UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES CHIMIQUES

CNRS 7199 - Laboratoire de Conception et Application de Molécules Bioactives,
Équipe de Pharmacie Biogalénique

THÈSE

présentée par :

Asad Ur REHMAN

soutenue le : **17 Octobre 2019**

pour obtenir le grade de :

Docteur de l'université de Strasbourg

Discipline/ Spécialité : Sciences Pharmaceutiques

Développement de nouvelles stratégies pour la formulation et la fonctionnalisation de nano-transporteurs lipidiques, destinés à des applications biomédicales

THÈSE dirigée par :

Pr. VANDAMME Thierry	Professeur, Université de Strasbourg

Co-Directeur de Thèse

Dr. ANTON Nicolas	HDR, Université de Strasbourg

RAPPORTEURS :

Pr. OURJOUMTSEV Alexandre	Professeur, Université de Lorraine, Nancy (France)
Pr. BUKHARI Nadeem Irfan	Professeur, Université de Punjab, Lahore (Pakistan)

I would like to dedicate my thesis to my beloved parents, especially my deceased mother (may Allah keep her soul in eternal peace), family members, friends and teachers for their endless love, prayers, guidance and encouragement, which made me able to get such success and honor

TABLE OF CONTENTS

ACKNOWLEDGMENT

All praise is to Allah, the lord of the entire universe, the gracious and the merciful. First and foremost, I would like to thank Almighty Allah, for giving me opportunity, capability, courage and strength to undertake this research study and complete it satisfactorily. Without HIS blessings, no achievement would have been possible. This thesis in its current form is due to the support, guidance, encouragement and assistance of several people, and this is a good opportunity for me to express my sincere thanks to all of them.

I would like to express my gratitude to my advisor Prof. Thierry VANDAMME and co-adviser Dr. Nicolas ANTON for their heartfelt continuous support throughout all my PhD study and research. Their kind suggestions, guidance, immense knowledge, unwavering support, encouragement and constructive criticism helped me immensely during the project and enabled me to develop the skills needed through this journey. I would like to express my special appreciations and thanks to Dr. Nicolas Anton for his help in experimental designs, continuous follow-ups, proof reading of all my publications and also for the long discussions that helped me sort out the technical details of my work. I shall eternally be grateful to both of them for always being there to listen and give advice.

Here, I would avail this opportunity to state that I am also very thankful to Prof. Alexandre OURJOUMTSEV and Prof. Nadeem Irfan BUKHARI for accepting our request to evaluate this thesis. Their comments will certainly be an added value to our work.

I would like to thank Dr. Mayuel COLLOT for his valuable time, worthy advices, help in experimental design and insightful comments regarding this thesis. His cool mindedness and his calm approach are commendable. I am also very grateful to Eugenia BELCASTRO for her contribution in the *in vitro*, *ex vivo* and *in vivo* studies. Many thanks to our other collaborators, Prof. Valerie SCHINI-KERTH, Dr. Halina ANTON, Dr. Andrey KLEMCHENKO and Madame Nadia MESSADEQUE as well. I really appreciate their collaboration and the impressive work achieved together.

I would also like to take this opportunity to thank permanent staff of CAMB 7199, notably Nathalie HAMMERSCHMITT, Sophie SIEGEL and Frederique DINHOF for their help in administrative procedures.

Sincere thanks to the past and current labmates Ikram Ullah Khan, Mohamed Attia, Shukai Ding, Justine Wallyn, Trinh Thi Lan Nguyen, Salman Akram, Bilal Mustafa, Usama Jamshaid, Imtiaz Ali, Aidar Seralin, Xinyue Wang, Sidy Mouhamed Dieng, Papa Mady, Riccardo Pedron and Germain Brou, Alphonse Rodrigue and also Minjie Zhao for generously sharing their knowledge, for all the fun we have had all the time, besides the respect and love

for each other and interesting research collaboration. I have lots of memories here, which I will be cherishing for the rest of my life.

My acknowledgement would be incomplete without thanking my family. I would like to begin by thanking my parents, and I know any amount of gratitude shown to them is woefully inadequate. My father's unconditional support and help is largely the reason that I have managed to reach at this stage. No words are sufficient to describe my late mother's contribution to my life (may Allah keep her soul in eternal peace). I owe every bit of my existence to her. I would like to express my deepest gratitude to all my family members for their love, care, support and encouragement throughout my life.

Words are inadequate to express my feeling and to thank all my friends, here in Strasbourg, for their moral support, encouragement, love and care, which made this journey easier for me and made my stay in France a memorable experience of my life. I would also like to acknowledge the Higher Education Commission of Pakistan and Campus France for providing me the financial support to pursue my studies in France.

ABBREVIATIONS

Abbreviations:

DOX	Doxorubicin
CF	Carboxyfluorescein
NP	Nanoparticle
NE	Nano-emulsion
SLN	Solid lipid nanoparticles
NLC	Nanostructured lipid carriers
DM	Dendrimer
MRI	Magnetic resonance imaging
O/W	Oil-in-water
W/O	Water-in-oil
NC	Nano-carrier
α-TOS	alpha-tocopheryl succinate
MCT	Medium chain triglycerides
SCT	Short chain triglycerides
HLB	Hydrophilic lipophilic balance
PDI	Polydispersity index
SNEDDS	Self-nanoemulsifying drug delivery systems
PMAO	Poly (maleic anhydride-alt-1-octadecene)
OSA	Octadecyl succinic anhydride
J-1000	Jeffamine® M-1000
J-2000	Jeffamine® M-2070
POE	Polyoxyethylene
PIT	Phase inversion temperature
PEG	Polyethylene glycol
PPG	Polypropylene glycol
GI	Gastro-intestinal
MPS	Mononuclear phagocyte system
HPH	High pressure homogenization
NSAID	Non-steroidal anti-inflammatory drugs
PO/EO	Propylene oxide/Ethylene oxide
TEA/NEt3	Triethylamine
THF	Tetrahydrofuran

DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
TFA	Trifluoroacetic acid
HCl	Hydrochloric acid
PBS	Phosphate buffer saline
$CDCl_3$	Deuterated chloroform (Chloroform-d)
NMR	Nuclear magnetic resonance
TEM	Transmission electron microscopy
SOR	Surfactant-oil-ratio
POR	Polymer-oil-ratio
DLS	Dynamic light scattering
HATU	1-[Bis (dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3- oxid hexafluorophosphate
DMF	Dimethyl formamide
CY	Cyanine
NR	Nile red
WHO	World health organization
EC	Endothelial cell
NO	Nitric oxide
Ang II	Angiotensin II
ULV	Unilamellar vesicle
MLV	Multilamellar vesicle
LUV	Large unilamellar vesicle
SUV	Small unilamellar vesicle
PE	Phosphatidyl ethanolamine
DOPE	Dioleoyl phosphatidyl ethanolamine
POPE	Palmitoyl-oleoyl phosphatidyl ethanolamine
DMPE	Dimyristoyl phosphatidyl ethanolamine
CHOL	Cholesterol
MDR	Multidrug resistance
CHEMS	Cholestryl hemisuccinate

RÉSUMÉ DE THÉSE

1. Introduction:

La faible solubilité dans l'eau des médicaments a toujours été un sujet d'étude important, et rend difficile le développement de formes pharmaceutiques appropriées pour administrer des médicaments lipophiles au corps humain. Différents types de nano-cargos ont été mis au point, et parmi eux, ceux à base de lipides ont montré des résultats encourageants du fait de leurs caractéristiques particulières, telles qu'une capacité de charge élevée, une biocompatibilité élevée (non toxique, stable dans les milieux physiologiques, inerte dans les tissus), surface adaptable pour appliquer tout type de stratégie de ciblage et un profil amélioré de biodistribution et de pharmacocinétique.

Cette thèse porte sur le développement, la fonctionnalisation, la caractérisation et les applications biomédicales de nano-cargos lipidiques, notamment à base de nano-emulsions. Les nano-émulsions sont des émulsions fines, stables, généralement huile dans eau, optiquement claires et translucides, stabilisées par un agent tensioactif non ionique. La taille des gouttelettes est inférieure à 200-300 nm. Les nano-émulsions sont généralement formées en présence d'un tensioactif approprié et, parfois, un solvant organique est également utilisé lors de leur préparation (par exemple, dans le cas d'une émulsification spontanée). Ces deux facteurs pourraient, dans une certaine mesure, constituer une limitation en termes de biocompatibilité, de toxicité, de efficacité du la modification de surface, de développement d'incompatibilités avec le temps et parfois compliquent l'ensemble du processus [12]. Les nano-émulsions, dotées d'interfaces dynamiques liquide / liquide, sont généralement des systèmes très intéressants, mais qui entrainent des difficultés lorsque l'on souhaite contrôler avec précision leur chimie et composition de surface. Pour résoudre ce problème, nous avons développé une plateforme polymère capable de former des nano-émulsions stables en l'absence de tensioactifs et offrant une solution simple et efficace pour la fonctionnalisation des interfaces dynamiques liquide / liquide, pouvant être transposées en production à grande échelle. Dans cette thèse, j'ai présenté des nouvelles techniques pour développer et fonctionnaliser les nano-émulsions et encapsuler une grande variété d'agents de contraste, de ligands et d'anticorps, pouvant être liés de manière covalente à ces nano-émulsions. Enfin, nous avons étudié les applications de ces nano-transporteurs fonctionnalisés en surface comme systèmes d'imagerie, de délivrance thérapeutique et de délivrance ciblée de médicaments par des études *in vitro*, *ex vivo* et *in vivo*.

Parallèlement aux études mentionnées ci-dessus, ce travail de thèse de doctorat décrit le développement de liposomes sensibles au pH en tant que transporteurs de médicaments anticancéreux. Les liposomes sont considérés comme le type le plus avancé de vecteurs de médicaments et ont gagné en importance en tant que système de distribution de médicaments traditionnel en raison du fait que des médicaments hydrophiles, lipophiles et amphiphiles peuvent être piégés dans les liposomes [3,4]. Les liposomes sensibles au pH sont d'une importance primordiale car ils subissent une transition de phase et acquièrent des propriétés fusogéniques en milieu acide, ce qui entraîne la libération de leur contenu aqueux, ce qui en fait des supports idéaux pour la délivrance de médicaments anticancéreux, puisque le pH extracellulaire des tissus cancéreux étant légèrement acide en raison de la forte activité métabolique des cellules cancéreuses [5]. Cette étude examine le rôle respectif des paramètres de formulation dans le développement de liposomes sensibles au pH afin d'optimiser les conditions permettant une encapsulation efficace d'un médicament anticancéreux, la doxorubicine (DOX)[6].

2. Résultats et discussions:

L'étude commence par la conception et la synthèse de polymères amphiphiles spécialement conçus pour présenter une propriété tensioactive importante, suivie de l'évaluation de sa capacité à générer des nano-émulsions par la méthode d'émulsification spontanée en l'absence de tensioactif et aussi de solvant organique [7,8]. La synthèse de polymère amphiphile repose sur la modification du poly(anhydride maléique-alt-1-octadécène) (PMAO), qui est un polymère hydrophobe à chaînes octadécènes aliphatiques (C18) à fonctions réactives anhydride, en effectuant une réaction systématique de Jeffamine ® Polyétheramine (copolymère polyéthylène glycol / polypropylène glycol) sur chaque fonction anhydride, afin de générer un motif octadécène / Jeffamine® sur chaque élément monomère (fig.1a). Il en résulte un polymère parfaitement conçu pour stabiliser les interfaces huile / eau, à la manière d'un assemblage de tensioactifs non ioniques. En parallèle, nous avons conçu des amphiphiles similaires à J1000 et J2000, mais en utilisant le monomère parent de PMAO, à savoir l'anhydride octadécylsuccinique (OSA) (fig.1b) au lieu de PMAO, et avons mené une étude comparative des capacités relatives du monomère et du polymère à former des nano-émulsions et également leur impact sur la taille des nano gouttelettes.

Un objectif secondaire était d'identifier les principaux paramètres ayant un impact sur le processus d'émulsification spontanée et, à cette fin, différents paramètres de formulation, chimiques et physico-chimiques inhérents au processus d'émulsification, ainsi que leur

impact sur les propriétés, la taille et la polydispersité des gouttelettes ont été soigneusement analysées. Les résultats ont montré que le polymère amphiphile ainsi que le monomère provoquaient la formation de nano-émulsions stables et parmi toutes les conditions explorées, les meilleurs résultats ont été obtenus, en termes de taille moyenne et de PDI, avec l'amphiphile de PMAO lorsqu'il était utilisé avec une Jeffamine® plus longue (c'est-à-dire, J-2000) (fig.1b). Il a en outre été observé que la taille des gouttelettes formées pouvait varier de manière significative avec l'évolution de la nature de l'huile. Le processus d'émulsification spontanée est lié aux affinités du polymère ou monomère amphiphile, pour l'huile et l'eau. Dans le cas du polymère, l'originalité réside dans sa non-dispersion dans l'eau et sa précipitation dans la région interfaciale, formant une coquille sphérique, confirmée par microscopie électronique à transmission (TEM). La nouveauté de ce travail réside dans le fait que l'émulsification spontanée n'a jamais été rapportée avec un polymère seul en l'absence de surfactant. Il constitue une base de travail intéressante pour la fonctionnalisation des gouttes.

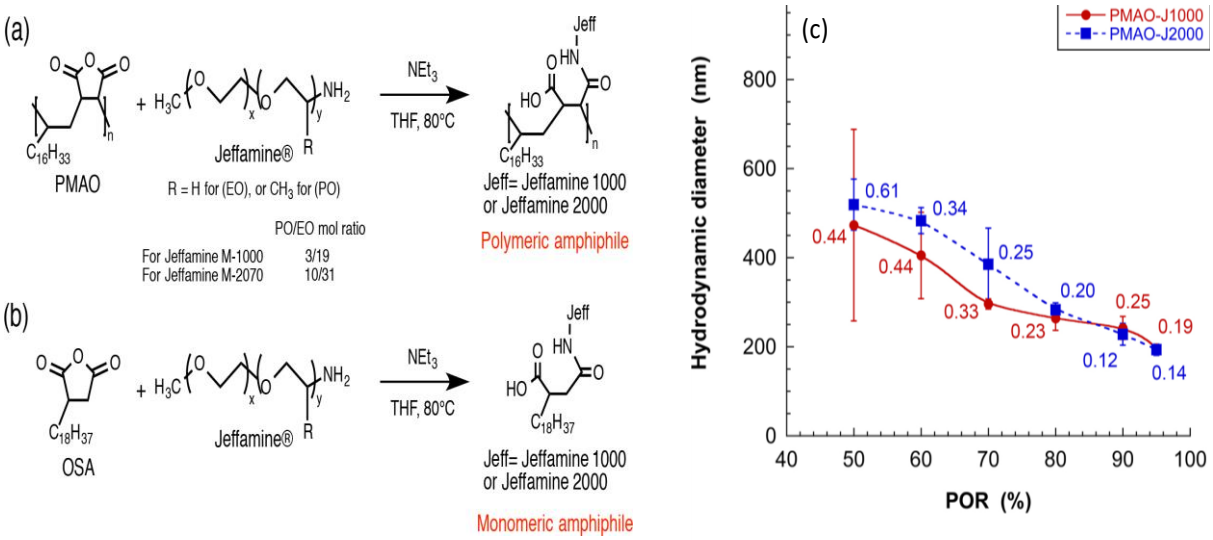


Figure 1: (a) Synthèse de polymères amphiphiles, (b) Synthèse de monomère amphiphile, (c) Effet du rapport polymère sur huile (POR) sur la taille moyenne des nano gouttelettes.

Au-delà d'une telle description pionnière de la nano-émulsification spontanée réalisée en utilisant uniquement des molécules polymère, cette étude propose plusieurs idées nouvelles sur la manière potentielle de conduire à la fonctionnalisation des nano-émulsions. À cette fin, nous avons utilisé le même polymère amphiphile avec une légère modification de la structure, c'est-à-dire qu'une partie (20%) de la Jeffamine® utilisée ici a été remplacée par un autre composé contenant du polyéthylène glycol (PEG) terminé par un réactif (par exemple : NH_2-PEG_4-N_3) va potentiellement réagir spécifiquement avec certains ligands. Les nano-émulsions ont été préparées à l'aide de ce polymère nouvellement synthétisé, par ultrasons, avec un

agent de contraste lipophile (dérivé du Nile red (NR668) ou du cyanine 5.5 (CY5.5)) encapsulé à l'intérieur des nano-gouttelettes. Ces nano-émulsions ont ensuite été incubées avec un ligand, à savoir le DBCO-PEG4-biotine, ce qui a permis d'établir la liaison covalente entre le ligand et la surface des nano-gouttelettes. Différentes techniques ont été utilisées pour caractériser ces formulations de nano-émulsions, à savoir la diffusion dynamiques de la lumière (DLS), la spectroscopie UV-visible, la microscopie électronique à transmission (TEM), la résonance magnétique nucléaire (RMN) et l'électrophorèse sur gel. Les résultats ont montré que ces systèmes de nano-émulsions avaient tendance à se lier de manière covalente à de tels ligands (biotine) et pouvaient être utilisés comme supports efficaces de médicaments pour la délivrance ciblée. Une incubation avec une cible modèle marquée (streptavidine) a permis de montrer la décoration de ces molécules sur la surface des nano-émulsions, et ainsi de prouver le greffage des ligands et les interactions spécifiques.

Après avoir obtenu des résultats prometteurs pour la fixation de ligands modèles à la surface des nano-émulsions, nous avons ensuite exploré le potentiel de ces nano-transporteurs spécialement conçus pour l'imagerie et les applications biomédicales. Notre objectif principal était de tirer parti des caractéristiques uniques de la membrane cellulaire des cellules endothéliales sénescentes (CE) pour l'évaluation précoce *in vivo* des sites artériels à risque, ainsi que pour l'administration sélective d'agents à des fins préventives et thérapeutiques, afin de restaurer des conditions vasculaires optimales de protection par une approche théranostique et de nano-médecine [9,10]. À cette fin, des nano-gouttelettes ont été développées et décorées avec le polymère à base de PMAO décrit ci-dessus, mais contenant cette fois une fonction maléimide décorant les gouttelettes, pour réagir avec la fonction cystéine d'un anticorps (anti-VCAM pour cibler les cellules sénescentes). Les formulations ont été optimisées en termes de rapport lipide / polymère de revêtement, PEG / PEG-anticorps, optimisation des sites fonctionnels, taille du nano-porteur et stabilité en température. La formulation a également été optimisée en relation étroite avec les résultats de ciblage *in vitro*, réalisés avec des protéines membranaires de ciblage d'anticorps (VCAM-1) d'EC sénescentes (fig. 2). Les nano-gouttes ont été chargées avec une sonde fluorescente (CY5.5 et NR668) pour permettre la détection des cellules endothéliales sénescentes, couvrant des sites athéro-sensibles à risque, ainsi qu'avec un agent vasoprotecteur thérapeutique (pour la délivrance et la régénération de la fonction endothéliale native).

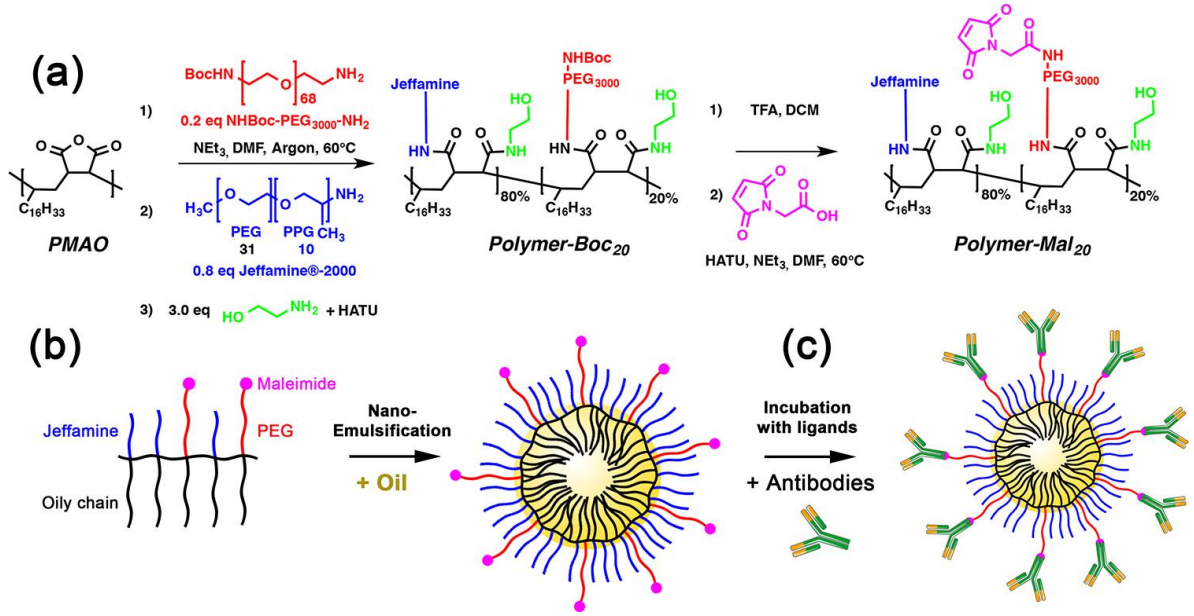


Figure 2: (a) synthèse du polymère, (b) préparation de nano-émulsions, et (c) fixation de l'anticorps à la surface de la nano-gouttelette.

L'évaluation biologique des nano-transporteurs théranostiques a été réalisée à trois niveaux différents, c'est-à-dire *in vitro* sur des cellules endothéliales en culture, *ex vivo* sur des segments artériels isolés à risque faible et élevé et *in vivo* sur des rats présentant un vieillissement artériel et caractéristiques de leur potentiel de régénération. Les résultats obtenus avec des études *in vitro* et *ex vivo* ont montré que les nano-transporteurs théranostiques développés ciblent spécifiquement (en une heure, et à la température corporelle) les cellules endothéliales sénescentes natives et modifiées (par des inducteurs pro-sénescents, angiotensine II) (figure 3) et que les nano-porteurs théranostiques s'accumulent de préférence sur des sites bien définis, caractérisés par un écoulement perturbé et un faible cisaillement (*i.e.* correspondant aux zones à risque de développement de plaques d'athéromes).

Les études *in vivo* sont toujours en cours et on s'attend à ce que l'administration de nano-cargos théranostiques ait une incidence minime sur les paramètres hémodynamiques et suite à l'accumulation locale, et délivreront sur le site l'agent thérapeutique contribuant à restaurer la fonction endothéliale et l'homéostasie vasculaire grâce à l'action combinée de normalisation du niveau du stress oxydatif et des gènes sensibles à l'oxydoréduction.

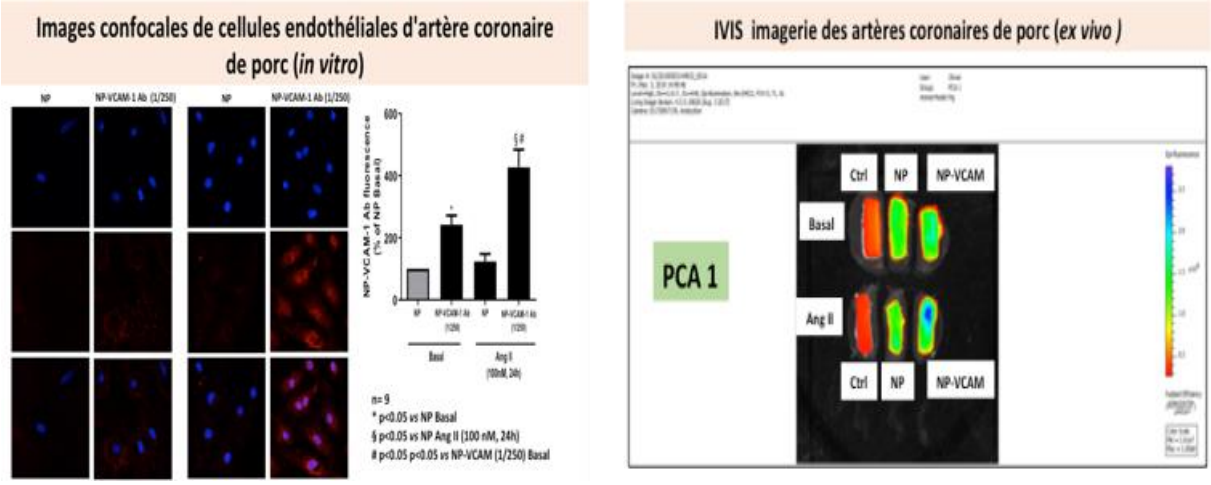


Figure 3: Imagerie in vitro et ex vivo

Parallèlement à l'étude susmentionnée, nous avons mis au point des liposomes sensibles au pH en tant que nano-transporteurs d'un médicament anticancéreux, la doxorubicine (DOX). La condition la plus importante pour ces liposomes est qu'ils soient stables au pH physiologique et qu'ils perturbent les milieux légèrement acides, tels que le microenvironnement tumoral, pour libérer leur charge de DOX. Le principal défi pour encapsuler la DOX dans les liposomes sensibles au pH réside dans le fait que ce médicament est soluble à pH faible (lorsque les liposomes sensibles au pH ne sont pas stables), mais que la solubilité aqueuse de la DOX diminue dans les conditions de pH correspondant à la stabilité des liposomes. L'étude de la sensibilité des liposomes au pH a été réalisée en utilisant de la carboxyfluorescéine (CF) encapsulée à une concentration élevée, conditions dans lesquelles la fluorescence de la sonde fluorescence est auto-desactivée, et qui augmentera lorsque le liposome sera détruit et le colorant libéré. Il s'agit d'un capteur de l'intégrité du liposome. Différents lipides (dioléoyl phosphatidyl éthanolamine (DOPE), palmitoyl-oléoyl phosphatidyl éthanolamine (POPE) et dimyristoyl phosphatidyl éthanolamine (DMPE)) ont été utilisés, associés à l'alpha-tocophéryl succinate (α-TOS) en tant qu'agent stabilisant pour la préparation de liposomes par extrusion manuelle sur membrane de polycarbonate. L'impact de (i) la nature chimique des lipides et (ii) du rapport lipide / agent stabilisant sur la sensibilité des liposomes au pH a été étudié. Ces combinaisons « lipides / α-TOS » se sont révélées former des liposomes stables à pH 7,4, avec une efficacité d'encapsulation maximale obtenue avec le POPE. L'intégrité des liposomes était significativement dépendante du pH avec une modulation possible en fonction de la concentration de α-TOS. Lorsque la concentration en α-TOS augmente, la dégradation des liposomes et la libération de CF est ralentie. La durée d'incubation a également un impact sur la libération de la sonde, car une incubation plus

longue augmente l'ampleur de la perturbation de la membrane des vésicules. Des formulations de liposomes optimisées ont ensuite été sélectionnées pour l'encapsulation de DOX par une procédure de chargement actif, c'est-à-dire entraînées par une différence de pH à l'intérieur et à l'extérieur des liposomes. De nombreuses conditions expérimentales ont été explorées, en fonction du gradient de pH et de la composition des liposomes, ce qui a permis d'identifier des paramètres critiques pour une encapsulation efficace de la DOX dans des liposomes sensibles au pH. Les valeurs du gradient de pH se sont révélées être un paramètre crucial du chargement de DOX. Il a en outre été observé que l'efficacité d'encapsulation de la DOX dans les liposomes sensibles au pH dépend de la nature du lipide -PE (plus élevée avec le POPE). Et parmi différentes formulations liposomales de POPE, on obtient des efficacités d'encapsulation plus élevées avec les combinaisons α-TOS et α-TOS / CHOL par rapport aux combinaisons CHEMS et CHEMS / CHOL. De plus, en fonction de la composition, de la présence de cholestérol augmente également l'encapsulation de la DOX et contribue à la rétention de la DOX à l'intérieur des liposomes.

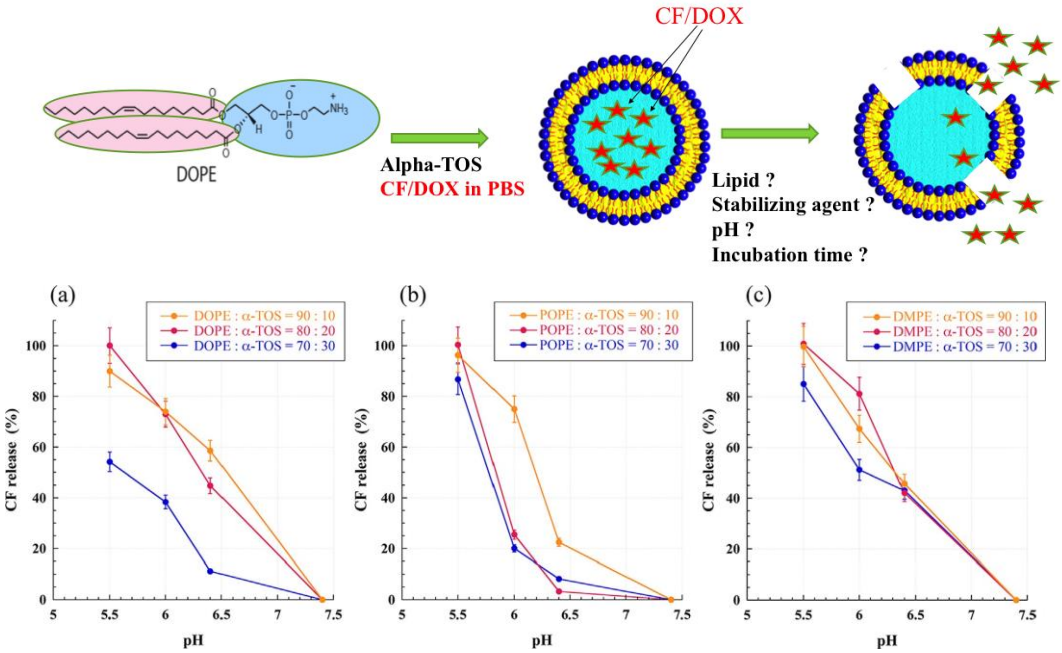


Figure 4: Synthèse et caractérisation de liposomes sensibles au pH

La dernière partie de cette thèse vise à combiner la modification de surface des nano-émulsions (avec le polymère basé sur le PMAO) avec la formulation de liposomes. Les liposomes ont donc été fabriqués par une technique d'extrusion membranaire en polycarbonate en utilisant la dioléoyle phosphatidylcholine (DOPC) contenant le polymère fonctionnel. Les premiers résultats sont encourageants et vont dans le sens de ceux obtenus avec les nano-émulsions.

3. Conclusion:

Les gouttelettes de nano-émulsion sont des nano-cargos à fort potentiel pour des centaines d'applications en raison de leur capacité à encapsuler des médicaments lipophiles. Les principaux inconvénients étant la difficulté de leur modification de surface. La solution présentée ici illustre bien que des solutions simples peuvent exister, être facilement développées et transposées à la production à grande échelle. Nous avons développé et optimisé les nouvelles techniques pour formuler et fonctionnaliser les nano-émulsions et avons montré avec succès que ces nano-émulsions fonctionnalisées offraient une plateforme pour une grande variété d'agents de contraste, de ligands et d'anticorps, pouvant être liés de manière covalente à ces nano-émulsions, en faisant des outils de ciblage pour des applications en imagerie et en thérapeutiques. Les résultats obtenus avec des études *in vitro* et *ex vivo* ont montré que les nano-cargos théranostiques développés ciblent spécifiquement (dans l'heure à la température corporelle) les cellules endothéliales sénescentes. La nouveauté du projet est de cibler très tôt le processus d'athérogénèse, caractérisé par l'induction de la sénescence et du dysfonctionnement endothélial en tirant parti des caractéristiques de surface distinctes apparaissant à la surface des cellules sénescentes, telles que l'expression des molécules d'adhésion VCAM-1 et ICAM-1. Le potentiel diagnostique et thérapeutique de l'approche théranostique peut être augmenté en utilisant un mélange de nano-transporteurs ciblant plusieurs caractéristiques distinctes de la surface cellulaire des CE sénescentes.

4. Références:

1. Hou, W. & Xu, J. Surfactant-free microemulsions. *Current Opinion in Colloid & Interface Science* **25**, 67–74 (2016).
2. Kaci, M. *et al.* Efficiency of emulsifier-free emulsions and emulsions containing rapeseed lecithin as delivery systems for vectorization and release of coenzyme Q10: physico-chemical properties and in vitro evaluation. *Colloids and Surfaces B: Biointerfaces* **147**, 142–150 (2016).
3. Sapra, P. & Allen, T. M. Ligand-targeted liposomal anticancer drugs. *Progress in Lipid Research* **42**, 439–462 (2003).
4. Eloy, J. O. *et al.* Liposomes as carriers of hydrophilic small molecule drugs: Strategies to enhance encapsulation and delivery. *Colloids and Surfaces B: Biointerfaces* **123**, 345–363 (2014).
5. Simões, S. On the formulation of pH-sensitive liposomes with long circulation times. *Advanced Drug Delivery Reviews* **56**, 947–965 (2004).
6. Gerweck, L. E. The pH difference between tumor and normal tissue offers a tumor specific target for the treatment of cancer. *Drug Resistance Updates* **3**, 49–50 (2000).
7. Anton, N., Benoit, J.-P. & Saulnier, P. Design and production of nanoparticles formulated from nano-emulsion templates—A review. *Journal of Controlled Release* **128**, 185–199 (2008).
8. Anton, N. & Vandamme, T. F. The universality of low-energy nano-emulsification. *International Journal of Pharmaceutics* **377**, 142–147 (2009).
9. Warboys, C. M. *et al.* Disturbed Flow Promotes Endothelial Senescence via a p53-Dependent Pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology* **34**, 985–995 (2014).
10. Abbas, M. *et al.* Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging and Thrombogenicity: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways. *Circulation* **135**, 280–296 (2017).

AIMS AND OBJECTIVES

This thesis focuses on the development, functionalization, characterization and biomedical applications of two most important types of lipid nano-carriers, namely nano-emulsions and liposomes. The overall research study can be classified into two projects, which are:

1) Nano-emulsions as carrier of lipophilic molecules for biomedical applications
2) Liposomes as carrier of drug molecules for biomedical applications

Project 1: Nano-emulsions as carrier of lipophilic molecules for biomedical applications

The main objectives of this study are as follows,

Objective 1: Development of a new strategy to formulate nano-emulsions in the absence of the surfactant as well as organic solvent

Nano-emulsions are fine, stable, and in general optically clear and translucent oil-in-water or water-in-oil dispersions, stabilized by a nonionic surfactant, with droplets size ranging below 200-300 nm. Nano-emulsions are generally formed in the presence of a suitable surfactant and sometimes an organic solvent is also used during their preparation (*e.g.*, in the case of spontaneous emulsification), both these factors could, constitute a limitation in terms of biocompatibility, efficiency of the surface modification, toxicity, need of additional steps (to remove solvent), development of incompatibilities with time *etc*. The first objective of my research project is to design an amphiphilic polymer, specially tailored to exhibit an important surface-active property. Then, this polymer will be used to generate nano-emulsions through the spontaneous emulsification method, in the absence of a surfactant as well as an organic solvent, in order to overcome all the incompatibilities and limitations associated with their incorporation in the preparation of nano-emulsions. A secondary objective of this study is to identify the main parameters having an impact on the spontaneous emulsification process by analyzing the effects of different chemical, physico-chemical and formulation parameters (inherent to the emulsification process) on the properties, size and polydispersity of the formed droplets.

Objective 2: Preparation and characterization of tunable surface functionalized oil-in-water nano-emulsions

Nano-emulsions, having dynamic liquid / liquid interfaces, are generally very powerful systems, but suffer from difficulties to precisely control the chemical modification of their surface. The second objective of this project is to perform tunable functionalization of the

nano-emulsions by developing a polymer derivative, capable of forming stable nano-emulsions and providing an easy but very efficient solution to functionalize the dynamic liquid / liquid interfaces and can be transposed to large scale production.

Objective 3: Development of a novel theranostic approach to target endothelial senescence: Nano-emulsions as targeted theranostic nano-carriers

The next objective of our study is to develop nano-emulsions and analyze their potential for imaging and targeted drug delivery applications. Firstly, different kinds of contrast agents and therapeutic agents will be encapsulated in the nano-emulsions, along with grafting of the ligands and antibodies at their surface. And then, their potential as theranostic nano-carriers will be evaluated by *in vitro*, *ex vivo* and *in vivo* studies.

Project 2: Liposomes as carrier of drug molecules for biomedical applications

The main objectives of this research project are as follows,

Objective 1: Development and characterization of pH-sensitive liposomes

Liposomes are considered as the most advanced type of particulate drug carriers and have gained importance as the mainstream drug delivery system because of the fact that hydrophilic, lipophilic as well as amphiphilic drugs can be entrapped in the liposomes. The pH-sensitive liposomes undergo phase transition and acquire fusogenic properties in acidic environment, leading to the release of their aqueous contents, which makes them ideal carriers for delivery of anticancer drugs since the extracellular pH of cancer tissues is slightly acidic due to the high metabolic activity of cancer cells. The objective of this study is to develop pH-sensitive liposomes and to study the impact of (i) the chemical nature of lipids and (ii) the lipid/stabilizing agent ratio on the pH sensitivity of the liposomes.

Objective 2: Encapsulation of anti-cancer drugs in pH-sensitive liposomes

Optimized liposomal formulations will be selected for the encapsulation of an anti-cancer drug, doxorubicin (DOX). The major challenge for encapsulating DOX in pH-sensitive liposomes lies in the fact that this drug is soluble at low pH (when the pH-sensitive liposomes are not stable), but the DOX aqueous solubility decreases in the pH conditions corresponding to the stability of the pH-sensitive liposomes. Numerous experimental conditions will be explored, in function of the pH gradient and liposome composition (nature and amount of the

lipid as well as stabilizer) to identify critical parameters for the efficient DOX encapsulation in pH-sensitive liposomes.

CHAPTER ONE

INTRODUCTION TO LIPID NANO-CARRIERS

1. Introduction

The word "Nano" is a Greek word and refers to tiny dimension beyond the limit of visibility for naked eye. It makes all objects described with "nano" as nanometric-sized entity. The term nanoparticle (NP) was used for the first time during the 1980s.[1] Since then, great interests have developed from the scientific community with the development of nanotechnology devoted to nanoscience and nanomedicine application. Nanoparticles have been developed with different kinds of materials such as organic, inorganic, hybrid, alloy, composite etc. and are usually produced in different morphologies like spherical, rods, cylinders, stars, cages etc. The most interesting features of NPs are their smaller sizes and high surface-to-volume ratio that offer large surface available for electrostatic attachment /anchoring of ligands or specific moieties, to design NPs for vast variety of applications.

The low-aqueous solubility and low bioavailability of the drugs have always been serious concerns because it creates many problems in developing a suitable dosage form to deliver lipophilic drugs to the human body. As many of the newly discovered drugs (about 40%) have low solubility in water or are completely hydrophobic, explaining why the research interest has been developed, for the past couple of decades, to design relatively inert and non-toxic carriers of drugs which can deliver the drugs efficiently to the living cells. Drugs can be loaded onto or into the core-shell assembly of the nanocarriers via encapsulation and surface attachment or entrapment. Different kind of nanocarriers have been developed but lipid-based nanocarriers have gained much importance for the delivery of the lipophilic drugs because of their special characteristics like, high drug loading capacity, high biocompatibility (non-toxic, stable in physiological media, inert to tissue), tailorable surface to apply any kind of targeting strategy and an improved biodistribution and pharmacokinetic profile.

2. Types of lipid nanocarriers

The most commonly employed lipid nanocarriers are liposomes, nano-emulsions, hybrid lipid / polymeric NPs (HPNPs), solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and dendrimers.

