



Nanovecteurs lipidiques pour la délivrance orale de peptides

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Nanovecteurs lipidiques pour la délivrance orale de peptides

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Résumé

Nanovecteurs lipidiques pour la délivrance orale de peptides

Les peptides thérapeutiques peuvent traiter de nombreuses pathologies de manière efficace et sélective. Leur biodisponibilité orale est limitée par une forte dégradation liée à l'action de protéases dans le lumen intestinal et par une faible absorption par la barrière intestinale. Nous avons évalué la capacité des nanoparticules lipidiques solides (SLN) et des vecteurs lipidiques nanostructurés (NLC) à augmenter la biodisponibilité orale d'un peptide modèle : le Leuprolide (LEU). L'augmentation de sa lipophilie par formation d'une paire d'ions hydrophobe (HIP) avec le docusate de sodium permet d'augmenter significativement le taux d'encapsulation du LEU dans les nanovecteurs. Ceux-ci sont obtenus par homogénéisation haute pression avec une taille de 120 nm et une structure en plaquettes. Malgré leur stabilité dans les milieux gastro-intestinaux simulés, une libération importante de la substance active est observée dans le milieu intestinal à jeun. Vis-à-vis de la dégradation protéolytique, les NLC montrent une protection significative du LEU en présence de trypsine. L'évaluation du passage intestinal sur des monocouches de Caco-2 (modèle entérocyte) et de Caco-2/HT29-MTX (modèle sécrétant des mucines) révèle une internalisation des nanovecteurs mais aucune amélioration de l'absorption. En effet, la morphologie en plaquettes des SLN et NLC associée à la faible stabilité de l'HIP dans le milieu provoquent une libération importante du LEU, annulant la capacité de transport des nanovecteurs à travers la barrière intestinale. Il convient d'améliorer la stabilité de l'HIP pour augmenter la biodisponibilité orale des peptides *via* l'encapsulation dans des nanovecteurs lipidiques solides.

Mots-clés : Peptides, Oral, Nanoparticules Lipidiques Solides, Vecteurs Lipidiques Nanostructurés, Paire d'Ions Hydrophobe, Protéases, Perméabilité intestinale, Formulation

Abstract

Lipid nanocarriers for oral delivery of peptides

Therapeutic peptides are able to treat a wide variety of diseases with selective and potent action. Their oral bioavailability is strongly limited by an important proteolytic activity in the intestinal lumen and poor permeation across the intestinal border. We have evaluated the capacity of Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid carriers (NLC) to overcome both oral bioavailability limiting aspects, using Leuprolide (LEU) as model peptide. Lipidization of LEU by formation of a Hydrophobic Ion Pair (HIP) with sodium docusate enables a significant increase of peptide encapsulation efficiency in both SLN and NLC. The nanocarriers, obtained by high pressure homogenization, measured 120 nm and were stable in simulated gastro-intestinal fluids. However, due to particles platelet-shape, an important quantity of LEU is released in simulated fasted state intestinal fluid. Regarding the protective effect towards proteolytic degradation, only NLC maintain LEU integrity in presence of trypsin. Intestinal transport, evaluated on Caco-2 (enterocyte-like model) and Caco-2/HT29-MTX (mucin-secreting model) monolayers, show nanocarriers internalization by enterocytes but no improvement of LEU permeability. Indeed, the combination of nanoparticles platelet-shape with the poor stability of the HIP in the transport medium induces a high burst release of the peptide, limiting nanoparticles capacity to transport LEU across the intestinal border.

Stability of peptide lipidization needs to be improved to withstand biorelevant medium to benefit from the advantages of encapsulation in solid lipid nanocarriers and consequently improve their oral bioavailability

Keywords: Peptides, Oral delivery, Solid Lipid Nanoparticles, Nanostructured Lipid Carriers, Hydrophobic Ion Pair, Proteases, Intestinal permeability, Formulation

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Préambule

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Ce manuscrit est basé sur trois publications constituant les trois premiers chapitres de la thèse. Chaque publication est précédée d'un résumé substantiel en français. Les références de ces publications sont :

1. C. Dumont, S. Bourgeois, H. Fessi, V. Jannin, Lipid-based nanosuspensions for oral delivery of peptides, a critical review, *International Journal of Pharmaceutics* 541 (2018) 117-135, DOI: 10.1016/j.ijpharm.2018.02.038
2. C. Dumont, V. Jannin, C. Miolane, Q. Lelong, J-P. Valour, S. Urbaniak, H. Fessi, S. Bourgeois, A proof-of-concept for developing oral lipidized peptide nanostructured lipid carrier formulations, *Journal of Drug Delivery Science and Technology* 54 (2019) 101394, DOI: <https://doi.org/10.1016/j.jddst.2019.101394>
3. C. Dumont, S. Bourgeois, H. Fessi, P-Y. Dugas, V. Jannin, *In-vitro* evaluation of solid lipid nanoparticles : Ability to encapsulate, release and ensure effective protection of peptides in the gastrointestinal tract, *International Journal of Pharmaceutics* 565 (2019) 409-418, DOI: 10.1016/j.ijpharm.2019.05.037

Ce travail a également été présenté sous la forme de présentation orale ou de posters lors de cinq congrès internationaux :

- Formulation Days 2019, 10-11 janvier 2019, Lyon, France. C. Dumont, V. Jannin, H. Fessi, S. Bourgeois, Solid Lipid Nanocarriers: high potential formulations for oral delivery of peptides (poster)
- CRS Annual Meeting and Exposition, 21-24 juillet 2019, Valence, Espagne. C. Dumont, S. Bourgeois, H. Fessi, V. Jannin, *In vitro* studies indicate promising properties of solid lipid nanosuspensions for oral peptide delivery (poster)

- GERLI 15th International Lipidomics Meeting 2019, 30 septembre – 2 octobre 2019, Compiègne, France. C. Dumont, V. Jannin, A. Beloqui, V. Prémat, S. Bourgeois, H. Fessi, Solid lipid nanocarriers for oral delivery of peptides (presentation orale)
- AAPS 2019 Pharmsci 360, 3-5 novembre 2019, San Antonio, Texas, USA. C. Dumont, V. Jannin, A. Beloqui, V. Prémat, H. Fessi, S. Bourgeois, *In vitro* evaluation of leuprolide-containing solid lipid-based nanosuspensions: ability to encapsulate, release, protect and permeate in the gastrointestinal tract (poster)
- SFNano C'Nano joint meeting 2019, 10-12 décembre 2019, Dijon, France. C. Dumont, V. Jannin, A. Beloqui, V. Prémat, S. Bourgeois, H. Fessi. Evaluation of solid lipid nanocarriers to increase peptide oral bioavailability (poster)

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Liste des abréviations

ACN	Acetonitrile
ANOVA	Analysis Of Variance
C888	Compritol® 888 ATO (glyceryl dibéhénate)
C90	Capryol® 90 (propylene glycol monocaprylate)
CEH	Cholesteryl Ester Hydrolase
CPP	Cell Penetrating Peptide
CSLM	Confocal Laser Scanner Microscopy
DAG	Diacylglycerol
DES	Desmopressin
DiD	1,1'-Diocetadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt
D _L	Drug Loading
DLS	Dynamic Light Scattering
DMEM	Dulbecco Modified Eagle Medium
EE	Encapsulation Efficiency
FA	Fatty Acids
FaSSIF-V2	Fasted State Simulated Intestinal Fluid, 2 nd version
FDA	Food and Drug Administration
FeSSIF-V2	Fed Stated Simulated Intestinal Fluid, 2 nd version
GI	Gastro-Intestinal
GRAS	Generally Recognized As Safe

Liste des abréviations

GSH	Reduced glutathione
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HIP	Hydrophobic Ion Pair
HLB	Hydrophilic-Lipophilic Balance
HPH	High Pressure Homogenization
HPL	Human Pancreatic Lipase
HPLC	High Performance Liquid Chromatography
LEU	Leuprolide
LLOQ	Lower Limit Of Quantification
MAG	Monoacylglycerol
MDCK	Mardin-Darby Canine Kidney
MeOH	Methanol
NLC	Nanostructured Lipid Carriers
PATO5	Préciprol® ATO5 (glyceryl distearate)
PDI	Polydispersity Index
PE	Precipitation Efficiency
PEG	Polyethylene glycol
PIT	Phase Inversion Temperature
PLRP1 and PLRP2	Pancreatic Lipase-Related Proteins
REAL	Reversible Aqueous Lipidization
RH40	Kolliphor® RH40 (PEG-40 hydrogenated castor oil)

Liste des abréviations

SCF	Supercritical Fluids
SEDDS	Self-Emulsifying Drug Delivery Systems
SEM	Standard Error of Mean
SGF	Simulated Gastric Fluid
SLN	Solid Lipid Nanoparticles
TAG	Triacylglycerol
TEER	TransEpithelial Electric Resistance
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
TJ	Tight Junction

Introduction Générale

Les peptides thérapeutiques sont une classe de molécules biologiquement actives connaissant un intérêt grandissant dans l'industrie pharmaceutiques. En 2008, l'usage de 60 peptides a été approuvé par la Food and Drug Administration (FDA). Actuellement, plus de 500 peptides sont en développement pré-clinique et 140 en phase de tests cliniques. Ce marché est estimé à 13 milliards de dollars et devrait atteindre 42 milliards de dollars en 2025.

Ces peptides thérapeutiques permettent de traiter un grand nombre de pathologies de manière sélective et efficace tout en réduisant le risque d'effets secondaires. Actuellement, la plupart des formes peptidiques présentes sur le marché sont administrées par voie parentérale, généralement intraveineuse. Malheureusement les peptides ont une demi-vie très courte dans les fluides biologiques humains. Par conséquent, les injections doivent être fréquemment répétées, résultant en un inconfort pour le patient et un coût élevé des traitements. De plus les formes pour administration parentérale doivent être stériles, rendant leur fabrication plus contraignante et coûteuse pour l'industriel ainsi que leur manipulation délicate. L'alternative idéale à ces injections serait une administration orale. Pour autant, seulement une dizaine de formes destinées à une administration orale, présentées dans le Tableau 1, sont actuellement sur le marché. Cette liste est à compléter par le Rybelsus® (Novo Nordisk, Danemark), qui a reçu l'approbation de la FDA le 20 septembre 2019 pour la délivrance orale du sémaglutide et qui devrait être mis sur le marché fin 2019.

Parmi ces formes pharmaceutiques destinées à la délivrance orale de peptides, la plupart sont administrées pour un effet local et seulement 4 sont destinées à atteindre la circulation systémique. Ces dernières montrent de faibles biodisponibilités orales (0.16% pour la desmopressine) sauf dans le cas de peptides particuliers présentant par exemple une hydrophobie importante ($\log P > 0$) ou ayant une faible masse moléculaire. En effet, la plupart des peptides thérapeutiques appartiennent à la classe III du Système de Classification Biopharmaceutique (BCS) caractérisant les substances de bonne solubilité aqueuse et de faible perméabilité. Constitués de 2 à 50 acides aminés, les peptides sont hydrophiles et de masse moléculaire importante (entre 500 et 5000 Da) ce qui explique leur faible perméabilité intestinale.

Introduction générale

Tableau 1: Formes pharmaceutiques commercialisées pour l'absorption orale de peptides, données issues de Pharmacircle Database, 2019 et de drugbank.com

Peptide	Nom commercial	Laboratoire	Activité	Stratégie	Masses molaire (g/mol)	LogP estimé
Colistine	Colomycin®	Pola Pharma Inc. (Japon)	Anti-infectieux intestinal	Action locale	1268	0.9
Cyclosporine	Neoral® / Sandimmune®	Novartis (Suisse) / Abbvie Inc. (USA) + others	Immunosuppression	Absorption systémique	1202	3.6
Desmopressine	Minirin® DDAVP®	Ferring Pharmaceuticals (Suisse)	Nycturie Diabète insipide	Absorption systémique	1183	-6.1
Fidaxomicine	Dificid®	Cubist Pharmaceuticals (USA)	Anti-infectieux Anti-diarrhéique	Action locale	1058	8.5
Glutathion	Migranade®	Migranade Inc. (USA)	Anti-migraineux	Absorption systémique	307	-4.9
Linaclotide	Linzess®	Ironwood Pharmaceuticals Inc. (USA)	Constipation Syndrome de l'intestin irritable	Action locale	1527	-11
Plecanatide	Trulance®	Bausch Health Companies Inc. (USA)	Constipation chronique Syndrome de l'intestin irritable	Action locale	1682	3.7
Taltirelin	Ceredist®	Mitsubishi Tanabe Pharma Corp (Japon)	Ataxie spinocérébelleuse	Absorption systémique	477	/
Vancomycin	Vancocin®	Flynn Pharmaceuticals Ltd. (USA)	Anti-infectieux	Action locale	1485	-4.4
Virginiamycin	Pyostacine®	Sanofi (France)	Anti-infectieux	Action locale	1350	3.9

Bien que largement étudiée, l'administration orale des peptides pose de nombreux problèmes biopharmaceutiques. Les peptides thérapeutiques n'étant pas stables dans les milieux acides, leur passage par l'estomac à jeun peut générer une dégradation des molécules. De plus, les enzymes naturellement présentes dans le tractus gastro-intestinal telles que la pepsine dans l'estomac et la trypsine, les carboxypeptidases et la chymotrypsine du lumen intestinal peuvent altérer la composition peptidique de ces molécules. Enfin, les peptides doivent être en mesure de passer la barrière intestinale composée du mucus et de l'épithélium intestinal. Le mucus est un gel viscoélastique négativement chargé, freinant le passage des macromolécules par interactions électrostatiques, ioniques, hydrogènes ou de Van der Waals. Une fois au contact de l'épithélium intestinal, le caractère hydrophile des peptides limite fortement leur passage par voie transcellulaire, excepté éventuellement par endocytose *via* des récepteurs spécifiques. Le passage paracellulaire est quant à lui restreint par les jonctions serrées et le faible espace intercellulaire (<50Å).

Des stratégies de formulation doivent être mises en place pour palier la totalité des limites biopharmaceutiques décrites ci-dessus. Ce projet est consacré à l'évaluation de l'une de ces stratégies, à savoir l'encapsulation des peptides dans des nanovecteurs lipidiques afin de les protéger de l'activité protéolytique duodénale et d'augmenter leur passage intestinal. Parmi les nombreux objets couverts par cette dénomination, les systèmes auto-émulsionnables (Self-Emulsifying Drug Delivery Systems, SEDDS) et les nanovecteurs lipidiques solides semblent les plus adaptés pour une délivrance orale de peptides. Des recherches ayant déjà été initiées par d'autres laboratoires pour évaluer l'augmentation de la biodisponibilité des peptides par encapsulation dans les SEDDS, nous avons focalisé notre travail sur l'étude des nanovecteurs lipidiques solides : les Nanoparticules Lipidiques Solides (Solid Lipid Nanoparticles, SLN) composées de lipides solides et les Vecteurs Lipidiques Nanostructurés (Nanostructured Lipid Carriers, NLC) constitués à la fois de lipides solides et liquides

Ce travail de thèse a donc pour objectif de développer des nanovecteurs lipidiques solides pour l'administration orale de 2 peptides modèles, le leuprolide et la desmopressine, et d'évaluer leur capacité à augmenter la biodisponibilité orale de peptides. Il vise notamment à répondre aux questions suivantes :

- Peut-on encapsuler un peptide hydrophile dans des nanovecteurs lipidiques ?
- Peut-on formuler des nanovecteurs lipidiques solides pour l'encapsulation de peptides *via* un procédé industrialisable ?
- Peut-on protéger les peptides de la dégradation protéolytique en les encapsulant dans des nanovecteurs lipidiques solides ?
- Peut-on améliorer le passage des peptides à travers l'épithélium intestinal en les encapsulant dans des nanovecteurs lipidiques solides ?
- Quel est l'impact du processus de lipolyse sur la biodisponibilité des peptides encapsulés ?
- Quel type de nanovecteurs lipidiques solides (SLN ou NLC) est le plus approprié pour la délivrance orale des peptides ?

Le présent manuscrit est divisé en deux grandes parties : une partie bibliographique (chapitre I) et une partie expérimentale elle-même découpée en 4 chapitres (chapitres II à V).

La première partie présente le fruit d'une recherche bibliographique approfondie sur le sujet ayant fait l'objet d'un article de revue publié dans International Journal of Pharmaceutics en 2018 (DOI : 10.1016/j.ijpharm.2018.02.038) ; revue bibliographique qui a été actualisée à la fin de ce chapitre. Cette synthèse bibliographique met en évidence plusieurs approches pour améliorer l'encapsulation de peptides hydrophiles dans des nanovecteurs lipidiques hydrophobes et décrit les différentes techniques existantes pour formuler ces systèmes. Ce chapitre détaille également les techniques pouvant être appliquées pour caractériser et évaluer le potentiel des nanovecteurs lipidiques solides pour augmenter la biodisponibilité orale des peptides thérapeutiques.

La deuxième partie du travail de thèse, composée de 4 chapitres, met en avant les études menées pour répondre aux questions et hypothèses formulées à l'issue de la recherche bibliographique. Ainsi le chapitre II décrit la mise au point du procédé pour encapsuler les

deux peptides hydrophiles modèles dans des nanovecteurs lipidiques solides. Le chapitre III présente une comparaison des propriétés physicochimiques des nanovecteurs formulés et une évaluation de leur capacité à protéger les peptides de la dégradation protéolytique intestinale. Le chapitre IV détaille l'étude menée pour évaluer la capacité des nanoparticules lipidiques à augmenter *in vitro* le passage intestinal des peptides encapsulés. Enfin, le chapitre V propose une étude préliminaire pour évaluer l'impact de la digestion lipidique sur le passage intestinal des peptides encapsulés.

Etude bibliographique

Chapitre I :

Nanosuspensions lipidiques pour la délivrance orale de peptides thérapeutiques

Publié sous la forme d'une revue dans

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Cette recherche bibliographique est consacrée aux nanoparticules lipidiques solides (Solid Lipid Nanoparticles, SLN) et aux vecteurs lipidiques nanostructurés (Nanostructured Lipid Carriers, NLC) dans l'objectif d'encapsuler des peptides thérapeutiques afin d'augmenter leur biodisponibilité orale. Pour cela, ces vecteurs doivent être capables de protéger le peptide encapsulé de la dégradation dans l'environnement gastro-intestinal et d'augmenter son passage à travers la paroi intestinale. Les SLN, développées dans les années 1990, sont constituées de lipides, solides à 37°C, et de tensioactifs. Les NLC ont ensuite été développés pour limiter la recristallisation des lipides dans le cœur des nanoparticules qui pouvait conduire une expulsion de la substance active encapsulée au cours du temps. Concrètement, une fraction lipidique, liquide à température ambiante, est ajoutée aux excipients solides afin de favoriser la mobilité des chaînes lipidiques.

Le caractère hydrophile de la majorité des peptides thérapeutiques est un frein à leur encapsulation dans des nanovecteurs lipidiques hydrophobes de type SLN ou NLC. Il convient donc d'augmenter leur lipophilie en amont de leur encapsulation. Cette augmentation peut se faire par formation d'une liaison covalente ou par interaction non-covalente avec un groupement hydrophobe. Dans le premier cas, plusieurs techniques sont décrites dans la littérature telles que la cyclisation du peptide ou la Lipophilisation Aqueuse Réversible (REAL). Toutefois, la création d'une liaison covalente a nécessairement un impact sur l'absorption, la distribution, le métabolisme et l'excrétion du médicament et nécessite l'approbation d'une nouvelle molécule à usage thérapeutique. En revanche, la formation d'une interaction non-covalente pour augmenter la lipophilie permet la conservation de l'activité thérapeutique à condition que l'interaction soit réversible. L'une des méthodes les plus employées consiste en la formation d'une paire d'ions hydrophobe par interaction électrostatique entre un peptide ionisable, sous certaines conditions, et un contre-ion hydrophobe. La lipophilie est généralement déterminée en évaluant le coefficient de partage des entités chimiques entre l'octanol et l'eau (Log P).

La plupart des nanovecteurs lipidiques solides sont obtenus par un procédé à chaud ou en utilisant des solvants organiques. Dans le premier cas, la formulation s'apparente à la préparation de nanoémulsions à une température supérieure de 5 à 10°C à la température de fusion des excipients lipidiques solides. Dans ces conditions, seuls quelques dispositifs sont capables de générer les forces de cisaillement nécessaires à la formation des nano-

gouttelettes : les sondes à ultrasons ou les homogénéisateurs haute pression. Les nanoparticules solides sont finalement obtenues après refroidissement des nanoémulsions.

Parmi les techniques utilisant des solvants organiques, la nanoprécipitation est une des méthodes les plus simples et des plus utilisées pour formuler des SLN ou des NLC. Celle-ci repose sur l'affinité entre solvants. Elle consiste en l'ajout contrôlé d'une solution d'excipients et de principe actif dissous dans un solvant organique miscible avec l'eau dans une phase aqueuse contenant un tensioactif. Cet ajout va entraîner la migration spontanée du solvant vers la phase aqueuse et la précipitation quasi instantanée des excipients sous la forme de nanoparticules solides. D'autres techniques, telles que les méthodes d'émulsion-diffusion ou d'émulsion-évaporation peuvent aussi être envisagées. Elles nécessitent toutefois de fort taux de cisaillement pour former des globules d'émulsion à l'échelle nanométrique.

Les nanovecteurs lipidiques solides sont généralement caractérisés par leur distribution de taille de particules, leur indice de polydispersité, leur morphologie, leur taux d'encapsulation et la concentration finale de substance active dans les nanoparticules. L'étude de la libération de la substance active dans les milieux gastro-intestinaux simulés est également fréquemment reportée.

L'une des principales raisons expliquant la faible biodisponibilité orale des peptides est l'importante dégradation pré-systémique subie lors d'une prise orale. En effet, lors de leur administration dans le tractus gastro-intestinal, les peptides subissent une forte dégradation enzymatique dans l'estomac par l'action de la pepsine et dans le lumen duodénal où se trouvent la trypsine, l' α -chymotrypsine et l'élastase. L'étude de l'effet protecteur des nanovecteurs est généralement évalué par incubation des nanoparticules dans des solutions contenant ces protéases. La quantité de substance active non dégradée est régulièrement quantifiée après arrêt de l'activité enzymatique. Quelques études ont montré l'efficacité des SLN et des systèmes auto-émulsionnables (Self-Emulsifying Drug Delivery System, SEDDS) à protéger les peptides encapsulés de l'activité protéolytique. Ces études ont ainsi montré que seuls les peptides libérés étaient dégradés par les protéases. Il est alors fort probable que les nanoparticules lipidiques non digérées, qui maintiennent leur structure dans les milieux intestinaux, assurent un environnement protecteur accru aux peptides encapsulés. Ces

formulations non-digestibles sont caractérisées par l'absence de groupement ester ou par l'utilisation de longues chaînes hydrocarbonées.

Des modèles cellulaires *in vitro* sont généralement utilisés pour évaluer le passage des molécules thérapeutiques et des vecteurs colloïdaux à travers l'épithélium intestinal. Celui-ci est majoritairement formé d'entérocytes (~99%), couverts de microvillosités et séparés par des jonctions serrées. Les cellules Caco-2, issues d'adénocarcinome colorectal, se différencient sous certaines conditions de culture pour présenter la plupart de ces caractéristiques. Il s'agit du modèle cellulaire intestinal le plus répandu. Les cellules caliciformes sont quant à elles responsables de la production des mucines et du glycocalyx dont est formé le mucus. Ce gel viscoélastique recouvrant la paroi intestinale est également responsable de la faible perméabilité des peptides. Son effet est étudié *in vitro* via l'utilisation de co-cultures Caco-2/HT29-MTX. Bien que faiblement présentes à la surface de la barrière épithéliale, les cellules M, moins couvertes en mucus et microvillosités, peuvent être le lieu d'absorption de molécules de taille importante, tels les peptides. Celles-ci sont alors transférées vers le système lymphatique, leur permettant d'éviter l'effet de premier passage hépatique. L'effet de ces cellules M peut être modélisé avec des co-cultures de Caco-2 et de lymphocytes Raji B, voire de tri-cultures Caco-2/HT29-MTX/Raji B. Les mesures de perméabilité des molécules thérapeutiques ou des vecteurs colloïdaux sont conduites sur des monocouches de cellules, cultivées sur des inserts, et dont l'intégrité est mesurée au préalable. Ces modèles cellulaires peuvent également servir à l'étude des mécanismes de transport.

En conclusion, cette étude bibliographique montre que les nanovecteurs lipidiques sont des systèmes prometteurs pour augmenter la biodisponibilité orale des peptides thérapeutiques. L'augmentation de la lipophilie des peptides est toutefois un prérequis pour augmenter leur taux d'encapsulation dans ces systèmes.



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Review

Lipid-based nanosuspensions for oral delivery of peptides, a critical review

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ABSTRACT

Peptides are therapeutic molecules that can treat selectively and efficiently a wide range of pathologies. However, their intrinsic properties cause their rapid degradation in the human gastrointestinal (GI) tract resulting in poor bioavailability after oral administration. Yet, their encapsulation in nanocarriers offers them protection from this harsh environment and increases their permeability across the epithelium border. In particular, Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC) have proven to improve peptide oral bioavailability.

This article details different techniques used to produce SLN and NLC with potential or effective peptide encapsulation. Basic principles of covalent and non-covalent lipidization are described and discussed as a prerequisite to improve hydrophilic peptide encapsulation in lipid-based nanosuspensions. The last part of this review provides the key evaluation techniques to assay SLN and NLC for peptide oral bioavailability enhancement. Methods to assess the protective effects of the carriers are described as well as the techniques to evaluate peptide release upon lipid digestion by lipases. Furthermore, this review suggests different techniques to measure permeability improvements and describes the main *in vitro* cell models associated.

1. Introduction

Therapeutic peptides and proteins are a new class of promising pharmaceutical ingredients that can treat a wide variety of affections with effective and potent action. Indeed, in 2008, > 130 different proteins or peptides have been approved for clinical use by FDA (Muheem et al., 2016). In 2013, this market was estimated to represent more than \$40 billion per year (Craik et al., 2013). This success comes from their composition and structure that enable very specific binding to therapeutic site hence increasing their efficiency and limiting side effects (Craik et al., 2013). In addition, they accumulate less in tissues and are supposed to be less toxic than classic chemical drug molecules (Craik et al., 2013; Morishita and Peppas, 2006). Most of the therapeutic peptides are administered parenterally and more specifically via intravenous route. Since they exhibit a very short half-life in human physiological fluids, this operation must be repeated frequently leading to real annoyance for patients (Almeida and Souto, 2007; Brayden and O'Mahony, 1998; Gupta et al., 2013). Development of new dosage

forms enabling alternative routes of administration is being widely investigated. Oral route is the preferred way of administration for patients. However, intrinsic properties of peptides generate numerous biopharmaceutical issues leading to low oral bioavailability (< 1%) (Brayden and O'Mahony, 1998). Nevertheless, in 2016, 8 peptides were already on the market for oral administration, among which 4 are systemically absorbed (Cyclosporin A, Desmopressin, Taltirelin and Glutathione) while the 4 others are intended for local intestinal effect (Linacotide, Vancomycin, Colistin and Tyrothricin) (Aguirre et al., 2016). In order to design an efficient drug delivery system for oral peptide delivery, some physical, chemical and biological barriers should be overcome. The main barriers limiting the oral bioavailability of peptides are the acidic pH of the stomach, the degradation by proteases and peptidases, the reduction by glutathione, and the difficulty to cross the mucus and intestinal epithelium.

The first barrier limiting peptide oral bioavailability is the stomach where the acid pH (2–3 in fasted condition) can cause denaturation of peptides. Indeed, below pH 3, acid-catalyzed hydrolysis of asparagine

Abbreviations: AOT, dioctyl sulfocinate; CEH, cholesteryl ester hydrolase; CPP, cell penetrating peptide; DL, drug load; EE, encapsulation efficiency; FA, Fatty Acid; GI, Gastrointestinal; GSH, reduced glutathione; HIP, hydrophobic ion pairing; HPH, high pressure homogenization; LHR, luteinizing hormone-releasing hormone; MDCK, Mardin-Darby canine kidney; NLC, nanostructured lipid carrier; PDI, polydispersity index; PEO, polyethoxylated; PIT, phase inversion temperature; REAL, reversible aqueous lipidization; RESS, rapid expansion of SCF solution; SAS, supercritical fluid anti-solvent; SCF, supercritical fluid; sCT, salmon calcitonin; SEDDS, self-emulsifying drug delivery system; SNEFDS, self-nanoemulsifying drug delivery system; SLN, solid lipid nanoparticle; TEER, transepithelial electric resistance

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and glutamine can lead to peptide deamination (Manning et al., 1989; Reubsæet et al., 1998). Furthermore, in very acidic medium ($\text{pH} < 4$), peptides are fully ionized which can lead to intrinsic electrostatic repulsions and potential loss of conformation resulting in loss of biological activity. In addition, the stomach secretes pepsinogen that transforms into pepsin – an aspartic peptidase – that cleaves bonds between hydrophobic amino acids and facilitates the action of proteases located in the small intestine (Malhaire et al., 2016; Rao et al., 1998).

Then the bolus reaches the duodenum where is located the main enzymatic barrier to peptide oral bioavailability. Duodenum exhibits a pH of 5–6 and is the receptor of bile and pancreatic juice secretions containing proteases: trypsin, α -chymotrypsin, elastase, exopeptidases, carboxypeptidases A&B (serine proteases) (Leonaviciute and Bernkop-Schnürch, 2015). These enzymes break peptides into smaller ones and free amino acids by nucleophilic attacks on specific bonds with different binding sites for each enzyme (Malhaire et al., 2016). Peptides displaying a disulfide bond can also be inactivated when subjected to thiol-disulfide exchange reaction with reduced glutathione (GSH) (Schmitz et al., 2006). Endopeptidases, carboxypeptidases and aminopeptidases, present at the brush border membrane add a supplementary barrier to absorption of intact peptides (Pereira de Sousa and Bernkop-Schnürch, 2014).

The human intestinal epithelium is designed to limit the absorption of pathogens in the systemic circulation and acts as the next and major barrier to peptide oral bioavailability. Three main pathways can be considered for peptide absorption depending on their specific physicochemical properties: transcellular, paracellular and receptor-mediated transports. The epithelium border is in great majority composed of enterocytes with lipophilic membranes. Peptides being generally highly hydrophilic molecules are then poor candidates for the transcellular pathway, except for some lipophilic peptides such as Cyclosporine A. These epithelium cells are separate by tight junctions allowing the transport of small hydrophilic molecule toward *lamina propria*. This paracellular pathway is limited for peptides whose size was arbitrary fixed under 50 amino acids (Craig et al., 2013; Li et al., 2012). The junctional space between cells displays a negative electrostatic field that may alter permeability of some molecules by charge-charge interactions. Active-transportation across enterocytes is only possible for small peptides (Pawar et al., 2014). In particular, PepT1 transporter limits absorption to di and tripeptides (Miner-Williams et al., 2014). Finally, the intestinal epithelium is covered by mucus. This viscoelastic gel can interact with peptides and prevent their permeation across the epithelium.

All the physiological barriers described above have been known for a long time and have urged formulators to develop strategies to overcome them. These strategies comprise the use of enteric protection, enzyme inhibitors, permeation enhancers, mucoadhesive polymers, the modification of the therapeutic peptide structure, or the use of advanced drug delivery systems.

Fragile molecules can be easily protected for acidic environment by using enteric protection (coating or capsules). They are generally composed of cellulosic or acrylic based-polymers that are very stable at acidic pH, in the stomach, and fast dissolving when pH increases. Thus, they release their content in the upper part of the small intestine where peptides are less susceptible to pH-induced degradation (Bruno et al., 2013; Gao et al., 1998; Leonaviciute et al., 2016). Enteric protection also prevent degradation from gastric enzymes and provide a gradual and steady drug release upon dissolution (Leonaviciute and Bernkop-Schnürch, 2015).

Proteases inhibitors can temporarily restrict the activity of proteolytic enzymes (Bernkop-Schnürch, 1998). Their use is nevertheless questioned because their action is not specific to therapeutic peptides and can disturb the metabolism of dietary proteins (Morishita and Peppas, 2006). Furthermore, repeated administration of these substances can induce an over-secretion of enzymes leading to pancreatitis overtime.

Permeation enhancers alter the natural anatomy of the intestinal membrane by changing mucus viscosity, increasing intestinal membrane fluidity or modifying structural integrity (Pawar et al., 2014). Despite the interesting results obtained by addition of permeation enhancers, one has to keep in mind that their use not only facilitates the passage of the drug but also of unwanted substances. Thus, their repeated use, in chronic diseases for example, has to be carefully considered before including them in dosage forms.

Mucoadhesive systems prolong residence time of biomolecules at the absorption site and enable an increased concentration gradient between the system and the intestinal membrane. Several polymers have been reported to allow adhesion of nanocarriers transporting peptides and proteins such as PEG, cyclodextrins, poly (ethyl cyanoacrylate) or polyallylamine (Gupta et al., 2013). Chitosan-based nanoparticles are one of the most used systems. Chitosan is a cationic polymer which allows electrostatic interactions with the negatively charged sialic acid residues of the mucosal surface (Mrsny, 2012). Thiolated polymers have also been widely studied due to their ability to form strong covalent bond with cysteine-rich subdomains of the mucus glycoprotein (Muheem et al., 2016). However, mucoadhesive systems are eliminated upon mucus degradation and regeneration. The intestinal epithelium is the most rapidly renewed body tissue with a complete renewal every 4–5 days (Brayden et al., 2015; Naudi, 2012).

Chemical conjugation of peptides with PEG (PEGylation) have also been reported as one of the most successful technique to increase peptides residence time by limiting their interaction with mucin. Furthermore, their long chains are able to increase solubility of the biomolecules while shielding them from enzymatic degradation. Peptides can be absorbed via associated transport mechanisms using membrane transporters or receptor mediated endocytosis by grafting specific ligands to macromolecules. Most reported ligands are cell-penetrating peptides (CPPs). CPPs are small peptides, capable of internalizing bioactive molecules into cells by perturbation of the lipid bilayer of enterocytes (Brooks et al., 2005).

As a minimum lipophilicity is required to cross the epithelial barrier through transcellular way, some trials were conducted to increase lipophilicity of biomolecules. This lipidization operation consists either in the covalent grafting of a hydrophobic moiety or the non-covalent interaction with a hydrophobic compound, thus enhancing the hydrophobicity of the peptides (Morishita and Peppas, 2006). Yet, to ensure a preservation of the peptide therapeutic efficacy, this operation has to be reversible once the epithelial membrane has been crossed. Increasing lipophilicity has also showed an increase association with chylomicrons, making them able to undergo a significant lymphatic transport. Yet, chemical modification of the active substances raises the question of therapeutic efficacy of peptides *in vivo* if structural modifications affect their capacity to bind with the receptor sites (Meyer and Manning, 1998).

Finally, advanced drug delivery systems can be used both to enhance protection of peptides against the harsh environment of the GI tract and to increase transport across the epithelial border. These vectors can be hydrogels, micelles, microspheres, and polymeric and/or lipid-based nanoparticles. Among them, nanosized carriers are of great interest as their dimension favors the transport across the epithelial border and are supposed to provide a better drug distribution at the apical surface of the intestinal epithelium compared to other solid dosage forms (Mrsny, 2012). When drugs are attached to carriers, their fate *in vivo* is determined by the carrier system properties (Almeida and Souto, 2007). Lipid-based nanocarriers are promising tools to deliver peptides as lipids are GRAS, biodegradable excipients and consequently do not accumulate in tissues. Furthermore, lipid-based molecules are known to increase transcellular transport by transient disruption of cells lipophilic bilayers. Furthermore, some of the lipid digestion products are known to alter tight junctions with a consequent increase of drug transport through the paracellular pathway (Niu et al., 2016). Numerous lipid-based nanocarriers are reported in the literature:

nanoeulsions, self-emulsifying drug delivery systems (SEDDS), liposomes, micelles, nanospheres and nanocapsules. Two solid-core lipid-based nanocarriers are widely described in the scientific literature: Solid Lipid Nanoparticles (SLNs) and Nanostructured Lipid Carriers (NLCs).

Development of SLNs started in the early 90s to replace liquid oil in o/w emulsions by solid or blend of solid lipids (Pardeike et al., 2009). Generally dispersed in water, they are composed of lipids in solid state at room and body temperature, typically long-chain triglycerides (stearate, palmitate...) or partial glycerides, fatty acids (FA), waxes and phospholipids. Surfactants (poloxamer 188, polysorbate 80, lecithin...) can be added to increase their stability in water (Matougui et al., 2016). The use of long-chain FA as lipidic phase might hinder the incorporation of hydrophilic peptides inside the nanocarriers. Hence, lipidization of the therapeutic peptide should be considered to increase the drug loading. Moreover, polymers can be associated with SLNs to grant additional functionalities. For example, chitosan coated SLNs have been shown to avoid phagocytosis after intestinal uptake compared to uncoated SLNs (Sarmiento et al., 2011).

NLCs were developed to overcome the problem of drug expulsion during crystallization or phase transition of lipids constituting SLNs (Almeida and Souto, 2007). Liquid lipids at room and body temperature are added to the solid ones in order to reduce the crystallization capacity of the material and conserve the encapsulated drug within particles (Beloqui et al., 2016b; Kathe et al., 2014; Muchow et al., 2008). NLCs system were compared to SLNs on drug release and stability for which NLCs demonstrated competitive advantages (Das et al., 2012).

This review focuses on solid lipid-based nanocarriers (SLNs and NLCs), which are said to have high drug loading capacities, long shelf-life stability and whose large scale production is made possible. However, high drug loading of hydrophilic peptides in lipid nanocarriers can only be achieved after increasing their lipophilicity. This paper aims to describe (i) the different lipidization methods of peptides, (ii) the SLNs and NLCs fabrication processes and their current and tentative applications to therapeutic peptide encapsulation, and (iii) the key evaluation techniques to assay their potential applications to peptide oral bioavailability enhancement.

2. Lipidization

The hydrophilic character of most peptides is a limiting factor for their inclusion in lipid-based nanocarriers and generally leads to poor entrapment efficiencies (EEs). Various techniques exist to increase their lipophilicity.

2.1. Lipophilicity measurements

Lipophilicity is generally measured via partitioning measurements between octanol and water (Log P). This test is conducted by incubating the compound in a biphasic system composed of water and n-octanol in equal ratios at 37 °C under constant shaking (Griesser et al., 2017; Sun et al., 2011). Peptide content in each phase allows the calculation of the partitioning coefficient Log P, Eq. (1). Positive Log P means that the compound prefers octanol vs. water; it is then considered as lipophilic. This coefficient is a good indicator of the release rate of complexes from lipid nanoparticles: the more lipophilic the drug the more sustained the release. Furthermore, a high Log P also indicates a potential improved permeability across the lipid bilayer of the cell membranes (Muchow et al., 2008).

Log P calculation

$$\text{LogP} = \log \left(\frac{C_{\text{peptide in n-octanol phase}}}{C_{\text{peptide in aqueous phase}}} \right) \quad (1)$$

As most peptides are ionized depending on pH, Log D, more

representative of ionized species, can also be applied (Zupančič and Bernkop-Schnürch, 2017), Eq. (2).

Log D calculation

$$\text{LogD} = \text{LogP} + \log \left(\frac{1}{1 + 10^{\text{pH} - \text{pKa}}} \right) \quad (2)$$

These parameters constitute useful tools to predict the solubility of a drug into lipid-based excipients (Alskär et al., 2016).

2.2. Covalent lipidization

Several studies report the covalent grafting of lipophilic moieties to increase peptides lipophilicity. The Reversible Aqueous Lipidization (REAL) technique consists in the reduction of disulfide bridges to create two thiols functionalities which are intended to react with a thiol-reactive, water-soluble, lipidizing agent. In case of pH sensitive peptides, the linker should be amine-reactive (Morishita and Park, 2016; Wang et al., 2003). This method was successfully used to lipidized desmopressin and increase by 250-fold its potency. The results were highly dependent on the chain length of the lipid and the structure of the linker (Wang et al., 2002).

Lipophilicity of peptides can also be enhanced by cyclization of the backbone, depending on exhibited residues: lactamization, lactonization or sulfide-based linkages (Goodwin et al., 2012). It leads to an increase in intramolecular hydrogen interactions, limiting intermolecular hydrogen interactions with aqueous environment (Gangwar et al., 1997).

However, grafting of covalent lipidization of peptides not only modify peptides lipophilicity but can also have dramatic incidence on their structure. This will consequently lead to modification in their absorption, distribution, metabolism and excretion (ADME) (Zhang and Bulaj, 2012). Most of all, peptides modified by covalent binding will be regarded as a new drug entity.

2.3. Non-covalent lipidization

Increasing lipophilicity of ionizable peptides can be obtained by complexation with ionic surfactants. Ion-paired compounds are obtained by electrostatic interactions between ionized peptide drugs and ionic surfactants or polymers (Cheng et al., 2010). These non-covalent interactions guarantee the preservation of peptides therapeutic efficiency as long as they are reversible at the action site.

Most peptides are composed of at least one ionizable amino acids: at physiological pH, arginine, histidine and lysine are positively charged whereas aspartic acid and glutamic acid exhibit negative charges (Zupančič and Bernkop-Schnürch, 2017). Then, the potential pairing ratio with ionic surfactant is governed by pH. For example, leuprolide acetate, a nonapeptide exhibiting two ionizable basic side-chains (one imidazole group of histidine with a pKa of 6.0 and one guanidine group of arginine with a pKa of 13.0) was complexed with dioctyl sulfocinate (AOT) in pH 6.5 and 4.0 aqueous medium, prior to incorporation in SLN. In pH 6.5, a 1:1 monoacetate:AOT ion pair was formed, resulting in an EE of 53.9% in SLN whereas in pH 4.0 a 1:2 ion pair was formed leading to an important increase of the lipophilicity and resulting in an EE of 86.8% (Gallarate et al., 2011), Fig. 1. Indeed, the Hydrophobic Ion Pairing (HIP) process involves the stoichiometric replacement of polar counter ions with an ionic surfactant of the same charge but less easily solvated in water (Meyer and Manning, 1998; Oliveira et al., 2016).

Choice on ionic surfactants is influenced by their biodegradability and lipophilicity. For instance, sodium deoxycholate is naturally produced in the liver and is reabsorbed from the site of action in the intestinal lumen to return to the liver (Sun et al., 2011). In particular, FA are preferred for their ability to open tight junctions and solubilize

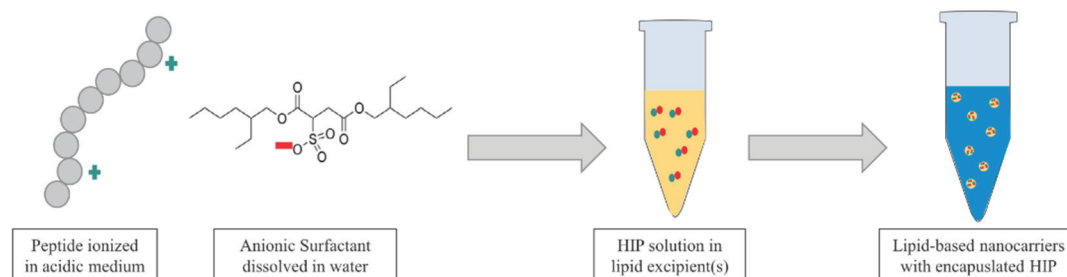


Fig. 1. Hydrophobic ion pairing enabling peptide loading in lipid-based nanocarriers.

enterocyte membranes. Furthermore, complexing peptides with long chain FA can disguise the drug into glycerides and limit their elimination by the immune system (Zupančič and Bernkop-Schnürch, 2017).

