptide ratio; $p<0.0001$ for hAG vs. hUAG within each condition tested.

3.3. LIPID-PEPTIDE INTERACTIONS: EFFECT OF AND ON MEMBRANE FLUIDITY

To determine if the binding of ghrelin to lipids was affected by the lipid state or affected the membrane fluidity, we investigated (i) the binding of ghrelin to liposomes exhibiting a phase transition; and (ii) the change of membrane fluidity.

The lipid state influence experiments were conducted at 4 and 37 °C with DMPC, DMPC/PI and DMPC/DMPG liposomes. The transition temperatures of DMPC, DMPC/PI (5.15:1, M/M) and DMPC/DMPG (5.15:1, M/M) liposomes (MLV) in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer were 20.1 °C, 17.6 °C and 22.9 °C, respectively, as determined by differential scanning calorimetry (from 0 to 55 °C at 1 °C/min). The binding experiments carried out by ultracentrifugation revealed no significant difference in acylated and unacylated ghrelin binding to these liposomes, at a lipid/peptide molar ratio of 50 to 1, below (4 °C) and above (37 °C) T_c (**Fig. 4**).

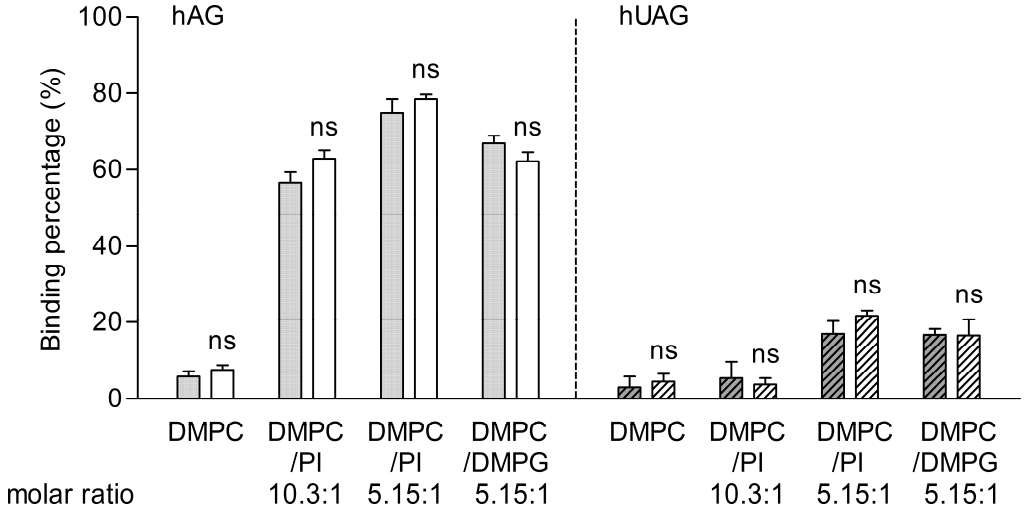


Figure 4 - Effect of lipid fluidity on ghrelin binding to liposomes. Acylated ghrelin (plain bars) and unacylated ghrelin (dashed bars) binding to DMPC or DMPC/PI $LUVET_{200}$ at a lipid/peptide molar ratio of 50:1 in 12.5 mM Na-citrate 14 mM NaCl pH 6 buffer (I_c = 0.06 M) at 4 °C (grey bars) and 37 °C (white bars). ns p>0.05 for 4 °C vs. 37 °C within each condition tested (n = 3)**.**

The change of membrane fluidity was assessed by the anisotropy change of fluorescent probes, DPH and TMA-DPH, which are located in the bilayer lipid compartment. The polarization of DPH and TMA-DPH in Chol/PC/SM/PI/PE liposomes ($LUVET_{200}$), at a 240 to 1 lipid/peptide molar ratio, were not statistically affected (p>0.05) by the presence of acylated or unacylated ghrelin at any of the investigated temperatures (**Fig. 5**).

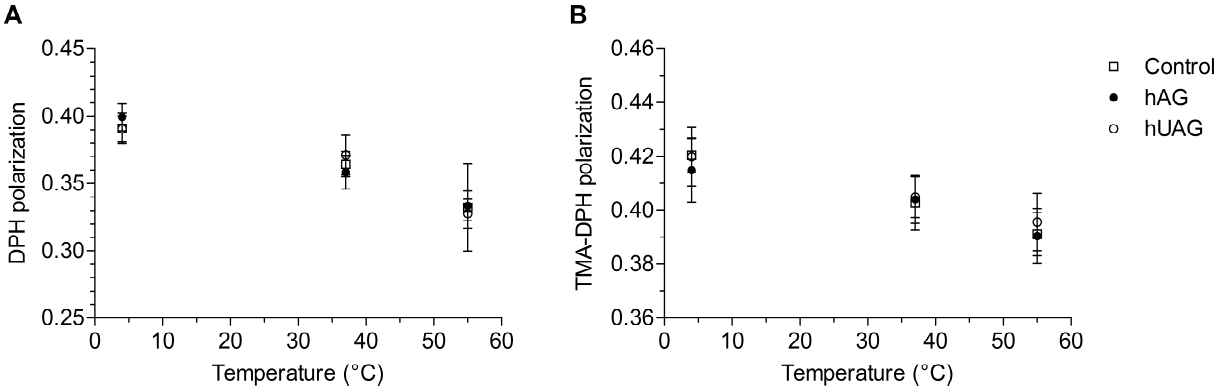


Figure 5 - Effect of acylated (hAG) and unacylated ghrelin (hUAG) on the polarization of fluorescence of (A) DPH and (B) TMA-DPH in Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.7, M/M) $LUVET_{200}$ in Na-citrate 12.5 mM NaCl 14 mM pH 6 buffer (I_c = 0.06 M) at a lipid/peptide molar ratio of 240 :1. Controls are liposomes with no peptide. No statistical difference compared to the control was observed for any probe at any temperature (one-way ANOVA, p>0.05).

3.4. HYDROPHOBIC INTERACTIONS

The overall partition coefficient of ghrelin into membranes, K_{app}, which takes into account the hydrophobic (K_p) and electrostatic (K_{el}) contributions, was quite easily determined from binding experiments. However, quantitative analysis of ghrelin hydrophobic interactions with negatively charged membranes was not straightforward due to the electrostatic interactions. Therefore we used a binding model, adapted from Seelig et al. [35,36,44] and described in Section 2.12., in order to calculate the chemical (hydrophobic) partition coefficient K_p from the binding and zeta potential measurements at 37 °C.

The acylated ghrelin apparent binding coefficient (Eq. (1)) to Chol/PC/SM/PI/PE liposomes in pH 6 citrate buffer (I_c = 0.06 M) varied from 775 to 8000 M^{-1} whereas the unacylated ghrelin apparent partition coefficient varied from 75 to 175 M^{-1} at external lipid/peptide molar ratios of 6:1 to 188:1. Although ghrelin theoretical charge is z = +5.5, we found that its effective charge perceived by the membrane was smaller (z_{eff} = +2.0), as it has already been shown for other basic peptides in presence of acidic lipids [44,59,60]. The effective charge is the number of charges of the peptide that are available for the binding. The acylated ghrelin hydrophobic partition coefficient, found after correction for electrostatic effects by means of the Gouy-Chapman theory, was 74 M^{-1}. However, with our method we were unable to determine the hydrophobic partition coefficient of unacylated ghrelin as on the one hand, the heats in lipid into peptide titration experiments were too small compared to the blanks, and on the other hand addition of unacylated ghrelin, even at lipid/peptide ratios as low as 1.2, to negatively charged liposomes did not induce any change in the zeta potential of these liposomes.

Thermodynamically, membrane partitioning is not only characterized by its partition coefficient, K, but also by the Gibbs energy of reaction, ΔG, the reaction enthalpy, ΔH, and the reaction entropy, ΔS. The Gibbs energy of reaction, ΔG, depends upon the partition coefficient as follows: $\Delta G = - RT \ln 55.5\, K$, where 55.5 accounts for the cratic contribution [61]. As the Gibbs energies of electrostatic and hydrophobic interactions are additive, the electrostatic contribution, K_{el}, can be calculated from the K_{app} and the K_p. Therefore for acylated ghrelin as we had $K_{app,max} = K_p\, K_{el,max}$ = 8000 M^{-1} and 300 M^{-1} in pH 6 citrate buffer (at an external lipid/peptide molar ratio of 120:1) with ionic strengths of 0.06 and 0.15 M, respectively, and a hydrophobic partition coefficient, K_p = 74 M^{-1}, thus $K_{el,max}$ were 108 M^{-1} and 4 M^{-1} at the ionic strengths of 0.06 and 0.15 M, respectively. This is a hint that the

electrostatic contribution at physiological ion concentration (I_c = 0.15 M) is rather small compared to the hydrophobic contribution but rather similar at a lower ionic strength.

For acylated ghrelin, hydrophobic binding alone led to ΔG_p = -5.1 kcal/mol, whereas electrostatic and hydrophobic bindings together yielded a ΔG_{app} max = -8.0 kcal/mol.

The binding of acylated and unacylated ghrelin to Chol/PC/SM/PI/PE liposomes, as measured by peptide-into-lipid isothermal titration calorimetry at high lipid/peptide ratios, were exothermic. The overall binding reaction enthalpies were - 4.22 ± 0.20 kcal/mol and - 0.73 ± 0.04 kcal/mol for acylated ghrelin at ionic strengths of 0.06 and 0.15 M, respectively, and - 0.19 ± 0.04 kcal/mol for unacylated ghrelin at a ionic strength of 0.06 M (**Fig. 6**). Since $\Delta G = \Delta H - T\Delta S$, the reaction entropy for acylated ghrelin binding to Chol/PC/SM/PI/PE liposomes was positive.