2.1. Polymeric and hybrid nanoparticles

Polymeric nanoparticles (PNPs) are submicron-sized colloidal particles and have attracted considerable interest over the last few years due to their unique properties and behavior resulting from their small size. Several synthetic or natural polymers have been used for the

preparation of PNPs, such as proteins, sugars or other natural macromolecules, biodegradable polymers and non-biodegradable, but pharmaceutically acceptable polymers. Polymer-based nanocarriers can be formulated as nanospheres and nanocapsules (figure 1). The first design is a rigid construct with an insoluble polymeric matrix as a core and the drug molecule can be directly grafted onto the polymer backbone either attached onto the surface or embedded within the matrix. In the case of nanocapsules, the inner core is a liquid phase surrounded by a rigid crosslinked polymeric membrane. As the particle morphology integrates a lipid core, their structure can be considered as polymer / lipid hybrid, and can serve as good carriers for lipophilic drugs. The outer hydrophilic part can be used as a stimuli-responsive block in order to promote controlled release of drug under specific conditions (like pH, temperature, redox, external conditions). The PNPs and HPNPs are widely used as theranostics and as targeted drug delivery systems. The targeted PEGylated polymeric micelles have been developed as a theranostic platform with SPIONs and doxorubicin co-loading for drug delivery to cancer cells and MRI monitoring.[2]

The PNPs have following major advantages,

- Allow controlled release of drugs
- Biodegradable and biocompatible
- Effective endosomal escape
- Protection of drug molecules
- Improve the therapeutic index of drugs

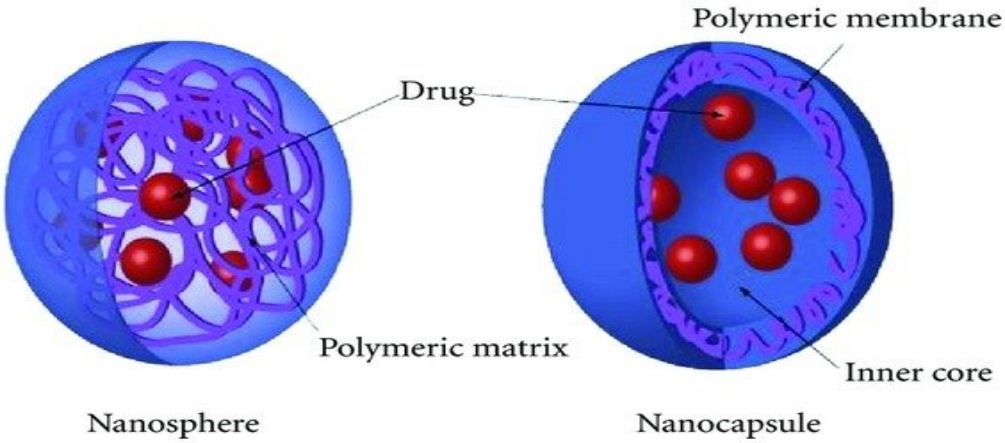


Figure 1. Structure of Polymeric nanoparticles (adapted from reference[3])

2.2. Solid lipid nanoparticles (SLNs) and Nanostructured lipid carriers (NLCs)

Solid lipid nanoparticles (SLNs) were introduced first time in early 1990s as novel drug carrier systems placing themselves at the interface between the already existing lipid systems (emulsions and liposomes) and polymeric nanoparticle systems because SLNs may combine interesting properties of both of these already existing systems. SLNs are produced by replacing the liquid lipid (oil) of an o/w emulsion by a solid lipid. The mean particle size of SLN is in the submicron rage, ranging from about 40 to 1000 nm.[4] The stability of the system is usually ensured by a surfactant. Solid lipid nanoparticles (SLN) are distinguishable from nanostructured lipid carriers (NLC) by the composition of the solid particle matrix. The NLC often referred to as the second generation of SLNs, were developed by Muller *et al*. in the late 1990s to overcome some potential limitations associated with SLN. NLCs typically range in size from 100 to 500 nm and are produced using blends of solid lipids and liquid lipids (oils), preferably in a ratio of 70:30 (up to a ratio of 99.9:0.1). As compared to the SLNs, NLC show higher drug loading capacity, lower water content of the particle suspension and avoid/minimize potential release of active compounds during storage. The overall solid content of NLC could be increased up to 95%. The hydrophobic cores of SLNs and NLCs provide a suitable environment for entrapment of hydrophobic drugs. The SLNs and NLCs can be prepared by using different techniques, however in cosmetic and pharmaceutical industries, they are most commonly prepared by the high-pressure homogenization technique.[4] Due to the production of lipid nanoparticles from physiological and/or biodegradable lipids, NLCs display a good biocompatibility and tolerability. SLNs and NLCs have been mostly used and are well known for oral and topical applications but recent studies have confirmed their potential as anticancer drug delivery vehicles as well.[4–7]

SLNs and NLCs exhibit many advantages over other nano carriers, which include

- Biocompatibility
- Increased penetration and hydration of the skin
- Drug protection against degradation
- Higher drug loading capacity
- Controlled drug release
- Occlusive properties
- The possibility to scale up

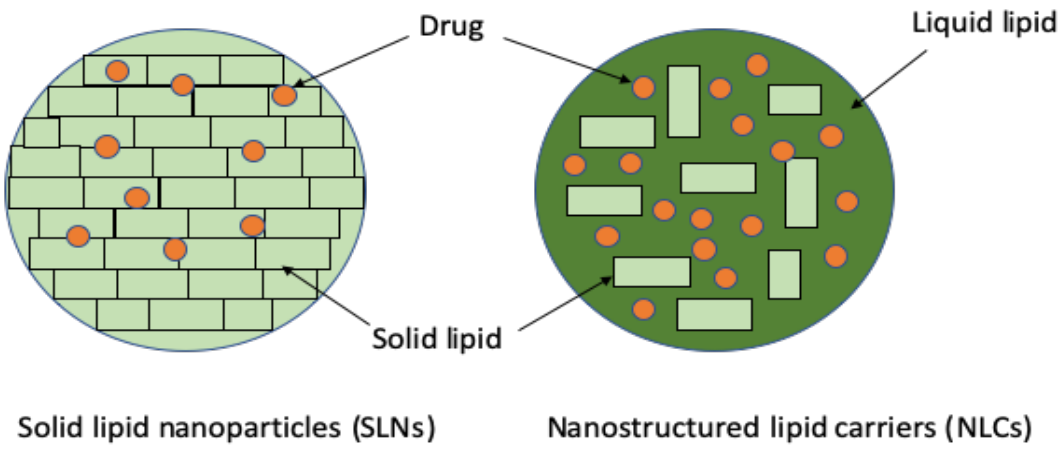


Figure 2. Structure of SLNs and NLCs

2.3. Dendrimers (DMs)

Dendrimers (DMs) are perfectly structured carriers relying on branched molecules with a globular shape. The name comes from the Greek word, “Dendron” which means a "tree" and “Meros” meaning “part”. The DMs are generally composed of three components, an inner core which trap or anchor a single element or a group, a multilayer made of repeating units, called generations, and an outer surface with peripheral functions directed outward available for functionalization. The DMs having hydrophobic core and hydrophilic periphery have shown to exhibit micelle-like behavior and can be used to deliver highly active pharmaceutical compounds that have limited water solubility. The monodispersity, water solubility, high encapsulation efficiency, and presence of large number of functionalizable peripheral groups, make these macromolecules appropriate candidates as drug delivery vehicles. The drugs can be loaded into the DMs by three different mechanisms, i.e., the drug can be covalently attached to the periphery of the dendrimer to form dendrimer prodrugs, the drug is coordinated to the outer functional groups via ionic interactions, or the dendrimer acts as a unimolecular micelle by encapsulating a drug through forming a dendrimer-drug supramolecular assembly.[8,9] The carboxylic acid and phenol-terminated water-soluble dendrimers have been synthesized to establish their utility in drug delivery as well as conducting chemical reactions in their interiors.[10] This strategy allows researchers to attach both targeting molecules and drug molecules to the same dendrimer, which will ultimately reduce the side effects of medications on healthy cells. The DMs have been used as carriers of anticancer and antiretroviral drugs.

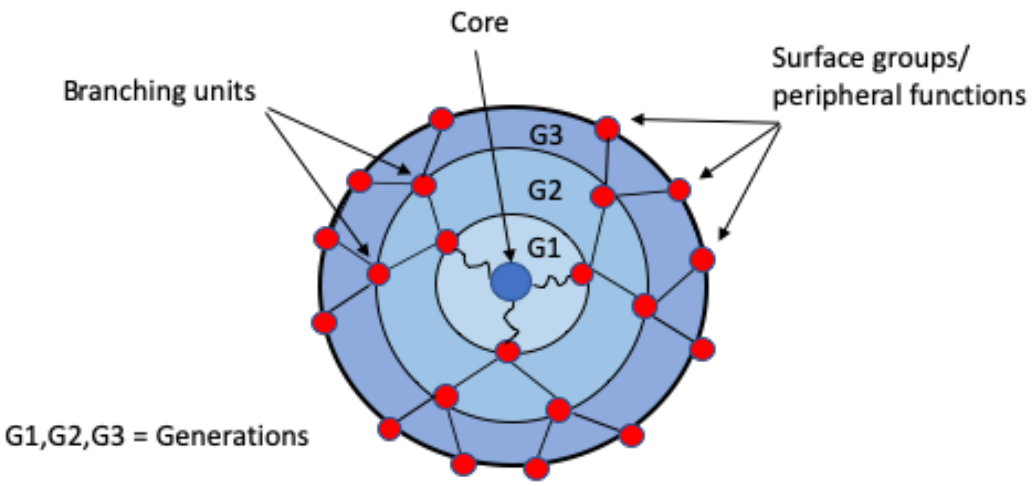


Figure 3. Structure of dendrimer

The major advantages associated with dendrimers are as follows,

- High encapsulation efficiency
- Availability of large number of functionalizable peripheral groups
- Controllable structural design
- Water solubility
- Shielded interior cores
- Capacity to incorporate bioactive agents chemically as well as physically

2.4. *Nano-emulsions*

Nano-emulsions are fine and clear oil-in-water or water-in-oil dispersions stabilized by an amphiphilic surfactant, with droplet sizes < 300 nm. The small droplet size results in long-term physical stability of nano-emulsions, because it impairs the destabilization phenomena like sedimentation, creaming and coalescence.

The major advantages of nano-emulsions are as follows,

- High encapsulation efficiency of lipophilic drugs
- Can be formulated in different dosage forms, enabling them to deliver the drugs by several routes of administration, like oral, topical, parenteral, ocular, transdermal etc.
- Due to smaller droplet sizes, nano-emulsions improve the absorption/bioavailability of drugs
- Taste masking
- Non-toxic and non-irritant
- Protection of drugs from hydrolysis and oxidation

The detailed explanation about the characteristics, types, methods of preparation and applications of nano-emulsions is given in chapter 2.1 of this thesis, however a schematic representation of a typical multifunctional nano-emulsion system is shown in the figure 4.

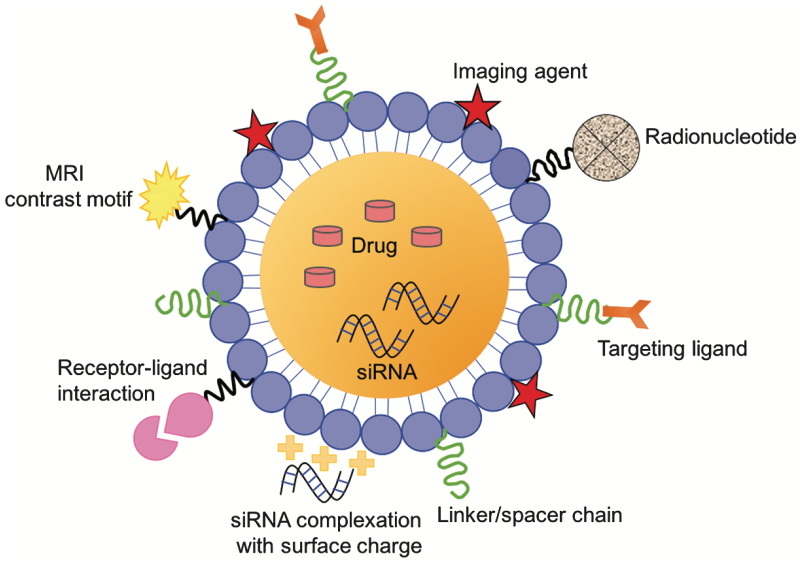


Figure 4. A multifunctional nano-emulsions system (adapted from reference[11])

2.5. Liposomes

The word liposome is derived from two Greek words, "lipos" meaning fat and "soma" meaning body. Liposomes were first described by Bangham *et al.*[12] as spherical structures made up of phospholipid and cholesterol, which upon hydration, self-associate to form bilayers surrounding an aqueous interior. Since then the liposomes have been explored extensively as carriers for drug delivery. Phospholipid forms the bilayered structures in the aqueous systems and cholesterol improves the stability of the bilayers and reduces the leakage of the active payload. Liposomes have widespread applications in pharmaceuticals. The major advantages of liposomes to be used as drug carriers are as follows,

- Liposomes are biocompatible, biodegradable and non-immunogenic
- Liposomes can be formulated in different dosage forms to facilitate administration through almost all routes of drug administration.
- The hydrophilic, lipophilic as well as amphiphilic drugs can be encapsulated/entrapped in the liposomes.
- The surface of the liposomes can be modified (ligand attachment) according to the desired properties, which result in the targeted delivery of the drugs.

The detailed explanation about the characteristics, types, methods of preparation and applications of liposomes is given in the chapter 3.1 of this thesis, however a schematic representation of conventional and functionalized liposomes is presented in figure 5.

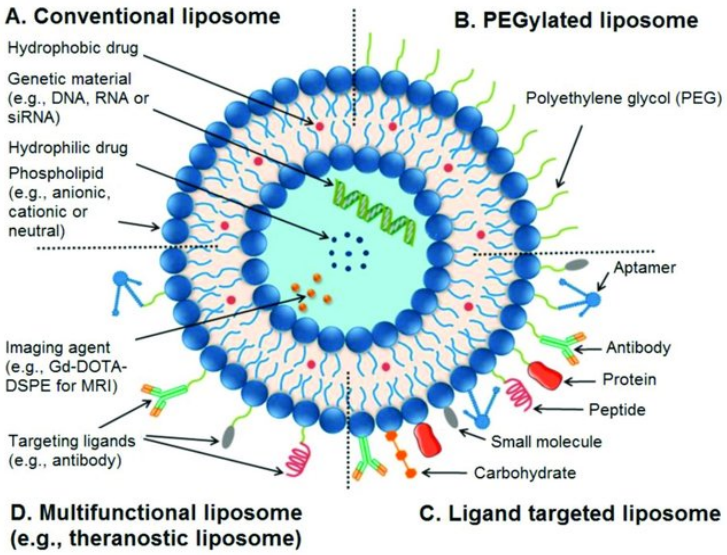


Figure.5. A schematic representation of conventional and functionalized Liposomes (reference [13]).

3. REFERENCES

(1) Henglein, A. Small-Particle Research: Physicochemical Properties of Extremely Small Colloidal Metal and Semiconductor Particles. *Chemical Reviews* **1989**, *89* (8), 1861–1873. https://doi.org/10.1021/cr00098a010.

(2) Jhaveri, A. M.; Torchilin, V. P. Multifunctional Polymeric Micelles for Delivery of Drugs and SiRNA. *Frontiers in pharmacology* **2014**, *5*, 77–77. https://doi.org/10.3389/fphar.2014.00077.

(3) Christoforidis, J. B.; Chang, S.; Jiang, A.; Wang, J.; Cebulla, C. M. Intravitreal Devices for the Treatment of Vitreous Inflammation. *Mediators of Inflammation* **2012**, *2012*, 1–8. https://doi.org/10.1155/2012/126463.

(4) Pardeike, J.; Hommoss, A.; Müller, R. H. Lipid Nanoparticles (SLN, NLC) in Cosmetic and Pharmaceutical Dermal Products. *International Journal of Pharmaceutics* **2009**, *366* (1), 170–184. https://doi.org/10.1016/j.ijpharm.2008.10.003.

(5) Martins, S.; Sarmento, B.; Ferreira, D. C.; Souto, E. B. Lipid-Based Colloidal Carriers for Peptide and Protein Delivery--Liposomes versus Lipid Nanoparticles. *International journal of nanomedicine* **2007**, *2* (4), 595–607.

(6) Zhang, X.-G.; Miao, J.; Dai, Y.-Q.; Du, Y.-Z.; Yuan, H.; Hu, F.-Q. Reversal Activity of Nanostructured Lipid Carriers Loading Cytotoxic Drug in Multi-Drug Resistant Cancer Cells. *International Journal of Pharmaceutics* **2008**, *361* (1), 239–244. https://doi.org/10.1016/j.ijpharm.2008.06.002.

(7) Bondì, M. L.; Fontana, G.; Carlisi, B.; Giammona, G. Preparation and Characterization of Solid Lipid Nanoparticles Containing Cloricromene. *Drug Delivery* **2003**, *10* (4), 245–250. https://doi.org/10.1080/drd_10_4_245.

(8) Morgan, M. T.; Nakanishi, Y.; Kroll, D. J.; Griset, A. P.; Carnahan, M. A.; Wathier, M.; Oberlies, N. H.; Manikumar, G.; Wani, M. C.; Grinstaff, M. W. Dendrimer-Encapsulated Camptothecins: Increased Solubility, Cellular Uptake, and Cellular Retention Affords Enhanced Anticancer Activity In Vitro</Em>. *Cancer Research* **2006**, *66* (24), 11913. https://doi.org/10.1158/0008-5472.CAN-06-2066.

(9) Tekade, R. K.; Dutta, T.; Gajbhiye, V.; Jain, N. K. Exploring Dendrimer towards Dual Drug Delivery: PH Responsive Simultaneous Drug-Release Kinetics. *J Microencapsul* **2009**, *26* (4), 287–296. https://doi.org/10.1080/02652040802312572.

(10) Kaanumalle, L. S.; Ramesh, R.; Murthy Maddipatla, V. S. N.; Nithyanandhan, J.; Jayaraman, N.; Ramamurthy, V. Dendrimers as Photochemical Reaction Media. Photochemical Behavior of Unimolecular and Bimolecular Reactions in Water-Soluble Dendrimers. *The Journal of Organic Chemistry* **2005**, *70* (13), 5062–5069. https://doi.org/10.1021/jo0503254.

(11) Mahato, R. Nanoemulsion as Targeted Drug Delivery System for Cancer Therapeutics. *Journal of Pharmaceutical Sciences and Pharmacology //*, *3* (2), 83–97. https://doi.org/10.1166/jpsp.2017.1082.

(12) Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids. *Journal of Molecular Biology* **1965**, *13* (1), 238-IN27. https://doi.org/10.1016/S0022-2836(65)80093-6.

(13) Riaz, M.; Riaz, M.; Zhang, X.; Lin, C.; Wong, K.; Chen, X.; Zhang, G.; Lu, A.; Yang, Z. Surface Functionalization and Targeting Strategies of Liposomes in Solid Tumor Therapy: A Review. *International Journal of Molecular Sciences* **2018**, *19* (1), 195. https://doi.org/10.3390/ijms19010195.

CHAPTER TWO

NANO-EMULSIONS AS CARRIER OF LIPOPHILIC MOLECULES FOR BIOMEDICAL APPLICATIONS

Chapter 2.1: Nano-emulsion: An advanced drug delivery system

1. Definition of nano-emulsions

Nanoemulsions are fine, stable, and in general optically clear dispersions of two immiscible liquids (oil-in-water or water-in-oil) stabilized by an amphiphilic surfactant, with droplet sizes < 300 nm.[1–5] The small droplet size results in long-term physical stability of nano-emulsions, because it impairs the destabilization phenomena like sedimentation, creaming and coalescence.[5,6]

2. Types of Nano-emulsions

Based on the constituents and relative distribution of dispersed phase and continuous phase, nano-emulsions can be classified as,

2.1. Biphasic nano-emulsions

Biphasic nano-emulsions are defined as the thermodynamically unstable colloidal dispersions consisting of two immiscible liquids, with one of the liquids being dispersed in the other liquid in the form of small spherical droplets. These nano-emulsions are further classified into oil-in-water (O/W) or water-in-oil (W/O) types depending upon whether the oil is dispersed as droplets in continuous water phase, or water is dispersed as droplets in continuous oil phase respectively. The O/W nano-emulsions are also called as "direct" or "water based" nano-emulsions and in such emulsions a water-soluble surfactant is used, whereas W/O nano-emulsions are also called as "inverse" or "oil based" nano-emulsions and in this case oil soluble surfactant is used.

2.2. Double nano-emulsions

Double nano emulsions are complex colloidal systems in which the droplets of dispersed phase are immersed in a continuous phase which itself is dispersed in another outermost continuous phase.

3. Components of Nano-emulsions

The major components of nanoemulsion are:

- **Aqueous phase:** The stability and droplet size of nanoemulsion is strongly influenced by the nature of aqueous phase. Generally, water is used as an aqueous phase, but apart from water, phosphate buffered saline, Ringer's solution, simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8) can be used as aqueous phase. During

the development of nano-emulsions, a special consideration should be given to the pH and ionic content of aqueous phase, because it is well documented that both the pH and electrolytes have strong influence on the properties of nano-emulsions, particularly on droplet sizes and physical stability of nano-emulsions.

- **Oil:** The oil content in the O/W nano-emulsions varies generally from 5-20%, however sometimes higher amounts can also be used to achieve desired properties of the nano-emulsions. The most commonly used oils are long chain triglycerides (LCT), medium chain triglycerides (MCT) and short chain triglycerides (SCT), e.g., labrafac oil, omega oil, corn oil, castor oil, linseed oil, olive oil, soybean oil, sesame oil, etc. The choice of oil is generally made based on its ability to solubilize the drugs and to facilitate formation of nanoemulsion of desired characteristics.
- **Emulsifying agent/Surfactants:** The emulsifying agents impart stability to these systems. The ideal properties of emulsifying agent are, to reduce the surface tension, to get adsorbed rapidly at the oil-water interface and be effective in a fairly low concentration to form nanoemulsion. The most commonly used surfactants are, sodium deoxycholate, cremophor EL, lecithin, polyoxyethylene sorbitan monolaurate (tween 20,40,60,80), sorbitan monolaurate (span 20,40,60,80), sodium dodecyl sulfate, solutol etc. The choice of the surfactant is critical for the nanoemulsion formation. Surfactants having hydrophilic-lipophilic balance (HLB) values less than 10 are hydrophobic (like sorbitan monoesters) and form w/o nanoemulsion where as those having HLB higher than 10, (like polysorbate 80) are hydrophilic and form o/w nanoemulsion. In some cases, mixture of lipophilic and hydrophilic surfactants may also be used to obtain nano-emulsions.

  The emulsifying agents can also be classified into following types,

  i) Natural emulsifying agents, for example acacia, tragacanth, pectin, agar, sodium alginate etc.

  ii) Synthetic emulsifying agents, like polyvinyl alcohol, polyethylene glycols, Tween, sodium stearate, lecithin, carbomers etc.

  iii) Semi-synthetic emulsifying agents, for example methylcellulose, sodium carboxymethylcellulose, hydroxyethyl cellulose etc.
- **Co-surfactants:** Sometimes the surfactant alone is not capable enough to lower the interfacial tension to form a nanoemulsion, that's why a co-surfactant (like ethanol, hexanol, pentanol, polyethylene glycol, propylene glycol, glycerine etc.) is used in

those cases to complement surfactants and form stable nano-emulsions. But the co-surfactant should not be capable of forming micelles on its own (unlike surfactants).

- **Other components:** Sometimes the preservatives, antioxidants, chelating agents and pH stabilizers are also added to increase the stability of the nano-emulsions in particular environment.

4. Methods of preparation of nano-emulsions

The methods used to prepare nano-emulsions can be classified into two main types,

4.1.High energy methods

High-energy methods make use of mechanical devices, like microfluidizers, ultrasonicators and high-pressure homogenizers, to create powerful disruptive forces that disrupt and intermingle the oil and water phases so that tiny droplets are formed.[1,3,5–8] Almost any oil can be subjected to nano-emulsification using these methods.

Following are the high-energy methods, which are used to produce nano-emulsions.

4.1.1. High pressure homogenization

a) High-pressure homogenizer

This method benefits from a high-pressure homogenizer to manufacture nano-emulsions with very small droplet sizes (<100nm). The process consists of two steps. The first step involves the preparation of macroemulsion by mixing oily phase, aqueous phase and surfactant in a simple batch stirrer system for a sufficient period of time. In the second step this macroemulsion is converted into nanoemulsion by passing it through a high-pressure homogenizer. The macroemulsion is forced to pass through a very small gap/orifice at an operating pressure of 500-5000 psi, which subjects the product to hydraulic shear and intense turbulence resulting in the formation of extremely small droplet sized nano-emulsions. This process can be repeated multiple times (number of passes) until the final product reaches the desired droplet size and polydispersity index (PDI). The droplet size obtained is directly proportional to the applied homogenization pressure as well as the number of homogenization cycles or passes (figure 1).

b) Piston gap homogenizer

Piston gap homogenizer works on the principle of a colloid mill. A coarse emulsion is prepared by mixing oily and aqueous phases in the presence of a surfactant and then this coarse emulsion is passed through a very narrow gap between a rapidly moving rotor and a

fixed stator. The high shear, stress and the grinding forces generated between the rotor and stator cause the size reduction of the droplets. The droplet size can be controlled by fixing the dissipation gap to required size, which will result in the formation of droplets having the sizes equal to or smaller than the gap between the rotor and the stator (figure 1).

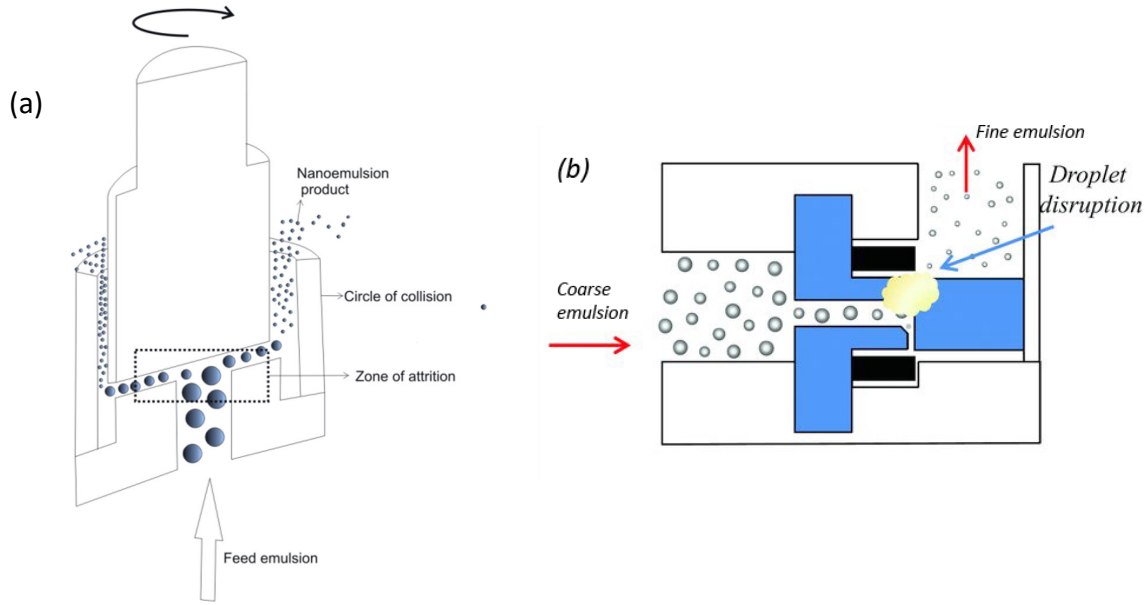


Figure 1. Schematic representation of, (a) piston gap homogenizer (reference[6]) (b) high-pressure valve homogenizer (reference[8])

4.1.2. Micro fluidization

Microfluidizers are widely used devices in pharmaceutical industry in order to manufacture very fine emulsions. The coarse emulsion is forced through an interaction chamber consisting of rigid stainless steel microchannels under the influence of a high-pressure displacement pump (500-50,000 psi) resulting in the formation of very fine droplets. The hydraulic shear, impact, attrition, cavitation and intense turbulence produced during the process cause the reduction in the droplet size. The coarse emulsion is repeatedly passed through the microfluidizer until the desired size is obtained. The energy generated by the collisions of the droplets dissipates in the form of heat and requires cooling by the use of heat exchanger without affecting the stability and size distribution. The major advantages of this high throughput method are high volume throughput of nanodroplets with reasonably uniform size distribution, extremely high peak shear rates and zero contamination of feed material.

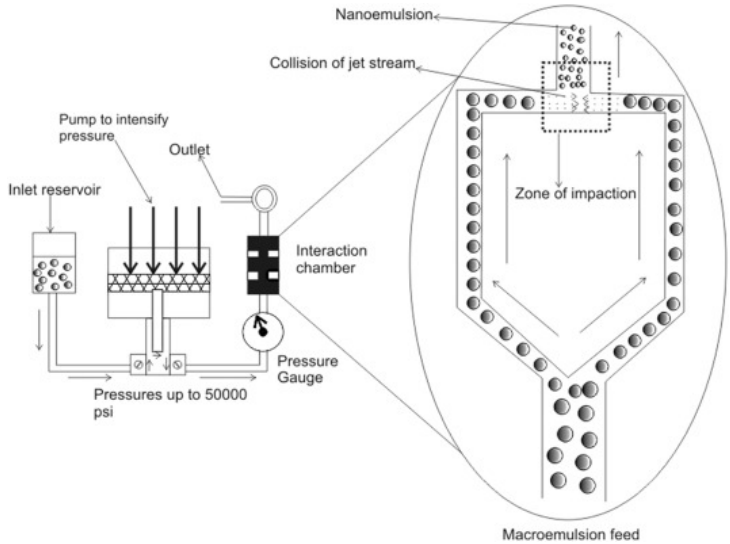


Figure 2. Schematic representation of a microfluidizer (Reference[6])

4.1.3. Ultrasonication

This method makes use of high frequency sound waves (equal to or higher than 20 KHz) to produce nano-emulsions *in situ* or to reduce the size of a pre-formed emulsion. A coarse emulsion is prepared by mixing a homogenous oily phase and aqueous phase in a mechanical stirrer system. Then this coarse emulsion is exposed to ultrasonic agitation to form nanoemulsion. When high power ultrasonic devices like focusing horns and pointed tips are dipped in the sample, cavitation bubbles are formed which continue to grow until they implode. This implosion creates a jet stream of surrounding liquid which in turn pressurizes dispersed droplets and causes reduction in the size of droplets (figure 3). In most of the ultrasonic systems, the emitted sound field is not homogeneous, that's why the emulsion is recirculated multiple times through the high-power region. As a result of which all the droplets experience the highest shear rate and nano-emulsions with uniform size distribution are obtained. The ultrasonicator probes are available in different dimensions, which affect their functionality. In case of small volume batches, the narrower probes are usually preferred. The depth to which a probe is dipped in the sample is also very important because it can affect the pressure distribution. It must be carefully monitored that the ultrasonicator probe should not touch any solid surface. Among all the high-energy methods, ultrasonication requires least expenditure of energy.

The major disadvantages associated with high-energy methods are:

i) High instrumental cost

ii) High operational temperature which rules out thermolabile drugs

iii) Contamination of the feed material induced by the probe

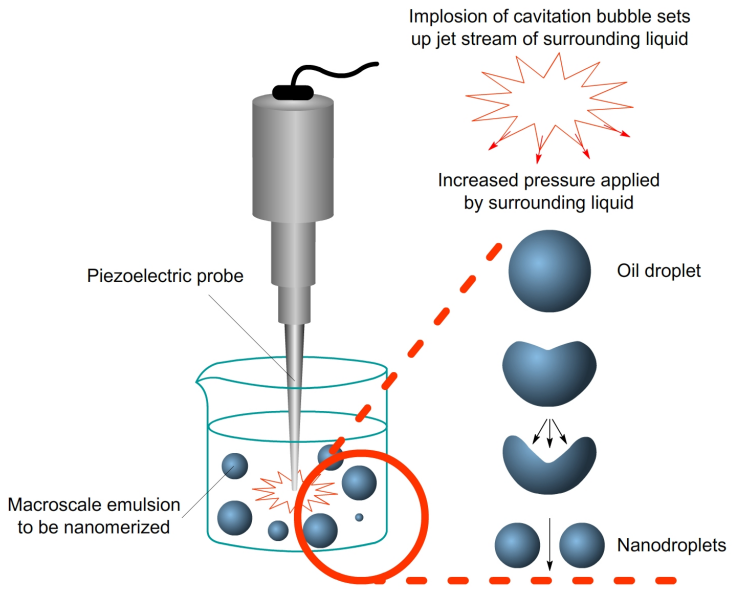


Figure. 3. Schematic representation of an ultrasonication (adapted from reference[6])

4.2.Low-energy methods

Low energy methods are more energy efficient, as only simple stirring is needed. A special character of these methods is the utilization of the energy stored in the system to produce ultra-fine droplets. Low-energy methods divert the intrinsic physicochemical properties of the surfactants, co-surfactants and excipients in the formulation, leading to the generation of emulsion droplets in the nanometric range.[2] Over the past twenty-five years, the research interest in the development of low energy methods has increased because of the numerous advantages of low energy methods like,

- Smaller droplet sizes
- Low cost
- High formulation yields
- Encapsulation of fragile active molecules
- Potential industrial scale up
- Wide range of applications in food, cosmetic and pharmaceutical industry

Low energy methods can be classified into two main types,

4.2.1. Spontaneous Emulsification

The spontaneous emulsification is a very simple method in which an aqueous phase is mixed with an oil phase composed of a mixture of oil, surfactant and/or water miscible solvent. By spontaneous emulsification, nano-emulsions can be produced at room

temperature without using special devices. Various mechanisms have been proposed in the literature for spontaneous emulsification, like, interfacial turbulence, development of transient values of interfacial tensions, diffusion and stranding, Marangoni flow and Rayleigh-Taylor instability.[9,10] However the general mechanism is the surfactant and/or solvent displacement from the oil phase to the aqueous phase that induces turbulence and super saturation at the oil/water interface, resulting in the formation of nanodroplets.[10,11] In pharmaceutical industry, this method is widely used to manufacture oil-in-water nano-emulsions as carriers of lipophilic drugs. Such systems are usually referred as self-nanoemulsifying drug delivery systems (SNEDDS). There is a drawback associated with this method, i.e., the use of toxic solvents like ethanol, acetone, etc. Some efforts have been put into dealing with this problem, like using a mixture of water miscible solvents.[12] Anton et al. have shown that the non-ionic surfactant itself can play the role of the displacing species in the spontaneous emulsification process, following a universal mechanism related to the displacement of the surfactants from the oily to the aqueous phase, due to the sudden change of surfactant solubility and thus avoiding the use of the toxic solvents.[2] Rehman *et al.*[13] have shown that the spontaneous emulsification – commonly performed with nonionic surfactants– can also be transposed with amphiphilic polymer based on poly (maleic anhydride-alt-1-octadecene) (PMAO) and jeffamine® (Figure 4).

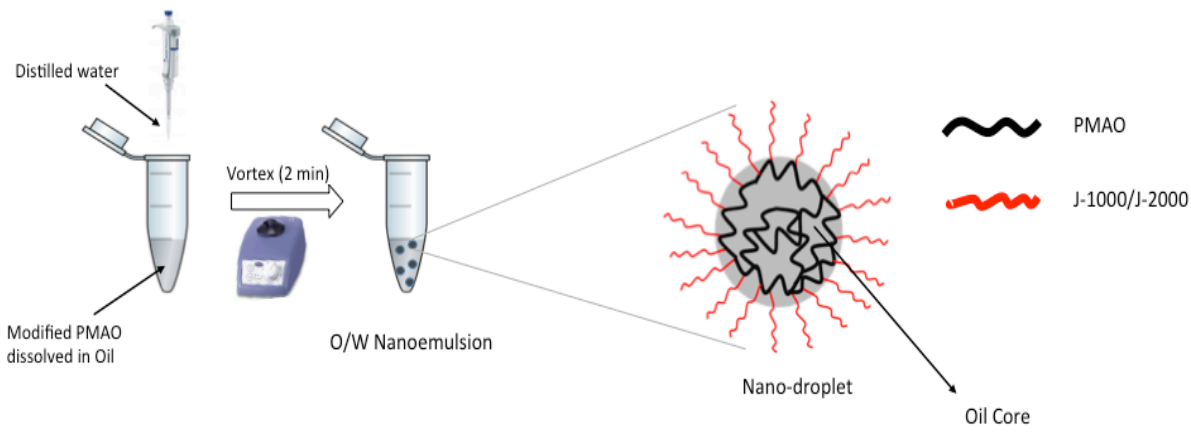


Figure 4. Schematic representation of preparation of polymeric nano-emulsions by spontaneous emulsification

4.2.2. Phase inversion methods

Phase inversion methods utilize the chemical energy, which is released as a result of phase transitions taking place during the emulsification process. These phase transitions occur following the changes either in composition (phase inversion composition method) or temperature (phase inversion temperature).[5,7] The phase behavior studies have shown that both these methods have the common general mechanism of emulsification because the phase transitions (bicontinuous microemulsions or lamellar liquid crystalline phases) occurring during the emulsification process are common for both these methods.

Phase inversion temperature method (PIT)

In this method, the phase transitions are triggered by changes in the temperature at constant composition. The emulsification occurs because of the changes in the solubility/affinity of the surfactant for water and oil phase as a function of temperature. The process involves an ordered conversion of W/O to O/W emulsion or vice versa through an intermediate bicontinuous phase. This method can only be applied to the systems having surfactants sensitive to changes in the temperature, like polyoxyethylated non-ionic surfactants, which undergo changes in the hydration of their polyoxyethylene (POE) chains due to changes in the temperature of the system, resulting in the inversion of their curvature and emulsion formation.[5–7,14] The surfactant behaves as hydrophilic at temperature below HLB temperature and as hydrophobic at temperature above HLB temperature. The first step of the procedure involves the preparation of an emulsion at its phase inversion temperature (also called hydrophile-lipophile balance (HLB) temperature). Although the formation of very small droplets is favored at this temperature but the spontaneous curvature of the surfactant is almost zero and the coalescence rate is very high, that's why the emulsions formed are very unstable (bicontinuous microemulsion). In the second step, the system is either cooled down or heated rapidly which causes the phase inversion, resulting in the formation of kinetically stable O/W or W/O emulsions respectively. The cooling or heating process must be very fast otherwise polydisperse nano-emulsions are formed due to predominant coalescence. The major drawback associated with this method is that the thermosensitive drugs cannot be used in these systems.

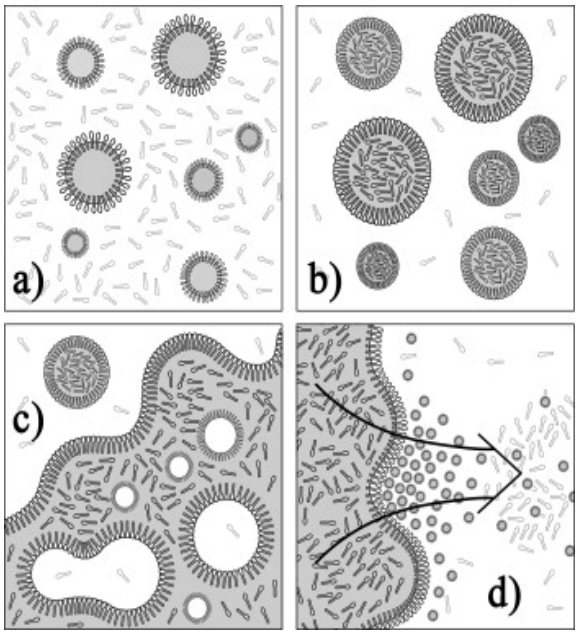


Figure. 5. Mechanism of the generation of nanoemulsion in a water/nonionic surfactant/oil system using the PIT method. (a) the ternary system presents a macro-emulsion and nonionic surfactants are mostly hydrophilic at temperature below the PIT, (b) the surfactants gradually become lipophilic and are solubilized by the oily phase as the temperature is increased, (c) bicontinuous microemulsions formation at PIT, (d) the emulsion is inverted at temperature, above the PIT, i.e., water is dispersed into the mixture of oil and lipophilic surfactant. The system is then rapidly cooled using water dilution, making the surfactant suddenly hydrophilic and inducing spontaneous and rapid migration to the aqueous phase.

Phase inversion composition method (PIC)

In this method, the phase transitions are triggered by changes in the composition of the formulation at constant temperature. The procedure consists of progressive addition of either water or oil to oil-surfactant or water-surfactant mixture respectively. The polyoxyethylene (POE) type surfactants are widely used to produce O/W or W/O nano-emulsions by this method as well. But this method is not limited to polyoxyethylene (POE) type surfactants. The initial system is usually a W/O microemulsion to which water is added progressively to form O/W nanoemulsion. As the water proportion increases, the POE chains of surfactant get more and more hydrated and the spontaneous curvature of the surfactant changes from negative to zero. This state is called the transition composition and just like at the HLB temperature, the hydrophilic-lipophilic characteristics of the surfactant are balanced at this composition, resulting in the formation of bicontinuous or lamellar structures. As soon as this transition composition is exceeded, the spontaneous curvature of the surfactant changes from zero to positive,

causing the formation of nanodroplets *i.e.,* phase inversion from W/O structures to an O/W nanoemulsion.

The major advantages of this method over PIT method are,

- Greater potential for large-scale production
- No restriction of using only POE-type surfactants
- Can be used for thermolabile products.

5. Drug release from nano-emulsions

The release of drugs from the nano-emulsions can be triggered by many stimuli like, temperature change, pH, enzyme activity etc. The lipophilic drugs are generally released from the nano-emulsions only after the digestion of the oily components of the emulsions. The release of drugs from the nano-emulsions follows Fick's first law of diffusion, which can be expressed mathematically as follows,

$$t_{1/2} = 0.0585r^2K_{ow}/D$$

Where, $t_{1/2}$ is the time taken by half of the drug to diffuse out from the oil, "r" is the radius of the droplet, "K_{ow}" is the oil-water partition coefficient and "D" is the diffusion coefficient of the drug. The nano-emulsions are usually digested more rapidly than the traditional emulsions because of very high oil/water interfacial area. And because of the smaller radii of the droplets, the drug diffuses very rapidly across oil. The release of the drug from the nano-emulsions can be delayed/controlled by using some vehicles like organogels[15] or by coating the nano-emulsions by a thick layer of a polymer (nanocapsules)[16] or by using suitable surfactants because a tightly packed interface serve as a barrier and turn the nanoemulsion into a drug reservoir having controlled rate of release of the drug.

6. Applications of nano-emulsions as nano-carrier for lipophilic drugs

A great deal of research is ongoing in developing oil-in-water nano-emulsions to incorporate lipophilic drugs to overcome the solubility problems of poorly soluble drugs. The water-insoluble drugs can be easily delivered to the patients through such kind of nanocarriers. Other advantages of using nano-emulsions as drug carriers are enhanced bioavailability, reduced toxicity, improved stability and pharmacological activity, more sustained delivery and protection from physical and chemical degradation.

Nano-emulsions can be administered by various routes like oral, ocular, topical, intravenous, pulmonary and intranasal route because of the compatibility of their physico-chemical properties with the different administration routes, as well as due to the possibility of

rendering them into different kinds of dosage forms, like creams, gels, liquids and aerosols. The recent advances in the use of nano-emulsions as carriers of lipophilic drugs are as follow.