FA with different functional groups and carbon chain (sodium docusate, sodium dodecylsulfate and sodium oleate) were used to form complexes with desmopressin, insulin and leuprolide (Griesser et al., 2017). Sodium docusate showed the highest precipitation efficiency with all peptides, up to 99.1% with leuprolide (ratio 2:1). This highlights the importance of the structure of surfactants in HIP. Indeed, the surfactant substructure seemed to have more impact on the complex stability than the charge neutralizing effect.

The amount of surfactant added to the peptide has to be controlled to obtain the best increase in lipophilicity. Indeed, the hydrophobicity of the complex will increase until reaching a plateau, then, any addition of surfactant will lead to the formation of micelles which dissolve complexes (Choi and Park, 2000; Hintzen et al., 2014; Thompson et al., 2009). The extent of formation of the ion-paired complexes is generally evaluated by subtraction. The concentration of the remaining peptide in the dispersion media is subtracted to the initial peptide concentration, leading to the amount of precipitated complex. Turbidity measurements taken on different dispersions of poly(allylamine) grafted with palmitoyl (comb-shaped polymer) and insulin in Tris buffer, also allow to evaluate the scope of complexation of the two entities (Thompson et al., 2009).

Peptides gain in lipophilicity leads to the improvement of their loading in lipid based carriers. Griesser et al. managed to load 10.67% of desmopressin, insulin and leuprolide in SEDDS by using the HIP approach with sodium docusate and appropriate solvents (Griesser et al., 2017). Furthermore, the loading of SLNs prepared by a solvent-diffusion method was largely improved with incorporation of leuprolide:sodium stearate complex (ratio 1:2) with a final EE of 46.1%, compared to 28.0% for free leuprolide. The same complex lead to an EE of 74.6% in SLNs obtained by an o/o (stearic acid in paraffin) emulsion-evaporation method, whereas only 53.1% was measured with free leuprolide, (Yuan et al., 2009).

Powers et al. showed an unexpected side effect of pair formation. Indeed, complexation of insulin with sodium dodecylsulfate contributed to an increase by 50 °C of the peptide denaturation temperature (from 65 °C to ~115 °C) (Powers et al., 1993). This interesting fact opens new perspectives for high temperature processes.

3. Methods for preparation of solid lipid-based nanoparticles

3.1. General principle

Nanospheres can be obtained by low or high energy processes. The former one is relying on the physicochemical characteristics of the selected excipients. Low energy processes have to play on the partitioning coefficient of the components in different solvents, on their conformation at various temperature or even on the use of dedicated apparatus to obtain particles with the desired properties.

Conception of nanospheres by high energy input mostly concerns

melt dispersion techniques. Lipid-based matricial systems are depending on the formation of nanoemulsions: a dispersion of lipidic phase into a continuous aqueous phase stabilized by surfactants (Couffin and Delmas, 2013). This process is handled at a temperature 5 to 10 °C above the melting point of the selected lipid excipients, solid at room and body temperature in the case of SLNs. From the immiscibility of both phases, resulting from the incapacity of the aqueous phase to establish enough hydrogen bonding with the oily phase, derives the interfacial tension between both liquids (Brochette, 2013). The existence of this interfacial energy leads to a pressure difference across both fluids that is well described by Young-Laplace equation. To form nanoemulsions, the shear stress applied to the biphasic system must exceed Laplace pressure (Mason et al., 2006) to counter Gibbs free energy and disperse one phase into the other (Couffin and Delmas, 2013). Thus, the energy brought to the system should be able to increase the interface, to overcome the resistance induced by both phases' viscosities while an important part is dissipated to the system by Joule effect. Taylors's equation enables to estimate the shear rate $\dot{\epsilon}$ (s^{-1}) to be applied to obtain the desired droplet diameter d_p as a function of the interfacial tension γ ($N.m^{-1}$) and the viscosity of the continuous phase η (Pa.s), Eq. (3) (Mason et al., 2006; Tiwari and Tiwari, 2013). This model is only valid for diluted systems.

Taylor's equation

$$d_p = \frac{2\gamma}{3\eta_c \dot{\epsilon}} \quad (3)$$

As soon as small droplets are formed, the surfactants generally solubilized in the aqueous phase are absorbed on the interface. This coating layer stabilizes the system.

The solid particles are finally obtained by cooling the system. The crystallinity of the nanospheres will depend on the cooling temperature and rate. However, the formation of supercooled melts in these systems is not unusual. They designate lipid-based nanoparticles for which solidification/crystallization does not occur at temperature lower than lipid melting point. Indeed, this crystallization phenomenon requires the presence of a critical number of nuclei to occur. Yet, it these event depends on particles size as nuclei are less likely to appear in small particles (Mehnert and Mäder, 2001, 2012). Solid state of particles can be easily monitored by Differential Scanning Calorimetry, X-ray diffraction and Nuclear Magnetic Resonance (Jores et al., 2003, 2004; Kathe et al., 2014).

Only a few devices such as High Pressure Homogenizers (HPH), microfluidizers or ultrasonic apparatus can apply enough shear rates to obtain nanosized particles via melt dispersion techniques.

An exhaustive review of process described in the literature to produce SLNs and NLCs is presented below. All attempt to encapsulate peptide in these lipid-based nanocarriers are also described and summarized in Tables 1 and 2.

Table 1

Melt encapsulation techniques used to produce lipid-based solid nanocarriers with peptides.

Peptide	Lipidic composition	Encapsulation technique used	Key characteristics	Reference
Calcitonin	Solid lips: Stearic acid or Glycerol mono stearate Surfactant: phosphatidylcholine Co-surfactant: butyric acid Steric stabilizer: dipalmitoyl phosphatidyl ethanolamine-PEG	Gasco method	High EE (83%) Low PDI	Gasco (1998)
Leuprolide	Solid lipid: stearic acid Stabilizer: Polyvinyl alcohol	Coacervation method	HIP, high EE, prolong drug release	Gallarate et al. (2011)
LHRH	Solid lipid: Stearic acid Surfactant: phosphatidylcholine Co-surfactant: butyric acid	Gasco method	High EE (80%) Low PDI	Gasco (1998)
Lysozyme	Solid lipids; Witexsol E85 and Softisan 142 (hard fats) Surfactants: Poloxamer 188, Tween 80 or sodium cholate	High Pressure Homogenization (cold process)	No degradation of the peptide	Almeida et al. (1997)
Thymopentin	Solid lipid: stearic acid Surfactant: egg lecithin, taurodeoxycholate sodium salts	Gasco method	Successful encapsulation of the peptide in SLNs after complexation Low EE (5%)	Morel et al. (1996)
Vancomycin	Solid lipid: Compritol 888 (glyceryl behenate) Surfactant: Lutrol F68	Ultrasonication	High EE after complexation (71%) Nanosuspensions stable for 3 months at 4 °C	Kalhapure et al. (2014)
Insulin	Solid lipid: stearic acid Stabilizer: Polyvinyl alcohol	Coacervation method	HIP, high EE, prolong drug release	Gallarate et al. (2011)

3.2. Melt dispersion

3.2.1. High pressure homogenizer (HPH)

A HPH is an apparatus enabling to push a liquid with high pressure (from 100 to 2000 bar) through a narrow gap (few microns). Therefore, the liquid will accelerate on a very short distance. The high shear stress induced disrupts the particle down to the nanosize range (Torchilin, 2006). This technology can be used either via hot or cold process.

In the hot HPH process, the drug is first homogeneously dispersed in the melted lipids (temperature 5–10 °C higher than lipid melting point).

Then, the aqueous surfactant solution heated at the same temperature is poured into the lipophilic solution, under high shear mixing. The pre-emulsion formed is then injected into the HPH. The intensifier pump continuously sucks portions of the pre-emulsified system and compresses it under high pressure into the interaction chamber. The resulting shear rates combined with the impact forces from collision with the walls and the fluid itself enable drastic size reduction. Nonetheless, several cycles should be run to obtain the desired particle size (Das and Chaudhury, 2011). As all high-energy intensification methods, hot HPH induce heat transfer to the material. However, the residence time of the

Table 2

Solvent-based encapsulation techniques used to produce lipid-based solid nanocarriers with peptides.

Peptide	Composition	Encapsulation technique used	Key characteristics	Reference
Calcitonin (salmon)	Solid lipid: glyceryl tripalmitate Surfactant: lecithin Solvent: dichloromethane	Emulsion evaporation method	Burst effect, prolonged drug release High EE (> 90%)	García-Fuentes et al. (2005)
Cyclosporine A	Solid lipid: Gelucire 44/14 (polyoxyglycerides) Stabilizer: Polyvinyl alcohol Solvent: ethyl acetate	Emulsion-diffusion method	Fast drug release High EE (92%) Unstable suspension – expulsion of the peptide	Urbán-Morlán et al. (2010)
Gonadorelin	Solid lipid: glyceryl mono stearate Stabilizer: polyvinyl alcohol	Emulsion-diffusion method	High EE (69%) Burst effect, prolonged drug release in simulated GI fluid	Hu et al. (2004)
Insulin	Solvent: acetone and ethanol Solid lipid: glyceryl mono stearate Stabilizer: polyvinyl alcohol Solvent: isovaleric acid	Emulsion-diffusion method	Burst effect Conservation of Insulin biological activity Protective effect toward trypsin	Battaglia et al. (2007)
Insulin	Solid lipid: Dynasan 116 Surfactants: L- α -lecithin (from soy) and Simulsol M52 Solvent: dichloromethane	Emulsion evaporation method	Burst effect	García-Fuentes et al. (2003)
Insulin	Solid lipid: cetyl palmitate Surfactant: Poloxamer 407 Solvent: dichloromethane	Emulsion evaporation method	Proven <i>in vivo</i> activity, 5% relative pharmacological bioavailability	Sarmiento et al. (2007)
Insulin	Solid lipid: Dynasan 114 Surfactant: soy lecithin Stabilizer: Polyvinyl alcohol Solvent: dichloromethane	Emulsion evaporation method	Low PDI (0.021) > 8% oral BA	Ansari et al. (2016)
Insulin	Solid lipid: glyceryl tristearate Surfactants: dioctyl sulfocinate and Tween 80 Solvent: CO ₂	Supercritical fluid	High EE, proven <i>in vivo</i> activity Release rate depending on presence of PEG-coating	Salmaso et al. (2009)

dispersion into the apparatus is relatively short, thus limiting the risk of degradation of thermosensitive drugs such as peptides (Müller et al., 2000). This method enables a restricted heating of peptides, most of them sensitive to high temperatures.

For thermosensitive drugs the cold process was developed. The drug is also dispersed in melted lipids but the mixture is rapidly placed in liquid nitrogen or dry ice to ensure fast cooling and to limit the exposure of the drug to heat. The solid obtained is milled into microparticles that are then suspended in a cold aqueous surfactant solution. These particles are then injected into the HPH to form the nanoparticles at low temperatures (Schwarz et al., 1994). Several cycles are also necessary to decrease particles size to an appropriate level. This method enables a restricted heating of peptides. It was employed to encapsulate lysozyme, a 130 amino acids protein, into SLN and vaccine applications. The drug was not degraded by the harsh process conditions and the EE was considered appropriate for the tested applications (Almeida et al., 1997). To our knowledge, this technique was not employed for the delivery of smaller biomolecules. There is a high risk of peptide partition into the aqueous phase during the dispersion phase of the milled particles. Indeed, a lipidization of hydrophilic peptides is highly encouraged to increase EE and DL. Furthermore, as the microparticles are milled we can expect that the created surface will not be covered by surfactant that might result in rapid aggregation of the nanoparticles.

HPH process is common in the pharmaceutical industry due to the ease of scaling-up. Indeed, larger scale equipment leads to an even better quality of the product with smaller particle size and increased homogeneity (Muchow et al., 2008). Furthermore, HPH has been reported more efficient than ultrasonication or high shear mixing to obtain nanosized particles (Mehnert and Mäder, 2001).

3.2.2. Ultrasonication

Ultrasonication is easy to conduct using a probe sonicator. The ultrasonic waves propagate through the pre-emulsified system, thus creating alternating low-pressure and high-pressure areas. This phenomenon causes cavitation and formation of the nanoparticles (Abismail et al., 1999). Using a response surface methodology, Siddiqui et al. have shown that increasing sonication time and pulse frequency contributed to decrease particle size with a greater impact of pulse frequency (Siddiqui et al., 2014). Indeed, size evolution of the nanoparticles can be described as a monoexponential function of the process time. Nonetheless, if the sonication time lasts long enough, the saturated size of the particles will be reached, independently of the sonication power (Delmas et al., 2011). Particle size is also highly dependent on the chain length of the selected lipids. Thus, when SLNs are prepared in the same conditions with Compritol® 888 ATO (glyceryl dibehenate, C22), Gelucire® 43/01 (mono-, di- and triglycerides esters of FA, C18), Glyceryl monostearate and Precirol® ATO 5 (glyceryl palmitostearate, C16-18), their sizes increase with the chain length (and melting point) of the excipients as a consequence of the increased viscosity (Elbahwy et al., 2017).

Vancomycin is a glycopeptide antibiotic used in treatments against *Staphylococcus aureus*. It was successfully encapsulated (EE = 70.73 ± 5.96% and Drug Load (DL) = 1.41 ± 0.2%) in Compritol® 888-based SLNs after a complexation with linoleic acid. Particles were obtained by homogenization at 6000 rpm for 15 min followed by ultrasonication during 30 min with an amplitude of 30% with both phases at 80 °C. Particles obtained after cooling at 20 °C displayed a particle size of 127 ± 0.25 nm with a Polydispersity Index (PDI) of 0.261 ± 0.01 (Kalhapure et al., 2014).

Publications reporting the use of the ultrasonic method to encapsulate peptides are scarce. Indeed, as all high energy methods, high shear induced by sonication also causes rapid heat increase of the system, especially as the process must be ran several minutes (sometimes dozen minutes). These harsh process conditions can potentially damage thermosensitive drugs (Siddiqui et al., 2014). Furthermore, the ultrasonication method can hardly be transposed to the industrial scale.

Finally, contamination of the emulsion by metallic residues during the process can occur which may prohibit its use in the pharmaceutical field (Das and Chaudhury, 2011).

3.3. Membrane contactors

This technique, patented in 2007 by Charcosset and Fessi, is also based on a melt dispersion technique although it is a low energy process (Charcosset et al., 2005). The lipid or lipid mixture is placed in a thermostated pressurized reservoir connected to a nitrogen bottle. The melted material is pushed into a continuous flow across a membrane contactor. The oily phase forms nanodroplets at the inner surface of the membrane where the heated aqueous solution of surfactant sweeps away the particles (Charcosset et al., 2005). Nanoparticles are obtained by cooling the nanoemulsion.

One of the main parameters to control particle size is the aqueous phase cross-flow velocity. Indeed, increasing circulation of the aqueous phase flow enables a faster detachment of the oily droplets consequently reducing their size. The second important parameter for controlling particle size is the lipid phase pressure that directly impacts lipid phase flux. Consequently, an increase in pressure leads to a decrease in particle size. Furthermore, particle size also decreases with the temperature (and therefore, lipid viscosity) and the amount of lipids (Charcosset et al., 2005).

Nowadays, no peptide-loaded SLNs were prepared by membrane contactor technique. However, this technique resulted in a 100% DL capacity of vitamin E, a lipophilic API and should have similar results with peptides after lipidization.

3.4. Gasco method

SLNs formulated by the Gasco method are based on the microemulsion template. Lipids containing the active substance are melted above their melting point. By contact with an aqueous solution of surfactants and eventually co-surfactants heated at the same temperature, o/w micro-droplets are formed. The microemulsion is poured into cold water under stirring and the particles are washed by diafiltration and lyophilized. The particles obtained are claimed to be between 50 and 800 nm, preferably between 100 and 400 nm, with a PDI between 0.06 and 0.90 (Gasco, 1993).

The lipid components selected comprise triglycerides, FA and alcohols. Surfactants are chosen among non-ionic surfactants (between 12 and 20%) and co-surfactants (3–7%) are preferably selected among low molecular weight alcohols or glycols and low molecular weight FA.

This method was employed to encapsulate thymopentin (TP-5), an immunostimulator pentapeptide, into SLNs. The hydrophilic drug was first complexed with sodium hexadecyl phosphate to increase its lipophilicity and then dispersed in melted stearic acid. This lipid mixture was added into the aqueous phase containing egg lecithin, taurodeoxycholate and butanol to form the o/w microemulsion. Then, the warm microemulsion was diluted (ratio 1:10) in water at 2 °C. After three washes by ultrafiltration and freeze-drying, the particles were dispersed anew and their size was evaluated around 100 nm with a PDI of 0.29, an encapsulation rate of 5.2% and a DL of 1%. In this study, a w/o/w was prepared by the same technique without HIP formation. The concentration of TP-5 in the SLNs was limited to 0.5% to obtain a clear microemulsion system. However, release rates were equivalent for both systems (Morel et al., 1996).

In another patent from Gasco, other examples of peptides encapsulated in SLNs are provided, with the additional claim of being capable to cross mucosal and brain blood barrier. Then, 80% of luteinizing hormone-releasing hormone (LHRH) was encapsulated in 96 nm SLNs from obtained w/o/w microemulsions based on stearic and butyric acids, stabilized by phosphatidylcholine. Calcitonin was also encapsulated in SLNs based on w/o/w microemulsion templates of stearic acid, monostearine and phosphatidylcholine. An EE of 83% was

reached in 93 nm particles (Gasco, 1998).

The Gasco method enables the formulation of SLNs with a solvent free process under gentle conditions that seems well adapted for peptides.

3.5. Jay and Mumper method

The method developed by Mumper and Jay is also based on the use of micro-emulsion templates to obtain lipid-based nanoparticles under 300 nm. These particles can be used to encapsulate drugs (proteins, peptides, plasmid DNA and small molecules), magnets or sensors and can be delivered to specific regions of the body by including cell-specific targeting ligands. The microemulsion templates can be either alcohol-in-fluorocarbons, liquid hydrocarbon-in-fluorocarbon or liquid hydrocarbon-in-water microemulsions and the encapsulated material is either located in the inner core of the particle or absorbed on its surface. For the specific production of drug-loaded SLNs, the material to be encapsulated (20 µg/mL–5 mg/mL) is dissolved or dispersed in the melted lipids (phospholipids, fatty acids, fatty alcohols...) (0.1–30 mg/mL). The mixture is added to the dispersed phase containing surfactants, eventually co-surfactants (with total surfactant concentration of 1–5000 mM, preferably 1–300 mM), and a film-forming substance. While particles are obtained by dilution in cold water in Gasco method, the process developed by Mumper and Jay uses a curing technique to solidify the pharmaceutical film-forming excipient and form a membrane suitable for drug delivery. Three methods were developed to cure this material: a) cooling of the undiluted o/w micro-emulsion from the process temperature to room temperature under continuous stirring, b) cooling of the undiluted o/w microemulsion from the process temperature by placing it in a refrigerator at 4 °C and c) dilution 1/10 of the microemulsion by addition of cold water at 4 °C. No significant difference was observed in particle size between these three curing procedures (Mumper and Jay, 2006).

3.6. Solvent-based techniques

3.6.1. Nanoprecipitation

Nanoprecipitation method enables the formation of spherical particles below 500 nm. It was first described by Fessi et al. for applications in pharmaceutical, medicinal and cosmetic fields (Fessi et al., 1988). In this process, a compound (polymer, protein, liposome) able to form the membrane (nanocapsules) or the core (nanospheres) of the particle is solubilized in a water-miscible solvent containing the material to be encapsulated, either solubilized or dispersed. This organic phase is then added to a non-solvent phase containing surfactants: the particles are formed by instantaneous migration of the solvent into the continuous phase, Fig. 2.

In the case of SLNs, nanoparticles are prepared by addition of a solution of lipids (e.g. triglycerides mixtures) dissolved in water-miscible solvent or solvent mixtures (e.g. isopropanol, ethanol, acetone...) in an aqueous surfactant solution (poloxamer 188, polysorbate 80...) (Schubert and Müller-Goymann, 2003). The spontaneous diffusion of the organic solvent in the aqueous phase upon the “emulsification step” will immediately decrease lipid solubility in the solution and lead to its precipitation. Thus, the main parameter to consider using this technique is the diffusion speed of the solvent into the aqueous phase. Playing on the amount of surfactant enables increase control of droplet size. Use of injection needles have also been reported (Das and Chaudhury, 2011; Schubert and Müller-Goymann, 2003). An evaporation step is required to eliminate the organic solvent.

This method has not yet been applied to the encapsulation of peptide, but two variations of this technique have been successfully tested with four therapeutic peptides.

3.6.2. Emulsion-diffusion method

The emulsification-diffusion method is based on a similar principle

as nanoprecipitation, playing on excipients miscibility with different solvents. Here, the chosen solvent must be partially miscible with the non-solvent, generally water. Solvents are mutually saturated to ensure initial thermodynamic equilibrium. Lipids and lipophilic drugs (or lipidized hydrophilic drugs) are dissolved into the organic phase saturated with water and emulsified under high shear stirring in the water phase saturated with the organic solvent. The organic solvent migrates into the outer water, leading to the precipitation of lipids and the formation of the nanoparticles, Fig. 3. As in the nanoprecipitation method, an evaporation step is also required to eliminate the solvent. The final suspension is generally diluted and needs to be concentrated by ultrafiltration or freeze-drying (Das and Chaudhury, 2011; Mora-Huertas et al., 2010).

The different parameters capable of influencing particle size in this technique were highlighted by Quintanar-Guerrero et al.: the higher the miscibility between solvents is, the smaller are the particles. Their work has also shown that increasing the lipid amount gives bigger particles. A lipid concentration of 10% is generally preferred (Quintanar-Guerrero et al., 2005).

The emulsion-diffusion method has been successfully employed for the formulation of peptide-loaded monostearin SLNs with gonadorelin, resulting in a drug EE of 50.4% at 25 °C and 69.4% at 0 °C for particles around 400 nm (Hu et al., 2004). However, the particles displayed an important burst release (24.4% in 6 h) in pH 6.8 buffer, indicating that an important amount of drug was located at the interface of the nanocarrier. However, the remaining encapsulated gonadorelin was slowly and regularly released over a period of 12 h.

For encapsulation of insulin, Battaglia et al. formulated nanoparticles of glyceryl monostearate using isovaleric acid and water as partially miscible solvents. Isovaleric acid is a solvent for both insulin and lipid: when the system is diluted after emulsification, they precipitate simultaneously leading to insulin encapsulation. The system was pre-emulsified by ultraturrax (15000 rpm for 1 min) or HPH (5000 psi). Particles size were 1035 ± 196 nm with a PDI of 0.26 and 205 ± 15 nm with a PDI of 0.08 respectively. Contrary to the dispersion size, the EE around 45% was not especially affected by the processing device employed. However, 30% of the encapsulated insulin was released *in vitro* within 5 h due a high proportion of the drug located at the surface of the nanospheres (Battaglia et al., 2007).

Encapsulation of cyclosporine A, a particular lipophilic peptide used as immunosuppressor in organ transplants, was made possible by using Gelucire® 44/14 (lauroyl polyoxyglycerides) as matrix excipient, ethylacetate and water as solvents and polyvinylalcohol as stabilizer. An EE above 92% was measured but the *in vitro* drug delivery was quite fast as 99.6% were released within 45 min (Urbán-Morlán et al., 2010). It should be due to the high water-solubility of the lipid-based excipient chosen as lipid matrix.

Studies reporting encapsulation of peptides in SLNs via an emulsion-diffusion method are conducted on hydrophilic peptides without alteration of their lipophilicity. Consequently, most of the drug migrates to the interface upon diffusion of the solvent into the aqueous phase that results in fast drug release and probably low protection towards digestive enzymes. This phenomenon could be reduced with the use of lipidized peptides.

3.6.3. Emulsion evaporation method

Emulsion-evaporation method is a variation of the two techniques described above. It also requires the use of a solvent capable of solubilizing the lipids and the active substance and a non-solvent. The solvent should have a lower evaporation temperature than the non-solvent. With the evaporation step, the fraction of non-solvent increases leading to the precipitation of the lipids, instantaneously stabilized by the surfactants. This method often uses w/o/w formulations. For example, Garcia-Fuentes et al. have succeeded in encapsulating insulin in tripalmitin-based SLNs using dichloromethane as solvent and water as non-solvent. However, an important burst release was recorded *in vitro*

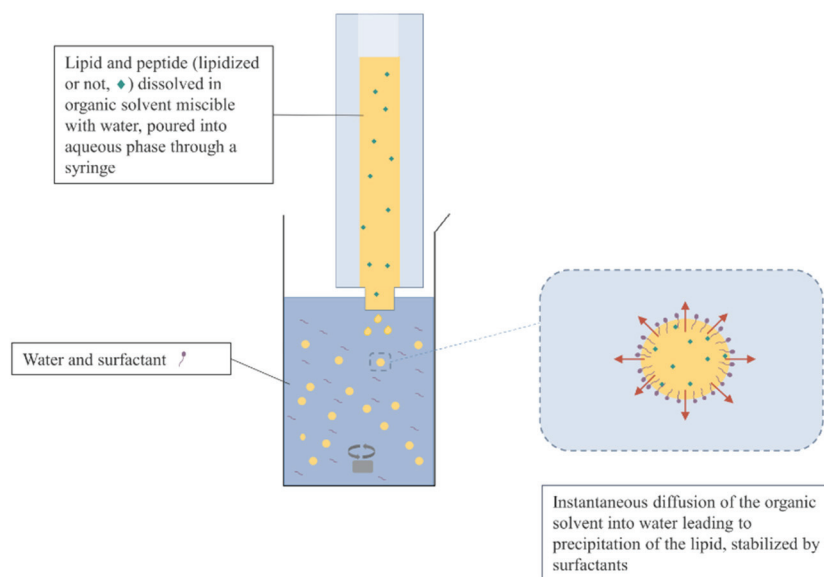


Fig. 2. Set up for preparation of SLN and NLC by nanoprecipitation method.

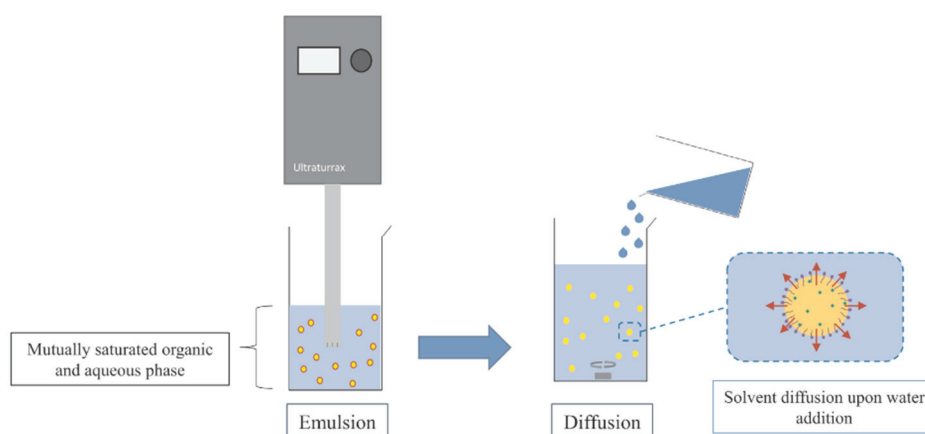


Fig. 3. Set up for preparation of SLN and NLC by emulsion-diffusion method.

studies (García-Fuentes et al., 2003). The same method was used with cetyl palmitate as lipid and exhibit a 43% EE. The *in vivo* activity of entrapped insulin was also proven (Sarmiento et al., 2007). A more recent study conducted in the same conditions claims a bioavailability 8.26% for insulin-loaded SLN versus 1.7% for the free insulin solution (Ansari et al., 2016). Emulsion-evaporation was also used to encapsulate salmon calcitonin in the inner aqueous phase of a w/o/w emulsion, forming SLNs or NLCs after evaporation of the solvent (dichloromethane). The system also showed an initial burst release followed by a continuous and slow release of the drug. This fact was explained by the affinity of the peptide for the lipid excipients used (García-Fuentes et al., 2005).

Yuan et al. described a variation of the emulsion-evaporation method. Their process is said to encapsulate hydrophilic or lipophilic compound in a lipid-based nanospheres by preparing an oil-in-oil emulsion (Yuan et al., 2009). This emulsion consists in dissolving the active substance and the lipid in a solvent prior to mixing with a non-miscible oily phase (e.g. paraffin oil). SLNs precipitate when evaporating the solvent. Relatively high EE rates were obtained even with a

hydrophilic active substance: the absence of aqueous phase prevented the migration of the macromolecule from the lipid phase. However, high burst effect was observed during *in vitro* release test as an important fraction of the active substance was located at the surface of the particle.

3.7. Phase inversion temperature techniques

The Phase Inversion Temperature (PIT) method was developed by Anton et al. in 2010 (Anton et al., 2010). This technique is based on the particular properties of some polyethoxylated (PEO) non-ionic surfactants that can be highly soluble in hydrophilic or lipophilic phases as a function of temperature. Thus, when reaching the phase inversion temperature, the relative affinity of the surfactant is inverted and the o/w emulsion turns into a w/o emulsion when increasing the temperature and vice versa (Anton et al., 2007). Furthermore, Heurtault et al. have proven that several cycles crossing the phase inversion zone lead to more reproducible nanoparticles (Heurtault et al., 2002). Thus, the particles are produced by mixing oil (generally capric or caprylic

triglycerides such as Labrafac® Lipophile WL 1349), PEO surfactant (polyethylene glycol hydroxystearate), water, NaCl and the active substance. The temperature of the formed emulsion is gradually increased until it reaches a temperature 8 °C above PIT leading to the phase inversion. Then, the temperature is decreased to a temperature slightly lower than PIT. The operation is repeated several times, most often three cycles are necessary. SLNs production through a PIT method should be possible by selecting a surfactant with a PIT above the melting point of the excipients (Anton et al., 2008). Finally, a sudden dilution by cold water causes the solidification of the lipid core conducting to the formation of SLNs.

The process was not reported to be used for encapsulation of peptides. One can imagine that the repetition of phase inversions creates high risks of peptides partition in the aqueous phase, leading to poor entrapment efficiency of the active substance.

This process can also be employed for the formation of reverse-micelles-in-oil-droplets-in-water nanoemulsions. These systems are kinetically stable, composed of a kinetically-stable nanoemulsion loaded with thermodynamically-stable reverse micelles. The first step of the process is similar to the classical phase inversion technique with the preparation of the emulsion stabilized by PEO surfactants. On the other hand, thermodynamically stable reversed micelles, capable of solubilizing hydrophilic molecules, are formed using lipophilic surfactants (e.g. Span®80, sorbitan monooleate). Drug loading of the micelles is made by simple incubation of the drug in the micellar system. The micelle-loaded oil is homogeneously incorporated to the oily continuous phase of the system described above at a temperature above PIT. The temperature is then progressively decreased to PIT. In a similar manner, the final particles are formed by dilution with cold water. The fast change in temperature provokes a rapid change in surfactant solubility and consequently a sudden displacement to the aqueous phase leading to the formation of a narrow monodispersed o/w nanoemulsion, regardless of the oil quantity.

This process has been successfully applied to encapsulate dyes such as fluorescein sodium salt (Anton et al., 2010), hydrophilic anti-cancer drugs doxorubicin hydrochloride and docetaxel (Vrignaud et al., 2011). We think that it could also be successfully applied to lipidized peptides.

3.8. Supercritical fluid extraction of emulsions

Supercritical fluids (SCFs), also called dense fluids or dense gases, are substances for which liquid and gas phases are not distinct. They are obtained when the temperature and pressure conditions are more elevated than the critical point of the material. The fluid obtained exhibits both liquid and gas properties: it can easily dissolve compounds and has high mobility into solids.

Several techniques were developed to produce nanoparticles based on different properties of the SCF. For Supercritical fluids Anti-Solvent (SAS) method, the ability of SCF to decrease the solvent capacity of organic solvents is exploited. Thus, injecting SCF in a solvent in which compounds (e.g. lipids) are dissolved leads to their precipitation and formation of nanoparticles in presence of surfactants (Knez and Weidner, 2003).

In Rapid Expansion of SCF Solution (RESS), the active substance or the active substance mixed with polymers is dissolved in a SCF and this solution is rapidly expanded via a capillary or a nozzle. The high supersaturation induced results in loss of the solvent capacity and therefore to nucleation and precipitation of the material with uniform conditions (Debenedetti et al., 1993). Shekunov et al. improved the technique to overcome agglomeration issues by addition of retardant compounds (sugar acetates or fluorocarbons) with SCF-philic and SCF-phobic groups and produce smaller particles, i.e. below 500 nm (Shekunov et al., 2010).

Chattopadhyay et al. patented a mechanism to obtain particles with polymers or lipids containing biologically active substance. The drug and the solid matrix excipient form a melt by contact with the SCF that

reduces solids viscosity. An emulsion is formed by contacting this melted phase with polar solvent containing surfactants. Further expansion of the emulsion forms particles whose size depends on the emulsion properties. The inventors claim that any peptide, protein, plant extract, nucleic acid, etc... can be encapsulated via this technique (Chattopadhyay et al., 2006).

Carbon dioxide is often used as SCF as the conditions for its production are quite soft with a temperature of 31 °C and a pressure of 72.9 bars (critical point). Furthermore, it is a cheap solvent with low toxicity and benign effects on the environment. It is also known to be miscible with numerous organic solvents making it ideal to extract them from o/w emulsion.

Tristearin-based SLNs were produced via atomization using SCF for encapsulation of insulin. Peptide and lipid were mixed under 150 bar of supercritical CO₂, reducing the melting point of the lipid from around 70 °C–35.4 °C which limited the risk of peptide denaturation. The melt was injected through a 100 µm nozzle under tangential air flux. Particles obtained were in the nanosize range (227 ± 158 nm) but very polydisperse. This method enabled the encapsulation of 95% of insulin and glycemic reduction after subcutaneous injection in rats proved the conservation of insulin bioactivity (Salmaso et al., 2009).

3.9. Coacervation method

Coacervation is based on the partial desolvation of two polyelectrolytes of opposite charges by modification of their environment (modification of pH or temperature). The compound to be encapsulated, liquid or solid, is first dispersed in a solution containing the two polymers. The desolvation is then initiated, leading to the formation of the coacervate by electrostatic attraction between the electrolytes.

Battaglia et al. have developed a specific technique to produce SLNs by a coacervation method in which particles are obtained by precipitation of FA from their sodium salts micelles (sodium stearate, sodium palmitate, sodium myristate, sodium behenate), Fig. 4. This phenomenon occurs above the FA Krafft point, at acidic pH, by proton exchange between the acidic solution and the salt of FA. Non-polymeric surfactant (PVA, HPMC) are used as stabilizer. The particles obtained by this technique are nearly monodisperse with regular and smooth surface and can be easily freeze-dried (Battaglia et al., 2010). Key parameters are the lipid concentration that will affect particles size and the type of stabilizer that will prevent aggregation and consequently particles polydispersity.

This technique is claimed to enable the loading of lipophilic substances in the micellar solution before acidification and resulting precipitation. Generally, it is applied to small lipophilic drug encapsulation such as amphotericin B (Bianco et al., 2010), cisplatin (Gallarate et al., 2010) or curcumin (Chirio et al., 2011).

Nonetheless, peptides with increased lipophilicity by use of HIP were successfully encapsulated in SLNs using this technique. The ion pairs (Leuprolide:dioctyl sulfocinate (AOT) 1:2 and Insulin:SDS 1:6) were dissolved in ethanol and added to a solution of sodium stearate dissolved in PVA aqueous solution at 50 °C. Particles precipitated by addition of an acidic coacervating solution to the system and cooling in water bath. An EE of 86.8% was measured with leuprolide:AOT ion pair with a mean particle size of 350 ± 30 nm and a PDI of 0.060. Insulin:SDS complex was encapsulated with an efficiency of 89.0% for particles of 418 ± 39 nm and a PDI of 0.055. Usual degradation products of insulin were not detected and sustained release profile was observed (Gallarate et al., 2011).

This coacervation technique has shown interesting results for the encapsulation of peptides. The method presents several process advantages such as an absence of toxic solvents, gentle stirring conditions compared to high energy processes, rather limited temperature which decreases the risk of peptides degradation.

Most common techniques to produce SLN and NLC were described

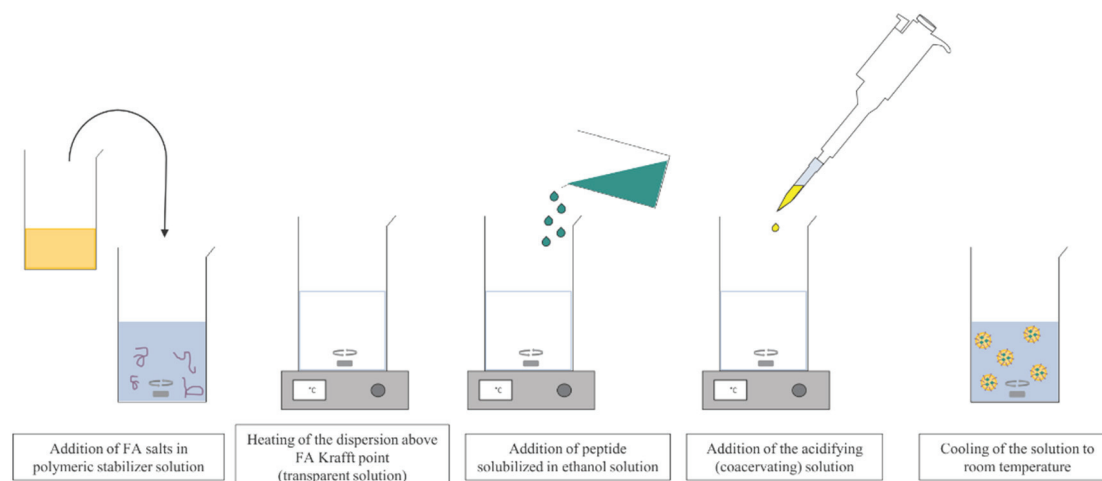


Fig. 4. Set up for preparation of SLN by coacervation method.

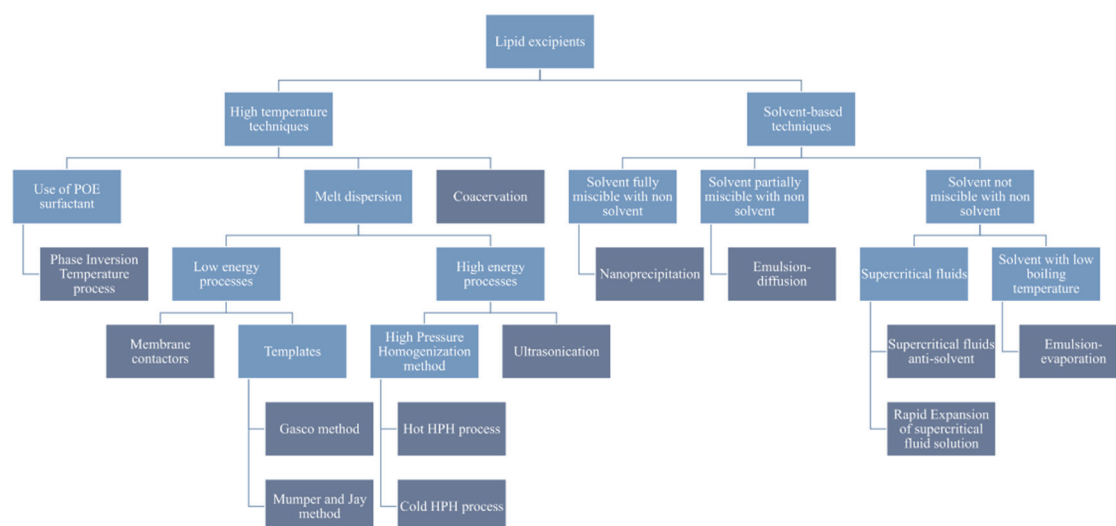


Fig. 5. Decision tree to select appropriate process to produce SLN or NLC.

in this section. The specificities of each process are summarized in Fig. 5. Table 3 provides a list of advantages and drawbacks inherent to each method in the case of peptide encapsulation.

4. Evaluation of the nanosuspensions for peptide oral delivery

The main issues of the nanoparticle-based systems are the protection of the peptide towards the harsh environment of the gastro-intestinal tract and the permeation improvement across the intestinal barrier without increased toxicity.

4.1. Protection towards proteases

Pepsin is a protease located in the stomach that specifically cleaves amino acids phenylalanine, leucine, methionine and triptorelin adjacent to a hydrophobic amino acid. Trypsin, α -chymotrypsin, elastase, exopeptidases, carboxypeptidases A&B (serine proteases) are secreted from the pancreas and are found in the intestinal tract with higher concentration in the duodenum (Leonaviciute and Bernkop-Schnürch,

2015). These free enzymes break peptides into smaller ones and free amino acids by nucleophilic attacks on specific bonds with different binding sites for each enzyme (Malhaire et al., 2016). For example, cleavage of peptides by trypsin occurs at carboxyl side of lysine and arginine while α -chymotrypsin hydrolyses amino acids with aromatic or large hydrophobic side chains (Leonaviciute et al., 2016). Elastase breaks amide bonds on carboxyl side of small hydrophobic amino acid such as glycine, alanine or valine. Carboxypeptidases hydrolyze peptides on their C-terminal, with carboxypeptidase A dedicated to aromatic and aliphatic chains while carboxypeptidase B action is located on positively charged amino acids (Ambler, 1972).

According to their length, composition and structure, peptides will be differently degraded by the enzymes described above. Thus, long-chain peptides (insulin, calcitonin) show extremely low stability in human gastric and simulated gastric medium whereas most of cyclic and short chain peptides were unaltered in the same conditions. Repeated trials in simulated gastric media without pepsin revealed no degradation of peptides, attributing the former results only to enzyme activity and not to low pH conditions. It was assumed that long peptide

Table 3

Main advantages and disadvantages of the principal techniques to obtain lipid nanosuspensions for peptide encapsulation.

Preparation technique	Advantages	Disadvantages
Coacervation method	● Gentle stirring conditions ● Solvent-free process ● Successful encapsulation of peptide ion pairs	● High temperature (depends on surfactant Krafft point)
Emulsion diffusion	● Room temperature ● Low energy process ● Larger choice of solvents than nanoprecipitation	● Solvent-based process ● Very diluted nanosuspensions
Emulsion evaporation	● Small particle size ● Low energy process ● Small particle size and PDI	● Solvent-based process
Gasco method	● Solvent free process ● Low energy process ● No specific equipment required	● Diluted nanosuspensions
Cold HPH	● Solvent free process ● Low cost process ● Reduced exposition of peptides to high temperature	● Risk of peptide partition in the aqueous phase ● Broad particle-size distribution
Hot HPH	● Ease of scaling up	● Risk of drug degradation induced by high temperature, heat transfer to the formulation and high shear rates
Jay and Mumper method	● Solvent free process ● Small particle size and low PDI ● Low cost process ● Low energy process ● No specific equipment required	● High temperature
Nanoprecipitation	● Room temperature ● Low energy process ● Rapidity of particles production	● Solvent-based process
Phase inversion temperature	● Gentle stirring conditions	● High temperature (above surfactant Krafft point) ● Risk of peptide partition in aqueous phase upon phase inversion if not lipidized ● No examples found with peptides (or proteins) ● Risk of peptide degradation under high energy process
Supercritical fluid extraction of emulsions	● Low temperature	
Ultrasonication	● Solvent with low toxicity (carbon dioxide) ● Solvent free process ● One step process ● Small particle size	● High temperature ● Heat transfer to formulation ● Laboratory scale ● Risk of metallic contamination of the samples

chains lead to an increased number of bonds susceptible to be cleaved and a higher polar surface (related to increased H bonds) leaving the opportunity for more interactions with pepsin. Concerning pancreatin, containing trypsin, α -chymotrypsin and elastase, results were more homogenous with rapid degradation of the sixteen hydrophilic peptides tested (Wang et al., 2015).