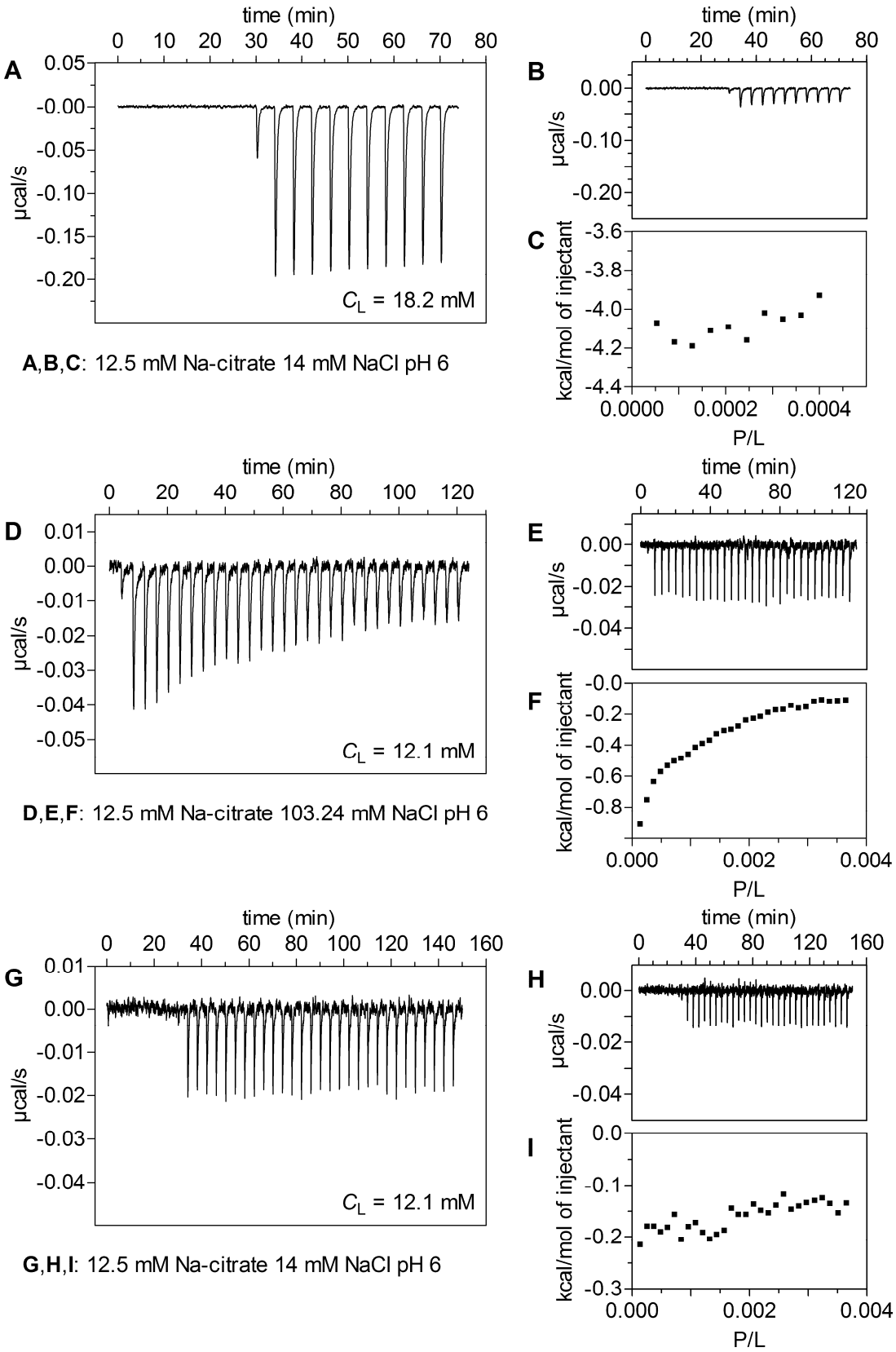


Figure 6 - Isothermal titration calorimetry of (A to F) acylated and (G to I) unacylated ghrelin (200µM) into Chol/PC/SM/PI/PE (5.7:4.0:1.8:2.7:2.7, M/M) $LUVET_{100}$ or buffer at T = 37°C. (A, D, G) Peptide-into-lipid calorimetric traces. (B,E,H) Peptide-into-buffer calorimetric traces. (C, F, I) Overall binding enthalpy (ΔH) as calculated by subtraction of peptide-into-buffer values from the peptide-into-lipid values in order to account for the heat of dilution. First V_{inj} = 2 µl then V_{inj} = (A to C) 5 µl or (D to I) 10 µl. C_L = lipid concentration, P/L = peptide-lipid molar ratio.

3.5. PEPTIDE CONFORMATION AND LIPID ORIENTATION STUDIES

To determine if the acylation of ghrelin modify the conformation of ghrelin in aqueous solutions, lipids and membranes, the conformation of ghrelin was investigated by (i) *in silico* modelling in an hydrophobic medium, (ii) ATR-FTIR and, (iii) CD spectroscopy.

Our *in silico* 3D model of acylated and unacylated ghrelin in a hydrophobic medium revealed an α-helix (Pro7 – Ser18) surrounded by more flexible arms. As it can be seen in **Fig. 7**, the acylation did not much affect the overall peptide structure.

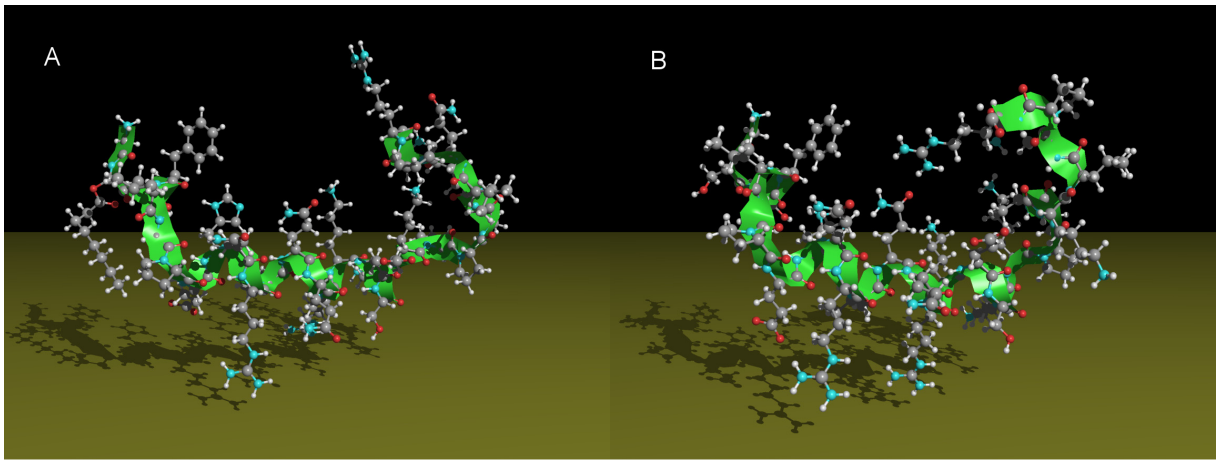


Figure 7 - *In silico* 3D model of ghrelin in a hydrophobic medium. (A) Acylated ghrelin and (B) unacylated ghrelin.

As displayed in Table 1 acylated and unacylated ghrelin structure, as determined by CD spectroscopy, were similar whatever the environment was: water, buffer, micelles (below, at, and above the micellar concentration) or a hydrogen bound stabilizing environment like trifluoroethanol (TFE). This α-helix content increased with the TFE content from 4 ± 1% in absence of TFE to 54 ± 5% in 100% TFE (**Table 1 and Fig. 8**).

Acylated and unacylated ghrelin also adopted an α-helix structure in negatively charged micelles made of sodium dodecyl sufate (SDS), e.g. 30 ± 8% in SDS 40 mM, whereas their conformation did not change in zwitterionic micelles made of dodecyl-phosphocholine (DPC), e.g. 6 ± 1% in DPC 5 mM, in comparison with water (4 ± 1%) and pH 6 buffer (8 ± 2%) (**Table 1 and Fig. 8**).

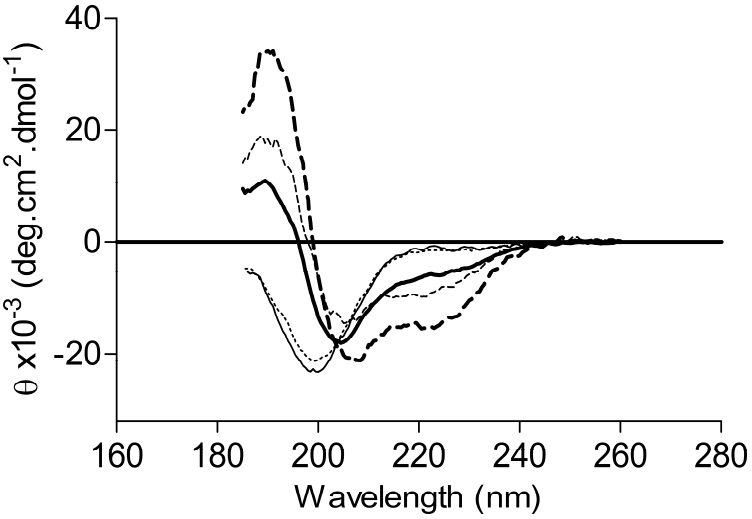


Figure 8 - Representative CD spectra of ghrelin. Acylated ghrelin in water (——), DPC 5 mM (·····), SDS 40 mM (——), TFE 80% (- - - -) or TFE 100% (— — ·) at 22 °C.

Table 1. Ghrelin structure by circular dichroism (CD). Secondary structural analysis of acylated ghrelin (hAG) and unacylated ghrelin (hUAG) in various environments as measured by CD spectroscopy and calculated using SELCON 3, CONTIN/LL and CDSSTR algorithms from the CDPRO software package. Values represent means of the 3 algorithms ± SD. Pipes pH 6 = Na-Na_2 Pipes 10mM pH 6 buffer; DPC, dodecyl-phosphocholine; SDS, sodium dodecyl sulphate; TFE, trifluoroethanol.

Environment	Secondary Structure (%)							
	hAG				**hUAG**			
	Helix	Strand	Turn	Random	Helix	Strand	Turn	Random
Water	4 ± 1	14 ± 4	10 ± 4	71 ± 8	4 ± 1	16 ± 6	11 ± 5	68 ± 8
Pipes pH 6	8 ± 2	16 ± 4	12 ± 2	63 ± 7	5 ± 1	15 ± 1	10 ± 2	69 ± 2
DPC 0.2 mM	4 ± 1	12 ± 1	10 ± 1	73 ± 3	4 ± 0	12 ± 4	10 ± 3	74 ± 7
DPC 1.1 mM	5 ± 1	12 ± 2	9 ± 1	75 ± 3	4 ± 0	9 ± 3	9 ± 2	78 ± 6
DPC 5 mM	6 ± 1	13 ± 4	10 ± 2	72 ± 5	4 ± 0	10 ± 4	9 ± 3	77 ± 8
SDS 8.1 mM	31 ± 7	17 ± 2	19 ± 4	32 ± 2	35 ± 7	12 ± 6	18 ± 5	34 ± 9
SDS 40 mM	30 ± 8	20 ± 7	21 ± 2	28 ± 1	32 ± 7	19 ± 0	18 ± 2	31 ± 3
TFE 20%	8 ± 2	19 ± 2	12 ± 1	61 ± 3	7 ± 2	17 ± 1	12 ± 1	64 ± 1
TFE 40%	19 ± 1	17 ± 6	15 ± 3	49 ± 9	16 ± 1	20 ± 3	15 ± 3	48 ± 5
TFE 50%	19 ± 1	21 ± 3	16 ± 2	44 ± 4	21 ± 2	18 ± 4	14 ± 2	48 ± 3
TFE 60%	25 ± 3	21 ± 3	18 ± 3	36 ± 8	29 ± 3	12 ± 3	16 ± 1	42 ± 6
TFE 80%	34 ± 3	16 ± 2	19 ± 2	29 ± 4	38 ± 2	12 ± 1	20 ± 5	30 ± 3
TFE 100%	54 ± 5	8 ± 3	14 ± 3	23 ± 5	47 ± 3	8 ± 5	15 ± 3	30 ± 6

Acylated and unacylated ghrelin conformations, as determined by ATR-FTIR, slightly changed towards the α-region in presence of negatively charged DMPC/DMPG liposomes (**Fig. 9**). However they did not induce any change in lipid orientation (data not shown). This α-shift was more important for acylated ghrelin than for unacylated ghrelin (**Fig. 9**).