6.1.Oral drug delivery

Oral administration is often the best route for drug delivery; however, oral bioactive agents experience the harsh environment of the gastrointestinal (GI) tract, which impairs their stability, solubility and absorption. Nano-emulsions offer many advantages over conventional oral formulation including enhanced stability in the harsh environment (pH and hydrolytic enzymes) of the gastrointestinal (GI) tract, improved clinical potency, increased absorption and bioavailability, and decreased drug toxicity.[6,17] That's why nano-emulsions are ideal nanocarriers for the oral delivery of lipophilic drugs like diuretics, antibiotics, steroids, hormones, antifungals etc. Few examples of the drugs, which have been incorporated in nano-emulsions for oral delivery, are given in the Table 1.

6.2.Parenteral drug delivery

The drugs that have poor water-solubility, lower bioavailability and narrow therapeutic indices can be administered by incorporating them in nano-emulsions. For intravenous administration of emulsions, the size of the dispersed oil droplets must be below the size of the smallest blood vessels (5 μm) to avoid embolisms.[18] The nano-emulsions are ideal candidates to be used as drug carriers for parenteral administration of lipophilic drugs because of very small sized droplets (below 300 nm). The lipophilicity of the drug is a very important factor for its biodistribution. If the lipophilicity of the drug is low, the drug may be released very quickly from the oil droplet into the blood stream. Whereas, if the lipophilicity is high, the drug will be retained in the oil droplet and the oil droplet will function as a drug reservoir for delayed/controlled release. The surface of the nanodroplets can be modified by incorporating a hydrophilic moiety like polyethylene glycol (PEG) chains, which prevents identification and opsonization by mononuclear phagocyte system (MPS) and enhances the circulation time of NE in the blood stream (i.e., stealth NE).[18,19] The NE can be used for targeted drug delivery by incorporating some ligands (like RGD peptide for Rvβ3-integrin receptor present on tumor surface)[20] at the surface of the nanodroplets. Some examples of the parenteral NE are given in the Table 1.

6.3. Topical drug delivery

Topical route of administration of drugs has many advantages over other routes of administration like, the avoidance of hepatic first pass metabolism of the drug and the direct delivery of the drug to affected area of the skin or eyes. But it has always been a challenge to enhance permeation of the drugs intended for topical application. These drugs are limited either by poor dispersibility in topical vehicles (creams, gels, patches) or due to skin irritant action. Nano-emulsions have been explored for topical delivery of such drugs and they provide enhanced penetration rates as well as deep skin delivery and also create a concentration gradient by acting as tiny reservoirs of drugs.[21,22] Nano-emulsions can also be employed to systemically deliver small molecules via topical route. Examples of topical nanoemulsion formulations are given in the Table 1.

6.4. Ocular drug delivery

Oil-in-water nano-emulsions have been developed to deliver water insoluble, poorly absorbed/poorly retained and environmentally sensitive drugs. Generally, transparency, refractive index and viscosity of the nano-emulsions are given special consideration in case of their use as ophthalmic drug delivery systems. The o/w nano-emulsions intended for ophthalmic drug delivery, have excellent wetting and spreading properties and also provide improved solubility and absorption of the poorly soluble drugs and facilitate prolonged retention and release of the drugs.[23–25] Patel *et al*. have developed temperature sensitive ophthalmic nano-emulsions, which transform into gel after administration and provide improved permeability, retention and bioavailability (Examples in Table 1).

6.5. Pulmonary drug delivery

The drug administration by pulmonary route has been extensively studied and nano-emulsions serve as a good carrier for the delivery of drugs by this route. Nano-emulsions ensure enhanced lung deposition and pulmonary retention of the drugs. The first pass metabolism can also be avoided by using pulmonary route of administration, which in turn improves the therapeutic efficacy.[26] An example for such kind of systems is the amphotericin-loaded nanoemulsion, which ensures direct delivery of the drug to the lungs to treat pulmonary aspergillosis (Table 1).

6.6.Intranasal drug delivery

The blood brain barrier is the major hurdle in the delivery of drugs to brain, as it restricts hydrophilic and high molecular weight molecules (like peptides). However, drug loaded nano-emulsions have been used to target brain by making use of the olfactory vein in the nasal mucosa which provides direct passage from nose to brain. A number of anti-parkinsonism, anti-alzheimer's and anti-psychotic drugs have been successfully developed in the form of intranasal nano-emulsions (Table 1), which provide direct transport of drugs to the brain.

6.7.In cosmetics

The nano-emulsions have been extensively used in cosmetics because of their aesthetic properties, i.e. low viscosity, transparent visual aspects of nanoemulsion, droplet sizes below 300 nm and high surface area allowing effective transport of the active ingredient to the skin. Because of such smaller droplet sizes, creaming, sedimentation, flocculation or coalescence are not observed which leads to more elegant and stable product. These properties make them useful for sun care products, hair care products, lotions, transparent milks and antiaging creams.[27]

Table 1: Examples of nano-emulsions developed for lipophilic drug delivery

Drug	Method of preparation	Purpose	Indication	Route of administration	Reference
Candesartan	Spontaneous emulsification	To Improve oral bioavailability	Hypertension	Oral	[28]
Primaquine	Homogenization	Dose reduction	Malaria	Oral	[29]
Paclitaxel	Spontaneous emulsification	To improve cellular uptake, bioavailability and antitumor activity	Cancer	Oral	[30]
Aspirin	Ultra-sonication	To Enhance analgesic and anti-inflammatory activity	Analgesic and anti-inflammatory	Oral	[31]
Cyclovirobu-xine D	Spontaneous emulsification	To Improve bioavailability	Arrythmias	Oral	[32]
Dabigatran etexilate	Spontaneous emulsification	To Improve oral bioavailability	Stroke and thromboembolism	Oral	[33]
Insulin	Homogenization	To enhance oral absorption	Diabetes	Oral	[34]

		and efficacy			
Carbamazepine	Spontaneous emulsification	To overcome poor solubility	Epilepsy	Parenteral	[35]
Docetaxel	Homogenization followed by ultra-sonication	To overcome poor solubility and hydrolytic instability	Cancer	Parenteral	[36]
Fisetin	PIT method	To improve bioavailability and anti-tumor activity	Cancer	Parenteral	[37]
Clotrimazole	Spontaneous emulsification	To improve bioavailability	Malaria	Parenteral	[38]
Nimesulide	Spontaneous emulsification	To modulate the skin penetration	Analgesic and anti-inflammatory	Topical	[39]
Turmeric oil	Spontaneous emulsification	To investigate potential for topical delivery	Psoriasis	Topical	[40]
Camphor, menthol and methyl salicylate	Ultraturrax followed by HPH	To enhance penetration ability	Analgesics and rubifacients	Topical	[21]
Lutein	Mild stirring followed by sonication	To enhance bioavailability	Macular degeneration	Ocular	[24]
Loteprednol etabonate	Spontaneous emulsification	To improve permeability, ocular bioavailability and sustained delivery	Inflammations of the eye	Ocular	[25]
Antisense oligonucleotides	Homogenization	To improve the overall effectiveness of the treatment	Ocular neovascularization	Ocular	[41]
Amphotericin B	Vortex mixing followed by sonication	To enhance pulmonary deposition and retention for prolonged periods	Pulmonary aspergillosis	Pulmonary	[26]
Saquinavir mesylate	Spontaneous emulsification	To improve penetration and brain targeting	AIDS	Intranasal	[42]
Paroxetine	Spontaneous emulsification	To enhance permeation and to get direct nose-to-brain delivery	Depression	Intranasal	[43]
Selegiline	High speed homogenization	To improve bioavailability to get direct nose-to-brain delivery	Parkinson's disease	Intranasal	[44]

6.8.In food industry

Nano-emulsions can be used in the food industry to develop smart foods with substances that are otherwise difficult to incorporate due to poor-water solubility. For example, β-carotene is a pigment abundant in plants and fruits (responsible for coloration), used as a dietary supplement and has very important health benefits. The research has been done on the development of β-carotene loaded nano-emulsions and also on the effect of temperature, pH and surfactant type on the size and stability of nano-emulsions.[45–48]

6.9.Biomedical imaging

The o/w nano-emulsions are of particular interest for developing fluorescent nanocarriers because their oily core work as a perfect reservoir for the encapsulation of lipophilic dyes. Dye-loaded nano-droplets provide an efficient alternative to inorganic nanoparticles as they provide high encapsulation of the dyes and at the same time prevent the self-quenching of the fluorescence and leakage of the encapsulated dye from the nano-droplets in the biological media. Klymchenko et al. have developed nano-emulsions encapsulating fluorescent dyes (based on 3-alkoxyflavone and Nile Red), in the oily core of the nano-droplets (at very high concentrations), and the dyes remained highly fluorescent within the nano-droplets even at high concentration.[49] Texier *et al*.[50] prepared lipid nanocarriers encapsulating cyanine dyes and showed that even at this high concentration in the oily core of nano-droplets, the dye preserved its efficient fluorescence, allowing successful cellular and *in vivo* animal imaging. Rapoport *et al*..[51] have demonstrated that biodegradable block copolymer stabilized perfluoro-15-crown-5-ether (PFCE) nano-emulsions are effective theranostic formulations with a very high potential for use in image-guided, ultrasound-mediated drug delivery. Attia *et al* have developed iodinated NEs for liver and spleen X-rays imaging.[52,53]

7. Commercially available Nano-emulsion products

As mentioned above in the applications of nano-emulsions, the nano-emulsions can be used as carrier for the delivery of a wide variety of drugs such as analgesics, parenteral nutrition, steroids, anesthetics, chemotherapeutics and tumor vaccines. A few examples of the marketed nanoemulsion products are listed in the Table 2.

Table 2: Commercially available nano-emulsion products:

Drug	Indication	Marketed name	Manufacturer
Propofol	Anesthetic	Diprivan®	Astra zeneca
Dexamethasone	Steroid	Limethasone®	Mitsubishi pharmaceuticals
Palmitate alprostadil	Vasodilator, platelet inhibitor	Liple®	Mitsubishi pharmaceuticals
Clevidipine	Ca-channel blocker	Cleviprex®	The medicine company
Vitamin D	Vitamin D supplement	Essential D®	Nano essentials
Vitamin A,D,E and K	Parentral nutrition	Vitalipid® and Intralipid®	Fresenius kabi
Etomidate	Anesthetic	Etomidate-lipuro®	Braun Melsungen
Flurbiprofen axetil	NSAID	Ropion®	Kaken pharmaceuticals
Cyclosporine	Dre eye disease	Restasis®	Allergan
Diazepam	Sedative	Diazemuls®	Kabipharmacia
Flurbiprofen axetil	NSAID	Lipfen®	Green cross
5-aminolevulinic acid	Precancerous keratosis	Ameluz®	Biofrontera

8. REFERENCES

(1) Mason, T. G.; Wilking, J. N.; Meleson, K.; Chang, C. B.; Graves, S. M. Nanoemulsions: Formation, Structure, and Physical Properties. *Journal of Physics: Condensed Matter* **2006**, *18* (41), R635–R666. https://doi.org/10.1088/0953-8984/18/41/r01.

(2) Anton, N.; Vandamme, T. F. The Universality of Low-Energy Nano-Emulsification. *International Journal of Pharmaceutics* **2009**, *377* (1–2), 142–147. https://doi.org/10.1016/j.ijpharm.2009.05.014.

(3) McClements, D. J. Nanoemulsions versus Microemulsions: Terminology, Differences, and Similarities. *Soft Matter* **2012**, *8* (6), 1719–1729. https://doi.org/10.1039/C2SM06903B.

(4) Mahato, R. Nanoemulsion as Targeted Drug Delivery System for Cancer Therapeutics. *Journal of Pharmaceutical Sciences and Pharmacology //*, *3* (2), 83–97. https://doi.org/10.1166/jpsp.2017.1082.

(5) Solans, C. Nano-Emulsions: Formation by Low-Energy Methods. *Current opinion in colloid & interface science* **2012**, *v. 17* (5), 246-254–2012 v.17 no.5. https://doi.org/10.1016/j.cocis.2012.07.003.

(6) Singh, Y.; Meher, J. G.; Raval, K.; Khan, F. A.; Chaurasia, M.; Jain, N. K.; Chourasia, M. K. Nanoemulsion: Concepts, Development and Applications in Drug Delivery. *J Control Release* **2017**, *252*, 28–49. https://doi.org/10.1016/j.jconrel.2017.03.008.

(7) Gupta, A.; Eral, H. B.; Hatton, T. A.; Doyle, P. S. Nanoemulsions: Formation, Properties and Applications. *Soft Matter* **2016**, *12* (11), 2826–2841. https://doi.org/10.1039/C5SM02958A.

(8) McClements, D. J.; Rao, J. Food-Grade Nanoemulsions: Formulation, Fabrication, Properties, Performance, Biological Fate, and Potential Toxicity. *Critical Reviews in Food Science and Nutrition* **2011**, *51* (4), 285–330. https://doi.org/10.1080/10408398.2011.559558.

(9) López-Montilla, J. C.; Herrera-Morales, P. E.; Pandey, S.; Shah, D. O. Spontaneous Emulsification: Mechanisms, Physicochemical Aspects, Modeling, and Applications. *Journal of Dispersion Science and Technology* **2002**, *23* (1–3), 219–268. https://doi.org/10.1080/01932690208984202.

(10) Solans, C.; Morales, D.; Homs, M. Spontaneous Emulsification. *Current Opinion in Colloid & Interface Science* **2016**, *22*, 88–93. https://doi.org/10.1016/j.cocis.2016.03.002.

(11) Miller, C. A. Spontaneous Emulsification Produced by Diffusion — A Review. *Colloids and Surfaces* **1988**, *29* (1), 89–102. https://doi.org/10.1016/0166-6622(88)80173-2.

(12) Bouchemal, K.; Briançon, S.; Perrier, E.; Fessi, H. Nano-Emulsion Formulation Using Spontaneous Emulsification: Solvent, Oil and Surfactant Optimisation. *International Journal of Pharmaceutics* **2004**, *280* (1), 241–251. https://doi.org/10.1016/j.ijpharm.2004.05.016.

(13) Rehman, A. U.; Collot, M.; Klymchenko, A. S.; Akram, S.; Mustafa, B.; Vandamme, T.; Anton, N. Spontaneous Nano-Emulsification with Tailor-Made Amphiphilic Polymers and Related Monomers. *European Journal of Pharmaceutical Research* **2019**, *1* (1), 27–36. https://doi.org/10.34154/2019-EJPR.01(01).pp-27-36/euraass.

(14) Anton, N.; Benoit, J.-P.; Saulnier, P. Design and Production of Nanoparticles Formulated from Nano-Emulsion Templates—A Review. *Journal of Controlled Release* **2008**, *128* (3), 185–199. https://doi.org/10.1016/j.jconrel.2008.02.007.

(15) Yu, H.; Huang, Q. Improving the Oral Bioavailability of Curcumin Using Novel Organogel-Based Nanoemulsions. *Journal of Agricultural and Food Chemistry* **2012**, *60* (21), 5373–5379. https://doi.org/10.1021/jf300609p.
(16) Asthana, S.; Jaiswal, A. K.; Gupta, P. K.; Pawar, V. K.; Dube, A.; Chourasia, M. K. Immunoadjuvant Chemotherapy of Visceral Leishmaniasis in Hamsters Using Amphotericin B-Encapsulated Nanoemulsion Template-Based Chitosan Nanocapsules. *Antimicrobial Agents and Chemotherapy* **2013**, *57* (4), 1714. https://doi.org/10.1128/AAC.01984-12.
(17) Hsu, C.-Y.; Wang, P.-W.; Alalaiwe, A.; Lin, Z.-C.; Fang, J.-Y. Use of Lipid Nanocarriers to Improve Oral Delivery of Vitamins. *Nutrients* **2019**, *11* (1), 68. https://doi.org/10.3390/nu11010068.
(18) Hörmann, K.; Zimmer, A. Drug Delivery and Drug Targeting with Parenteral Lipid Nanoemulsions — A Review. *Journal of Controlled Release* **2016**, *223*, 85–98. https://doi.org/10.1016/j.jconrel.2015.12.016.
(19) Fornaguera, C.; Grijalvo, S.; Galán, M.; Fuentes-Paniagua, E.; de la Mata, F. J.; Gómez, R.; Eritja, R.; Calderó, G.; Solans, C. Novel Non-Viral Gene Delivery Systems Composed of Carbosilane Dendron Functionalized Nanoparticles Prepared from Nano-Emulsions as Non-Viral Carriers for Antisense Oligonucleotides. *International Journal of Pharmaceutics* **2015**, *478* (1), 113–123. https://doi.org/10.1016/j.ijpharm.2014.11.031.
(20) Hak, S.; Helgesen, E.; Hektoen, H. H.; Huuse, E. M.; Jarzyna, P. A.; Mulder, W. J. M.; Haraldseth, O.; Davies, C. de L. The Effect of Nanoparticle Polyethylene Glycol Surface Density on Ligand-Directed Tumor Targeting Studied in Vivo by Dual Modality Imaging. *ACS nano* **2012**, *6* (6), 5648–5658. https://doi.org/10.1021/nn301630n.
(21) Mou, D.; Chen, H.; Du, D.; Mao, C.; Wan, J.; Xu, H.; Yang, X. Hydrogel-Thickened Nanoemulsion System for Topical Delivery of Lipophilic Drugs. *International Journal of Pharmaceutics* **2008**, *353* (1), 270–276. https://doi.org/10.1016/j.ijpharm.2007.11.051.
(22) Khandavilli, S.; Panchagnula, R. *Nanoemulsions as Versatile Formulations for Paclitaxel Delivery: Peroral and Dermal Delivery Studies in Rats*; 2007; Vol. 127. https://doi.org/10.1038/sj.jid.5700485.
(23) Kaur, I. P.; Kakkar, S. Nanotherapy for Posterior Eye Diseases. *Journal of Controlled Release* **2014**, *193*, 100–112. https://doi.org/10.1016/j.jconrel.2014.05.031.
(24) Lim, C.; Kim, D.; Sim, T.; Hoang, N. H.; Lee, J. W.; Lee, E. S.; Youn, Y. S.; Oh, K. T. Preparation and Characterization of a Lutein Loading Nanoemulsion System for Ophthalmic Eye Drops. *Journal of Drug Delivery Science and Technology* **2016**, *36*, 168–174. https://doi.org/10.1016/j.jddst.2016.10.009.
(25) Patel, N.; Nakrani, H.; Raval, M.; Sheth, N. Development of Loteprednol Etabonate-Loaded Cationic Nanoemulsified in-Situ Ophthalmic Gel for Sustained Delivery and Enhanced Ocular Bioavailability. *Drug Delivery* **2016**, *23* (9), 3712–3723. https://doi.org/10.1080/10717544.2016.1223225.
(26) Nasr, M.; Nawaz, S.; Elhissi, A. Amphotericin B Lipid Nanoemulsion Aerosols for Targeting Peripheral Respiratory Airways via Nebulization. *International Journal of Pharmaceutics* **2012**, *436* (1–2), 611–616. https://doi.org/10.1016/j.ijpharm.2012.07.028.
(27) Sonneville-Aubrun, O.; Simonnet, J. T.; L'Alloret, F. Nanoemulsions: A New Vehicle for Skincare Products. *Advances in Colloid and Interface Science* **2004**, *108–109*, 145–149. https://doi.org/10.1016/j.cis.2003.10.026.

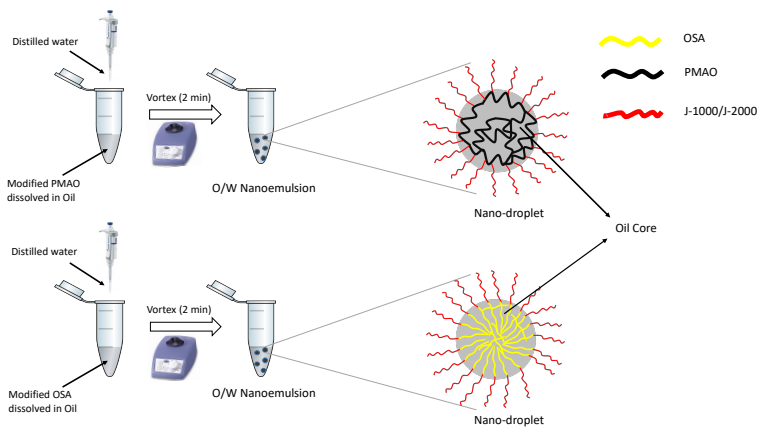
(28) Gao, F.; Zhang, Z.; Bu, H.; Huang, Y.; Gao, Z.; Shen, J.; Zhao, C.; Li, Y. Nanoemulsion Improves the Oral Absorption of Candesartan Cilexetil in Rats: Performance and Mechanism. *Journal of Controlled Release* **2011**, *149* (2), 168–174. https://doi.org/10.1016/j.jconrel.2010.10.013.
(29) Singh, K. K.; Vingkar, S. K. Formulation, Antimalarial Activity and Biodistribution of Oral Lipid Nanoemulsion of Primaquine. *International Journal of Pharmaceutics* **2008**, *347* (1), 136–143. https://doi.org/10.1016/j.ijpharm.2007.06.035.
(30) Choudhury, H.; Gorain, B.; Karmakar, S.; Biswas, E.; Dey, G.; Barik, R.; Mandal, M.; Pal, T. K. Improvement of Cellular Uptake, in Vitro Antitumor Activity and Sustained Release Profile with Increased Bioavailability from a Nanoemulsion Platform. *International Journal of Pharmaceutics* **2014**, *460* (1), 131–143. https://doi.org/10.1016/j.ijpharm.2013.10.055.
(31) Tang, S. Y.; Sivakumar, M.; Ng, A. M.-H.; Shridharan, P. Anti-Inflammatory and Analgesic Activity of Novel Oral Aspirin-Loaded Nanoemulsion and Nano Multiple Emulsion Formulations Generated Using Ultrasound Cavitation. *International Journal of Pharmaceutics* **2012**, *430* (1), 299–306. https://doi.org/10.1016/j.ijpharm.2012.03.055.
(32) Ke, Z.; Hou, X.; Jia, X.-B. Design and Optimization of Self-Nanoemulsifying Drug Delivery Systems for Improved Bioavailability of Cyclovirobuxine D. *Drug design, development and therapy* **2016**, *10*, 2049–2060. https://doi.org/10.2147/DDDT.S106356.
(33) Chai, F.; Sun, L.; Ding, Y.; Liu, X.; Zhang, Y.; Webster, T. J.; Zheng, C. A Solid Self-Nanoemulsifying System of the BCS Class IIb Drug Dabigatran Etexilate to Improve Oral Bioavailability. *Nanomedicine* **2016**, *11* (14), 1801–1816. https://doi.org/10.2217/nnm-2016-0138.
(34) Li, P.; Tan, A.; Prestidge, C. A.; Nielsen, H. M.; Müllertz, A. Self-Nanoemulsifying Drug Delivery Systems for Oral Insulin Delivery: In Vitro and in Vivo Evaluations of Enteric Coating and Drug Loading. *International Journal of Pharmaceutics* **2014**, *477* (1), 390–398. https://doi.org/10.1016/j.ijpharm.2014.10.039.
(35) Kelmann, R. G.; Kuminek, G.; Teixeira, H. F.; Koester, L. S. Carbamazepine Parenteral Nanoemulsions Prepared by Spontaneous Emulsification Process. *International Journal of Pharmaceutics* **2007**, *342* (1), 231–239. https://doi.org/10.1016/j.ijpharm.2007.05.004.
(36) Venkateshwarlu, I.; Kandadi, P.; Ali, M.; Veerabrahma, K. *Development and in Vitro Cytotoxic Evaluation of Parenteral Docetaxel Lipid Nanoemulsions for Application in Cancer Treatment*; 2010; Vol. 64.
(37) Ragelle, H.; Crauste-Manciet, S.; Seguin, J.; Brossard, D.; Scherman, D.; Arnaud, P.; Chabot, G. G. Nanoemulsion Formulation of Fisetin Improves Bioavailability and Antitumour Activity in Mice. *International Journal of Pharmaceutics* **2012**, *427* (2), 452–459. https://doi.org/10.1016/j.ijpharm.2012.02.025.
(38) Borhade, V.; Pathak, S.; Sharma, S.; Patravale, V. Clotrimazole Nanoemulsion for Malaria Chemotherapy. Part I: Preformulation Studies, Formulation Design and Physicochemical Evaluation. *International Journal of Pharmaceutics* **2012**, *431* (1), 138–148. https://doi.org/10.1016/j.ijpharm.2011.12.040.
(39) Alves, M. P.; Scarrone, A. L.; Santos, M.; Pohlmann, A. R.; Guterres, S. S. Human Skin Penetration and Distribution of Nimesulide from Hydrophilic Gels Containing

Nanocarriers. *International Journal of Pharmaceutics* **2007**, *341* (1), 215–220. https://doi.org/10.1016/j.ijpharm.2007.03.031.
(40) Ali, M. S.; Alam, M. S.; Imam, F. I.; Siddiqui, M. R. Topical Nanoemulsion of Turmeric Oil for Psoriasis: Characterization, Ex Vivo and in Vivo Assessment. *2012* **2012**, *4* (2), 14.
(41) Hagigit, T.; Abdulrazik, M.; Valamanesh, F.; Behar-Cohen, F.; Benita, S. Ocular Antisense Oligonucleotide Delivery by Cationic Nanoemulsion for Improved Treatment of Ocular Neovascularization: An in-Vivo Study in Rats and Mice. *Journal of Controlled Release* **2012**, *160* (2), 225–231. https://doi.org/10.1016/j.jconrel.2011.11.022.
(42) Mahajan, H. S.; Mahajan, M. S.; Nerkar, P. P.; Agrawal, A. Nanoemulsion-Based Intranasal Drug Delivery System of Saquinavir Mesylate for Brain Targeting. *Drug Delivery* **2014**, *21* (2), 148–154. https://doi.org/10.3109/10717544.2013.838014.
(43) Raj Pandey, Y.; Kumar, S.; Kumar Gupta, B.; Ali, J.; Baboota, S. *Intranasal Delivery of Paroxetine Nanoemulsion via the Olfactory Region for the Management of Depression: Formulation, Behavioural and Biochemical Estimation*; 2015; Vol. 27. https://doi.org/10.1088/0957-4484/27/2/025102.
(44) Kumar, S.; Ali, J.; Baboota, S. Design Expert®supported Optimization and Predictive Analysis of Selegiline Nanoemulsion via the Olfactory Region with Enhanced Behavioural Performance in Parkinson's Disease. *Nanotechnology* **2016**, *27* (43), 435101. https://doi.org/10.1088/0957-4484/27/43/435101.
(45) Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J. Nanoemulsion Delivery Systems: Influence of Carrier Oil on β-Carotene Bioaccessibility. *Food Chemistry* **2012**, *135* (3), 1440–1447. https://doi.org/10.1016/j.foodchem.2012.06.047.
(46) Qian, C.; Decker, E. A.; Xiao, H.; McClements, D. J. Physical and Chemical Stability of β-Carotene-Enriched Nanoemulsions: Influence of PH, Ionic Strength, Temperature, and Emulsifier Type. *Food Chemistry* **2012**, *132* (3), 1221–1229. https://doi.org/10.1016/j.foodchem.2011.11.091.
(47) Yuan, Y.; Gao, Y.; Mao, L.; Zhao, J. Optimisation of Conditions for the Preparation of β-Carotene Nanoemulsions Using Response Surface Methodology. *Food Chemistry* **2008**, *107* (3), 1300–1306. https://doi.org/10.1016/j.foodchem.2007.09.015.
(48) Yuan, Y.; Gao, Y.; Zhao, J.; Mao, L. Characterization and Stability Evaluation of β-Carotene Nanoemulsions Prepared by High Pressure Homogenization under Various Emulsifying Conditions. *Food Research International* **2008**, *41* (1), 61–68. https://doi.org/10.1016/j.foodres.2007.09.006.
(49) Klymchenko, A. S.; Roger, E.; Anton, N.; Anton, H.; Shulov, I.; Vermot, J.; Mely, Y.; Vandamme, T. F. Highly Lipophilic Fluorescent Dyes in Nano-Emulsions: Towards Bright Non-Leaking Nano-Droplets. *RSC Advances* **2012**, *2* (31), 11876–11886. https://doi.org/10.1039/C2RA21544F.
(50) Nogues, I. F. T.; Goutayer, M.; Silva, A. da; Guyon, L.; Djaker, N.; Josserand, V.; Neumann, E.; Bibette, J.; Vinet, F. *Cyanine-Loaded Lipid Nanoparticles for Improved in Vivo Fluorescence Imaging*; SPIE, 2009; Vol. 14.
(51) Rapoport, N.; Nam, K.-H.; Gupta, R.; Gao, Z.; Mohan, P.; Payne, A.; Todd, N.; Liu, X.; Kim, T.; Shea, J.; et al. *Ultrasound-Mediated Tumor Imaging and Nanotherapy Using*

Drug Loaded, Block Copolymer Stabilized Perfluorocarbon Nanoemulsions; 2011; Vol. 153. https://doi.org/10.1016/j.jconrel.2011.01.022.
(52) Attia, M. F.; Anton, N.; Akasov, R.; Chiper, M.; Markvicheva, E.; Vandamme, T. F. Biodistribution and Toxicity of X-Ray Iodinated Contrast Agent in Nano-Emulsions in Function of Their Size. *Pharmaceutical Research* **2016**, *33* (3), 603–614. https://doi.org/10.1007/s11095-015-1813-0.
(53) Attia, M. F.; Anton, N.; Chiper, M.; Akasov, R.; Anton, H.; Messaddeq, N.; Fournel, S.; Klymchenko, A. S.; Mély, Y.; Vandamme, T. F. Biodistribution of X-Ray Iodinated Contrast Agent in Nano-Emulsions Is Controlled by the Chemical Nature of the Oily Core. *ACS Nano* **2014**, *8* (10), 10537–10550. https://doi.org/10.1021/nn503973z.

Chapter 2.2: Spontaneous nano-emulsification with tailor-made amphiphilic polymers and related monomers

In this study, we have developed nano-emulsions using an amphiphilic polymer, synthesized from poly (maleic anhydride-alt-1-octadecene) (PMAO). The main objective of this investigation was to understand if the spontaneous emulsification, commonly performed with non-ionic surfactants alone or in combination with an organic solvent, can be transposed with amphiphilic polymers in the absence of both the surfactant as well as organic solvent or not. A secondary objective was to identify the main parameters influencing the spontaneous emulsification process, and to achieve this objective, different formulation and physico-chemical parameters inherent to the emulsification process were studied, and their impact on the properties, size and polydispersity of the droplets was carefully analyzed. First of all, PMAO was modified by grafting separately, two Jeffamine® polyetheramine (polyethylene glycol / polypropylene glycol copolymer) (*i.e.*, J-1000 and J-2000) on each anhydride function, in order to generate an octadecene / Jeffamine® pattern on each monomeric element. In parallel, similar amphiphiles with J1000 and J2000 were designed, but using the parent monomer of PMAO, i.e. octadecyl succinic anhydride (OSA), instead of PMAO and relative abilities of the monomer and polymer to form nano-emulsions by spontaneous emulsification were evaluated. The results showed that the developed amphiphilic polymer stabilizes the oil / water interfaces and forms nano-emulsions in the absence of other surfactants. The most efficient conditions were obtained with the combination of PMAO and longer Jeffamine® chains (J-2000), compared to OSA surfactant. It was further observed that the size of the formed droplets could significantly vary with the change in the nature of the oil. This study may have important implications in the development of surfactant-free nano-emulsions, and surface functionalized nano-emulsions.



EUROPEAN JOURNAL OF PHARMACEUTICAL RESEARCH - VOL. 1 ISSUE 1 PP. 27-36 (2019)

European Academy of Applied and Social Sciences – www.euraass.com

European Journal of Pharmaceutical Research

https://www.euraass.com/category/list-of-journals/european-journal-of-pharmaceutical-research/

Research Article

Spontaneous nano-emulsification with tailor-made amphiphilic polymers and related monomers

Asad Ur Rehman,[a,b] Mayeul Collot,[c] Andrey S. Klymchenko,[c] Salman Akram,[a] Bilal Mustafa,[a] Thierry Vandamme,[a] Nicolas Anton [a,]*

[a]*University of Strasbourg, CNRS, CAMB UMR 7199, F-67000 Strasbourg, France*

[b]*Bahauddin Zakariya University (BZU) Multan, Pakistan*

[c]*University of Strasbourg, CNRS, LBP UMR 7021, F-67000 Strasbourg, France*

Received: January 30th 2019 / Revised: February 21st 2019 / Accepted: March 8th 2019

Abstract

In general, nano-emulsions are submicron droplets composed of liquid oil phase dispersed in liquid aqueous bulk phase. They are stable and very powerful systems when it regards the encapsulation of lipophilic compounds and their dispersion in aqueous medium. On the other hand, when the properties of the nano-emulsions aim to be modified, *e.g.* for changing their surface properties, decorating the droplets with targeting ligands, or modifying the surface charge, the dynamic liquid / liquid interfaces make it relatively challenging. In this study, we have explored the development of nano-emulsions which were not anymore stabilized with a classical low-molecular weight surfactant, but instead, with an amphiphilic polymer based on poly(maleic anhydride-alt-1-octadecene) (PMAO) and Jeffamine®, a hydrophilic amino-terminated PPG/PEG copolymer. Using a polymer as stabilizer is a potential solution for the nano-emulsion functionalization, ensuring the droplet stabilization as well as being a platform for the droplet decoration with ligands (for instance after addition of function groups in the terminations of the chains). The main idea of the present work was to understand if the spontaneous emulsification –commonly performed with nonionic surfactants– can be transposed with amphiphilic polymers, and a secondary objective was to identify the main parameters impacting on the process. PMAO was modified with two different Jeffamine®, additionally different oils and different formulation conditions were evaluated. As a control, the parent monomer, octadecyl succinic anhydride (OSA) was also modified and studied in the similar way as that of polymer. The generated nano-emulsions were mainly studied by dynamic light scattering and electron microscopy, that allows discriminating the crucial parameters in the spontaneous process, originally conducted with polymers as only stabilizer.

Keywords:

Spontaneous emulsification; PMAO; nano-emulsion; surfactant; Jeffamine.

1. Introduction

Nano-emulsions are fine, stable, and in general optically clear and translucent oil-in-water or water-in-oil dispersions, stabilized by a nonionic surfactant. Droplets size range below 200-300 nm (N. Anton & Vandamme, 2009; Mahato, 2017; Mason, Wilking,

* *Corresponding author.* Tel.: +33(0)3-68-85-42-51; fax: +33(0)3-68-85-43-06.
E-mail address: nanton@unistra.fr (N. ANTON)
Available online: March 13rd 2019; doi: 10.34154/2019-EJPR.01(01).pp-27-36/euraass
Cite this: *Eur. J. Pharm. Res.* 2019, 1(1), 27-36.
ISSN-e: 2650-7501.

Meleson, Chang, & Graves, 2006; McClements, 2012; Solans, Morales, & Homs, 2016). The small droplet size results in long-term physical stability of nano-emulsions, because it impairs the destabilization phenomena like sedimentation, creaming and coalescence (Singh et al., 2017; Solans et al., 2016). Nano-emulsions can be administered by various routes like oral, ocular, topical, intravenous, pulmonary and intranasal route because of the compatibility of their physico-chemical properties with the different administration routes, as well as the possibility of rendering them into different kinds of dosage forms, like creams, gels, liquids and aerosols (Ammar, Salama, Ghorab, & Mahmoud, 2009; Hussain et al., 2016; Khani, Keyhanfar, & Amani, 2016; Nasr, Nawaz, & Elhissi, 2012; Pawar et al., 2014; Singh et al., 2017). The nano-emulsions are thermodynamically unstable and kinetically stable systems. Unlike nanostructures of microemulsions, the nano-emulsion droplets can remain stable in stress conditions as well like temperature and concentration (*e.g.* dilution) modifications (Nicolas Anton & Vandamme, 2011; Lefebvre et al., 2017; Singh et al., 2017). The methods used to prepare nano-emulsions can be classified into two main types, *(i)* high-energy methods and *(ii)* low-energy methods (Anton, Benoit, & Saulnier, 2008). Over the past twenty five years, the research interest in the development of low-energy methods has increased because of their numerous advantages like smaller droplet sizes, low cost, high formulation yields, encapsulation of fragile active molecules, potential industrial scale up and wide range of applications (food, cosmetic and pharmaceutical industry). The low-energy methods divert the intrinsic physicochemical properties of the surfactants, co-surfactants and excipients in the formulation, leading to the generation of stable emulsion droplets in the nanometric range (N. Anton & Vandamme, 2009). A special character of these methods is the utilization of energy stored in the system to produce ultra-fine droplets.

Among the various low-energy methods, spontaneous emulsification phenomenon has been the focus of attention due to its diverse applications and easy industrial scaling-up, particularly in pharmaceutical and cosmetics field. The spontaneous emulsification is a very simple method in which an aqueous phase is mixed with an oil phase –composed of a mixture of oil, surfactant and/or water miscible solvent. Various mechanisms related to the droplet formation process have been proposed in the literature, and the most important and well-documented methods are explained by the creation, during the spontaneous emulsification, of interfacial turbulence, development of transient values of interfacial tensions, diffusion and stranding, Marangoni flow and Rayleigh-Taylor instability (López-Montilla, Herrera-Morales, Pandey, & Shah, 2002; Solans et al., 2016). However the general mechanism is the rapid displacement of a solvent, from the oil phase to the aqueous phase that induces turbulence and super saturation at the oil/water interface (Miller, 1988; Solans et al., 2016). The major drawback associated with this method is in fact the use of the solvent itself, like ethanol, acetone, that can limit the application compatibilities and make the whole process complicated –*e.g.* if including solvent evaporation step. In this context, our group has already shown that non-ionic surfactants themselves can play the role of the displacing species in the spontaneous emulsification process, following an universal mechanism related to the sudden displacement of the surfactants from the oily to the aqueous phase, due to the sudden change of surfactant solubility (N. Anton & Vandamme, 2009).

In the context of the control of the chemical composition of the surface, we have reported preliminary studies that describe original methods to modify the water / oil interface, and reinforce the droplet with in situ synthesis of silica shell at the droplet interfaces (Attia et al., 2015), or by anchorage of polymeric amphiphiles (Attia et al., 2017). However, these methods still use surfactants to help the nano-droplet formation, and in some extents, it could constitute a limitation –in term of biocompatibility, toxicity, development of incompatibilities with time (Hou & Xu, 2016; Kaci et al., 2016; Nehilla, Bergkvist, Popat, & Desai, 2008; Sahoo, Panyam, Prabha, & Labhasetwar, 2002; Sheibat-Othman & Bourgeat-Lami, 2009)– or in term of efficiency of the surface modification.

To deal with this aspect of the nano-emulsion formulation related to surface modification, we propose in the current study, investigating how nano-emulsions can be formulated by low-energy emulsification when the nonionic surfactants are totally replaced for an amphiphilic polymer. The most innovative aspect lies in the fact that we describe for the first time that spontaneous nano-emulsification can be successfully performed using a macromolecule migrating from the oil phase to the liquid / liquid interface. In fact, the formulation of nano-emulsions stabilized by polymers presents numerous advantages respective to the conventional surfactant-stabilized droplets, mainly the surface functionalization –related to the chemical function present at the termination of the hydrophilic parts of the polymer– and secondly the droplet stability.

This study begins with the design and synthesis of amphiphilic polymers, specially tailored to exhibit an important surface-active property, followed by the evaluation of its ability to generate nano-emulsions through the spontaneous emulsification method. The synthesis of amphiphile polymer is based on the modification of poly(maleic anhydride-alt-1-octadecene) (PMAO), which is a hydrophobic polymer having aliphatic octadecene chains (C18) with reactive anhydride functions. Herein we start from a former study (Attia et al., 2017) on which we showed their solubility in the oil core and their ability to migrate at the interfacial layer driven by the reactivity of its anhydride function. PMAO is a low-cost lipophilic polymer, for which the monomeric group is composed of aliphatic octadecene chain and a reactive cyclic anhydride function. Our objective was to modify the PMAO by performing a systematic reaction of Jeffamine® polyetheramine (polyethylene glycol / polypropylene glycol copolymer) on each anhydride function, in order to generate an octadecene / Jeffamine® pattern on each monomeric element. As a result, it gives rise to a polymer perfectly designed to stabilize the oil / water interfaces, similarly to a high number of nonionic surfactants assembled together. The different

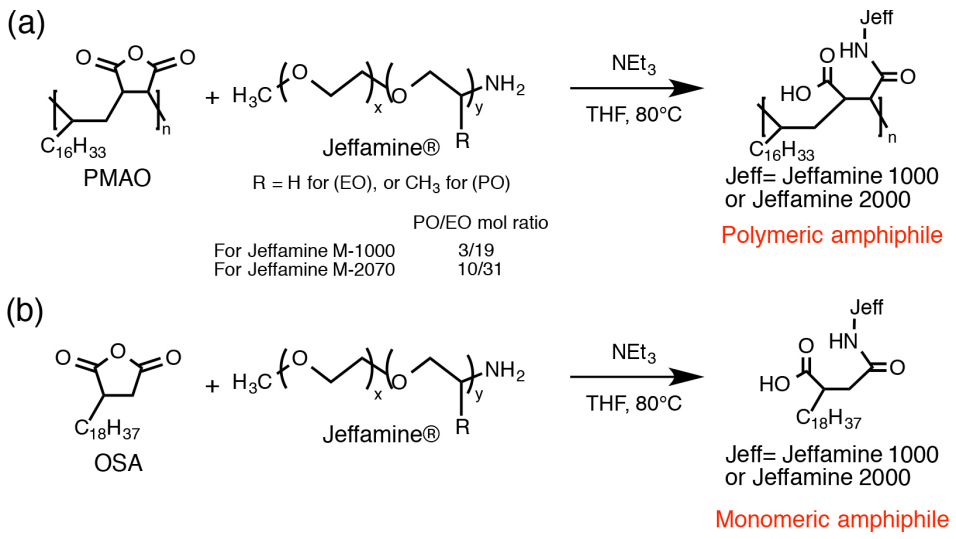


Figure 1: Synthesis of amphiphilic polymers (a) and monomers (b).

formulations, chemical and physico-chemical parameters inherent to the emulsification process were studied, and their impact on the properties, size and polydispersity of the droplets was carefully analyzed. In addition, Jeffamine® 1000 / 2000 was chosen because of the high amount of PEG in the chain composition. This will result in high interfacial stabilization creating steric repulsion among the neighboring nano-droplets (Hörmann & Zimmer, 2016), but also well-recognized to decrease the immune system uptake after in vivo administration, inhibiting opsonization and phagocytosis as observed with nano-emulsions (Cristina Fornaguera et al., 2015; Hörmann & Zimmer, 2016). In addition, and in order to compare the impact of the macromolecular structure, we have designed similar amphiphiles with J1000 and J2000, but using the parent monomer of PMAO, *i.e.* octadecyl succinic anhydride (OSA). The idea was to use it in place of PMAO based amphiphile, keeping the rest of the parameters constant, to observe in parallel the relative abilities of the monomer and polymer to form nano-emulsions and also their impact on the size of the nano droplets. The resulting species exhibit a structure close to nonionic surfactants, making a bridge between modified PMAO and literature.