Many papers report the protective effects of lipid-based nanocarriers towards these proteases. For example, insulin degradation in presence of trypsin was lowered by 70% by encapsulation in SLNs based on glyceryl monostearate (Battaglia et al., 2007). Particles with a similar composition were tested by the same team in presence of pepsin and managed to reduce insulin loss from 100 to 25% (Trotta et al., 2005). Cationic SLNs coated with PEG displayed several improvements compared with uncoated ones, among which the protection of insulin towards trypsin and pepsin. This effect may be attributed to steric hindrance caused by the presence of the PEG chains, thus preventing proteases approach (Hecq et al., 2016).

Moreover, the hydrophilic nature of the proteases prevents their attack on the peptide encapsulated in the oily particles or droplets of the formulation. Thus, 79% of insulin complexed with the anionic phospholipid dimyristoyl phosphatidylglycerol and loaded in a SEDDS (Labrafil® M 1944CS/Cremophor® EL/Lauroglycol™ FCC, 5/7/3) was preserved after 180 min incubation with trypsin. Similar effects were noted with α -chymotrypsin. Indeed, insulin presents five potential bonds that can be cleaved by this protease and, therefore, is completely degraded after 60 min of incubation. However, the inclusion of the insulin-complex enabled preservation of 73% of the insulin. The complexation of insulin was necessary to maintain the peptide in the oily

system since free insulin mixed with the blank SEDDS was as much degraded as free insulin (Karamanidou et al., 2015). Same results were found with a SEDDS with a different composition (tetraglycol/Peceol™/Labrasol® ALF, 3/3/4) in which insulin was loaded complexed with sodium docusate by HIP. The system managed to maintain insulin in concentration 4 to 5-fold higher than free insulin in presence of α -chymotrypsin and trypsin. This study also tested resistance to elastase and showed 4-fold higher amount of remaining insulin (Hetényi et al., 2017). By comparing data from drug release study and degradation by proteases, Hetényi et al. could demonstrate that only peptides that had already left the oily droplets of the SEDDS were degraded by proteases. Thus, a 100% protective effect of their system towards trypsin, α -chymotrypsin and elastase can be claimed.

4.2. Protection towards glutathione

Glutathione, a tripeptide based on glycine, glutamic acid and cysteine, is derived from biliary secretions. It is naturally occurring in most mammalian cells and intestinal lumen. It is present in both oxidized and reduced forms and is one of the most important antioxidant of the cells. The reduced form (GSH) can react by disulfide exchanges with peptides exhibiting a disulfide bridge like oxytocin, vasopressin or desmopressin and inhibit their activity by modification of their conformation and structure.

Indeed, Schmitz et al. have evaluated the degradation of desmopressin, a peptide bearing one disulfide bond, incubated in different glutathione concentration, reflecting the individual variation of intestinal concentration. They have shown the considerable impact of

glutathione on desmopressin degradation and the relative increase of the disulfide exchange when increasing the pH (Schmitz et al., 2006).

In a recent study, Hetényi et al. have demonstrated that encapsulation of desmopressin in SEDDS enabled a 100% protective effect of the peptide toward glutathione. The explanation lies in the insolubility of the hydrophilic enzyme into the oily droplets formed by contact of the formulation with water. However, desmopressin which had left the oily protective environment of the emulsion was degraded (Hetényi et al., 2017). Thus, one of the key parameter of peptides protection and delivery lies in their controlled release in the intestinal tract.

4.3. Release of peptides – Digestion of SLNs and NLCs

In vitro drug release tests generally show a sustained release of encapsulated peptides from lipid-based carriers. However, a burst effect is often noted and is attributed to absorption of peptide at the surface of the particles (Gallarate et al., 2011; Hu et al., 2004; Martins et al., 2009; Yang et al., 2011). Bernkop-Schnürch and Jalil have recently shown that, in the case of SEDDS, the sustained release effect observed is proportional to the partition coefficient of the drug between the lipophilic phase and the release medium (Bernkop-Schnürch and Jalil, 2018). This postulate can be extrapolated to the release of drugs from SLNs. The observed burst effect from nanoparticles could be due to the rapid diffusion of the hydrophilic peptide from the lipid core due to its positive partition coefficient. Then drug release is controlled by the rate of digestion of the carrier by lipases (Bernkop-Schnürch and Jalil, 2018).

Actually, orally administered lipid-based formulations are submitted to the same digestive process as dietary fat. Digestion of triglycerides is initiated in the stomach where gastric lipase is located. This enzyme cleaves their ester bonds in positions sn-1 and sn-3 to give diacylglycerides and free FA. Generation of these lipolysis products stimulates the production of biliary salts and pancreatic enzymes. Among these, lipid degradation is mainly controlled by human pancreatic lipase (HPL) and its co-lipase, cholesteryl ester hydrolase (CEH), and two pancreatic lipase-related proteins (PLRP1 and PLRP2) (Fernandez et al., 2007; Jannin et al., 2015).

In vitro evaluations of formulation behavior in gastric environment are generally restricted by the lack of commercially available gastric lipase, which limits the tests to only few laboratories. However, when tests can be conducted, care has to be taken to monitor kinetic of digestion, directly related to the amount of FA release. Indeed, FA are poorly ionized at low pH while gastric lipase exhibits its highest activity (pH = 5). It becomes then difficult to proceed to an accurate direct titration method and extent of digestion is evaluated by back titration (Bakala-N'Goma et al., 2015).

Evaluation of the digestibility of orally administered lipid-based formulations is usually limited to *in vitro* lipolysis by pancreatic lipases. A consortium of academic and industrial scientists, Lipid Formulation Classification System consortium, has settled the parameters to standardize a method to evaluate digestion of lipid-based formulation in simulated fasted intestinal media, in presence of pancreatic lipases. They describe the conditions to mimic the fasted small intestine (pH 6.5), to sample and to prepare aliquots. A schematic representation of the method is provided in Fig. 6: intestinal lipolysis is simulated in a pH-stat apparatus consisting of a reaction vessel equipped with a glass electrode linked with a pH-meter that is connected to an adjustable controlling device. This device operates a motor-driven burette dipping into the reaction system and supplying the system with sodium hydroxide to maintain the desired pH value. Digestion is initiated by adding porcine pancreatic extracts to the reaction medium containing the dispersed lipid-based formulations (Sassene et al., 2014; Williams et al., 2012a,b). Aliquots are withdrawn at given times and the lipase-induced digestion process is immediately stopped, either by addition of an inhibitor or freezing in liquid nitrogen (Fernandez et al., 2013). With

the first method, samples are centrifuged and the supernatant is collected to evaluate the amount of drug remaining in solution.

This method was used by Christophersen et al. to evaluate the release mechanism of a model protein, lysozyme, encapsulated in Solid Lipid Microparticles. Formulations based on Dynasan® 114 (glyceryl trimyristate), Dynasan® 118 (glyceryl tristearate) and Precirol® ATO 5 (glyceryl distearate type 1) were tested and a strong correlation between drug release and lipid hydrolysis by lipases could actually be established. The length of triglyceride chains was of great importance: the longer the chains are, the lower the digestion is and the slower the release is (Jannin et al., 2018). This study also highlighted that the measurements of hydrolyzed FAs to evaluate drug release is not always appropriate (Christophersen et al., 2013). Indeed, Heider et al. have compared two techniques to measure the extent of *in vitro* lipid digestion: pH-stat method and High Performance Thin Layer Chromatography (HPTLC). They concluded that, because of their different pKa related to their different chain lengths, all FAs are not quantitatively detected by the pH-stat. As a result, this method might either overestimate or underestimate the enzymatic digestion of drug delivery systems. On the other hand, HPTLC method offers a better separation of digestion compounds and is more specific. However, risk of evaporation of short chains FAs can occur (Heider et al., 2016).

Not only the chain length, but also the choice of surfactant has an impact on degradation velocity of SLNs by lipases. Indeed, incubation of SLNs formulated with different surfactants (cholic acid sodium salt, Lipoid E80, Poloxamer 407 and Tween® 80) in lipase/co-lipase medium has emphasized the role of the stabilizer in SLNs degradation. According to Olbrich et al., the velocity of degradation is linked with the number of ethylene oxide functionalities on surfactants chains: the more ethylene oxide groups are on the SLNs, the more they hinder the anchoring of the enzyme on the particle and consequently its degradation (Olbrich and Müller, 1999).

Finally, in a recent study conducted on lipid-based SEDDS, the authors tested different composition of ester substructures to evaluate their resistance to pancreatic lipase. A formulation containing triglycerides was fully hydrolyzed within 60 min whereas a formulation containing mono and diglycerides was hydrolyzed up to 20% and a formulation free of esters was not affected by pancreatic lipase. The same systems were loaded with hydrophobic leuprolide oleate and their protective effect evaluated in presence of both pancreatic lipase and proteases (trypsin, α -chymotrypsin and elastase). As expected the highest amount (48.14%) of remaining leuprolide was obtained with the ester-free SEDDS whereas all leuprolide was degraded when dispersed in the formulation based on monoglycerides (Leonaviciute et al., 2016). These results bring useful information for designing SEDDS for oral peptide delivery and may be applicable to SLNs and NLCs.

4.4. Permeation improvement

Many *in vitro* cells models were developed to mimic the complex structure of the human intestine epithelium and estimate the passage of biologically active substance.

4.4.1. *In vitro* cell models

The epithelium border displays a specific area of 200 m². It is mostly formed of enterocytes (~99%) whose surface is covered with microvilli (Pawar et al., 2014). Passage from the apical to the basal side of enterocytes can be driven by either transcellular or paracellular transport, Fig. 7.

The lipidic composition of enterocyte cell membranes limits the transcellular uptake by passive diffusion to lipophilic compounds. Thus, peptides are poor candidates for transcellular pathway if not carried by lipophilic vehicles. The internalization of nanocarriers occurs via a pinocytosis mechanism resulting from either macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis or clathrin- and caveolae-independent processes. In the case of micropinocytosis and

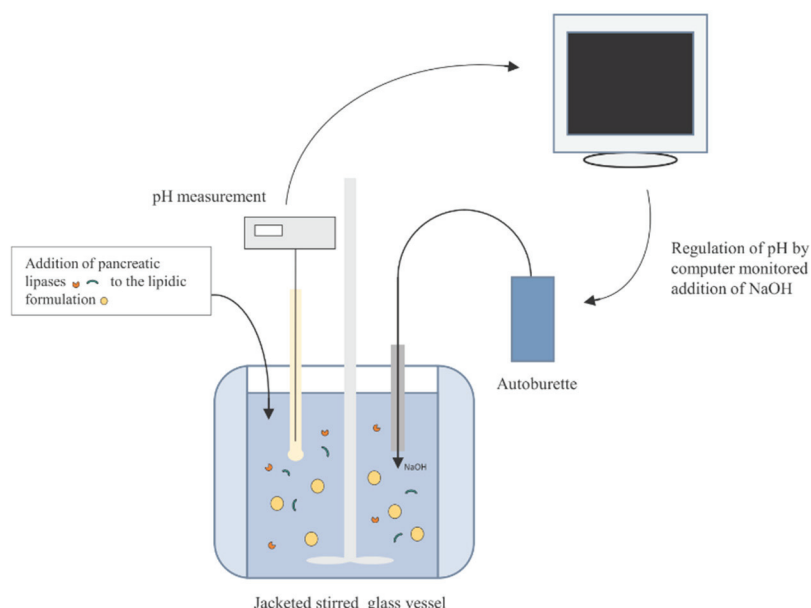


Fig. 6. pH-stat apparatus to evaluate *in vitro* the extent of digestion of lipid based formulation by pancreatic lipases.

clathrin-mediated endocytosis, a risk of fusion of the carrier with the lysosome of the cells prevails. The acidic environment of these organelles containing several enzymes can alter the SLNs and consequently the encapsulated drug (Beloqui et al., 2016a; Hillaireau and Couvreur, 2009). However, caveolae-mediated endocytosis avoid these degradations and the carriers cross the entire cells length before being discharged into the extra cellular matrix, beneath the basolateral layer. This movement is only driven by a concentration gradient (Pawar et al., 2014).

Epithelium cells are separate by tight junctions (TJs), also called zonula occludens. They are aqueous channels connecting the apical side of the epithelial border to the plasma on the basolateral side. This paracellular route is however estimated to have a pore radius of around

0.52 nm in humans, preventing the passage for large molecules such as peptides (Li et al., 2012). The junctional space between cells displays a negative electrostatic field that may alter permeability of some molecules by charge-charge interactions. Furthermore, due to their restricted size, TJs only represent 0.1% of the epithelial surface (Lundquist and Artursson, 2016).

Parallel artificial membrane permeability model (PAMPA) and Caco-2 cell models are the two most used models for *in vitro* evaluation of the permeation. However, the former is limited to passive permeability evaluation whereas the latter enables the assessment of both passive and active transport (Ciappellano et al., 2016).

Caco-2 cells are widely reported for permeation capacity evaluation of formulations. Originally obtained from human epithelial colorectal

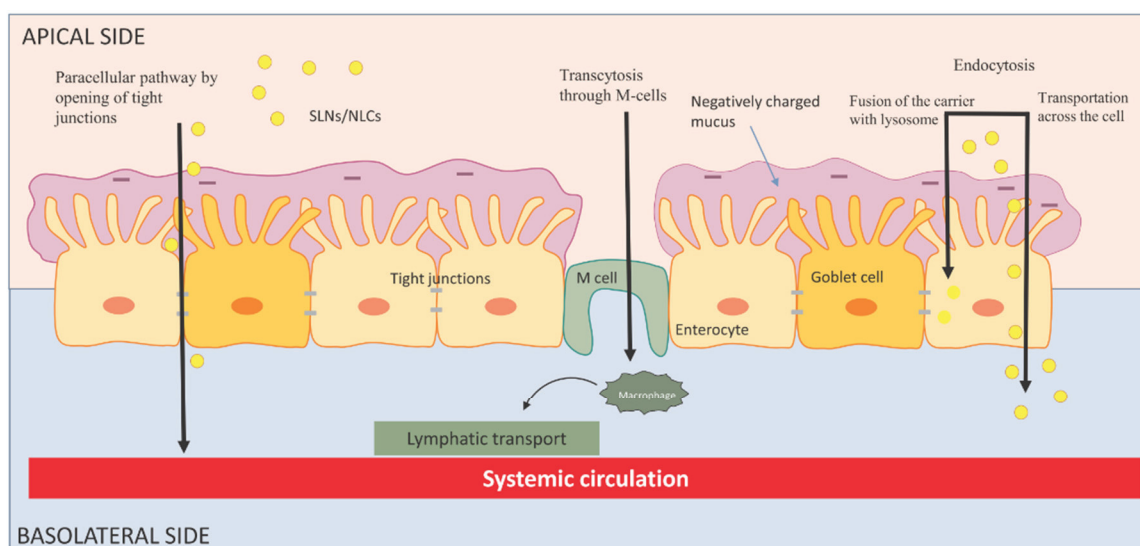


Fig. 7. Representation of lipid nanoparticles mechanisms of transport across the epithelium border.

adenocarcinoma cells, these heterogeneous cell lines differentiate in culture medium and express many characteristics of human enterocytes: tight junctions between adjacent cells, microvilli on apical face and small intestine hydrolase activities (Sambuy et al., 2005). Comparative tests for permeation evaluation of lipophilic drugs (naproxen, antipyrine and metoprolol) were performed on Caco-2 cells and in human jejunum. The drugs were rapidly transported and comparable permeation coefficients were obtained for both models. However, in the case of hydrophilic drugs, transportation across Caco-2 cells was much lower than in human jejunum. The low paracellular permeability observed was closer to the behavior of human colon which might be associated with the source of the cells (Lennernäs et al., 1996).

MDCK (Mardin-Darby Canine Kidney) cells are also used to evaluate the permeation capacity of formulations across enterocytes. These cells are able to differentiate into columnar epithelium and form tight junctions according to their culture mode. They are said to give more predictive apparent permeability coefficient of *in vivo* human intestinal than Caco-2 cells due to good morphological homogeneity, which also induces more inter-lab reproducibility. On a practical point of view, their culture time is shorter than Caco-2 cells which decrease the risk of contamination (3 days for MDCK cells and 3 weeks for Caco-2) (Balimane and Chong, 2005). They were used to evaluate the transportation of SEDDS formulations loaded with β -lactamase (29 kDa protein) grafted with FTIC (Rao et al., 2008a). All SEDDS tested have shown improved transportation with an increase up to 33% without disrupting the monolayer integrity. Same formulation injected to rats have shown augmentation of relative bioavailability (Rao et al., 2008b).

The intestinal epithelium is not only composed of enterocytes, but also exhibits Goblet cells that produces mucin and glycocalyx composing mucus. Mucins are the main component of mucus. These glycosylated molecules of high molecular weight, linked together via disulfide bonds, form a large chain resulting in a dynamic viscoelastic gel with shear-thinning properties (Pawar et al., 2014). Mucins are capable of interacting with peptides and proteins via electrostatic interactions (via sialic acids and sulfate residues), ionic interactions, Van der Waals interaction, hydrophobic forces and hydrogen bonding. These interactions stop macromolecules on mucus surface and prevent their permeation, especially for peptides with a molecular weight superior to 6.5 kDa (Leonaviciute and Bernkop-Schnürch, 2015). Glycocalyx, on the other side, constitute a hydrophilic acidic fibrous coating composed of sulfated mucopolysaccharides. The resulting mucus is a complex hydrogel covering the surface of the epithelial border of the small intestine. It contains proteins, carbohydrates, salts, lipids, antibodies and bacteria (Pawar et al., 2014) and exhibits a negative surface charge (permeation is hindered for compounds presenting overall positive charge). Two intestinal layers (areolar or submucosal) connect together mucus and muscular layer and form the strongest layer of the intestine and a hardly crossable barrier for peptides (Muheem et al., 2016).

The Caco-2 model cells are not covered with mucus layer and their low paracellular permeability limits the passage of hydrophilic drugs such as peptides. Furthermore, a lack of control of the expression of P-glycoprotein by Caco-2 cells was reported (Anderle et al., 1998). In the perspective of overcoming these drawbacks, development of models combining Caco-2 and HT29-MTX cells were developed (Béduneau et al., 2014; Chen et al., 2010). These latter cells are Goblet cells type with enhanced paracellular permeability and limitation of P-glycoprotein (P-gp) expression (Hilgendorf et al., 2000). This combination allows the use of cell-based model with biorelevant media containing bile components and pancreatin (Antoine et al., 2015) and limits the effects of lipid-based excipients on P-gp observed on Caco-2 (Dubray et al., 2016).

These cells models are particularly useful for evaluation of particles coated with mucoadhesive compounds. In the case of chitosan-coated SLNs loaded with insulin, the use of Caco-2/HT29 monolayer model highlights the prolonged absorption of the active substance *in vitro*

(Fonte et al., 2011). The same model was also used to prove the permeation capacity improvement of SLNs coated with PEG moieties. The results were comparable with *ex vivo* permeation test on rat intestinal tissues (Yuan et al., 2013).

Despite their lower occurrence in the intestinal border (< 1% of GI tract coverage) M cells are of great importance in the uptake of larger entities. They are only present in specific region known as Peyer's patches. They are less covered in mucus and microvilli and, are more conductive for the passage of large molecules. Once absorbed, these macromolecules are transferred to the lymph system. This absorption route presents the advantage of avoiding the first pass effect. Moreover, M cells are not restricted to small molecules so as enterocytes (limited to 50–100 nm) (Gangwar et al., 1997; Maher et al., 2009). Thus, they represent of potential transportation route for peptides and nanoparticles.

The co-culture of Caco-2 cells with Raji B lymphocytes was developed by Gullberg et al. The combination enables Caco-2 cells to express M cells morphologic characteristics (Gullberg et al., 2000). This model was used to evaluate the influence of nanoparticles characteristics on their transport in *in vitro* models. The study revealed the importance of surface functional groups and hydrophobicity in absorption of the nanoparticles (Rieux et al., 2005).

Finally, the development of Caco-2/HT29/Raji B co-cultured cell models by Antunes et al. enables an even more precise *in vitro* model to estimate nanoparticles fate in human intestine (Antunes et al., 2013). This system was used for comparative study permeation evaluation of SLNs, poly(lactic-co-glycolic acid) (PLGA) and porous silicon nanoparticles loaded with Glucagon-like-peptide-1 (GLP-1), a 30 amino acids peptides used in type 2 diabetes mellitus treatment (Araújo et al., 2014).

4.4.2. Toxicity evaluation

Toxicity evaluation of nanomaterial is essential prior conduction of any permeation study. Indeed, one has to ensure the preservation of the intestinal epithelium to assess correctly the passage of formulations. Despite the absence of standardized methods for toxicology assessment of nanomaterial on human health, many have been described in the literature. They were reported in a recent review (Ciappellano et al., 2016). Briefly, *in vitro* toxicology assessment can be divided in two types: evaluation of cellular viability or mortality or by investigate materials mode of actions on *in vitro* cell models. Various assays evaluate cell metabolic activity using tetrazolium dyes (MTT, XTT, MTS and WST) that are reduced by enzymes present in viable cells. The colored reduced form of the dye is quantified by colorimetric measurement, proportional to the number of viable cells (Riss et al., 2004). ATP quantification using the reaction with firefly luciferase is also one of the most common method. Another existing colorimetric test is the Alamar blue® assay, which is also based on an oxidation-reduction reaction of a reagent (resazurin) with enzymes of metabolically active cells. This test enabled to prove the cell-depending toxicity of insulin-loaded SLNs made of Softisan® 100 (C10-18 triglycerides). Caco-2 cells viability was not altered by SLNs until 3 or 6 days while HepG-2 (*in vitro* epithelial cell line) showed immediate cell mortality (Severino et al., 2014).

Several methods also exist to evaluate the integrity of cell layers such as paracellular passage measurements of extracellular markers (inulin, mannitol, fluorescein...) (Zucco et al., 2005) or TransEpithelial Electrical Resistance (TEER). The latter measure reflects the integrity of tight junctions and cell monolayer. The measurement is taken by placing two electrodes on each side of the cell model and applying an alternative current to them as represented in Fig. 8. This measurement is non-invasive and can be handle at different stages of the cell development. The Caco-2 cell monolayer generates a TEER around 150–400 $\Omega \cdot \text{cm}^2$ but results vary from one laboratory to another (Srinivasan et al., 2015).

Regarding SLN/NLC toxicity towards *in vitro* cell lines, damages are generally concentration-dependent and it was proven that most of them remain viable in presence of up to 1 mg/mL SLN (Doktorovova et al.,

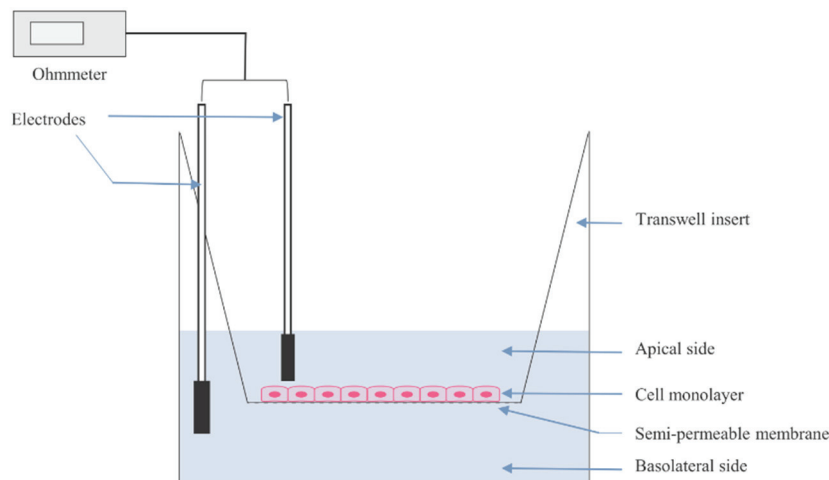


Fig. 8. Set up for transepithelial electrical resistance measurements.

2014; Doktorová et al., 2016). Nonetheless, 7.5 mg/mL of SLN could be tolerated by Caco-2 cells (Soddu et al., 2016).

4.4.3. Permeation studies of lipidic nanoparticles

As mentioned above, the two main routes that can be followed to cross the intestinal epithelium are the paracellular and the transcellular pathways. The former one is constituted by tight junctions forming channels of a few angstroms, limiting this route to substances presenting at least one dimension in this order of magnitude. The latter one can be followed by passive diffusion, carrier-mediated transportation or transcytosis. This route of absorption is not only limited to enterocytes cells but also can be followed by M cells if the follicle-associated epithelium of Peyer's patches. They can represent an easier route because of the absence of microvilli and the reduced concentration of glycocalyx compared to enterocytes. Furthermore, the absorbed moieties are delivered to the lymphatic system thus reducing first-pass metabolism (Yáñez et al., 2011). However it is generally limited to lipophilic substances with a size of 500–1000 nm (Galindo-Rodríguez et al., 2005).

One of the main interesting feature of lipid-based formulations is their ability to promote intestinal absorption of active molecules. This is especially appropriate for medium chain FA: capric, caprylic and lauric acids, despite their different mode of action (Lindmark et al., 1995, 1998). Sodium salt of capric acid (C10), in particular, has been widely studied *in vitro*, *ex vivo* and *in vivo* for its permeation enhancing properties (Maher et al., 2009). The ability of capric acid to increase paracellular flux was proven by various methods (TEER, MTT assay...). This modification of epithelium permeability is assumed to be reversible and concentration-dependent. *In vitro* studies conducted on Caco-2 monolayers have shown that sodium caprate induced disruption of 42% TJs whose integrity was recovered after 1 h (Lindmark et al., 1995). Four hours after removal of the surfactant, the monolayers had improved morphological appearance and the recovery was complete after 24 h (Brayden et al., 2015). However, repeated applications progressively lead to membrane alteration (Brayden et al., 2015; Shima et al., 1999). Yet, repeated applications of C10 can be considered as meaningless considering its rapid *in vivo* absorption: 7 min following intra-intestinal administration (Raoof et al., 2002). The action of C10 on tight junctions is mediated by changing perijunctional actin and localization of the proteins composing and forming TJs: zonula occludens-1, occluding and claudin-1 (Brayden and O'Mahony, 1998; Li et al., 2012; Maher et al., 2009). The use of surfactants has also been reported to enhance intestinal permeation but correlated with epithelial damages. The extent of these damages depends on the type and quantity of surfactant

used as well as the time of exposure (Rama Prasad et al., 2004). Transcellular permeability improvement induced by C10 has also been reported. The mechanism of action seems to be driven by changes in hydrophobic and polar domains, resulting in increased fluidity of enterocytes membrane (Maher et al., 2009). Hence, it would have the same effects as longer chain FA, supposed to created destabilization in the lipophilic bilayer of epithelial cells (Niu et al., 2016; Scott Swenson and Curatolo, 1992).

Chen et al. have shown the benefits of adding tripalmitin to stearic acid based SLNs on bioavailability of salmon calcitonin (sCT). Indeed, pharmacokinetic experiments showed 13% bioavailability in rats after intra-duodenal administration. This represents a 6-fold increase compared to free-sCT, 3-fold compared to SLN based only on stearic acid. Mechanism of transport studied on Caco-2 cells monolayers demonstrated that an active transport phenomenon via clathrin and caveolae-dependent endocytosis was involved (Chen et al., 2012). The same mechanism was highlighted for the transport of paclitaxel, a poorly water-soluble drug loaded in NLCs. The nanocarrier was based on Captex® 8000 (tricaprylin), Lipoid® S74-3 (Lecithin) and Solutol® HS15 (polyoxyl 15 hydroxystearate). It enabled a 3.5-fold improvement of permeability across Caco-2 compared to a suspension of the free drug. The authors demonstrated that the NLCs did not open tight junctions of the Caco-2 cell lines (Roger et al., 2009).

5. Conclusions

SLN and NLC are promising systems to overcome the barriers inherent to oral peptide delivery. Several processes have proven their ability to encapsulate peptides after lipidization, without denaturation of the active substance. Furthermore, the number of existing techniques enable formulators to adapt their processing method to the intrinsic properties of peptides and produce loaded nanoparticles in large scale. Additionally, these particles have been proven to protect peptides from protease-induced degradation and increase their transportation across epithelium border.

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Mise à jour de la revue

La revue « Lipid-based nanosuspensions for oral delivery of peptides, a critical review” a été soumise à International Journal of Pharmaceutics en janvier 2018 et publiée en février 2018. Le document suivant est une mise à jour de cette revue, prenant en compte les articles de recherche publiés ultérieurement à sa publication.

I. Hydrophobisation

En 2017, Griesser *et al.* ont montré que des peptides hydrophiles pouvaient être incorporés dans des systèmes auto-émulsionnables (SEDDS) avec des taux d'encapsulation élevés après formation de paires d'ions hydrophobes (HIP) (Griesser et al., 2017). L'Analyse de Dispersion de Taylor (TDA) a été utilisée par Chamieh *et al.* pour montrer l'importance du Log P de l'HIP sur le taux de chargement en peptides dans les SEDDS après dilution en milieu aqueux (Chamieh et al., 2019). En effet, l'HIP leuprolide-docusate (ratio molaire 1 :2 et Log P=3) a été incorporée en totalité dans les SEDDS tandis que le taux d'encapsulation de l'HIP desmopressine-docusate (ratio molaire 1 :1.5, Log P=0.5) dans le même système n'était que de 30% après dilution 1/36^e dans l'eau.

L'octréotide, un octapeptide cyclique, a été incorporé dans des SEDDS après formation de HIP avec le déoxycholate de sodium, le docusate de sodium et le décanoate de sodium dans des ratio molaires 1 :3 (octréotide : tensioactif anionique) (Bonengel et al., 2018). Les HIP octréotide-docusate et octréotide-déoxycholate ont montré une augmentation de la biodisponibilité orale équivalent à respectivement 4,2 et 17,9 fois celle du contrôle (octréotide en solution) après administration à des porcs (n=1).

La formation d'HIP peut également être utilisée pour augmenter la lipophilie de peptides de masse molaire plus importante, voire de protéines. Ainsi le Log P de l'exénatide (peptide linéaire de 39 acides aminés) a été augmenté de -1.1 à 2.1 après formation d'une HIP avec le docusate de sodium au ratio molaire 1 :4 (peptide :docusate) (Menzel et al., 2018). L'HIP a été incorporée dans des SEDDS (taux de chargement de 1%) et les systèmes ont montré une augmentation de la biodisponibilité orale de 14% chez le rat en comparaison avec une injection sous-cutanée d'une solution d'exénatide. Dans le cas de la peroxydase de raifort

(HPR) (protéine de 44 kDa), le taux de précipitation de plus important (87,5%) a été obtenu une fois de plus avec le docusate de sodium (ratio molaire protéine : docusate = 1 :45). L'incorporation de cette HIP dans des SEDDS a permis de multiplier par 4 la perméabilité de la protéine à travers une monocouche de Caco-2 et par 2,5 à travers des intestins de rats fraîchement excisés, en comparaison avec l'HRP en solution.

II. Formulations lipidiques pour délivrance orale de peptides

Shrestha et al. ont récemment encapsulé avec succès deux analogues peptidiques du GLP-1, le liraglutide et l'exénatide, dans des NLC produits par HPH (Shrestha et al., 2018). Des taux d'encapsulation de 95,4% pour le liraglutide et de 87,5% pour l'exénatide ont été obtenus. Les NLC étaient chargés négativement, mesuraient entre 160 et 225 nm, et ont multiplié par 2,9 la perméabilité de l'exénatide à travers une monocouche de Caco-2. En revanche, les études *ex vivo* sur des tissus jéjunaux humains ont montré une importante adhésion des nanoparticules au mucus.

Dans le cas des SEDDS, Griesser *et al.* ont démontré que les paramètres clefs pour augmenter leur passage à travers le mucus sont la taille des particules, avec une augmentation de la perméabilité observée pour des particules de taille inférieure à 200 nm, et un potentiel zeta négatif (Griesser et al., 2018)

Bien que de nombreuses études soient menées sur l'utilisation des nanoparticules comme système de délivrance de substance active, et en particulier sur les nanoparticules lipidiques, il n'existe en réalité aucun produit pharmaceutique commercialisé contenant ces nanoparticules lipidiques (O'Driscoll et al., 2019).

III. Caractérisation des nanoparticules lipidiques pour la délivrance orale de peptides

Le taux d'encapsulation est un des paramètres clefs pour évaluer le potentiel des nanoparticules comme système de délivrance de substance active. Dans le cas des nanoparticules solides, le taux d'encapsulation est généralement calculé *via* l'Équation 1.

Équation 1: Calcul du taux d'encapsulation

$$\text{Taux d'encapsulation(\%)} = \frac{\text{Quantité totale de substance active} - \text{Quantité de substance active libre}}{\text{Quantité totale de substance active}}$$

La quantité totale de substance dans la formule peut facilement être déterminée après dissolution des nanoparticules dans un solvant approprié. En revanche, la quantification de la substance active libre nécessite une séparation optimale des nanoparticules du milieu de dispersion. Lv *et al.* ont récemment comparé trois techniques communément employées pour séparer des SLN, des nanoparticules de polycaprolactone et des nanoparticules de polyéthylène glycol-acide polylactique de leur milieu de dispersion (Lv *et al.*, 2018). Cette étude a montré qu'une faible séparation était obtenue par centrifugation, en particulier avec les SLN, en dépit de forces centrifuges élevées. La chromatographie par perméation de gel a permis une bonne séparation des nanoparticules des macromolécules. Néanmoins, la substance active peut adhérer aux billes constituant la colonne et biaiser les résultats. Enfin, la technique d'ultrafiltration, combinant centrifugation et filtration sur membrane de porosité prédéfinie, a permis une bonne séparation des nanoparticules, avec un relargage de seulement 0,32-0,93% de la substance active des nanoparticules dans le milieu de dispersion.

L'évaluation de la libération de la substance active depuis n'importe quelle forme posologique est nécessaire pour estimer son devenir après administration au patient. Contrairement aux systèmes dimensionnés à l'échelle macroscopique (comprimés, gélules...), il n'existe pas de méthodes standardisées pour évaluer la libération de substance active encapsulées dans des nanoparticules. Nothnagel *et al.* ont récemment recensé et décrit les différentes techniques existantes pour évaluer cette libération dans différents milieux (Nothnagel and Wacker, 2018). Toutes les techniques présentées possèdent des avantages et des inconvénients qu'il convient d'évaluer avec soin avant de sélectionner une de ces méthodes. Par exemple, plusieurs techniques requièrent l'intervention de membranes pour séparer les particules du milieu de libération. Celles-ci sont rapides et simples à mettre en œuvre. Elles impliquent toutefois deux processus : la libération de la substance active des nanoparticules et la diffusion de la molécule à travers la membrane, qui sont généralement impossible à différencier (Bernkop-Schnürch and Jalil, 2018).

IV. Mise au point de procédés permettant des études simultanées de lipolyse et de perméation intestinale

La digestion *in vitro* de formulations lipidiques peut facilement être réalisée à l'aide de procédures de lipolyse standardisées. De la même manière, de nombreuses études décrivent l'utilisation de modèle cellulaires pour évaluer le transport des substances actives à travers l'épithélium intestinal. Cependant, la combinaison de ces deux analyses n'a été étudiée que récemment.

En 2016, Crum *et al.* ont proposé un modèle combinant lipolyse *in vitro* et perfusion intestinale *in situ* sur des rats anesthésiés (Crum *et al.*, 2016). Bien que ce modèle permette de prédire de manière plus précise le devenir de la substance active dans le tractus gastro-intestinal, sa mise en œuvre reste longue, compliquée et l'utilisation du modèle animal risque d'engendrer d'importantes variabilités. Deux modèles conjuguant digestion lipidique *in vitro* et évaluation de la perméabilité ont récemment été proposés pour pallier ces contraintes.

Le premier de ces modèles utilise Permeapad®, une membrane hydrophile d'hydrate de cellulose, combiné avec une couche lipidique constituée de phosphatidycholine de soja S-100 (Bibi *et al.*, 2017). Cette barrière est placée entre deux chambres communicantes : l'une d'elles constituant le compartiment donneur contient le milieu de lipolyse et l'autre constituant le compartiment accepteur et contient du FaSSIF-V2. L'intégrité de la membrane est maintenue en présence du milieu de lipolyse, de SEDDS et des produits de digestion. Ce modèle a été testé avec de la cinnarizine contenue dans des SEDDS. Les résultats ont montré une augmentation de la perméabilité de la substance active lorsque la formulation était digestible. Cependant, les propriétés de perméabilité du dispositif (TEER, perméabilité de marqueurs spécifiques...) n'ont pas été comparées avec celles des modèles cellulaires utilisés classiquement pour déterminer la perméabilité (modèles entérocytes de type Caco-2 ou MDCK par exemple).

Le second modèle a été proposé par Keemink *et al.* et est également composé de deux chambres : la lipolyse est menée dans la partie supérieure tandis que la substance active transportée est prélevée dans la chambre inférieure (Keemink *et al.*, 2019). Ces deux chambres sont séparées par une monocouche de cellules Caco-2 dont l'intégrité est conservée au cours de la lipolyse. En raison de l'incompatibilité entre les cellules Caco-2 et la pancréatine

de porc habituellement utilisée pour évaluer la digestibilité des formules lipidiques, celle-ci a été remplacée par une lipase immobilisée sur un support hydrophobe. Le degré de lipolyse mesuré avec cette lipase était plus faible qu'avec la pancréatine mais était néanmoins plus important que lors du processus *in vivo*. Les résultats obtenus *in vitro* avec ce dispositif ont montré une bonne corrélation avec l'absorption *in vivo* du fénofibrate incorporé dans des formulations lipidiques administrée par voie orale à des cochons.

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Partie expérimentale

-

Introduction et objectifs de la thèse

Bien qu'il existe de nombreuses techniques de formulation de nanovecteurs lipidiques solides pouvant être appliquées à l'encapsulation de peptides thérapeutiques, la plupart d'entre elles sont restreintes à une utilisation à l'échelle de laboratoire. La technique d'homogénéisation haute pression, sans solvant et transposable à l'échelle industrielle, apparaît pourtant comme une technique de choix pour cette application. Toutefois, lorsque ce travail de thèse a été initié, aucune étude reportant son utilisation pour l'encapsulation de peptides hydrophiles dans des nanovecteurs lipidiques solides n'avait été publiée.

Par ailleurs, il a été montré que l'un des facteurs clés pour augmenter la capacité d'encapsulation des peptides dans des systèmes lipidiques auto-émulsionnables (SEDDS) réside dans l'augmentation du caractère lipophile de ces molécules. Pour cela, la formation d'une paire d'ions hydrophobe (HIP) entre un peptide hydrophile ionisable et un contre ion hydrophobe semble le meilleur compromis. En effet, cette méthode permet une augmentation significative de leur lipophilie sans altérer leur structure chimique et leur activité thérapeutique. Ainsi, la première partie de ce travail de thèse vise à mettre au point une procédure industrialisable d'encapsulation de peptides sous forme de paires d'ions dans des nanovecteurs lipidiques solides.

De récentes études ont montré *in vivo* la faculté des SEDDS à augmenter la biodisponibilité orale de peptides thérapeutiques hydrophobisés. Ces vecteurs ont en effet permis de protéger les peptides de la dégradation protéolytique, et d'augmenter leur capacité à franchir le mucus intestinal. Pour autant, la capacité des nanovecteurs solides à conférer ces mêmes bénéfices aux peptides hydrophobisés n'a pas été étudiée. La deuxième partie de ce travail de thèse consiste donc à évaluer la capacité des nanovecteurs lipidiques solides à protéger un peptide modèle vis-à-vis de la dégradation enzymatique induite par les protéases intestinales et à augmenter son passage à travers le mucus et l'épithélium intestinal.

Les vecteurs lipidiques nanostructurés (NLC) ont été développés dans les années 1990 pour améliorer certaines propriétés des nanoparticules lipidiques solides (SLN). Néanmoins, il n'existe pas d'étude comparant l'efficacité des deux systèmes utilisés dans le cadre d'une administration orale de peptides. Ainsi le comportement de ces deux types de nanoparticules est comparé tout au long de l'étude.

Hypothèses et objectifs

Les hypothèses et objectifs de chaque chapitre expérimental, basés sur l'état des connaissances actuelles, sont présentés ci-dessous.

Chapitre II : Mise au point de vecteurs lipidiques nanostructurés pour l'administration orale de peptides hydrophobisés

Hypothèses :

1. Le procédé d'homogénéisation haute pression (HPH) permet de formuler des nanoparticules lipidiques nanostructurées (NLC) de petite taille avec un faible indice de polydispersité
2. Après augmentation de leur lipophilie par appariement ionique hydrophobe, des peptides initialement hydrophiles peuvent être encapsulés dans des NLC
3. L'intégrité des peptides est conservée lors de leur encapsulation dans des NLC par un procédé de HPH

Objectifs :

1. Développer un procédé de formulation de des SLN et des NLC par HPH permettant d'obtenir des nanoparticules de taille inférieure à 200 nm avec un indice de polydispersité inférieur à 0,2
2. Utiliser le procédé développé pour la formulation de NLC pour y encapsuler des peptides modèles sous forme d'une paire d'ions hydrophobe
3. Comparer l'efficacité d'encapsulation des peptides dans les NLC en fonction de leurs propriétés physico-chimiques

La desmopressine et le leuprolide, deux peptides hydrophiles, ionisables en milieu acide, ont été retenus comme peptides modèles dans le cadre de ce chapitre. Par la suite, seul le leuprolide a été utilisé lors des études présentées dans les chapitres III, IV et V.

Chapitre III : Evaluation *in vitro* des nanovecteurs lipidiques solides : capacité à encapsuler, libérer et protéger un peptide modèle de l'environnement gastro-intestinal

Hypothèses :

1. L'augmentation du caractère lipophile des peptides par appariement ionique hydrophobe permet d'augmenter l'efficacité d'encapsulation (EE) et le taux de chargement (D_L) dans les nanovecteurs lipidiques solides : SLN et NLC
2. L'efficacité d'encapsulation et le taux de chargement des peptides est potentiellement supérieure dans les NLC dans lesquelles la paire d'ions hydrophobe peut être dissoute dans la fraction lipidique liquide de ces nanoparticules
3. Les nanovecteurs lipidiques solides peuvent procurer aux peptides un environnement les protégeant de la protéolyse intestinale

Objectifs :

1. Evaluer l'impact de la hydrophobisation d'un peptide modèle sur l'efficacité d'encapsulation et le taux de chargement des SLN et des NLC
2. Comparer les propriétés physico-chimiques des SLN et des NLC
3. Etudier le comportement des nanoparticules dans les milieux gastro-intestinaux simulés
4. Evaluer la capacité protectrice des SLN et des NLC vis-à-vis de la dégradation enzymatique d'un peptide modèle, induite par la trypsine et l' α -chymotrypsine.