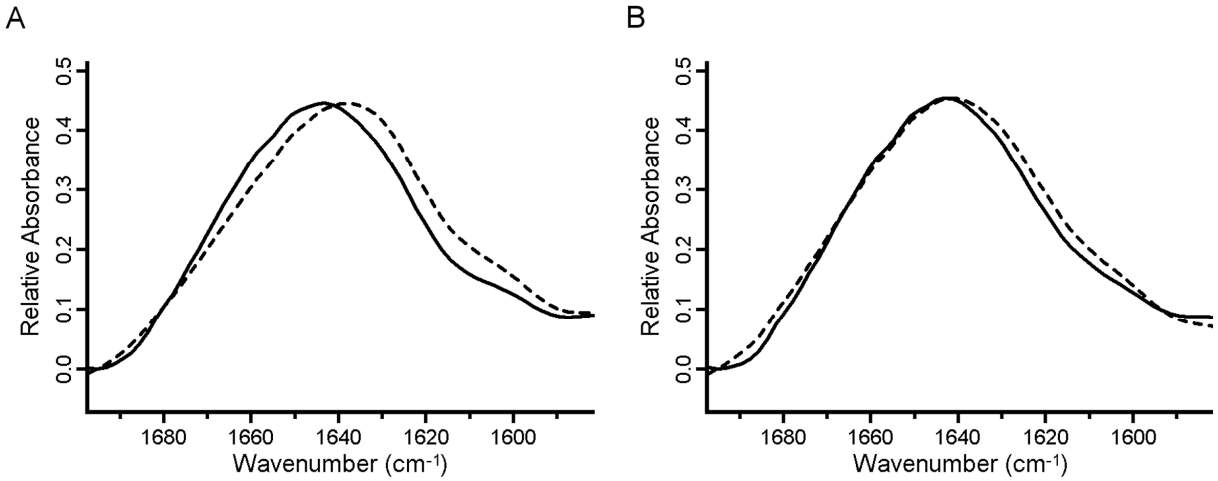


Figure 9 - Acylation influence on ghrelin structure in aqueous and membrane environments by infrared spectroscopy. ATR-FTIR spectra (1700-1580 cm^{-1}) at room temperature in NaCl 3 mM (– –) or in presence of DMPC/DMPG (5.15:1, M/M) $LUVET_{200}$ in NaCl 3 mM (—). (A) Acylated ghrelin; (B) unacylated ghrelin.

3.6. TRANSMEMBRANE AND CELLULAR TRANSPORT STUDIES

As ghrelin strongly binds to membranes, we tested whether ghrelin could cross lipid bilayers and/or model epithelia and how the acylation influenced these phenomena. The ability of acylated and unacylated ghrelin to diffuse passively through lipid bilayers was assessed by PAMPA with lipid bilayers composed of either DOPC or a lecithin mixture with an excess of negative charges. Neither acylated nor unacylated ghrelin was detected in the receptor compartments (LOQ = 10 pg/ml; n = 6). The calculated P_{app} was thus below 2.5×10^{-12} cm/s for both peptides. Experiments to demonstrate the ability of ghrelin to cross intestinal epithelia were conducted on monocultures of Caco-2 and co-cultures of Caco-2/M-like cells. Both acylated and unacylated ghrelin were able to cross cell monolayers (**Fig. 10**). As acylated ghrelin is easily converted into unacylated ghrelin both peptides were dosed in the receptor compartments. M-like cells presence in the *in vitro* cell co-culture model resulted in a 4.3-fold increase in the transport of acylated ghrelin and a 2.9-fold increase for unacylated ghrelin, in comparison with cell monolayers consisting exclusively of Caco-2 cells. This increase was even more pronounced for intact acylated ghrelin (5.7-fold). The transport of unacylated ghrelin was higher than acylated ghrelin transport in mono-cultures but not in co-cultures.

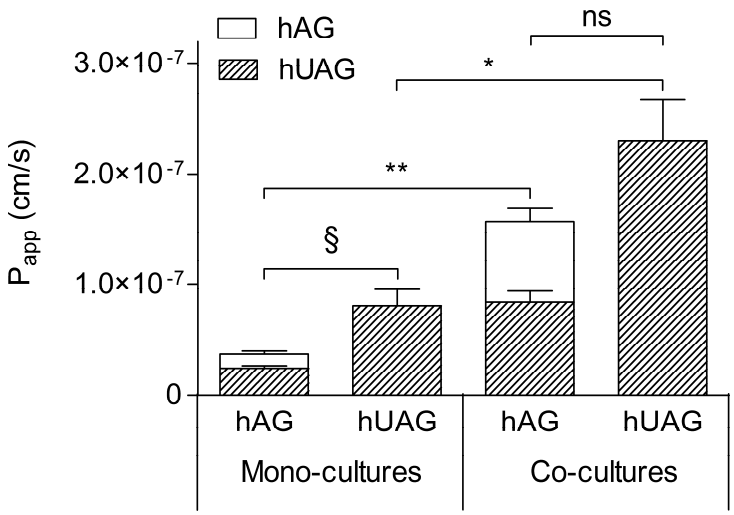


Figure 10 - Influence of acylation and presence of M-like cells on ghrelin transport by human intestinal cells. Transport of acylated ghrelin (hAG) and unacylated ghrelin (hUAG) in mono-culture of Caco-2 cells and co-cultures of Caco-2 and Raji cells at 37 °C (n = 6). Peptides on the abscissa are the peptides deposited in the donor compartments. Bars represent the means values ± SEM of the collected peptides in the receptor compartments that were dosed by EIA. Statistical analysis by unpaired t-test. ns, §: comparison of hAG total vs. hUAG on the same cell model, ns: no statistical difference ($p>0.05$), § $p<0.05$; *,**: comparison between the mono- and co-cultures for each peptide, *$p<0.005$, **$p<0.001$.

4. DISCUSSION

It is known that ghrelin binds to the ghrelin receptor and that the Ser^3-acylation is necessary to activate it [1,62]. However, little is known about the role of the octanoyl in ghrelin membrane binding and transport [14]. Therefore, the aim of this paper was to investigate the roles of this ghrelin octanoyl moiety in membrane binding and associated phenomena in order to give new insights about its role in ghrelin receptor affinity.

After showing that the octanoyl significantly enhanced the binding of ghrelin to model and biological membranes, we further studied the underlying mechanisms and possible biological implications. The electrostatic attraction/ membrane adsorption of ghrelin were investigated, as well as the peptides penetration in the headgroup region or in the hydrophobic backbone of the membrane and the conformational change of the peptides. Based on these observed phenomena we will try to explain the differences in acylated and unacylated ghrelin receptor binding.

4.1. ELECTROSTATIC-HYDROPHOBIC INTERACTIONS

Knowing that acylated and unacylated ghrelin are highly basic peptides we first investigated the possible interactions between ghrelin and neutral or acidic lipids. Acylated and unacylated ghrelin both interacted electrostatically with the membrane as the presence and the amount of acidic lipids like PI, PS and PG greatly enhanced their binding to liposomes. Our results suggest that electrostatics play a more important role in unacylated ghrelin binding to liposomes than for the acylated form as: (i) unacylated ghrelin did not bind to neutral liposomes whereas acylated ghrelin did (**Fig. 2A**); (ii) doubling the number of negative charges of the liposomes induced a larger increase in binding for unacylated ghrelin than for acylated ghrelin (**Fig. 2B**); (iii) increasing the peptide charge by about 2 units only increased the binding of unacylated ghrelin to the acidic liposomes (**Fig. 3**); (iv) increasing the screening of the liposomes surface charge by addition of NaCl decreased the binding of acylated ghrelin to the liposomes but suppressed it for unacylated ghrelin (**Fig. 3**).

Complementary results such as very weak binding heat in ITC studies and no zeta potential modification further indicate that unacylated ghrelin does not specifically adsorb to negatively charged liposomes and that the nature of their interactions are Coulombic which are long-range electrostatic interaction forces [38]. Indeed, on the one hand the very low enthalpy

measured by ITC suggested pure electrostatic interactions [63]. On the other hand (i) zeta potential of negatively charged liposomes was unaffected by unacylated ghrelin even at high concentrations (**Fig 2D**) and (ii) the zeta potential is a technique that measures the potential difference between the hydrodynamic plane of shear of the liposomes and the bulk solution; (iii) the Stern layer is located closer within the plane of shear; (iv) specifically adsorbed ions charges are located within the Stern layer [38]. Acylated ghrelin is also electrostatically attracted to acidic liposomes and results indicate that at least a part of it must be located very close to the surface as the zeta potential is measured near the vesicles surface i.e. 2 to 8.2 Å [43,64,65]. We have also shown that acylated ghrelin also interacts with the lipids with a hydrophobic partition coefficient ($74M^{-1}$) comparable to that of penetratin [66], somatostatin analogues [67] and magainin 2 amide [61]. Additionally the binding of acylated ghrelin to negatively charged liposomes is entropy and enthalpy driven as it does for other acylated peptides [68,69]. The calculated ΔG = -5.1 kcal/mol is in good agreement with the octanoyl penetration into hydrocarbon region of the membrane [69,70]. However, acylated ghrelin is unlikely to be deeply inserted in the membrane as (i) it did neither interact with DPH nor with TMA-DPH and that both these probes have a deep DPH location within the bilayer [71]; (ii) ghrelin binding to the liposomes was not affected by the membrane fluidity state; (iii) its hydrophobic partition coefficient is not large [61]. As ghrelin is an endogenous hormone, it is not so surprising that it does not perturb the membrane organization.
Therefore, we hypothesize that the octanoyl penetrates into the hydrophobic backbone while the ghrelin α-helix lies in the headgroup region of the membrane.

4.2. PEPTIDE CONFORMATION

Ghrelin does neither disturb the membrane, nor does it cross lipid bilayers as shown in our PAMPA experiments. Nevertheless does ghrelin become structured in contact with membranes, even though it has been reported to adopt a random coil conformation in water [72]? It is known that peptides and proteins with a random coil conformation in water can adopt an α-helical or β-sheet structure in membranes [73-75]. CD and ATR-FTIR spectroscopy studies as well as computational modelling revealed that both acylated and unacylated ghrelin adopt mainly the same structure in any of the environments tested although acylated ghrelin in presence of model membranes adopted a slightly more structured conformation than unacylated ghrelin towards the α-helical region. The absence of structuring

effect of DPC in opposition to SDS confirmed the necessity of a negative charge. Our *in silico* 3D model of acylated ghrelin in a hydrophobic medium obtained using the stochastic procedure PepLook algorithm is in accordance with the molecular dynamics simulation studies of Beevers and Kukol in 2006 [76]. Indeed, our simulations also revealed a central α-helix surrounded by more flexible arms that confers a "staple" shape aspect to the peptide (**Fig. 7**). Our computational modelling is, as far as we know, the first that compared both forms of ghrelin and is in accordance with our experimental results.