Beyond such a pioneer description of spontaneous nano-emulsification performed using only polymeric molecule –without low molecular weight surfactants– this study proposes several new ideas on the potential route that can serve to the functionalization of emulsion, that is to say functionalizing dynamic liquid / liquid interfaces. As an example, a proportion of the Jeffamine® herein used can be terminated with another reactive group and finally covalently linked with, for instance, a ligand. Emulsions droplets constitute a high-potential nano-carrier for hundred applications owing to their reservoir capability, with a main drawback in their surface modification and stability: the solution herein presented is an illustration that simple solutions can exist, can be easily developed and transposed to large-scale production.

2. Materials and Methods

2.1. Chemicals

Poly (maleic anhydride-alt-1-octadecene) (PMAO), triethylamine (TEA), tetrahydrofuran (THF) octadecyl succinic anhydride (OSA) and dichloromethane (DCM) were purchased from Sigma Aldrich. Jeffamine® M-1000 (J-1000) and Jeffamine® M-2070 polyetheramine (J-2000) were kindly offered by Huntsman corporation (Texas, U.S.A.). Medium chain triglycerides (Labrafac WL®) and Plurol oleic were purchased from Gattefossé (Saint Priest, France). The rest of the reagents and solvents used were of analytical grade.

2.2. Polymer chemical modification

The global synthesis is illustrated in Fig. 1 (a). To a solution of Jeffamine® (J-1000 or J-2000, 2.85 mmol) in THF (about 7 mL) was added PMAO (1.00 g, 2.85 mmol based on repeating unit) Then the volume of THF was completed up to 50 mL. After total solubilization of the PMAO, 1.2 mL of TEA (3 equivalents) was added into the solution that was warmed at 80°C and allowed to stir at this temperature overnight. Next, the THF was evaporated using rotavapor, and the resulting product was dried under vacuum.

Jeffamine® M-1000 and M-2070 polyether amine (J-1000 and J-2000) are monoamines based on a copolymer backbone with an average molecular weight of 1000 and 2000, respectively. Their propylene oxide / ethylene oxide (PO/EO) molar ratio is 3/19 and 10/31, respectively.

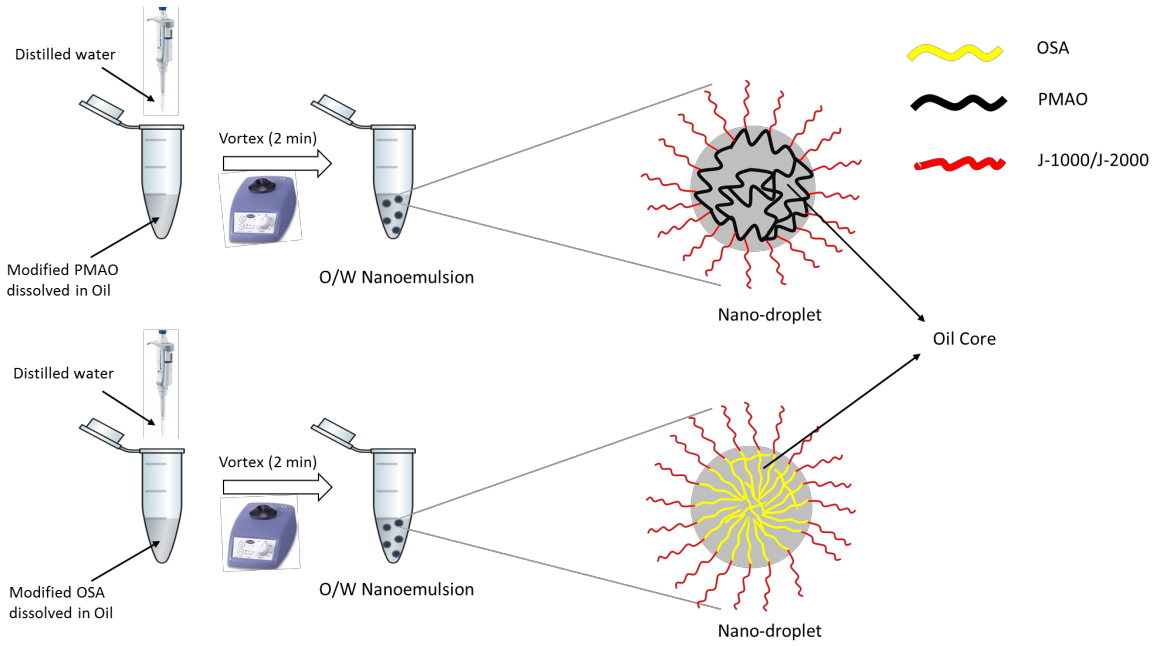


Figure 2: Schematic illustration of the nano droplet formation. The amphiphilic polymers or monomers are solubilized inside the oily core whereas the hydrophilic moiety (Jeffamine® M-1000/Jeffamine® M-2000) are displayed at the droplet surface.

2.3. Monomer chemical modification

The global synthesis is illustrated in Fig. 1 (b). The experimental procedure followed was strictly the same as described above for the polymer, After solvent evaporation by rotary evaporator, the obtained product was dissolved in DCM and then washed using a separatory funnel after mixing different aqueous phases *(i)* first 1M HCl aqueous phase, *(ii)* then with saturated NaCl solution, and *(iii)* finally dried by using sodium sulfate. The mixture was filtered under filter paper, and DCM evaporated using rotary evaporator to collect the final product.

2.4. ^{1}H-NMR Analysis

To confirm the structure of the amphiphilic polymers / monomers, 25 mg of these products were dissolved in 600 µL of deuterated chloroform ($CDCl_3$) and 1H-NMR spectrum was studied with a Bruker Top Spin 3.0 operating at 400 MHz. The chemical shifts (δ) were expressed in parts per million.

2.5. Preparation of Nano-emulsions

First of all, both oil and aqueous phases were prepared separately and heated at 90°C for 20 min. Oil phase consists of monomer or polymer dissolved in oil (medium chain triglycerides, Labrafac WL®), while the aqueous phase was distilled water. The second step is the spontaneous emulsification process (N. Anton & Vandamme, 2009): aqueous and oil phases were rapidly mixed together and homogenized by vortex for 2 min, giving rise to the formation of nano-emulsions (Fig. 2).

2.6. Dynamic light scattering

The hydrodynamic diameters of the nano emulsion droplets were measured, in triplicate, by dynamic light scattering using a Malvern Nano ZS instrument, equipped with a helium-neon laser 4 mW operating at 633 nm, with the scatter angle fixed at 173° and the temperature was maintained at 25° C. The polydispersity index (PDI) was also measured which shows the quality of the dispersion. Generally, the PDI values ⩽ 0.2 indicate good mono dispersity and a good quality of the nano-emulsions.

2.7. Microscopic observations

The morphology and structure of the nano droplets formed were studied by transmission electron microscopy (TEM). The nano-emulsion samples were diluted (1/100) with Milli-Q water. One drop of the diluted nano-emulsion was introduced on a carbon grid (carbon type A, 300 mesh, copper, Ted Pella Inc. Redding, PA) and dried at 40°C. The observations were carried out by a Philips Morgagni 268D electron microscope.

2.8. Optimization of the formulation parameters

Nano-emulsions were generated by the addition of aqueous phase into the oil phase. As it is an important parameter for common spontaneous emulsification of nano-emulsions with surfactant (surfactant-to-oil weight ratio, SOR expressed in percen-

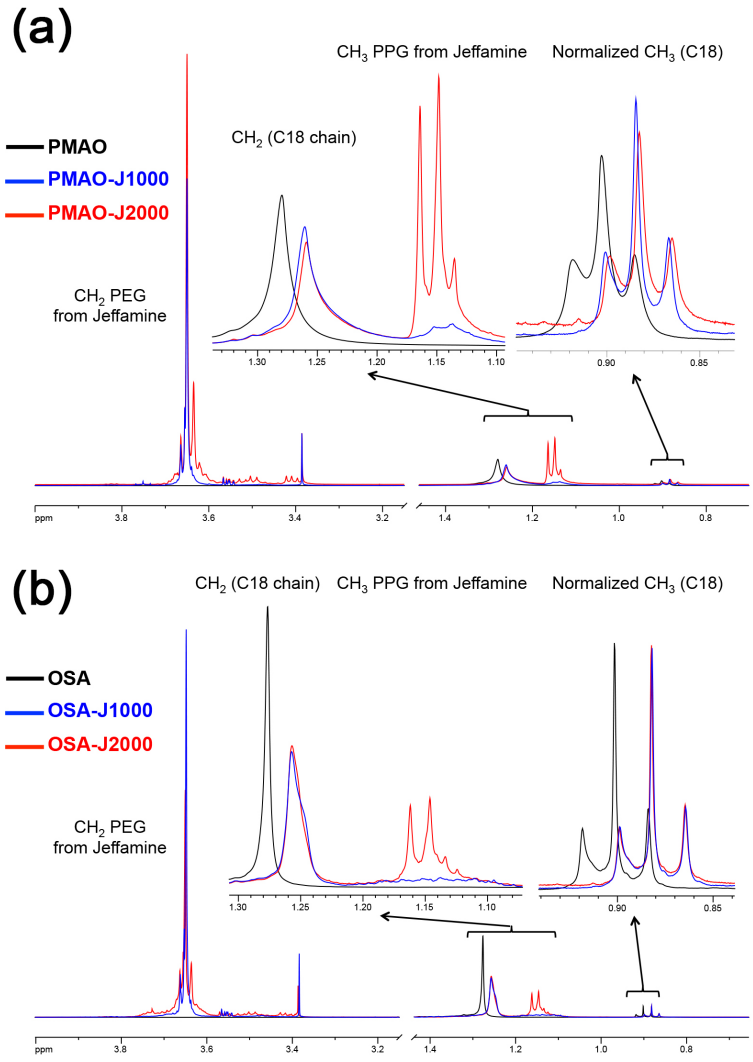


Figure 3: Superimposed NMR spectra of amphiphilic polymers and PMAO (a) and amphiphilic monomers and OSA (b) showing the shifts of the signals and the increasing amount of PEG and PPG compared to the starting materials. The NMR spectra were normalized to the CH_3 signal of the C_{18} chain at 0.8-0.9 ppm. The spectra were cropped for a better visibility.

-tage) similar parameter were herein defined when using the polymer: the polymer-to-oil weight ratio (POR, in %) –while SOR is used when using the monomer, similar to a surfactant. Impact of POR and SOR on size distribution and PDI were followed, keeping the rest of the parameters constant (temperature, mixing time, volume of aqueous phase). The experiments were performed using both the Jeffamine® derivatives (J1000 and J2000), grafted on both polymer and monomer.

2.9. Effect of nature of oil on emulsification

To study the effect of nature of oil on the formation of nano-emulsions, size and polydispersity, the formulation was followed with different oils, soy bean oil, fish oil and Plurol oleic. Based on the results obtained with different POR and SOR ratios, two values were selected: 50% and 80%. The rest of the parameters were kept constant and nano-emulsions were prepared using the same procedure described above.

3. Results and discussion

First, the amphiphilic monomers and polymers were synthesized from OSA and PMAO respectively, by mean of addition of Jeffamine® 1000 or 2000 in the presence of

triethylamine (Fig. 1). The obtained amphiphiles were characterized by 1H NMR spectroscopy (Fig. 3). The spectra showed significant shifts of characteristic signals compared to the starting materials along with increasing PEG and PPG signals from the grafted Jeffamine®, thus proving the chemical efficient modifications.

Nonionic surfactants are used in the preparation of nano-emulsions and the literature has extensively shown their crucial role in the emulsification processes (N. Anton, Akram, & Vandamme, 2018; N. Anton & Vandamme, 2009). After mixing homogeneously the {oil + surfactant} phase, the spontaneous emulsification process was indeed driven by the turbulent solubilization of the surfactant by the aqueous phase and during homogenization of these three compounds. Making nano-emulsions using amphiphilic polymer as stabilizer –or core-shell nano-droplets– is a real advantage towards the classical surfactant based emulsions: at first sight the first advantage appears as an improvement of the droplet stability. In addition, and importantly, a second advantage lies in the potential functionalization of the droplet surface, much more strongly anchored when it is performed onto a polymeric shell, compared to surfactant anchorage lonely made by molecular adsorption. On the other hand, when making nano-emulsions by spontaneous emulsification is simple, the corresponding processes using polymers are not comparatively simple (N. Anton, Benoit, & Saulnier, 2008; Nehilla et al., 2008; Sahoo et al., 2002). Through this study, we propose to investigate whether spontaneous emulsification can be performed with an amphiphilic polymer as it is the case with common nonionic surfactants, but this time without surfactant. In is also important to note that such a formulation method, besides following a simple and scalable process, is able to produce ready-to-use suspensions, that is to say without need of further purification –*e.g.* for removing chemical precursors when compared with the in situ polymerization with residual monomer or initiator, or undesired by-products (Calderó, García-Celma, & Solans, 2011; C Fornaguera, Feiner-Gracia, Calderó, García-Celma, & Solans, 2016).

Both Jeffamine® polyetheramines (J-1000 and J-2000) used in this study are predominantly composed of PEG, making their structure highly similar to the polar head of nonionic surfactants generally used in the spontaneous nano-emulsification processes (N. Anton et al., 2018), and therefore should induce, similarly, efficient stabilizer properties of the species synthetized in the present study. In the case of nonionic surfactants, right after mixing, the water phase immediately and suddenly penetrate the oily phase to solubilize the amphiphilic molecules. Due to the sudden composition change, the physico-chemical properties of the global system undergoes a drastic change making the surfactant much more hydrophilic than before mixing –thanks to the effect of temperature and/or dilution (N. Anton & Vandamme, 2009; Nicolas Anton & Vandamme, 2011; Hörmann & Zimmer, 2016; Miller, 1988; Solans et al., 2016). The water breaks-up the oil phase and the surfactants are assumed to be shared between the water/oil interface and solubilized in water (Fig. 2). Regarding the amphiphilic polymer, the principle proposed is close, playing on its own partitioning between the oily and aqueous phases, before and after their mixing. The expected results would be the fractionation of oily phase by the water, generating nano-droplets, but with the difference –compared with nonionic surfactants– that the polymeric molecules stays trapped at the oil/water interface since it is not soluble neither in oil nor in water at room temperature, making a

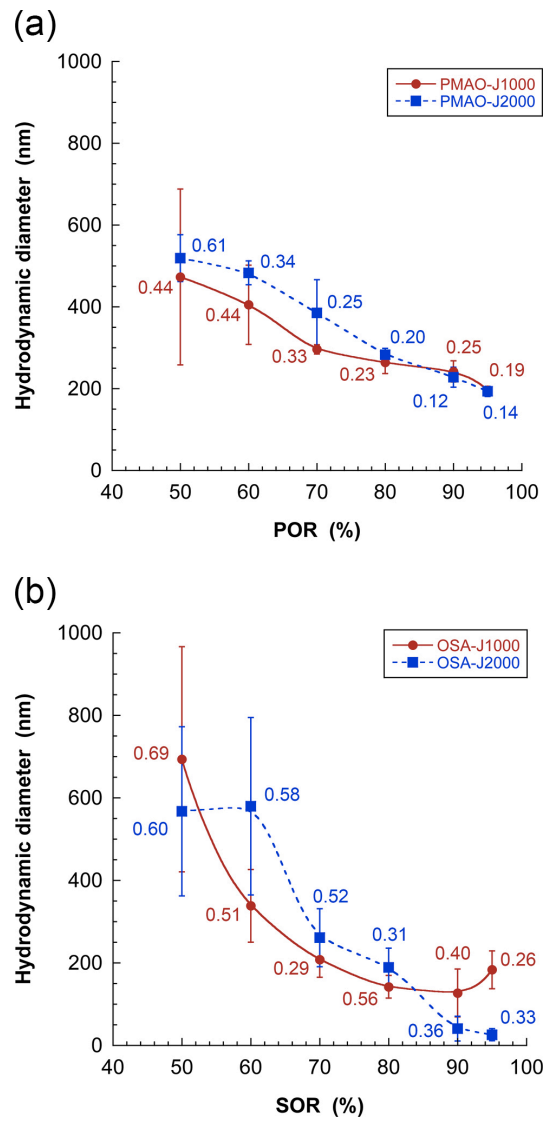


Figure 4: Spontaneous emulsification (a) PMAO-Jeffamine® (1000 or 2000), or (b) OSA-Jeffamine® (1000 or 2000), using medium chain triglycerides (Labrafac WL®) as oil phase. Effect of the formulation parameters polymer-to-oil weight ratio or surfactant-to-oil weight ratio (POR and SOR, respectively) on the resulting droplet size and polydispersity suspension (PDI are indicated in the graph for each point).

core-shell droplet structure at final (Fig. 2).

Figure 4 summarizes the main results obtained, comparing the nano-emulsification performed with polymer (PMAO) and monomer (OSA) for different size of Jeffamine® grafted (J1000 and J2000). Through the values of the droplet sizes and the PDI, these results give the efficiency of the process itself, along with a fine comparison of the different experimental configurations.

These results present the main polymer-oil and monomer-oil ratios allowing to getting the optimum conditions for the formation of stable nano-emulsions, with size in the desired range (*i.e.* below 300 nm) and good monodispersity. The first very important point to note when observing Fig. 4 owes to the fact that the spontaneous emulsification process without surfactant, *i.e.* only with amphiphilic polymer, works, and works according to a similar way to the ones generally observed with classical nonionic surfactants (N. Anton & Vandamme, 2009). Globally the results showed that increasing the amount of the polymer or surfactant induces a decrease in the size of the nano-suspensions. The results are in accordance with our hypothesis that our modified surfactant (OSA-Jeffamine®) or polymer (PMAO-Jeffamine®) plays the role of surfactant itself as it is described in literature, and as well, seems to induce a size control on the dispersion (Calderó et al., 2011; Lefebvre et al., 2017; Saberi, Fang, & McClements, 2013).

It is interesting to note that the best results arise for the polymer formulation: indeed, even if the size range are close when we compare PMAO and OSA, the main difference is given by both the data fluctuation –values of standard deviation–, and the PDI values. It follows therefrom that the process with OSA-Jeffamine® is not reproducible and dispersion not monodisperse (PDI > 0.3-0.4), while PMAO-Jeffamine® are much more reproducible with PDI < 0.2. The best results were obtained (for both PMAO-J1000 and PMAO-J2000) for the values of POR equal to 80% and 90%, that generates nanodroplets having sizes lower than 300 nm and PDI < 0.2. The results are clear, and appear *(i)* much better with the polymeric compounds compared to the corresponding OSA surfactants, and *(ii)* slightly better with a longer Jeffamine® chain. In fact, this is a surprising point since nonionic surfactants are known for showing very good surface activity, and intuitively one can expect a better efficiency of the low molecular weight species in the spontaneous emulsification process compared to the polymers. However, the better reproducibility and monodispersity of the nanoparticles formed with the PMAO based disclosed a better interfacial synergy. Several explanations can be found: *(i)* the first comes in the fact that in general, "polar head" of nonionic surfactants are made with pure PEG (and not co-polymer PEG/polypropylene glycol) as it is the case here; even if the ratio is largely favorable to the proportion of PEG (19/3 and 31/10, as PEG/polypropylene ratios glycol for J-1000 and J-2000, respectively), the introduction of polypropylene glycol could impact their water solubility and thus the process efficiency. *(ii)* A second reason could come from the negatively charged carboxylate present on the amphiphile molecules once in aqueous solution– its

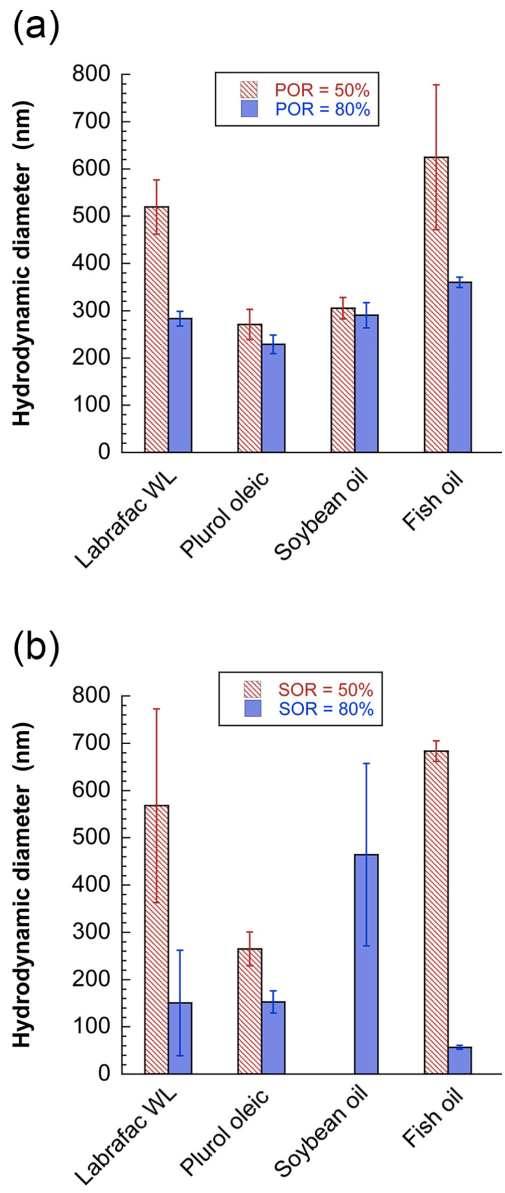


Figure 5: Effect of nature of oil on the mean size of the nano-droplets generated by spontaneous emulsification, for (a) PMAO-J2000 and (b) OSA-J2000, and for two representative values of POR and SOR= 50% and 80%. In figure (b), SOR = 50% with Soybean oil is not present as no emulsion was obtained.

impact on the interfacial behavior of the molecule does not appear negligible. In both cases, the better emulsification observed with the polymer could come from the global interfacial inertia of the molecule, higher with higher molecular weight, thus forming more stable interfaces when adsorbed at interface.

Based on these results, the next step we decided to investigate and optimize the impact of the nature of the oil on the spontaneous

emulsification. In fact, we discussed above the fact that the affinity of the polymers (or of the surfactants) for the water phase –by changing the PEG length– importantly impacts on the emulsification process, but their affinity with the nature of the oil can also be affected. To this regard, representative compounds were selected as J-2000 derivatives of PMAO and OSA, with two representative values of POR and SOR: 50% and 80%. Then, several different oils were used instead of medium chain triglyceride (Labrafac WL®), and the spontaneous emulsification process followed was strictly similar to the one described above. The mean sizes of the nano-droplets obtained are reported in Fig. 5.

It clearly appears that significant variations arise, especially impacted by the POR/SOR variations (confirming the results observed in Fig. 4). A slightly more regular behavior is provided by the polymer in the same line as that of the previous results. To summarize, the effect on the size of the polymer or surfactant-to-oil ratio is similar as the one observed with Labrafac WL®, but the medium chain triglycerides still remain one of the best candidate, along with Plurol oleic (a lipophilic amphiphile molecule). Even if all these oily phases are able to fully solubilize PMAO and OSA modified Jeffamine®, their impact on the emulsification processes indicate that their affinity –chemically speaking– is able to impact on the turbulent molecular migrations at the origin of the emulsifications.

An additional characterization technique, important in the formulation of nanoparticulate carrier, is their direct visualization in electron microscopy. The results are presented in Fig. 6, for the PMAO-J2000, POR = 80%. The first information is the confirmation of the very spherical morphology of the particles fabricated, and this clearly proves that the nano-emulsions have a capsular –core-shell– structure, since when it is classically stabilized with surfactant, the TEM measurement generally destroys the spherical structure (Attia et al., 2016) and make the nano-emulsions droplets spread and merge each other onto the carbon support. In this case, the very spherical shape is conserve, showing that the strong stabilization of the interface is effectively achieved by the precipitation of the polymer in the form of a shell. The second observation regards the sizes revealed by the TEM pictures: they appear in line with the ones shown with DLS in the previous figures, with an average value between 200 and 300 nm. In addition, such TEM pictures also give a visual illustration of the dispersity of the nano-emulsion suspension, showing the important presence of droplets much smaller than the average size of the log-normal distribution –in fact, as expected.

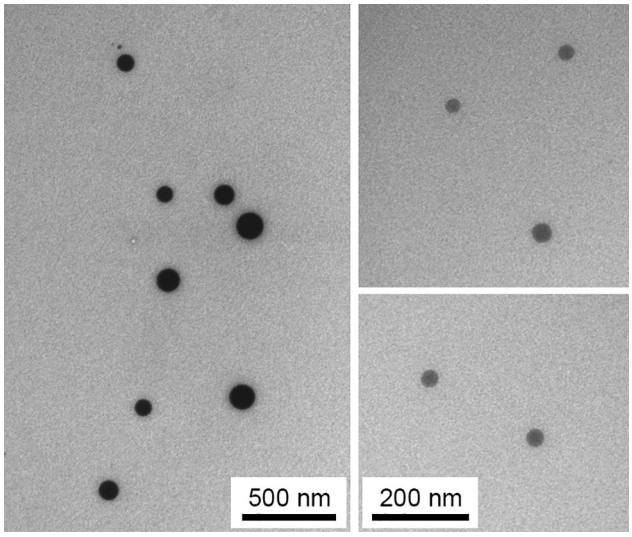


Figure 6: TEM analysis of PMAO-J2000 (POR = 80%) nano-emulsions.

4. Conclusion

In this study, we have explored the development of nano-emulsions using an amphiphilic polymer, synthetized from PMAO on which Jeffamine® was grafted. Nano-emulsions composed of dynamic liquid / liquid interfaces are powerful system, but suffer from difficulties to precisely control the chemical modification of their surface. Using a polymer as stabilizer –instead of nonionic surfactant– is a potential solution for this issue, ensuring the droplet stabilization as well as being a platform for the droplet decoration with cell targeting moiety like ligands or antibodies.. The main idea of this investigation was to understand if the spontaneous emulsification –commonly performed with nonionic surfactants– can be transposed with amphiphilic polymers and compared to the same experiment conducted with the parent monomer of PMAO also modified with Jeffamine®. A secondary objective was to identify the main parameters impacting on the spontaneous emulsification process. To this end, PMAO was grafted with two Jeffamines® of different length (average Mw of 1000 and 2000) compared with surfactant made with the PMAO monomeric entity (OSA). The main results clearly show that the spontaneous emulsification process works, discriminating that the most efficient conditions were obtained with longer Jeffamine® and the PMAO compared to OSA surfactant. During the spontaneous process, as soon as the aqueous phase comes in contact with the oily phase –containing the amphiphiles species–, the modified polymers rearrange themselves in order to displace and orientate the hydrophilic Jeffamine® towards the aqueous phase, *i.e.* at water / oil interface. On the one hand, Jeffamines® are covalently attached to the polymer backbone and thus to the nano-emulsion droplet, and on the other hand, these chains are available at the surface of the droplets, ensuring the stability of the nano-emulsions, and potentially can be functionalized. It was further observed that the size of the formed droplets could significantly vary with the change in the nature of the oil. Eventually, the spontaneous emulsification process is related to the affinities of the stabilizer, polymer or

surfactant, for oil and water. In the case of polymer, the originality comes with its non-dispersion in water and precipitation in the interfacial region, forming a spherical shell shape, confirmed by TEM. To finish, this study may have important implications in the development of surfactant-free nano-emulsions, as well as in the development of functional emulsions and nano-emulsions, along with the fact that spontaneous emulsification has never been reported with polymer without surfactant.

5. Acknowledgments

The authors would like to acknowledge the financial support provided by the Higher Education Commission (HEC) of Pakistan.

6. References

Ammar, H. O., Salama, H. A., Ghorab, M., & Mahmoud, A. A. (2009). Nanoemulsion as a Potential Ophthalmic Delivery System for Dorzolamide Hydrochloride. *AAPS PharmSciTech*, *10*(3), 808. https://doi.org/10.1208/s12249-009-9268-4

Anton, N., Akram, S., & Vandamme, T. F. (2018). Transitional Nanoemulsification Methods. *Nanoemulsions*, 77–100. https://doi.org/10.1016/B978-0-12-811838-2.00004-7

Anton, N., Benoit, J.-P., & Saulnier, P. (2008). Design and production of nanoparticles formulated from nano-emulsion templates-A review. *Journal of Controlled Release*, *128*(3). https://doi.org/10.1016/j.jconrel.2008.02.007

Anton, N., & Vandamme, T. F. (2009). The universality of low-energy nano-emulsification. *International Journal of Pharmaceutics*, *377*(1), 142–147. https://doi.org/https://doi.org/10.1016/j.ijpharm.2009.05.014

Anton, Nicolas, Benoit, J.-P., & Saulnier, P. (2008). Design and production of nanoparticles formulated from nano-emulsion templates—A review. *Journal of Controlled Release*, *128*(3), 185–199. https://doi.org/https://doi.org/10.1016/j.jconrel.2008.02.007

Anton, Nicolas, & Vandamme, T. F. (2011). Nano-emulsions and micro-emulsions: Clarifications of the critical differences. *Pharmaceutical Research*, *28*(5), 978–985. https://doi.org/10.1007/s11095-010-0309-1

Attia, M. F., Anton, N., Akasov, R., Chiper, M., Markvicheva, E., & Vandamme, T. F. (2016). Biodistribution and toxicity of X-ray iodinated contrast agent in nano-emulsions in function of their size. *Pharm. Res.*, *33*(3), 603–614. https://doi.org/10.1007/s11095-015-1813-0

Attia, M. F., Anton, N., Bouchaala, R., Didier, P., Arntz, Y., Messaddeq, N., … Vandamme, T. F. (2015). Functionalization of nano-emulsions with an amino-silica shell at the oil-water interface. *RSC Advances*, *5*(91). https://doi.org/10.1039/c5ra12676b

Attia, M. F., Dieng, S. M., Collot, M., Klymchenko, A. S., Bouillot, C., Serra, C. A., … Anton, N. (2017). Functionalizing Nanoemulsions with Carboxylates: Impact on the Biodistribution and Pharmacokinetics in Mice. *Macromolecular Bioscience*, *17*(7). https://doi.org/10.1002/mabi.201600471

Calderó, G., García-Celma, M. J., & Solans, C. (2011). Formation of polymeric nano-emulsions by a low-energy method and their use for nanoparticle preparation. *Journal of Colloid and Interface Science*, *353*(2), 406–411. https://doi.org/https://doi.org/10.1016/j.jcis.2010.09.073

Fornaguera, C, Feiner-Gracia, N., Calderó, G., García-Celma, M. J., & Solans, C. (2016). PLGA nanoparticles from nano-emulsion templating as imaging agents: Versatile technology to obtain nanoparticles loaded with fluorescent dyes. *Colloids and Surfaces B: Biointerfaces*, *147*, 201–209. https://doi.org/https://doi.org/10.1016/j.colsurfb.2016.08.001

Fornaguera, Cristina, Grijalvo, S., Galán, M., Fuentes-Paniagua, E., de la Mata, F. J., Gómez, R., … Solans, C. (2015). Novel non-viral gene delivery systems composed of carbosilane dendron functionalized nanoparticles prepared from nano-emulsions as non-viral carriers for antisense oligonucleotides. *International Journal of Pharmaceutics*, *478*(1), 113–123. https://doi.org/https://doi.org/10.1016/j.ijpharm.2014.11.031

Hörmann, K., & Zimmer, A. (2016). Drug delivery and drug targeting with parenteral lipid nanoemulsions — A review. *Journal of Controlled Release*, *223*, 85–98. https://doi.org/https://doi.org/10.1016/j.jconrel.2015.12.016

Hou, W., & Xu, J. (2016). Surfactant-free microemulsions. *Current Opinion in Colloid & Interface Science*, *25*, 67–74. https://doi.org/https://doi.org/10.1016/j.cocis.2016.06.013

Hussain, A., Samad, A., Singh, S. K., Ahsan, M. N., Haque, M. W., Faruk, A., & Ahmed, F. J. (2016). Nanoemulsion gel-based topical delivery of an antifungal drug: in vitro activity and in vivo evaluation. *Drug Delivery*, *23*(2), 642–657. https://doi.org/10.3109/10717544.2014.933284

Kaci, M., Arab-Tehrany, E., Dostert, G., Desjardins, I., Velot, E., & Desobry, S. (2016). Efficiency of emulsifier-free emulsions and emulsions containing rapeseed lecithin as delivery systems for vectorization and release of coenzyme Q10: physico-chemical properties and in vitro evaluation. *Colloids and Surfaces B: Biointerfaces*, *147*, 142–150. https://doi.org/https://doi.org/10.1016/j.colsurfb.2016.07.036

Khani, S., Keyhanfar, F., & Amani, A. (2016). Design and evaluation of oral nanoemulsion drug delivery system of mebudipine. *Drug Delivery*, *23*(6), 2035–2043. https://doi.org/10.3109/10717544.2015.1088597

Lefebvre, G., Riou, J., Bastiat, G., Roger, E., Frombach, K., Gimel, J.-C., … Calvignac, B. (2017). Spontaneous nano-emulsification: Process optimization and modeling for the prediction of the nanoemulsion's size and polydispersity. *International Journal of Pharmaceutics*, *534*(1), 220–228. https://doi.org/https://doi.org/10.1016/j.ijpharm.2017.10.017

López-Montilla, J. C., Herrera-Morales, P. E., Pandey, S., & Shah, D. O. (2002). Spontaneous Emulsification: Mechanisms, Physicochemical Aspects, Modeling, and Applications. *Journal of Dispersion Science and Technology*, *23*(1–3), 219–268. https://doi.org/10.1080/01932690208984202

Mahato, R. (2017). Nanoemulsion as Targeted Drug Delivery System for Cancer Therapeutics. *Journal of Pharmaceutical Sciences and Pharmacology*, *3*(2).

Mason, T. G., Wilking, J. N., Meleson, K., Chang, C. B., & Graves, S. M. (2006). Nanoemulsions: formation, structure, and physical properties. *Journal of Physics: Condensed Matter*, *18*(41), R635--R666. https://doi.org/10.1088/0953-8984/18/41/r01

McClements, D. J. (2012). Nanoemulsions versus microemulsions: terminology, differences, and similarities. *Soft Matter*, *8*(6), 1719–1729. https://doi.org/10.1039/C2SM06903B

Miller, C. A. (1988). Spontaneous Emulsification Produced by Diffusion — A Review. *Colloids and Surfaces*, *29*(1), 89–102. https://doi.org/https://doi.org/10.1016/0166-6622(88)80173-2

Nasr, M., Nawaz, S., & Elhissi, A. (2012). Amphotericin B lipid nanoemulsion aerosols for targeting peripheral respiratory airways via nebulization. *International Journal of Pharmaceutics*, *436*(1), 611–616. https://doi.org/https://doi.org/10.1016/j.ijpharm.2012.07.028

Nehilla, B. J., Bergkvist, M., Popat, K. C., & Desai, T. A. (2008).

Purified and surfactant-free coenzyme Q10-loaded biodegradable nanoparticles. *International Journal of Pharmaceutics*, *348*(1), 107–114. https://doi.org/https://doi.org/10.1016/j.ijpharm.2007.07.001

Pawar, V. K., Panchal, S. B., Singh, Y., Meher, J. G., Sharma, K., Singh, P., … Chourasia, M. K. (2014). Immunotherapeutic vitamin E nanoemulsion synergies the antiproliferative activity of paclitaxel in breast cancer cells via modulating Th1 and Th2 immune response. *Journal of Controlled Release*, *196*, 295–306. https://doi.org/https://doi.org/10.1016/j.jconrel.2014.10.010

Saberi, A. H., Fang, Y., & McClements, D. J. (2013). Fabrication of vitamin E-enriched nanoemulsions: Factors affecting particle size using spontaneous emulsification. *Journal of Colloid and Interface Science*, *391*, 95–102. https://doi.org/https://doi.org/10.1016/j.jcis.2012.08.069

Sahoo, S. K., Panyam, J., Prabha, S., & Labhasetwar, V. (2002). Residual polyvinyl alcohol associated with poly (d,l-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *Journal of Controlled Release*, *82*(1), 105–114. https://doi.org/https://doi.org/10.1016/S0168-3659(02)00127-X

Sheibat-Othman, N., & Bourgeat-Lami, E. (2009). Use of Silica Particles for the Formation of Organic−Inorganic Particles by Surfactant-Free Emulsion Polymerization. *Langmuir*, *25*(17), 10121–10133. https://doi.org/10.1021/la900895z

Singh, Y., Meher, J. G., Raval, K., Khan, F. A., Chaurasia, M., Jain, N. K., & Chourasia, M. K. (2017). Nanoemulsion: Concepts, development and applications in drug delivery. *Journal of Controlled Release*, *252*, 28–49. https://doi.org/https://doi.org/10.1016/j.jconrel.2017.03.008

Solans, C., Morales, D., & Homs, M. (2016). Spontaneous emulsification. *Current Opinion in Colloid & Interface Science*, *22*, 88–93. https://doi.org/https://doi.org/10.1016/j.cocis.2016.03.002

Chapter 2.3. A novel strategy to formulate tunable functionalized oil-in-water nano-emulsions

1. INTRODUCTION

Nano-emulsions are heterogenous, kinetically stable, optically clear and very fine dispersions of two immiscible liquids (oil-in-water or water-in-oil), generally stabilized by a surfactant with average size of the droplets ranging from 200-300 nm.[1–3] The nano-emulsions have gained much importance in pharmaceutical industry because of their high loading capacity, low manufacturing cost, ease of manufacturing, good stability and their potential to encapsulate lipophilic, hydrophilic as well as amphiphilic drugs. Nano-emulsions have been widely studied as drug delivery systems[4–8], but nano-emulsions are yet under explored as tunable therapeutic carriers for targeted drug delivery because of the challenges involved in the surface functionalization of nano-emulsions, major challenge being the stabilization of the interface. Some efforts have been put in to functionalize and control the chemical composition of the surface of nano-emulsions for targeted drug delivery, *e.g.*, *in situ* synthesis of silica shell at the droplet interfaces[9], use of cationic lipids or surfactants[10,11], use of preformed polymers followed by solvent evaporation[12,13] *etc*. But the problem associated with these strategies is the use of surfactants or an organic solvent or sometimes both in the preparation and stabilization of nano-emulsions, which could cause some serious limitations in terms of biocompatibility, efficiency of the surface modification, toxicity, need of additional steps (like solvent evaporation, purification *etc*.) and development of incompatibilities with time.[14–18]

Our research group has already shown that the poly (maleic anhydride-alt-1-octadecene) (PMAO) itself as well as its derivatives with Jeffamine polyetheramines (J-1000 & J-2000), after being solubilized in the oil core, has the ability to migrate at the oil-water interface upon addition of aqueous phase and produce stable oil-in-water nano-emulsions in the absence of an organic solvent as well as surfactant.[14,19] And now we are making use of this property of PMAO, to functionalize the dynamic oil/water interface of nano-emulsions by synthesizing a chemically modified PMAO having tunable surface-active property which will help in improving the current strategies for targeted drug delivery and will limit the off-target interactions. The synthesis of functionalized polymer is based on the chemical modification of poly (maleic anhydride-alt-1-octadecene) (PMAO), a hydrophobic polymer having aliphatic octadecene chains with reactive anhydride

functions, by performing a systematic reaction of Jeffamine polyetheramine (Jeffamine® M2070) and a polyethylene glycol (PEG)-containing compound terminated by a reactive azide group (i.e., NH_2-PEG-N_3) on each anhydride function, in order to generate a pattern with Jeffamine® M2070 and NH_2-PEG-N_3 being attached on each monomeric element of PMAO. The nano-emulsions are prepared, using this newly synthesized polymer, by ultrasonication method. Then, the potential of these azide decorated nano-emulsions to graft contrast agents and ligands (covalently) at the surface of the nano-droplets has been evaluated. For this purpose, we have used a contrast agent (JS-050) and a biotin-containing ligand and studied their interaction and the extent of binding with the azide groups available at the surface of the nano-droplets. The nano-emulsions were optimized in relation to the size of the nano-droplets, ratio between jeffamine and azide-containing component in the formulation and the amount of the contrast agent required to completely saturate the azide groups at the surface of the nano-droplets. Different techniques were used to characterize these nano-emulsion formulations namely differential light scattering (DLS), UV-visible spectroscopy, transmission electron microscopy (TEM) and nuclear magnetic resonance (NMR).