Chapitre IV : Evaluation de la capacité des nanovecteurs lipidiques solides à augmenter le passage intestinal des peptides

Hypothèses :

1. Les nanoparticules lipidiques solides sont capables de passer la couche de mucus dont est recouvert l'épithélium intestinal
2. Les nanoparticules lipidiques solides peuvent passer la barrière formée par les entérocytes, cellules composant majoritairement l'épithélium intestinal
3. L'encapsulation d'un peptide modèle dans les SLN et les NLC permet d'augmenter son passage à travers la barrière intestinale
4. L'inclusion d'une partie lipidique liquide contenant des esters d'acide caprylique, connu pour sa capacité à augmenter la perméabilité d'actifs, dans les NLC peut accroître le transport du peptide encapsulé en comparaison avec les SLN

Objectifs :

1. Evaluer la capacité des nanoparticules à interagir avec des cellules utilisées comme modèles de l'épithélium intestinal : les Caco-2, utilisées pour simuler les mécanismes d'absorption relatifs aux entérocytes, et les HT29-MTX, cellules caliciformes permettant la production de mucine à la surface des entérocytes.
2. Evaluer la capacité des nanoparticules à transporter un peptide modèle à travers des monocouches cellulaires de Caco-2, des monocouches cellulaires composées de Caco-2 et de HT29-MTX co-cultivées
3. Comparer la capacité de transport du peptide modèle hydrophobisé par les SLN et les NLC

Chapitre V : Impact de la lipolyse sur l'absorption intestinale des peptides encapsulés dans des NLC, étude préliminaire

Hypothèses :

1. Le procédé de formulation de NLC développé chapitre II avec du Precirol®ATO5 peut être adapté pour formuler des nanoparticules composées d'autres excipients lipidiques solides
2. La composition des nanovecteurs lipidiques solides à un effet sur leur digestibilité
3. La digestibilité des nanoparticules à un effet sur l'absorption de l'actif encapsulé à travers la barrière intestinale

Objectifs :

1. Adapter le procédé de formulation par homogénéisation haute pression développé chapitre II pour produire des NLC composé d'un excipient solide non digestible
2. Evaluer *in vitro* la digestibilité des nanoparticules de Precirol®ATO5, supposées digestibles, et de Compritol®888 ATO, supposées non digestibles
3. Evaluer l'impact de la digestibilité des nanoparticules sur la perméabilité intestinale d'un peptide hydrophobisé encapsulé *via* l'utilisation de modèles cellulaires

Chapitre II

Mise au point de vecteurs lipidiques nanostructurés pour l'administration orale de peptides hydrophobisés

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L'encapsulation des peptides thérapeutiques dans des nanovecteurs semble être une solution prometteuse pour pallier les limitations biopharmaceutiques rencontrées lors de leur administration orale, à savoir leur rapide dégradation par les protéases de l'intestin et leur faible perméabilité intestinale. En particulier, les formes lipidiques, tels les vecteurs lipidiques nanostructurés (NLC), constituées d'excipients biocompatibles et biodégradables apparaissent comme une solution sûre. Ces NLC sont des nanoparticules solides, composées majoritairement de lipides solides à température ambiante et à 37°C, dont la cristallisation est limitée par l'addition d'une fraction lipidique liquide.

Parmi les nombreuses méthodes disponibles pour formuler les NLC, l'Homogénéisation Haute Pression (HPH) présente les avantages d'une formulation en l'absence de solvants organiques et la possibilité d'une transposition à l'échelle industrielle. Concrètement, l'actif est ajouté à la phase lipidique chauffée 5 à 10°C au-dessus du point de fusion des lipides. Une pré-émulsion est formée sous agitation par ajout de la phase aqueuse, contenant le ou les tensioactifs, chauffée à la même température. La pré-émulsion formée est versée dans le réservoir de l'homogénéisateur haute pression dans lequel elle sera soumise à un certain nombre de cycles à pression constante. La nanoémulsion obtenue est ensuite refroidie pour obtenir les nanoparticules solides.

Le caractère hydrophile de la plupart des peptides thérapeutiques limite leur encapsulation dans les nanovecteurs lipidiques. Il est alors recommandé d'augmenter leur lipophilie, par exemple *via* la formation d'une paire d'ions hydrophobe (HIP) par complexation entre un peptide et un contre-ion hydrophobe. Cette stratégie a notamment permis d'augmenter de manière significative le taux d'encapsulation de peptides modèles dans des systèmes auto-émulsionnables (SEDDES).

Cette première partie expérimentale vise à développer un protocole pour formuler des NLC de Precirol® ATO5 (PATO5, distearate de glycerol) par HPH avec une taille de particules de 200 nm ou moins et un indice de polydispersité inférieur à 0,2. En effet ces propriétés ont montré une amélioration du passage de la barrière de mucus intestinal par les SEDDES. La méthode de formulation développée pour les NLC blanches (sans encapsulation de principe actif) a ensuite été appliquée à deux peptides modèles, la desmopressine (DES) et le leuprolide (LEU), sous forme d'HIP.

La DES est un peptide hydrophile ($\log P = -2,5$) de 9 acides aminés, partiellement cyclique, utilisé dans le traitement du diabète insipide. Le LEU est un peptide hydrophile ($\log P = -2$) linéaire de 8 acides aminés, utilisé dans le traitement du cancer de la prostate. Le docusate de sodium a été utilisé avec chacun de ces deux peptides pour former des HIP. Un ratio molaire peptide : docusate de 1 :1,5 pour la DES a conduit à un taux de précipitation de 95% tandis qu'un ratio molaire 1 :2 a permis de précipiter 99,9% du LEU.

La solubilité de ces HIP a été évaluée dans différents lipides liquides. Le Capryol®90 (C90, monocaprylate de propylène glycol) s'est révélé le meilleur solvant pour ces deux HIP et a donc été choisi pour constituer la fraction lipidique liquide des NLC.

Afin de limiter le nombre de paramètres, le protocole de formulation a dans un premier temps été mis au point pour des nanoparticules lipidiques solides (SLN), composées uniquement de lipides solides et d'un tensioactif. Trois tensio-actifs, le Kolliphor® RH40 (RH40, PEG-40 hydrogenated castor oil), le Tween® 80 (T80, polysorbate 80) et le Pluronic® F68 (PF68, poloxamer 188), ont été sélectionnés au regard de leur capacité à former des pré-émulsions de PATO5 reproductibles après 3 minutes d'émulsification à l'ultra-turrax® à 11 000 rpm. Ces tensioactifs ont été utilisés à une concentration de 4% (w/v) (pour 10% de PATO5, w/v) afin d'évaluer l'importance des paramètres d'HPH (nombre de cycles et pression appliquée) sur la taille et le PDI des nanoparticules. Le RH40 est le seul tensio-actif ayant permis d'obtenir des SLN inférieures à 200 nm avec un PDI en dessous de 0,2 dans des conditions de procédé modérées : 5 cycles à 500 bars. La variation du nombre de cycles à cette pression a montré qu'un nombre minimal de 4 cycles était nécessaire pour obtenir des particules de taille inférieure à 200 nm. En revanche, l'augmentation du nombre de cycles au-delà de 5 n'a pas montré d'impact significatif sur la taille ni sur le PDI.

Pour développer les NLC, différents volumes de C90 (300 – 1200 μL) ont été ajoutés au PATO5 avant formation de la pré-émulsion. La stabilité des NLC a été évaluée visuellement et par mesure de la taille des nanoparticules pendant un mois. L'observation visuelle a montré que la stabilité des formules était inversement proportionnelle à la proportion de lipide liquide. En effet, bien que la taille des nanoparticules, mesurée par Diffraction Dynamique de la Lumière (DLS) soit stable, un phénomène d'exsudation de la fraction lipidique liquide a été constaté. Pour les formules les plus concentrées en C90, cette observation a été faite dès la première

semaine de stockage à 4°C. Seules les nanoparticules formulées avec 300 µL de C90 ont montré une stabilité supérieure à 28 jours. De manière à réduire le risque de relargage des peptides encapsulés par exsudation rapide de la fraction lipidique liquide, il a été décidé de fixer le volume de C90 à 300 µL pour la formulation de NLC chargées en actif.

Afin d'évaluer le risque de dégradation des peptides modèles par le procédé de fabrication des NLC, des solutions de DES et de LEU ont été soumises aux mêmes contraintes que les formulations lipidiques puis analysées par HPLC. Les chromatogrammes obtenus se superposaient avec ceux de solutions standards et ne montraient pas d'apparition de pics de dégradation, démontrant ainsi la stabilité de ces peptides dans les conditions du procédé.

La méthode développée a été utilisée pour formuler des NLC chargées en peptide. Les HIP ont été encapsulées avec succès, sans impact sur le PDI mais avec une légère augmentation du diamètre moyen des particules, de 95 nm pour les particules blanches à 111 nm pour les NLC de DES et 114 nm pour les NLC de LEU. A dose équivalente, les taux d'encapsulation mesurés étaient proportionnels au log P des HIP avec 10,7 % pour la DES (log P = 0,5) et 84,5 % pour la LEU (log P=3).

En conclusion, la méthode de HPH, sans solvant organique et transposable à l'échelle industrielle, peut être utilisée pour encapsuler dans des nanovecteurs lipidiques solides des peptides thérapeutiques dont la lipophilie aura suffisamment été augmentée en amont. Les caractéristiques de ces nanoparticules doivent être analysées pour évaluer leur capacité à augmenter la biodisponibilité orale de ces peptides.



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A proof-of-concept for developing oral lipidized peptide Nanostructured Lipid Carrier formulations

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ABSTRACT

The aim of the study was to develop a protocol to encapsulate lipidized-peptides in Nanostructured Lipid Carrier (NLC) by High Pressure Homogenization (HPH) for oral delivery. The impact of composition, pressure and number of cycles on particle size, polydispersity index (PDI), and stability was studied. The lipophilicity of two model peptides, Desmopressin (DES) and Leuprolide (LEU) was increased by formation of a Hydrophobic Ion Pair (HIP) with sodium docusate. The precipitation efficiency was $94.96 \pm 0.27\%$ and $99.92 \pm 0.08\%$ respectively. Their solubility was evaluated in lipidic excipients. NLC were formed by pre-emulsion and HPH at 70°C . Blank NLC of 95 ± 3 nm with a PDI of 0.14 ± 0.01 were obtained using 10% (w/v) Precirol®ATO5, 4% Kolliphor®RH40 and 5% Capryol®90. The particles were obtained after 3 min pre-emulsion at 11 000 rpm followed by 5 cycles at 500 bar on Microfluidizer® at 70°C and finally 10 min in at 4°C under stirring. The complexes were successfully encapsulated in the developed NLC. HPLC analysis attested that peptides were not degraded by the process. The encapsulation efficiency (EE) was highly dependent on the Log P of the HIP with an EE of $10.7 \pm 2.2\%$ for DES-docusate HIP (log P = 0.5) and $84.7 \pm 2.0\%$ for LEU-docusate HIP (log P = 3).

1. Introduction

The interest for therapeutic peptides has been growing over the last decade, representing a market estimated to 40 billion dollars in 2013 with 140 peptides currently evaluated in clinical trials and 500 in preclinical development [1–3]. The reasons of this interest can be explained by a higher selectivity and efficiency than smaller traditional therapeutic molecules due to specific binding with precise receptors. Moreover, peptides present good patient tolerance and low toxicity.

Most peptides currently on the market are formulated to be parenterally administered. However, due to short *in-vivo* half-life of peptides, the injections have to be frequently repeated, leading to poor patient compliance. Among the needle-free alternative routes being evaluated, development of oral dosage forms would be beneficial either regarding patient adherence and decrease of treatment costs. However, many hurdles related to peptide nature strongly limit their oral bioavailability. In the stomach, peptides can be degraded by the acidic environment or the presence of pepsin. In the duodenum, a strong enzymatic activity induced by trypsin, α -chymotrypsin and elastase can

cleave peptides at different chemical sites. Finally, peptides low permeability hinders their passage across the intestinal epithelium to reach the systemic circulation. Several solutions are currently studied by formulators to overcome these limitations. Among them, encapsulation of peptides in nanocarriers seems a promising solution to shield encapsulated molecules from enzymatic degradation, increase their distribution at the apical border of the epithelium and improve their passage across the intestinal barrier. In particular, lipid-based nanocarriers are composed of biocompatible and biodegradable excipients and, consequently, do not accumulate in tissues. These carriers can be nanoemulsions, Self Emulsifying Drug Delivery Systems (SEDDS), nanocapsules, liposomes or solid lipid-based nanocarriers. At first, this last denomination referred to Solid Lipid Nanoparticles (SLN), composed of a solid matrix at room and body temperature surrounded by surfactants. These surfactants are added to reduce the size and increase the stability of the nanoparticles in the dispersion medium [4]. However, even if the addition of surfactants limits the phenomenon, crystallization or phase transition of lipids often results in drug expulsion upon storage [5,6]. In order to limit this issue, Nanostructured Lipid Carriers

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(NLC) were developed by adding liquid lipid excipients during the formation of SLN, thus limiting the crystallization inside particle cores and increasing the solubilization capacity of the systems [7]. Moreover, presence of a liquid lipid surfactant has recently shown prolonged resistance to trypsin-induced proteolytic degradation of peptides [8].

Most therapeutic peptides being hydrophilic, their encapsulation in lipid-based nanocarriers might results in low drug loading. To overcome this limitation, it is recommended to increase the lipophilicity of the drug. In particular, the formation of a Hydrophobic Ion Pair (HIP) by complexation with an anionic surfactant enables increasing peptides octanol/water partition coefficient (log P) without alteration of peptide structure and therapeutic effect. This technique has been successfully used to increase peptide drug loading in SEDDS [9–11]. These systems were effective in protecting encapsulated lipidized peptides from proteolytic degradation [12]. Moreover, it has been shown that SEDDS were able to permeate across mucus, in particular when displaying small particle size (< 200 nm) [13]. However, one of the reported drawbacks of SEDDS is their potential phase inversion leading to drug leakage upon dilution in the GI tract [14], which is not the case with solid carriers. Then, developing NLC below 200 nm and evaluate the possibility to encapsulate various lipidized peptides in these nanocarriers could provide an interesting solution for oral delivery of peptides.

Several methods exist to produce SLN and NLC. Among them, hot High Pressure Homogenization (HPH) enables a scalable solvent free production of solid nanoparticles with low polydispersity index (PDI) [15]. The method consists in heating the lipid phase containing the Active Pharmaceutical Ingredient 5–10 °C above the melting point of the solid lipid excipient. The aqueous phase containing the surfactant is heated at the same temperature and poured into the lipid phase under high shear mixing, so as to obtain a coarse pre-emulsion in the micrometer range. This pre-emulsion is then injected into the HPH where it is driven under higher pressure into the interaction chamber consisting of a small capillary of pre-determined shape. Size reduction of the oily droplets are obtained by the combination of two phenomena: high shear rates induced by velocity differences within the chamber and impact of the droplets with walls (caused by chamber geometry) and between the droplets themselves. Several cycles are generally required to obtain a satisfying size and PDI [16]. The final nanoparticles are formed by cooling down the nanoemulsion. The composition, the number of cycles and the pressure applied are the main parameters impacting nanoparticles attributes.

The aim of this study was (i) to develop a protocol for NLC production by HPH in order to encapsulate lipidized peptides, (ii) to evaluate the feasibility of lipidized-peptides encapsulation in the designed NLC and (iii) to compare the encapsulation efficiency of two model peptides: Desmopressin (DES) and Leuprolide (LEU), which chemical structures are depicted in Fig. 1.

2. Material and methods

2.1. Materials

DES and LEU (Glentham Life Sciences Ltd, Corsham, UK) were selected as model hydrophilic peptides. Excipients tested in this study are presented in Table 1. They are all approved for oral administration and listed in the Inactive Ingredient Database of the Food and Drug Administration. Tween® 80 was purchased from Fisher Scientific (MA, USA). Trifluoroacetic acid (TFA), sodium docusate and all other surfactants were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). All solid and liquid lipids were produced by Gattefossé S.A.S. (Saint Priest, France). Solvents were all in the HPLC grade.

2.2. Methods

2.2.1. Formation of Hydrophobic Ion Pair

DES-docusate and LEU-docusate HIPs were formed using the technique described by Griesser et al. [9]. 10 mg of DES were placed in Eppendorfs (20 mg in the case of LEU) and dissolved in 1 mL of 0.01 M HCl (pH = 2) solution to ionize the peptides (isoelectric point of 10.22 and 10.07 for DES and LEU, respectively [17]). Then, 1 mL of a docusate aqueous solution were added to reach a final molar ratio of 1:1.5 DES:docusate (1:2 in the case of LEU:docusate) with a limited effect on pH (pH = 2.3). In these acid conditions, the charged residues Histidine (pKa = 6) and Arginine (pKa = 13) of peptides are positively charged. Also the sulfonate group of docusate (pKa = −1) is negatively charged. The immediate formation of a white precipitate indicated the formation of the insoluble complexes. Samples were centrifuged for 10 min at 2800 g and 25 °C (Universal Centrifuge 320 R, Hettich). Supernatants were withdrawn and precipitates were plunged in liquid nitrogen before freeze-drying (Heto Drywinner FD 3). The HIPs were stored at −20 °C until further use.

Quantification of peptides in the supernatants by HPLC (see method below, II.2.2) enabled the calculation of the precipitation efficiency (PE), Equation (1), which indicates the efficiency of HIP formation.

Equation (1): Precipitation efficiency calculation

$$\text{Precipitation efficiency (\%)} = 100 - \left(\frac{\text{peptide concentration in the supernatant}}{\text{peptide concentration before HIP formation}} \times 100 \right)$$

2.2.2. Peptides quantification with HPLC

DES and LEU were quantified with an Alliance 2695D HPLC (Waters, Saint-Quentin-en-Yvelines, France) equipped with a UV detector at 217 nm (2487, Waters). Separation was made on Kinetex EVO C18 100 Å 2.6 µm 50 × 4.6 mm column (Phenomenex, Le Pecq, France) at 40 °C.

For DES quantification, the mobile phase was 80% water with 0.1% TFA (v/v) and 20% acetonitrile, with a flow rate of 0.7 mL/min. The

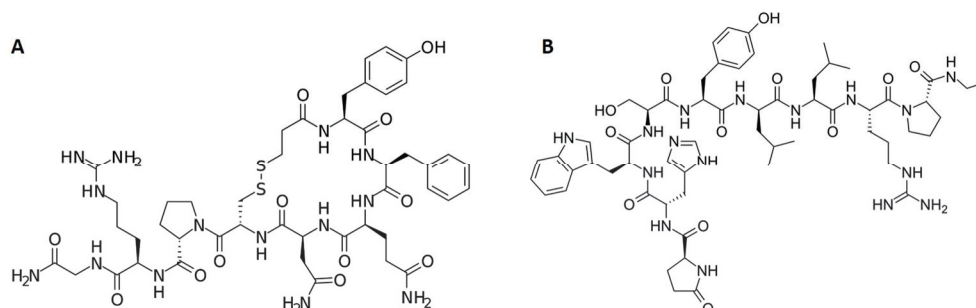
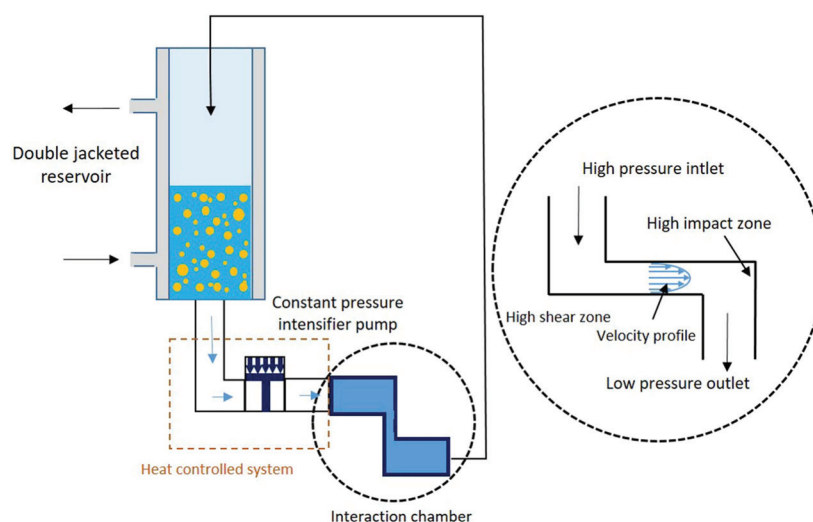


Fig. 1. Chemical structures of (A) Desmopressin and (B) Leuprolide.

Table 1

Excipients classified regarding their functionality in the study.

FUNCTIONALITY	CHEMICAL NAME	COMMERCIAL NAME	ABBREVIATION
SOLID LIPIDS	Glyceryl distearate	Precirol® ATO 5	PATO5
LIQUID LIPIDS	Glyceryl monolinoleate	Maisine® CC	MCC
	Medium chain triglycerides	Labrafac™ lipophile WL 1349	L1349
	Polyglyceryl-3 dioleate	Plurol® oléique CC 497	PO
	Propylene glycol monocaprylate	Capryol™ 90	C90
	Propylene glycol monolaurate	Lauroglycol™ 90	L90
WATER-SOLUBLE SURFACTANTS (HLB > 12)	Poloxamer 188	Pluronic® F68	PF68
	Poloxamer 407	Pluronic® F127	PF127
	Polyoxyl-35 castor oil	Cremophor® ELP	CELP
	Polyoxyl-40 hydrogenated castor oil	Kolliphor® RH40	RH40
	Polysorbate 80	Tween® 80	T80

**Fig. 2.** Schematic representation of the HPH set up.

injection volume was 20 μL . The calibration curve was established between 5 and 100 $\mu\text{g/mL}$ and the Lower Limit of Quantification (LLOQ) was evaluated at 1.5 $\mu\text{g/mL}$.

For LEU quantification, a linear gradient of elution was applied from 15% solvent A/85% solvent B to 50% solvent A/50% solvent B (solvent A: water with 0.1% (v/v) TFA/solvent B: acetonitrile). The flow rate was 0.7 mL/min and the injection volume was 20 μL . The calibration curve was established between 10 and 100 $\mu\text{g/mL}$ and the LLOQ was evaluated at 1 $\mu\text{g/mL}$.

2.2.3. Selection of liquid lipids for the solubilization of HIPs

Solubility of both HIPs was first evaluated in the solid lipid excipient: PATO5. For this purpose, 100 mg of PATO5 were placed in Eppendorfs and 10 mg of HIP (10% w/w) were added. The mixture was heated at 70 °C to ensure the melting of PATO5. Sample was regularly vortexed. After 1 h, if complete dissolution was not observed, 100 mg of PATO5 were added and the operation repeated until the concentration of HIP was 0.625% (w/w).

Solubility of complexes was evaluated in a series of liquid lipid excipients: C90, L1349, L90, MCC and PO. Different HIP concentrations (10%, 5%, 2.5% and 1.25% (w/v)) were evaluated. The mixtures were placed at 37 °C for 3 h with regular vortex agitation. Dissolution of the complexes was visually assessed.

2.2.4. Designing Precirol® ATO 5 based nanoparticles

In order to limit the number of parameters, the study was first restricted to the formulation of blank SLN. The effect of the addition of

various volumes of the pre-determined liquid lipids on particle size, PDI and stability was evaluated in a second time.

2.2.4.1. Set up description. Nanoparticles were mainly composed of PATO5, a mixture of palmitic and stearic glycerol esters (mainly diesters), which displays a melting range from 50 to 60 °C. Then, both solid lipid and aqueous phase containing a surfactant were heated at 70 °C to ensure complete melting of excipients. A pre-emulsion was formed by mixing both phases at 70 °C under high shear agitation induced by 3 min mixing at 11 000 rpm with Ultra-turrax® (T25 Ultra-turrax®, IKA). This coarse pre-emulsion was then poured into the reservoir of a Microfluidizer® LM20 (Microfluidics) equipped with a Z-type chamber G10Z (87 μm internal diameter).

In order to prevent clogging in the interaction chamber by unexpected cooling of PATO5, modifications were brought to the system. First, the standard glass reservoir was changed to a double jacketed glass reservoir connected to a temperature-controlled water bath at 70 °C. Then, a heating system was also added around the pump to maintain the entrance of the compression chamber at 70 °C during the process. Finally, a metallic tube was connected from the output of the chamber to the top of the reservoir. The formed loop enabled to automatically process several cycles (Fig. 2).

2.2.4.2. Particle size measurements. Nanoparticle size and polydispersity index (PDI) were evaluated on diluted samples (1 droplet in 2 mL deionized water) by Diffraction Light Scattering (DLS) at 25 °C on a Zetasizer Nano ZS (Malvern). This

characterization technique is based on Rayleigh diffusion, which states that light diffuses in all direction as long as the particles are small compared to the wavelength (633 nm). Particle size distributions are expressed in intensity.

Particle size of objects larger than 600 nm was measured by laser diffraction using a Mastersizer 3000 (Malvern). Measurements were performed under an agitation of 1500 rpm. Refraction index and absorption index were set at 1.48 and 0.001 respectively (based on experiments – data not shown). Particle size distributions are expressed as volume moment mean ($D[3,4]$, De Brouckère Mean Diameter). This number is calculated as described in Equation (2), in which i corresponds to the size in range of the whole particle size distribution, N_i the number of particles in the size i range and D_i the middle diameter of the size range i . It is widely used as it is sensitive to the presence of large particles in the particle size distribution, providing relevant information regarding bulk composition.

Equation (2): Mean volume moment calculation

$$D[4; 3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i}$$

2.2.4.3. Selection of surfactant concentration based on pre-emulsion formation. Pre-emulsions were formed as described in the set up description (II.2.4.1). Efficacy of various surfactants: CELP, RH40, PF68, PF127 and T80 was first screened using 5% (w/v) (1.6 g) surfactant and 10% PATO5 (4 g). Particle size distribution was analyzed on Mastersizer 3000. To standardize the procedure, analyses were performed on solid microparticles obtained by placing the hot emulsion in a 4 °C ice/water bath for 10 min under 400 rpm magnetic stirring. Surfactants that enabled formation of microparticles with a $D[3,4]$ below 10 μm were selected and used to reduce surfactant concentrations.

2.2.4.4. Screening of HPH parameters to obtain SLN with selected surfactants. A first screening was conducted to determine the range of parameters that should be applied to obtain nanoparticles with the targeted particle size and PDI. Pre-emulsions were formed using the method described above, with 4% (w/v) surfactant dispersed in the 40 mL aqueous phase. The pre-emulsions were then poured in the HPH and submitted to either 5 or 10 cycles at 500 or 1000 bar. The formed nanoemulsions were collected and placed 10 min in a 4 °C ice/water bath under 400 rpm magnetic stirring. Particles size dispersion was measured by DLS.

Parameters were then optimized with the best formulation to obtain nanoparticles less than 200 nm in size with a PDI lower than 0.2.

2.2.4.5. Addition of liquid lipid excipients: impact on particle size and stability of nanoparticles. Volumes from 300 μL to 1200 μL of the selected liquid lipids (II.2.3) were added to solid PATO5. NLC were formulated as detailed in the set up description (II.2.4.1). Their particle size was measured by DLS on the day of the production and every week during one month to control the stability of the formulations.

2.2.5. Encapsulation of peptide-docosate HIP in NLC

Equal quantities of DES and LEU were dissolved in the selected liquid lipid under their HIP form and added to 4g (10%, w/v) of PATO5. Nanoparticles were formed using the protocol described above (II.2.4.5).

The quantity of peptide encapsulated in NLC was expressed regarding the encapsulation efficiency (EE) and the drug loading (D_L), which calculation are described in Equation (3) and Equation (4), respectively. To measure the total amount of drug (encapsulated and non-encapsulated drug), the formulations were first diluted 1/3 in water followed by a dilution 1/4 in methanol supplemented with 0.1% TFA. Solutions were then placed in Eppendorfs equipped with 0.1 μm PVDF

filters (Ultrafree®-MC VV, Merck Millipore) and centrifuged at 4 °C during 15 min at 12 000 g (Universal Centrifuge 320 R, Hettich). The filtrates were analyzed with HPLC using the methods described above (II.2.2). To quantify the amount of non-encapsulated peptides, the suspensions were diluted 1/3 in water and filtered using the same filters and centrifuge parameters as for the total amount of peptides measurements. To obtain the total solid content, a known mass of suspension was dried using a vacuum drying oven.

Equation (3): Encapsulation efficiency calculation

Encapsulation Efficiency,

$$\% = \frac{\text{total amount of drug} - \text{nonencapsulated amount of drug}}{\text{total amount of drug}}$$

Equation (4): Drug loading calculation

Drug Loading, $\mu\text{g}/\text{mg}$

$$= \frac{\text{total amount of drug} - \text{nonencapsulated amount of drug}}{\text{total solid content}}$$

The stability of the peptide-loaded NLC was evaluated over 1 month, with weekly controls of EE and particle size distribution.

In order to evaluate whether peptides were degraded by the developed method, peptide solutions were prepared with the same initial concentrations as in the formulations. The solutions were heated at 70 °C for 10 min under 400 rpm magnetic stirring, agitated during 3 min at 11 000 rpm by Ultra-Turrax®, submitted to 5 cycles at 500 bar in the Microfluidizer® LM20 and finally cooled at 4 °C under magnetic stirring during 10 min. The solutions were then analyzed by HPLC and chromatograms obtained were compared with those of standard solutions.

2.2.6. Statistical analysis

All experiments were performed in triplicates and are expressed as mean $\pm$ standard error of mean (SEM). The normal distribution of data was evaluated by a Shapiro-Wilk normality test. Then one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test or two-way ANOVA followed by Tukey's post-hoc test were conducted. Data were treated and analyzed with the GraphPad Prism 8 program (CA, USA).

3. Results and discussion

3.1. Formation of HIP

To increase the lipophilicity of the two hydrophilic model peptides used in this study, the method of HIP formation was applied to DES and LEU. DES is a 9 amino acids partially cyclic peptide, used to treat insipidus diabetes and LEU is a short linear peptide of 8 amino acids used to treat endometriosis, breast and prostate cancers (structure are shown in Fig. 1). A precipitation efficiency of $94.96 \pm 0.27\%$ (mean $\pm$ SEM, $n = 30$) was obtained with DES using a docosate:DES ratio of 1.5:1. For LEU the precipitation efficiency was calculated to be $99.92 \pm 0.08\%$ (mean $\pm$ SEM, $n = 118$). These results were similar to the one obtained by Griesser et al. [9]. According to their study [9], the log P of DES was increased by 3 units (from -2.5 to $+0.5$) using a 1.5:1 docosate:DES molar ratio and the log P of LEU by 5 units (from -2 to $+3$) with a 2:1 docosate:LEU molar ratio. The difference in log P increase might be explained by a difference in the number of ionizable functions: only one for DES (arginine) while LEU has two charged residues (arginine and histidine), limiting the interaction sites with the anionic surfactant. Moreover, the cyclic conformation of DES may also sterically hinder the approach of the docosate counterion.

3.2. Selection of liquid lipids for the solubilization of HIPs

Both HIPs were not soluble in melted PATO5 at 70 °C for

Table 2

Solubility of peptide-docosate HIP in various liquid lipids in the 1.125–10% (m/v) range tested.

Liquid lipids	Maximum concentration of dissolved peptide (% w/v)	
	DES-docosate	LEU-docosate
MCC	2.50	5.00
L1349	< 1.25	< 1.25
PO	< 1.25	< 1.25
C90	5.00	10.00
L90	1.25	10.00

concentration as low as 0.625%.

Various liquid lipids were tested to dissolve the HIPs, covering a range of HLB from 1 to 5. Results show that LEU-Docosate HIP, with higher log P than DES-docosate HIP, exhibited a higher solubility in all liquid lipids. In particular, high solubility was measured in monoesters of propylene glycol: C90 and L90 with respective HLB of 5 and 3 (Table 2). DES-docosate HIP was less soluble in the tested liquid lipids, with a maximum of 5% (m/v) in C90.

C90, presenting the highest solvent capacity for both HIP, was further used in the study for the development of NLC. Moreover, the use of an ester of propylene glycol and caprylic acid should help enhancing the passage of the nanocarriers across the epithelial border. Indeed, medium chain fatty acids, and in particular caprylic (C8) and capric (C10) acids, salts and esters are known to promote the absorption of active molecules [18–21].

3.3. Development of NLC

In order to limit the number of parameters, the first stages of the nanoparticles development were limited to the formulation of SLN, without addition of the liquid lipid excipient.

3.3.1. Selection of appropriate surfactants and concentration

A pre-selection of non-ionic surfactants was made, regarding their precedence of use in marketed products or their ability to increase permeability in monolayer of intestinal cells [22,23]. The usual surfactant content in NLC being below or equal to 5% (w/v) [24], a first screening was made using the maximal 5% concentration of surfactants to evaluate their impact on pre-emulsion particle size.

Large microparticles were obtained with PF127 and CELP, displaying D [3,4] of 39 and 251 μm respectively. On the contrary, D [3,4] below 10 μm were measured with PF68 (9.3 μm), RH40 (5.3 μm) and T80 (9.8 μm).

Formation of these pre-emulsions was repeated with these three latter surfactants to evaluate the reproducibility of the process and the possibility to reduce the percentage of surfactant. Results, displayed in Fig. 3, show that the smallest microparticles were obtained with RH40. This surfactant also enabled the highest batch-to-batch reproducibility. When T80 and PF68 were used as surfactants, a slight, but not significant, decrease in particle size was observed, especially between 3 and 4% (w/v) surfactant. Nevertheless, results have to be handled with care. Indeed, they correspond to the size of the particles obtained after cooling the pre-emulsion and may not be representative of the droplets size of the emulsion that will be injected in the HPH apparatus.

As no significant difference in particle size was observed using 4 or 5% surfactants, it was decided to use the lowest concentration of surfactant (i.e. 4%) in order to prevent damages on the intestinal mucosa associated with an excess of surfactant.

3.3.2. Impact of HPH parameters on particle size distribution

Both pressure and number of cycles can have an impact on particle size distribution and PDI. A first screening was performed with the 3 selected surfactants with at a concentration of 4% (w/v). The pre-

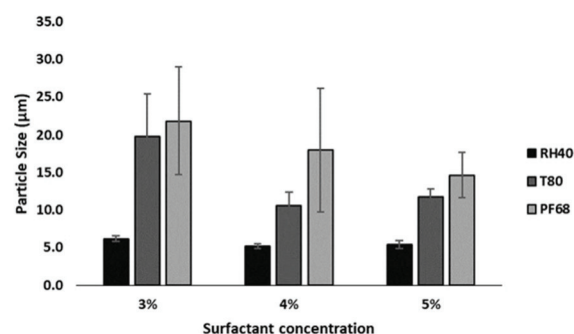


Fig. 3. Impact of surfactants concentration (w/v) on pre-emulsion particle size (results expressed as mean $\pm$ SEM, n = 3).

emulsions were submitted to various combinations of pressure and number of cycles. Particle size distributions and PDI measured after solidification of the nanoemulsions are presented in Table 3. All conditions enabled the formation of nanoparticles below 300 nm.

For nanoparticles formed using T80 and PF68, modifying the parameters had a dramatic impact on size reduction. Indeed, a very significant difference in particle size ($p < 0.001$) was observed between the gentler parameters (5 cycles at 500 bar) and the other sets of parameters. It was possible to obtain nanoparticles below 200 nm only with the higher number of cycles or by increasing the applied pressure. Same observations can be made regarding the PDI measured with nanoparticles obtained with T80: it was necessary to increase the number of cycles or the pressure to measure PDI below 0.2. However, when PF68 was used as surfactant, all nanoparticles exhibited higher polydispersity, with a minimal PDI of 0.26 obtained after 10 cycles at 500 bar. When RH40 was used as surfactant all nanoparticles presented a particle size around 100–110 nm with no significant difference observed between the different conditions applied. Regarding PDI, they were below 0.2, for all the conditions applied.

As a risk of peptide degradation exists using harsh HPH conditions, it was decided to use RH40 as surfactant, with which it is possible to obtain particles around 100 nm and PDI below 0.2 with moderated conditions. In order to evaluate the possibility of reducing the number of cycles, pre-emulsions containing 4 g (10%, w/v) of PATO5, 1.6 g RH40 (4%, w/v) in 40 mL of water were formed and then submitted to various numbers of cycles at 500 bar. Particle size dispersion was measured after solidification of the hot nanoemulsions in an ice/water bath. The results, displayed in Table 4, show that a minimum of 4 cycles at 500 bar is required to obtain reproducible nanoparticles below 200 nm and PDI inferior to 0.2. Increasing the number of cycles from 4 to 5 had a small effect on particle size (from 118 to 110 nm) and no effect on PDI. Further increases in the number of cycles had no impact on nanoparticles dispersion.

3.3.3. Addition of liquid lipid: impact on particle size and stability of NLC

The parameters set for the development of the SLN (i.e. RH40 as surfactant, ratio RH40/PATO5 and the use of 5 cycles at 500 bar in the HPH device) were used to develop the NLC. Usual solid to liquid lipids ratio in NLC are from 70:30 to 99.9:0.1 [23]. However, high volumes of liquid can lead to structure alteration of nanoparticles. In the current system, the maximal quantity of the selected liquid lipid, C90, would be 1.7 g (~1650 μL). This quantity was added to the lipid phase prior to melting at 70 $^{\circ}\text{C}$. The formed pre-emulsion was submitted to 5 cycles at 500 bar in the Microfluidizer[®]LM20. Large particles in the micrometer range were clearly visible in the heterogeneous suspension. A screening with smaller volumes of C90 was performed in the same conditions and particle size distributions were compared with the ones measured for SLN. Results presented in Table 5 show a slight decrease (not

Table 3Impact of surfactant, pressure and number of cycles on particle size distribution and PDI using an HPH process (results expressed as mean $\pm$ SEM, n = 3).

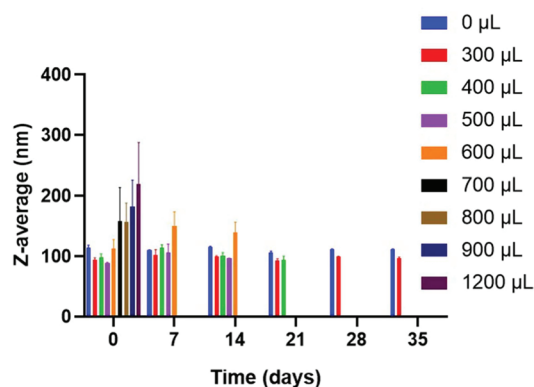
		5 cycles, 500 bar	10 cycles, 500 bar	5 cycles, 1000 bar	10 cycles, 1000 bar
PF68	Z-average (nm)	235 $\pm$ 10	149 $\pm$ 14	160 $\pm$ 8	160 $\pm$ 13
	PDI	0.34 $\pm$ 0.05	0.26 $\pm$ 0.05	0.31 $\pm$ 0.03	0.29 $\pm$ 0.05
RH40	Z-average (nm)	114 $\pm$ 4	103 $\pm$ 2	101 $\pm$ 3	100 $\pm$ 5
	PDI	0.19 $\pm$ 0.01	0.16 $\pm$ 0.03	0.16 $\pm$ 0.01	0.14 $\pm$ 0.03
T80	Z-average (nm)	225 $\pm$ 1	102 $\pm$ 1	110 $\pm$ 1	111 $\pm$ 2
	PDI	0.32 $\pm$ 0.03	0.17 $\pm$ 0.01	0.19 $\pm$ 0.01	0.16 $\pm$ 0.02

Table 4Impact of the number of cycles at 500 bar on particle size and PDI of SLN (results expressed as mean $\pm$ SEM, n = 3).

	3	4	5	6	7	8	9	10
Z-average (nm)	207.6 $\pm$ 45.1	118.3 $\pm$ 0.6	109.6 $\pm$ 0.4	106.2 $\pm$ 2.8	111.0 $\pm$ 0.4	109.3 $\pm$ 0.2	109.7 $\pm$ 1.4	99.1 $\pm$ 4.7
PDI	0.29 $\pm$ 0.09	0.19 $\pm$ 0.01	0.18 $\pm$ 0.01	0.16 $\pm$ 0.01	0.14 $\pm$ 0.01	0.12 $\pm$ 0.01	0.14 $\pm$ 0.01	0.14 $\pm$ 0.03

Table 5Impact of increasing volume of C90 on particle size distribution of NLC obtained by HPH (results expressed as mean $\pm$ SEM, n = 3).

Volume of C90 (μ L)	0	300	400	500	600	700	800	900	1200
Z-average (nm)	114 $\pm$ 4	95 $\pm$ 3	98 $\pm$ 6	89 $\pm$ 1	113 $\pm$ 15	158 $\pm$ 56	157 $\pm$ 32	184 $\pm$ 44	220 $\pm$ 69
PDI	0.19 $\pm$ 0.01	0.14 $\pm$ 0.01	0.18 $\pm$ 0.03	0.11 $\pm$ 0.01	0.14 $\pm$ 0.03	0.18 $\pm$ 0.04	0.25 $\pm$ 0.07	0.31 $\pm$ 0.15	0.29 $\pm$ 0.10

**Fig. 4.** Particle size evolution of PATO5 NLC formulated with different amount of C90 (results expressed as mean $\pm$ SEM, n = 3).

significant) of the particle size upon addition of small volumes of C90 to the system. However, with the addition of 700 μ L (15% of particle weight) and more, larger particles were obtained, with less batch-to-batch reproducibility.

Stability controls were made by assessing an eventual evolution of particle size upon storage at 4 °C. For NLC formed with 900 and 1200 μ L C90, an important gelation of the formulation was visible after one-week storage. It has been shown that solid lipid contained in nanoparticles reorganize rapidly from the unstable α organization with nanoparticles with spherical shape to the more stable β -polymorph leading to nanoparticles with a platelet-shape [25,26]. Although NLC were developed to limit this issue, this modification of morphology, associated with a decrease of nanoparticles volume, could lead to an expulsion of the liquid lipid, especially when high volumes of liquid lipid are added to the system. With a HLB of 5, C90 moieties may remain at the surface of the particles and eventually partially solubilize PATO5. This phenomenon may explain the formation of bridges between particles and gelation by aggregation.

For the ones formed with 700 and 800 μ L C90, an oily film was visible at the surface of the nanosuspensions after one week. Then, DLS

measurements were only performed on suspensions presenting the same aspect as the one visible on the day of production (D0). After 3 weeks at 4 °C, NLC formulated with 500 and 600 μ L C90 exhibited, in turn, a similar oily film at the surface of the formulations. Particle size measurements, displayed in Fig. 4, show that for volumes of C90 of 500 μ L and below, particle size measurements remained stable over 3 weeks.

In order to reduce the risk of drug leakage upon NLC storage, it was decided to use a volume of 300 μ L of C90 to encapsulate lipidized peptides.

3.4. Proof-of-concept encapsulation of lipidized peptides in optimal NLC formulation

3.4.1. Peptide recovery after process

To evaluate if peptides could resist the different steps of the developed process, solutions of DES and LEU were prepared and submitted to the same conditions as NLC formulation process. The chromatograms of peptides solutions retrieved after cooling down the solution are presented in Fig. 5. Overlaying the chromatograms obtained with the ones of standard solutions show good superposition, with equal retention times and no degradation peaks.