4.3. CELLULAR TRANSPORT

We have shown that ghrelin is unable to cross lipid bilayers by itself. The reason for this is probably that the octanoyl arm is too short for the peptide to translocate. Indeed, peptides able to cross the bilayer usually need an acyl modification of at least 14 to 16 carbons which roughly corresponds to half the bilayer thickness [77,78]. In Caco-2 cells, the unacylated form of ghrelin was better transported than the acylated form. This is in contradiction with previous publications about the transport of other acylated peptides like tetragastrin and thyrotropin releasing hormone by Caco-2 cells [18,79]. This is not, however, highly surprising in the case of ghrelin. Indeed, Banks et al. already reported in 2002 that, in mice, unacylated ghrelin was transported through the blood brain barrier by an unsaturable mechanism in the blood-to-brain direction whereas acylated ghrelin was transported by a saturable mechanism in the brain-to-blood direction [15]. M-like cells presence not only enhanced the transport of both forms of ghrelin but also the proportion of intact ghrelin. These results are in agreement with previously published results from our laboratory on helodermin transport [80].

4.4. GHRELIN ACYLATION VS. RECEPTOR ASSOCIATION

As the acylation of ghrelin seems to be essential for efficient membrane binding, we were interested in the possible implications for receptor binding. Indeed, the membrane could act as a catalyst for ghrelin receptor binding as it does for other peptides [9,10,81]. The ghrelin third amino acid residue has to carry a bulky hydrophobic group for its maximum activity mediated by the ghrelin receptor [62,82]. Concerning the amino acid sequence, it has been shown *in vitro* that only the first five amino acids are necessary for full activation of the ghrelin receptor [62]. If we look at the hydrophilicity/hydrophobicity profile of ghrelin, we see that even though ghrelin is predominantly hydrophilic, these five amino acids along with the

octanoyl belong to the most hydrophobic portion of the peptide. Moreover, in mammals, the first ten amino acids are identical. Altogether these data indicate that the N-terminal region of ghrelin is of central importance to the activity of ghrelin [83]. Furthermore, mutational analysis of the ghrelin receptor revealed that the ghrelin binding pocket is likely to be located in transmembrane domains III, VI and VII which implies that ghrelin needs access to it [84-88]. Besides, even though electrostatics played a determinant role in ghrelin attraction towards the membrane, acylation induced a 120-fold increase, as compared to unacylated ghrelin, in ghrelin local concentration in the membrane and hence in the vicinity of the receptor [10,67,70]. Moreover, at a physiologically relevant ionic strength the observed decrease of ghrelin binding to the liposomes indicates that unacylated ghrelin is much less likely to encounter the membrane, and hence its receptor, than acylated ghrelin. All these could partly explain the difference in the concentrations necessary for the full activation of the ghrelin receptor by unacylated (μM) and acylated (nM) ghrelin observed by Gauna and co-workers [8]. Even if ghrelin is directed to the membranes, it is unlikely that the octanoyl plays a role in ghrelin secretion as in GOAT-null mice unacylated ghrelin is detected in the bloodstream [3]. We have thus demonstrated that close association of ghrelin with membranes requires Ser^3 *O*-octanoylation as well as the presence of basic residues. These results are in accordance with those obtained for other peptides like the myristoylated alanine-rich C kinase substrate effector domain (MARKS ED) [57,89].

In conclusion, the unacylated ghrelin seems to be only driven towards the membrane by electrostatic attraction between its basic residues and the negatively charged lipids of the membrane, leading to a lower affinity for the membrane than acylated ghrelin. Acylated ghrelin is also electrostatically attracted but in addition, it penetrated into the headgroup and lipid backbone regions. The membrane probably acts as a "catalyst" for ghrelin binding to its receptor. In this process the *O*-octanoylation of ghrelin is assumed to (i) partition ghrelin into the lipids; (ii) increase local concentration in the vicinity of the receptor; (iii) bring ghrelin to membrane where its binding pocket is located; (iv) optimize the conformation of ghrelin for improving its docking to the ghrelin receptor. This latest hypothesis needs to be confirmed. Moreover, ghrelin conformational flexibility may be associated with its various roles in different tissues. However, it seems that *O*-octanoylation of ghrelin does not help its transport across membranes and epithelia.

5. REFERENCES

[1] Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H., and Kangawa K., Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature, 402 (1999) 656-660.

[2] Hosoda H., Kojima M., Mizushima T., Shimizu S., and Kangawa K., Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing, J. Biol. Chem., 278 (2003) 64-70.

[3] Gutierrez J.A., Solenberg P.J., Perkins D.R., Willency J.A., Knierman M.D., Jin Z., Witcher D.R., Luo S., Onyia J.E., and Hale J.E., Ghrelin octanoylation mediated by an orphan lipid transferase, Proc. Natl. Acad. Sci. U. S. A, 105 (2008) 6320-6325.

[4] Yang J., Brown M.S., Liang G., Grishin N.V., and Goldstein J.L., Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone, Cell, 132 (2008) 387-396.

[5] Kojima M. and Kangawa K., Ghrelin: structure and function, Physiol. Rev., 85 (2005) 495-522.

[6] Leite-Moreira A.F. and Soares J.B., Physiological, pathological and potential therapeutic roles of ghrelin, Drug Discov. Today, 12 (2007) 276-288.

[7] Soares J.B. and Leite-Moreira A.F., Ghrelin, des-acyl ghrelin and obestatin: three pieces of the same puzzle, Peptides, 29 (2008) 1255-1270.

[8] Gauna C., van de Zande B., van Kerkwijk A., Themmen A.P., van der Lely A.J., and Delhanty P.J., Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R), Mol. Cell Endocrinol., 274 (2007) 30-34.

[9] Sargent D.F. and Schwyzer R., Membrane lipid phase as catalyst for peptide-receptor interactions, Proc. Natl. Acad. Sci. U. S. A, 83 (1986) 5774-5778.

[10] Castanho M.A. and Fernandes M.X., Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the "membrane catalysis" model, Eur. Biophys. J., 35 (2006) 92-103.

[11] Schwyzer R., 100 years lock-and-key concept: are peptide keys shaped and guided to their receptors by the target cell membrane?, Biopolymers, 37 (1995) 5-16.

[12] Seelig J., Titration calorimetry of lipid-peptide interactions, Biochim. Biophys. Acta, 1331 (1997) 103-116.

[13] Seelig J., Thermodynamics of lipid-peptide interactions, Biochim. Biophys. Acta, 1666 (2004) 40-50.

[14] Dehlin E., Liu J., Yun S.H., Fox E., Snyder S., Gineste C., Willingham L., Geysen M., Gaylinn B.D., and Sando J.J., Regulation of ghrelin structure and membrane binding by phosphorylation, Peptides, 29 (2008) 904-911.

[15] Banks W.A., Tschop M., Robinson S.M., and Heiman M.L., Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure, J. Pharmacol. Exp. Ther., 302 (2002) 822-827.

[16] Staes E., Rozet E., Ucakar B., Hubert P., and Préat V., Validation of a method for the quantitation of ghrelin and unacylated ghrelin by HPLC, J. Pharm. Biomed. Anal., 51 (2010) 633-639.

[17] Asada H., Douen T., Waki M., Adachi S., Fujita T., Yamamoto A., and Muranishi S., Absorption characteristics of chemically modified-insulin derivatives with various fatty acids in the small and large intestine, J. Pharm. Sci., 84 (1995) 682-687.

[18] Fujita T., Kawahara I., Quan Y., Hattori K., Takenaka K., Muranishi S., and Yamamoto A., Permeability characteristics of tetragastrins across intestinal membranes using the Caco-2 monolayer system: comparison between acylation and application of protease inhibitors, Pharm. Res., 15 (1998) 1387-1392.

[19] Hope M.J., Bally M.B., Webb G., and Cullis P.R., Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta, 812 (1985) 55-65.

[20] Mathot F., Schanck A., Van Bambeke F., Arien A., Noppe M., Brewster M., and Préat V., Passive diffusion of polymeric surfactants across lipid bilayers, J. Control. Release, 120 (2007) 79-87.

[21] Bartlett G.R., Phosphorus assay in column chromatography, J. Biol. Chem., 234 (1959) 466-468.

[22] Chanteux H., Paternotte I., Mingeot-Leclercq M.P., Brasseur R., Sonveaux E., and Tulkens P.M., Cell handling, membrane-binding properties, and membrane-penetration modeling approaches of pivampicillin and phthalimidomethylampicillin, two basic esters of ampicillin, in comparison with chloroquine and azithromycin, Pharm. Res., 20 (2003) 624-631.

[23] Shinitzky M. and Barenholz Y., Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate, J. Biol. Chem., 249 (1974) 2652-2657.