Finally, after having proof of the concept, these systems were studied for the targeting applications by reaction of these biotinylated nano-emulsions with streptavidin. This interaction was studied by gel electrophoresis. Biotin, also known as vitamin B7, is a water-soluble vitamin and has been widely used in biochemical assays to conjugate proteins and because of its small size, the biological activity of the protein is generally unaffected. Streptavidin is a protein which is purified from a bacterium *Streptomyces avidinii* and it is widely used for the purification or detection of various biomolecules (like proteins) and to attach various biomolecules to one another or onto a solid support (as biotin-streptavidin complex)**.** The biotin-streptavidin complex is commonly used in molecular biology and bio-nanotechnology as well as in pre-targeted immunotherapy**.** This complex is resistant to the organic solvents, detergents (like Triton), extremes of pH and temperature, denaturants (like guanidinium chloride) and proteolytic enzymes.

These tunable functionalized nano-emulsion systems provide a platform for encapsulation and conjugation of a large variety of contrast agents, drugs, ligands, proteins and antibodies and thus can be used for imaging, therapeutic and targeted drug delivery applications.

2. MATERIAL AND METHODS

2.1. Chemicals used

The Poly (maleic anhydride-alt-1-octadecene) (PMAO), triethylamine (TEA), ethanolamine, 1-[Bis (dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU) and dimethyl formamide (DMF) were purchased from Sigma Aldrich. Jeffamine® M-2070 polyetheramine (J-2000) was kindly offered by Huntsman corporation (Texas, U.S.A.). The N_3-PEG_{3000}-NH_2 was purchased from Iris pharma®. The DOBC-PEG_4-biotin was purchased from Broadpharm®. Medium chain triglyceride-Labrafac WL®, was purchased from Gattefossé (Saint Priest, France). The dyes, CY 5.5TPB and JS-050 were kindly provided by Dr. Mayeul Collot. The rest of the reagents and solvents used were of analytical grade.

2.2. Chemical modification of the polymer

First of all, 514.3 mg of N_3-PEG-NH_2 (0.2 equivalent of PMAO) and 1371.4 mg of J-2000 (0.8 equivalent of PMAO) were taken and dissolved in 20 ml of DMF in a round bottom flask. To this solution, PMAO (300 mg) was added and the final volume was adjusted up to 50 mL with DMF. Once the components were completely solubilized, 0.6 mL (5 equivalents) of triethylamine was added into the flask and this solution was then continuously stirred overnight at 60°C under reflux condenser. Then, HATU (651 mg) and ethanolamine (261.5 mg or 0.26 ml) were added into this solution and the solution was again stirred overnight at 60°C under reflux condenser. The solvent (DMF) was evaporated (using rotavapor) and the resulting product was dried under vacuum. Next, the polymer was dissolved in 10 mL ethanol and this solution was subjected to dialysis (MWCO of dialysis membrane=3500) for 24 hours. Finally, the solvent (ethanol) was evaporated (using rotavapor) and the resulting product was dried under vacuum.

The same procedure was used to prepare another derivative of the polymer with only two changes, *i.e.*, using 0.1 equivalent (instead of 0.2) N_3-PEG-NH_2 and 0.9 equivalent (instead of 0,8) J-2000. The overall scheme is illustrated in figure 1.

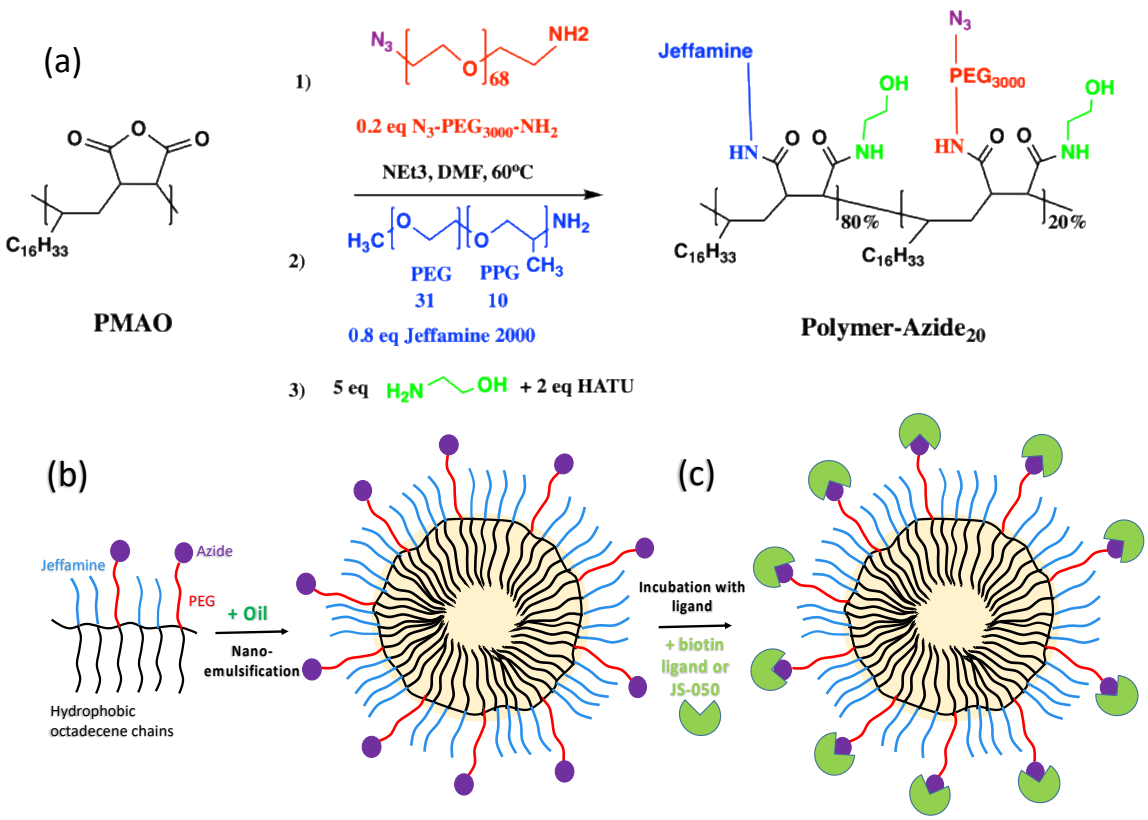


Figure 1: Schematic illustration of, a) polymer synthesis, b) Formation of nano-emulsions, and c) Grafting of ligand/dye at the surface of nano-droplets

2.3. ^{1}H-NMR Analysis

To study the successful grafting of the jeffamine 2000 and azide-containing component on the polymer and to confirm the structure of the modified polymer, took 25 mg of the final product and dissolved in 600 µL of deuterated chloroform ($CDCl_3$). The ^{1}H-NMR spectrum was then studied with a Bruker Top Spin 3.0 (operating at 400 MHz). The chemical shifts (δ) are expressed in parts per million.

2.4. Preparation of azide-decorated nano-emulsions

First of all, the aqueous and oil phases were prepared separately and heated at 90°C for 30 min. Oil phase consists of polymer dissolved in oil (Labrafac WL®), while the aqueous phase used was distilled water. The hot aqueous and oil phases were then rapidly mixed together and homogenized by vortex for 30 seconds, followed by ultrasonication for 4 min and 10 seconds. The ultrasonicator was operated at 20 % amplitude (intensity of ultrasonicator), with repeated cycles of "pulse ON" for 10 seconds and "pulse OFF" for 20 seconds, which resulted in the formation of nano-emulsions with the surface of droplets being decorated with azide functions (figure 1b).

2.5. Dynamic light scattering and zeta potential measurements

The hydrodynamic diameters and polydispersity index (PDI) of the nano-emulsion droplets were measured, in triplicate, by dynamic light scattering (DLS) along with zeta potential measurement using a Malvern Nano ZS instrument, equipped with a helium-neon laser (4 mW) operating at 633 nm, with the scatter angle 173° and the temperature was maintained at 25°C.

2.6. Microscopic analysis

The transmission electron microscopy (TEM) was used to study the morphology and structure of the nano-droplets formed. The nano-emulsion samples were diluted (1/100) with Milli-Q water. One drop of the diluted nano-emulsion was introduced on an ultrathin carbon support film (carbon type A, 400 mesh, copper, Ted Pella Inc. Redding, PA) and then the sample was dried at 40°C for ten minutes. The observations were carried out by a Philips Morgagni 268D electron microscope.

2.7. Grafting of JS-050 (dye) on azide-decorated nano-emulsions

In this step, our objective was to graft JS-050 at the surface of nano-emulsions by a reaction between the alkyne group of the dye JS-050 and the azide groups available at the surface of the nano-carriers. First of all, we calculated, theoretically, the molar concentration of the azide available (in the case of PMAO:N_3-PEG-NH_2:J-2000, with 1:0.2:0.8 ratio, respectively) to react with JS-050. Then, we took a fixed volume of nano-emulsions and diluted with distilled water to have final concentration of azide as 10 µM, followed by addition of a specified volume (10 µL) of JS-050 stock solution (in DMSO) into the diluted nano-emulsion. The JS-050 stock solutions having different molar concentrations (*i.e.*, 2.5 µM, 5 µM, 7.5 µM, 10 µM, 15 µM, 20 µM, and 30 µM) were prepared and used to study the effect of increasing concentration of the dye on the extent of grafting and also to measure the point of saturation of all the azide functional groups available at the surface. After addition of JS-050, the nano-emulsions were incubated for 2 hours at room temperature and protected from light (figure 1c). Then, the JS-050 grafted nano-emulsions were separated from the unreacted/free JS-050 by size exclusion chromatography, using PD10 Sephadex® G-25M column, pre-equilibrated with distilled water. The fractions obtained were then analysed by UV-visible spectrophotomer to measure the extent of attachment of JS-050 at the surface of the nano-carriers.

The same procedure was used to graft JS-050 on the nano-emulsions formulated with the polymer consisting of PMAO:N_3-PEG-NH_2:J-2000, 1:0.1:0.9, respectively, but same stock solutions of the JS-050 were used to study the effect of the change in the number of available azide sites on the grafting of JS-050 at the surface of the nano-carriers.

As a control formulation, we prepared nano-emulsions with a polymer without having the N_3-PEG-NH_2 component, *i.e.*, PMAO:J-2000, 1:1 respectively. Then, these nano-emulsions were incubated for 2 hours with the same dilutions of JS-050 solution (in DMSO), as used above, to study the non-specific binding of the dye with nano-emulsions.

2.8. Grafting of biotin-containing ligand on azide-decorated nano-emulsions

After successful grafting of JS-050 at the surface of nano-emulsions, we moved towards our main objective, which was to graft a ligand at the surface of lipid nano-carriers. To achieve this objective, a biotin containing ligand was selected. The nano-emulsions were formulated using the same procedure as described above with only slight change in the composition of oil phase, *i.e.*, 1 % Cy 5.5 solution in Labrafac® was used instead of Labrafac®. Then, a fixed volume of nano-emulsions was diluted with PBS (1X, pH 7.4) to have a final concentration of azide as 100 µM, followed by addition of a specified volume (100 µL) of DOBC-PEG_4-biotin stock solution (1 mM in DMSO) into the diluted nano-emulsion, to have the final concentration of this ligand as 100 µM. The nano-emulsions were then incubated for 2 hours at room temperature and protected from the light (figure 1c). After two hours of incubation, the DOBC-PEG_4-biotin grafted nano-emulsions were separated from the unreacted DOBC-PEG_4-biotin by size exclusion chromatography, using PD10 Sephadex® G-25M column pre-equilibrated with PBS (1X, pH 7.4). The resulting DOBC-PEG_4-biotin grafted nano-emulsions were then analysed by gel electrophoresis (described below) to confirm the successful grafting of DOBC-PEG_4-biotin at the surface of the nano-carriers.

The azide-decorated nano-emulsions, without grafting of the biotin ligand at the surface of the nano-carriers, were prepared by the same procedure as described above and were used as a control formulation (NEc).

2.9. Gel electrophoresis

To perform gel electrophoresis, following solutions were prepared:

- Streptavidin (S): diluted 2 times (20µl in 20µl of PBS)

- Nano-emulsions (NEs) (with/without biotin, *i.e.*, NE/NEc): diluted 6 times (10µl in 50µl of PBS)
- Nano-emulsions (NEs) (with/without biotin) + Streptavidin (S): 30µl of S + 10 µl NEs + 20µl of PBS
- NEs (with biotin) + Biotin: 10µL of NEs + 20µl of PBS/Biotin + 30µl of PBS
- NEs (with biotin) + Biotin + Streptavidin: 10µl of NEs + 30µl of S + 20µl of PBS/Biotin
- The solution of Biotin was prepared by dissolving 1 mg of it in 1 ml of PBS by vortex, followed by decantation.

Procedure: First of all, took 0.5 g of agarose in a conical flask (250 mL) and then 100 mL of tris acetate EDTA (TAE) (1x) was added into it. This mixture was heated for 3 min in a micro-wave (at 800 W), while kept stirring the solution manually after every minute. The solution was cooled down to room temperature and poured into special moulds having combs placed in them. The comb was removed after 30 minutes, which resulted in the formation of wells in the gel. Then, 12 µL of each solution was added into the wells and subjected to electrophoresis for 1h at 125 V and the machine was covered with aluminium foil.

Then, the gels were analyzed with LAS 4000 using two different wavelengths/channels, one corresponding to cyanine 3 (CY3) and the other one to cyanine 5 (CY5), with the exposure time for each picture being fixed at 10 seconds. The images were processed and analyzed by using ImageJ software.

3. RESULTS AND DISCUSSIONS

3.1. ^{1}H-NMR Analysis

The results obtained with both the polymer derivatives are shown in the figure 1. By applying the integration on the NMR spectra, it was found that there was 81 % grafting of the Jeffamine 2000 component in the case of PMAO:N_3-PEG-NH_2:J-2000, with 1:0.2:0.8 ratio and the rest of the signal was from N_3-PEG-NH_2 component.

The same pattern is observed in the case of PMAO:N_3-PEG-NH_2:J-2000, with 1:0.1:0.9 ratio, but here the % grafting of the jeffamine 2000 was found to be 87 % and the rest of the signal was from N_3:PEG:NH_2. These results confirmed the successful chemical modification of the polymer.

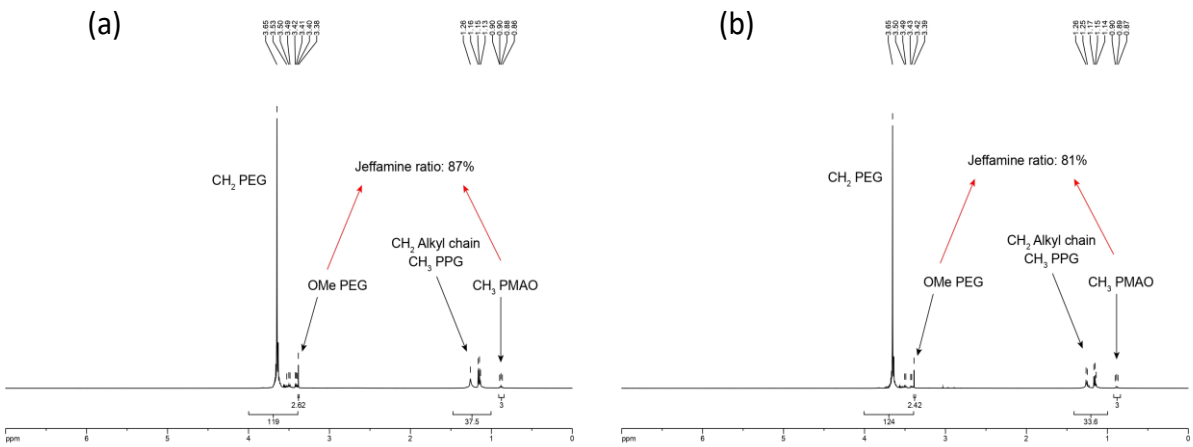


Figure 2: NMR spectra of, a) PMAO:N3-PEG-NH2:J2000 (1:0.1:0.9) and b) PMAO:N3-PEG-NH2:J2000 (1:0.2:0.8)

3.2. Dynamic light scattering and zeta potential measurements

The nano-emulsions were formulated by ultrasonication technique. As soon as the aqueous phase comes in contact with the oil phase, the hydrophilic part of the polymer (Jeffamine® and azide-terminated PEG chains) moves towards the continuous aqueous phase and the hydrophobic chains (octadecene chains) remain anchored in the oil phase, forming a core-shell structure.[14] This results in the formation of nano-droplets decorated with azide functions. The nano-emulsions were characterized by dynamic light scattering technique and the results obtained are shown in the figure 3(a). The average sizes obtained with both the azide-containing derivatives of PMAO were more or less the same, around 128 ± 3 nm, and this size range is ideal for the desired applications. However, the size was slightly smaller in case of PMAO:N_3-PEG-NH_2:J-2000 having a ratio 1:0.2:0.8, as compared to the polymer with a ratio as 1:0.1:0.9. The average size of the nano-droplets obtained in the case of control formulation was considerably larger (around 160 nm) as compared to the azide-decorated nano-droplets and this increase in the size might be due to the relatively higher amount of the jeffamine (having long chains) in the control formulation.

The zeta-potential measurements have shown that the surface charge on the nano-emulsion droplets is negative in all the three cases, with maximum negative charge (-39 mV±1) on the control formulations (figure 3b). As the azide component (N_3-PEG-NH_2) is introduced into the polymer structure (*i.e.*, PMAO:N_3-PEG-NH_2:J-2000, 1:0.1:0.9, respectively) the surface charge on the resulted nano-droplets becomes less negative (-29 mV±1). On further increasing the proportion of azide moiety in the polymer structure

(*i.e.*, 1:0.2:0.8), the surface charge on the resulted nano-droplets moves further towards lesser negativity (-26 mV±1).

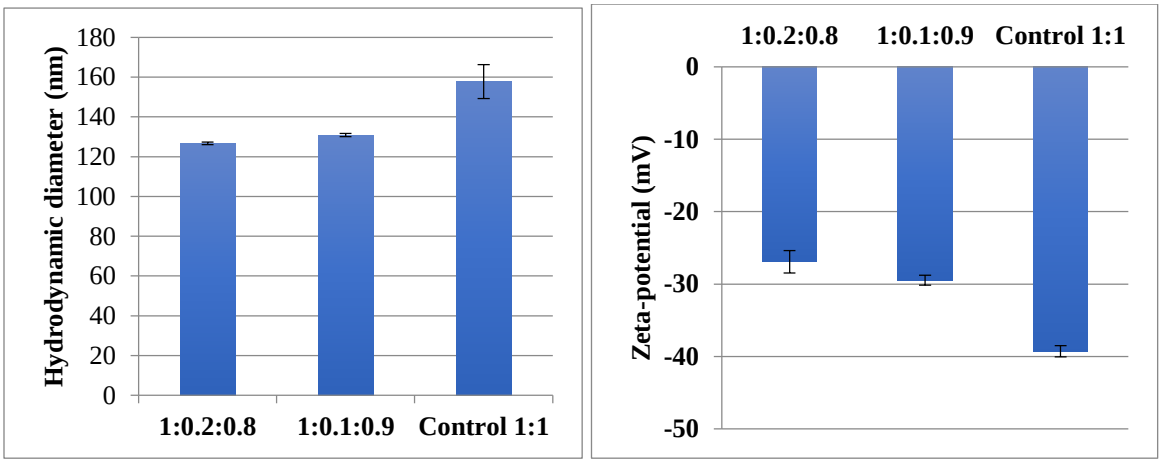


Figure 3: Physico-chemical characterization of nano-emulsions, (a) Hydrodynamic diameter (nm), (b) zeta-potential (mV)

3.3. Transmission electron microscopic (TEM) analysis

The results obtained with transmission electron microscopy (TEM) for the PMAO:N_3PEGNH_2:J2000 sample (ratio 1:0.2:0.8), are presented in figure 4, which show that the particles formed are spherical in shape and the results are also coherent with the ones obtained with DLS, with an average size of major population of the droplets ranging from 120-130 nm. The interface of the droplets is strongly stabilized due to the precipitation of the polymer in the form of a shell, that confirms the capsular core-shell structure of the nano-emulsions.

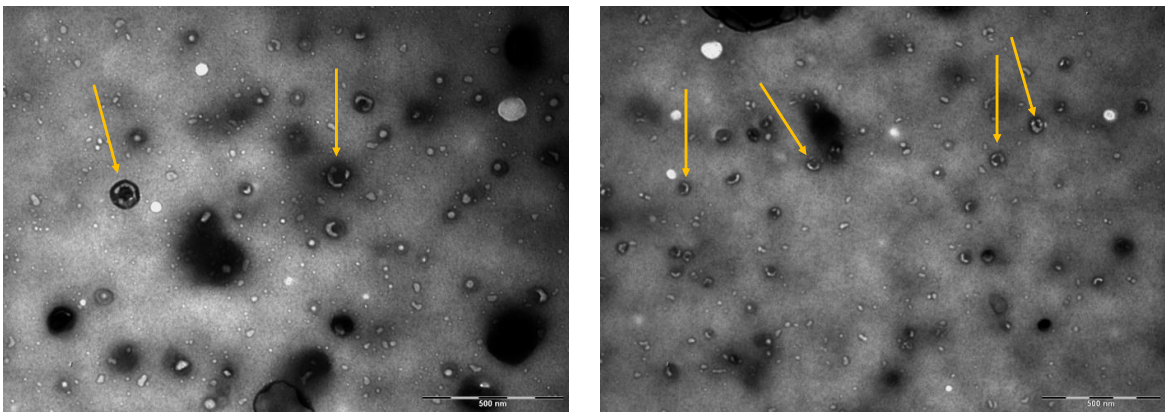


Figure 4: TEM images for the PMAO:N_3PEGNH_2:J2000 sample (ratio 1:0.2:0.8)

3.4. Grafting of JS-050 (dye) on azide-decorated nano-emulsions

It was observed that the binding of the dye (JS-050) with the azide functional groups, present at the surface of nano-droplets, was increased with the increase in the amount of the dye added to the formulation. Higher values of binding of the dye were obtained in the case of nano-emulsions, 20 % decorated with azide function (1:0.2:0.8) as compared to those having 10 % decorated azide function (1:0.1:0.9). That's why it can be concluded that the extent of dye attachment on the nano-droplets is directly proportional to the number of available azide sites. We further checked the attachment of the dye with the control formulation, *i.e.*, nano-emulsions formed by using the polymer not having any azide functions (PMAO:J-2000, 1:1), to see whether there was some non-specific binding of the dye or not. It was observed that there was some non-specific binding of the dye, as well, with nano-droplets, as shown in the "figure 5(a)" and "5(b)". But there was a significant difference in the extent of binding of the dye with the azide-decorated nano-droplets and azide-free nano-droplets (*i.e.*, control formulation). Because of this non-specific binding tendency of the dye, we subtracted the values of the binding of the dye obtained with control formulations from the values obtained with azide-decorated nano-emulsions. The results presented in "figure 5(c)" represent the binding of the dye specifically to the azide functions available at the surface of the nano-droplets. Moreover, it was observed that the increase in the binding of the dye was very prominent up to 15 µM concentration of the added solution of the dye. Beyond this point, the increase in the extent of the binding of the dye was not consistent with the added amount of the dye, which might be related to the saturation of the azide sites at the surface of the nano-droplets and unavailability for the further reaction (figure 5c).

3.5. Grafting of DOBC-PEG4-biotin on azide-decorated nano-emulsions

The different conditions explored and all the results obtained with gel electrophoresis are presented in the figure 6 and it can be seen that in the CY3 laser channel there was a signal from streptavidin in wells A, C, D and G, but there was a clear difference in the elution rate of well C from the rest of the wells (figure 6a). The streptavidin decorated nano-emulsions (well C) are eluted slightly slower, showing an intense signal at a different position as compared to the other cases, having only streptavidin *i.e.*, (A, D and G).

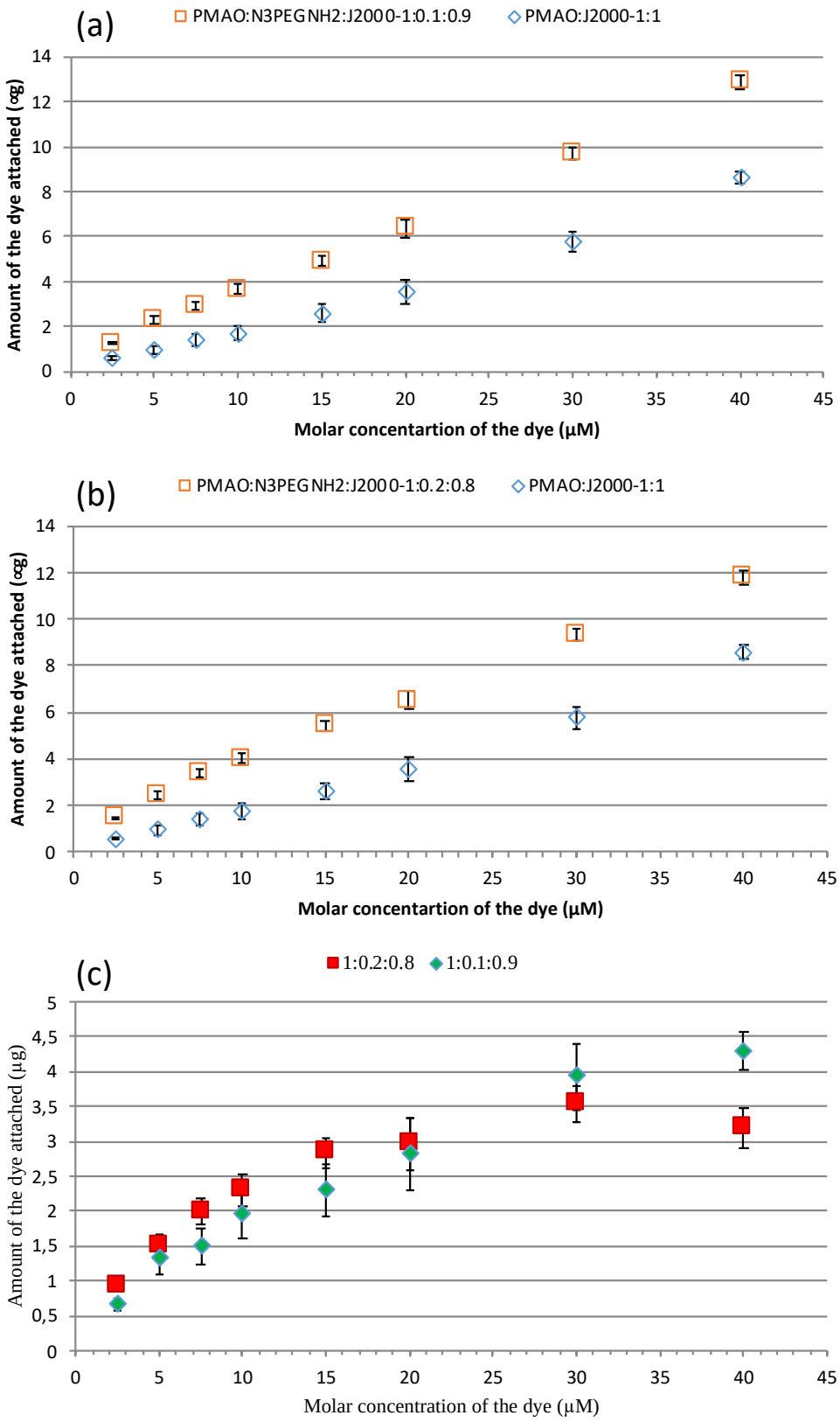


Figure 5: a) Comparison of the binding of the dye with 10 % azide-decorated and control nano-droplets, b) Comparison of the binding of the dye with 20 % azide-decorated and control nano-droplets, c) Measurement of the binding of the dye, specifically with azide functions available at the surface of the nano-droplets

In figure 6b, the results correspond to the other laser channel (CY5) and the grafting of the biotin on nano-emulsions was quite evident because of the signal obtained in the well C corresponding to “NE + Streptavidin”, while there was no signal at the same position from other solutions which showed that the NE were not eluted and stayed in the well in all the rest of the cases. The “figure 6c” corresponds to a merge of the figures 6a, and 6b, and it can be seen that there was a combined elution signal for NE as well as for streptavidin in well C, which confirmed the successful grafting of the streptavidin on the nano-emulsions. There was no combined signal in the rest of the wells. The absence of the signal in “well A” was also a confirmation of the concept, as in that case, the reaction between biotin and streptavidin occurred before the addition of NE and when the NE were added there were no free streptavidin molecules to react with the biotin available at the surface of the NE, that’s why no signal was observed in this case.

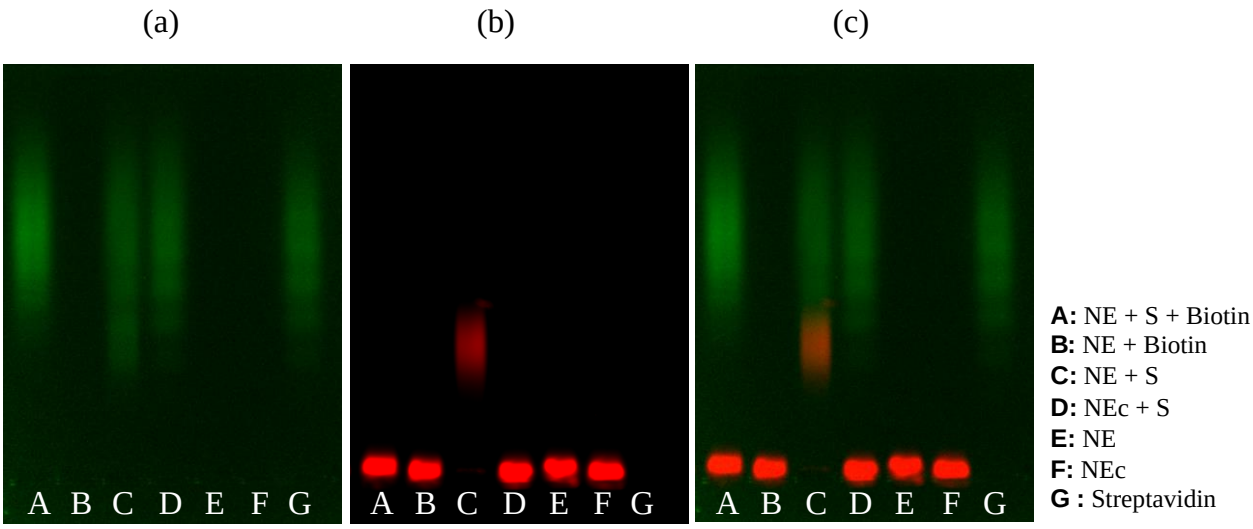


Figure 6: Gel electrophoresis-studying the interaction between the biotin grafted nano-emulsions and streptavidin, (a) streptavidin signal from the CY3 laser channel, (b) CY 5.5 signal from the CY5 laser channel, (c) Merge of the signals from both laser channels

4. Conclusion:

This research aims at developing a new strategy for surface functionalization of nano-emulsions to enable them to graft ligands at their surface. For that purpose, we have formulated nano-emulsions using a non-ionic polymer, poly (maleic anhydride-alt-1-octadecene) (PMAO). The polymer was first modified in such a way that, upon emulsification, it provided reactive azide groups at the interface of the nano-emulsions to facilitate the chemical grafting of the contrast agents or ligands at the surface of the nano-

droplets. The morphology and size of the nano-droplets were studied by DLS and TEM techniques, which showed that the formed nano-droplets have a spherical core shell structure with average sizes ranging from 120-130 nm and surface zeta potential values between -28 ± 2 mV. The structure and chemical modifications of the polymer were verified by NMR studies. It was observed that the binding of the dye (JS-050) with the nano-emulsions was dependent on the amount of the clickable sites (azide groups) available at the surface of the nano-emulsions, lower in the case of PMAO:N_3PEGNH_2:J-2000, with 1:0.1:0.9 ratio and higher with PMAO:N_3PEGNH_2:J-2000, having 1:0.2:0.8 ratio. After successful grafting of the sample dye (JS-050), biotin containing ligands were grafted on the nano-droplets. The attachment of the biotinylated ligand on the surface of the nano-droplets was confirmed by gel electrophoresis, by performing a reaction between these biotin-decorated nano-emulsions and streptavidin. The use of Jeffamine® and PEG (in this system) has a great significance because the PEG-chains not only create steric repulsion among the neighboring nano-droplets and improve the interfacial stabilization but also have a well-recognized ability to reduce the uptake of the nano-emulsions (after *in-vivo* administration) by the immune system, inhibiting opsonization and phagocytosis (as observed with conventional nano-emulsions), which increases the circulation time of nano-emulsions in the body.[12,20] This kind of nano-emulsions provide an excellent carrier of the contrast agents, ligands, proteins, antibodies *etc.*, and can be used for imaging and biomedical applications.

5. References

(1) Anton, N.; Vandamme, T. F. The Universality of Low-Energy Nano-Emulsification. *International Journal of Pharmaceutics* **2009**, *377* (1–2), 142–147. https://doi.org/10.1016/j.ijpharm.2009.05.014.

(2) Solans, C.; Morales, D.; Homs, M. Spontaneous Emulsification. *Current Opinion in Colloid & Interface Science* **2016**, *22*, 88–93. https://doi.org/10.1016/j.cocis.2016.03.002.

(3) McClements, D. J. Nanoemulsions versus Microemulsions: Terminology, Differences, and Similarities. *Soft Matter* **2012**, *8* (6), 1719–1729. https://doi.org/10.1039/C2SM06903B.

(4) Hussain, A.; Samad, A.; Singh, S. K.; Ahsan, M. N.; Haque, M. W.; Faruk, A.; Ahmed, F. J. Nanoemulsion Gel-Based Topical Delivery of an Antifungal Drug: *In Vitro* Activity and *in Vivo* Evaluation. *Drug Delivery* **2016**, *23* (2), 642–657. https://doi.org/10.3109/10717544.2014.933284.

(5) Nasr, M.; Nawaz, S.; Elhissi, A. Amphotericin B Lipid Nanoemulsion Aerosols for Targeting Peripheral Respiratory Airways via Nebulization. *International Journal of Pharmaceutics* **2012**, *436* (1–2), 611–616. https://doi.org/10.1016/j.ijpharm.2012.07.028.

(6) Pawar, V. K.; Panchal, S. B.; Singh, Y.; Meher, J. G.; Sharma, K.; Singh, P.; Bora, H. K.; Singh, A.; Datta, D.; Chourasia, M. K. Immunotherapeutic Vitamin E Nanoemulsion Synergies the Antiproliferative Activity of Paclitaxel in Breast Cancer Cells via Modulating Th1 and Th2 Immune Response. *Journal of Controlled Release* **2014**, *196*, 295–306. https://doi.org/10.1016/j.jconrel.2014.10.010.

(7) Saberi, A. H.; Fang, Y.; McClements, D. J. Fabrication of Vitamin E-Enriched Nanoemulsions: Factors Affecting Particle Size Using Spontaneous Emulsification. *Journal of Colloid and Interface Science* **2013**, *391*, 95–102. https://doi.org/10.1016/j.jcis.2012.08.069.

(8) Kretzer, I. F.; Maria, D. A.; Maranhão, R. C. Drug-Targeting in Combined Cancer Chemotherapy: Tumor Growth Inhibition in Mice by Association of Paclitaxel and Etoposide with a Cholesterol-Rich Nanoemulsion. *Cellular Oncology* **2012**, *35* (6), 451–460. https://doi.org/10.1007/s13402-012-0104-6.

(9) Attia, M. F.; Anton, N.; Bouchaala, R.; Didier, P.; Arntz, Y.; Messaddeq, N.; Klymchenko, A. S.; Mély, Y.; Vandamme, T. F. Functionalization of Nano-Emulsions with an Amino-Silica Shell at the Oil–Water Interface. *RSC Advances* **2015**, *5* (91), 74353–74361. https://doi.org/10.1039/C5RA12676B.

(10) Khachane, P. V.; Jain, A. S.; Dhawan, V. V.; Joshi, G. V.; Date, A. A.; Mulherkar, R.; Nagarsenker, M. S. Cationic Nanoemulsions as Potential Carriers for Intracellular Delivery. *Saudi Pharmaceutical Journal* **2015**, *23* (2), 188–194. https://doi.org/10.1016/j.jsps.2014.07.007.

(11) Fraga, M.; Bruxel, F.; Diel, D.; de Carvalho, T. G.; Perez, C. A.; Magalhães-Paniago, R.; Malachias, Â.; Oliveira, M. C.; Matte, U.; Teixeira, H. F. PEGylated Cationic Nanoemulsions Can Efficiently Bind and Transfect PIDUA in a Mucopolysaccharidosis Type I Murine Model. *Journal of Controlled Release* **2015**, *209*, 37–46. https://doi.org/10.1016/j.jconrel.2015.04.013.

(12) Fornaguera, C.; Grijalvo, S.; Galán, M.; Fuentes-Paniagua, E.; de la Mata, F. J.; Gómez, R.; Eritja, R.; Calderó, G.; Solans, C. Novel Non-Viral Gene Delivery Systems

Composed of Carbosilane Dendron Functionalized Nanoparticles Prepared from Nano-Emulsions as Non-Viral Carriers for Antisense Oligonucleotides. *International Journal of Pharmaceutics* **2015**, *478* (1), 113–123. https://doi.org/10.1016/j.ijpharm.2014.11.031.

(13) Chaix, C.; Pacard, E.; Elaïssari, A.; Hilaire, J.-F.; Pichot, C. Surface Functionalization of Oil-in-Water Nanoemulsion with a Reactive Copolymer: Colloidal Characterization and Peptide Immobilization. *Colloids and Surfaces B: Biointerfaces* **2003**, *29* (1), 39–52. https://doi.org/10.1016/S0927-7765(02)00177-7.

(14) Rehman, A. U.; Collot, M.; Klymchenko, A. S.; Akram, S.; Mustafa, B.; Vandamme, T.; Anton, N. Spontaneous Nano-Emulsification with Tailor-Made Amphiphilic Polymers and Related Monomers. *European Journal of Pharmaceutical Research* **2019**, *1* (1), 27–36. https://doi.org/10.34154/2019-EJPR.01(01).pp-27-36/euraass.

(15) Hou, W.; Xu, J. Surfactant-Free Microemulsions. *Current Opinion in Colloid & Interface Science* **2016**, *25*, 67–74. https://doi.org/10.1016/j.cocis.2016.06.013.

(16) Kaci, M.; Arab-Tehrany, E.; Dostert, G.; Desjardins, I.; Velot, E.; Desobry, S. Efficiency of Emulsifier-Free Emulsions and Emulsions Containing Rapeseed Lecithin as Delivery Systems for Vectorization and Release of Coenzyme Q10: Physico-Chemical Properties and in Vitro Evaluation. *Colloids and Surfaces B: Biointerfaces* **2016**, *147*, 142–150. https://doi.org/10.1016/j.colsurfb.2016.07.036.

(17) Sahoo, S. K.; Panyam, J.; Prabha, S.; Labhasetwar, V. Residual Polyvinyl Alcohol Associated with Poly (d,l-Lactide-Co-Glycolide) Nanoparticles Affects Their Physical Properties and Cellular Uptake. *Journal of Controlled Release* **2002**, *82* (1), 105–114. https://doi.org/10.1016/S0168-3659(02)00127-X.

(18) Lunn, M.; Fausnight, T. Hypersensitivity to Total Parenteral Nutrition Fat-Emulsion Component in an Egg-Allergic Child. *PEDIATRICS* **2011**, *128* (4), e1025–e1028. https://doi.org/10.1542/peds.2010-2771.

(19) Attia, M. F.; Dieng, S. M.; Collot, M.; Klymchenko, A. S.; Bouillot, C.; Serra, C. A.; Schmutz, M.; Er-Rafik, M.; Vandamme, T. F.; Anton, N. Functionalizing Nanoemulsions with Carboxylates: Impact on the Biodistribution and Pharmacokinetics in Mice. *Macromolecular Bioscience* **2017**, *17* (7), 1600471. https://doi.org/10.1002/mabi.201600471.

(20) Hörmann, K.; Zimmer, A. Drug Delivery and Drug Targeting with Parenteral Lipid Nanoemulsions — A Review. *Journal of Controlled Release* **2016**, *223*, 85–98. https://doi.org/10.1016/j.jconrel.2015.12.016.

Chapter 2.4. Development of targeting theranostic nanocarriers for early detection and treatment of endothelial senescence

1. INTRODUCTION

According to a recent report (2018) published by the world health organization (WHO), cardiovascular diseases are the leading cause of death globally for the last 15 years. Atherothrombosis is a major cause of cardiovascular diseases and can lead to some serious conditions like myocardial infarction and stroke. The underlying atherogenesis process starts with the appearance of early lesions of atherosclerosis which can develop into mature plaques impeding blood flow and optimal perfusion of target organs, ultimately triggering an atherothrombotic event (myocardial infarction, stroke, peripheral artery diseases). The studies have shown that the atherogenesis process is not a generalized alteration of the cardiovascular system but is targeting well-defined arterial sites at risk such as bifurcations and curvatures predominantly in the aortic arch and branches, carotid bifurcations, aorta-renal bifurcations, and aorto-iliac bifurcations. The preferential targeting of these athero-susceptible arterial sites is most likely a consequence of their particular abnormal local flow behavior characterized by a disturbed flow and low shear versus the atheroprotective sites characterized by a laminar flow and high shear.[1] Such a region often arises at sites of arterial branching. At atheroprotective arterial sites, laminar flow-related high shear stress triggers key protective mechanisms in endothelial cells (ECs) especially the formation of nitric oxide (NO), a potent vasodilator and inhibitor of platelet activation that also very effectively prevents the expression of NF-κB-controlled pro-atherothrombotic factors including adhesion molecules, pro-inflammatory factors and the activator of the coagulation cascade, tissue factor etc. Therefore, ECs at arterial sites at risk and exposed to disturbed flow and low shear are unable to protect the arterial wall in an efficient manner as indicated by the appearance of early endothelial dysfunction, local inflammation, oxidative stress and expression of several atherothrombotic factors.