3.4.2. Peptide encapsulation

The process developed above to produce stable blank NLC was used to encapsulate HIP of DES and LEU. To this end, complexes were first dissolved in C90 and then added to melted PATO5. Composition of the formulations is given in Table 6. Particle size of the obtained loaded-NLC were slightly but significantly ($p < 0.05$) higher compared to blank NLC, probably due to the presence of HIP in nanoparticles (Fig. 6).

To calculate EE and DL, supernatants were isolated from NLC using an ultrafiltration method. The results are depicted in Table 7. Both peptides were successfully encapsulated in the NLC. However, in the case of LEU the EE was of 84.7% while it was only 10.7% in the case of DES. This can be explained by the difference in lipophilicity of the formed HIP: LogP LEU-docusate = +3 and LogP DES-docusate = +0.5 [9]. Then, the encapsulation of lipidized LEU, being around 1000 times more hydrophobic than lipidized DES, resulted in 8-fold higher EE and DL.

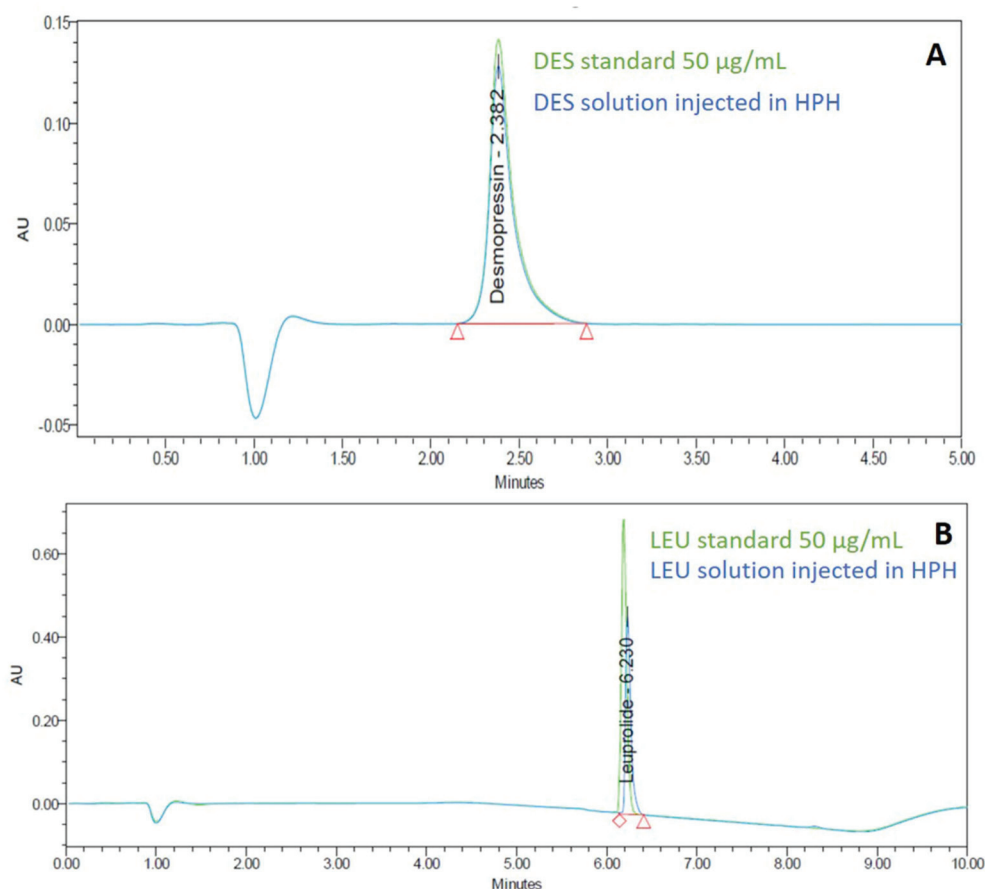


Fig. 5. Superposition of chromatograms corresponding to A: DES standard solution (50 µg/mL) and a solution of DES submitted to the protocol of NLC formulation by HPH; B: LEU standard solution (50 µg/mL) and a solution of LEU submitted to the protocol of NLC formulation by HPH.

Table 6

Composition of the formulations containing HIP.

Composition	NLC-HIP-DES	NLC-HIP-LEU
PAT05	4g	4g
C90	300 µL	300 µL
HIP	15 mg	16 mg
Corresponding peptide	9 mg	9 mg
RH40	1.6g	1.6g
Water	40 mL	40 mL

3.4.3. HIP-loaded NLC stability

Stability of NLC formulations was evaluated over one month. Particle size analyses showed no difference between the day of production and after 28 days storage at 4 °C, Fig. 7. Same observations were made concerning PDI (data not shown).

EE and DL of LEU-loaded NLC were stable over 28 days (Fig. 8). However, for NLC containing DES, a significant increase ($p < 0.05$) in the calculated EE and DL was observed after 21 days. This can be explained by a lack of stability of DES in the acid dispersing phase over time as previously described in Ref. [8]. Then, the calculation of the EE is altered by a decrease of the quantity of intact non-encapsulated DES remaining in the dispersing phase. However, this does not imply a degradation of the peptide actually encapsulated within the nanocarriers. Unfortunately, no direct method exists to measure the EE.

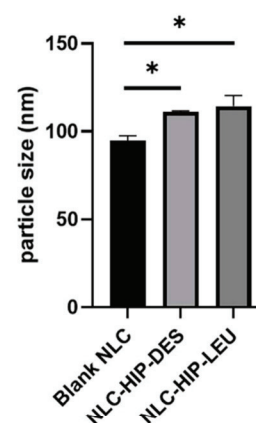


Fig. 6. Particle size of blank and peptide-loaded NLC (data expressed as mean $\pm$ SEM, $n = 3$).

After one-month storage the same phenomenon as for blank NLC started to be visible with an oily film at the surface of the nanosuspensions, indicating an exudation of the liquid lipid from the core of the NLC. C90 is a liquid surfactant with a HLB value of 5 that has some

Table 7

Encapsulation efficiency and drug loading of DES and LEU in NLC-HIP-DES and NLC-HIP-LEU respectively (data expressed as mean $\pm$ SEM, n = 3).

	NLC-HIP-DES	NLC-HIP-LEU
EE (%)	10.7 $\pm$ 2.2	84.7 $\pm$ 2.0
D _L (μ g/mg)	0.2 $\pm$ 0.06	1.58 $\pm$ 0.06
Log P HIP	0.5	3

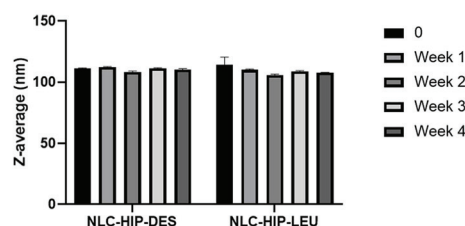


Fig. 7. Weekly evolution of HIP-loaded NLC particle size over one month at 4 °C (data shown as mean $\pm$ SEM, n = 3).

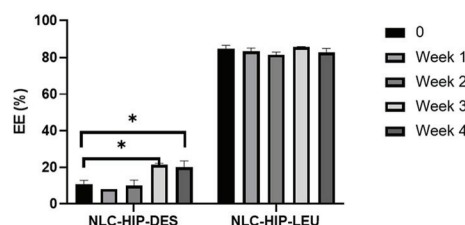


Fig. 8. Weekly evolution of the EE of HIP-loaded NLC over one month at 4 °C (data shown as mean $\pm$ SEM, n = 3) (* p < 0.05 when compared to the initial value on D0).

affinity for water and tends to migrate to the water phase upon storage. In order to avoid this phenomenon an additional drying step (e.g. freeze-drying) could be appended to remove water and stabilize the formulations.

4. Conclusion

The first objective of this study was to develop a protocol enabling the reproducible formation of NLC with mean particle size lower than 200 nm using a hot HPH process. NLC composed of PATO5, C90 and RH40 (68.5:27% w/w) were successfully formulated with 5 cycles at 500 bar in the Microfluidizer®LM20 leading to a mean particle size of 95 nm and a PDI below 0.2. These NLC were stable for at least 35 days at 4 °C.

The second objective of the study was to evaluate the possibility to encapsulate two hydrophilic model peptides (Desmopressin and Leuprolide) in the designed NLC and compare their encapsulation efficiency (EE). Peptides lipophilicity was increased by formation of a Hydrophobic Ion Pair (HIP) with sodium docusate, leading to high precipitation efficiency revealing a strong complexation. The solubility of both complexes in lipophilic solvents was dependent on their log P and was higher in propylene glycol monocaprylate (C90) exhibiting higher polarity. The HIP were successfully encapsulated in the developed NLC with only a slight but significant increase of particle size and the NLC were stable over one month. The swift developed process did not degrade the model peptides. The EE of the HIP in NLC was highly dependent on their lipophilicity confirming that this is a key material attribute for achieving efficient encapsulation of peptides.

Author contribution

Camille Dumont: Investigation, Formal analysis, Writing-original draft, Vincent Jannin: Conceptualization, Writing-review & editing, Supervision, Cédric Miolane: Investigation, Quentin Lelong: Investigation, Jean-Pierre Valour: Investigation, Sebastien Urbaniak: Investigation, Hatem Fessi: Supervision, Sandrine Bourgeois: Writing-review & editing, Supervision

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jddst.2019.101394>.

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Chapitre III

Evaluation *in vitro* des nanovecteurs lipidiques solides :
capacité à encapsuler un peptide modèle et à le protéger de
l'environnement gastro-intestinal

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La biodisponibilité orale de la plupart des peptides thérapeutiques est faible, dépassant rarement 1%. Deux raisons sont avancées pour justifier cette observation : la faible perméabilité intestinale des peptides et leur rapide dégradation par l'activité enzymatique du tractus gastro-intestinal. L'objectif de cette étude est d'évaluer la capacité des nanovecteurs lipidiques solides, à savoir les nanoparticules lipidiques solides (SLN) et les vecteurs lipidiques nanostructurés (NLC), à protéger un peptide modèle de la dégradation protéolytique. Le leuprolide (LEU), peptide linéaire de 9 acides aminés utilisé dans le traitement du cancer de la prostate, a été utilisé comme modèle dans le cadre de cette évaluation.

Afin d'augmenter l'affinité du LEU pour les nanovecteurs lipidiques, sa lipophilie a été modifiée par formation d'une paire d'ion hydrophobe (HIP) avec le docusate de sodium. Cette complexation, réalisée avec un ratio molaire LEU : docusate = 1 :2, a permis d'hydrophobiser la quasi-totalité du peptide avec un taux de précipitation de 99,9%. Ces HIP ont augmenté de manière significative le taux d'encapsulation du peptide, que ce soit dans les SLN (44% pour le LEU et 76% pour l'HIP) ou les NLC (52% pour le LEU et 78% pour l'HIP). Les nanoparticules, obtenues de manière reproductible, mesuraient entre 115 et 125 nm avec un PDI inférieur à 0,2 (mesures par diffusion dynamique de la lumière) et présentaient une forme de plaquettes. Ni la structure, ni la taille des particules n'ont été altérées par l'encapsulation du peptide, que ce soit sous forme hydrophobisée ou non.

Afin d'observer d'éventuels phénomènes d'agrégation ou de dégradation des nanoparticules dans les milieux gastro-intestinaux, l'évolution de leur taille a été observée à 37°C après dispersion dans des fluides gastro-intestinaux simulés : le fluide gastrique simulé (SGF), le milieu intestinal simulé à jeun (FaSSIF-V2) et le milieu intestinal simulé nourri (FeSSIF-V2). La comparaison des données mesurées avec celles obtenues dans les mêmes conditions dans de l'eau ultra pure, a démontré la stabilité des nanoparticules dans ces milieux.

Le milieu FaSSIF-V2 a été utilisé pour étudier la cinétique de libération du peptide à 37°C sur 6 heures. La quantification par HPLC du LEU dans le milieu a montré une libération très rapide du peptide dès les premières minutes. Celle-ci s'explique par la morphologie des nanovecteurs lipidiques solides. En effet, la structure en plaquettes implique une surface très importante en comparaison du volume. Ainsi, la majorité de l'actif encapsulé se retrouve proche de la surface des particules. Par ailleurs, cette structure implique une interface conséquente avec le milieu

de dispersion. Dans le cas du FaSSIF-V2, celui-ci contient des phospholipides et des sels biliaires pouvant facilement accroître la dissolution du peptide dans la phase aqueuse.

Le profil de dégradation du LEU en présence de trypsine ou d' α -chymotrypsine a été établi à pH 6,8. La dégradation du LEU par la trypsine est rapide avec seulement 16% d'actif subsistant après 90 minutes d'incubation et une dégradation totale observée après 2 heures. L'encapsulation du peptide dans les NLC a permis de préserver 23% du peptide après 90 minutes d'incubation et jusqu'à 35% lorsque le peptide était encapsulé sous sa forme hydrophobisée. En revanche, les SLN n'ont pas démontré d'effet protecteur vis-à-vis de la trypsine. Il semblerait alors que ce soit la fraction lipidique liquide des NLC, dans laquelle les protéases ne sont pas solubles, qui ait permis de prolonger la présence du peptide dans cet environnement. Pour autant, aucun des deux nanovecteurs lipidiques solides n'a permis de protéger le LEU de la dégradation induite par l' α -chymotrypsine. De manière surprenante, la dégradation du LEU a été plus rapide lorsque celui-ci était encapsulé dans les nanovecteurs lipidiques. Il semblerait donc que l'activité enzymatique ait été renforcée en présence des nanoparticules.

En conclusion, cette étude a permis de mettre en avant l'intérêt de la formation d'une HIP pour augmenter le taux d'encapsulation de peptides hydrophiles dans des nanovecteurs lipidiques solides. Les deux types de vecteurs, SLN et NLC, ont été obtenus de manière reproductible *via* le procédé d'HPH. Elles présentaient une taille, un PDI et une morphologie similaires. La pré-dissolution du peptide dans la fraction lipidique liquide n'a pas eu d'impact sur le taux d'encapsulation. Néanmoins, celle-ci a permis d'augmenter de manière significative la protection du LEU vis-à-vis de l'activité enzymatique de la trypsine. La structure en plaquettes des nanoparticules s'est avérée désavantageuse au regard de la libération rapide du peptide dans les milieux intestinaux simulés. Cependant, une quantité non-négligeable de LEU demeure tout de même encapsulée dans les nanovecteurs et donc temporairement protégée de la dégradation enzymatique et absorbable par la barrière intestinale. En effet, *in vivo*, les nanoparticules ne restent pas en permanence dans le lumen intestinal avec un risque élevé de dégradation protéolytique du peptide. Si leur taille et leur composition le permet, les nanovecteurs vont pouvoir franchir la barrière de mucus et atteindre l'épithélium intestinal. A ce niveau, l'activité enzymatique est bien moins importante que dans le lumen. L'amélioration de l'absorption du peptide dans la circulation systémique sera alors le résultat

de la compétition entre la cinétique de dégradation protéolytique et celle de diffusion à travers le mucus puis l'épithélium intestinal. Il est alors nécessaire de mesurer la capacité des nanoparticules à augmenter le passage intestinal des peptides.



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In-vitro evaluation of solid lipid nanoparticles: Ability to encapsulate, release and ensure effective protection of peptides in the gastrointestinal tract

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ABSTRACT

Peptides are rarely orally administrated due to rapid degradation in the gastrointestinal tract and low absorption at the epithelial border. The objective of this study was to encapsulate a model water-soluble peptide in biodegradable and biocompatible solid lipid-based nanoparticles, i.e. Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC) in order to protect it from metabolic degradation. Leuprolide (LEU) and a LEU-docusate Hydrophobic Ion Pair (HIP) were encapsulated in SLN and NLC by High Pressure Homogenization. The particles were characterized regarding their Encapsulation Efficiency (EE), size, morphology, peptide release in FaSSIF-V2, and protective effect towards proteases. Nanoparticles of 120 nm with platelet structures were obtained. Formation of HIP led to a significant increase in LEU EE. Particle size was moderately affected by the presence of simulated fluids. Nonetheless, an important burst release was observed upon dispersion in FaSSIF-V2. NLC were able to improve LEU-HIP resistance to enzymatic degradation induced by trypsin but presented no advantages in presence of α -chymotrypsin. SLN provided no protection regarding both proteases. Despite an increased amount of encapsulated peptide in solid lipid-based nanoparticles following HIP formation, the important specific surface area linked to their platelet structures resulted in an important peptide release upon dispersion in FaSSIF-V2 and limited protection towards enzymatic degradation.

1. Introduction

Peptides represent a class of Active Pharmaceutical Ingredients (API) with effective and specific action over a wide variety of diseases. Currently, due to their poor oral bioavailability, most peptides are parenterally administered (Brayden and O'Mahony, 1998). However, due to the short half-life of these biomolecules in human physiological fluids, injections must be frequently repeated, leading to poor patient compliance (Almeida and Souto, 2007; Brayden and O'Mahony, 1998; Gupta et al., 2016). Development of non-invasive routes, especially oral, for peptides absorption would notably improve patient's medication. Yet, because of their biological nature, therapeutic peptides are strongly metabolized by proteases in the human gastrointestinal tract (Leonaviciute and Bernkop-Schnürch, 2015).

Numerous strategies have been considered by formulators such as the use of enteric coatings, addition of protease inhibitors, chemical modification of peptides or use of advanced drug delivery carriers. This last option seems to be a promising approach to deliver peptides via oral route. In particular, lipid-based nanocarriers are of great interest because of their GRAS status and biodegradability (Dumont et al., 2018). These systems can be either micelles, nanoemulsion, nanospheres, nanocapsules or liposomes.

One of the major limitations for the encapsulation of peptides in these systems lies in the hydrophilic character of most peptides and the lipophilic character of solid lipid-based nanoparticles. However, this constraint can easily be overcome by the formation of a Hydrophobic Ion Pair (HIP) with a lipophilic counterion. This reversible technique has shown improved Encapsulation Efficiency (EE) of peptide when

Abbreviations: Cryo-TEM, cryogenic Transmission Electronic Microscopy; D_L, Drug Loading; EE, Encapsulation Efficiency; DLS, Dynamic Light Scattering; FaSSIF, Fasted State Simulated Intestinal Fluid; FeSSIF, Fed State Intestinal Fluid; GRAS, Generally Recognized As Safe; HIP, Hydrophobic Ion Pair; HPLC, High Pressure Liquid Chromatography; LEU, Leuprolide; NLC, Nanostructured Lipid Carrier; PDI, Polydispersity Index; SEDDS, Self-Emulsifying Drug Delivery System; SGF, Simulated Gastric Fluid; SLN, Solid Lipid Nanoparticle

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used with Self-Emulsifying Drug Delivery Systems (SEDDS) (Griesser et al., 2017) and Solid Lipid Nanoparticles (SLN) (Gallarate et al., 2011; Kalhapure et al., 2014). If these SLN present many advantages such as controlled drug release or possibility of production without solvent, their low drug-loading capacities and their tendency to eject APIs upon storage has also been denounced (Almeida and Souto, 2007; Mehnert and Mäder, 2001). A second type of solid lipid-based nanoparticles, Nanostructured Lipid Carriers (NLC), was developed to address these issues. In the same way as for SLN, they are formulated with solid lipid at room and body temperature but, in the case of NLC, liquid lipids are added to the system to reduce the crystallization behavior and to maintain the drug within particles (Beloqui et al., 2016; Kathe et al., 2014; Muchow et al., 2008).

NLC have proven their ability to increase the EE of lipophilic drugs by pre-dissolving them in the liquid excipients prior to encapsulation rather than only dispersing them in the solid matrix (Jenning et al., 2000; Jennings and Gohla, 2001). Thus, we hypothesize that encapsulating lipidized peptides by HIP in NLC would result in a higher EE than in SLN. Furthermore, SEDDS, only composed of liquid excipients, were able to provide peptides a high protective environment towards enzymatic degradation by luminal proteases and glutathione (Hetényi et al., 2017). Hence, dissolving HIP in the liquid lipid part of NLC might ensure a similar peptide protection. If the encapsulation of peptide-anionic surfactant HIP in SLN have been detailed in several publications, to our knowledge, the encapsulation of peptide-based HIP in NLC has never been reported. The SLN were either prepared by solvent-based techniques (Gallarate et al., 2010, 2011; Yuan et al., 2009), or using a microemulsion template (Morel et al., 1996). Although an increase in peptide temperature stability has been proved by Power et al., only one article refers to the use of melt dispersion, with an ultrasonication technique (Kalhapure et al., 2014; Powers et al., 1993). However, the use of High-Pressure Homogenization (HPH), an industrial process which enables the production of large volume of homogeneous solid lipid-based nanoparticles had never been reported for this purpose before.

Leuprolide (LEU) was chosen as model peptide for this study. It is a linear hydrophilic nonapeptide (log P = -2) used to treat various hormonal diseases. It has two ionizable functions, necessary for the formation of the HIP (guanidine group of arginine (pKa = 13) and imidazole group of histidine (pKa = 6)).

In this study, we first compared the ability of NLC and SLN, prepared by HPH, to encapsulate LEU and LEU-anionic surfactant HIP. Then, an eventual modification of the solid lipid-based nanoparticles in the simulated gastrointestinal (GI) fluids was controlled over time. Next, drug release studies from the solid lipid-based nanoparticles were performed to evaluate the capacity of the systems to maintain the drug within the particles. Finally, the ability of solid lipid-based nanoparticles to protect LEU from enzymatic degradation was studied in presence of two intestinal proteases: trypsin and α -chymotrypsin.

2. Materials and methods

2.1. Materials

Leuprolide acetate (LEU) was purchased from Glentham Life Sciences Ltd (batch 110YXI, Corsham, UK). Sodium docusate, used for the design of HIP, was obtained from Sigma Aldrich (batch MKCC5209, Saint-Quentin-Fallavier, France).

For the preparation of solid lipid-based nanoparticles, the following excipients were used: Kolliphor® RH40 (RH40, polyoxyl-40 hydrogenated castor oil, batch BCB56251V) obtained from Sigma Aldrich, Capryol™ 90 (C90, propylene glycol monocaprylate, batch 157349) and Precirol® ATO 5 (PATO5, glyceryl distearate, batch 160460) both obtained from Gattefossé S.A.S. (Saint-Priest, France).

For preparation of simulated GI fluids, L- α -phosphatidylcholine (batch BCBV3947) and maleic acid (batch 018K5400) were obtained

from Sigma Aldrich. FeSSIF-V2 was purchased from BioRelevant (batch V2FES-0618-A, London, UK), sodium chloride from VWR (batch 13L180015, Bourges, France), pepsin (batch 5S010575, Darmstadt, Germany), sodium hydroxide (batch B1581782829) and sodium taurocholate (NaTC, batch 2007100274) from Sigma Aldrich (Saint-Quentin-Fallavier, France).

The evaluation of the protection of LEU towards proteases was handled in Tris-HCl buffer made with Tris obtained from Chimie-Plus (Tris(hydroxymethyl)aminomethane, 8389658) and HCl 37% from Merck. The proteases used were α -chymotrypsin from bovine pancreas (TLCK treated, SLBQ8063V) and trypsin from bovine pancreas (TPCK treated, SLBS8956), both purchased from Sigma Aldrich. All solvents were HPLC grade. Ultrapure water was obtained from MilliQ (Millipore).

2.2. Methods

2.2.1. Hydrophobic ion pairing

LEU was complexed via the formation of a HIP with sodium docusate in a 1:2 M ratio. The complexes were prepared according to the method previously described by Griesser et al. (Griesser et al., 2017). Briefly, 20 mg of LEU were dissolved in 1 mL 0.01 M HCl solution. Sodium docusate was dissolved in demineralized water at a concentration of 14 mg/mL and 1 mL of this solution was added to the peptide solution. The immediate appearance of a white precipitate indicated the formation of the insoluble complexes. Then samples were centrifuged at 15,000g with a Universal Centrifuge 320 R (Hettich) at 25 °C for 10 min. Supernatants were separated from the precipitate to measure the remaining fraction of soluble LEU via the HPLC method described below. Precipitates were frozen in liquid nitrogen, lyophilized and stored at -20 °C. Precipitation efficiency was described using the following equation:

Precipitation efficiency calculation

Precipitation efficiency

$$= 100 - \left(100 \times \frac{\text{Leuprolide concentration after HIP}}{\text{Leuprolide concentration before HIP}} \right) \quad (1)$$

2.2.2. HPLC analysis

Leuprolide was quantified on an Alliance 2695D (Waters, Saint-Quentin-en-Yvelines, France) equipped with a UV detector (2487, Waters). Separation was made on Kinetex EVO C18 100 Å 2.6 μ m 50x4.6 mm column (Phenomenex, Le Pecq, France) at 40 °C. A linear gradient of elution was applied from 15% solvent A/85% solvent B to 50% solvent A/50% solvent B (solvent A: water with 0.1% (v/v) TFA/solvent B: acetonitrile). The flow rate was 0.7 mL/min, the injection volume was 20 μ L and the wavelength of the detector was set at 217 nm. The calibration curve was established between 10 and 100 μ g/mL and the Lower Limit of Quantification (LLOQ) was evaluated at 1 μ g/mL.

2.2.3. Preparation of NLC and SLN

NLC and SLN were prepared using HPH technique. Their composition is given in Table 1.

In the case of SLN, PATO 5 (10%, w/v) was melted at 70 °C. LEU or LEU-HIP were added into the melted lipid phase and let to homogenize for 10 min under magnetic stirring at 400 rpm. The aqueous phase, containing RH40 was also heated at 70 °C and poured into the lipid phase. The O/W pre-emulsion was formed using an ultra-turrax (ultra-turrax T18, IKA) at 11000 rpm for 3 min. The pre-emulsion was subsequently introduced to the HPH (Microfluidizer®LM20, Microfluidics) where it was homogenized during 5 cycles at 500 bars. The SLN were formed by placing the hot nanoemulsion in a water/ice-cold bath at 4 °C under controlled magnetic agitation at 400 rpm. SLN containing pure LEU were referred as SLN-LEU while formulations containing its

Table 1
Composition of solid lipid-based nanoparticles.

	Blank NLC	NLC-LEU	NLC-HIP	Blank SLN	SLN-LEU	SLN-HIP
Lipid phase						
PATO5	4 g	4 g	4 g	4 g	4 g	4 g
C90	300 µL	300 µL	300 µL	–	–	–
LEU	–	20 mg	–	–	15 mg	–
HIP	–	–	32 mg	–	–	27 mg
Aqueous phase						
RH40	1.6 g	1.6 g	1.6 g	1.6 g	1.6 g	1.6 g
Water	40 mL	40 mL	40 mL	40 mL	40 mL	40 mL

complexed form were named SLN-HIP.

NLC were prepared using the same HPH method by adding Capryol™ 90 (C90) in the pre-emulsion oily phase. In the case of HIP loaded NLC, the complex was dissolved in C90 prior addition to melted PATO5 and let to homogenize under magnetic stirring for 10 min. These NLC were reported as NLC-HIP. In the case of LEU loaded NLC, LEU was added to the melted lipid phase containing PATO5 and C90. These NLC were referred as NLC-LEU.

The SLN and NLC were stored at 4 °C until further evaluation.

In order to assay the chemical stability of LEU during the preparation of solid lipid-based nanoparticles, a solution of LEU was submitted to the same protocol, i.e. 10 min at 70 °C under magnetic stirring, mixing with an ultra-turrax for 3 min, then 5 cycles at 500 bars in the microfluidizer followed by cooling at 4 °C. Leuprolide was quantified by HPLC and the amount was equivalent to the initial concentration. In addition, no degradation peak was observed on the chromatograms. Therefore, it can be concluded that the integrity of LEU was preserved during the SLN and NLC preparation.

2.2.4. Solid lipid-based nanoparticles characterization

2.2.4.1. Encapsulation efficiency and drug loading. The amount of peptide loaded in solid lipid-based nanoparticles was characterized regarding encapsulation efficiency (EE) and drug loading (DL), which calculation are respectively described in Eqs. (2) and (3). The non-encapsulated peptide was isolated from the solid lipid-based nanoparticles by centrifugation. 150 µL of formulation were mixed with 300 µL of MilliQ water in Eppendorfs equipped with 0.1 µm PVDF filters (Ultrafree®-MC VV, Merck Millipore). Then samples were centrifuged at 4 °C during 15 min at 12,000g. The filtrate was analyzed by HPLC according to the method described above (Section 2.2.2). To measure the total amount of LEU in the solid lipid-based nanoparticles, 500 µL of formulation were diluted in 500 µL of THF and vortexed to dissolve the solid lipid-based nanoparticles. 500 µL of this mixture were then diluted in a solution of methanol with 0.1% TFA to dissociate the complex. The final solution was filtered on 0.22 µm PVDF syringe filters and analyzed according to the HPLC method described above (Section 2.2.2). As different amounts of API were added in the systems, the loading capacity was introduced to compare the SLN and NLC, avoiding these initial differences. Its calculation takes into account the mass yield (Eq. (4)) and is described in Eq. (5).

Encapsulation efficiency calculation

Encapsulation Efficiency, %

$$= \frac{(\text{total amount of drug} - \text{unencapsulated amount of drug})}{(\text{total amount of drug})} \quad (2)$$

Drug loading calculation:

Drug loading, µg/mg

$$= \frac{\text{total amount of drug} - \text{unencapsulated amount of drug}}{\text{total solid content}} \quad (3)$$

Mass yield

$$\text{Mass yield} = \frac{\text{total final solid content}}{\text{total initial solid content}} \quad (4)$$

Loading capacity calculation

$$\text{Loading capacity, \%} = \text{EE} * \text{mass yield} \quad (5)$$

2.2.4.2. Size distribution and solid lipid-based nanoparticles morphology. Particle size and Polydispersity Index (PDI) of the solid lipid-based nanoparticles were measured at 25 °C on diluted samples via Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern). Solid lipid-based nanoparticles size was confirmed by cryogenic Transmission Electron Microscopy (cryo-TEM). To this end, formulations were diluted 10-fold to better analyze separate particles. 6 µL of diluted formulation were placed on a carbon grid (Quantifoil® R2/1 300 mesh copper grids) hydrophilized for 50 s. The grid was blotted between two paper filter and automatically plunged in liquid ethane using a cryo-plunge workstation (designed at LPS Orsay). The sample was then transferred to the cryo-Electron Microscope (Philips CM 120) using a precooled cryo-transfer holder (Gatan 626) observed at 120 kV. The grids were observed under low-dose conditions. Images were acquired via a Gatan Orius 200 camera.

2.2.4.3. Solid lipid-based nanoparticles stability. After production, formulations were stored in closed glass bottles at 4 °C. The visual aspect of the formulations as well as the EE were regularly assessed for up to two months.

2.2.5. In vitro evaluation of solid lipid-based nanoparticles

2.2.5.1. Impact of simulated gastrointestinal fluids on particle size stability. In order to evaluate the stability of NLC and SLN in both gastric and intestinal simulated fluids, particle size and PDI evolution were evaluated at 37 °C in Simulated Gastric Fluid (SGF), Fasted State Simulated Intestinal Fluid (FaSSIF-V2) and Fed State Simulated Intestinal Fluid (FeSSIF-V2) and compared with the values obtained in water.

SGF was prepared according to USP recommendations. SGF contained 0.2% (w/v) sodium chloride dissolved in 0.7% (v/v) hydrochloric acid aqueous solution to which 3.2 mg/mL pepsin were added. The final pH was 1.2. FaSSIF-V2 and FeSSIF-V2 were prepared according to Jantratid et al. recommendations (Jantratid et al., 2008). FaSSIF-V2 was prepared by dissolving 1.613 g/L sodium taurocholate, 0.151 g/L phosphatidylcholine, 2.220 g/L maleic acid, 1.182 sodium hydroxide and 4.010 g/L of sodium chloride water. The final pH of FaSSIF-V2 was adjusted to 6.5 with a 0.2 mM NaOH solution. FeSSIF-V2 was prepared by dissolving FeSSIF-V2 preparation to which were added 7.330 g/L sodium chloride, 6.390 g/L maleic acid and 3.270 g/L sodium hydroxide. The final pH was 5.8.

To evaluate the impact of the simulated gastrointestinal fluids on particle size, 300 µL of blank NLC or SLN were dispersed in 30 mL simulated fluids. The dispersions were placed in an incubator at 37 °C, under 80 rpm agitation. Samples were withdrawn every hour and directly analyzed by DLS on a NICOMP™ 380 ZLS (PSS.NICOMP, Santa Barbara, CA, USA). The study was conducted for 2 h in SGF and for 6 h in FaSSIF-V2 and FeSSIF-V2.

2.2.5.2. In vitro release studies. The release studies were conducted in FaSSIF-V2 on both NLC and SLN, containing either LEU or HIP. They were performed under gentle stirring (80 rpm), at 37 °C. Peptide release from these systems was studied by dispersing 1 mL of formulation in 10 mL of FaSSIF-V2. At predetermined time points, 600 µL aliquots were withdrawn and filtered on 0.1 µm PVDF filters (Ultrafree®-MC VV, Merck Millipore) via centrifugation at 12,000g during 15 min at 4 °C. The filtrates were immediately analyzed via HPLC according to the method described above (Section 2.2.2). The release studies were conducted for 6 h and sink conditions were respected.

2.2.5.3. Degradation by trypsin and α -chymotrypsin. Degradation studies were adapted from Hetényi et al. method developed for SEDDS (Hetényi et al., 2017). Enzyme solutions were prepared with a final concentration of 0.3 mg/mL for trypsin and 0.0006 mg/mL for α -chymotrypsin. The degradation studies were performed on samples with an overall peptide concentration of 100 μ g/mL (sum of encapsulated and unencapsulated LEU in the formulation). Then the volume of each formulation was adapted and completed with pH 6.8 Tris-HCl buffer to reach a final volume of 1 mL. 500 μ L of each enzyme solution were added to each formulation and placed in an incubator at 37 °C under agitation of 80 rpm. The enzymatic activity was stopped at predetermined time points by addition of 600 μ L of a solution of methanol + 0.1% TFA to 200 μ L withdrawn aliquots. The samples were then filtered on 0.1 μ m PVDF filters (Ultrafree[®]-MC VV, Merck Millipore) via centrifugation at 12,000g during 15 min at 4 °C and further analyzed by HPLC according to the method described above (Section 2.2.2). Controls were prepared following the same procedure but replacing the 500 μ L enzyme solution by 500 μ L ultra-pure water.

2.3. Statistical analysis

All experiments were performed in triplicates and are expressed as mean $\pm$ standard error of mean (SEM). The normal distribution of data was evaluated by a Shapiro-Wilk normality test. Then a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was conducted. Two-way ANOVA followed by Dunnett's post-hoc test was performed to statistically analyze data concerning particle size stability in simulated gastrointestinal fluids and leuprolide degradation by proteases. Data were treated and analyzed with the GraphPad Prism 8 program (CA, USA).

3. Results

3.1. Nanoparticles characterization

3.1.1. Encapsulation efficiency and drug loading

The technique of non-covalent lipidization described by Griesser et al. was reproduced and enabled the immediate formation of a leuprolide-docusate ion pair white precipitate. The precipitation efficiency obtained was 99.91 $\pm$ 0.07%.

LEU-sodium docusate HIP were encapsulated in NLC and SLN. The quantity of liquid C90 used was determined as the minimum volume required to dissolve the mass of HIP formed using 20 mg LEU. Neither LEU nor the HIP were soluble in PATO5.

EE and DL measurements of LEU in NLC and SLN were based on separation by ultracentrifugation method, which has been established as a method of choice for the separation of SLN around 100 nm (Lv et al., 2018). Results are shown in Table 2. When LEU was complexed with sodium docusate, the EE were very significantly increased, compared to neat LEU. These results were verified for both solid lipid-based nanoparticle types since the EE improved from 44% to 76% and 52% to 78% for NLC and SLN respectively. However, as the amounts of peptide introduced were not the same in the NLC and SLN, the loading capacities were calculated. It corresponded to the mass ratios between the final amount of peptide in the solid lipid-based nanoparticles and the

Table 2

EE, DL and Loading capacity of NLC and SLN containing HIP or LEU, (n = 3 $\pm$ SEM).

	LEU NLC	HIP NLC	LEU SLN	HIP SLN
EE (%)	44.3 $\pm$ 0.3	75.7 $\pm$ 1.2	53.7 $\pm$ 2.3	78.3 $\pm$ 0.7
DL (μ g/mg)	1.72 $\pm$ 0.03	2.51 $\pm$ 0.19	1.64 $\pm$ 0.29	2.36 $\pm$ 0.43
Loading capacity (%)	34 $\pm$ 1	64 $\pm$ 5	44 $\pm$ 6	59 $\pm$ 10

initial amount of peptide introduced in the lipid phase and provided more comparable data to evaluate formulations. Indeed, loading capacity was significantly different between NLC-LEU and NLC-HIP, in which the quantity of LEU was almost doubled. An important difference was also noted between SLN-LEU and SLN-HIP.

3.1.2. Size distribution and solid lipid-based nanoparticles morphology

The characteristics of HIP- and LEU-loaded solid lipid-based nanoparticles were compared to the blank ones. According to DLS measurements presented in Table 3, blank NLC and NLC were in the same size range, around 120 nm. Then, the addition of a small portion of liquid lipid to the solid lipid did not impact the size of the particles. Moreover, neither NLC nor SLN particle sizes were affected by the encapsulation of the peptide, either neat or in its complexed form, more sterically hindered. Concerning the size distribution, the systems presented PDI around 0.2 which is representative of a small polydispersity. These results were further confirmed by cryo-TEM measurements.

Cryo-TEM measurements enable a direct observation of solid lipid-based nanoparticles in the frozen-state and thus provide information regarding their structure. As displayed in Fig. 1, they appeared as deformed circular or ellipsoidal platelets in top-view, and darker rods edge-on. In the case of NLC these rods or "needle-like" structures presented a "sack" on one of their edges, shown by blue triangles on Fig. 1. SLN also presented these dark rods, without the oily compartments, highlighted by red rectangles in Fig. 1. The size of the objects observed by cryo-TEM investigations was in the same range as the one measured by light scattering technique.

3.1.3. Solid lipid-based nanoparticles stability

After 21-day storage, NLC-LEU formulation started to show instabilities with oily droplets visible at the surface of the aqueous phase. These instabilities were associated with important variations in the calculation of EE (Fig. 2). On the other hand, visual stability of NLC-HIP was prolonged of one week (total of 28 days), with less variability within the physical aspect of the formulation and an EE of 75% after 28 days (closed to the 76% measured after formulation production).

Surprisingly, the EE of SLN-HIP was found to increase over time. This was a consequence of a decrease of LEU concentration in the dispersing phase. Simultaneously, degradation peaks appeared in HPLC chromatographs. However, as the gradient used during HPLC analyses induced a change in baseline, these degradation peaks could not be integrated properly and allow the calculation of the effective EE.

SLN-LEU formulations were stable over 64 days even if the disparity between formulations was becoming more important over time. Once more, these disparities can be related to the instability of the unencapsulated peptide in solution. Leuprolide, like other gonadorelin analogs, can degrade in acidic aqueous solutions, which is the case of our formulations (measured pH = 5). The main degradation mechanism is a hydrolysis at the N-terminal site of the serine residue (Hall et al., 1999; Hoitink et al., 1997).

3.2. In-vitro evaluation of the solid lipid-based nanoparticles

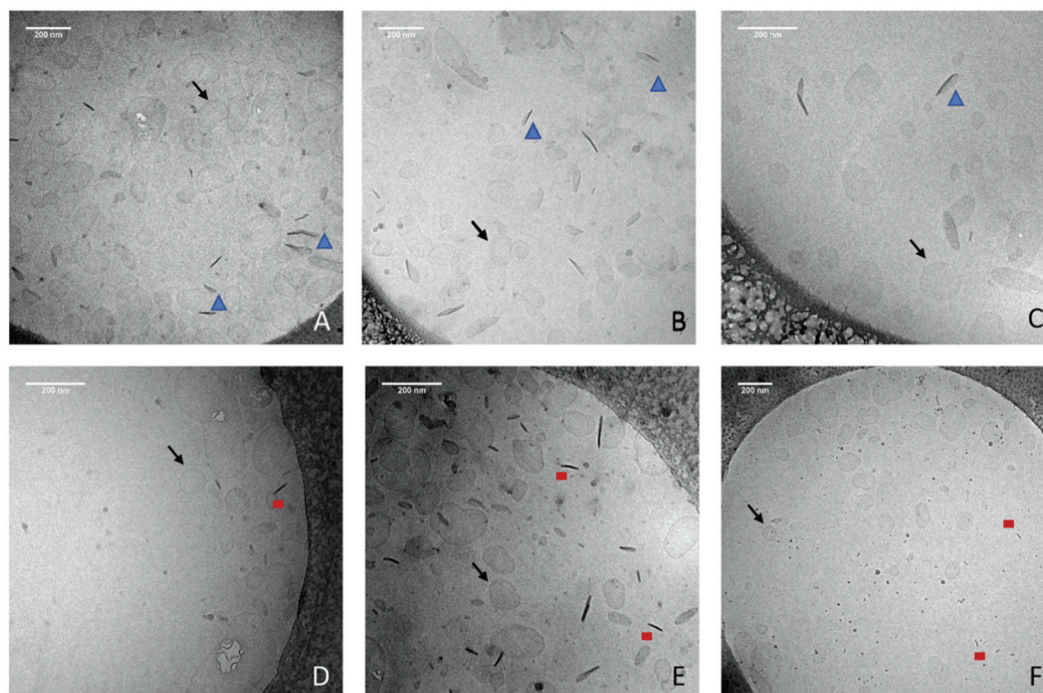
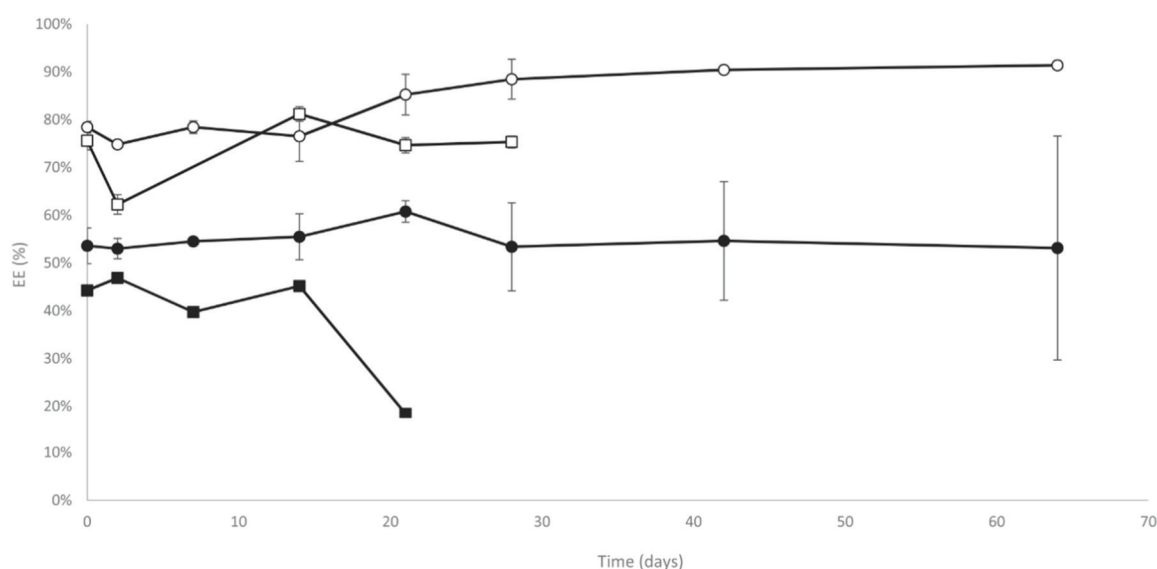
3.2.1. Impact of simulated intestinal fluids on particles size stability

Evaluation of solid lipid-based nanoparticles behavior in simulated gastrointestinal fluids provides valuable information to predict their fate *in vivo* and consequently the fate of the encapsulated molecules. Based on approximate transit time in the GI tract, solid lipid-based nanoparticles were incubated for 2 h in SGF and 6 h in FaSSIF-V2 and FeSSIF-V2. The results, presented in Fig. 3, showed that blank NLC and blank SLN were stable under gastric conditions. However, for both types of solid lipid-based nanoparticles, particle sizes were slightly, but significantly, increased right after dispersion in FaSSIF-V2 media. The size augmentation was conserved during the 6 h of incubation. Similarly, a small increased was visible immediately upon incubation in FeSSIF-V2. The size of the dispersion kept growing during the first hour

Table 3

Particle size distribution of the solid lipid-based nanoparticles measured by DLS at 25 °C (n = 3, N = 3), mean $\pm$ Standard Error of Mean (SEM).