[24] Mingeot-Leclercq M.P., Lins L., Bensliman M., Van Bambeke F., Van Der Smissen P., Peuvot J., Schanck A., and Brasseur R., Membrane destabilization induced by beta-amyloid peptide 29-42: importance of the amino-terminus, Chem. Phys. Lipids, 120 (2002) 57-74.
[25] Andrushchenko V.V., Vogel H.J., and Prenner E.J., Optimization of the hydrochloric acid concentration used for trifluoroacetate removal from synthetic peptides, J. Pept. Sci., 13 (2007) 37-43.
[26] Haris P.I. and Chapman D., The conformational analysis of peptides using Fourier transform IR spectroscopy, Biopolymers, 37 (1995) 251-263.
[27] Lins L. and Brasseur R., Tilted peptides: a structural motif involved in protein membrane insertion?, J. Pept. Sci., 14 (2008) 416-422.
[28] Goormaghtigh E., Raussens V., and Ruysschaert J.M., Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes, Biochim. Biophys. Acta, 1422 (1999) 105-185.
[29] Lins L., Brasseur R., Rosseneu M., Yang C.Y., Sparrow D.A., Sparrow J.T., Gotto A.M., Jr., and Ruysschaert J.M., Structure and orientation of apo B-100 peptides into a lipid bilayer, J. Protein Chem., 13 (1994) 77-88.
[30] Thomas A., Deshayes S., Decaffmeyer M., Van Eyck M.H., Charloteaux B., and Brasseur R., Prediction of peptide structure: how far are we?, Proteins, 65 (2006) 889-897.
[31] Lagarias J.C., Reeds J.A., Wright M.H., and Wright P.E., Convergence properties of the Nelder-Mead simplex method in low dimensions, SIAM J. Optim., 9 (1998) 112-147.
[32] Nelder J.A. and Mead R., A simplex method for function minimazation, Comput. J., 7 (1965) 308-313.
[33] Wiseman T., Williston S., Brandts J.F., and Lin L.N., Rapid measurement of binding constants and heats of binding using a new titration calorimeter, Anal. Biochem., 179 (1989) 131-137.
[34] Ziegler A., Blatter X.L., Seelig A., and Seelig J., Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis, Biochemistry, 42 (2003) 9185-9194.
[35] Beschiaschvili G. and Seelig J., Peptide binding to lipid bilayers. Binding isotherms and zeta-potential of a cyclic somatostatin analogue, Biochemistry, 29 (1990) 10995-11000.
[36] Wieprecht T. and Seelig J., Isothermal Titration Calorimetry for Studying Interactions between Peptides and Lipid Membranes, in: Simon S.A. and McIntosh T.J. (Eds.), Peptide-lipid interactions, Academic Press, San Diego, California, (2002) 31-56.
[37] McLaughlin S., Electrostatic Potentials at Membrane-Solution Interfaces, in: Bronner F. and Kleinzeller A. (Eds.), Current Topics in Membranes and Transport, Volume 9, Academic Press, New York, (1977) 71-144.
[38] Delgado A.V., Gonzalez-Caballero F., Hunter R.J., Koopal L.K., and Lyklema J., Measurement and interpretation of electrokinetic phenomena, J. Colloid Interface Sci., 309 (2007) 194-224.
[39] Rasmusson M., Polymer Particles and Polyelectrolytes: Surface Electrical Properties, in: Somasundaran P. and Hubbard A. (Eds.), Encyclopedia of Surface and Colloid Science, Taylor & Francis, London, (2006) 4900-4920.
[40] Hiemenz P.C. and Rajagopalan R., Principles of colloid and surface chemistry, Marcel Dekker, New York (1997).
[41] Seelig A., Local anesthetics and pressure: a comparison of dibucaine binding to lipid monolayers and bilayers, Biochim. Biophys. Acta, 899 (1987) 196-204.
[42] Ruso J.M., Besada L., Martinez-Landeira P., Seoane L., Prieto G., and Sarmiento F., Interactions between liposomes and cations in aqueous solution, J. Liposome Res., 13 (2003) 131-145.
[43] McLaughlin S., The electrostatic properties of membranes, Annu. Rev. Biophys. Biophys. Chem., 18 (1989) 113-136.
[44] Klocek G., Schulthess T., Shai Y., and Seelig J., Thermodynamics of melittin binding to lipid bilayers. Aggregation and pore formation, Biochemistry, 48 (2009) 2586-2596.
[45] Matos C., de Castro B., Gameiro P., Lima J.L., and Reis S., Zeta-potential measurements as a tool to quantify the effect of charged drugs on the surface potential of egg phosphatidylcholine liposomes, Langmuir, 20 (2004) 369-377.
[46] des Rieux A., Ragnarsson E.G., Gullberg E., Préat V., Schneider Y.J., and Artursson P., Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, Eur. J. Pharm. Sci., 25 (2005) 455-465.
[47] des Rieux A., Fievez V., Théate I., Mast J., Préat V., and Schneider Y.J., An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells, Eur. J. Pharm. Sci., 30 (2007) 380-391.
[48] Cunderlikova B. and Moan J., Electrostatic properties of cells estimated by absorption and fluorescence spectroscopy, Cell Biochem. Biophys., 41 (2004) 1-10.
[49] Wall J., Ayoub F., and O'Shea P., Interactions of macromolecules with the mammalian cell surface, J. Cell Sci., 108 (Pt 7) (1995) 2673-2682.

[50] Zachowski A., Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement, Biochem. J., 294 (1993) 1-14.
[51] Beaumont N.J., Skinner V.O., Tan T.M., Ramesh B.S., Byrne D.J., MacColl G.S., Keen J.N., Bouloux P.M., Mikhailidis D.P., Bruckdorfer K.R., Vanderpump M.P., and Srai K.S., Ghrelin can bind to a species of high density lipoprotein associated with paraoxonase, J. Biol. Chem., 278 (2003) 8877-8880.
[52] De Vriese C., Hacquebard M., Gregoire F., Carpentier Y., and Delporte C., Ghrelin interacts with human plasma lipoproteins, Endocrinology, 148 (2007) 2355-2362.
[53] Lockridge O., Xue W., Gaydess A., Grigoryan H., Ding S.J., Schopfer L.M., Hinrichs S.H., and Masson P., Pseudo-esterase activity of human albumin: slow turnover on tyrosine 411 and stable acetylation of 82 residues including 59 lysines, J. Biol. Chem., 283 (2008) 22582-22590.
[54] De Vriese C., Gregoire F., Lema-Kisoka R., Waelbroeck M., Robberecht P., and Delporte C., Ghrelin degradation by serum and tissue homogenates: identification of the cleavage sites, Endocrinology, 145 (2004) 4997-5005.
[55] Sedlackova D., Dostalova I., Hainer V., Beranova L., Kvasnickova H., Hill M., Haluzik M., and Nedvidkova J., Simultaneous decrease of plasma obestatin and ghrelin levels after a high-carbohydrate breakfast in healthy women, Physiol Res., 57 Suppl 1 (2008) S29-S37.
[56] Machaidze G. and Seelig J., Specific binding of cinnamycin (Ro 09-0198) to phosphatidylethanolamine. Comparison between micellar and membrane environments, Biochemistry, 42 (2003) 12570-12576.
[57] DiNitto J.P., Cronin T.C., and Lambright D.G., Membrane recognition and targeting by lipid-binding domains, Sci. STKE., 2003 (2003) re16.
[58] Cevc G., Phospholipids handbook, Marcel Dekker, Inc, New York (1993).
[59] Montich G., Scarlata S., McLaughlin S., Lehrmann R., and Seelig J., Thermodynamic characterization of the association of small basic peptides with membranes containing acidic lipids, Biochim. Biophys. Acta, 1146 (1993) 17-24.
[60] Seelig A., Blatter X.L., Frentzel A., and Isenberg G., Phospholipid binding of synthetic talin peptides provides evidence for an intrinsic membrane anchor of talin, J. Biol. Chem., 275 (2000) 17954-17961.
[61] Wenk M.R. and Seelig J., Magainin 2 amide interaction with lipid membranes: calorimetric detection of peptide binding and pore formation, Biochemistry, 37 (1998) 3909-3916.
[62] Bednarek M.A., Feighner S.D., Pong S.S., McKee K.K., Hreniuk D.L., Silva M.V., Warren V.A., Howard A.D., Van Der Ploeg L.H., and Heck J.V., Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a, J. Med. Chem., 43 (2000) 4370-4376.
[63] Arseneault M. and Lafleur M., Isothermal titration calorimetric study of calcium association to lipid bilayers: influence of the vesicle preparation and composition, Chem. Phys. Lipids, 142 (2006) 84-93.
[64] Charlton I.D. and Doherty A.P., Locating the micellar shear plane and its relationship with the Debye screening length, J. Phys. Chem. B, 103 (1999) 5081-5083.
[65] Yang Z., Zhao J., Lu Y., Du Z., and Yang Z., Ultramicroelectrode voltammetric measurement of the hydrodynamic radii and shear plane of micellar particles for sodium dodecylsulfonate in aqueous NaCl solution, Chemical Physics, 307 (2004) 71-75.
[66] Binder H. and Lindblom G., Charge-dependent translocation of the Trojan peptide penetratin across lipid membranes, Biophys. J., 85 (2003) 982-995.
[67] Seelig J., Nebel S., Ganz P., and Bruns C., Electrostatic and nonpolar peptide-membrane interactions. Lipid binding and functional properties of somatostatin analogues of charge z = +1 to z = +3, Biochemistry, 32 (1993) 9714-9721.
[68] Hanakam F., Gerisch G., Lotz S., Alt T., and Seelig A., Binding of hisactophilin I and II to lipid membranes is controlled by a pH-dependent myristoyl-histidine switch, Biochemistry, 35 (1996) 11036-11044.
[69] Peitzsch R.M. and McLaughlin S., Binding of acylated peptides and fatty acids to phospholipid vesicles: pertinence to myristoylated proteins, Biochemistry, 32 (1993) 10436-10443.
[70] Simon S.A. and McIntosh T.J., Peptide-lipid interactions, Academic Press, San Diego, Calif (2002).
[71] Kaiser R.D. and London E., Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth, Biochemistry, 37 (1998) 8180-8190.
[72] Silva Elipe M.V., Bednarek M.A., and Gao Y.D., 1H NMR structural analysis of human ghrelin and its six truncated analogs, Biopolymers, 59 (2001) 489-501.
[73] Dike A. and Cowsik S.M., Membrane-induced structure of scyliorhinin I: a dual NK1/NK2 agonist, Biophys. J., 88 (2005) 3592-3600.
[74] Satheeshkumar K.S. and Jayakumar R., Conformational polymorphism of the amyloidogenic peptide homologous to residues 113-127 of the prion protein, Biophys. J., 85 (2003) 473-483.

[75] Sui S.F., Membrane-induced conformational change of proteins, Adv. Colloid Interface Sci., 85 (2000) 257-267.
[76] Beevers A.J. and Kukol A., Conformational flexibility of the peptide hormone ghrelin in solution and lipid membrane bound: a molecular dynamics study, J. Biomol. Struct. Dyn., 23 (2006) 357-364.
[77] Chopineau J., Robert S., Fenart L., Cecchelli R., Lagoutte B., Paitier S., Dehouck M.P., and Domurado D., Monoacylation of ribonuclease A enables its transport across an in vitro model of the blood-brain barrier, J. Control. Release, 56 (1998) 231-237.
[78] Harishchandran A. and Nagaraj R., Interaction of a pseudosubstrate peptide of protein kinase C and its myristoylated form with lipid vesicles: only the myristoylated form translocates into the lipid bilayer, Biochim. Biophys. Acta, 1713 (2005) 73-82.
[79] Yamamoto A., [Improvement of intestinal absorption of peptide and protein drugs by chemical modification with fatty acids], Nippon Rinsho, 56 (1998) 601-607.
[80] des Rieux A., Fievez V., Momtaz M., Detrembleur C., Alonso-Sande M., Van Gelder J., Cauvin A., Schneider Y.J., and Préat V., Helodermin-loaded nanoparticles: characterization and transport across an in vitro model of the follicle-associated epithelium, J. Control. Release, 118 (2007) 294-302.
[81] Lopes S.C., Fedorov A., and Castanho M.A., Lipidic membranes are potential "catalysts" in the ligand activity of the multifunctional pentapeptide neokyotorphin, Chembiochem., 6 (2005) 697-702.
[82] Matsumoto M., Hosoda H., Kitajima Y., Morozumi N., Minamitake Y., Tanaka S., Matsuo H., Kojima M., Hayashi Y., and Kangawa K., Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides, Biochem. Biophys. Res. Commun., 287 (2001) 142-146.
[83] Kojima M. and Kangawa K., Structure and function of ghrelin, Results Probl. Cell Differ., 46 (2008) 89-115.
[84] Holst B., Holliday N.D., Bach A., Elling C.E., Cox H.M., and Schwartz T.W., Common structural basis for constitutive activity of the ghrelin receptor family, J. Biol. Chem., 279 (2004) 53806-53817.
[85] Holst B., Lang M., Brandt E., Bach A., Howard A., Frimurer T.M., Beck-Sickinger A., and Schwartz T.W., Ghrelin receptor inverse agonists: identification of an active peptide core and its interaction epitopes on the receptor, Mol. Pharmacol., 70 (2006) 936-946.
[86] Holst B., Mokrosinski J., Lang M., Brandt E., Nygaard R., Frimurer T.M., Beck-Sickinger A.G., and Schwartz T.W., Identification of an efficacy switch region in the ghrelin receptor responsible for interchange between agonism and inverse agonism, J. Biol. Chem., 282 (2007) 15799-15811.
[87] Liu G., Fortin J.P., Beinborn M., and Kopin A.S., Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities, J. Pharmacol. Exp. Ther., 322 (2007) 1036-1043.
[88] Pantel J., Legendre M., Cabrol S., Hilal L., Hajaji Y., Morisset S., Nivot S., Vie-Luton M.P., Grouselle D., de Kerdanet M., Kadiri A., Epelbaum J., Le B.Y., and Amselem S., Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature, J. Clin. Invest, 116 (2006) 760-768.
[89] Zhang W., Crocker E., McLaughlin S., and Smith S.O., Binding of peptides with basic and aromatic residues to bilayer membranes: phenylalanine in the myristoylated alanine-rich C kinase substrate effector domain penetrates into the hydrophobic core of the bilayer, J. Biol. Chem., 278 (2003) 21459-21466.