The endothelial senescence has been recently studied as a very important early event leading to the development of atherogenesis, since early signs are detected at arterial sites of disturbed flow in young adult animals, and they are more prominent in the presence of major risk factors such as ageing, hypercholesterolemia, diabetes and hypertension.[1–4] Thus, the endothelial layer appears to be the most pertinent target for both preventive and therapeutic interventions due to its major role in atherogenesis.

The main objectives of this study are: i) to take advantage of the unique features of the cell membrane of senescent ECs, such as expression of the adhesion molecules VCAM-1 and ICAM-1, for the *in-vivo* early assessment of arterial sites at risk, ii) to develop a new method to functionalize nano-emulsions, for the selective delivery of agents to the ECs for preventive and/or therapeutic purposes, iii) to evaluate the potential of the developed nano-carriers to target and restore optimal vascular protection using a nanomedicine theranostic approach.

A wide range of targeted nanoparticles have been reported in the literature, like polymeric nanoparticles (*e.g*., PLGA), liposomes, nano-emulsions etc., to specifically accumulate in ECs, for imaging or drug delivery applications.[5] The targeted drug delivery through such nano-carriers have many advantages including improved bioavailability, improved stability, reduced side effects, and the facility for co-encapsulation of imaging agent for theranostic applications. All these nano-carriers have different advantages and disadvantages associated with them, in terms of their loading capabilities, stability, surface modification in order to graft targeting ligands etc. In comparison with the other nano-carriers, nano-emulsions have been extensively studied as drug delivery systems because of their high loading capacity, good stability, low manufacturing cost and their potential to encapsulate hydrophilic, lipophilic, as well as amphiphilic drugs.[6–10] Nano-emulsions are kinetically stable, heterogenous, very fine and optically clear dispersions of two immiscible liquids (water-in-oil or oil-in-water), generally stabilized by a surfactant with average size of the droplets ranging from 200-300 nm.[11–13] However, it's not easy to chemically modify the surface of the nano-emulsions due to the challenges involved in the stabilization of the interface. In this study, we have developed a polymer network, based on the chemical modification of poly (maleic anhydride-alt-1-octadecene) (PMAO), by performing a systematic reaction of Jeffamine polyetheramine (Jeffamine® M2070) and a polyethylene glycol (PEG)-containing compound terminated by a reactive maleimide on each anhydride function of the polymer. The nano-emulsions are then developed by ultrasonication method using this newly synthesized polymer, with some PEG presenting maleimide at the surface of the nano-droplets to react with the cysteine function of the antibody. These nano-carriers are loaded with a fluorescent probe to allow detection of senescent ECs covering athero-susceptible sites at risk for diagnosis and monitoring purposes using fluorescence imaging, and/or a therapeutic vasoprotective agent for delivery and regeneration of the protective endothelial function. And then the potential of these maleimide decorated nano-emulsions to graft antibodies at the surface of the formed

nano droplets has been evaluated. For this purpose, we have used anti-VCAM antibodies and studied their interaction and the extent of binding with the maleimide groups available at the surface of the nano droplets. The selection of these Abs is done to target the surface markers of senescent ECs (VCAM-1), to induce locally a specific concentration of the nano-carriers and, thus, an on-site specific accumulation of the imaging probe and the therapeutic agent intended to restore the endothelial function. The nano-emulsions have been optimized in relation to the size of the nano-droplets and the amount of the antibody required to completely saturate the maleimide groups available at the surface of the nano droplets. Different techniques are used to characterize these nano-emulsion formulations namely differential light scattering (DLS), UV-visible spectroscopy, transmission electron microscopy (TEM) and nuclear magnetic resonance (NMR). The biological evaluation of theranostics nano-carriers have been performed at two different levels, *i.e.*, *in vitro* on cultured ECs and *ex vivo* on isolated low and high-risk arterial segments, to analyze their EC regenerative potential.

2. MATERIAL AND METHODS

2.1. Chemicals used

The Poly (maleic anhydride-alt-1-octadecene) (PMAO), triethylamine (TEA), ethanolamine, 1-[Bis (dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluorophosphate (HATU) and dimethyl formamide (DMF) were purchased from Sigma Aldrich. Jeffamine® M-2070 polyetheramine (J-2000) was kindly offered by Huntsman corporation (Texas, U.S.A.). The BOC-NH-PEG-NH_2 was purchased from Iris pharma®. The maleimide reagent was purchased from Ark Pharm Inc®. Medium chain triglyceride-Labrafac WL®, was purchased from Gattefossé (Saint Priest, France). The dyes, CY 5.5 TPB and NR 668 were kindly provided by Dr. Mayeul Collot. The rest of the reagents and solvents used were of analytical grade.

2.2. Chemical modification of the polymer

First of all, 514.3 mg of BOC-NH-PEG-NH_2 (0.2 equivalent of PMAO) and 1371.4 mg of J-2000 (0.8 equivalent of PMAO) were taken and dissolved in 20 ml of DMF in a round bottom flask. To this solution, PMAO (300 mg) was added and the final volume was adjusted up to 50ml with DMF. Once the components were completely solubilized, 0.6 mL (5 equivalents) of triethylamine was added into the flask and this solution was

then continuously stirred overnight at 60°C under reflux condenser. Then HATU (651 mg) and ethanolamine (261.5 mg or 0.26 ml) were added into this solution and the solution was again stirred overnight at 60°C under reflux condenser. The solvent (DMF) was evaporated (using rotavapor) and the resulting product was dried under vacuum for 2 hours to ensure complete removal of the solvent. Next, the polymer was dissolved in 10 mL ethanol and this solution was poured into the dialysis membrane (MWCO of dialysis membrane=3500) and subjected to dialysis in a 1000 mL beaker filled with ethanol (800 ml) for 24 hours. The solvent (ethanol) was evaporated, the resulting product was dried under vacuum and small quantity was used to perform NMR. The remaining product was dissolved in 5 mL TFA and 30 mL DCM and was then continuously stirred overnight at room temperature. The solvent was evaporated (using rotavapor) and the resulting product was dried under vacuum. To completely remove TFA from the product, the product was dissolved in methanol and then the solvent was evaporated again, this step was repeated three times. At this stage, took 25 mg of the resulting polymer, dissolved in 0.6 mL $CDCl_3$ and performed NMR. The remaining product was dissolved in 5-10 mL of DMF (dimethyl formamide) followed by addition of 2 equivalents of the 2-(2,5-dioxo-2,5-dihydro-1H-pyrol-1-yl) acetic acid (maleimide reagent), 5 equivalents of triethylamine and 2 equivalents of HATU into the flask and kept it overnight at 60°C with continuous stirring. Finally, 10 equivalents of acetic anhydride were added into the mixture and stirred for 30 minutes. The solvent (DMF) was then evaporated and the resulting product was dried under high vacuum (for 2 hours). The final product obtained was dissolved in 10 mL ethanol and this solution was poured into the dialysis membrane (MWCO of dialysis membrane=3500) and subjected to dialysis for 24 hours. The solvent (ethanol) was evaporated, the resulting product was dried under high vacuum and then 25 mg of the resulting polymer were used to perform NMR. The overall scheme is illustrated in figure 1(a).

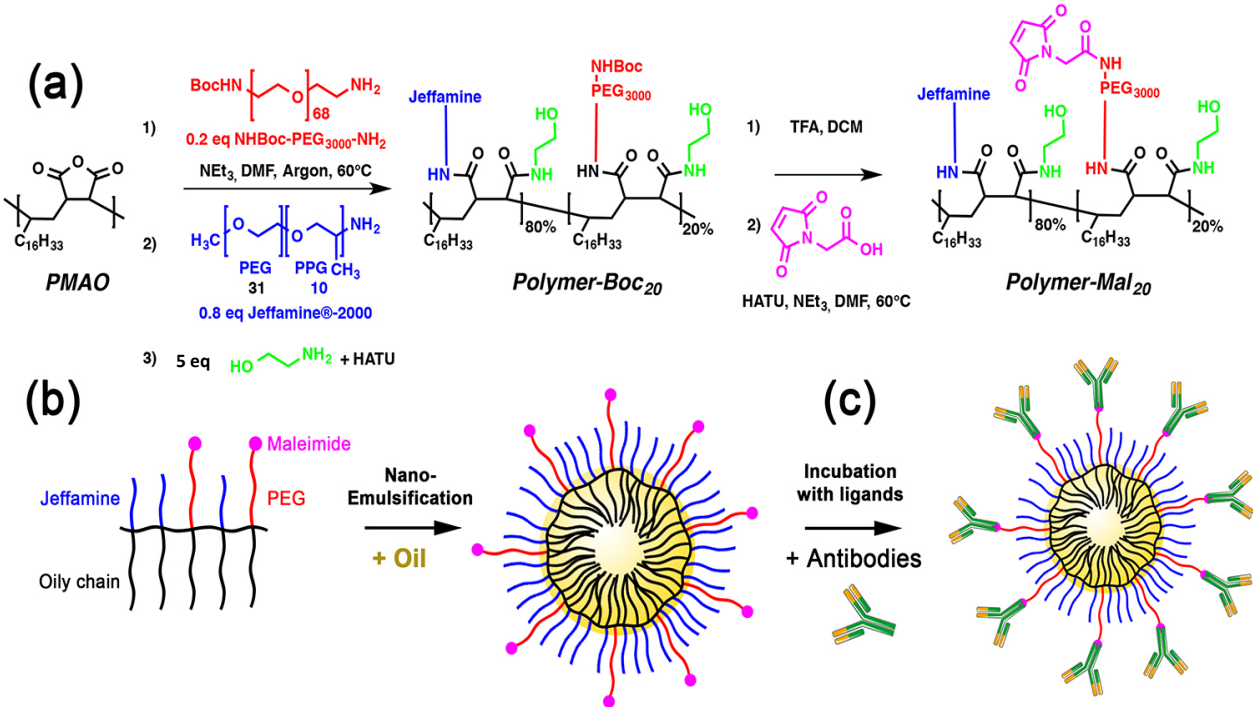


Figure 1: Schematic illustration of, (a) synthesis of the maleimide decorated polymer, (b) formation of nano-emulsions and (c) grafting of the antibody on the nano-droplets

2.3. ^{1}H-NMR Analysis

To study the progress of the reaction and to confirm the structure of the modified polymer, took 25 mg of the product at three different steps to perform ^{1}H-NMR *i.e.*, first after reaction of PMAO with J-2000 and BOC-NH-PEG-NH_2, then after deprotection with TFA/DCM and finally after the reaction with maleimide reagent. Then, this polymer was dissolved in 600 µL of deuterated chloroform ($CDCl_3$) and the ^{1}H-NMR spectrum was studied with a Bruker Top Spin 3.0 (operating at 400 MHz). The chemical shifts (δ) were expressed in parts per million.

2.4. Preparation of maleimide-decorated nano-emulsions

First of all, both oil and aqueous phases were prepared separately and heated at 90°C for 30 min. Oil phase consists of polymer dissolved in 1 % dye (NR 668 or CY 5.5 TPB) solution in Labrafac WL®, while the aqueous phase used was distilled water. The hot aqueous and oil phases were then rapidly mixed together and homogenized by vortex for 30 seconds, followed by ultrasonication for 4 min and 10 seconds, with repeated cycles of "pulse ON" for 10 seconds and "pulse OFF" for 20 seconds, at 20 % amplitude (intensity of ultrasonicator), which resulted in the formation of nano-emulsions with the surface of droplets being decorated with maleimide functions (figure 1b). For the evaluation of

therapeutic potential of the nano-carriers, these were formulated with a vasoprotective agent, an optimized formulation of Omega 3, i.e., EPA:DHA 6:1 (instead of labrafac) as dispersed phase and distilled water was used as continuous phase, without any fluorescent probe.

2.5. Dynamic light scattering and zeta potential measurements

The hydrodynamic diameter and polydispersity index (PDI) of the nano-emulsion droplets were measured by dynamic light scattering (DLS), in triplicate, along with zeta potentials using a Malvern Nano ZS instrument, equipped with a helium-neon laser (4mW) operating at 633 nm. The scatter angle was fixed at 173° and the temperature was maintained at 25°C.

2.6. Microscopic analysis

The morphology and structure of the nano-droplets formed were studied by transmission electron microscopy (TEM). The nano-emulsion samples were diluted (1/100) with Milli-Q water. One drop of the diluted nano-emulsion was introduced on an ultrathin carbon support film (carbon type A, 400 mesh, copper, Ted Pella Inc. Redding, PA) and then the sample was dried at 40°C for 10 minutes. The measurements and analysis were carried out by a Philips Morgagni 268D electron microscope.

2.7. Grafting of antibody on maleimide-decorated and dye loaded nano-emulsions to selectively target VCAM-1 on the senescent endothelial cells

First of all, we took a fixed volume of maleimide-decorated, dye loaded nano-emulsions (100 µL) and diluted 10 times with PBS (1X, pH 7.4). The antibody solutions having different antibody concentration (*i.e.*, 1/250, 1/500, 1/1000 and 1/2000) were added into the diluted nano-emulsions to study the effect of increasing concentration of the antibody on the extent of grafting. After addition of antibody (Ab), the nano-emulsions were incubated for 24 hours at 4-6°C, protected from light, which caused attachment of the Ab onto the maleimide functions at the surface of the nano-droplets. Then, the antibody grafted nano-emulsions were subjected to size exclusion chromatography, using PD10 Sephadex® G-25M column, pre-equilibrated with PBS (1X, pH 7.4). The nano-emulsion fraction obtained after this process was used for *in vitro* and *ex vivo* studies. The schematic illustration of grafting of antibodies on the surface of the nano-droplets is presented in figure 1c.

The maleimide-decorated nano-emulsions, without grafting of the antibody at the surface of the nano-carriers, were used as a control formulation for *in vitro* and *ex vivo* studies.

2.8. *In vitro* studies

To perform *in vitro* evaluation, two ageing-related models of EC senescence were developed: the premature senescence and replicative senescence model (figure 2). The objective of the *in vitro* studies was to evaluate the targeting of the fluorescent VCAM-1 targeting nano-carriers, into the normal and senescent ECs (in culture), using fluorescence microscopy techniques. The endothelial cells, from the porcine coronary artery, were cultured in glass chamber slides by the procedure mentioned in the figure 2. In the stress-induced premature senescence model, the senescence was induced by angiotensin-II (100 nM). The Ab-functionalized and non-functionalized nano-carriers, labelled with specially designed lipophilic dyes (NR668, Cy5.5 TPB) were prepared and incubated with the endothelial cell culture for 1 hour (4°C or 37°C) and then the potential for VCAM-1-targeting was studied by confocal microscopy using a Leica SP2 UV DM Irbe confocal microscope. The fluorescent probes were used to track nano-carriers and the DAPI was used to stain the nuclei of the cells. The excitation wavelengths were set at 405 nm for DAPI, 635 nm for CY5.5-TPB and 543 nm for NR668 and the fluorescence was measured in following wavelength ranges, 431 - 481 nm for DAPI, 650 - 711 nm for CY5.5-TPB and 560 - 650 nm for NR668. The images obtained were processed by imageJ software. Different parameters were studied, like polymer/antibody ratio, average size of the nano-carriers and temperature change, to optimize the conditions in order to ensure, i) maximum internalization of Ab-functionalized nano-carriers into target senescent ECs, and ii) minimum non-specific interactions among the (a) Ab-functionalized nano-carriers with normal cells, (b) non-functionalized nano-carriers with different healthy and senescent ECs. A competition experiment to confirm and validate the targeting specificity of the nano-carriers has been performed by using also the replicative senescence model, as shown in figure 2.

In parallel with these experiments, the non-functionalized nano-carriers were formulated with a vasoprotective agent, an optimized formulation of Omega 3, *i.e.*, EPA:DHA 6:1 (instead of Labrafac®) as dispersed phase and distilled water as continuous phase, without any fluorescent probe. Then the regenerative potential of Omega 3 was evaluated *ex vivo*, by studying the level of prothrombotic markers. And nano-carriers were also prepared

using corn oil as dispersed phase (instead of Omega 3 oil) and used this formulation as control formulation.

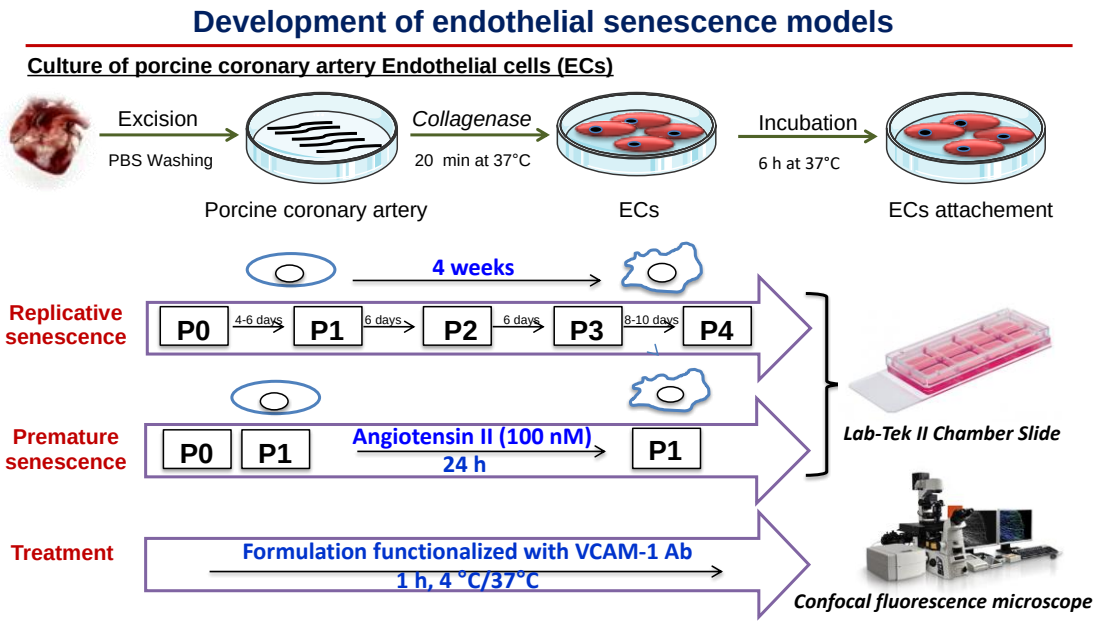


Figure 2: Endothelial senescence models

2.9. *Ex vivo* studies

To overcome the limitations inherent to artificial cell culture, we proceeded towards *ex vivo* studies, with the nano-carriers which showed most promising results *in vitro*. The Ab-functionalized nano-carriers, loaded with fluorescent probes, were studied using isolated healthy porcine coronary arteries either under control conditions or after exposure to senescence inducer (*i.e.*, Ang II) for 24 h. The visualization of the nano-carrier location and accumulation was performed *ex vivo*, using cryo-sectioning from the different artery senescent models by classical confocal microscope.

3. RESULTS AND DISCUSSIONS

3.1. Synthesis of the amphiphilic polymer and evaluation of the structure by ^{1}H-NMR Analysis

As a polymeric platform, poly(maleic anhydride-alt-1-octadecene) (PMAO) is a polymer (average M_n 30,000-50,000) with a repeating unit composed of a hydrophobic hydrocarbon chain (C_{18}) and a succinic anhydride function. As illustrated in Figure 1(a), an amphiphilic polymer (Polymer-Boc20) was first synthesized through a reaction of

PMAO with an amino-PEG_{3000} bearing a protected amino function (Boc-NH-PEG_{3000}-NH_2) on 20% of the anhydride functions. The remaining 80% of anhydride functions were then reacted with Jeffamine®2000, an amino/methoxy terminated PEG/PPG (poly(ethylene glycol)/poly(propylene glycol)) copolymer, to reduce the polymer hydrophobicity. Then the deprotection of the amino functions of Polymer-Boc_{20} was performed followed by coupling of the amino groups with the maleimidoacetic acid to result in the formation of Polymer-Mal_{20} (figure 1a). The progress of the reaction as well as the structure of the polymer was studied by performing NMR at each step of the synthesis of this amphiphilic polymer derivative. The NMR results have confirmed the attachment of Jeffamine® 2000 (80 %) and PEG-NH_2 (20%) with the polymer network. After the addition of the maleimide, we have got a clear signal for maleimide indicating the binding of maleimide with the available NH_2 at the end of the PEG-NH_2 chains.

3.2. Preparation of nano-carriers loaded with fluorescent probes

The nano-carriers loaded with either CY 5.5-TPB or NR 668 were formulated by ultrasonication method and in this process, the amphiphilic polymer derivative stabilized the nano-emulsions and also provided the facility to functionalize the nano-carriers after their formation. The mechanism involved is simple but very efficient, *i.e.*, during the formation of nano-droplets the amphiphilic polymer re-organizes itself in such a way, that the hydrophobic hydrocarbon chains rest in the hydrophobic interior of the nano-droplets (oil phase) whereas the hydrophilic part of the polymer (J-2000 and maleimide-decorated PEG chains) move towards the interface of the droplets (towards aqueous phase).[14] This provides active maleimide functions at the surface of the nano-droplets which will ensure a strong anchorage of the ligands since it will be a part of the nano-carriers as a “shell” (figure 1c). Such surface functionalized systems, having core shell structure and an internal oil reservoir, can serve as excellent targeted nano-carriers for lipophilic compounds (either therapeutic or imaging agents or both) with very high loading capacities. The use of Jeffamine®2000 and PEG in this system, has great significance because the PEG-chains not only create steric repulsion among the neighboring nano-droplets and improve the interfacial stabilization but also have a well-recognized ability to reduce the uptake of the nano-emulsions (after *in vivo* administration) by the immune system, inhibiting opsonization and phagocytosis (normally seen with conventional nano-emulsions), which increases the circulation time of nano-emulsions in the body[1516]. Another important feature of this formulation is the type of fluorescent probes used. These

probes (namely NR668 and Cy5.5TPB) are very stable in oil, and can be highly concentrated without excessive decrease of quantum yield[17–19]. As a result, nano-carriers are ultrabright and allow their fine tracking during evaluation of their interactions with biological systems.

3.3. Dynamic light scattering and zeta potential measurements

The nano-carriers loaded with fluorescent probes (NR668 or CY 5.5 TPB) were characterized by dynamic light scattering technique and the results obtained are shown in the figure 3 (a). The average hydrodynamic diameter of the maleimide-decorated nano-emulsions was 95 ± 7 nm. The hydrodynamic diameters obtained for nano-carriers developed by using omega oil and corn oil as dispersed phase (instead of Labrafac) were 80 nm and 190 nm respectively. The size distribution observed with the corn oil was different from the rest of the formulations, which was mostly related to the nature of this oil, but this formulation was only used as control formulation in one of our experiments that's why it did not have any effect on our overall approach. The most important formulations, under investigation, were the ones with labrafac and with Omega oil as dispersed phases, and in these cases the average hydrodynamic diameters of the nano-droplets are below 100 nm, and this size range is ideal for the desired applications.

The zeta-potential measurements have shown that the surface charge on the nano-emulsion droplets is positive and ranges from 10-15 mV, irrespective of the nature of the oil used to formulate the nano-carriers (figure 3(b)).

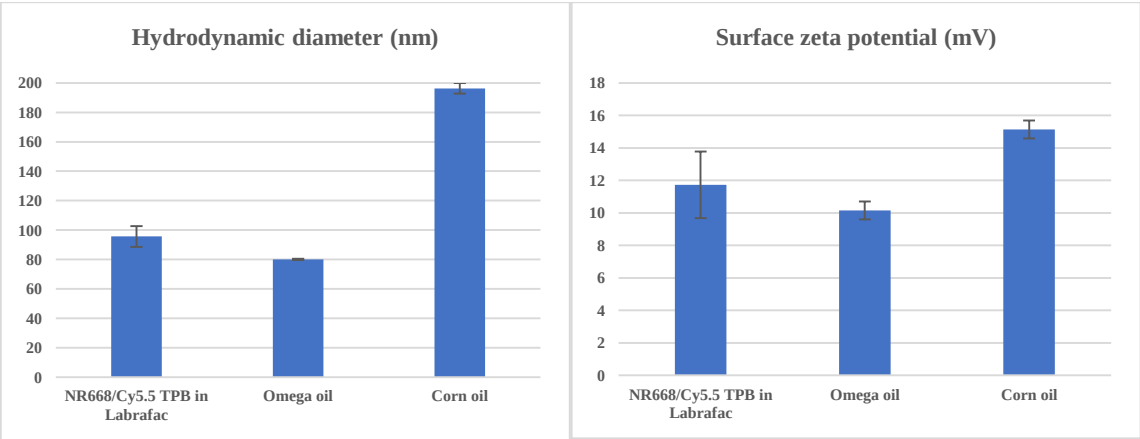


Figure 3: Physico-chemical characterization of nano-emulsions, (a) Hydrodynamic diameter (nm), (b) zeta-potential (mV), whereas, NR668/ CY 5.5 TPB in Labrafac, Omega oil and Corn oil represent different oil phases used to prepare nano-emulsions

3.4. Microscopic analysis

The results, illustrated in figure 4, represent the morphology and average size of the nano-emulsions formed by using polymer dissolved in CY5.5-TPB solution in Labrafac® (1%) as oil phase and distilled water as aqueous phase. These results are coherent with the ones obtained with DLS, showing homogenous droplet dispersion with an average size below 100 nm. Moreover, the nano-droplets formed are spherical in shape and the interface of the nano-droplet is strongly stabilized due to the precipitation of the polymer in the form of a shell *i.e.*, the nano-emulsions have a spherical core-shell structure.

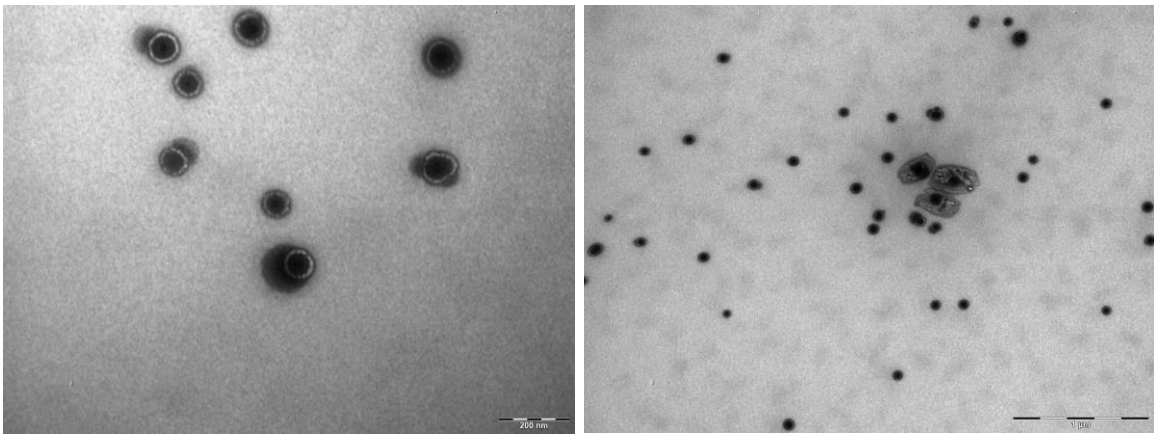


Figure 4: TEM characterization of nano-emulsions formed by using polymer dissolved in 1% CY5.5-TPB solution in Labrafac® as oil phase and distilled water as aqueous phase

3.5. Grafting of antibodies onto the surface of nano-carriers loaded with fluorescent probes and evaluation of their theranostic potential

The maleimide-decorated nano-emulsions were incubated with anti-VCAM-1 antibody solution for 24 hours at 4-6°C, which resulted in the attachment of the Ab at the surface of the nano-droplets. It gave rise to Ab-functionalized nano-carriers, loaded with fluorescent probes. The theranostic potential of these nano-carriers was evaluated by *in vitro* and *ex vivo* studies.

3.5.1. *In vitro* studies

The VCAM-1 is normally expressed by the ECs in basal conditions, however it was observed that it is significantly over-expressed by the senescent ECs. The targeting of the Ab-decorated nano-carriers, loaded with a fluorescent probe (NR668, CY 5.5-TPB), were studied (*in vitro)* by confocal microscopy. The dyes, which we selected and used to track the nano-carriers, are highly lipophilic and do not show any leakage from the nano-

emulsions which ensures their successful tracing *in vitro* as well as in the biological fluids[17]. The Ab-decorated nano-carriers showed their efficiency to selectively and specifically target VCAM-1 overexpressed at the membrane of both replicative and Ang II-induced premature senescent ECs compared to the healthy cultured ECs. First of all, we studied the effect of the average size of the nano-carriers on their targeting potential, by confocal microscopy. Nano-carriers having hydrodynamic diameters in the below 100 nm, 100-150 nm and 150-200 nm ranges were prepared, by changing the parameters for ultrasonication process. It was observed that the obtained fluorescence signal increases with the decreasing size of the VCAM-1 Ab-decorated nano-carriers, *i.e.*, the maximum signal was observed with the nano-carriers having sizes less than 100 nm (95 nm) as compared to those having sizes ranging from 100-150 nm and 150-200 nm (figure 5). And as it has already been well documented that the cell internalization is higher for the particles having sizes around 100 nm as compared to the larger particles[20], so the optimized size distribution (<100 nm) of these VCAM-1 Ab-decorated nano-carriers could be ideal for their applications involving cellular uptake and cell internalization.

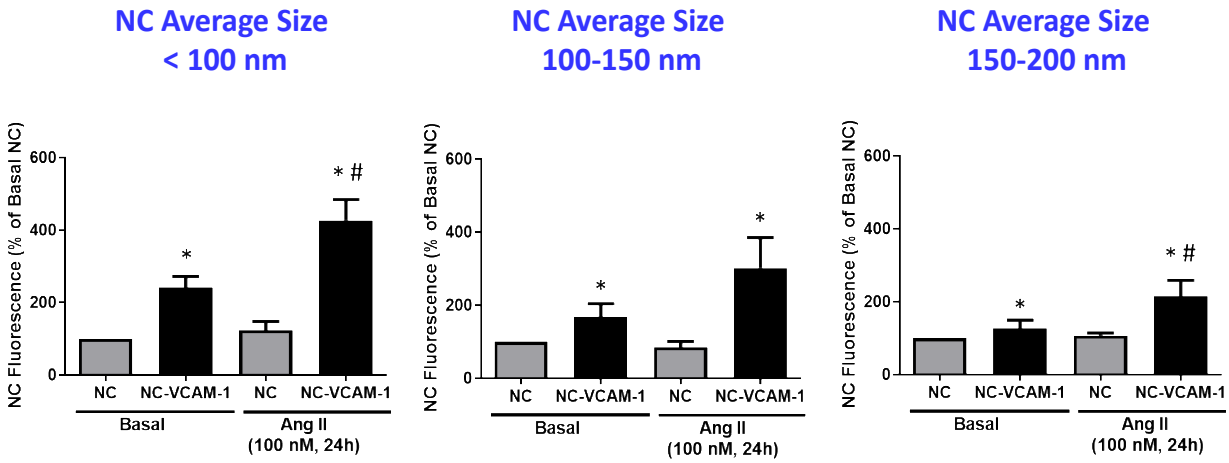


Figure 5: The fluorescence signals observed using Ab-decorated nano-carriers having different size

After selecting the appropriate size distribution (<100 nm), different concentrations of the anti-VCAM-1 Abs were tested and analysed to find the optimum concentration of the Ab required to saturate the available maleimide active sites at the surface of the nano-carriers and to obtain maximum targeting. The initial concentration of antibody aliquot was 0.437mg/ml. The maximum fluorescence signals were obtained with the formulation having highest antibody concentration (1/250 dilution) as compared to those having lower amounts of the Ab, *i.e.*, 1/500, 1/1000 and 1/2000 (figure 6). That's why, we selected 1/250 concentration of the Ab to proceed for the next experiments.

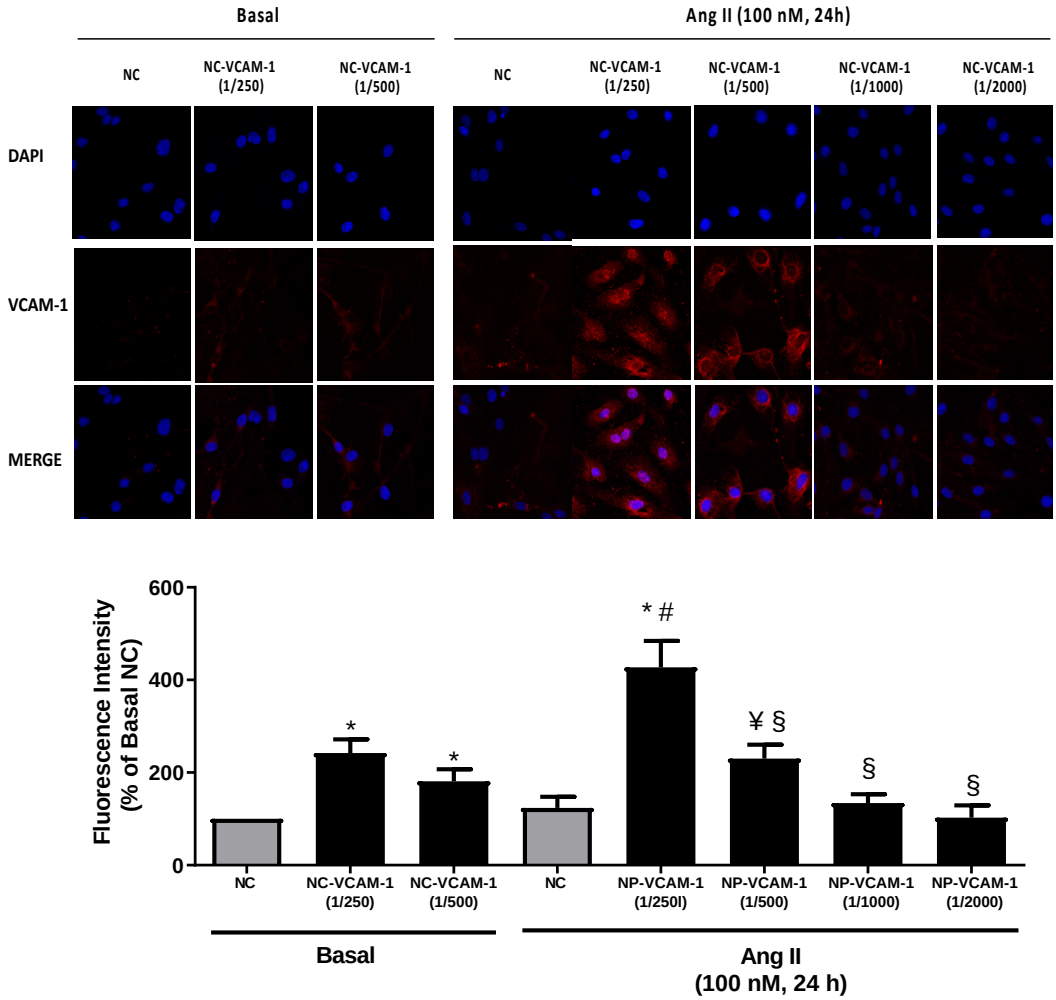


Figure 6: Effect of the concentration of the Ab on the targeting

We have also performed experiments to observe the targeting of the developed formulations at different temperatures. The results obtained are shown in the figure 7, which indicate that the fluorescent Ab-decorated nano-carriers selectively target cultured senescent ECs (premature senescence), within 1 h, at 4°C as well as at 37°C. That means, the increase in temperature from 4°C to 37°C did not affect the targeting potential of our formulations. This experiment was performed to form the basis for the *in vivo* experiments, where the formulation will be exposed to and evaluated at 37°C.

The experiments have been performed in "replicative senescent model" as well and it was found that the Ab-decorated nano-carriers showed almost similar behaviour as in the case of premature senescent model. Thus, it can be concluded that the fluorescent Ab-functionalized nano-carriers selectively target cultured and native senescent ECs induced by the pro-senescent inducer (*i.e.*, "Ang II") and by replicative senescence and this effect is observed rapidly (within 1 h), at 4°C as well as at 37°C, with minimal off-target cell surface interactions (figure 8).

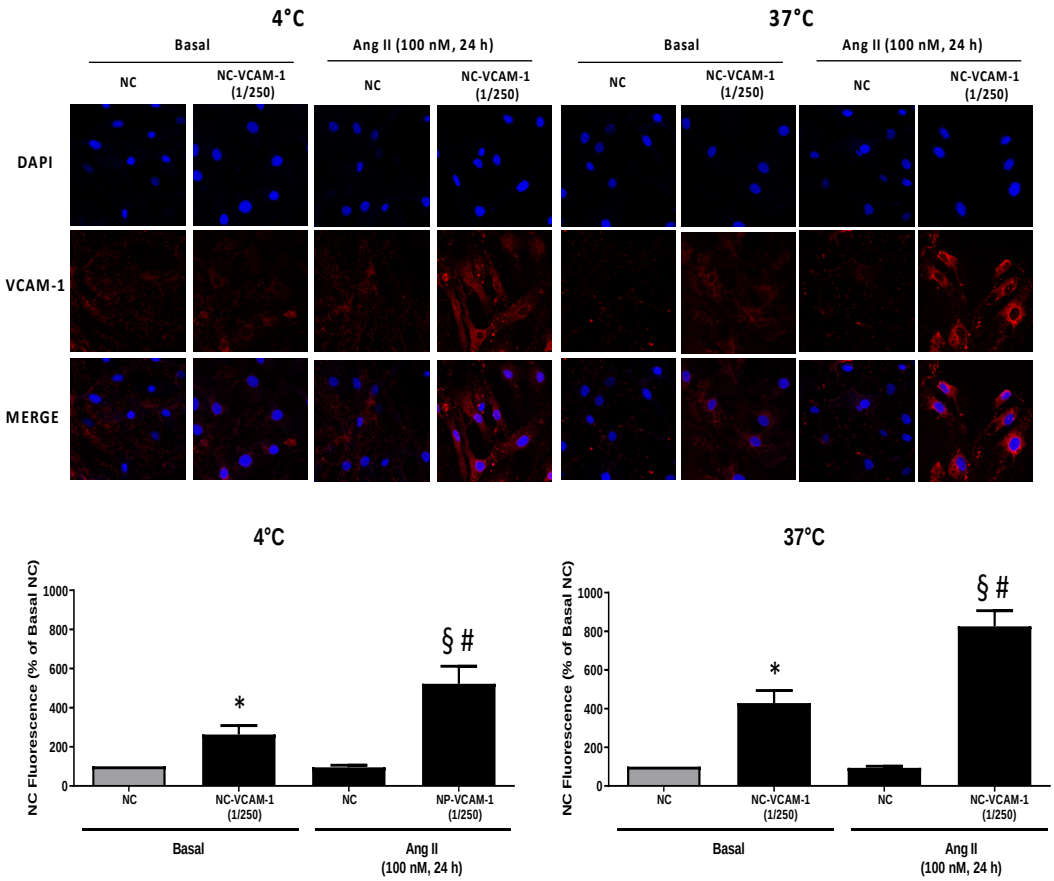


Figure 7: Effect of the temperature on the targeting of VCAM-1 Ab-decorated nano-carriers in basal and Ang II-induced senescent conditions

To further validate the specificity of targeting of Ab-decorated nano-carriers, the senescent ECs were first pre-treated by an antibody directed against VCAM-1 and then the Ab-decorated nano-carriers were incubated with these pre-treated senescent ECs. It was observed by the confocal microscopy that the pre-treatment with the anti-VCAM-1 antibody prevents the Ab-decorated nano-carriers to target the senescent ECs (Figure 8) which confirms, i) the expression of VCAM-1 in endothelial cell senescence and its potential for targeting and drug delivery, and ii) the targeting specificity of the Ab-decorated nano-carriers.

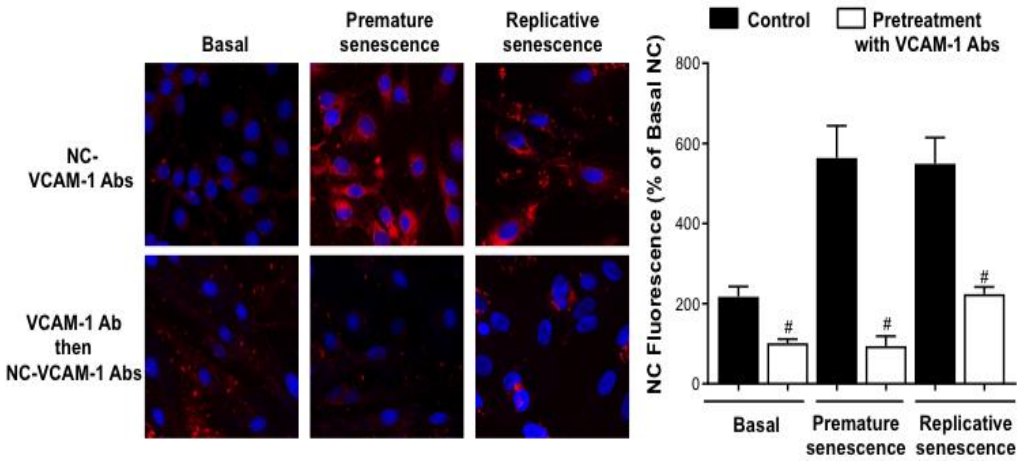


Figure 8: Targeting and cell internalization of the Ab-decorated nano-carriers in senescent ECs (both premature and replicative)

After having all these promising results, the non-functionalized nano-carriers, loaded with the therapeutic agent Omega 3 (EPA: DHA) were prepared without any fluorescent probe, in order to observe the potential of these nano-carriers to prevent VCAM-1 upregulation induced by Ang-II in porcine coronary artery. Nano-carriers were also prepared using corn oil as dispersed phase (instead of Omega 3 oil) and used as control formulation. It was observed that the nano-carriers loaded with Omega 3 prevented significantly the Ang II-induced VCAM-1 upregulation in the porcine coronary artery, as compared to the control formulation with corn oil, where no such effects were observed (figure 9).