	Blank NLC	NLC-LEU	NLC-HIP	Blank SLN	SLN-LEU	SLN-LEU
Z-average (nm)	114 $\pm$ 6	113 $\pm$ 1	125 $\pm$ 1	119 $\pm$ 2	124 $\pm$ 0.2	127 $\pm$ 0.4
PDI	0.16 $\pm$ 0.01	0.16 $\pm$ 0.01	0.21 $\pm$ 0.01	0.19 $\pm$ 0.01	0.16 $\pm$ 0.02	0.21 $\pm$ 0.01


Fig. 1. Cryo-TEM images of blank NLC (A), LEU loaded NLC (B), HIP loaded NLC (C), blank SLN (D), LEU loaded SLN (E) and HIP loaded SLN (F). Black arrows point at top-view particles, the blue triangles indicate edge-on “nanospoon” platelets, red rectangles indicate edge-on platelet. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. EE of LEU in NLC-HIP (□), NLC-LEU (■), SLN-HIP (○) and SLN-LEU (●) calculated over time. Data expressed as mean $\pm$ SEM (n = 3).

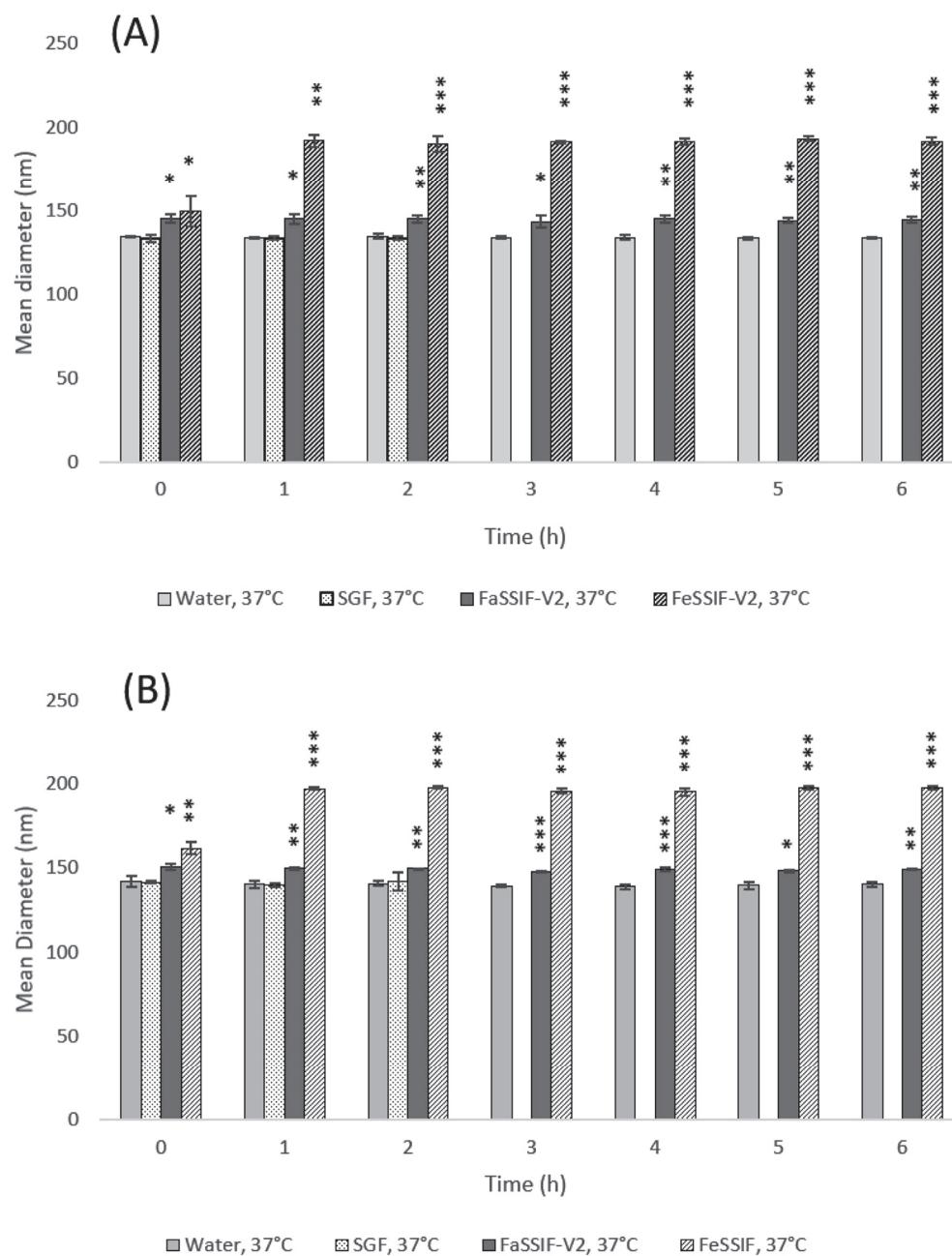


Fig. 3. Particle size evolution of solid lipid-based nanoparticles dispersed in simulated gastrointestinal fluids at 37 °C. (A) NLC and (B) SLN. Data expressed as mean $\pm$ SEM ($n = 3$) (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to solid lipid-based nanoparticles dispersed in water).

before stabilizing around 200 nm for both systems (40% increase compared to dispersion in water). According to DLVO aggregation theory, the interactions between particles is the sum of Van der Waals and Electrostatic Double Layer (EDL) forces. The latter partly depends on the composition of the media surrounding the particles. Indeed, an increase in ionic strength results in the compression of the EDL and, consequently, aggregation of the particles (Hotze et al., 2010). Thus, as FeSSIF-V2 presents a higher ionic strength than FaSSIF-V2, particle aggregation was more important in this media. However, as the mean

particle size distribution remained in the same size range, the interaction of particles with gastrointestinal media should be of limited importance in the release of the peptide.

3.3. *In vitro* released studies

Peptide release studies from NLC and SLN were performed in FaSSIF-V2 over 6 h. The results, expressed as weight percentage of LEU released, showed an important burst effect as soon as the solid lipid-

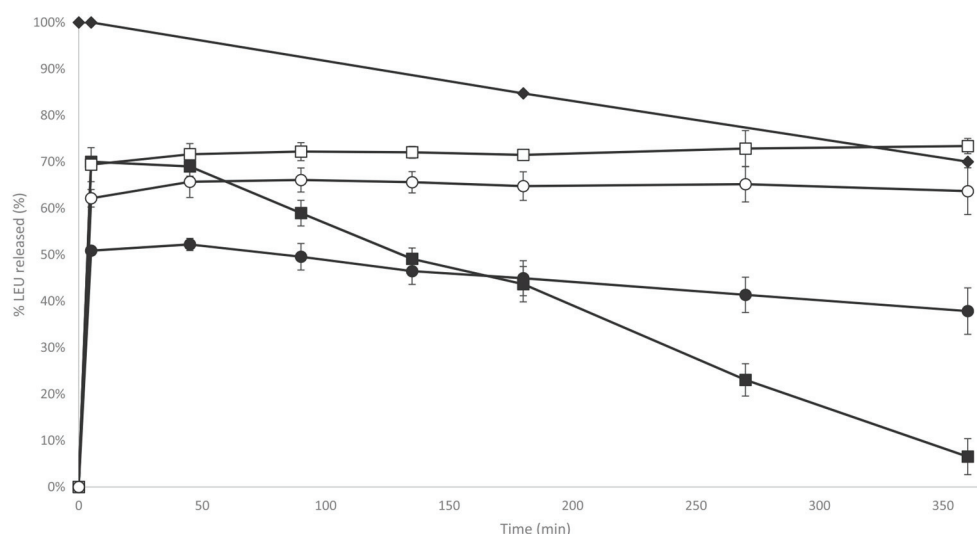


Fig. 4. Release profiles of LEU from NLC LEU (■), NLC HIP (□), SLN LEU (●) and SLN HIP (○), compared to free LEU (◆) in FaSSIF-V2.

based nanoparticles were introduced in the medium, Fig. 4. However, the release was limited to this first step as no further increase of peptide concentration was observed in FaSSIF-V2. In the case of solid lipid-based nanoparticles containing neat peptide, as well as for LEU control solution, an important decrease of the concentration of LEU was observed. Indeed, for these samples, degradation peaks were visible in the HPLC-chromatographs. By integration and subtraction of these degradation peaks to the total concentration of peptides present in the media, the real EE value could be calculated. It was visible that the amount of encapsulated peptide remained stable in all formulations after the initial burst release (Fig. S11). Degradation peaks were only observed when LEU was not complexed. Then, it can be supposed that the presence of sodium docusate provided a protective effect to LEU.

The relative amount of peptide released was similar for both NLC systems, around 70%, while a more important difference was noticeable between SLN-HIP (65%) and SLN-LEU (50% released). However, when data were reported as D_L , it appeared that HIP enabled to maintain more peptide within the solid lipid-based nanoparticles with a final D_L of 0.66 $\mu\text{g}/\text{mg}$ in NLC and 1.03 $\mu\text{g}/\text{mg}$ in SLN while it was 0.55 $\mu\text{g}/\text{mg}$ and 0.74 $\mu\text{g}/\text{mg}$ respectively for neat LEU, (Fig. S12).

3.3.1. Degradation by trypsin and α -chymotrypsin

Degradation profiles of LEU by trypsin and chymotrypsin were reported in Figs. 5 and 6. Samples were prepared in separated containers and represented as scatter charts. The dotted lines are just guides for the eyes to facilitate graphical reading. The formulations and LEU control solution were introduced in a mixture of pH 6.8 Tris-HCl buffer and enzymatic solution for 180 min. However, in most cases, the quantity of remaining LEU measured was inferior to the LLOQ after 10 min, explaining the absence of results in the Figures.

LEU was rapidly degraded by trypsin: 16% were remaining after 90 min of incubation while it was completely degraded after 120 min. However, when it was encapsulated into NLC, a non-significant difference was observed, with a remaining quantity of LEU of 23% after 90 min of incubation and 8% after 150 min. Furthermore, when the peptide was encapsulated in its complexed form, the presence of LEU in the system was even more prolonged with 8% still present after 180 min of incubation, providing a very significant difference of protection compared to the control. However, in the case of α -chymotrypsin, both NLC systems were inefficient in protecting LEU from the enzymatic degradation.

Concerning SLN, it can be noticed that for both tested proteases, the degradation curves were below the control. Then, SLN did not provide any protection to the peptide towards trypsin and α -chymotrypsin.

4. Discussion

4.1. Hydrophobic ion pairing is the main driver for high peptide payload in solid lipid-based nanoparticles

LEU is a nonapeptide used to treat various hormonal diseases. As the hydrophilic character of LEU might limit its encapsulation in lipid-based nanocarriers, LEU lipophilicity was increased by formation of a HIP, a non-covalent lipidization process. This technique is based on the formation of electrostatic interactions between ionized peptides at acidic pH and anionic surfactants or polymers. It is reversible and guarantees the conservation of peptide therapeutic activity. In the case of LEU, the electrostatic interactions are formed between the guanidine group of arginine ($pK_a = 13$) and the imidazole group of histidine ($pK_a = 6$). Griesser et al. have shown that the formation of a HIP using sodium docusate as anionic surfactant showed a precipitation efficiency of 99.1% with an increase of water/octanol partition coefficient ($\log P$) from -2 to 3 (Griesser et al., 2017). In the present study, the precipitation efficiency was of 99.9% using sodium docusate as counterion. The encapsulation of LEU in NLC and SLN was significantly increased after LEU complexation. Neat LEU was encapsulated in NLC with an EE of 44% (1.72 $\mu\text{g}/\text{mg}$) and 52% in SLN (1.64 $\mu\text{g}/\text{mg}$) and HIP was encapsulated in NLC with an EE of 76% (2.51 $\mu\text{g}/\text{mg}$) and of 78% in SLN (2.36 $\mu\text{g}/\text{mg}$). Then, the combination of solvent-free, high-pressure process and formation of peptide-anionic surfactant HIP enabled the formation of solid lipid-based nanoparticles with high EE. The chosen process provided a high level of inter and intra batch reproducibility, verified both by EE and size dispersion results.

NLC were formulated to increase HIP stability by avoiding drug expulsion upon lipids reorganization. However, an opposite trend was observed with relatively rapid exudation of the liquid oily excipient in which the drug was dissolved. Then, the proportion of liquid excipient might have been too high and disrupted the initial stability of the SLN. Furthermore, the inclusion of liquid excipient did not provide significant difference in terms of solid lipid-based nanoparticles loading capacity. Then, despite absence of solubilizing capacity the dispersion of HIP in the solid matrix was efficient to encapsulate LEU.

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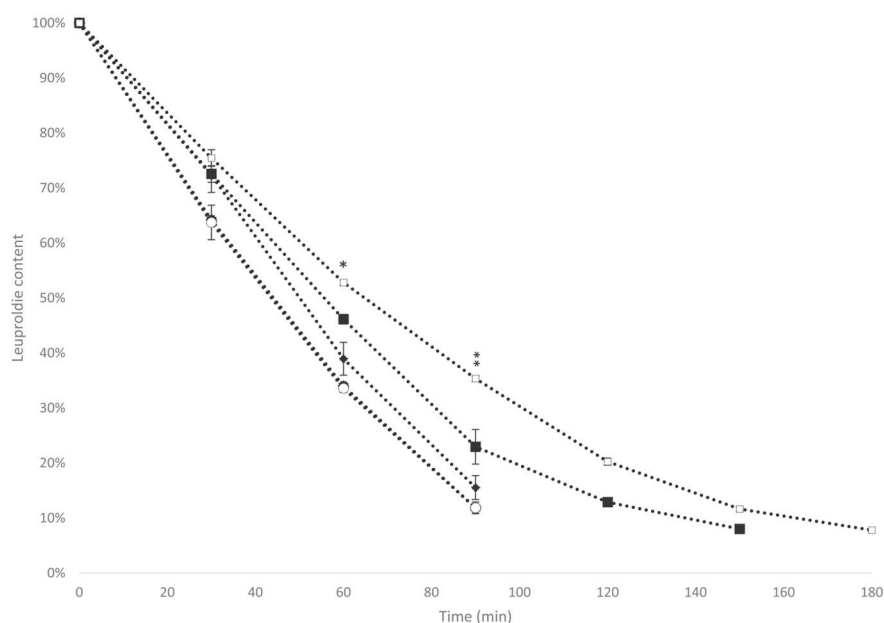


Fig. 5. Degradation profile of LEU from NLC LEU (■), NLC HIP (□), SLN LEU (●) and SLN HIP (○) by trypsin, compared to free LEU (◆). Data expressed as mean $\pm$ SEM (n = 3) (*p < 0.05 and **p < 0.01 when compared to free LEU solution).

4.2. Solid lipid-based nanoparticles' platelet shape is a disadvantage to efficient peptide protection

NLC and SLN were formed by melt dispersion. This technique consisted in the formation of an emulsion at temperature above lipid excipient melting temperature. The emulsion oily droplets were reduced to reach the nanosize range before formation of the solid dispersion by placing the nanoemulsion in a cold bath under stirring. Nevertheless, although solid lipid-based nanoparticles were formed from oil nanodroplets, the final particles presented a platelet shape with a "sack" on one edge of the NLC.

These arrangements have already been described and can be associated to the presence of liquid oil, C90, compartments at the surface of the platelets (Bunjes et al., 2001; Esposito et al., 2013; Jores et al., 2004; Kuntsche et al., 2011). This platelet shape was attributed to inherent polymorphic behavior of lipids which tend to rapidly reorganize from the α organization to the more anisometric and more stable β -structure (Bunjes et al., 2003; Bunjes, 2011). Contrary to spherical particles, these platelets structures present a more important surface to volume ratio. Consequently, the probability of having API located at the surface of the particle rather than encapsulated in its core is increased, resulting in the important burst release observed in FaSSiF-V2. This

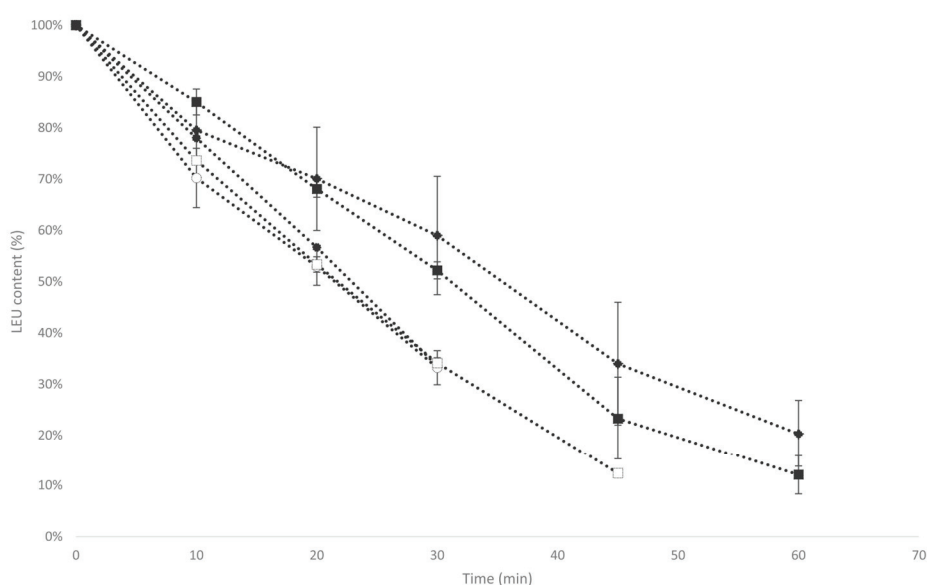


Fig. 6. Degradation profile of LEU from NLC LEU (■), NLC HIP (□), SLN LEU (●) and SLN HIP (○) by α -chymotrypsin compared to free LEU (◆). Data expressed as mean $\pm$ SEM (n = 3).

initial strong release rate was frequently reported in the case of nanoparticles containing peptides (Battaglia et al., 2007; Garcia-Fuentes et al., 2005b,a; Hu et al., 2004; Shrestha et al., 2018; Yuan et al., 2009). It can be attributed to the presence of bile salts and phospholipids in FaSSIF-V2 which can easily enhance the dissolution of the peptide present at the surface of the particles in the aqueous phase.

Compared to SLN the morphology of NLC was slightly modified by the inclusion of a liquid lipid excipient, Fig. 1. It seems that the major part of this liquid fraction was located at the surface of the particles. Consequently, the volume, but also the surface, of the particles was extended. As in the case of NLC HIP, HIP was first dispersed in C90, the API was probably mostly located at the surface of the structure and justifies the more important release measured in the case of NLC.

Thus, if encapsulation in NLC is linked with an increased release of the peptide in FaSSIF-V2, it can be postulated that its degradation will be more important in presence of proteases: the protective effect sought will only concern the small quantity of LEU remaining inside the small inner core of the particle.

However, the dispersion of the solid lipid-based nanoparticles in presence of trypsin have shown opposite results. Another phenomenon should then be considered to explain the results.

4.3. Liquid lipid environment is key for peptide protection towards enzymatic degradation

During the evaluation of the protective effect of the solid lipid-based nanoparticles towards LEU degradation by trypsin, NLC have demonstrated the ability to prolong LEU-HIP integrity. On the contrary, SLN provided no protection at all to LEU. As the only difference between these formulations lies in the inclusion of C90, the addition of this liquid excipient might be the key to an effective peptide protection. Several studies have demonstrated the efficiency of SEDDS to protect encapsulated peptides from trypsin, α -chymotrypsin, elastase and thiol-disulfide exchange by reduced glutathione (Hetényi et al., 2017; Hintzen et al., 2014; Karamanidou et al., 2015; Leonaviciute et al., 2016; Zupančič et al., 2016). Indeed, the lack of solubility of these enzymes in a lipophilic environment prevents the degradation of biomolecules in the core of the emulsions (Hetényi et al., 2017). However, very few studies report the efficiency of SLN to protect encapsulated peptides (Battaglia et al., 2007; Trotta et al., 2005). Furthermore, to our knowledge, no studies concerning the use of NLC for this purpose have been published. Contrary to emulsions composed of a continuous liquid phase, these NLC and SLN are mostly made of lipids solid at room and body temperature. This difference may impact the solubility of intestinal proteases inside the carriers. Indeed, unless if the particles are composed of pure lipids forming perfect crystalline arrangements, most lipid excipients used for their formulations are complex mixtures of mono-, di- and triglycerides leading to more imperfect structures (Müller et al., 2000). Then, the formation of an inner porous network can be considered, with the associated risk of facilitating the penetration of proteases inside the particles and potential hydrolysis of encapsulated peptides. Nevertheless, in the case of NLC, the inclusion of a liquid lipid should fill the capillaries induced by the solid lipid crystallization.

However, in the case of α -chymotrypsin, NLC have been found to be as inefficient as SLN to protect LEU from the enzymatic degradation.

4.4. Activity of trypsin and α -chymotrypsin was boosted in presence of solid-phase lipids

Trypsin and α -chymotrypsin, like many other proteases or esterases (e.g. lipases), are industrially used to catalyze chemical reactions such as esterifications or transesterifications (Cao et al., 2016). To increase their activity, their stability and to make them reusable, these enzymes are generally immobilized on porous substrates.

As put forward above, the crystallization of PATO5 by cooling the

solid lipid-based nanoparticles may have led to the formation of a porous structure within the particles. Then, it offered the possibility for proteases to be absorbed on the surface of the particles. As the porosity should be less important in NLC in which the liquid lipid must fill the formed capillaries, the adsorption of enzymes might be reduced at the surface of these particles. This hypothesis corroborates the enhanced degradation of LEU-HIP by trypsin in SLN while a protective effect was observed in the case of NLC. Indeed, the probable porous structure of SLN, enabled the docking of trypsin at the surface of the particles or within the pores, depending on their size. Thus, the hydrolysis of the peptide is made easier by closer proximity between the enzyme and the substrate. On the contrary, the reduction of porosity combined with the insolubility of trypsin in C90 might reduce the enzyme immobilization process and can explain the protective effect of NLC on LEU-HIP towards trypsin degradation.

In the case of α -chymotrypsin, none of the solid lipid-based nanoparticles were able to provide LEU protection. On the contrary, it appeared that LEU was less degraded when it remained free. The only possible explanation is an interaction of the protease with the particles. Indeed, several studies were found in which α -chymotrypsin is used with PEG to increase its activity (Calderón and Lissi, 2013; Castellanos et al., 2005; Chiu et al., 1993). Then a possible interaction between α -chymotrypsin with the PEG moieties of the surfactant RH40 can be considered to explain the increased activity of the proteases in presence of the solid lipid-based nanoparticles. Furthermore, the stimulation of the enzymatic activity of pancreatic enzymes in presence of lipid-based excipients (Gelucire® 44/14, a PEG-based surfactant) has been patented in 2014 (Shlieout, 2014).

5. Conclusion

Formation of HIP led to an increase in peptide encapsulation in solid lipid-based nanoparticles. Surprisingly, the inclusion of a liquid lipid fraction did not improve the EE. NLC and SLN presented a similar size and morphology that was not beneficial for peptide protection. Indeed, both systems presented platelet-like shapes. These structures possess large exchange surface. Then, the encapsulated peptides were easily released in the dispersing simulated GI media and rapidly degraded in presence of proteases. However, the presence of a liquid lipid fraction impeded significantly the degradation of the peptide by the enzymatic degradation induced by trypsin. Then, it can be supposed that increasing this proportion of liquid excipient should lead to increased protective effect, but it might simultaneously destabilize the structure of the particles and cause faster solid/liquid partition.

It was recently shown that SEDDS carriers with size inferior to 200 nm were able to rapidly cross the mucus barrier (Griesser et al., 2018). On the other side of this natural physical border the protease activity is far less important than in the intestinal lumen. Then, permeation studies on these particles should be conducted. Indeed, it is the competition between protease degradation and mucus diffusion kinetics that will be the key to peptide absorption.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2019.05.037>.

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Chapitre IV :

Evaluation de la capacité des nanovecteurs lipidiques solides
à augmenter le passage intestinal des peptides

Ce chapitre présente l'étude menée pour évaluer la capacité des nanovecteurs lipidiques solides, à savoir les nanoparticules lipidiques solides (SLN) et les vecteurs lipidiques nanostructurés (NLC), à augmenter le passage intestinal des peptides encapsulés. La barrière intestinale est en effet un des principaux freins à leur absorption systémique. Celle-ci est composée de deux éléments pouvant chacun limiter voire empêcher le passage des peptides : le mucus et l'épithélium intestinal. Le premier, composé de mucines et de glycocalyx sécrétés par les cellules caliciformes, forme un gel recouvrant la totalité de l'épithélium. Chargé négativement il peut interagir avec des molécules, dont les peptides, et bloquer leur passage. L'épithélium intestinal est composé en très grande majorité d'entérocytes. Ces cellules, par leur membrane constituée d'une bicouche phospholipidique, bloquent le passage transcellulaire des molécules hydrophiles. Le passage des peptides par voie paracellulaire *via* les jonctions serrées séparant les entérocytes est quant à lui limité par leur importante masse moléculaire. Dans le cadre de cette étude, les cellules Caco-2 ont été utilisées pour modéliser le comportement des entérocytes dont elles présentent les principales caractéristiques. Afin de modéliser la présence de mucus, les cellules Caco-2 ont été co-cultivées avec des cellules caliciformes HT29-MTX, capables de sécréter des mucines.

Le leuprolide (LEU) a été choisi comme peptide modèle dans le cadre de cette étude. Afin d'accroître son taux d'encapsulation dans les SLN et les NLC, sa lipophilie a été augmentée par complexation avec le docusate de sodium *via* la formation d'une paire d'ions hydrophobe (HIP) par interaction électrostatique. Cette HIP a été obtenue avec un taux de précipitation de 99,9%. Une dose de 29 mg, soient 16 mg de LEU a été utilisée pour formuler les SLN et les NLC par homogénéisation à haute pression. Les particules obtenues mesuraient entre 105 et 125 nm, avec un PDI inférieur à 0.2. Des taux d'encapsulation similaires de 84% (NLC) et 89% (SLN) ont été mesurés. Dans le but d'observer les interactions entre les nanoparticules et les cultures cellulaires, les HIP ont été co-encapsulées avec du DiD un fluorophore lipophile (DiD, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, sel de 4-chlorobenzenesulfonate) (Log P=10,2). Cette co-encapsulation n'a pas eu d'incidence sur les propriétés des nanoparticules, que ce soit sur la taille, le PDI ou le taux d'encapsulation.

La cytotoxicité des nanoparticules blanches et chargées en HIP a été évaluée *via* un test MTT sur les 2 modèles cellulaires. Bien que les mêmes concentrations en nanoparticules aient été

utilisées, seules les NLC ont démontré un effet cytotoxique sur les cellules avec une légère augmentation de l'IC₅₀ lorsque les particules étaient chargées en actif.

Les interactions entre les nanoparticules et les modèles cellulaires ont été évaluées de manière qualitative par microscopie confocale en fluorescence (CSLM) et quantitative par cytométrie en flux. Pour l'analyse qualitative, les nanoparticules chargées d'HIP et de DiD, ont été incubées pendant 2 heures avec les monocouches de Caco-2 ou de Caco-2/HT29-MTX cultivées sur inserts. Les monocouches ont ensuite été rincées, fixées et teintées avant observation. Les images obtenues par CSLM ont permis de visualiser l'internalisation des nanoparticules dans les deux modèles cellulaires, mettant en avant leur capacité à passer la bicouche phospholipidique et la barrière de mucus. Les analyses de cytométrie en flux ont été conduites uniquement avec les Caco-2. Les analyses, conduites après avoir incubé les nanoparticules avec les cellules pendant 2 heures, ont montré une très forte internalisation des SLN (82%) et des NLC (98.8%).

L'évaluation du transport des nanoparticules a été menée sur les monocouches de Caco-2 et de Caco-2/HT29-MTX cultivées sur des inserts en utilisant le HBSS comme milieu de transport. Dans le cas des co-cultures, la présence de mucus a été attestée par coloration des mucines par du bleu alcian. L'intégrité des monocouches a été vérifiée avant incubation des formules dilués par mesure de la résistance électrique transépithéliale. Les nanoparticules ou le LEU ont été dilués dans du HBSS puis incubés sur la face apicale des monocouches de cellules. Après 2 heures d'incubation, le HBSS du côté basolatéral des monocouches cellulaires a été collecté afin de doser la quantité de LEU transportée. Malheureusement, alors qu'une forte internalisation des particules avait été mise en avant, le passage du peptide s'est révélé très faible avec seulement 0.3% de la dose incubée quantifiée côté basolatéral. De plus, aucune différence statistique n'a pu être mise en avant entre le transport du LEU en solution et du LEU encapsulé, que ce soit avec les Caco-2 ou les co-cultures Caco-2/HT29-MTX.

Ces résultats ont pu être justifiés après étude du comportement des formulations dans le milieu de transport. En effet, bien que la taille des nanoparticules soit stable dans le HBSS, un relargage quasi instantané de près de 90% de l'actif a été mesuré lors de la dispersion des nanoparticules dans ce milieu. Ce phénomène s'explique par la forme en plaquette des nanoparticules. En effet ces structures présentent une importante surface d'échange avec le

milieu, facilitant la libération de l'actif, principalement localisé en surface des particules. De plus, il a récemment été montré que l'HIP LEU-docusate était instable en milieu de force ionique élevée, tel que le HBSS (150 mM).

En conclusion, il convient d'améliorer la stabilité l'HIP pour maintenir le peptide encapsulé dans les nanovecteurs lipidiques solides lors de leur dispersion dans les milieux biologiques. Ainsi il pourrait bénéficier de leur capacité à franchir la barrière de mucus et à être internalisés par les entérocytes et par conséquent potentiellement augmenter leur biodisponibilité orale.

Evaluation of solid lipid nanocarriers ability to improve intestinal permeability of lipidized peptide using cell-based models

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I. Abstract

Context: Peptides are a class of therapeutic molecules with high potential to treat a wide variety of diseases with few adverse effects. Most of them are parenterally administered due to poor oral bioavailability. As a matter of fact, most peptides are large hydrophilic molecules which passage across the intestinal border is limited by the epithelial border covered by mucus.

Objective: The aim of this study was to evaluate the potential of solid lipid nanocarriers, namely Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC), to improve peptide transport across the intestinal border using Caco-2 cell monolayers (enterocyte-like model) and Caco-2/HT29-MTX co-cultured monolayers (mucin-secreting model).

Methods: The lipophilicity of Leuprolide (LEU), a linear hydrophilic model peptide, was increased by formation of a Hydrophobic Ion Pair (HIP). The lipidized peptide was encapsulated in SLN and NLC and the nanoparticles were characterized regarding their size, polydispersity index (PDI), encapsulation efficiency (EE) and drug loading. The abilities of the particles to increase the transport of LEU was evaluated on Caco-2 cell monolayers. The effect of mucus was evaluated using Caco-2/HT29-MTX co-culture monolayers. Interactions of the nanoparticles (containing HIP and a fluorescent dye) with the cells was assessed qualitatively by confocal microscopy (CSLM) and quantitatively by flow cytometry (FACS).

Results: SLN and NLC measuring between 105 and 125 nm were obtained with a PDI of 0.2. LEU was successfully complexed (precipitation efficiency = 99.9%) and encapsulated in nanoparticles. Both systems presented similar EE (84% for NLC and 85% for SLN). SLN and NLC were internalized by Caco-2 cell monolayers but also by Caco-2/HT29-MTX co-cultured monolayers, demonstrating their ability to cross mucus. These observations were confirmed by flow cytometry indicating that 82% of the incubated SLN and 99% of the NLC were internalized by Caco-2.

However, encapsulation of LEU in SLN and NLC has shown no improvement of peptide permeability. Indeed, a burst release of LEU from the nanoparticles was measured in the transport medium, limiting the potential benefits of the nanocarriers. This high release was imputed to the combination of the platelet-shape morphology of the nanocarriers, which implies a large surface of exchange with the surrounding medium, and the low stability of the HIP in the transport medium.

Conclusion: The nanoparticles were highly internalized. Then, the stability of the HIP needs to be improved to maintain the peptide in the nanocarrier and enhance its passage across the intestinal barrier.

II. Introduction

Interest in the use of peptides as therapeutic molecules has been growing over the last decades. This is particularly due to their specific binding capacity with the *in vivo* target, enabling high potency with few adverse effects. Currently, most of these molecules are parenterally administered to reach acceptable levels of efficacy. Although oral administration would result in higher patient compliance and reduced industrial costs, peptides exhibit poor oral bioavailability (generally inferior to 1%) (Brayden and O'Mahony, 1998). Indeed, upon oral administration, peptides encounter a succession of barriers limiting their possibility to reach the systemic circulation. The first barrier is a chemical barrier: peptides undergo enzymatic degradation initiated by proteases which degrade macromolecules into absorbable nutrients. The second barrier corresponds to the mucus layer. Mucus is produced by goblet cells and is about 100 to 200 μm thick in human adults. This negatively charged gel layer can interact with biomolecules, blocking them at its surface, and prevent their passage (Leonaviciute and Bernkop-Schnürch, 2015). The third barrier, the epithelial layer, is mainly composed of enterocytes (~99%), separated by tight junctions. The passage of peptides across this cell layer is hindered for two reasons: their hydrophilic character prevents their transcellular permeation across the lipophilic plasma membrane of the cells, and their important size limits the paracellular pathway through tight junctions whose diameter is evaluated at 0.52 nm in human duodenum (Li et al., 2012).

Several solutions are currently evaluated to enhance peptide oral bioavailability: utilization of permeation enhancers, administration of proteases inhibitors, use of mucoadhesive systems, modification of peptides properties by chemical conjugation or encapsulation of peptides in nanocarriers (Bernkop-Schnürch, 1998; Brooks et al., 2005; Gupta et al., 2013; Morishita and Peppas, 2006). Lipid-based solid nanocarriers, composed of biocompatible and biodegradable excipients seems a promising and safe answer to address peptides oral limitations (Almeida et al., 1997; Dumont et al., 2018; Geszke-Moritz and Moritz, 2016; Li et al., 2012). These compounds can either refer to Solid Lipid Nanosuspensions (SLN), composed of lipids solid at room and body temperature, or Nanostructured Lipid Carriers (NLC), in which the solid lipids are supplemented with liquid lipids. Nevertheless, to optimize the drug loading of hydrophilic peptides in lipid-based nanocarriers, it is recommended to increase their lipophilicity. The formation of Hydrophilic Ion Pairs (HIP) by complexation with a counterion (e.g. an anionic

surfactant in the case of peptides with positively charged residues) enables a reversible increase in lipophilicity without altering the structure of the therapeutic drug. This technique was used to increase the lipophilicity of leuprolide (LEU), a hydrophilic nonapeptide, from a log P of -2 to +3, by complexation with sodium docusate in a 1:2 peptide:surfactant molar ratio (Griesser et al., 2017). The technique was reproduced and enabled a significant increase in Encapsulation Efficiency (EE) of LEU in SLN and NLC (Dumont et al., 2019). The nanosuspensions were obtained by hot High Pressure Homogenization (HPH), a scalable and solvent-free technique (Dumont et al., 2018). The nanoparticles were neutral, platelet-shaped, measured around 100 nm and have shown a protective effect towards trypsin-induced degradation *in vitro*.

The present study aims to evaluate if the designed nanoparticles are able to overcome the second and third barriers to oral peptide delivery leading to low permeability across the intestinal barrier. The platelet structure of the nanoparticles should be an advantage to increase their passage across the intestinal epithelium. Indeed, in the case of polymeric nanoparticles, absorption was higher for platelet-shape nanoparticles compared to spherical ones (Banerjee et al., 2016; Doshi Nishit and Mitragotri Samir, 2010). Furthermore, some anionic surfactants have been shown to increase the passage of molecules by fluidization of the enterocytes membranes (Anderberg et al., 1992). It was the case of sodium docusate which was used as counter-ion for the formation of the LEU-based HIP in the current study.

Intestinal cell models were used to evaluate the intestinal passage of the formulations. Caco-2 cells are widely used as enterocyte-like model in *in-vitro* intestinal permeability. Originally obtained from human epithelial colorectal adenocarcinoma cells, these cells are widely used as they spontaneously differentiate in human enterocytes, exhibiting tight junctions, microvilli on the apical surface and small intestine hydrolase activities (Sambuy et al., 2005). Mucin-secreting intestinal cell models were developed to better mimic the intestinal border, using co-cultures of Caco-2 and HT29-MTX (goblet cells) (Béduneau et al., 2014; Chen et al., 2010). The mucus layer present at the apical side of the coculture allows a more biorelevant use of the cell-based membrane. Indeed, this coculture can be used with biorelevant media, pancreatic enzyme (Antoine et al., 2015) and lipid-based excipients exhibiting surfactant properties (Dubray et al., 2016). An even more complex follicle associated epithelium model can be used to evaluate the influence of M cells in the transport, using a triple culture of Caco-

2, HT29-MTX and Raji cells (Antunes et al., 2013; Beloqui et al., 2017; Lozoya-Agullo et al., 2017). However, two recent studies have recently demonstrated that M-cells do not influence the transport of lipid nanocarriers (Beloqui et al., 2013; Memvanga et al., 2013).

In this study, LEU was encapsulated by HPH in NLC and SLN after formation of a HIP with sodium docusate. The nanoparticles were characterized regarding their size, polydispersity index (PDI), EE and drug loading (D_L). The ability of these lipid nanosuspensions to transport LEU across the mucus layer and cell-based membrane was evaluated using either Caco-2 cell monolayers or the Caco-2/HT29-MTX mucin-secreting model. The transport capacity of NLC and SLN was compared as well as their internalization by the cells.

III. Material and methods

III.1. Materials

Leuprolide acetate was obtained from Glentham Life Sciences (LEU, Corsham, UK). Sodium docusate, used for the preparation of the HIP, was purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France).

Nanoparticles were formulated with Precirol® ATO 5 (glyceryl distearate, PATO5, Gattefossé), Capryol® 90 (propylene glycol monocaprylate, C90, Gattefossé) and Kolliphor® RH40 (PEG-40 hydrogenated castor oil, RH40, Sigma Aldrich).

1,1'-Diocadecyl-3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD, Invitrogen) and rhodamine-phalloidin (Invitrogen) were purchased from Thermofisher. Vectashield mounting medium was obtained from Labconsult. Alcian Blue 8GX, Thiozol Blue Tetrazolium Bromide and Triton X-100 were obtained from Sigma Aldrich. Matrigel™ was purchased to BD Bioscience.

The Caco-2 cells lines were kindly provided by Dr Maria Rescigno (University of Milano-Bicocca, Milano, Italy) and used from passages X+25 to 30. The HT29-MTX cell lines were kindly donated by Dr. Thécla Lesuffleur from INSERM UMR S 938 in Paris, France, and used between passages X+50 to 62. Dulbecco's Modified Eagle Medium (DMEM, ref 41965-039), Dulbecco's Phosphate Buffered Saline (DPBS, ref 14190-094), Hank's Balanced Salt Solution (HBSS, ref

14175-053), Minimum Essential Medium Non-Essential Amino Acids (MEM NEAA), L-glutamine and Penicillin-Streptomycin were purchased from Gibco.

Fetal Bovine Serum (FBS) HyClone™ purchased from GE Healthcare

Leuprolide ELISA kit (S-1144) was obtained from BMA Biomedicals.

Trifluoroacetic acid (TFA) was obtained from Sigma Aldrich.

Tetrahydrofuran (THF) and Acetonitrile used were in the HPLC grade.

III.2. Methods

III.2.1. HPLC analysis

LEU was quantified using an Alliance 2695D (Waters, Saint-Quentin-en-Yvelines, France) equipped with a UV detector (2487, Waters). Separation was made on Kinetex EVO C18 100 Å 2.6 µm 50x4.6 mm column (Phenomenex, Le Pecq, France) at 40°C. A linear gradient of elution was applied from 15% solvent A/85% solvent B to 50% solvent A/50% solvent B (solvent A: water with 0.1% (v/v) TFA / solvent B: acetonitrile). The flow rate was 0.7 mL/min, the injection volume was 20 µL and the wavelength of the detector was set at 217 nm. The calibration curve was established between 10 and 100 µg/mL and the Lower Limit of Quantification (LLOQ) was evaluated at 1 µg/mL.

III.2.2. Formation of HIP

The method used for the formation of the leuprolide-docusate HIP (molar ratio 1:2 LEU:docusate) has been previously described (Dumont et al., 2019). Briefly, 20 mg of LEU were ionized in 1 mL of 0.01 M HCl. Then, 1 mL of a 14 mg/mL sodium docusate solution was added to LEU solutions. The immediate formation of a white precipitate confirmed the formation of the ion pair. The complexes were centrifuged for 10 minutes at 15000 g, 4°C on a Universal Centrifuge 320 R (Hettich). The supernatants were collected and analyzed *via* HPLC (see III.2.1) to quantify the amount of LEU remaining free. The results were used to calculate the precipitation efficiency, Equation 2.

The HIP were frozen in liquid nitrogen, lyophilized and stored at -20°C until further use.

Equation 2: Calculation of precipitation efficiency of HIP formation

$$\text{Precipitation efficiency, \%} = 100 - \left(100 \times \frac{[LEU]_{\text{supernatant}}}{[LEU]_{\text{initial}}}\right)$$

III.2.3. NLC and SLN formulation

A HPH technique was used to prepare both type of nanoparticles, as previously described by (Dumont et al., 2019). The composition of the nanoparticles is given in Table 1. PATO5 and DiD (when specified), were melted under magnetic stirring at 70°C in a water bath. The HIP was added to the melted lipid phase either as a solid, for SLN formulations, or dissolved in C90 for NLC formulations. The lipid phase was let to homogenize under 400 rpm magnetic stirring for 10 minutes. The aqueous phase containing RH40, also heated at 70°C, was added to the mixture and homogenized for 3 minutes at 11 000 rpm (Ultra-Turrax® T18, IKA). Next, the formed O/W emulsion was introduced in the high pressure homogenizer (Microfluidizer® LM20, Microfluidics) and submitted to 5 cycles at 500 bar. The nanoemulsion was then collected and placed for 10 minutes at 4°C in an ice/water bath under 400 rpm to form the solid nanoparticles. The nanosuspensions were stored at 4°C.