Chapter V.

DISCUSSION AND PERSPECTIVES

Ghrelin is unique in four ways: (i) it is the only hormone peptide to be modified by a fatty acid on one of its lateral chains; (ii) *O*-octanoylation is a medium chain length fatty acid (C_8) modification whereas other acylated peptides are usually acylated with either much shorter (C_2) or much longer fatty acids such as myristoylate (C_{14}) and palmitoylate (C_{16}); (iii) other acylated peptides are usually *N*- or *S*-linked; (iv) its unacylated form is also endogenous and also seems to play physiological roles, and the charge of both forms is identical.

The aims of this thesis were to determine the importance of the unique post-translational modification of ghrelin i.e. a *n*-octanoyl moiety located on its Ser^3 residue, as well as of its basic residues (i) in the extent of ghrelin binding to membranes; (ii) on the mechanisms that drive acylated and unacylated ghrelin to the membrane and hence to the ghrelin receptor; (iii) on ghrelin transmembrane and transepithelial transport.

The originality of this work is that we gained some insights into the mechanisms of action of a unique hormone peptide modification, *O*-octanoylation, and the concomitant role of its basic residues, especially at the level of peptide-membrane interactions and their possible biological implications. Moreover, whenever possible, we worked with natural lipids mixtures which is quite unusual in the field of peptide-lipid interactions.

In this work we have shown differential behaviours of acylated and unacylated ghrelin towards the membrane. In **Fig. 1** is represented the way I think acylated and unacylated ghrelin interact with the membrane in the light of our results. Unacylated ghrelin only seems to interact with the lipids by Coulombic interactions, which are long range electrostatic interactions. Acylated ghrelin, however, is not only driven to the membrane by electrostatic attraction between the positively charged residues of ghrelin and the negatively charged lipids, but acylated ghrelin binding to the membrane is also accompanied by: (i) partitioning into the lipid headgroup and into the lipid backbone regions; (ii) peptide concentration in the membrane; (iii) change in peptide conformation.

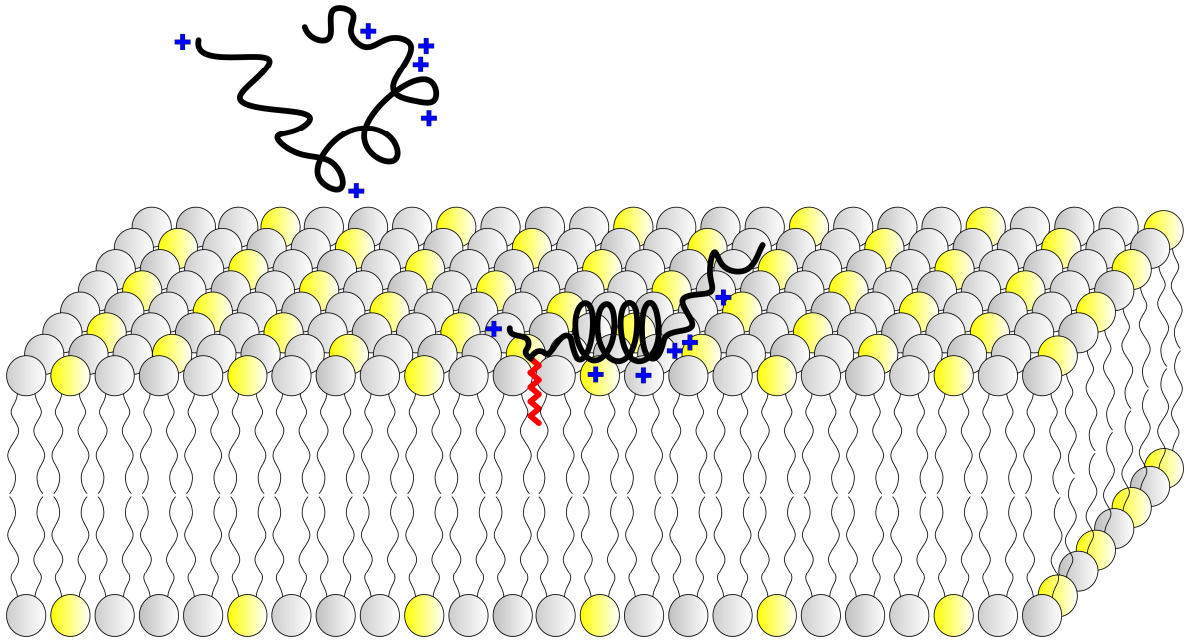


Figure 1- Schematic representation of acylated ghrelin (backbone in black; serine side chain and octanoyl in red) and unacylated ghrelin (backbone in black) interactions with the lipid bilayer of liposomes composed of zwitterionic (grey) and negatively charged phospholipids (yellow). Only 4 carbons of the octanoyl penetrate into the lipid backbone region. Net positive charges of ghrelin at pH 6 are given in blue.

Besides, based on our results along with the fact that the ghrelin binding pocket is thought to be located in the transmembrane domains of the ghrelin receptor [1-5], I hypothesized that the membrane probably acts as a "catalyst" in acylated ghrelin-ghrelin receptor interactions, as it does for other peptides [6-8]. Physiologically our findings also imply that unacylated ghrelin is less likely to encounter and thus to bind to the ghrelin receptor than acylated ghrelin.

On the basis of our results and of the literature, I will expose the roles that octanoylation have or may have on peptide-membrane interactions in terms of: hydrophobic partitioning into the lipids, membrane binding kinetics, plasma membrane targeting, membrane raft targeting, peptide conformation, peptide translocation, peptide secretion, membrane destabilization and peptide transport through epithelia.

Hydrophobic partitioning into the lipids

In 2010, two papers that focused on ghrelin-liposome formulations with highlights on peptide-lipid interactions were published. Their results corroborate the ones we have obtained.

Indeed, they determined acylated ghrelin binding rates to zwitterionic liposomes made of dipalmitoylphosphatidylcholine (DPPC) or DPPC/Chol (70:30 mol %) at L/P~50 by capillary electrophoresis. Binding rates were less than 4% for DPPC and ~9% for DPPC/Chol [9]. This

is close to the value of the binding rate of acylated ghrelin to DMPC that we have found at the same L/P ratio i.e. 7.7 ± 1.3%. They also determined that acylated and unacylated ghrelin binding rates to liposomes made of DPPC and dipalmitoylphosphatidylserine (DPPS) (70:30 mol%) at L/P~50 were 73 ± 5 % and 48 ± 4 % [9]. These results are similar to ours [10] considering that our liposomes only contained 16.4 mol% of negatively charged lipids (PI). Their conclusions reinforced ours i.e. that electrostatic interactions were not solely responsible for the observed acylated ghrelin-negatively charged liposomes interactions.

From our results showing the absence of ghrelin effect on the membrane fluidity together with the small hydrophobic partitioning coefficient (K_p = 74 M^{-1}) as well as the lack of change in lipid orientation and thus in lipid chain order, we concluded that acylated ghrelin is unlikely to be deeply inserted into the lipid bilayer. Østergaard and Moeller drew the same conclusions by showing fast association and dissociation kinetics of ghrelin-DPPC/DPPS by capillary electrophoresis and no significant effect on the T_m of DPPC/DPPS liposomes by DSC [9]. The fast on–off kinetics of ghrelin-DPPC/DPPS that they found also indicated that these interactions are fully reversible [9].

Another study that assessed the binding of another highly basic (histidine rich) but *N*-octanoylated decapeptide to lipids by tryptophan fluorescence also revealed that this peptide only slightly bound to zwitterionic liposomes (DPPC) but more effectively bound to negatively charged liposomes (DPPC and DPPS) [11].

Altogether, efficient membrane binding of octanoylated peptides seems to necessitate the presence of a basic cluster and of negatively charged lipids. This is not highly surprising since, as already mentioned in the introduction, strong and long lasting interactions with the membrane of peptides and proteins that lack a proteinaceous membrane anchor often require double palmitoylation (C_{16}) [12,13]. Even acylation of peptides and proteins with a myristoyl group (C_{14}) without the presence of highly basic residues or of an additional hydrophobic group only provides a transient attachment to the membrane [14].

Yet, another study published in 2010 also showed the importance of the octanoyl group, but also of the Phe^4 side chain, in ghrelin binding to a zwitterionic membrane mimicking model (DPC micelles) [15]. Nevertheless, their calculated free energy of binding of acylated ghrelin to DPC micelles were very high i.e. ΔG = -11.9 kcal/mol compared with the ones we found i.e. ΔG_{app} max = -8.0 kcal/mol (electrostatic and hydrophobic interactions) and ΔG_p = -5.1 kcal/mol (hydrophobic interactions only), but also with the ones previously reported for

myristoylated (C_{14}) peptide-zwitterionic vesicles interactions i.e. ΔG = -8.0 kcal/mol, as well as with many antimicrobial peptides whose free energy of binding to palmitoyloleoyl-phosphatidylcholine (POPC) bilayers range from -5.5 to – 8.1 kcal/mol [16]. Therefore, these results should be considered with great care and further studies should be performed.

Further experiments should be carried out in order to confirm the hydrophobic penetration of acylated ghrelin. The peptide penetration depth could roughly be determined by quartz crystal microbalance experiments since the record of the mass change at different overtones, of the sensor chip natural frequency, gives a way to determine the peptide localization as a function of distance from the lipid bilayer surface. It could therefore confirm the relative positions of acylated and unacylated ghrelin regarding to the bilayer surface. Moreover, ^{1}H NMR experiments with partially deuterated lipids i.e. deuterated glycerol and headgroup as well as deuterated acyl chain, except at positions either close to the glycerol or close to the end of the acyl chain, could on the other hand confirm the octanoyl penetration into the hydrophobic region of the phospholipids.