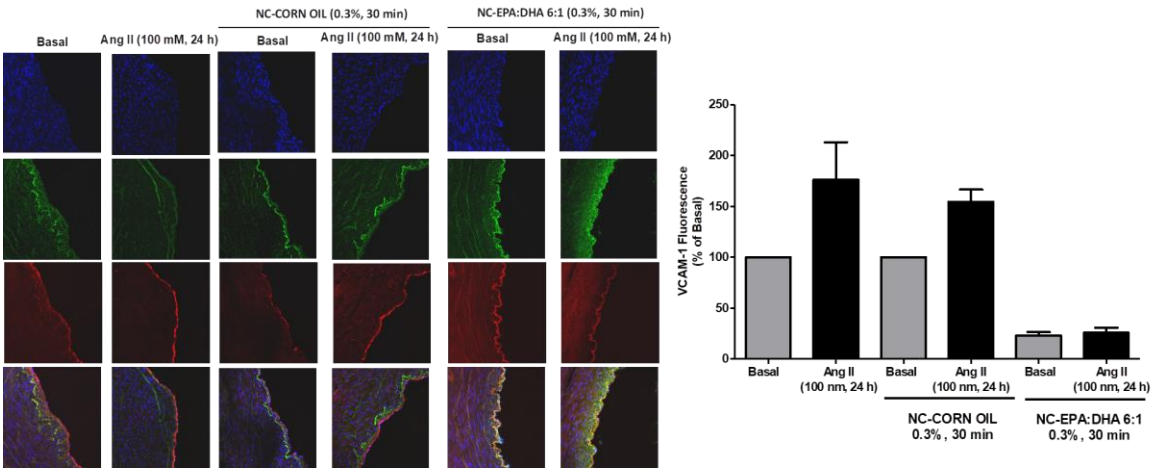


Figure 9: Prevention of Ang II-induced VCAM-1 upregulation in the porcine coronary artery, wherein "NC-corn oil" shows the control formulation while "NC-EPA:DHA 6:1" shows the formulation having therapeutic agent (Omega 3)

Currently the experiments are in progress, where we have prepared Ab-decorated nano-carriers loaded with the fluorescent probe (CY 5.5 TPB) as well as the therapeutic agent (Omega 3) and are studying the theranostic potential of these nano-carriers *ex vivo* and *in vivo* in order to target and prevent the endothelial senescence at very early stages.

3.5.2. *Ex vivo* studies

The porcine coronary artery rings were first exposed to Ang II (100 nM) for 24 h to induce premature senescence to promote senescence-associated expression of VCAM-1 selectively at the endothelium overlying the arterial wall. Then these coronary artery rings were incubated separately with the nano-carriers decorated with VCAM-1-Abs and with VCAM-1 Abs alone. The results obtained by confocal microscopy have shown that the nano-carriers decorated with VCAM-1 Abs (NC-VCAM-1 Abs) stain predominantly the endothelium of the porcine coronary artery rings. A similar pattern of staining is also observed with the VCAM-1 Ab alone (figure 10 a). These experiments confirmed that the VCAM-1 is over expressed at the endothelium overlying the arterial wall by the treatment of the coronary artery rings with the Ang-II and that the nano-carriers decorated with VCAM-1 Abs specifically target the VCAM-1 expressed at the ECs. In order to evaluate the arterial sites at risk, the expression level of VCAM-1 was observed and compared at the aortic arch characterized by disturbed flow and at the thoracic aorta characterized by laminar flow, in young adult rats by Western blot analysis. These investigations have indicated that this marker VCAM-1 is highly expressed at the arterial site exposed to disturbed flow (figure 10 b). That's why, the aortic arch and other such sites having disturbed flow can be successfully targeted, by using the developed theranostic nano-carriers, to restore the endothelial function and prevent endothelial senescence at very early stages, thus contributing to the prevention of atherogenesis development.

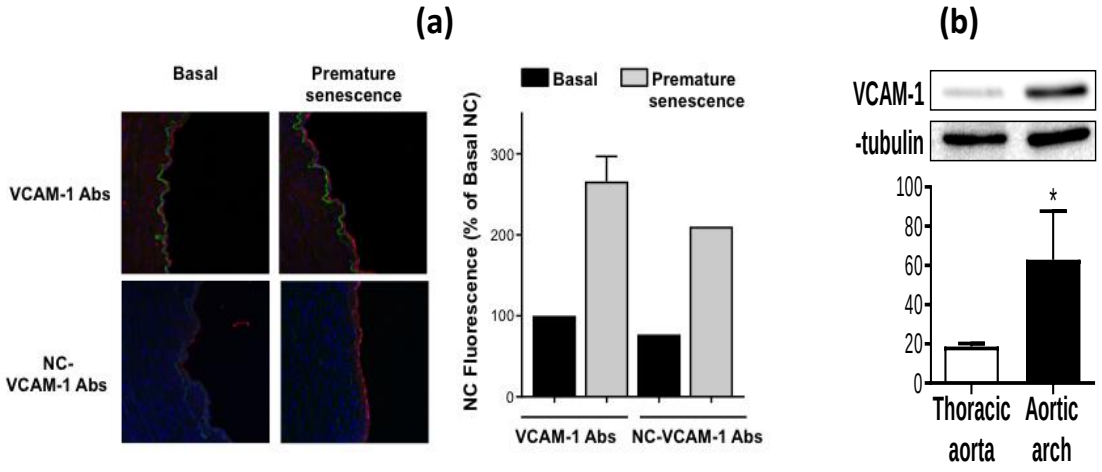


Figure 10: (a) The confocal images of the endothelium of porcine coronary artery rings in basal conditions as well as in premature senescent conditions, obtained after treatment with the nano-carriers decorated with VCAM-1 Abs (NC-VCAM-1 Abs) and with the VCAM-1 Ab alone (VCAM-1 expression is shown by the red staining), (b) Western blot analysis of the expression level of VCAM-1 in the aortic arch (disturbed flow) and the thoracic aorta (laminar flow) of young adult rats.

4. Conclusion

The aim of this research was to develop a new strategy to formulate targeting theranostic nanocarriers for early detection and treatment of endothelial senescence. To achieve this objective, we have formulated nano-emulsions using a non-ionic polymer, poly (maleic anhydride-alt-1-octadecene) (PMAO). The polymer was modified in such a way that, upon emulsification, it provides reactive maleimide groups at the surface of the nano-droplets to facilitate the grafting of the anti-VCAM-1 antibodies. The cell membrane of senescent ECs has some unique features, such as expression of the adhesion molecules VCAM-1 and ICAM-1. In this study, one of these features *i.e.*, the expression of VCAM-1, was used for the assessment of arterial sites at risk and to evaluate the potential of the developed nano-carriers to target and restore optimal vascular protection using a nanomedicine theranostic approach, in order to reduce the risk of development of a subsequent advanced calcified atherosclerotic plaque, which is poorly sensitive to drug treatment. The nano-carriers decorated with VCAM-1 Abs and loaded with a fluorescent probe (NR668, Cy5.5 TPB) showed their efficiency to selectively and specifically target VCAM-1 overexpressed at the membrane of both replicative senescent and Ang II-induced premature senescent ECs, compared to healthy cultured ECs. It was further observed that the targeting was directly dependent on the amount of VCAM-1 Abs decorating the nano-carriers. Among the nano-carriers having different size distributions, the nano-carriers with average size around 95 nm showed maximum fluorescence signals as compared to those with average sizes above 100 nm. The effect of change in temperature from 4°C to 37°C did not show any significant effect on the targeting potential of the Ab-decorated nano-carriers, which is very promising as it will not create any complications when we will use these nano-carriers for the *in vivo* studies. The competition experiment, involving pre-treatment of the senescent ECs with an antibody directed against VCAM-1, followed by the addition of Ab-decorated nano-carriers, verified the expression of VCAM-1 in endothelial cell senescence and the targeting specificity of the Ab-decorated nano-carriers. The targeting specificity of the nano-carriers was also validated by the *ex vivo* experiments. When the coronary artery rings were treated with the Ang-II, the VCAM-1 was over expressed at the endothelium overlying the arterial wall and the nano-carriers decorated with VCAM-1 Abs specifically target the VCAM-1 expressed at the ECs. Based on the *in vitro and ex vivo* results obtained, it can be concluded that these nano-carriers have an excellent potential to serve as a candidate for the theranostic approach to target endothelial senescence at very early

stages and to reduce the risk of development of a subsequent advanced calcified atherosclerotic plaque.

Perspectives

The *ex vivo* studies of athero-resistant (thoracic aorta, carotid artery) and athero-susceptible sites including the carotid bifurcation, the aortic arch and branches and the iliac bifurcations in rat *ex vivo* models are in progress. The *in vivo* evaluation of the optimized nano-carriers (following intravenous administration) will be undertaken in models of rats exhibiting arterial ageing: at arterial sites of premature endothelial ageing (inner versus outer curvature of the aortic arch, already established) in healthy young adult rats and in the arterial circulation of aged rats.

We are expecting (on the basis of *in vitro* and initial *ex vivo* results) that the theranostic nano-carriers will accumulate preferentially at well-defined sites characterized by disturbed flow and low shear, and following targeting, the nano-carrier load will be delivered towards senescent ECs to reach levels sufficient to re-establish the protective endothelial function.

5. References

(1) Warboys, C. M.; de Luca, A.; Amini, N.; Luong, L.; Duckles, H.; Hsiao, S.; White, A.; Biswas, S.; Khamis, R.; Chong, C. K.; et al. Disturbed Flow Promotes Endothelial Senescence via a P53-Dependent Pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2014**, *34* (5), 985–995. https://doi.org/10.1161/ATVBAHA.114.303415.

(2) Abbas, M.; Jesel, L.; Auger, C.; Amoura, L.; Messas, N.; Manin, G.; Rumig, C.; León-González, A. J.; Ribeiro, T. P.; Silva, G. C.; et al. Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging and Thrombogenicity: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways. *Circulation* **2017**, *135* (3), 280–296. https://doi.org/10.1161/CIRCULATIONAHA.116.017513.

(3) Kim, C.-S.; Jung, S.-B.; Naqvi, A.; Hoffman, T. A.; DeRicco, J.; Yamamori, T.; Cole, M. P.; Jeon, B.-H.; Irani, K. P53 Impairs Endothelium-Dependent Vasomotor Function Through Transcriptional Upregulation of P66shc. *Circulation Research* **2008**, *103* (12), 1441–1450. https://doi.org/10.1161/CIRCRESAHA.108.181644.

(4) Khemais-Benkhiat, S.; Idris-Khodja, N.; Ribeiro, T. P.; Silva, G. C.; Abbas, M.; Kheloufi, M.; Lee, J.-O.; Toti, F.; Auger, C.; Schini-Kerth, V. B. The Redox-Sensitive Induction of the Local Angiotensin System Promotes Both Premature and Replicative Endothelial Senescence: Preventive Effect of a Standardized *Crataegus* Extract. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **2016**, *71* (12), 1581–1590. https://doi.org/10.1093/gerona/glv213.

(5) Flores, A. M.; Ye, J.; Jarr, K.-U.; Hosseini-Nassab, N.; Smith, B. R.; Leeper, N. J. Nanoparticle Therapy for Vascular Diseases. *Arteriosclerosis, Thrombosis, and Vascular Biology* **2019**. https://doi.org/10.1161/ATVBAHA.118.311569.

(6) Hussain, A.; Samad, A.; Singh, S. K.; Ahsan, M. N.; Haque, M. W.; Faruk, A.; Ahmed, F. J. Nanoemulsion Gel-Based Topical Delivery of an Antifungal Drug: *In Vitro* Activity and *in Vivo* Evaluation. *Drug Delivery* **2016**, *23* (2), 642–657. https://doi.org/10.3109/10717544.2014.933284.

(7) Nasr, M.; Nawaz, S.; Elhissi, A. Amphotericin B Lipid Nanoemulsion Aerosols for Targeting Peripheral Respiratory Airways via Nebulization. *International Journal of Pharmaceutics* **2012**, *436* (1–2), 611–616. https://doi.org/10.1016/j.ijpharm.2012.07.028.

(8) Pawar, V. K.; Panchal, S. B.; Singh, Y.; Meher, J. G.; Sharma, K.; Singh, P.; Bora, H. K.; Singh, A.; Datta, D.; Chourasia, M. K. Immunotherapeutic Vitamin E Nanoemulsion Synergies the Antiproliferative Activity of Paclitaxel in Breast Cancer Cells via Modulating Th1 and Th2 Immune Response. *Journal of Controlled Release* **2014**, *196*, 295–306. https://doi.org/10.1016/j.jconrel.2014.10.010.

(9) Saberi, A. H.; Fang, Y.; McClements, D. J. Fabrication of Vitamin E-Enriched Nanoemulsions: Factors Affecting Particle Size Using Spontaneous Emulsification. *Journal of Colloid and Interface Science* **2013**, *391*, 95–102. https://doi.org/10.1016/j.jcis.2012.08.069.

(10) Kretzer, I. F.; Maria, D. A.; Maranhão, R. C. Drug-Targeting in Combined Cancer Chemotherapy: Tumor Growth Inhibition in Mice by Association of Paclitaxel and Etoposide

with a Cholesterol-Rich Nanoemulsion. *Cellular Oncology* **2012**, *35* (6), 451–460. https://doi.org/10.1007/s13402-012-0104-6.
(11) Anton, N.; Vandamme, T. F. The Universality of Low-Energy Nano-Emulsification. *International Journal of Pharmaceutics* **2009**, *377* (1–2), 142–147. https://doi.org/10.1016/j.ijpharm.2009.05.014.
(12) Solans, C.; Morales, D.; Homs, M. Spontaneous Emulsification. *Current Opinion in Colloid & Interface Science* **2016**, *22*, 88–93. https://doi.org/10.1016/j.cocis.2016.03.002.
(13) McClements, D. J. Nanoemulsions versus Microemulsions: Terminology, Differences, and Similarities. *Soft Matter* **2012**, *8* (6), 1719–1729. https://doi.org/10.1039/C2SM06903B.
(14) Rehman, A. U.; Collot, M.; Klymchenko, A. S.; Akram, S.; Mustafa, B.; Vandamme, T.; Anton, N. Spontaneous Nano-Emulsification with Tailor-Made Amphiphilic Polymers and Related Monomers. *European Journal of Pharmaceutical Research* **2019**, *1* (1), 27–36. https://doi.org/10.34154/2019-EJPR.01(01).pp-27-36/euraass.
(15) Fornaguera, C.; Grijalvo, S.; Galán, M.; Fuentes-Paniagua, E.; de la Mata, F. J.; Gómez, R.; Eritja, R.; Calderó, G.; Solans, C. Novel Non-Viral Gene Delivery Systems Composed of Carbosilane Dendron Functionalized Nanoparticles Prepared from Nano-Emulsions as Non-Viral Carriers for Antisense Oligonucleotides. *International Journal of Pharmaceutics* **2015**, *478* (1), 113–123. https://doi.org/10.1016/j.ijpharm.2014.11.031.
(16) Hörmann, K.; Zimmer, A. Drug Delivery and Drug Targeting with Parenteral Lipid Nanoemulsions — A Review. *Journal of Controlled Release* **2016**, *223*, 85–98. https://doi.org/10.1016/j.jconrel.2015.12.016.
(17) Klymchenko, A. S.; Roger, E.; Anton, N.; Anton, H.; Shulov, I.; Vermot, J.; Mely, Y.; Vandamme, T. F. Highly Lipophilic Fluorescent Dyes in Nano-Emulsions: Towards Bright Non-Leaking Nano-Droplets. *RSC Advances* **2012**, *2* (31), 11876. https://doi.org/10.1039/c2ra21544f.
(18) Kilin, V. N.; Anton, H.; Anton, N.; Steed, E.; Vermot, J.; Vandamme, T. F.; Mely, Y.; Klymchenko, A. S. Counterion-Enhanced Cyanine Dye Loading into Lipid Nano-Droplets for Single-Particle Tracking in Zebrafish. *Biomaterials* **2014**, *35* (18), 4950–4957. https://doi.org/10.1016/j.biomaterials.2014.02.053.
(19) Attia, M. F.; Anton, N.; Chiper, M.; Akasov, R.; Anton, H.; Messaddeq, N.; Fournel, S.; Klymchenko, A. S.; Mély, Y.; Vandamme, T. F. Biodistribution of X-Ray Iodinated Contrast Agent in Nano-Emulsions Is Controlled by the Chemical Nature of the Oily Core. *ACS Nano* **2014**, *8* (10), 10537–10550. https://doi.org/10.1021/nn503973z.
(20) Attia, M. F.; Anton, N.; Akasov, R.; Chiper, M.; Markvicheva, E.; Vandamme, T. F. Biodistribution and Toxicity of X-Ray Iodinated Contrast Agent in Nano-Emulsions in Function of Their Size. *Pharmaceutical Research* **2016**, *33* (3), 603–614. https://doi.org/10.1007/s11095-015-1813-0.

CHAPTER THREE

LIPOSOMES AS CARRIER OF DRUG MOLECULES FOR BIOMEDICAL APPLICATIONS

Chapter 3.1. Introduction, properties, preparation and applications of liposomes

1. Definition:

The word liposome is derived from two Greek words, "lipos" meaning fat and "soma" meaning body. The liposomes can be defined as the spherical structures consisting of an internal aqueous compartment enclosed by one or more phospholipidic bilayers. These bilayers are also known as lamellae. Liposomes were first described by *Bangham et al.*[1] as spherical structures made up of phospholipid and cholesterol, which upon hydration, self-associate to form bilayers surrounding an aqueous interior. Phospholipid forms the bi-layered structures in the aqueous systems and cholesterol improves the stability and fluidity of the bilayers and reduces the leakage of the active payload. Such kind of liposomes are known as the classical or conventional liposomes. Since then the liposomes have been explored extensively as carriers for drug delivery, having a wide range of applications in pharmaceutical, food and cosmetic industry. Due to the unique bilayer structure of the liposomes, these are used as carriers for hydrophilic, lipophilic as well as amphiphilic molecules.

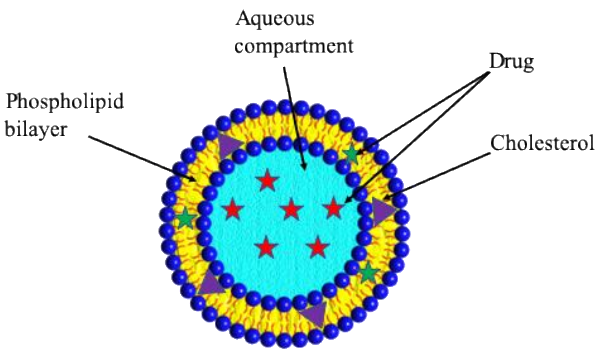


Figure 1. Structure of liposome

2. Classification of liposomes:

There are different ways of classifying liposomes, like based on the size and number of bilayers (lamellarity), on the method of preparation, on the structure, or on the composition and application. On the basis of the size and lamellarity, liposomes are generally classified into following types:

2.1. Unilamellar vesicles (ULVs)

The unilamellar vesicles are composed of only one phospholipid bilayer enclosing an inner aqueous compartment. These liposomes are further classified into two types,

i) Small unilamellar vesicles (SUVs) with a size < 100 nm

ii) Large unilamellar vesicles (LUVs) with a size > 100 nm[23]

2.2. Multilamellar vesicles (MLVs):

The multilamellar vesicles are composed of two or more concentric phospholipid bilayers separated by aqueous compartments and organized in an arrangement similar to that of an onion skin. These vesicles have an average diameters above 500 nm.[2,3]

Another commonly used method to classify liposomes is based on their composition and application. The liposomes can be classified into following main groups based on their composition and application,

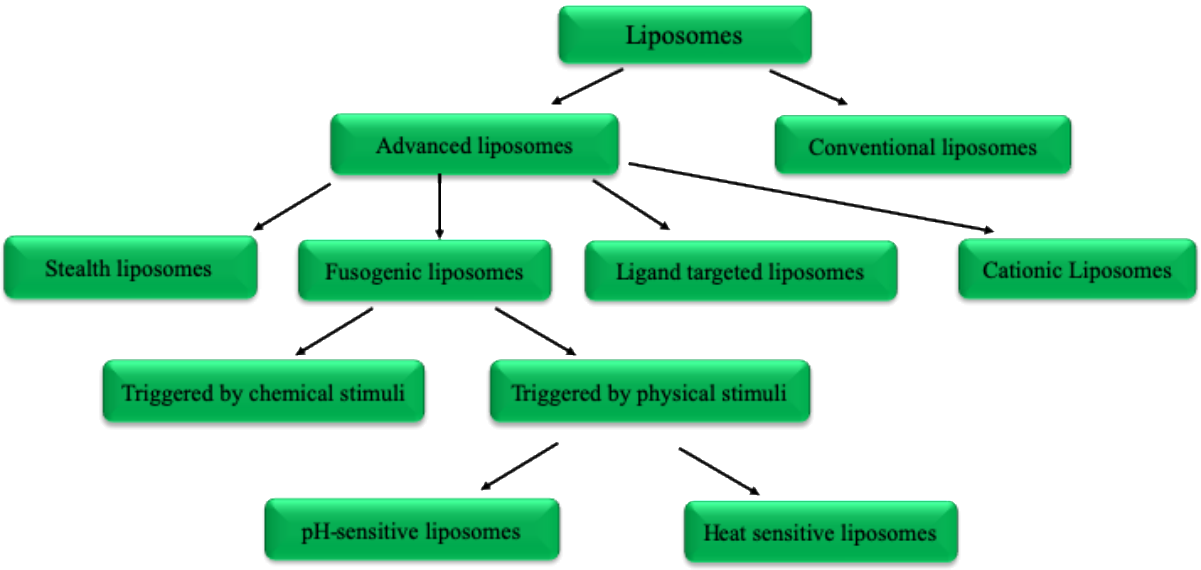


Figure 2. Classification of liposomes

3. Methods for the preparation of liposomes:

Liposomes can be prepared by a number of different methods with each method influencing the properties of the resulting liposomes. The average size, cost, reproducibility, the type of aqueous medium to form the liposomes, physicochemical properties of the components to be used and the substance to be loaded, and the toxicity of the active ingredients to be loaded are the most important factors to be considered to choose the appropriate method to prepare liposomes.[4] Liposomes are thermodynamically unstable and kinetically stable nano carriers, like nano-emulsions, that is why many of the principles and techniques used to prepare nano-emulsions can also be used to prepare liposomes.[5] The methods which are commonly used to prepare liposomes are:

3.1. Bangham's method or thin film hydration method

This method is the first method ever used to prepare the liposomes. In this method, a lipid or a mixture of lipids is dissolved in an organic solvent in a round bottom flask and then the solvent is evaporated, which results in the formation of a dry lipid film. This lipid film is then hydrated using a suitable aqueous phase with continuous shaking, which causes the formation of heterogenous multilamellar vesicles (MLVs) dispersed in the aqueous phase.[1,6] This method requires some additional techniques like sonication or extrusion (through membrane extruder), to obtain the unilamellar vesicles with homogenous size distribution. The drug can be either added to the aqueous phase (hydrophilic drug) during hydration step or it can be entrapped in the lipid film (lipophilic drug). Then encapsulated drugs in liposomes can be separated from the free drug (in the bulk, outside of liposomes), either by size exclusion column chromatography, centrifugation or by gel filtration.[7] The advantages associated with this method are: low energy input, easy and quick formation of liposomes and higher stabilities of the formulations as compared to other methods for liposome preparation. A major drawback of this method is the low encapsulation of the drugs.

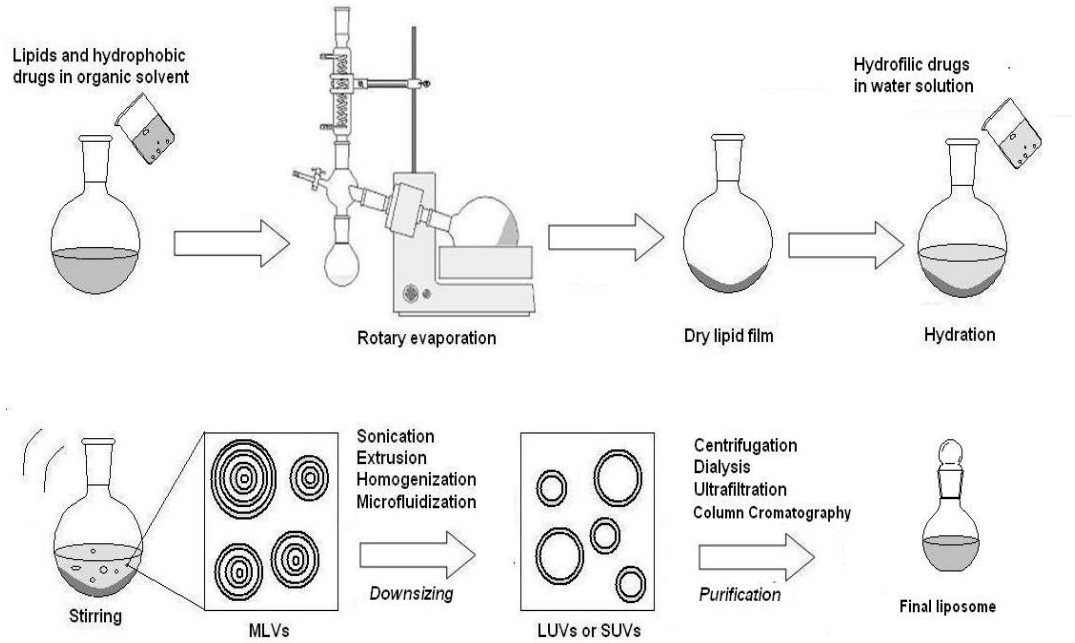


Figure 3. Preparation of liposomes by thin film hydration method coupled with sonication and extrusion[1]

3.2. Ultrasonication method

This method is a widely used to prepare homogenous dispersions of small/large unilamellar vesicles (SUVs or LUVs) from multilamellar vesicles (MLVs), prepared through the conventional thin film hydration method, by high frequency sound waves. The

principle involves the propagation of high frequency sound waves through the dispersion of MLVs, which induces cavitation phenomena (spontaneous formation and collapse of the small cavities). This produces very high pressure and turbulent flow in the proximities of the collapsing cavities. These conditions exert very strong shear forces on the MLVs causing their disruption and conversion into the smaller vesicles (SUVs). This method can be optimized to generate liposomes of the desired sizes. The two techniques used for sonication are :

a) Probe sonication technique/Direct tip sonicators

In this technique, the tip of a probe sonicator is dipped directly into the dispersion of the MLVs and a high input sonic energy is supplied, which produces smaller structures in a very short period of time (figure 4). This method is the preferred method to convert MLVs into SUVs and also result in higher encapsulation efficiencies but this technique has many drawbacks like, contamination with metal (titanium) from the probe tip, risk of exposure of the sample to high temperature resulting in the degradation of the lipids or the substance to be encapsulated, constant cooling requirements, limitation of the volume of the sample, non-homogenous size distribution and low encapsulation efficiencies etc.[5,7]

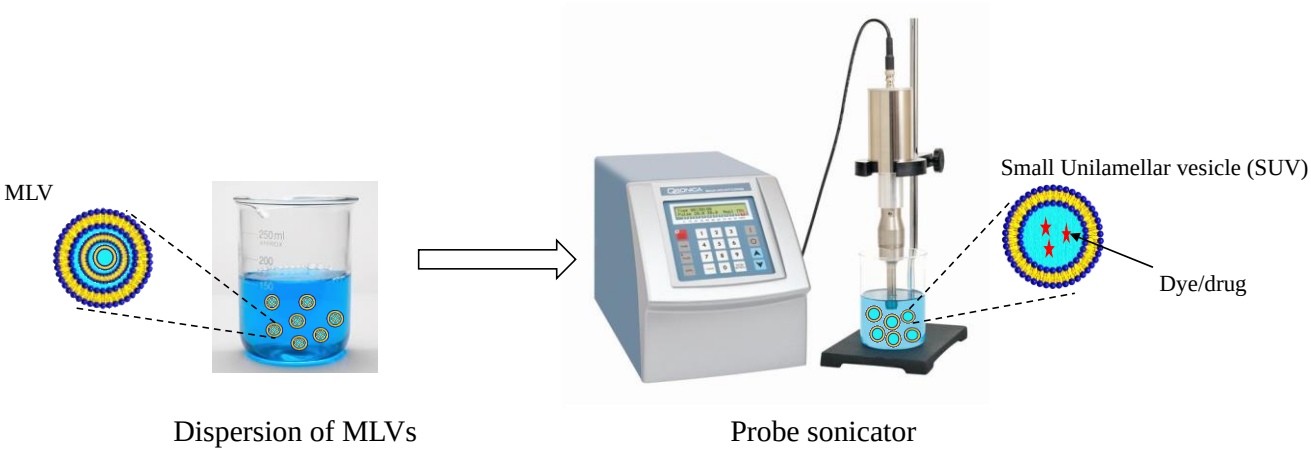


Figure 4. Preparation of SUVs by ultrasonication

b) Bath sonication technique/Indirect bath sonicators

The dispersion of the MLVs is placed in a flask or a tube and introduced into the bath sonicator and subjected to sonication for few minutes (5-15 minutes) with adjusted sonication parameters (like temperature, sonication time, level of water in the bath and the level of dispersion in the tube etc.). This method has some advantages over probe

sonication, which include higher reproducibility, more homogenous particle size distribution due to uniform distribution of the energy throughout the dispersion, capacity to accommodate more sample volume, better control over temperature of the sample, comparatively less destructive to liposomes than probe sonication and can be used in the case of thermolabile components to prevent their degradation.[5] But this method does require an extensive sonication to achieve the desired size limit and sometimes it is necessary to use another technique like centrifugation or chromatography to get rid of the larger vesicles formed along with the smaller vesicles.

3.3. Polycarbonate membrane extrusion technique

In this technique MLVs are repeatedly forced through an extruder, having a polycarbonate membrane with very small pore size (50-150 nm), by applying high pressure either manually or by using a pump. When liposome suspension is passed repeatedly through the membrane filters with high pressure, the successive lamellae are removed one by one, generating a uniform population of small unilamellar vesicles. The desired size of the liposomes can be obtained very easily using this method by selection of the appropriate pore size of the polycarbonate membranes and by optimization of the number of extrusion cycles. This method has many advantages over other methods like improved homogenization of the formed vesicles, suitable for small as well as large batches and preparation of unilamellar vesicles from MLVs in very short period of time. But there are some drawbacks as well with this method, which include possibility of leakage/loss of the sample during extrusion, temperature must be above the transition temperature of the polar lipids and limitation towards the volume of the sample (1 mL) in case of manual extruders.[5,8] The figure 5 demonstrate the assembly of a typical extruder, used for the preparation of liposomes.

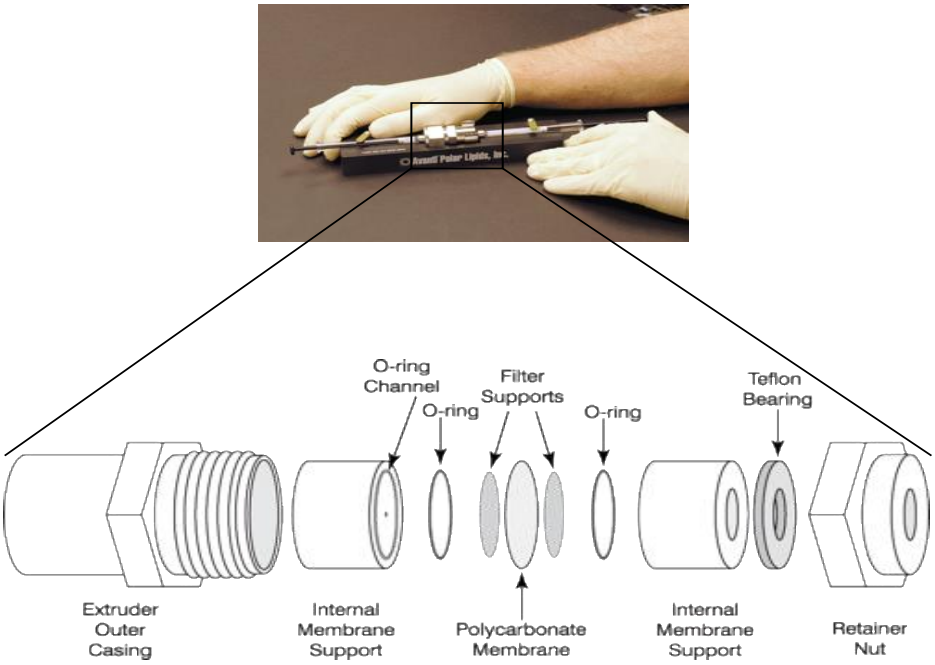


Figure 5. The Liposofast® mini-extruder assembly

3.4. High pressure homogenization technique

This technique is used to produce very small sized liposomes suitable for parenteral applications. This method has many advantages over other methods of liposome production, which include, high reproducibility, large-scale production of liposomes, processing of higher lipid concentrations etc. Following are the most commonly used high pressure homogenizers for the size reduction of liposomes.

a) *High-Pressure valve homogenizer*

The high-pressure valve homogenizer is used extensively to reduce the size of the liposomes. The pro-liposomes are dry granules and form a dispersion of liposomes when come in contact with an aqueous solution. These pro-liposomes are forced through the small orifice, known as homogenization valve, at very high pressure (10-100 MPa) which causes disruption of pro-liposomes into very small liposomes due to the cavitations and turbulence produced by the shear forces.

b) *Microfluidizer*

Microfluidizer is a special kind of high-pressure homogenizer which is used to produce small vesicles. The lipid is dissolved in a suitable organic solvent (*e.g.* ethanol) and is passed through microfluidic channels into the interaction chamber, where this stream interacts with the aqueous streams and causes the formation of liposomes due to the collisions of streams of the fluids at very high velocity and

dilution of the organic phase. The microfluidizers produce liposomes of very small size and have high efficiencies. The size of the liposomes can be controlled by regulating the pressure and the number of passes through the microfluidic channels.[9,10]

c) *Colloid mills*

These are rotor-stator systems which are generally used for size reduction of the pro-liposome dispersions in aqueous medium. The dispersion is forced through a narrow gap between rotor and stator with a high pressure. The shear forces created by the movement of the rotor and stator causes the size reduction of the dispersion. The width of the gap between rotor and the stator can be adjusted to obtain the liposomes of the desired size.

3.5. Reverse phase evaporation (REV) technique

The liposomes were first prepared by reverse-phase evaporation technique by Szoka and Papahadjopoulo.[11] In this technique, the polar lipids are dissolved in a suitable organic solvent having low boiling point (like chloroform, methanol etc.) and the substance to be encapsulated in the liposomes is dissolved in water. Then an emulsion is prepared by the addition followed by homogenization of aqueous phase into the organic phase either by vortex or by sonication. At this stage, the structure of the emulsion resembles to that of the water-in-oil microemulsion or reverse micellar system, with hydrophilic groups of the lipids attached with the aqueous droplets and the hydrophobic chains spreading towards the organic solvent. The organic solvent is then evaporated under reduced pressure followed by vigorous agitation, which causes the conversion of the water-in-oil type system, through the formation of a viscous gel, to the aqueous dispersion of the vesicles. This technique has been widely used to encapsulate small as well as macromolecules like enzymes and RNA. High encapsulation efficiencies are obtained with this method; however, it has some drawback as well, like heterogenous size distribution of the vesicles (MLVs and ULVs), requires additional extrusion step to generate smaller vesicles with uniform size distribution and the breakage and denaturation of the biological molecules like RNA, DNA, enzymes etc. due to the exposure to organic solvent and mechanical agitation.[3,5,12]

3.6. Solvent injection method

The lipid is first dissolved in an organic solvent and is then injected into the aqueous phase containing the drug/substance to be encapsulated, which causes formation of

liposomes. This method is generally classified into two types, based on the nature of the organic solvent used.

a) *Ethanol injection method*

This method, first described by Batzri and Korn[13], involves the dissolution of the lipid in ethanol, followed by injection of this solution into the aqueous solution of substance to be encapsulated, which at once causes formation of liposomes (MLVs) with uniform size distribution. This method has advantages over ether injection method, like reproducibility, no requirement of high temperature to get rid of the solvent and is simple and easy to perform. However, this method does have some drawbacks, like heterogenous population of liposomes in case of lipids having poor solubility in ethanol, impossible to remove ethanol completely and the risk of denaturation of biological molecules due to presence of ethanol.[14] The modern and improved versions of this method are the ink-jet method, developed by Hauschild *et al*[15], and crossflow injection method, developed by Wagner et al[16], these methods have excellent scale-up potential with great control over the particle size distribution.

b) *Ether injection method*

In this method, the lipid is dissolved in diethyl ether or sometimes mixture of ether and methanol, and then injected gradually into the aqueous solution of the substance to be encapsulated at high temperature (50-60°C) or reduced pressure or sometimes both, to remove ether (water immiscible) from the system. The removal of ether causes the formation of concentrated liposomal dispersion. This method has the advantage of high encapsulation efficiencies over ethanol injection method. However, there are some limitations of this method, like heterogenous particle size distribution and the exposure of fragile substances to organic solvents and high temperatures.[14,17]

3.7. Detergent removal method

In this method of liposome production, a micellar mixture of lipids and a suitable detergent (cationic, anionic or non-ionic) is prepared in an aqueous medium containing the substance to be encapsulated, and then the detergent is removed from this preparation. The removal of the detergent helps the micelles progressively become richer in lipid content and transform initially into a mixed detergent-lipid vesicle structure and then finally into detergent free bilayer structures/vesicles upon complete removal of the detergent[18]. The methods which are most commonly used to remove the detergent from the liposome preparation are dialysis, column chromatography, dilution and adsorption

onto the beads. The advantages associated with this method are, uniform distribution of the size, simple design, and can be used to encapsulate biological compounds (like proteins) without risk of denaturation. The limitations of this method are, low encapsulation efficiencies, very time consuming process and possibility of having some detergent residues in the system.[5,12]

4. Drug loading in liposomes:

The drugs or other substances like proteins, enzymes etc. can be loaded into the liposomes by two different techniques, either during the formation of liposomes (called as passive loading) or into the intact liposomes, after their formation (known as active loading). The selection of the loading technique is related to different parameters which include, concentration of the lipid, drug/lipid ratio, liposome stability, encapsulation efficiency, stability of the drugs, production cost, potential for scale-up and retention of drugs inside the liposomes.

4.1. Passive loading

The passive loading involves the loading of the drug during the preparation of liposomes. This technique can be used to encapsulate hydrophilic, lipophilic as well as amphiphilic drugs inside the liposomes. In all the methods of liposome preparation, described above, drugs can be dissolved either in the aqueous medium (hydrophilic drugs) or in the lipidic solutions in organic solvents (hydrophobic drugs). During the process of the liposome production, the hydrophilic drugs are encapsulated inside the core of liposomes, i.e., in the aqueous phase, while hydrophobic drugs are entrapped in the lipidic bilayer of liposomes. The amphiphilic drugs are entrapped in the liposomes in such a way that the hydrophilic part of the drugs stays in the aqueous phase while the hydrophobic part gets entrapped in the lipidic bilayers. For hydrophilic drugs, the encapsulation efficiency depends on the volume of aqueous medium enclosed by the liposomes, the concentration of the phospholipids, hydrophobic chain length of the phospholipids (higher loading with phospholipids having shorter chain lengths, due to decreased membrane thickness and increased drug penetration[19]) as well as on the morphology and lamellarity of the vesicles[12]. Another important factor which affects the loading efficiencies is the permeability of the lipid bilayer, which is more permeable in liquid crystalline phase as compared to the gel state.[20] The addition of cholesterol into the bilayers of the liposomes exerts strong impact on the organization, functioning and the dynamics of the membrane. The cholesterol reduces the free rotation of phospholipid chains, contributing to the

stability of the lipid bilayer as well as retention of the hydrophilic drugs inside liposomes and on the other hand it increases the entrapped volume of the liposomes by increasing the size of the liposomes, both these factors help in improving the hydrophilic drug loading.[21–23] The hydrophobic drugs can be very efficiently loaded into the liposomes by this technique with higher encapsulation efficiencies because of their embedding in the lipid bilayer during self-assembly of liposomes. The loading efficiency depends mainly on the membrane composition, *e.g.*, the addition of cholesterol in the membrane displaces the lipophilic dye and therefore decreases the loading of the hydrophobic drugs.[24,25] A schematic representation of the passive loading by thin film rehydration method (coupled with extrusion) used by Rehman *et al.*[6] for the encapsulation of the dye/drug is given in the figure 6.