Table 1: Nanoparticles composition

	NLC-HIP	NLC-HIP-DiD	SLN-HIP	SLN-HIP-DiD
PATO5	4.00 g	4.00 g	4.00 g	4.00 g
C90	300 µL	300 µL	-	-
RH40	1.60 g	1.60 g	1.60 g	1.60 g
HIP	29 mg	29 mg	29 mg	29 mg
(LEU)	(16 mg)	(16 mg)	(16 mg)	(16 mg)
DiD	-	1 mg	-	1 mg

III.3. Nanoparticles characterization

III.3.1. Particles size

The particle size distribution and PDI of the nanoparticles were measured *via* Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern) at 25°C on samples diluted in water, one droplet in 2 mL (dynamic viscosity: 8.90×10^{-4} Pa.s). Results are expressed as mean Z-average $\pm$ standard error of mean (SEM).

III.3.2. Encapsulation efficiency and drug loading

The EE and D_L are defined in Equation 3 and Equation 4, respectively. To determine the total amount of drug, 500 µL of nanoparticles were dissolved in 500 µL of THF to dissolve PATO5. Then, 500 µL of this solution were mixed with 500 µL MeOH + 0.1% TFA. This solution was filtered on 0.45 µm before analysis by HPLC (see III.2.1). To quantify the unencapsulated amount of LEU, the suspensions were diluted in MilliQ water and filtered upon centrifugation at 12 000 g for 15 minutes at 4°C using Eppendorfs equipped with 0.1 µm PVDF filters (Ultrafree®-MC VV, Merck Millipore).

Equation 3 : Encapsulation efficiency calculation

$$\begin{aligned} \text{Encapsulation efficiency, \%} \\ = 100 \times \frac{\text{Total amount of drug} - \text{unencapsulated amount of drug}}{\text{Total amount of drug}} \end{aligned}$$

Equation 4: Drug loading calculation

$$\text{Drug loading, } \mu\text{g/mg} = \frac{\text{Total amount of drug} - \text{unenapsualted amount of drug}}{\text{Solid content}}$$

III.4. Cell culture studies

III.4.1. Culture medium

Caco-2 and HT29-MTX cell lines were cultured in DMEM supplemented with 10% FBS HyClone™, 1% Non-Essential Amino Acids, 1% L-Glutamine and 1% Penicillin/Streptomycin. Cells were grown in incubator at 37°C with 10% CO₂. Medium was changed every other day.

III.4.2. Cytotoxicity evaluation

The impact of the nanosuspensions on Caco-2 and Caco-2/HT29-MTX cells viability was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) colorimetric assay. To that end, 96 well plates were first coated with Matrigel™ (10 µL/mL of DMEM) to ensure a proper adhesion of the cells and emulate the conditions of the inserts. The wells were rinsed twice with DMEM before seeding 2x10⁴ cells per well of Caco-2 or Caco-2:HT29-MTX (ratio 3:1). Cells were allowed to grow overnight at 37°C, 10% CO₂. They were then

washed twice with pre-warmed HBSS at 37°C. 100 µL of nanosuspensions diluted in HBSS were added in each well to reach final concentrations between 0.5 and 18 mg/mL of blank particles. The same particle concentrations were used in the case of NLC-HIP and SLN-HIP (corresponding to a total LEU concentration between 0.8 and 60 µg/mL). The negative control corresponded to 4 wells containing only HBSS and the positive control corresponded to 4 wells containing a 0.5% Triton solution. The formulations were incubated with the cells during 2 hours at 37°C. The wells were then washed twice with HBSS and 100 µL MTT solution in DMEM (0.5 mg/mL) were added in each well. The plates were incubated for 3 hours at 37°C. The formazan purple crystals formed by the reaction of MTT with NAD(P)H of alive cells were dissolved in 200 µL DMSO and the absorbance was measured at 450 nm on a Multiskan EX plate reader (Thermo Fisher Scientific, MA, USA). The IC₅₀ were calculated using GraphPad Prism 8 program (CA, USA).

III.4.3. Transport study

The transport of LEU encapsulated in NLC and SLN was evaluated on both Caco-2 and Caco-2:HT29-MTX (3:1) cell monolayers. To this end, PET cell culture inserts with 1.0 µm pore size (ref 353103, Falcon) were coated with 200 µL Matrigel™ solution (10 µL/mL in DMEM). They were rinsed 3 times with DMEM before seeding 5x10⁵ Caco-2 cells or Caco-2:HT29-MTX (3:1) cells in 500 µL culture medium. The cells were cultured for 21 days with medium replacement every other day. On day 21, the inserts were washed 3 times with pre-warmed HBSS at 37°C and the integrity of the cell monolayer was evaluated by measuring the TransEpithelial Electric Resistance (TEER) with an epithelial voltohmmeter (EVOM², World Precision Instrument, Berlin). Transport experiment was conducted only on inserts with TEER values over 200 Ω.cm².

The transport of LEU and LEU encapsulated in NLC and SLN across Caco-2 and Caco-2/HT29-MTX cell monolayers was evaluated by addition of 500 µL LEU, NLC-HIP-DiD and SLN-HID-DiD diluted in HBSS on the apical compartment. A concentration equivalent to the IC₅₀ (11 µg/mL of LEU) was used, considering that the number of cells used for the transport study is much higher than for cytotoxicity measurements. The basolateral chamber was filled with 1000 µL HBSS. The cells were incubated with the solutions during 2 hours at 37°C. HBSS on the apical

side was collected to quantify transported LEU by ELISA. The results enabled the calculation of the apparent permeability (P_{app} , cm.s^{-1}) as described in Equation 5, where dQ/dt is the transport rate ($\mu\text{g/s}$), A is the surface area of the insert (cm^2) and C_0 is the initial concentration at the apical side ($\mu\text{g/mL}$).

Equation 5: Apparent permeability calculation

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

The inserts were rinsed with cold HBSS to stop the transport and the cell monolayers were fixed with a 4% paraformaldehyde solution for further observation by Confocal Scanning Laser Microscope (CSLM), see III.4.4.

To demonstrate the presence of mucus on the co-culture monolayers, some inserts were plunged in Methacarn (60% methanol, 30% chloroform, 10% acetic acid) for 30 minutes at 4°C to fix the cells. They were gently rinsed in DPBS before addition of 200 μL of a 1% Alcian blue solution in acidic medium (3% acetic acid, pH 2.5). After 45 minutes at room temperature, the inserts were washed, cut and observed under microscope (Axioskop 40, Carl Zeiss Microscopy, USA). Images were taken with the Zen lite 2012 software.

III.4.4. Nanoparticles interactions with cell cultures

Interactions of the nanoparticles with Caco-2 cell culture and Caco-2/HT29-MTX were evaluated both qualitatively and quantitatively.

Interactions of the nanoparticles and the cells were qualitatively evaluated using the Zeiss™ confocal microscope (LSM 150, Zeiss, Germany) which enables to localize the NLC-HIP-DiD and the SLN-HIP-DiD within the cell monolayers. The observation and images were done on the inserts used for the transport study (III.4.3). Cell monolayers, fixed in paraformaldehyde, were gently washed in DPBS. Actin was stained by addition of 200 μL of a 0.5% Triton solution in DPBS supplemented with Rhodamine-Phalloidin (1.3 $\mu\text{L/mL}$) to each of them. After 30 minutes in the dark, the inserts were cut and placed on microscope slides with one drop of Vectashield® mounting medium containing DAPI, staining cell nuclei in blue. Data were analyzed by ZEN Blue software to obtain x-y, y-z and x-z images.

To quantify the amount of nanoparticles uptake by Caco-2, flow cytometry was used. The samples were prepared in a similar manner as for the transport study. Thus, 500 μL of cell medium containing $5 \cdot 10^5$ Caco-2 cells per well were seeded in a 24 well plate. The cells were placed at 37°C overnight. After 3 washes with pre-warmed HBSS, 500 μL of HBSS (control), NLC-HIP-DiD or SLN-HIP-DiD (particles concentration of 4 mg/mL) were added in each well. After 2 hours of incubation at 37°C , cells were detached by addition of 200 μL trypsin-EDTA per well. After 5 minutes, trypsin-EDTA activity was stopped by addition of 1 ml DMEM. The samples were then centrifuged, the medium aspirated and replaced by 500 μL HBSS. The cells were analyzed using the BD FACSVerse™ flow cytometer (BD Bioscience, USA). To visualize the cell viability, 1 μL propidium iodide was added to the samples before analysis. The FlowJo analysis software was used to treat the data.

III.5. Nanoparticles behavior in transport medium

III.5.1. Particle size

To evaluate the stability of the nanoparticles dispersed in HBSS, particle size distribution was monitored during 2 hours. Therefore, 300 μL of NLC-HIP-DiD or SLN-HIP-DiD were dispersed in 30 mL HBSS at 37°C . The suspensions were placed in an incubator at 37°C under 80 rpm agitation. Samples were withdrawn every hour and directly analyzed by DLS at 37°C on a NICOMP™ 380 ZLS (PSS.NICOMP, Santa Barbara, CA, USA) using the dynamic viscosity of water at 37°C (8.90×10^{-4} Pa.s). Data are expressed as mean diameter $\pm$ SEM.

III.5.2. Drug release

Release studies in HBSS were performed in the same concentrations as for the transport study: 11 $\mu\text{g/mL}$. The corresponding volume of nanosuspensions was dispersed in HBSS pre-warmed at 37°C so as to have a global volume (HBSS plus formulation) of 10 mL. Samples were placed in an incubator at 37°C with an agitation of 80 rpm. Every 30 minutes, 300 μL aliquots were withdrawn from the vial and filtered on 0.1 μm PVDF filters (Ultrafree®-MC VV, Merck Millipore) *via* centrifugation at 12 000 g during 15 minutes at 4°C . The filtrates were immediately analyzed *via* HPLC according to the method described above (III.2.1). The release studies were conducted for 2 hours and sink conditions were respected.

III.6. Statistical analysis

All the experiments were performed in triplicate and are presented as mean $\pm$ standard error of mean (mean $\pm$ SEM). The normal distribution of the data was determined using a Shapiro-Wilk statistic test. Analyses of variance (ANOVA) followed by Bonferroni's *post hoc* test were conducted to compare EE, DL and cytotoxicity of the formulations. For other analyses, Mann-Whitney test was applied. The level of significance was set at probabilities of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with the control. Data were treated and analyzed with the GraphPad Prism 8 program (CA, USA).

IV. Results and discussion

IV.1. Nanoparticles characterization

LEU lipophilicity was modified by formation of a HIP by electrostatic interaction with sodium docusate. The precipitation efficiency was 99.93 ± 0.02 (n=44), indicating that almost all LEU was under a lipophilic complexed form.

The HIP was encapsulated in NLC and SLN formed by HPH using 5 cycles at 500 bar. EE and DL values are shown in Figure 1. No significant difference was observed between NLC and SLN concerning the EE of LEU ($84 \pm 3\%$ for NLC-HIP and $89 \pm 2\%$ for SLN-HIP). Moreover, no significant difference in EE with original formulations was measured when DiD was co-encapsulated with HIP ($85 \pm 1\%$ for NLC-HIP-DiD and $85 \pm 2\%$ for SLN-HIP-DiD). DiD being highly lipophilic ($\log P = 10.2$, (Mérien et al., 2015)), it was considered completely encapsulated in the nanoparticles.

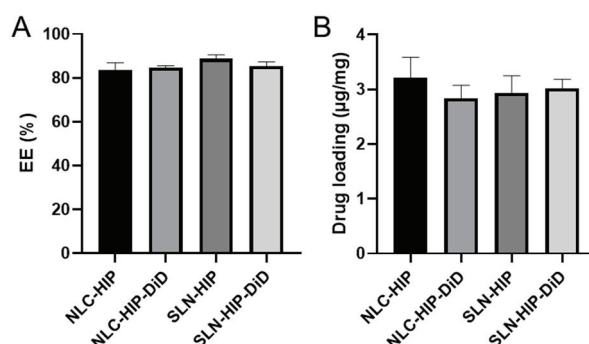


Figure 1: Encapsulation efficiency (A) and drug loading (B) of LEU in NLC-HIP, NLC-HIP-DiD, SLN-HIP and SLN-HIP-DiD (Data shown as mean $\pm$ SEM, n=3)

The nanoparticles were also characterized regarding their particle size and PDI which are displayed in Table 2. Both blank nanosuspensions presented a particle size around 115 nm. The loading of HIP or HIP and DiD had no significant impact on particle size ($p>0.05$). The PDI of both type of blank nanoparticles was around 0.2 and was not affected by the loading of the complex alone or co-encapsulated with the fluorophore.

Table 2: Particle size distribution of the solid lipid-based nanoparticles measured by DLS at 25 °C (results expressed as mean $\pm$ Standard Error of Mean (SEM), n=3, N=3)

	Blank NLC	NLC-HIP	NLC-HIP-DiD	Blank SLN	SLN-HIP	SLN-HIP-DiD
Z-average (nm)	114 $\pm$ 14	104 $\pm$ 12	109 $\pm$ 7	115 $\pm$ 11	124 $\pm$ 5	122 $\pm$ 6
PDI	0.2	0.2	0.2	0.2	0.2	0.2

IV.2. Cell culture studies

IV.2.1. Cytotoxicity evaluation

The viability of Caco-2 and co-cultures of Caco-2 and HT29-MTX cells in presence of blank or loaded nanoparticles was evaluated using a MTT assay. The same range of nanoparticle concentrations, 0.5-18 mg/mL (i.e. 2.3-75.0 μ g/mL of LEU for loaded nanoparticles), were incubated with the cells. No impact on cells viability was observed upon SLN incubation within this range of concentrations. The measured IC₅₀ values for blank and loaded NLC are presented in Figure 2. Regarding both cells type, a slight but not significant increase of the IC₅₀ was observed when loaded NLC were incubated compared to blank nanoparticles (from 2.3 mg/mL to 3.2 mg/mL for Caco-2 and from 4.1 mg/mL to 4.3 mg/mL for Caco-2/HT29-MTX). The IC₅₀ measured with loaded nanoparticles corresponded to a total LEU concentration of 14.8 μ g/mL in the case of Caco-2/HT29-MTX and 11.3 μ g/mL when only Caco-2 cells were used. The ratio between the IC₅₀ values measured with Caco-2/HT29-MTX and Caco-2 is equivalent to the cells ratio used in the wells, indicating that only Caco-2 viability was affected by the incubation with NLC. The difference of behavior between NLC and SLN can be explained by the presence of C90 in NLC. This oily compound is a water-insoluble surfactant that has already shown a slight concentration-dependent cytotoxicity over Caco-2 cells, induced by the high content of propylene glycol monoester (~90%) (Ujhelyi et al., 2012).

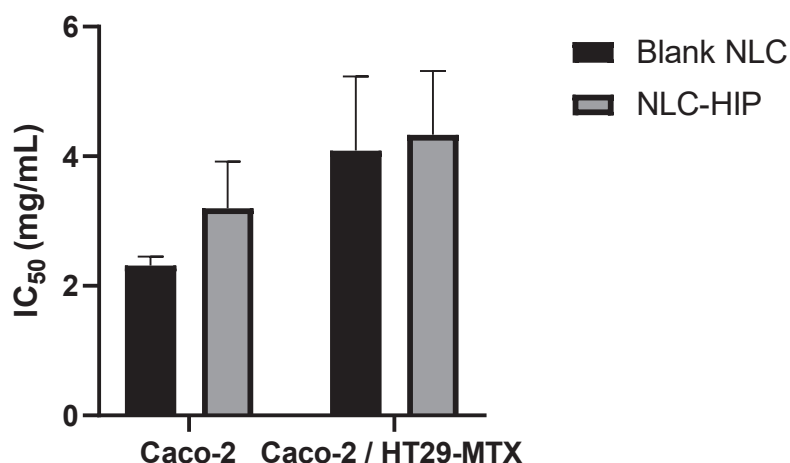


Figure 2: IC_{50} values of blank and HIP-loaded NLC after 2 hours incubation with Caco-2 and Caco-2/HT29-MTX cells (data expressed as mean $\pm$ SEM, $n=3$)

IV.2.2. Presence of mucus

Presence of mucus on Caco-2/HT29-MTX cell monolayers (mucin-secreting model) was assessed by staining the cell monolayers at the surface of the inserts by Alcian Blue. The positive charges induced by the basic isothiuronium groups of this dye were attracted by the anionic sites of mucus. The images taken by microscope attested the homogeneous presence of mucus on co-cultured monolayers, Figure 3.

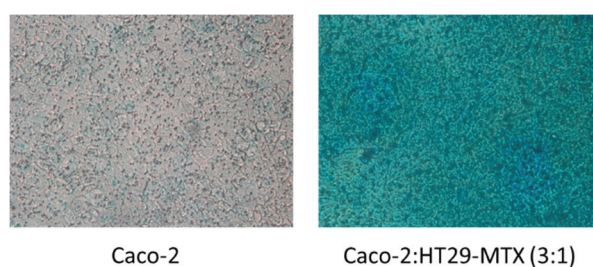


Figure 3: Microscope images of Caco-2 and Caco-2:HT29-MTX cell monolayers after 45 minutes incubation in Alcian blue (magnification x400)

IV.2.3. Intracellular uptake

The interactions of the nanocarriers with the cells were investigated qualitatively by confocal microscopy and quantitatively by flow cytometry.

The confocal microscopy images, shown in Figure 4 (A), were taken on cell monolayers incubated with the DiD-loaded nanosuspensions for 2 hours at 37°C, which were then fixed and stained. The green channel corresponds to actin stained by Rhodamine-Phalloidin and the red channel indicates the SLN-HIP-DiD and NLC-HIP-DiD. The images show the presence of both type of nanoparticles inside the two cells monolayers, indicating the ability of the nanocarriers to penetrate the enterocytes. Furthermore, both NLC and SLN nanoparticles were able to cross the mucus layer present at the surface of the co-cultured cell monolayers.

The flow cytometry results conducted on Caco-2 cells are expressed in Figure 4 (B). The results confirmed quantitatively that both formulations were internalized by Caco-2 cells with 82.0 % of the cells indicating positive to DiD-loaded SLN and 98.8% of the cells indicating positive to DiD-loaded NLC. The significant difference (**, $p < 0.01$) between the uptake of DiD-loaded SLN and DiD-loaded NLC was induced by the presence of C90, a monoester of caprylic acid. These compounds are known to promote the intestinal absorption of active molecules by various modes of action: by enhancing paracellular transport, by transient opening of the tight junctions or by facilitating the transcellular transport by fluidization of the cells membrane (Brayden et al., 2014; Lindmark et al., 1995; Maher et al., 2018).

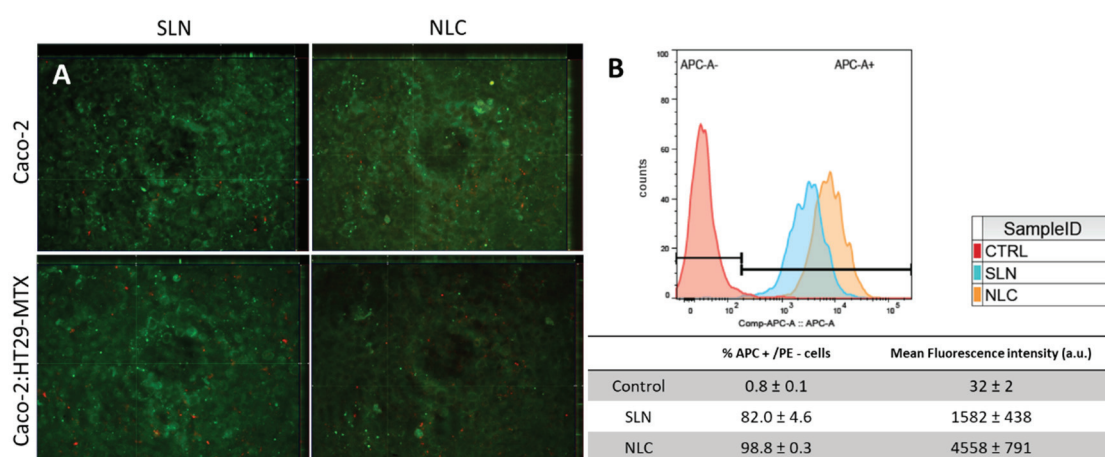


Figure 4: (A) CLSM images of SLN-HIP-DiD and NLC-HIP-DiD with Caco-2 and Caco-2/HT29-MTX co-cultured cells monolayers after 2h incubation at 37°C (x25). The green channel indicates Rhodamine-Phalloidin and the DiD-loaded nanoparticles are indicated by the red channel. (B) Cellular uptake in Caco-2 cells of SLN-HIP-DiD (blue) and NLC-HIP-DiD (orange) measured by flow cytometry, untreated cells are shown as controls. The table presents the percentage of APC positive (DiD-loaded nanoparticles) and PE negative (living cells) cells and the mean fluorescence intensity of the APC channel ($n=10$, $N=3$, data expressed as mean $\pm$ SEM)

IV.2.4. In-vitro evaluation of Leuprolide intestinal transport

The in-vitro evaluation of the intestinal transport of LEU across the epithelial barrier was conducted on Caco-2 cell monolayers (enterocyte-like model). The effect of mucus was

investigating using co-cultures of Caco-2 with HT29-MTX (mucin-secreting model). The passage of LEU and LEU encapsulated in lipid nanoparticles under a lipidized form was compared by quantification of the LEU content in the basolateral reservoir after incubation of the formulations for 2 hours on cell monolayers grown on inserts. TEER values very significantly decreased upon incubation with the formulations, Figure 5. The nanoparticles were not responsible for this decrease as the same phenomenon was observed in the same proportions with the controls, where only HBSS was incubated with the cells. Moreover, final TEER values were still equivalent or above $200 \Omega \cdot \text{cm}^2$ which is acceptable for this kind of experiments and superior to the *in vivo* values of the human intestinal epithelium ($12\text{--}69 \Omega \cdot \text{cm}^2$ (Béduneau et al., 2014)). The quantification of the transported LEU enabled the calculation of the apparent permeability, which results are depicted in Figure 66. The data showed no significant difference between the transport of free and encapsulated LEU across Caco-2 cell monolayers, regardless of the formulations. Moreover, there were no statistical differences between the Papp of free or encapsulated LEU across Caco-2 and across Caco-2/HT29-MTX cell monolayers although the presence of the mucus barrier had been attested, see Figure 3.

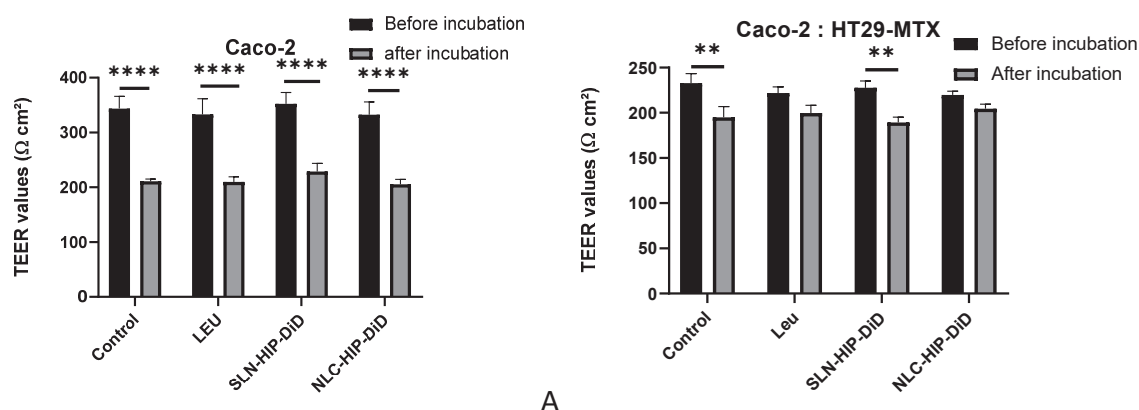


Figure 5: TEER values of Caco-2 (A) and Caco-2/HT29-MTX cell monolayers, before and after incubation with formulations

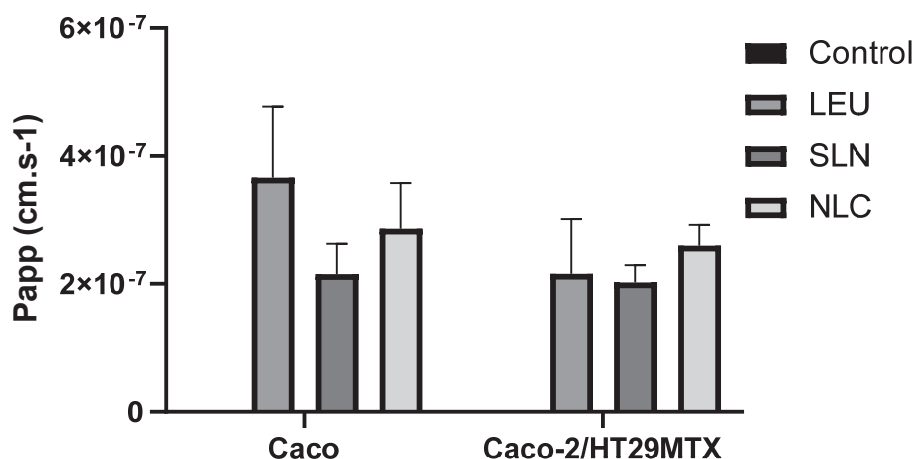


Figure 6: Papp of neat and encapsulated LEU in SLN and NLC after 2 hours incubation on Caco-2 and Caco-2/HT29-MTX cell monolayers (n=3, N=3, data expressed as mean ± SEM)

The values of apparent permeability depicted in Figure 6, are weak and correspond to a transport of LEU below 0.3% of the incubated dose. Regarding the results from nanoparticles uptake, showing that more than 80% of nanoparticles were internalized by Caco-2 cells (in the conditions of the experiments), a higher LEU transportation rate across cell monolayers was expected.

Actually, the results of interactions between formulations and cells obtained by CSLM and flow cytometry testified the ability of the nanoparticles to cross the apical membrane of the enterocytes. However, even if these results demonstrated high internalization, they did not guarantee the capacity of SLN and NLC in crossing the basolateral cell membrane. Indeed, by measuring the fluorescence of the samples collected on the basolateral compartment of Caco-2 cell monolayers, it was possible to estimate the passage of DiD-loaded SLN to be 1.8 % of the nanoparticles incubated with Caco-2 monolayers and 1.2% for Caco-2/HT29-MTX co-cultured monolayers. In the case of NLC, the fluorescent measurements have shown that less than 1% nanoparticles had crossed the monolayers. These results suggested that the higher internalization, and consequently the higher affinity, between NLC and enterocyte cells was not necessarily a good indication of the transport of the nanoparticles across the entire cell monolayer.

Another explanation to the low concentration of LEU in the basolateral compartments after transportation studies could lie in an unappropriated analytical method. Indeed, the ELISA method only enables the quantification of free LEU. Yet, if the particles are intact, the encapsulated LEU cannot be detected by the dosing method. Addition of solvent would be required to disrupt the solid lipid matrix, which is not compatible with the chosen ELISA method.

Finally, the absence of difference in apparent permeability between free and encapsulated LEU could be the result of an important drug release from the nanoparticles, limiting the presumed advantages of peptide encapsulation in the nanocarriers.

IV.3. Nanoparticles behavior in transport medium

IV.3.1. Particle size stability in HBSS

HBSS was used as transport media. The stability of nanoparticles was evaluated over 2 hours in this medium by monitoring particle size evolution using DLS, at 37°C. The results, Figure 7, showed no aggregation of NLC nor SLN. Indeed, the size of the nanoparticles was not affected by the dispersion medium and neither were PDI (data not shown).

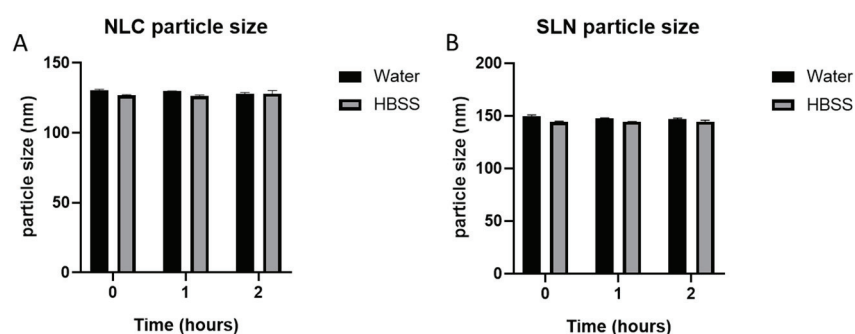


Figure 7: Particle size evolution of NLC-HIP-DiD and SLN-HIPC-DiD dispersed in water or HBSS at 37°C over 2 hours (data expressed as mean $\pm$ SEM, n=3)

IV.3.2. Drug release in transport medium

Concerning LEU liberation in HBSS, a burst release could be observed as soon as the nanoparticles were dispersed in the medium, Figure 8. Indeed, within the first five minutes, 89% LEU was released from NLC and 92% from SLN. However, there was no further release of the peptide during the remaining 2 hours. This important initial drug release can be attributed to the platelet shape of solid lipid nanoparticles (Dumont et al., 2019). Indeed, this particular structures present an important surface to volume ratio, with drug leakage facilitated by their important surface of exchange. Furthermore, the formation of HIP is based on an electrostatic interaction between the peptide and an anionic surfactant. This electrostatic attraction can be modified by the surrounding medium. Indeed, a recent study using Taylor Dispersion Analysis has shown that the release of LEU from HIP-loaded SEDDS was favored with increasing ionic strength (Chamieh et al., 2019). Then, in HBSS, with an ionic strength above 150 mM, it can be postulated that a majority of the HIP has dissociated leading to a change in LEU lipophilicity and, consequently, let to an easier release from the solid lipid matrix of the nanoparticles.

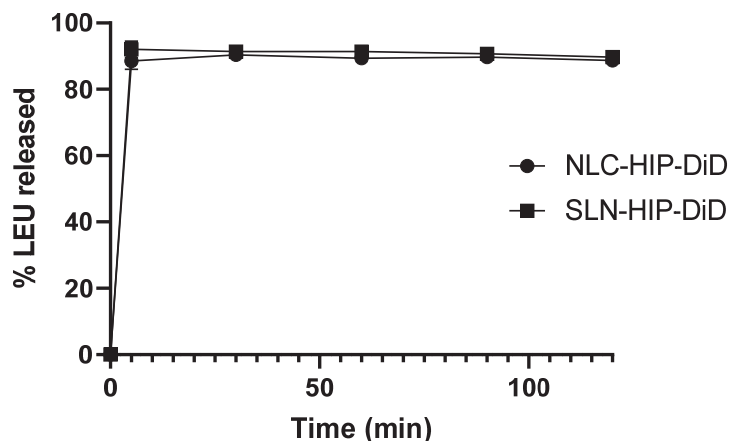


Figure 8: In-vitro release profile of LEU from NLC-HIP-DiD and SLN-HIP-DiD in HBSS at 37°C over 2 hours (data expressed as mean $\pm$ SEM)

This important drug release explains the lack of differences between the transport free LEU and encapsulated LEU across Caco-2 and Caco-2/HT29-MTX cell monolayers as the great majority of the peptide had left the particles before reaching the apical side of the cells.

As the HIP is dissociated in the transport medium, both LEU and the water-soluble anionic surfactant docusate are released. This latter compound can fluidize enterocyte membrane if present under its Critical Micellar Concentration (2.37 mM at 35°C, (Chakraborty et al., 2007)). In the present study, the maximal concentration of docusate in the medium (if completely released from the nanoparticles) would be $1.78 \cdot 10^{-5}$ mM. However, as no improvement of peptide transport was observed, probable interactions of docusate with the surrounding medium of high ionic strength should be taken into account.

V. Conclusion

In this study the lipophilicity of Leuprolide, a hydrophilic linear peptide model, was increased by formation of a Hydrophobic Ion Pair (HIP) with sodium docusate. The hydrophobic complex was loaded in Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers obtained by High Pressure Homogenization process with a high Encapsulation Efficiency. Both particle types showed similar physicochemical properties. The cytotoxicity of the nanoparticles with Caco-2 cells and co-cultures of Caco-2 and HT29-MTX cells was evaluated. The results have shown that only NLC presented toxicity over the cells in the range of concentration tested. This toxicity was attributed to the presence propylene glycol monocaprylate, which probably dissolve cell walls better than esters with longer chains.

The aim of the study was to evaluate *in-vitro* the potential of the designed lipid nanoparticles to cross the intestinal barrier. In this objective, the interactions of the nanoparticles with Caco-2 cells (enterocyte-like model) and co-cultures of Caco-2 and HT29-MTX cell lines (mucin-secreting model) were studied. Moreover, the transport of free LEU across the cell monolayers was compared to the transport of encapsulated LEU in lipid nanoparticles.

Images of the cell monolayers by Confocal Scanning Laser Microscopy after incubation of the formulation during 2 hours in HBSS have shown that both SLN and NLC were internalized by Caco-2 cells. Internalization was also observed in the presence of mucus on Caco-2/HT29-MTX cell monolayers. Flow cytometry measurements confirmed these observations with 82% SLN and 98.8% NLC being internalized by Caco-2 cells. However, despite these promising results, the transport of LEU across the cell monolayers was not improved by the encapsulation in lipid nanocarriers. Indeed, most of the peptide was released in the transport medium due to the

platelet-shape of the lipid nanoparticles and a loss of lipophilicity of the peptide induced by the ionic strength of the medium.

In conclusion, the stability of the HIP needs to be improved to maintain the peptide inside the lipid nanocarriers upon dispersion in the biorelevant media. Then, as the designed SLN and NLC were able to cross the mucus barrier and were internalized by the enterocytes, the encapsulation of stabilized HIP should increase the transport of peptides across the intestinal barrier.

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Chapitre V :

Impact de la lipolyse sur l'absorption intestinale des peptides
encapsulé dans des NLC,

Etude préliminaire

Les formulations lipidiques administrées par voie orale sont soumises au même processus de digestion que les lipides contenus dans l'alimentation. Elles peuvent facilement être protégées de l'action de la lipase gastrique *via* un enrobage entérique. De ce fait, l'évaluation *in vitro* de la digestibilité des formules est généralement limitée à une étude en milieu intestinal *via* l'activité des enzymes pancréatiques. La lipolyse des formules lipidiques a d'importantes conséquences sur le devenir des actifs thérapeutiques qu'elles contiennent. En effet, ceux-ci peuvent précipiter dans le cas des principes actifs insolubles ou être maintenus en solution après intégration dans des micelles mixtes composées de phospholipides, de sels biliaires et des produits de dégradation des formules lipidiques. Les conditions de lipolyse *in vitro* des formules lipidiques ont été standardisées par un groupe d'industriels et de scientifiques, le consortium LFCS (Lipid Formulation Classification System).

La digestibilité des formulations lipidiques est impactée par plusieurs facteurs : le type de polyol, la tension interfaciale, la surface offerte par les nanovecteurs, le nombre de fonctions ester et la longueur des chaînes glycérides. Dans le cas des systèmes auto-émulsionnables, ceux formulés avec des éthers et ne contenant pas de fonctions esters ne sont pas dégradés en présence de lipase pancréatique. Par conséquent lorsqu'un peptide est encapsulé dans ces systèmes, il n'est pas libéré. Dans le cas des nanoparticules solides, il a été montré que l'augmentation de la longueur des chaînes carbonées limitait, voire empêchait leur digestion. Ainsi, pour des SLN de Compritol® 888 ATO (C888, dibéhénate de glycérol, C₂₂) aucune altération de la composition glycéridique n'a été observée au cours de la lipolyse *in vitro* contrairement aux SLN de Précirol® ATO 5 (PATO5, mélange de stéarate de glycérol, C₁₆, et de palmitate de glycérol, C₁₈). Ces deux excipients pourraient donc être utilisés pour encapsuler un peptide modèle dans des nanoparticules et évaluer l'impact de la digestibilité sur son passage à travers la barrière intestinale. Les NLC diffèrent des SLN par l'addition d'une fraction lipidique liquide. L'utilisation du Carpyol® 90 (monocaprylate de propylène glycol) pourrait augmenter la perméabilité du peptide par formation d'acide caprylique après dégradation des liaisons ester par action de la lipase pancréatique.

Dans un premier temps cette étude vise à évaluer la possibilité d'adapter le procédé développé dans le chapitre II pour la formation de NLC de Précirol® ATO 5 à la formation de NLC de Compritol® 888 ATO pour l'encapsulation d'un peptide modèle hydrophobisé (le

Leuprolide, LEU). La digestibilité *in vitro* de ces nanoparticules sera ensuite évaluée. Enfin des stratégies pour évaluer l'impact du passage intestinal de peptides modèles seront proposées.

Les NLC de C888 ont été formulées en utilisant les mêmes proportions en excipients que pour les NLC de PATO5 présentées chapitre II et III (excipient solide : Capryol® 90: Kolliphor® RH40 = 68:5:27 %, w/w). Le procédé a été légèrement adapté pour garantir l'obtention de nanoparticules reproductibles. Pour cela, la température de travail a été augmentée (de 70 à 80°C) pour assurer la fonte complète de l'excipient (plage de fusion = 65-77°C) et le nombre de cycles a été étendu (de 5 à 10 cycles). Les NLC de C888 blanches présentaient un diamètre moyen plus important (141 ± 2 nm) que les NLC de PATO5 blanches (96 ± 1 nm).

Le LEU a été encapsulé dans les deux types de NLC après augmentation de sa lipophilie par formation d'une paire d'ions hydrophobe (HIP) en utilisant la méthode décrite dans les chapitre II et III. Les nanoparticules obtenues mesurent 107 ± 4 nm dans le cas des NLC de C888 et 115 ± 10 nm pour les NLC de PATO5 pour des taux d'encapsulation respectifs de 67 ± 3 % et 82 ± 4 %.

Les recommandations du LFCS concernant la composition des milieux de lipolyse *in vitro* ont été adaptées pour reproduire des conditions équivalentes à celles utilisées pour les SEDDS. L'évaluation de la digestibilité des formules a été conduite dans un pH-stat maintenant le pH du milieu à 6,5 et le processus de lipolyse a été initié par ajout de pancréatine porcine. Des échantillons ont été prélevés à des temps de mesure prédéfinis et immédiatement congelés dans l'azote liquide pour stopper l'activité enzymatique. La composition en mono-, di- et triglycérides a été déterminée par chromatographie par exclusion stérique. Les résultats n'ont montré aucun changement de la composition en glycérides dans le milieu au cours de la lipolyse des NLC de C888. En revanche, avec les NLC de PATO5, la composition en glycérides est fortement impactée par l'activité de la pancréatine avec une diminution progressive du pourcentage en diesters et triesters de glycérol et une augmentation de la proportion d'acides gras.

Malheureusement les études de lipolyse menées sur les nanoparticules chargées en LEU hydrophobisé n'ont pas permis de conclure concernant le maintien ou non du peptide sous forme solubilisée. En effet, la pancréatine n'est pas une lipase pancréatique pure et contient

des protéases. Ainsi, dans les conditions de l'expériences (non représentatives de l'activité enzymatique *in vivo*), celles-ci ont dégradé le LEU en quelques minutes.

De plus, la pancréatine n'est pas compatible avec les cellules Caco-2 utilisées pour modéliser *in vitro* les entérocytes de l'épithélium intestinal et quantifier la perméabilité intestinale. Il n'est donc pas possible de mettre en présence le lipolysat (contenant encore la pancréatine) et les monocouches de Caco-2 pour quantifier le passage des substances actives en présence des produits de digestion.

Une étude publiée en 2019 a montré que l'utilisation d'une enzyme supportée pourrait résoudre ces problèmes en ne dégradant que les lipides, avec un niveau d'efficacité similaire à la pancréatine tout en étant compatibles avec les monocouches de Caco-2. Les auteurs ont ainsi proposé un système permettant l'évaluation simultanée de la lipolyse et de la perméabilité sur monocouche de Caco-2. L'utilisation de ce modèle avec les NLC de C888 et de PATO5 permettrait d'observer l'impact de la digestibilité des formules sur le passage intestinal de l'actif encapsulé.

Impact of lipolysis on intestinal absorption of peptides loaded in NLC, preliminary study

I. Introduction

Orally administered lipid-based formulations are submitted to the same digestive process as dietary fat. Digestion of triglycerides is initiated in the stomach where is located gastric lipase. This enzyme cleaves their ester bonds in positions sn-1 and sn-3 to give diacylglycerides and free fatty acids. Generation of these lipolysis products stimulates the production of biliary salts and pancreatic enzymes. Of these, lipid degradation is mainly controlled by human pancreatic lipase (HPL) and its co-factor, cholesteryl ester hydrolase (CEH), and two pancreatic lipase-related proteins (PLRP1 and PLRP2) (Jannin et al., 2015b).

Formulations are generally protected from the gastric lipase by an enteric coating that physically separates the lipid particles from the harsh gastric media. This method was indeed proven as efficient for the protection of SEDDS loaded with insulin complexed with phospholipids. The formulation was dispersed into hard gelatin capsules prior to a coating step using Eudragit L100, which dissolves when pH is above 6.0, enabling the release of its content in simulated intestinal medium (Li et al., 2014). Recently, Capsugel developed capsules based on pharmaceutical grades of cellulosic enteric derivatives, enTRinsic™ DDT, thus overstepping the coating step (Benameur, 2015).

Thus, evaluation of *in vitro* orally administered lipid based formulation fate is usually focused on small intestine and its pancreatic lipases. A group of scientists and industrialists, Lipid Formulation Classification System (LFCS) consortium, has settled the parameters to standardize a method to evaluate digestion rate of lipid-based formulation in simulated fasted intestinal media, in presence of pancreatic lipase. They describe the conditions to represent fasted small intestine (buffer medium pH 6.5), to sample and to prepare aliquots. In the method, intestinal lipolysis is simulated in a pH-stat apparatus which consists of a reaction vessel equipped with a glass electrode linked with a pH-meter that is connected to an adjustable controlling device. This device operates a motor-driven burette dipping into the reaction system and supplying the system with sodium hydroxide to maintain the desired pH value (schematic representation of the set up page 34). Digestion is initiated by adding porcine pancreatic extracts to the reaction medium containing the dispersed lipid-based formulations

(Williams et al., 2012a, 2012b). Aliquots are withdrawn at given times and the lipase-induced digestion process is immediately stopped by addition of an inhibitor. Samples are then centrifuged and the supernatant is collected to evaluate the amount of drug remaining in solution. In other studies, the enzymatic activity is stopped by freezing the samples in liquid nitrogen. The frozen samples are freeze-dried prior to dilution in an appropriate solvent for quantification.

The extent of lipolysis on lipid particles can play an important role on the bioavailability of an encapsulated substance which can either remain encapsulated in non-digestible formulations or precipitate in the case of non-soluble drugs or be maintained in solution in mixed micelles of phospholipids, bile salts and products of digestion when incorporated in digestible lipid particles (Jannin et al., 2015a; Williams et al., 2012a).