Membrane binding kinetics

Kinetics of acylated ghrelin binding to membranes i.e. kinetics of on-off association with membranes, as indicated by the results of Moeller et al., seems to be fast [17]. Besides, ghrelin Ser^{18} is an excellent substrate for protein kinase C [18]. Moreover, phosphorylation of acylated basic peptides or proteins can favour the detachment of the peptide from the membrane like it does for myristoylated proteins such as MARCKS [19]. Therefore, we could conceive that phosphorylation could further regulate the kinetics of acylated ghrelin attachment to the membrane. However, as protein kinase C is intracellular, it might be unlikely. Nevertheless, the binding kinetics of acylated and unacylated ghrelin, whether phosphorylated or not, could be investigated by surface plasmon resonance or quartz crystal microbalance.

Plasma membrane, raft targeting and membrane organization

The octanoyl, unlike the myristoyl and the palmitoyl moieties, is unlikely to target ghrelin to the cytoplasmic face of the plasma membrane as the acylated ghrelin binding pocket is located in the transmembrane domain of the extracellular face of the plasma membrane [1,3]. Still no generalization for other octanoylated peptides can be made as there is, to my best knowledge, no other available data about octanoylated peptides on this subject.

Besides, even though octanoylation provides hydrophobic partitioning of acylated ghrelin, it is improbable that it could drive ghrelin or other octanoylated peptides to membranes rafts. Indeed, even single myristoylation or prenylation does not usually seem to drive peptides and proteins to rafts; only palmitoylation does but even so not in all cases [12]. It seems that another unknown factor is sometimes necessary to drive peptides and proteins to rafts [13]. Yet, upon T cells activation, the ghrelin receptor and acylated ghrelin associate with GM1+ lipid rafts but it has neither been shown that they co-localize in rafts, nor that they migrate together [20,21]. The role of these associations with rafts is not yet known. However, ghrelin receptor is coupled to G proteins and raft association has been shown to be important in some GPCR-G protein signalling [22,23]. Therefore, ghrelin and ghrelin receptor associations with rafts could also be important in ghrelin signal transduction. Knowing that many GPCR associate with rafts it is most likely that it is the ghrelin receptor that could drive acylated ghrelin to the rafts. This hypothesis should of course be experimentally checked. In the same line of thinking, the ghrelin membrane effects on lateral membrane organization should also be investigated as well as the possible clustering effects of the negatively charged lipids by ghrelin [24].

Peptide conformation

For some peptides it has been shown that acylation can induce a change in the peptide conformation in phosphate buffers in absence of lipid vesicles, as for some acylated magainins (antimicrobial peptide) and some acylated endothelia nitric oxide synthase enzyme derivatives [25,26]. Though we have shown by our experiments that octanoylation of ghrelin does not induce any change in its conformation in either water (pH ~4.7) or sodium Pipes buffer at pH 6.0, it could affect the conformation of other peptides. Indeed, there is evidence that laurylation (C_{12}) of antimicrobial peptides can induce a conformational change of peptide P, but not of LL-37 [27].

Besides, while all published results on ghrelin structure in water agree on the fact that ghrelin shows characteristics of an unstructured peptide in water [10,28,29], there are discrepancies about ghrelin structure in phosphate buffer as determined by ^{1}H NMR experiments published in 2010. Indeed, Martín-Pastor et al. suggested “the presence of a certain population of α-helix” for ghrelin at 5°C in phosphate buffer pH 7.2 [28], while for Großauer et al. their NMR data indicated a typical random coil conformation at 25°C in phosphate buffer pH 5.0 [15]. As low temperature decreases conformational exchange, it could explain some of the observed

differences. However, complementary experiments would be necessary to get a better picture of ghrelin structural behaviour in aqueous solution at different pH.

In the membrane, the change in conformation of acylated ghrelin that we have observed is most likely due to lipid binding, as acylated and unacylated ghrelin conformation did not differ in membrane mimetic environments.

Additional information about ghrelin conformation in presence of cells expressing the ghrelin receptor (GHS-R1a) was recently brought by the NMR study of Martín-Pastor. They suggested that "the GHS-R1a receptor is acting as a prolyl-*cis/trans* isomerase and that, prior to bind to its receptor, acylated ghrelin needs to adopt a specific conformation" [28]. This reinforces our membrane catalysis hypothesis.

Peptide translocation and secretion

Octanoylation of a peptide is unlikely to be sufficient for its translocation across a lipid bilayer by passive diffusion. Indeed, so far it has been shown that the minimum carbon chain length required for such a process to occur was C_{14}-C_{16} [30-33]. This acyl chain length roughly corresponds to half the membrane lipid bilayer thickness. This hypothesis was corroborated by our experiments that revealed the inability of both forms of ghrelin to cross either zwitterionic or negatively charged lipid bilayers by passive diffusion. Moreover, the highly basic profile of ghrelin moiety does not seem to be sufficient for the peptide translocation, even in combination with the octanoyl moiety, unlike it seems to be the case for some cell penetrating peptides, even though their mechanisms of transport are still a subject to controversy [34].

Besides, the octanoyl moiety is not likely to help in ghrelin secretion. Indeed, in mice that lack the unique enzyme capable of transforming unacylated into acylated ghrelin, i.e. ghrelin *O*-acyl transferase-null mice, unacylated (but not acylated ghrelin) was detected in the bloodstream which implies that it had to be secreted [35].

Membrane destabilization

As we had expected, ghrelin did not lead to drastic membrane destabilization effects showing that, unlike endogenous peptides in pathological states such as the beta-amyloid peptide or the islet amyloid peptide [36,37], and unlike several antimicrobial peptides such as nisin, magainin 2 or alamethicin [38], the ghrelin mechanism of action does not seem to rely on membrane integrity disruption. Indeed, neither have we observed an effect on lipid orientation

and thus on lipid chain order, nor calcein release was observed from calcein loaded liposomes in contact with acylated ghrelin (Mingeot et al., unpublished data).
This is consistent with the ghrelin biological effects that have been observed so far.

Peptide transport through epithelia

As already mentioned in the introduction, acylation can enhance the *in vitro* and/or *in vivo* intestinal transport of short and long peptides [39-42]. Therefore, we hypothesized that ghrelin octanoylation could enhance its transport through model intestinal epithelia. However, our results showed that in Caco-2 monolayers unacylated ghrelin transport was higher than acylated ghrelin transport. Nevertheless, there could be a way to explain these findings. Indeed, it has been shown that human unacylated and acylated ghrelin were transported through the blood brain barrier of mice by an unsaturable mechanism and saturable mechanism, respectively [43]. Moreover, human acylated ghrelin also exhibited saturable binding and endocytosis in a rat cerebral microvessel endothelial cell line [44]. Yet, in the Caco-2 cells, ghrelin cannot be transported by the known peptides transporters as they only carry di- or tri-peptides. However, it has already been shown that larger intact peptides can cross the intestinal barrier and preferentially at the large intestine level [45] by a still unclear mechanism [46]. As we have shown that acylated and unacylated ghrelin cannot cross lipid bilayers by passive diffusion, ghrelin could therefore get through the Caco-2 cells via the paracellular or transcellular (via endocytosis) route. Ghrelin diameter is 19.6 Å as calculated on the basis of its molecular weight and hence globular volume [47]. Several studies on paracellular transport showed that the functional tight junction pore size should comprised between 3 to 14 Å [48,49]. Moreover, a recent and systematic study using PEGs of increasing molecular weights even showed that Caco-2 cell monolayers present high capacity and size-restrictive pores with a diameter of 8 Å [50]. Ghrelin should therefore not be transported by this pathway. Nevertheless, it seems that another size-independent paracellular pathway of low capacity seems to exist [50] and could participate in ghrelin transport. Whereas the observed difference in acylated and unacylated ghrelin transport through Caco-2 monolayers could be explained by the greater plasma membrane retention of acylated ghrelin in the case of paracellular transport, the paracellular pathway could not explain a saturable transport mechanism. Preferential ghrelin transport pathway should thus be determined by performing further transport experiments with endocytosis inhibitors and tight junction modulators. The role of efflux proteins should also be investigated. Moreover, it would be interesting to see if

the ghrelin receptor truncated form, GHS-R1b, plays a role in ghrelin transport as, unlike the active form of the ghrelin receptor, GHS-R1a, this receptor is almost ubiquitous [51].

More generally, a systematic study with different acylated peptides and their unacylated forms should be conducted as it does not exist so far and that it could bring light to the various effect of acylation described in the literature. This study would ideally look at the influence of (i) the acylation site (terminal or in the peptide sequence, *N*- or *S*-acylation); (ii) the acyl bond (ester, thioester, amide); (iii) the fatty acyl chain length vs. the hydrophilic lipophilic balance of the peptide, as it seems that, for each peptide, there exists an optimum number of carbons for the fatty acid in order to enhance the peptide transport and activity [42,52,53].

In conclusion, we have brought some answers concerning the role of the peptide octanoylation in peptide-membrane interactions, but a lot of work remains to be done in order to reveal all the biological roles of this unusual peptide modification.

REFERENCES

[1] Liu G., Fortin J.P., Beinborn M., and Kopin A.S., Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities, J. Pharmacol. Exp. Ther., 322 (2007) 1036-1043.

[2] Holst B., Mokrosinski J., Lang M., Brandt E., Nygaard R., Frimurer T.M., Beck-Sickinger A.G., and Schwartz T.W., Identification of an efficacy switch region in the ghrelin receptor responsible for interchange between agonism and inverse agonism, J. Biol. Chem., 282 (2007) 15799-15811.

[3] Holst B., Lang M., Brandt E., Bach A., Howard A., Frimurer T.M., Beck-Sickinger A., and Schwartz T.W., Ghrelin receptor inverse agonists: identification of an active peptide core and its interaction epitopes on the receptor, Mol. Pharmacol., 70 (2006) 936-946.

[4] Pantel J., Legendre M., Cabrol S., Hilal L., Hajaji Y., Morisset S., Nivot S., Vie-Luton M.P., Grouselle D., de Kerdanet M., Kadiri A., Epelbaum J., Le B.Y., and Amselem S., Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature, J. Clin. Invest, 116 (2006) 760-768.

[5] Holst B., Holliday N.D., Bach A., Elling C.E., Cox H.M., and Schwartz T.W., Common structural basis for constitutive activity of the ghrelin receptor family, J. Biol. Chem., 279 (2004) 53806-53817.

[6] Biaggi M.H., Riske K.A., and Lamy-Freund M.T., Melanotropic peptides-lipid bilayer interaction. Comparison of the hormone alpha-MSH to a biologically more potent analog, Biophys. Chem, 67 (1997) 139-149.

[7] Castanho M.A. and Fernandes M.X., Lipid membrane-induced optimization for ligand-receptor docking: recent tools and insights for the "membrane catalysis" model, Eur. Biophys. J., 35 (2006) 92-103.

[8] Lee J.Y., Moon J.S., Eu Y.J., Lee C.W., Yang S.T., Lee S.K., Jung H.H., Kim H.H., Rhim H., Seong J.Y., and Kim J.I., Molecular interaction between kisspeptin decapeptide analogs and a lipid membrane, Arch. Biochem. Biophys., 485 (2009) 109-114.

[9] Ostergaard J. and Moeller E.H., Ghrelin-liposome interactions: characterization of liposomal formulations of an acylated 28-amino acid peptide using CE, Electrophoresis, 31 (2010) 339-345.

[10] Staes E., Absil P.A., Lins L., Brasseur R., Deleu M., Lecouturier N., Fievez V., Rieux A., Mingeot-Leclercq M.P., Raussens V., and Preat V., Acylated and unacylated ghrelin binding to membranes and to ghrelin receptor: Towards a better understanding of the underlying mechanisms, Biochim. Biophys. Acta, 1798 (2010) 2102-2113.

[11] Pedersen T.B., Sabra M.C., Frokjaer S., Mouritsen O.G., and Jorgensen K., Association of acylated cationic decapeptides with dipalmitoylphosphatidylserine-dipalmitoylphosphatidylcholine lipid membranes, Chem Phys. Lipids, 113 (2001) 83-95.

[12] Bijlmakers M.J. and Marsh M., The on-off story of protein palmitoylation, Trends Cell Biol., 13 (2003) 32-42.

[13] Levental I., Grzybek M., and Simons K., Greasing their way: lipid modifications determine protein association with membrane rafts, Biochemistry, 49 (2010) 6305-6316.

[14] Resh M.D., Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins, Biochim. Biophys. Acta, 1451 (1999) 1-16.

[15] Grossauer J., Kosol S., Schrank E., and Zangger K., The peptide hormone ghrelin binds to membrane-mimetics via its octanoyl chain and an adjacent phenylalanine, Bioorg. Med. Chem, 18 (2010) 5483-5488.

[16] Almeida P.F. and Pokorny A., Mechanisms of antimicrobial, cytolytic, and cell-penetrating peptides: from kinetics to thermodynamics, Biochemistry, 48 (2009) 8083-8093.

[17] Moeller E.H., Holst B., Nielsen L.H., Pedersen P.S., and Ostergaard J., Stability, liposome interaction, and in vivo pharmacology of ghrelin in liposomal suspensions, Int. J. Pharm., 390 (2010) 13-18.

[18] Dehlin E., Liu J., Yun S.H., Fox E., Snyder S., Gineste C., Willingham L., Geysen M., Gaylinn B.D., and Sando J.J., Regulation of ghrelin structure and membrane binding by phosphorylation, Peptides, 29 (2008) 904-911.

[19] McLaughlin S. and Aderem A., The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions, Trends Biochem. Sci., 20 (1995) 272-276.

[20] Dixit V.D., Schaffer E.M., Pyle R.S., Collins G.D., Sakthivel S.K., Palaniappan R., Lillard J.W., Jr., and Taub D.D., Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells, J. Clin. Invest, 114 (2004) 57-66.

[21] Dixit V.D., Yang H., Cooper-Jenkins A., Giri B.B., Patel K., and Taub D.D., Reduction of T cell-derived ghrelin enhances proinflammatory cytokine expression: implications for age-associated increases in inflammation, Blood, 113 (2009) 5202-5205.

[22] Patel H.H., Murray F., and Insel P.A., G-protein-coupled receptor-signaling components in membrane raft and caveolae microdomains, Handb. Exp. Pharmacol., (2008) 167-184.

[23] Allen J.A., Halverson-Tamboli R.A., and Rasenick M.M., Lipid raft microdomains and neurotransmitter signalling, Nat. Rev. Neurosci., 8 (2007) 128-140.
[24] Rusu L., Gambhir A., McLaughlin S., and Radler J., Fluorescence correlation spectroscopy studies of Peptide and protein binding to phospholipid vesicles, Biophys. J., 87 (2004) 1044-1053.
[25] Yelamos B., Roncal F., Albar J.P., Rodriguez-Crespo I., and Gavilanes F., Influence of acylation of a Peptide corresponding to the amino-terminal region of endothelial nitric oxide synthase on the interaction with model membranes, Biochemistry, 45 (2006) 1263-1270.
[26] Avrahami D. and Shai Y., Conjugation of a magainin analogue with lipophilic acids controls hydrophobicity, solution assembly, and cell selectivity, Biochemistry, 41 (2002) 2254-2263.
[27] Radzishevsky I.S., Rotem S., Zaknoon F., Gaidukov L., Dagan A., and Mor A., Effects of acyl versus aminoacyl conjugation on the properties of antimicrobial peptides, Antimicrob. Agents Chemother., 49 (2005) 2412-2420.
[28] Martin-Pastor M., De C.A., Alvarez C.J., az-Hernandez M.D., Jimenez-Barbero J., Casanueva F.F., and Pazos Y., Interaction between ghrelin and the ghrelin receptor (GHS-R1a), a NMR study using living cells, Bioorg. Med. Chem, 18 (2010) 1583-1590.
[29] Silva Elipe M.V., Bednarek M.A., and Gao Y.D., 1H NMR structural analysis of human ghrelin and its six truncated analogs, Biopolymers, 59 (2001) 489-501.
[30] Chopineau J., Robert S., Fenart L., Cecchelli R., Lagoutte B., Paitier S., Dehouck M.P., and Domurado D., Monoacylation of ribonuclease A enables its transport across an in vitro model of the blood-brain barrier, J. Control. Release, 56 (1998) 231-237.
[31] Gallina A. and Milanesi G., Trans-membrane translocation of a myristylated protein amino terminus, Biochem. Biophys. Res. Commun., 195 (1993) 637-642.
[32] Harishchandran A. and Nagaraj R., Interaction of a pseudosubstrate peptide of protein kinase C and its myristoylated form with lipid vesicles: only the myristoylated form translocates into the lipid bilayer, Biochim. Biophys. Acta, 1713 (2005) 73-82.
[33] Takada R., Satomi Y., Kurata T., Ueno N., Norioka S., Kondoh H., Takao T., and Takada S., Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion, Dev. Cell, 11 (2006) 791-801.
[34] Heitz F., Morris M.C., and Divita G., Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics, Br. J. Pharmacol., 157 (2009) 195-206.
[35] Gutierrez J.A., Solenberg P.J., Perkins D.R., Willency J.A., Knierman M.D., Jin Z., Witcher D.R., Luo S., Onyia J.E., and Hale J.E., Ghrelin octanoylation mediated by an orphan lipid transferase, Proc. Natl. Acad. Sci. U. S. A, 105 (2008) 6320-6325.
[36] Matsuzaki K., Physicochemical interactions of amyloid beta-peptide with lipid bilayers, Biochim. Biophys. Acta, 1768 (2007) 1935-1942.
[37] Knight J.D., Hebda J.A., and Miranker A.D., Conserved and cooperative assembly of membrane-bound alpha-helical states of islet amyloid polypeptide, Biochemistry, 45 (2006) 9496-9508.
[38] Hale J.D. and Hancock R.E., Alternative mechanisms of action of cationic antimicrobial peptides on bacteria, Expert. Rev. Anti. Infect. Ther., 5 (2007) 951-959.
[39] Fujita T., Kawahara I., Quan Y., Hattori K., Takenaka K., Muranishi S., and Yamamoto A., Permeability characteristics of tetragastrins across intestinal membranes using the Caco-2 monolayer system: comparison between acylation and application of protease inhibitors, Pharm. Res., 15 (1998) 1387-1392.
[40] Asada H., Douen T., Waki M., Adachi S., Fujita T., Yamamoto A., and Muranishi S., Absorption characteristics of chemically modified-insulin derivatives with various fatty acids in the small and large intestine, J. Pharm. Sci., 84 (1995) 682-687.
[41] Wang J., Hogenkamp D.J., Tran M., Li W.Y., Yoshimura R.F., Johnstone T.B., Shen W.C., and Gee K.W., Reversible lipidization for the oral delivery of leu-enkephalin, J. Drug Target, 14 (2006) 127-136.
[42] Yodoya E., Uemura K., Tenma T., Fujita T., Murakami M., Yamamoto A., and Muranishi S., Enhanced permeability of tetragastrin across the rat intestinal membrane and its reduced degradation by acylation with various fatty acids, J. Pharmacol. Exp. Ther., 271 (1994) 1509-1513.
[43] Banks W.A., Tschop M., Robinson S.M., and Heiman M.L., Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure, J. Pharmacol. Exp. Ther., 302 (2002) 822-827.
[44] Pan W., Tu H., and Kastin A.J., Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin, Peptides, 27 (2006) 911-916.
[45] des Rieux A., Fievez V., Momtaz M., Detrembleur C., Alonso-Sande M., Van Gelder J., Cauvin A., Schneider Y.J., and Préat V., Helodermin-loaded nanoparticles: characterization and transport across an in vitro model of the follicle-associated epithelium, J. Control. Release, 118 (2007) 294-302.
[46] Fricker G. and Drewe J., Current concepts in intestinal peptide absorption, J. Pept. Sci., 2 (1996) 195-211.

[47] Peptide property calculator, Center for Biotechnology - Northwestern University, http://www.basic.northwestern.edu/biotools/proteincalc.html (Last access date: 2010).
[48] des Rieux A., Fievez V., Théate I., Mast J., Préat V., and Schneider Y.J., An improved in vitro model of human intestinal follicle-associated epithelium to study nanoparticle transport by M cells, Eur. J. Pharm. Sci., 30 (2007) 380-391.
[49] Nellans H.N., Mechanisms of Peptide and Protein-Absorption .1. Paracellular Intestinal Transport - Modulation of Absorption, Adv. Drug Deliv. Rev., 7 (1991) 339-364.
[50] Van Itallie C.M., Holmes J., Bridges A., Gookin J.L., Coccaro M.R., Proctor W., Colegio O.R., and Anderson J.M., The density of small tight junction pores varies among cell types and is increased by expression of claudin-2, J. Cell Sci., 121 (2008) 298-305.
[51] Gnanapavan S., Kola B., Bustin S.A., Morris D.G., McGee P., Fairclough P., Bhattacharya S., Carpenter R., Grossman A.B., and Korbonits M., The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans, J. Clin. Endocrinol. Metab., 87 (2002) 2988-2991.
[52] Wang J., Wu D., and Shen W.C., Structure-activity relationship of reversibly lipidized peptides: studies of fatty acid-desmopressin conjugates, Pharm. Res., 19 (2002) 609-614.
[53] Uchiyama T., Kotani A., Tatsumi H., Kishida T., Okamoto A., Okada N., Murakami M., Fujita T., Fujiwara Y., Kiso Y., Muranishi S., and Yamamoto A., Development of novel lipophilic derivatives of DADLE (leucine enkephalin analogue): intestinal permeability characateristics of DADLE derivatives in rats, Pharm. Res., 17 (2000) 1461-1467.