Both the ester content in a formulation and glycerides chain length have a significant impact on lipase activity (Jannin et al., 2018; Leonaviciute et al., 2016). For instance, ester-free formulations are not hydrolyzed in presence of pancreatic lipase. As the drug release from a lipid carrier is controlled by the extend of the digestion process (Bernkop-Schnürch and Jalil, 2018), no peptide release was observed from a ether-based Self-Emulsifying Drug Delivery System (SEDDS) loaded with leuprolide in presence of pancreatic lipase. Moreover, in presence of both lipase and proteases (trypsin and α -chymotrypsin), the protective effect of the SEDDS was the highest for ester-free formulations (48%). Increasing the chain length of the fatty acids used in the formulations also affects the digestion extent. Jannin *et al.* have shown that no variation of glycerides composition upon *in vitro* lipolysis of Solid Lipid Nanoparticles (SLN) of Compritol®888 ATO (glyceryl dibehenate) (Jannin et al., 2018) while SLN of Precirol®ATO5 (glyceryl distearate) were rapidly digested (Jannin et al., 2015b). These two solid lipid excipients be used to formulated peptide-loaded solid nanocarriers and evaluate the impact of digestion on protection towards proteolytic-induced degradation, as well as the effect on peptide permeation across the intestinal epithelium.

NLC differs from SLN by addition of a liquid lipid fraction to the solid excipient. The use of Capryol®90 (propylene glycol monocaprylate) could increase the permeation ability of the system by generating caprylic acid, a known permeation enhancer, by cleavage of the ester bond by action of the pancreatic protease.

First, this study aims to evaluate the possibility to adapt the process developed for the formulation of Precirol®ATO5 NLC to Compritol®888 ATO for the encapsulation of a lipidized peptide (Leuprolide, LEU) and to compare the digestibility *in vitro* of the blank formulations. Then, in a second part, strategies to evaluate the impact of particles digestibility on the protective effect towards proteases or on the intestinal absorption of the encapsulated peptide will be proposed.

II. Material and methods

II.1. Material

Leuprolide acetate was obtained from Glentham Life Sciences (LEU, Corsham, UK). Sodium docusate, used for the preparation of the HIP, was purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France).

For the preparation of the formulations, Capryol®90 (propylene glycol monocaprylate, C90), Compritol® 888 ATO (glyceryl dibehenate, C888) and Precirol® ATO 5 (Glyceryl distearate, Pato5) were obtained from Gattefossé SAS (Saint Priest, France). Kolliphor® RH40 (polyoxyl 40 castor oil, RH40) was purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France).

For lipolysis experiments, HCl, NaOH and NaCl were obtained from Merck KGaA (VWR, Bourges, France). CaC₂ and Tris (hydroxymethylaminomethane) were bought to Chimie Plus Laboratoire (Décines, France). L- α -phosphatidylcholine, pancreatin, sodium taurodeoxycholate (NaTDC) and trybutyrine were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

II.2. Preparation and characterization of SLN and NLC

II.2.1. Formation of Leuprolide docusate Hydrophobic Ion Pairs

The method used for the formation of the leuprolide-docusate HIP (molar ratio 1:2 LEU:docusate) has been previously described (Dumont et al., 2019a, 2019b; Griesser et al., 2017). Briefly, 20 mg of LEU were ionized in 1 mL of 0.01 M HCl. Then; 1 mL of a 14 mg/mL sodium docusate solution was added to LEU solutions. The immediate formation of a white precipitate confirmed the formation of the ion pair. The complexes were centrifuged for 10 minutes at 15000 g, 4°C on a Universal Centrifuge 320 R (Hettich). The supernatants were

collected and analyzed *via* HPLC (see III.2.1) to quantify the amount of LEU remaining free. The results were used to calculate the precipitation efficiency, Equation 2.

The HIP were frozen in liquid nitrogen, lyophilized and stored at -20°C until further use.

Equation 6: Calculation of precipitation efficiency of HIP formation

$$\text{Precipitation efficiency, \%} = 100 - \left(100 \times \frac{[LEU]_{\text{supernatant}}}{[LEU]_{\text{initial}}} \right)$$

II.2.2. Formulation of solid nanoparticles

The hot High Pressure Homogenization technique was used to produce NLC whose composition is given in Table 3. Briefly, the lipid phase, composed of the solid lipid excipient supplemented with C90, was heated 10°C above the melting point of the solid excipient (i.e.: 80°C for C888 and 70°C for PATO5) under magnetic stirring. The aqueous surfactant solution was heated at the same temperature and added to the melted lipid phase. A hot emulsion was formed by high shear mixing during 3 minutes at 11000 rpm using an Ultra-turrax® (T25, IKA). This premix was injected in a Microfluidizer LM 20 (Microfluidics) where it was submitted to 10 cycles at 500 bar in the case of C888 NLC and 5 cycles at 500 bar with PATO5 NLC to enable the formation of a hot nanoemulsion. The final solid nanoparticles were obtained by cooling down the nanoemulsions in a 4°C ice/water bath, under magnetic stirring at 400 rpm during 10 minutes.

Loaded-NLC were prepared according to the same protocol with the HIP first dissolved in C90 before addition to the melted lipid phase.

Table 3: NLC composition

	C888 Blank NLC	HIP loaded C888 NLC	PATO5 blank NLC	HIP loaded PATO5 NLC
C888	4 g	4 g	-	-
PATO5	-	-	4 g	4 g
C90	300 µL	300 µL	300 µL	300 µL
LEU-HIP	-	30 mg	-	30 mg
Corresponding LEU	-	16 mg	-	16 mg
RH40	1.6 g	1.6 g	1.6 g	1.6 g
Water	40 mL	40 mL	40 mL	40 mL

II.2.3. Characterization

II.2.3.1. Particle size

Particle size and polydispersity index (PDI) were analyzed by Diffraction Light Scattering on diluted samples (one droplet in 2 mL deionized water) with a zetasier Nano-ZS (Malvern) at 25°C.

II.2.3.2. Encapsulation efficiency and Drug Loading

The encapsulation efficiency (EE) and drug loading (D_L) are defined in Equation 3 and Equation 4, respectively. To determine the total amount of drug, 500 μ L of nanoparticles were dissolved in 500 μ L of THF to dissolve PATO5. Then, 500 μ L of this solution were mixed with 500 μ L MeOH + 0.1% TFA. This solution was filtered on 0.45 μ m before analysis by HPLC (see III.2.1). To quantify the unencapsulated amount of LEU, the suspensions were diluted in MilliQ water and filtered upon centrifugation at 12 000 g for 15 minutes at 4°C using Eppendorfs equipped with 0.1 μ m PVDF filters (Ultrafree®-MC VV, Merck Millipore).

Equation 7 : Encapsulation efficiency calculation

$$\text{Encapsulation efficiency, \%} = 100 \times \frac{\text{Total amount of drug} - \text{unencapsulated amount of drug}}{\text{Total amount of drug}}$$

Equation 8: Drug loading calculation

$$\text{Drug loading, } \mu\text{g/mg} = \frac{\text{Total amount of drug} - \text{unenapsualted amount of drug}}{\text{Solid content}}$$

II.3. *In vitro* lipolysis of nanoparticles

II.3.1. Preparation of lipolysis medium

The pancreatin solution was prepared according to the Lipid Formulation Classification System (LFCS) Consortium (Williams et al., 2012a, 2012b). The lipolysis buffer was prepared by solubilizing CaCl_2 (0.208 g/L), NaCl (8.810 g/L) and Tris (0.474 g/L) in ultrapure water. The pH of the buffer was adjusted to 6.5 by addition of NaOH 0.6M.

The pancreatin solution was obtained by dispersion of 1 g of pancreatin powder in 5 mL of lipolysis buffer. After 10 minutes of magnetic stirring, the mixture was centrifuged at 5°C during 10 minutes at 2 800 g. The supernatant was collected to be used for the lipolysis tests.

The lipolysis media was prepared by adapting the protocol from the LFCS consortium: the amounts of the components were adapted to reach the same prescribed concentrations after dilution of the media by addition of the nanosuspensions. Then, the concentrated lipid buffer was prepared by solubilizing 0.288 g/L CaCl₂, 12.128 g/L NaCl and 0.656 g/L Tris in ultrapure water. The pH of the buffer was adjusted to 6.5 by addition of NaOH 0.6 M. The concentrated lipolysis media was obtained by addition of L- α -phosphatidylcholine (0.79 g/L) and NaTDC (2.160 g/L) to the lipolysis medium.

II.3.2. *In vitro* lipolysis testing

The experimental set up consists in a pH-stat apparatus (Mettler Toledo) equipped with a Titrando 802 propeller stirrer/804 Ti, a double jacketed 60 mL vessel, a pH electrode and one 800 Dosino dosing unit coupled to 5 mL autoburette. The apparatus is connected to a computer that regulates the pH at 6.5 by addition of NaOH 0.2 M.

Before initiating the *in vitro* digestion of the formulations, the enzymatic activity of pancreatin was tested with tributyrin (model substrate). To this end, 500 μ L of tributyrine were added to 30 mL of lipolysis medium (without L- α -phosphatidylcholine) heated at 37°C. After 150s homogenization, 1.5 μ L of pancreatin solution were introduced in the system. The specific activity of pancreatin should be above 900 U/mg.

Ten milliliters of the nanosuspensions (i.e. 0.750 g of NLC-C888 and 0.770 g of NLC-PATO5) were added to 26 mL of the concentrated lipolysis media. The pancreatin solution, 4 mL, were added once the 37°C were reached. Aliquots of 1 mL were withdrawn just before addition of the pancreatic solution (t=0) and 5, 15, 30 and 60 minutes after the addition.

II.3.3. Analysis of glycerol esters composition by Size Exclusion Chromatography

The withdrawn samples were immediately frozen in liquid nitrogen to stop the enzymatic activity of the lipases and freeze-dried (Heto Drywinner FD 3, Thermo). The freeze-dried

samples were dissolved in 4 mL of THF and filtered 0.45 μm PVDF filters. The solvent was evaporated and the residues were dissolved in 1 mL of THF.

The composition in mono-, di- and triesters of glycerol was analyzed by Size Exclusion Chromatography (SEC, Pump L2130, Injector L2200, Refractometer L2490, VWR Hitachi Lachrom Elite) equipped with a refractometer and a 600×7.5 mm PL Gel 100 Å (5 μm) analytical column (Agilent Technologies, Les Ulis, France) at 35°C according to the compendial assays described in the United State Pharmacopeia and the National Formulary (USP-NF). The mobile phase was THF (1 mL/min) and the injection volume was 10 μL . The retention times of the mono-, di- and triglycerides under these conditions are summarized in Table 4.

The lipolysis tests were performed in triplicate and the results were expressed as percentage of glycerol esters.

Table 4: retention time of glycerol esters

COMPONENTS	RETENTION TIME (MIN)
C90 MONOESTERS	14.9
C90 DIESTERS	14.3
C888 MONOGLYCERIDES	12.9
C888 DIGLYCERIDES	12.1
C888 TRIGLYCERIDES	11.7
PATO5 MONOGLYCERIDES	13.6
PATO5 DIGLYCERIDES	12.9
PATO5 TRIGLYCERIDES	12.4
PATO5 FATTY ACIDS	16.1

III. Results and discussion

III.1. Nanoparticles production and characterization

Hot HPH is a solvent-free scalable process which is said to enable the formulation of reproducible SLN and NLC with low particle size and low PDI. The technique described by Dumont et al. to obtain NLC of PATO 5 was adapted in the case of C888 by increasing the process temperature to 80°C to ensure a complete melting of the solid excipient and extending the number of cycles at 500 bar (Dumont et al., 2019b). Nanoparticle size and PDI were measured by DLS. The results, displayed in Table 5, confirmed that the formulation technique can be adapted to C888 to produce reproducible NLC. Surprisingly, particle size of loaded C888 nanoparticles was significantly lower than for blank C888 NLC.

NLC of C888 were larger than NLC based on PATO 5. Indeed, the production of the nanosuspensions by hot HPH is based on the formation of a hot nanoemulsion with high energy inputs. This energy is used to increase the interface between the lipid and the aqueous phases, to overcome the viscous resistance of the material and finally an important part of this energy is dissipated by Joule effect (Couffin and Delmas, 2013). The longer carbonated chains contained in C888 (C22), compared to PATO5 (C18), may result in higher viscosity of the melted product. Then, with same levels of energy applied to the systems, the size reduction was less important with C888.

Table 5: Z-average and PDI of C888 and PATO5 NLC (results expressed as mean $\pm$ SEM)

FORMULATION	BLANK C888 NLC	HIP LOADED C888 NLC	BLANK PATO5 NLC	HIP LOADED PATO5 NLC
Z-AVERAGE (NM)	141 $\pm$ 2	107 $\pm$ 4	96 $\pm$ 1	115 $\pm$ 10
PDI	0.14 $\pm$ 0.01	0.20 $\pm$ 0.01	0.15 $\pm$ 0.01	0.18 $\pm$ 0.02

The results concerning EE and D_L are displayed in Table 6. They show a significantly higher encapsulation of LEU in NLC of PATO5 compared to NLC of C888. The latter one is composed of longer lipid chains which might results in more entanglement in the particles core and, consequently less free space for encapsulation of the peptide.

Table 6: EE and DLI of C888 and PATO5 loaded NLC (results expressed as mean $\pm$ SEM)

FORMULATION	HIP LOADED C888 NLC	HIP LOADED PATO5 NLC
EE, %	67 $\pm$ 3	82 $\pm$ 4
D _L (μG/MG)	1.95 $\pm$ 0.8	2.54 $\pm$ 0.30

III.2. *In-vitro* lipolysis of NLC

The *in-vitro* lipolysis protocol was adapted from the recommendations of the LFCS consortium for 1 g SEDDS, to be in accordance with the prescribed conditions with nanosuspensions.

Traditionally, the extent of lipid digestion is evaluated by titration with the pH-stat maintaining the pH at 6.5. Indeed, lipase cleaves triglycerides in positions *sn1* and *sn3* of triglycerides, leading to the formation of one 2-monoglyceride and two fatty acids. These fatty acids will decrease the pH of the medium, giving the signal to pH-stat to readjust the pH by NaOH

addition. However, this technique was found to underestimate the extent of digestion as long fatty acids with high apparent pKa were not titrated. Furthermore, the back-titration method proposed to counteract this effect by dosing all the released fatty acids at pH 9 were found to overestimate the lipolysis of the formulations (Heider et al., 2016). Then, it was decided to use the technique proposed by Jannin et al. by directly dosing the glycerides composition of the medium at predefined times (Jannin et al., 2018).

Figure 9**Erreur ! Source du renvoi introuvable.** shows the composition in fatty acids, mono-, di- and triglycerides during the *in vitro* lipolysis of the C888 and PATO5 NLC. Only degradation products from the lipolysis of C888 and PATO5 excipients were taken into account in the calculations. Regarding the C888 NLC, the composition remained stable throughout the duration of the lipolysis experiment, which confirms the non-digestibility of C888 (Jannin et al., 2018). On the contrary, important changes can be observed in the glycerides composition of PATO5 NLC. Indeed, the percentages of di- and tri-glycerides has constantly decreased while fatty acids were produced to reach a final content of 55% of the total lipid content. However, after 1 hour in presence of pancreatin, triglycerides were still present in the medium (9%). Thus, although PATO5 NLC undergo a strong lipolysis, a small fraction remained intact after one hour in these harsh conditions.

Lipolysis of the loaded formulation could not lead to interesting results as the pancreatin used for the experiment also contains proteases which completely degraded LEU.

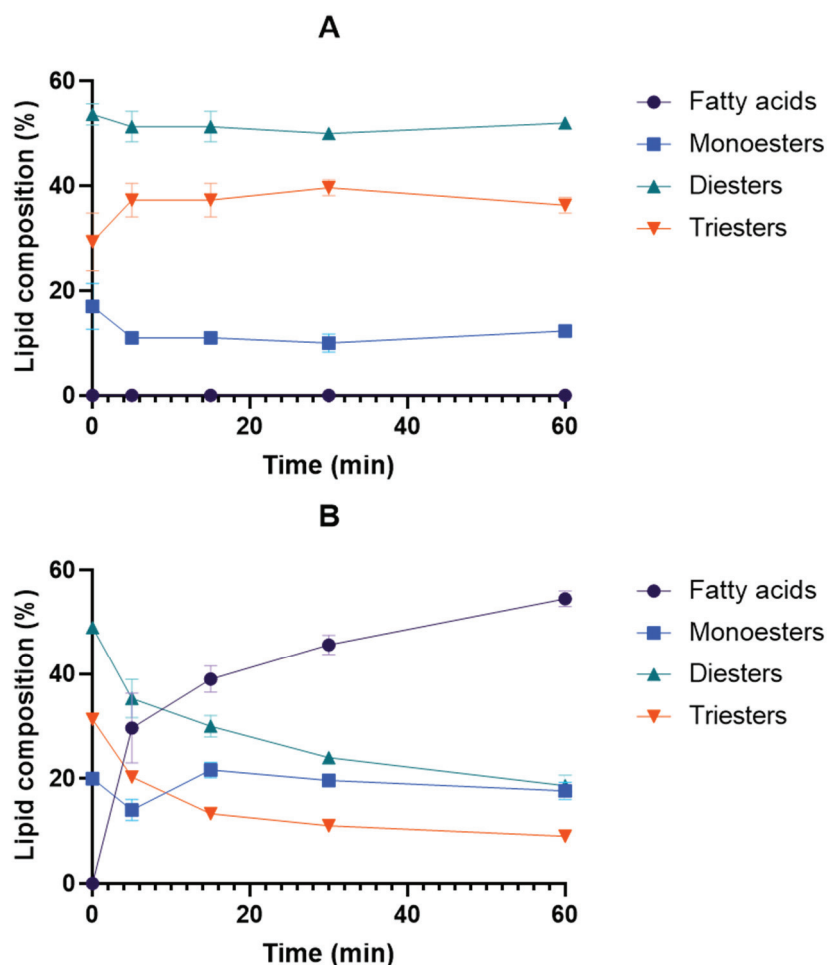


Figure 9: Evolution of ester composition during the in vitro dispersion/digestion of (A) C888 NLC and (B) Pato5 NLC (results expressed as mean $\pm$ SEM)

III.3. Perspectives

The digestion process completely or partially transforms the initial solid particles in mixed micelles of biliary salts and products of digestion. The fate of the encapsulated peptide is hard to anticipate in these conditions: is it completely released from the lipid structure or integrated in mixed micelles? Both options may influence the permeation properties of the peptide. Then, evaluate the permeation properties of the peptide contained in the digested formulation would provide valuable indications on the fate of the drug.

The LFCS consortium prescribes the use of pancreatic extract to conduct lipolysis studies which, in the present case, leads to two major problems. The first one is that the pancreatic extract not only contains the lipases involved in lipid digestion, but also proteases. These

proteases completely degrade the peptide released in the medium, especially in the conditions of the experiment which are more severe than the *in vivo* conditions. The second drawback is related to the instability of the Caco-2 cells in presence of the pancreatic lipase.

Keemink et al. recently proposed a solution to these issues by replacing pancreatic extract by Novozym® 435 (Novozymes, Denmark), a non-specific lipase originating from *Candida Antarctica B* immobilized on acrylic resin. The extent of digestion induced by both lipase was compared regarding the pH regulation, but not the effect on the ester composition. The authors proposed a setup, using this immobilized lipase, which enables simultaneous lipolysis of lipid-based formulations and evaluation of permeation on Caco-2 cell monolayers (Keemink et al., 2019).

After increasing the stability of the lipidized peptide, which should maintain the peptide inside the nanoparticles, the evaluation of the capacity of the nanoparticles (or their digested product) to improve peptide permeability should be conducted on a similar device as the one mentioned above. In particular, a recent review enlightens the lack of studies regarding the transportation of indigestible formulations (Wang and Luo, 2019). As *in vitro* lipolysis of blank PATO5 and C888 NLC has shown different behavior regarding lipolysis, both formulations loaded with the stabilized peptide should be used to evaluate the impact of lipolysis on its permeation rate.

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Discussion générale

Les peptides thérapeutiques permettent de traiter de manière efficace et sélective un grand nombre d'affections et présentent peu d'effets secondaires comparativement à d'autres substances actives d'origine chimique. En raison de leur faible stabilité en milieu acide, de la forte dégradation enzymatique dans le tractus gastro-intestinal et de leur faible perméabilité intestinale, la plupart de ces peptides sont administrés par voie parentérale. Cependant, même par ce mode d'administration, la demi-vie des peptides reste très faible. Les injections doivent être fréquemment répétées, parfois plusieurs fois par jour, pour atteindre l'effet thérapeutique recherché. A terme, cela peut générer une diminution de l'observance thérapeutique et *a fortiori* une réduction de l'efficacité du traitement.

Le but de ce projet de thèse est d'envisager l'encapsulation de peptides dans des nanovecteurs lipidiques solides comme stratégie de formulation pour en améliorer leur biodisponibilité orale.

La première partie de l'étude est consacrée à la conception des nanovecteurs. Le procédé d'homogénéisation à haute pression à chaud a été utilisé pour mettre au point des nanoparticules lipidiques solides (SLN) composées de glycérides d'acides stéarique et palmitique (Precirol® ATO5, glyceryl distearate), et d'huile de ricin hydrogénée éthoxylée (Kolliphor® RH40, PEG-40 hydrogenated castor oil) dans les pourcentages massiques 71 :29%. Le même procédé a été utilisé pour les vecteurs lipidiques nanostructurés (NLC), dans lesquels la phase lipidique est complétée par ajout de monocaprylate de propylène glycol (Capryol®90, propylene glycol monocaprylate, type II) pour une composition finale en pourcentage massique 68 :5 :27 % (Precirol®ATO5 : Capryol®90 : Kolliphor®RH40). L'optimisation des paramètres de procédé permet de formuler des nanoparticules d'une centaine de nanomètres, avec un indice de polydispersité (PDI) inférieur à 0,2. Cette méthode de formulation peut être adaptée en fonction du lipide solide utilisé. Ainsi des NLC de 120 nm sont également obtenus avec des glycérides d'acide béhénique (Compritol®888 ATO, glyceryl dibehenate) en optimisant les paramètres de procédé comme démontré Chapitre V.

La capacité d'encapsulation des NLC de Precirol®ATO5 a été évaluée pour deux peptides modèles hydrophiles : la desmopressine (DES) et le leuprolide (LEU). Afin d'augmenter leur lipophilie, et de fait leur affinité pour les nanovecteurs lipidiques, le docusate de sodium est utilisé pour former des paires d'ions hydrophobes (HIP). Les deux complexes sont encapsulés

dans les NLC sans dégradation des peptides et le taux d'encapsulation des HIP dans les NLC est fonction de la lipophilie (Log P) des paires d'ions.

Le Capryol®90 est choisi comme lipide liquide dans les NLC pour son pouvoir solvant des deux HIP. Cependant le taux d'encapsulation de la paire d'ion LEU-docusate est équivalent après dissolution dans le C90 et inclusion dans les NLC ou simplement dispersée dans la matrice solide (SLN). En revanche, pour les deux types de particules, une augmentation significative du taux d'encapsulation a été observée en utilisant les HIP avec plus de 75% du peptide encapsulé dans les nanovecteurs, soit une concentration finale en actif de 2.5 µg par mg de nanoparticules. Après lyophilisation, 500 mg de ces nanoparticules intégrées dans une capsule entérique de taille 0 serait une alternative au LEU actuellement disponible sur le marché sous l'appellation Leupron Depot® (Abbvie), destiné à une administration intra-musculaire mensuelle de 3.75 mg.

Dans un deuxième temps, les caractéristiques physico-chimiques des nanovecteurs ont été étudiées. La distribution en tailles des deux types de nanovecteurs, mesurée par diffusion dynamique de la lumière, montre que les nanoparticules mesurant entre 115 et 125 nm n'ont pas été affectées par l'encapsulation du LEU, sous forme hydrophobisé ou non. Aucune agrégation des nanoparticules n'est observée lors de leur dispersion dans les fluides gastro-intestinaux simulés (fluide gastrique simulé (SGF), fluide intestinal simulé à jeun (FaSSIF-V2) et fluide intestinal simulé nourri (FeSSIF-V2)).

L'analyse morphologique des nanoparticules par cryo-microscopie par transmission électronique (cryo-TEM) a confirmé une taille des nanoparticules de l'ordre de 100 nanomètres. Cette analyse met en avant la morphologie en plaquettes des nanoparticules, due à la rapide réorganisation des chaînes lipidiques de la forme α vers la forme cristalline β (Mehnert and Mäder, 2012). Cette morphologie en plaquettes, confère aux particules une importante surface spécifique et par conséquent une importante proportion de l'actif présent à la surface. Cette caractéristique des nanovecteurs lipidiques solides explique l'important relargage du principe actif observé lors de la dispersion des formules dans le milieu FaSSIF-V2.

Dans un troisième temps, l'effet protecteur des nanovecteurs lipidiques solides vis-à-vis de la dégradation protéolytique a été évalué en présence de trypsine et d' α -chymotrypsine. Seuls les NLC protègent le LEU d'une dégradation par la trypsine, particulièrement lorsque celui-ci

a été encapsulé sous forme d'HIP. Ces nanoparticules doublent la quantité de peptide présent dans le milieu d'incubation après 90 minutes comparativement au peptide en solution. En revanche, aucun des deux types de nanovecteurs lipidiques solides ne limite la dégradation du peptide par l' α -chymotrypsine.

L'effet protecteur des NLC vis-à-vis de la dégradation induite par la trypsine peut être attribué à la présence de la fraction lipidique liquide. On peut donc supposer qu'une augmentation de cette fraction permettrait d'augmenter l'effet protecteur, à condition que les nanoparticules soient stables. Dès lors, si l'augmentation de la quantité de lipide liquide dans les NLC est en effet proportionnelle à l'accroissement de l'effet protecteur vis-à-vis de l'activité des protéases, alors les SEDDS, complètement liquides, sont probablement plus adaptés pour cet effet (Hetényi et al., 2017). De plus, la structure sphérique des gouttelettes huileuses formées lors de la dispersion des formules dans les milieux aqueux offre une surface d'échange moins importante que les nanovecteurs lipidiques solides en forme de plaquettes et pourrait ainsi limiter la libération du peptide. Par ailleurs, les SEDDS en tant que forme pharmaceutique sont plus faciles à mettre en œuvre que les nanovecteurs lipidiques solides. En effet les SEDDS peuvent être directement mise en gélule ou capsule gastro-résistante tandis que pour les nanoparticules solides une étape supplémentaire de séchage (lyophilisation par exemple) est nécessaire afin de pouvoir les inclure dans une capsule ou gélule entérique ou dans des comprimés.

Néanmoins, l'utilisation des SEDDS requiert la dissolution de l'actif dans la formule. Or, dans le cadre des nanovecteurs lipidiques solides, il a été montré dans le chapitre III que les taux d'encapsulation du LEU dissous dans la phase lipidique liquide des NLC ou dispersé dans la matrice solide des SLN étaient équivalents. Dès lors les nanovecteurs lipidiques permettraient des taux d'encapsulation plus élevés par simple dispersion de la substance active dans la matrice lipidique sans passer par une étape préalable de dissolution dans une phase liquide lipidique. Il serait alors intéressant d'évaluer la capacité d'encapsulation maximale des SLN et des NLC. L'augmentation du taux d'encapsulation pourrait accroître la proportion de peptide maintenue au sein de la matrice lipidique lors de la dispersion des formules dans les milieux biologiques avec pour conséquences un accroissement de l'effet protecteur des nanovecteurs ainsi qu'une l'amélioration de la perméabilité intestinale. De plus, contrairement aux SEDDS,

les nanovecteurs lipidiques solides offrent la possibilité de greffer des ligands permettant le ciblage spécifique de sites d'actions thérapeutiques.

Dans un quatrième temps, la capacité des nanovecteurs lipidiques solides à améliorer le passage épithélial des peptides a été évaluée *in vitro* en utilisant des monocouches de cellules Caco-2 (modèle cellulaire de type entérocyte). L'effet du mucus sur le passage a quant à lui été évalué *via* des monocouches de Caco-2 et de HT29-MTX co-cultivées (modèle cellulaire sécrétant de la mucine). L'internalisation des nanoparticules contenant un fluorophore a été démontrée après incubation des formules avec les Caco-2. Ce même phénomène a été observé avec les co-cultures de Caco-2/HT29-MTX, démontrant la capacité des nanovecteurs lipidiques solides à franchir la barrière de mucus présente à la surface de ce modèle cellulaire. Néanmoins, malgré l'internalisation des nanoparticules par les cellules épithéliales, aucun bénéfice de l'encapsulation des peptides dans les nanovecteurs lipidiques solides n'a pu être montré. En effet, environ 90% du LEU encapsulé est libéré quasi instantanément dans le milieu HBSS utilisé pour évaluer la perméabilité intestinale. Cela s'explique par la combinaison de deux facteurs : l'importante surface d'échange des nanovecteurs lipidiques solides (NLC ou SLN) en forme de plaquettes avec le milieu environnant et la faible stabilité de la paire d'ion hydrophobe dans cet environnement présentant une forte charge ionique. En effet, la stabilité de l'HIP est gouvernée par la loi de Coulomb, présentée dans l'Équation 1, avec F la force d'attraction électrostatique, K la constante de Coulomb ($8.99 \cdot 10^9 \text{ Nm}^2\text{C}^{-2}$), r la distance entre les deux charges ponctuelles q1 et q2, et ϵ la constante diélectrique du milieu.

Équation 1: Loi de Coulomb

$$F = k \frac{q_1 q_2}{r^2 \epsilon}$$

La plupart des lipides liquides ont une faible constante diélectrique ($\epsilon < 5$) (Griesser et al., 2017; Phan et al., 2019). Dans cet environnement la force d'attraction électrostatique est importante et permet de maintenir la cohésion de la paire d'ion hydrophobe. En revanche, dans l'eau, cette constante diélectrique s'élève à 80 et facilite la dissociation des paires d'ions hydrophobes par diminution de F. Par conséquent, la stabilité des paires d'ions hydrophobes est maintenue dès lors qu'elles demeurent dans un environnement lipidique. Cependant, cette stabilité peut être perturbée par les entités ioniques présentes dans les fluides biologiques. Bien que ces ions soient généralement trop hydrophiles pour pénétrer les

nanovecteurs lipidiques, ils peuvent interagir avec les paires d'ions en surface des nanoparticules et favoriser la dissociation de l'HIP. En raison de l'importante force ionique dans les fluides biologiques, le phénomène de décomplexation semble inéluctable. Toutefois, Nazir et al. ont récemment démontré qu'une HIP LEU-acide pamoïque ($pK_a=2,7$) dans un tampon phosphate ($pH=7,4$, 154 mM NaCl) était relativement stable avec un taux de dissociation du complexe de seulement 25% après 6 heures d'incubation (Nazir et al., 2019). Cette stabilité est vérifiée lors des études de libération dans un milieu de force ionique importante dans lequel la majorité du peptide hydrophobisé (60%) est resté encapsulée dans les SEDDS. Elle peut s'expliquer par la présence de deux doubles cycles benzéniques, volumineux, capables de limiter les interactions entre les charges du peptide et les ions du milieu. Il serait intéressant d'évaluer dans notre cas si cette paire d'ion, plus stable en milieu ionique que l'association leuprolide-docusate, pourrait être davantage maintenue dans les nanovecteurs lipidiques solides.

Enfin, au cours de cette thèse des travaux ont été amorcés pour évaluer l'impact de la lipolyse des nanoparticules lipidiques sur le devenir des peptides encapsulés. Ces études ont montré que la digestibilité des formules pouvait être modifiée en jouant sur la composition lipidique des nanovecteurs. Ainsi la quasi-totalité des NLC composées de Precirol® ATO5 a été dégradée après une heure en présence de pancréatine tandis que les NLC de Compritol® 888 ATO n'ont pas été affectés par la présence des lipases pancréatiques. La mise au point de nouveaux modèles détaillés dans des publications parues récemment ouvre des perspectives pour la poursuite de cette étude.

L'un des objectifs de cette thèse était de comparer l'efficacité des NLC et des SLN dans le cadre d'une administration orale de peptides. Concernant l'efficacité d'encapsulation, les deux types de nanovecteurs ne sont pas différenciés avec des taux de chargement équivalents, que ce soit pour le peptide seul ou sous forme d'HIP. Néanmoins, sous forme de nanosuspensions dans l'eau, les SLN développées dans cette étude sont plus stables que les NLC qui présentent une exsudation de la partie lipidique liquide après quelques semaines à 4°C. D'autre part, il est démontré que les NLC protègent le peptide modèle encapsulé de la dégradation protéolytique induite par la trypsine tandis qu'aucun effet protecteur n'est mesuré pour les SLN. Concernant les interactions avec les cellules Caco-2, les NLC sont davantage internalisés par ces cellules mais possèdent une toxicité plus élevée que les SLN. Il n'est pas possible de

conclure sur la capacité des nanovecteurs à transporter le peptide encapsulé en raison du relargage brutal de l'actif dans les milieux utilisés.

Ainsi, bien que les NLC augmentent la stabilité du LEU vis-à-vis de la trypsine, l'absence d'augmentation nette de la perméabilité intestinale, rend difficile la comparaison entre les NLC ou les SLN pour la délivrance orale des peptides sans stabilisation préalable de la hydrophobisation. Les deux types de nanoparticules nécessiteront une opération de séchage pour pouvoir être administrées sous leur forme finale : dans une capsule entérique ou incluses dans un comprimé avec un pelliculage gastro-résistant.

Conclusions et perspectives

Ce travail de thèse a permis de mettre au point une stratégie de développement et d'évaluation de nanovecteurs lipidiques solides pour augmenter la biodisponibilité orale de peptides thérapeutiques modèles. De nombreux bénéfices de ces nanoparticules ont été mis en lumière, tels que la capacité à limiter la dégradation protéolytique et l'internalisation par les cellules épithéliales. Néanmoins, bien que la formation d'une HIP améliore le taux d'encapsulation dans ces nanovecteurs, une augmentation de sa stabilité dans les milieux biologiques est nécessaire afin de bénéficier pleinement des avantages liés à l'encapsulation dans ces nanoparticules.

Trois axes sont proposés pour la suite de ce projet : l'amélioration de la stabilité de la hydrophobisation des peptides dans les milieux biologiques, la modification de la formulation et l'évaluation de l'impact de la digestibilité des formules sur la biodisponibilité orale.

Afin d'augmenter les avantages liés à l'encapsulation des peptides thérapeutiques dans les nanovecteurs lipidiques solides, il est nécessaire d'assurer leur maintien dans ces nanoparticules. Pour cela plusieurs stratégies peuvent être envisagées :

- Former une HIP plus stable en milieu ionique en utilisant par exemple l'acide pamoïque à la place du docusate de sodium comme mentionné précédemment
- Jouer sur l'aptitude du contre-ion à former des liaisons hydrogènes avec le peptide. En effet, d'après la « règle de 5 » de Lipinski, la lipophilie n'est pas le seul facteur influençant la biodisponibilité orale de molécules thérapeutiques. En effet, celle-ci est également liée au nombre de liaisons hydrogènes que peuvent former une molécule et son environnement. Ainsi pour promouvoir la perméabilité d'un principe actif, le nombre de groupements accepteurs de liaisons hydrogène doit être inférieur à 10 et celui de donneurs de liaisons hydrogène doit être supérieur à 5. Par conséquent, lors de la formation d'une liaison non-covalente, le contre-ion ne devrait pas être choisi uniquement sur sa capacité à former une liaison électrostatique mais également sur son aptitude à former des liaisons hydrogènes avec le peptide, afin de limiter les interactions avec les milieux. Il pourrait ainsi être envisagé de combiner la formation de paires d'ions hydrophobes par interactions électrostatiques avec la formation de « paires de liaisons hydrogène hydrophobes » avant encapsulation dans des nanovecteurs lipidiques.

- Former une prodrogue : bien que cette méthode soit peptide-dépendante et nécessite la validation d'une nouvelle pro-droque, l'augmentation de la lipophilie des peptides par formation liaison covalente avec une entité hydrophobe doit être considérée. Toutefois, il est nécessaire de s'assurer que cette liaison sera clivée dans les milieux biologiques afin de garantir l'activité thérapeutique du peptide encapsulé. On peut par exemple envisager la formation d'une liaison covalente de type ester sur le groupement hydroxyle de la tyrosine par une réaction d'estérification avec un acide carboxylique à longue chaîne hydrocarbonée (ex : acide palmitique). Celle-ci pourrait facilement être hydrolysée *in vivo* par action de lipases pancréatiques. La formation d'une liaison covalente peut cependant engendrer de nombreuses étapes de réactions et de purifications supplémentaires générant un coût additionnel important lors de la mise au point du médicament.

Les formules de SLN et NLC développées et utilisées dans ces travaux de thèse pourraient être améliorées afin d'augmenter leurs bénéfices pour augmenter la biodisponibilité orale des peptides. Plusieurs aspects peuvent être considérer :

- Modifier la fraction lipidique liquide des NLC. En effet, les travaux ont mis en avant la capacité protectrice des NLC vis-à-vis de la dégradation protéolytique. Cet effet protecteur a été attribué à la présence de la fraction lipidique liquide dans laquelle les protéases ne sont pas solubles. On peut donc supposer que l'augmentation de la proportion de lipidique liquide dans ces nanoparticules accentuerait cet effet protecteur. Or, il a été montré dans le chapitre II que l'augmentation de la fraction de Capryol®90 déstabilisait les formules. Il est alors envisageable de changer d'excipient liquide afin d'augmenter la quantité de lipides dans les NLC. On peut ainsi considérer le Capryol® PGMC (propylène glycol monocaprylate, type I) dont la proportion plus importante en diesters moins hydrophiles peut maintenir l'excipient et l'HIP au cœur de la matrice lipidique. L'utilisation d'excipients lipidiques liquides plus hydrophobes (HLB=1) tels que le Peceol™ (glyceryl monooleate), la Maisine®CC (glyceryl monolinoleate) ou le Labrafac™ lipophile WL 1349 (medium chain triglycerides) pourraient également limiter le phénomène d'exsudation observé avec le Capryol®90.
- Modifier la forme des nanovecteurs lipidiques solides par mis au point d'autres procédés de formulation afin de les rendre plus sphériques diminuer la surface

d'échange de la particule. Cela nécessiterait d'optimiser la formulation ainsi que le procédé d'encapsulation par homogénéisation haute pression ou la mise au point d'autres procédés de fabrication.

- Evaluer la capacité de chargement maximal en peptides hydrophobisés des nanovecteurs lipidiques solides développés au cours de l'étude. En effet, la concentration en peptide hydrophobisé utilisé dans le cadre de l'étude a été déterminée par des mesures de solubilité dans les excipients lipidiques liquides. Or, il n'a pas été montré de bénéfice de cette pré-solubilisation vis-à-vis d'une dispersion comme c'est le cas pour les SLN. Dès lors, la capacité de chargement des vecteurs développés est probablement bien plus importante que celle qui a pu être mesurée.

Lors de l'utilisation de formulations lipidiques, la digestibilité des formulations est un facteur important à prendre en compte. Après optimisation de la stabilité du peptide hydrophobisé afin qu'il demeure dans les nanovecteurs lipidiques solides, il serait intéressant d'observer son devenir lors de la digestion des formules, et notamment d'étudier l'impact de la dégradation de la matrice lipidique sur la protection de l'activité protéolytique et le passage intestinal. Cette étude pourrait être menée *via* l'utilisation de modèles *in vitro* récemment développés offrant la possibilité d'évaluer l'impact de la digestibilité des formulations lipidiques sur la protection des peptides et sur leur passage intestinal. Les propriétés de formules digestibles composées de Precirol®ATO5 pourraient être comparées à celles de formules non-digestibles de Compritol®888 ATO (cf. Chapitre V).

La non-digestibilité de la formule peut garantir la protection du peptide dans le milieu duodénal, néanmoins il est tout de même nécessaire que l'actif soit libéré avant d'atteindre sa cible pour garantir l'effet thérapeutique. Cependant, seules des études *in vivo* de pharmacologiques avec un modèle animal adapté permettront de conclure sur la relation entre digestibilité de la formule et augmentation de la biodisponibilité orale.

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Nanovecteurs lipidiques pour la délivrance orale de peptides

Les peptides thérapeutiques peuvent traiter de nombreuses pathologies de manière efficace et sélective. Leur biodisponibilité orale est limitée par une forte dégradation liée à l'action de protéases dans le lumen intestinal et par une faible absorption par la barrière intestinale. Nous avons évalué la capacité des nanoparticules lipidiques solides (SLN) et des vecteurs lipidiques nanostructurés (NLC) à augmenter la biodisponibilité orale d'un peptide modèle : le Leuprolide (LEU). L'augmentation de sa lipophilie par formation d'une paire d'ions hydrophobe (HIP) avec le docusate de sodium permet d'augmenter significativement le taux d'encapsulation du LEU dans les nanovecteurs. Ceux-ci sont obtenus par homogénéisation haute pression avec une taille de 120 nm et une structure en plaquettes. Malgré leur stabilité dans les milieux gastro-intestinaux simulés, une libération importante de la substance active est observée dans le milieu intestinal à jeun. Vis-à-vis de la dégradation protéolytique, les NLC montrent une protection significative du LEU en présence de trypsine. L'évaluation du passage intestinal sur des monocouches de Caco-2 (modèle entérocyte) et de Caco-2/HT29-MTX (modèle sécrétant des mucines) révèle une internalisation des nanovecteurs mais aucune amélioration de l'absorption. En effet, la morphologie en plaquettes des SLN et NLC associée à la faible stabilité de l'HIP dans le milieu provoquent une libération importante du LEU, annulant la capacité de transport des nanovecteurs à travers la barrière intestinale. Il convient d'améliorer la stabilité de l'HIP pour augmenter la biodisponibilité orale des peptides *via* l'encapsulation dans des nanovecteurs lipidiques solides.

Mots-clés : Peptides, Oral, Nanoparticules Lipidiques Solides, Vecteurs Lipidiques Nanostructurés, Paire d'Ions Hydrophobe, Protéases, Perméabilité intestinale, Formulation

Lipid nanocarriers for oral delivery of peptides

Therapeutic peptides are able to treat a wide variety of diseases with selective and potent action. Their oral bioavailability is strongly limited by an important proteolytic activity in the intestinal lumen and poor permeation across the intestinal border. We have evaluated the capacity of Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid carriers (NLC) to overcome both oral bioavailability limiting aspects, using Leuprolide (LEU) as model peptide. Lipidization of LEU by formation of a Hydrophobic Ion Pair (HIP) with sodium docusate enables a significant increase of peptide encapsulation efficiency in both SLN and NLC. The nanocarriers, obtained by high pressure homogenization, measured 120 nm and were stable in simulated gastro-intestinal fluids. However, due to particles platelet-shape, an important quantity of LEU is released in simulated fasted state intestinal fluid. Regarding the protective effect towards proteolytic degradation, only NLC maintain LEU integrity in presence of trypsin. Intestinal transport, evaluated on Caco-2 (enterocyte-like model) and Caco-2/HT29-MTX (mucin-secreting model) monolayers, show nanocarriers internalization by enterocytes but no improvement of LEU permeability. Indeed, the combination of nanoparticles platelet-shape with the poor stability of the HIP in the transport medium induces a high burst release of the peptide, limiting nanoparticles capacity to transport LEU across the intestinal border. Stability of peptide lipidization needs to be improved to withstand biorelevant medium to benefit from the advantages of encapsulation in solid lipid nanocarriers and consequently improve their oral bioavailability

Keywords: Peptides, Oral delivery, Solid Lipid Nanoparticles, Nanostructured Lipid Carriers, Hydrophobic Ion Pair, Proteases, Intestinal permeability, Formulation

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