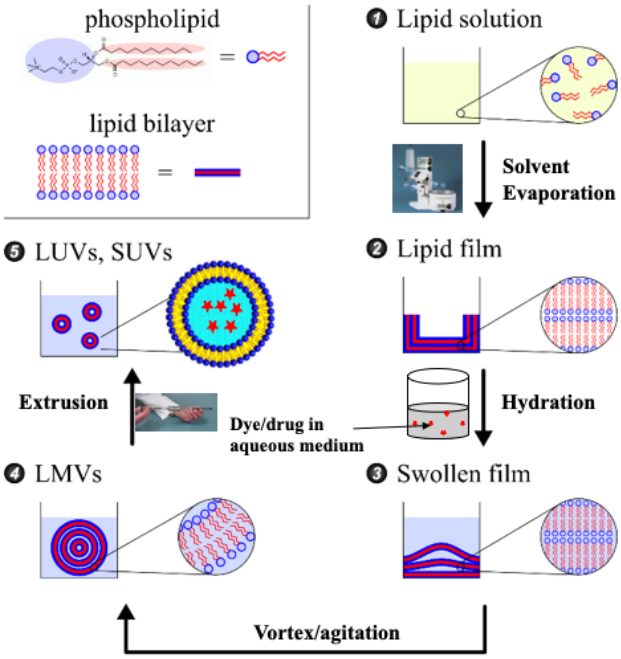


Figure 6. Schematic representation of preparation of liposomes by thin film hydration method, involving passive loading of the dye/drug

4.2. Active loading

This technique is also called as remote loading and involves the encapsulation of the drugs into the intact liposomes, *i.e.* the empty liposomes are first formed followed by the addition of the drug into the liposome dispersion, either directly or its concentrated solution. The active loading technique has some advantages over passive loading technique, like higher drug loading and no risk of denaturation of substance to be encapsulated. The loading of the drugs depends on different parameters based on their solubility. The penetration of hydrophobic drugs in the liposomes depends on the nature

of the lipid and the packing restrictions of the bilayer of the liposomes. The hydrophilic drugs interact with polar head groups of the phospholipids and are captured and retained by the liposomes. But the amphiphilic drugs cannot be loaded and retained into the liposomes by active loading technique because such drugs permeate rapidly through the lipid bilayers[26]. However, this method is restricted only to a very small group of drugs that can penetrate the lipid bilayers only in uncharged form, i.e., weak amphiphilic acids or bases. The penetration of the drugs inside the liposomes is triggered and controlled by one of these mechanisms, concentration gradient, pH-gradient or ion gradient.

In the case of loading of drugs caused by concentration gradient, the concentrated solution of drug is added into the liposome dispersion and during the incubation period the drug diffuses through the lipidic bilayers into the liposomes and this process continues until the equilibrium condition is attained.

The pH-gradient method has been evolved as a very efficient method for loading of the drugs. This method involves the preparation of the liposomes in a solution having certain pH and then the pH of the solution outside the liposomes is increased either by addition of an alkaline solution or by exchanging the external solution with another solution having desired pH by using column chromatography, gel filtration or dialysis.[27,28] This pH imbalance serves as a driving force for drug loading. The mechanism of drug loading involves diffusion of the drug (in uncharged state) through the lipid bilayer and once it goes inside the liposomes it gets protonated and entrapped inside due to inability of the charged state to diffuse through the bilayer of the liposomes. A group of amine-containing drugs, like doxorubicin, daunorubicin, dopamine, quinidine, serotonin, diphenhydramine, chloroquine etc., can be successfully loaded into the liposomes by using this transmembrane pH-gradient.

Another technique of remote loading of the drugs makes use of ammonium sulphate gradient, which is very efficient method and provides very high encapsulation efficiencies. This method was developed by Barenholz *et al* [29] to enhance the loading of doxorubicin (DOX) into the liposomes, which has proven to be a breakthrough in the field of liposomal drug loading. This method has led to development of the first nano drug "Doxil®", approved by FDA.[30] The overall procedure they adapted for the loading of the DOX is shown in the figure 7 and the driving force for the loading of DOX into the liposomes is the transmembrane ammonium sulphate gradient, because the DOX, being the weak base and present in the outer solution, gets exchanged with the ammonium ions present inside the liposomes. And once the drug goes inside it gets precipitated as a

sulphate salt. This method is advantageous over pH-gradient method as it does not require the fabrication of the liposomes at acidic pH and there is no need to alkalinize the aqueous phase outside the liposomes.

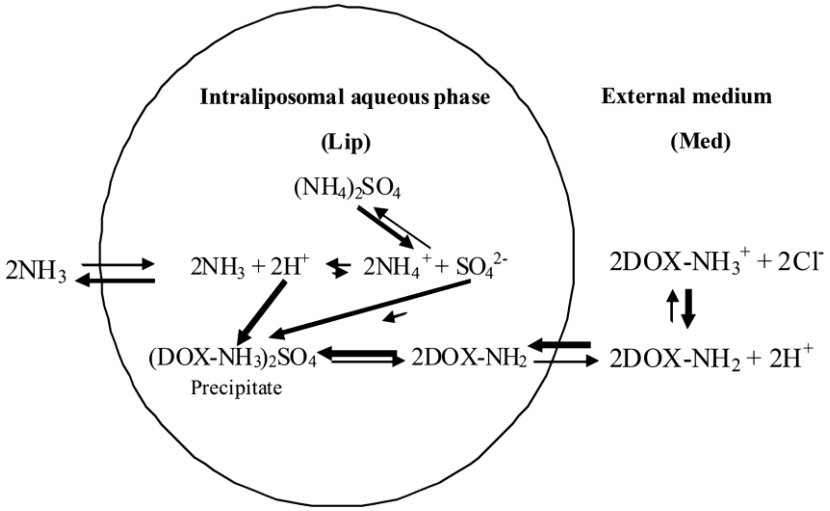


Figure 7. Doxorubicin remote loading by a transmembrane ammonium ion gradient, the bold arrows (➡) represent the processes which occur during drug loading, while simple arrows (→) represent the processes which occur during drug release.

After the discovery of this method, different ammonium salts have been used to load the weak bases actively into the liposomes, like citrate, phosphate and acetate, and there are studies being done to explore the drug loading potential of sodium salts (like phosphate, citrate, acetate and sulphate) as well.[31]

5. Applications of liposomes:

There has been considerable progress in the development of suitable dosage forms for the treatment of the diseases in recent years, however the constraints in the diagnosis and treatment, like low sensitivity, toxicity of drug, side effects etc., have yet to be dealt with. And continuous efforts have been put in to solve these problems, like development of nano carriers for drug delivery. And among different available drug carriers, liposomes have gained importance as the most advanced particulate drug carriers due to their capacity to encapsulate lipophilic, hydrophilic as well as amphiphilic drugs and also because of their biodegradability and biocompatibility.[6] Since their discovery in the 1960s, many drugs, enzymes, peptide hormones, genetic materials, vaccines, chelating agents etc. have been successfully incorporated into the inner aqueous compartment or in the lipid bilayers of liposomes. Following are some of the major applications of the liposomes as drug delivery vehicles.

5.1. Oral drug delivery

Since the discovery of liposomes, there has been growing investigations on the liposomal delivery of drug by oral route. Initially it did not work out for the researchers to develop oral liposomal formulations due to a number of constraints like poor understanding of absorption mechanisms, stability of the liposomes in the gastrointestinal tract, variations from one batch to another causing unsatisfactory mass productions etc.[32] However recent studies, with advanced manufacturing and modification techniques, have overcome these challenges by the application of the polymer coatings[33,34] or by modulating the composition of the liposomes[35,36] and have shown that the liposomes have great potential to be used as oral drug delivery systems, *e.g.* for insulin, bile salts, calcitonin, proteins, peptides etc.[4,37]

5.2. Systemic drug delivery

This is the most widely used route of drug administration for the liposomal formulations. As soon as they come in contact with the blood circulation, after systemic (mostly intravenous) administration, the liposomes are recognized and endocytosed by mononuclear phagocytic cells (mostly kupfer cells) in spleen and liver. The liposomes can be used as for the delivery of drugs into these cells. However, to prevent this phagocytosis of the liposomes, they are stabilized sterically, most commonly by incorporation of PEG in the bilayer, which results in the increased accumulation of these liposomes in the trauma sites, like tumors, inflammation or infection. The accumulation of the liposomes in these sites is due to the prolonged circulation caused by the steric stabilization or stealth properties.[38] Liposomes have been developed to protect the drug (*e.g.* antibiotics sensitive to β-lactamase), whilst in blood circulation, against the enzymatic degradation. Liposomes are the excellent candidates for the targeted drug delivery especially in cancer treatment, for example Doxil® is a FDA approved commercially available liposomal formulation of doxorubicin.[30] Liposomes are also very useful for the delivery of immunotherapeutic agents[39] and to increase the drug solubilization, to make a complex with DNA to transfect the cells for gene therapy.[40]

5.3. Topical drug delivery

The liposomes have all the properties needed for an ideal drug carrier for topical applications due to the similarities between the natural membranes and the lipid bilayer of the liposomes (e.g., their ability to fuse with the cell membrane, to alter fluidity of cell

membrane etc.[2]) and due to their ability to penetrate the skin. The properties and composition of the liposomes have an important role in their penetration through the skin. A wide range of liposomal formulations have been developed for dermal as well as transdermal applications, *e.g.* liposomal preparation as non-invasive DNA vaccine[41], for restoring and moisturizing the skin[42], to reduce the adverse effects associated with corticosteroid therapy[43], antiviral therapy[44] etc.

5.4. Pulmonary drug delivery

The liposomes have been widely used pulmonary drug delivery and the liposomal formulations have numerous advantages over other pulmonary drug delivery systems, like no local irritation, sustained release, reduced toxicity and improved stability[45]. The liposomes are perfect candidates as pulmonary delivery vehicles for anticancer drugs[46], antimicrobial agents[47], steroids[48], DNA, insulin[49], proteins[50] etc.

5.5. Brain-targeted drug delivery

Liposomes have been studied and used for drug delivery to the brain due to their biocompatibility, biodegradability and reduced-toxicity. The liposomal formulations increase the permeability of drugs through the blood brain barrier and also provide targeted drug delivery, due to the possibility of grafting small ligand molecules, monoclonal antibodies or peptides on the surface of the liposomes, for the treatment of brain tumor (glioma).[51,52]

5.6. Combination therapy

The combination therapy provides the increased therapeutic outcomes as compared to the individual outcomes. A number of different liposomal combination therapies have been developed in the recent years using different strategies, including: combination of therapeutic agents to overcome multidrug resistance[53], combination of two different drugs targeted against two different antigens to enhance the therapeutic efficiency[54] or combination of ligand targeted delivery with a remotely triggered method in order to enhance the transfection efficiency.[55]

5.7. In cosmetics

The liposomes have found many applications in cosmetics due to the lipid component in their composition. The lipids are generally well hydrated, can humidify the skin and also

improve its elasticity which will reduce dryness of skin thus preventing it from ageing. The first marketed cosmetic liposomal product, "Capture" was launched in 1986 by a French company "Christian Dior", as an anti-ageing cream. Since then, several liposomal cosmetic products have been marketed including creams, ointments, gels, moisturizers, perfumes, aftershaves, hair conditioners, anti-hair fall, lipsticks, etc.

5.8. In biomedical imaging

Liposomes have widespread applications in many medical imaging techniques, including;

- Fluorescence imaging: Cancer imaging by DOPC-supported functionalized liposomes having PEG-coated quantum dots (QDs) inside the aqueous phase.[56]
- Magnetic resonance imaging (MRI): Liposomes have shown the capacity to load a wide range of MRI contrast agents and provide controlled and effective delivery of these MRI probes for improved imaging.[57,58]
- Ultrasound imaging: Acoustic liposomes (ALs), the liposomes containing perfluoro propane gas, serve as efficient ultrasound imaging probes.[59]
- Nuclear imaging: A large number of liposomal formulations encapsulating radionuclide tracers either inside the aqueous compartment or within a chemically engineered lipid bilayer have been reported.[60,61]

6. Commercially available products:

As mentioned above in the applications, the liposomes can be used as carrier for the delivery of a wide variety of drugs such as analgesics, steroids, anesthetics, chemotherapeutics and tumor vaccines. A few examples of the marketed liposomal products are listed in the Table 1.

Table 1: Commercially available liposomal products

Drug	Route of administration	Indication	Marketed name	Year of approval	Manufacturer
Inactivated hepatitis A virus (strain RGSB)	Intramuscular	Hepatitis A	Epaxal®	1993	Crucell, Berna Biotech
Amphotericin B	Intravenous	Fungal infections	Abelcet®	1995	Sigma-Tau Pharmaceuticals
Doxorubicin	Intravenous	Ovarian cancer, breast cancer, Kaposi's sarcoma	Doxil®	1995	Sequus Pharmaceuticals
Daunorubicin	Intravenous	AIDS-related Kaposi's sarcoma	DaunoXome®	1996	NeXstar Pharmaceuticals

Amphotericin B	Intravenous	Severe fungal infections	Amphotec®	1996	Ben Venue Laboratories Inc.
Amphotericin B	Intravenous	Presumed fungal infections	Ambisome®	1997	Astellas Pharma
Inactivated hemaglutinine of Influenza virus strains A and B	Intramuscular	Influenza	Inflexal® V	1997	Crucell, Berna Biotech
Cytarabine/Ara-C	Spinal	Neoplastic meningitis	Depocyt®	1999	Sky Pharma Inc.
Verteporfin	Intravenous	Choroidal neovascularisation	Visudyne®	2000	Novartis
Doxorubicin	Intravenous	Combination therapy with cyclophosphamide in metastatic breast cancer	Myocet®	2000	Elan Pharmaceuticals
Morphine sulfate	Epidural	Pain management	DepoDur™	2004	Sky Pharma Inc.
Mifamurtide	Intravenous	High-grade, resectable, non-metastatic osteosarcoma	Mepact®	2004	Takeda Pharmaceutical Limited
Bupivacaine	Intravenous	Pain management	Exparel®	2011	Pacira Pharmaceuticals, Inc.
Vincristine	Intravenous	Acute lymphoblastic leukaemia	Marqibo®	2012	Talon Therapeutics, Inc.
Irinotecan	Intravenous	Metastatic adenocarcinoma of the pancreas	Onivyde™	2015	Merrimack Pharmaceuticals Inc.

7. References:

(1) Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids. *Journal of Molecular Biology* **1965**, *13* (1), 238-IN27. https://doi.org/10.1016/S0022-2836(65)80093-6.

(2) Laouini, A.; Jaafar-Maalej, C.; Limayem-Blouza, I.; Sfar, S.; Charcosset, C.; Fessi, H. Preparation, Characterization and Applications of Liposomes: State of the Art. *Journal of Colloid Science and Biotechnology* **2012**, *1* (2), 147–168. https://doi.org/10.1166/jcsb.2012.1020.

(3) Pattni, B. S.; Chupin, V. V.; Torchilin, V. P. New Developments in Liposomal Drug Delivery. *Chemical Reviews* **2015**, *115* (19), 10938–10966. https://doi.org/10.1021/acs.chemrev.5b00046.

(4) Bozzuto, G.; Molinari, A. Liposomes as Nanomedical Devices. *International Journal of Nanomedicine* **2015**, 975. https://doi.org/10.2147/IJN.S68861.

(5) Taylor, T. M.; Weiss, J.; Davidson, P. M.; Bruce, B. D. Liposomal Nanocapsules in Food Science and Agriculture. *Critical Reviews in Food Science and Nutrition* **2005**, *45* (7–8), 587–605. https://doi.org/10.1080/10408390591001135.

(6) Rehman, A. U.; Omran, Z.; Anton, H.; Mély, Y.; Akram, S.; Vandamme, T. F.; Anton, N. Development of Doxorubicin Hydrochloride Loaded PH-Sensitive Liposomes: Investigation on the Impact of Chemical Nature of Lipids and Liposome Composition on PH-Sensitivity. *European Journal of Pharmaceutics and Biopharmaceutics* **2018**, *133*, 331–338. https://doi.org/10.1016/j.ejpb.2018.11.001.

(7) Uhumwangho, M.; Okor, R. Current Trends in the Production and Biomedical Applications of Liposomes: A Review. *Journal of Medicine and Biomedical Research* **2009**, *4* (1). https://doi.org/10.4314/jmbr.v4i1.10663.

(8) Mui, B.; Chow, L.; Hope, M. J. Extrusion Technique to Generate Liposomes of Defined Size. In *Methods in Enzymology*; Elsevier, 2003; Vol. 367, pp 3–14. https://doi.org/10.1016/S0076-6879(03)67001-1.

(9) Barnadas-Rodríguez, R.; Sabés, M. Factors Involved in the Production of Liposomes with a High-Pressure Homogenizer. *International Journal of Pharmaceutics* **2001**, *213* (1–2), 175–186. https://doi.org/10.1016/S0378-5173(00)00661-X.

(10) Jahn, A.; Vreeland, W. N.; DeVoe, D. L.; Locascio, L. E.; Gaitan, M. Microfluidic Directed Formation of Liposomes of Controlled Size. *Langmuir* **2007**, *23* (11), 6289–6293. https://doi.org/10.1021/la070051a.

(11) Szoka, F.; Papahadjopoulos, D. Procedure for Preparation of Liposomes with Large Internal Aqueous Space and High Capture by Reverse-Phase Evaporation. *Proceedings of the National Academy of Sciences* **1978**, *75* (9), 4194–4198. https://doi.org/10.1073/pnas.75.9.4194.

(12) Maherani, B.; Arab-Tehrany, E.; R. Mozafari, M.; Gaiani, C.; Linder, M. Liposomes: A Review of Manufacturing Techniques and Targeting Strategies. *Current Nanoscience* **2011**, *7* (3), 436–452. https://doi.org/10.2174/157341311795542453.

(13) Batzri, S.; Korn, E. D. Single Bilayer Liposomes Prepared without Sonication. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1973**, *298* (4), 1015–1019. https://doi.org/10.1016/0005-2736(73)90408-2.

(14) Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S. W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: Classification, Preparation, and Applications. *Nanoscale Research Letters* **2013**, *8* (1). https://doi.org/10.1186/1556-276X-8-102.
(15) Hauschild, S.; Lipprandt, U.; Rumplecker, A.; Borchert, U.; Rank, A.; Schubert, R.; Förster, S. Direct Preparation and Loading of Lipid and Polymer Vesicles Using Inkjets. *Small* **2005**, *1* (12), 1177–1180. https://doi.org/10.1002/smll.200500093.
(16) Wagner, A.; Vorauer-Uhl, K.; Kreismayr, G.; Katinger, H. THE CROSSFLOW INJECTION TECHNIQUE: AN IMPROVEMENT OF THE ETHANOL INJECTION METHOD. *Journal of Liposome Research* **2002**, *12* (3), 259–270. https://doi.org/10.1081/LPR-120014761.
(17) Schieren, H.; Rudolph, S.; Finkelstein, M.; Coleman, P.; Weissmann, G. Comparison of Large Unilamellar Vesicles Prepared by a Petroleum Ether Vaporization Method with Multilamellar Vesicles. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1978**, *542* (1), 137–153. https://doi.org/10.1016/0304-4165(78)90240-4.
(18) Schubert, R. Liposome Preparation by Detergent Removal. In *Methods in Enzymology*; Elsevier, 2003; Vol. 367, pp 46–70. https://doi.org/10.1016/S0076-6879(03)67005-9.
(19) Chaudhury, A.; Das, S.; Lee, R. F. S.; Tan, K.-B.; Ng, W.-K.; Tan, R. B. H.; Chiu, G. N. C. Lyophilization of Cholesterol-Free PEGylated Liposomes and Its Impact on Drug Loading by Passive Equilibration. *International Journal of Pharmaceutics* **2012**, *430* (1–2), 167–175. https://doi.org/10.1016/j.ijpharm.2012.04.036.
(20) Eloy, J. O.; Claro de Souza, M.; Petrilli, R.; Barcellos, J. P. A.; Lee, R. J.; Marchetti, J. M. Liposomes as Carriers of Hydrophilic Small Molecule Drugs: Strategies to Enhance Encapsulation and Delivery. *Colloids and Surfaces B: Biointerfaces* **2014**, *123*, 345–363. https://doi.org/10.1016/j.colsurfb.2014.09.029.
(21) Taylor, K. M. G.; Taylor, G.; Kellaway, I. W.; Stevens, J. Drug Entrapment and Release from Multilamellar and Reverse-Phase Evaporation Liposomes. *International Journal of Pharmaceutics* **1990**, *58* (1), 49–55. https://doi.org/10.1016/0378-5173(90)90286-D.
(22) Manojlovic, V.; Winkler, K.; Bunjes, V.; Neub, A.; Schubert, R.; Bugarski, B.; Leneweit, G. Membrane Interactions of Ternary Phospholipid/Cholesterol Bilayers and Encapsulation Efficiencies of a RIP II Protein. *Colloids and Surfaces B: Biointerfaces* **2008**, *64* (2), 284–296. https://doi.org/10.1016/j.colsurfb.2008.02.001.
(23) Glavas-Dodov, M.; Fredro-Kumbaradzi, E.; Goracinova, K.; Simonoska, M.; Calis, S.; Trajkovic-Jolevska, S.; Hincal, A. A. The Effects of Lyophilization on the Stability of Liposomes Containing 5-FU. *International Journal of Pharmaceutics* **2005**, *291* (1–2), 79–86. https://doi.org/10.1016/j.ijpharm.2004.07.045.
(24) Tsotas, V.-A.; Mourtas, S.; Antimisiaris, S. G. Dexamethasone Incorporating Liposomes: Effect of Lipid Composition on Drug Trapping Efficiency and Vesicle Stability. *Drug Delivery* **2007**, *14* (7), 441–445. https://doi.org/10.1080/10717540701603530.
(25) Bhardwaj, U.; Burgess, D. J. Physicochemical Properties of Extruded and Non-Extruded Liposomes Containing the Hydrophobic Drug Dexamethasone. *International Journal of Pharmaceutics* **2010**, *388* (1–2), 181–189.

https://doi.org/10.1016/j.ijpharm.2010.01.003.
(26) Mayer, L. D.; Bally, M. B.; Hope, M. J.; Cullis, P. R. Techniques for Encapsulating Bioactive Agents into Liposomes. *Chemistry and Physics of Lipids* **1986**, *40* (2–4), 333–345. https://doi.org/10.1016/0009-3084(86)90077-0.
(27) Ishida, T.; Takanashi, Y.; Doi, H.; Yamamoto, I.; Kiwada, H. Encapsulation of an Antivasospastic Drug, Fasudil, into Liposomes, and in Vitro Stability of the Fasudil-Loaded Liposomes. *International Journal of Pharmaceutics* **2002**, *232* (1–2), 59–67. https://doi.org/10.1016/S0378-5173(01)00896-1.
(28) Hwang, S.; Maitani, Y.; Qi, X.-R.; Takayama, K.; Nagai, T. Remote Loading of Diclofenac, Insulin and Fluorescein Isothiocyanate Labeled Insulin into Liposomes by PH and Acetate Gradient Methods. *International Journal of Pharmaceutics* **1999**, *179* (1), 85–95. https://doi.org/10.1016/S0378-5173(98)00392-5.
(29) Haran, G.; Cohen, R.; Bar, L. K.; Barenholz, Y. Transmembrane Ammonium Sulfate Gradients in Liposomes Produce Efficient and Stable Entrapment of Amphipathic Weak Bases. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1993**, *1151* (2), 201–215. https://doi.org/10.1016/0005-2736(93)90105-9.
(30) Barenholz, Y. (Chezy). Doxil® — The First FDA-Approved Nano-Drug: Lessons Learned. *Journal of Controlled Release* **2012**, *160* (2), 117–134. https://doi.org/10.1016/j.jconrel.2012.03.020.
(31) Fritze, A.; Hens, F.; Kimpfler, A.; Schubert, R.; Peschka-Süss, R. Remote Loading of Doxorubicin into Liposomes Driven by a Transmembrane Phosphate Gradient. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2006**, *1758* (10), 1633–1640. https://doi.org/10.1016/j.bbamem.2006.05.028.
(32) He, H.; Lu, Y.; Qi, J.; Zhu, Q.; Chen, Z.; Wu, W. Adapting Liposomes for Oral Drug Delivery. *Acta Pharmaceutica Sinica B* **2019**, *9* (1), 36–48. https://doi.org/10.1016/j.apsb.2018.06.005.
(33) Thirawong, N.; Thongborisute, J.; Takeuchi, H.; Sriamornsak, P. Improved Intestinal Absorption of Calcitonin by Mucoadhesive Delivery of Novel Pectin–Liposome Nanocomplexes. *Journal of Controlled Release* **2008**, *125* (3), 236–245. https://doi.org/10.1016/j.jconrel.2007.10.023.
(34) Takeuchi, H.; Matsui, Y.; Yamamoto, H.; Kawashima, Y. Mucoadhesive Properties of Carbopol or Chitosan-Coated Liposomes and Their Effectiveness in the Oral Administration of Calcitonin to Rats. *Journal of Controlled Release* **2003**, *86* (2–3), 235–242. https://doi.org/10.1016/S0168-3659(02)00411-X.
(35) Conacher, M.; Alexander, J.; Brewer, J. M. Oral Immunisation with Peptide and Protein Antigens by Formulation in Lipid Vesicles Incorporating Bile Salts (Bilosomes). *Vaccine* **2001**, *19* (20–22), 2965–2974. https://doi.org/10.1016/S0264-410X(00)00537-5.
(36) Niu, M.; Lu, Y.; Hovgaard, L.; Guan, P.; Tan, Y.; Lian, R.; Qi, J.; Wu, W. Hypoglycemic Activity and Oral Bioavailability of Insulin-Loaded Liposomes Containing Bile Salts in Rats: The Effect of Cholate Type, Particle Size and Administered Dose. *European Journal of Pharmaceutics and Biopharmaceutics* **2012**, *81* (2), 265–272. https://doi.org/10.1016/j.ejpb.2012.02.009.
(37) Yamano, E.; Miyauchi, M.; Furusyo, H.; Kawazoe, A.; Ishikado, A.; Makino, T.; Tanne, K.; Tanaka, E.; Takata, T. Inhibitory Effects of Orally Administrated Liposomal

Bovine Lactoferrin on the LPS-Induced Osteoclastogenesis. *Laboratory Investigation* **2010**, *90* (8), 1236–1246. https://doi.org/10.1038/labinvest.2010.80.
(38) Jain, R. K. Delivery of Molecular and Cellular Medicine to Solid Tumors1PII of Original Article: S0169-409X(97)00027-6. The Article Was Originally Published in Advanced Drug Delivery Reviews 26 (1997) 71–90.1. *Advanced Drug Delivery Reviews* **2001**, *46* (1–3), 149–168. https://doi.org/10.1016/S0169-409X(00)00131-9.
(39) Sullivan, S. M.; Gieseler, R. K. H.; Lenzner, S.; Ruppert, J.; Gabrysiak, T. G.; Peters, J. H.; Cox, G.; Richer, L.; Martin, W. J.; Scolaro, M. J. Inhibition of Human Immunodeficiency Virus-1 Proliferation by Liposome-Encapsulated Sense DNA to the 5′ *TAT* Splice Acceptor Site. *Antisense Research and Development* **1992**, *2* (3), 187–197. https://doi.org/10.1089/ard.1992.2.187.
(40) Lasic, D. Novel Applications of Liposomes. *Trends in Biotechnology* **1998**, *16* (7), 307–321. https://doi.org/10.1016/S0167-7799(98)01220-7.
(41) Shi, Z.; Curiel, D. T.; Tang, D. DNA-Based Non-Invasive Vaccination onto the Skin. *Vaccine* **1999**, *17* (17), 2136–2141. https://doi.org/10.1016/S0264-410X(98)00488-5.
(42) Betz, G.; Aeppli, A.; Menshutina, N.; Leuenberger, H. In Vivo Comparison of Various Liposome Formulations for Cosmetic Application☆. *International Journal of Pharmaceutics* **2005**, *296* (1–2), 44–54. https://doi.org/10.1016/j.ijpharm.2005.02.032.
(43) Mezei, M.; Gulasekharam, V. Liposomes - a Selective Drug Delivery System for the Topical Route of Administration I. Lotion Dosage Form. *Life Sciences* **1980**, *26* (18), 1473–1477. https://doi.org/10.1016/0024-3205(80)90268-4.
(44) Horwitz, E.; Pisanty, S.; Czerninski, R.; Helser, M.; Eliav, E.; Touitou, E. A Clinical Evaluation of a Novel Liposomal Carrier for Acyclovir in the Topical Treatment of Recurrent Herpes Labialis. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **1999**, *87* (6), 700–705. https://doi.org/10.1016/S1079-2104(99)70164-2.
(45) Rudokas, M.; Najlah, M.; Alhnan, M. A.; Elhissi, A. Liposome Delivery Systems for Inhalation: A Critical Review Highlighting Formulation Issues and Anticancer Applications. *Medical Principles and Practice* **2016**, *25* (2), 60–72. https://doi.org/10.1159/000445116.
(46) Verschraegen, C. F. Clinical Evaluation of the Delivery and Safety of Aerosolized Liposomal 9-Nitro-20(S)-Camptothecin in Patients with Advanced Pulmonary Malignancies. *Clinical Cancer Research* **2004**, *10* (7), 2319–2326. https://doi.org/10.1158/1078-0432.CCR-0929-3.
(47) Corcoran, T. E.; Venkataramanan, R.; Mihelc, K. M.; Marcinkowski, A. L.; Ou, J.; McCook, B. M.; Weber, L.; Carey, M.-E.; Paterson, D. L.; Pilewski, J. M.; et al. Aerosol Deposition of Lipid Complex Amphotericin-B (Abelcet) in Lung Transplant Recipients. *American Journal of Transplantation* **2006**, *6* (11), 2765–2773. https://doi.org/10.1111/j.1600-6143.2006.01529.x.
(48) Waldrep, J. C.; Scherer, P. W.; Hess, G. D.; Black, M.; Knight, V. Nebulized Glucocorticoids in Liposomes: Aerosol Characteristics and Human Dose Estimates. *Journal of Aerosol Medicine* **1994**, *7* (2), 135–145. https://doi.org/10.1089/jam.1994.7.135.
(49) Jendle, J.; Karlberg, B. E.; Persliden, J.; Franzén, L.; Arborelius, M. Delivery and Retention of an Insulin Aerosol Produced by a New Jet Nebulizer. *Journal of Aerosol Medicine* **1995**, *8* (3), 243–254. https://doi.org/10.1089/jam.1995.8.243.
(50) Ten, R. .; Anderson, P. .; Zein, N. .; Temesgen, Z.; Clawson, M. L.; Weiss, W.

Interleukin-2 Liposomes for Primary Immune Deficiency Using the Aerosol Route. *International Immunopharmacology* **2002**, *2* (2–3), 333–344. https://doi.org/10.1016/S1567-5769(01)00143-6.
(51) Peng, Y.; Zhao, Y.; Chen, Y.; Yang, Z.; Zhang, L.; Xiao, W.; Yang, J.; Guo, L.; Wu, Y. Dual-Targeting for Brain-Specific Liposomes Drug Delivery System: Synthesis and Preliminary Evaluation. *Bioorganic & Medicinal Chemistry* **2018**, *26* (16), 4677–4686. https://doi.org/10.1016/j.bmc.2018.08.006.
(52) Zhao, G.; Huang, Q.; Wang, F.; Zhang, X.; Hu, J.; Tan, Y.; Huang, N.; Wang, Z.; Wang, Z.; Cheng, Y. Targeted ShRNA-Loaded Liposome Complex Combined with Focused Ultrasound for Blood Brain Barrier Disruption and Suppressing Glioma Growth. *Cancer Letters* **2018**, *418*, 147–158. https://doi.org/10.1016/j.canlet.2018.01.035.
(53) Patel, N. R.; Rathi, A.; Mongayt, D.; Torchilin, V. P. Reversal of Multidrug Resistance by Co-Delivery of Tariquidar (XR9576) and Paclitaxel Using Long-Circulating Liposomes. *International Journal of Pharmaceutics* **2011**, *416* (1), 296–299. https://doi.org/10.1016/j.ijpharm.2011.05.082.
(54) Loi, M.; Marchiò, S.; Becherini, P.; Di Paolo, D.; Soster, M.; Curnis, F.; Brignole, C.; Pagnan, G.; Perri, P.; Caffa, I. Combined Targeting of Perivascular and Endothelial Tumor Cells Enhances Anti-Tumor Efficacy of Liposomal Chemotherapy in Neuroblastoma. *Journal of Controlled Release* **2010**, *145* (1), 66–73. https://doi.org/10.1016/j.jconrel.2010.03.015.
(55) Un, K.; Kawakami, S.; Suzuki, R.; Maruyama, K.; Yamashita, F.; Hashida, M. Enhanced Transfection Efficiency into Macrophages and Dendritic Cells by a Combination Method Using Mannosylated Lipoplexes and Bubble Liposomes with Ultrasound Exposure. *Human Gene Therapy* **2010**, *21* (1), 65–74. https://doi.org/10.1089/hum.2009.106.
(56) Al-Jamal, W. T.; Al-Jamal, K. T.; Bomans, P. H.; Frederik, P. M.; Kostarelos, K. Functionalized-Quantum-Dot-Liposome Hybrids as Multimodal Nanoparticles for Cancer. *Small* **2008**, *4* (9), 1406–1415. https://doi.org/10.1002/smll.200701043.
(57) Soenen, S. J.; Velde, G. V.; Ketkar-Atre, A.; Himmelreich, U.; De Cuyper, M. Magnetoliposomes as Magnetic Resonance Imaging Contrast Agents: Magnetoliposomes as MRI Contrast Agents. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **2011**, *3* (2), 197–211. https://doi.org/10.1002/wnan.122.
(58) Kamaly, N.; Kalber, T.; Thanou, M.; Bell, J. D.; Miller, A. D. Folate Receptor Targeted Bimodal Liposomes for Tumor Magnetic Resonance Imaging. *Bioconjugate Chemistry* **2009**, *20* (4), 648–655. https://doi.org/10.1021/bc8002259.
(59) Deckers, R.; Moonen, C. T. W. Ultrasound Triggered, Image Guided, Local Drug Delivery. *Journal of Controlled Release* **2010**, *148* (1), 25–33. https://doi.org/10.1016/j.jconrel.2010.07.117.
(60) Seo, J. W.; Zhang, H.; Kukis, D. L.; Meares, C. F.; Ferrara, K. W. A Novel Method to Label Preformed Liposomes with ^{64}Cu for Positron Emission Tomography (PET) Imaging. *Bioconjugate Chemistry* **2008**, *19* (12), 2577–2584. https://doi.org/10.1021/bc8002937.
(61) Henriksen, J. R.; Petersen, A. L.; Hansen, A. E.; Frankær, C. G.; Harris, P.; Elema, D. R.; Kristensen, A. T.; Kjær, A.; Andresen, T. L. Remote Loading of $^{64}Cu^{2+}$ into Liposomes without the Use of Ion Transport Enhancers. *ACS Applied Materials & Interfaces* **2015**, *7* (41), 22796–22806. https://doi.org/10.1021/acsami.5b04612.

Chapter 3.2: Development of doxorubicin hydrochloride loaded pH-sensitive liposomes: Investigation on the impact of chemical nature of lipids and liposome composition on pH-sensitivity

In this present study, we have developed pH-sensitive liposomes as nano-carriers of anticancer drug, doxorubicin (DOX). The most important requirement for these liposomes is that, they should be stable at physiological pH, and disrupt in slightly acidic media, such as the tumor microenvironment, to release the loaded DOX. Different lipids (dioleoyl phosphatidyl ethanolamine (DOPE), palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) and dimyristoyl phosphatidyl ethanolamine (DMPE)) were used, associated with alpha-tocopheryl succinate (α-TOS) as stabilizing agent to prepare liposomes by polycarbonate membrane extrusion method. The impact of (i) the chemical nature of lipids and (ii) the lipid/stabilizing agent ratio, on the pH sensitivity of the liposomes was studied. The study of pH-sensitivity of liposomes was conducted using carboxyfluorescein (CF) encapsulated in high concentration, *i.e.,* quenched, and following the dye dequenching as a sensor of the liposome integrity. These lipid:α-TOS combinations formed stable liposomes at pH 7.4. The resulting liposomes were found to be very sensitive to pH and maximum release of the dye/drug was obtained at pH 5.5. The breakdown of liposomes as well as release of dye from the liposomes was inversely related to the amount of the stabilizer. Optimized liposomal formulations were then selected for the encapsulation of DOX by an active loading procedure, *i.e.,* driven by a difference in pH inside and outside the liposomes. Numerous experimental conditions were explored, in function of the pH gradient and liposome composition, which allowed identifying critical parameters for the efficient DOX encapsulation in pH-sensitive liposomes. The loading of dye/DOX was mainly dependent on the values of the pH gradient and the nature of lipid and stabilizer. Moreover, in function of the composition, the presence of cholesterol also caused an increase in the encapsulation as well as retention of the DOX inside the liposomes.

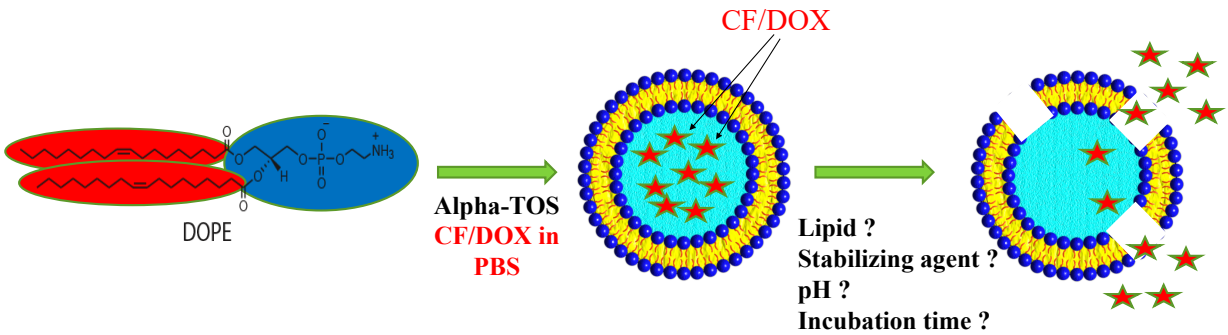


Figure: Schematic illustration of the project

European Journal of Pharmaceutics and Biopharmaceutics 133 (2018) 331–338

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Development of doxorubicin hydrochloride loaded pH-sensitive liposomes: Investigation on the impact of chemical nature of lipids and liposome composition on pH-sensitivity

Asad Ur Rehman[a,b], Ziad Omran[c,*], Halina Anton[d], Yves Mély[d], Salman Akram[a], Thierry F. Vandamme[a,*], Nicolas Anton[a,*]

[a] Université de Strasbourg, CNRS, CAMB UMR 7199, F-67000 Strasbourg, France
[b] Bahauddin Zakariya University (BZU) Multan, Pakistan
[c] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Umm AlQura University, Kingdom of Saudi Arabia
[d] Université de Strasbourg, CNRS, LBP UMR 7213, F-67000 Strasbourg, France

ARTICLE INFO

Keywords:
Liposomes
pH-sensitivity
Phosphatidyl ethanolamine lipids
Alpha-tocopheryl succinate
Carboxyfluorescein
Doxorubicin

ABSTRACT

This study investigates the impact of the chemical nature of lipids and additive on the formulation and properties of pH sensitive liposomes. The objective is to understand the respective role of the formulation parameters on the liposome properties in order to optimize the conditions for efficient encapsulation of doxorubicin (DOX). These liposomes should be stable at physiological pH, and disrupt in slightly acidic media such as the tumor microenvironment to release their DOX load. The major challenge for encapsulating DOX in pH sensitive liposomes lies in the fact that this drug is soluble at low pH (when the pH-sensitive liposomes are not stable), but the DOX aqueous solubility decreases in the pH conditions corresponding to the stability of the pH-sensitive liposomes. The study of pH-sensitivity of liposomes was conducted using carboxyfluorescein (CF) encapsulated in high concentration, *i.e.* quenched, and following the dye dequenching as sensor of the liposome integrity. We studied the impact of (i) the chemical nature of lipids (dioleoyl phosphatidyl ethanolamine (DOPE), palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) and dimyristoyl phosphatidyl ethanolamine (DMPE)) and (ii) the lipid/stabilizing agent ratio (alpha-tocopheryl succinate), on the pH sensitivity of the liposomes. Optimized liposome formulations were then selected for the encapsulation of DOX by an active loading procedure, *i.e.* driven by a difference in pH inside and outside the liposomes. Numerous experimental conditions were explored, in function of the pH gradient and liposome composition, which allowed identifying critical parameters for the efficient DOX encapsulation in pH-sensitive liposomes.

1. Introduction

Cancer is second only to the cardiovascular diseases as a cause of mortality. The clinical use of chemotherapeutic agents to treat cancer is successful in many cases. However, the lack of selectivity of chemotherapeutic agents which cause severe side effects and the emergence of multidrug resistance (MDR) are two major drawbacks for the effective use of these agents in clinic [1]. MDR is a complex phenomenon resulting from synergism of many factors. One of the most important factors is the change of pH gradient across the cell membrane, *i.e.* acidification of the tumoral extracellular (pHe) fluid and alkalization of the cytosol (pHi), which results from the Warburg effect [2–4].

Liposomes are considered as the most advanced type of particulate drug carriers and have gained importance as the mainstream drug delivery system. The importance of liposomes lies in the fact that hydrophilic, lipophilic as well as amphiphilic drugs can be entrapped in the liposomes [5–7]. Of particular interest are fusogenic liposomes that show triggered phase transitions and release properties promoted by various chemical and physical stimuli, *e.g.*, temperature, pH, light etc. [8–11]. pH-sensitive liposomes are of prime importance because they undergo phase transition and acquire fusogenic properties in acidic environment, leading to the release of their aqueous contents [12]. This property is of particular interest for delivery of anticancer drugs since the extracellular pH of cancer tissues is slightly acidic due to the high metabolic activity of cancer cells [13–15].

Different classes of pH-sensitive liposomes have been proposed

* Corresponding authors at: University of Strasbourg, CNRS 7199, Laboratoire de Conception et Application de Molécules Bioactives, équipe de Pharmacie Biogalénique, route du Rhin No.74, F–67401 Illkirch Cedex, France (T. Vandamme, N. Anton).
E-mail addresses: zhomran@uqu.edu.sa (Z. Omran), vandamme@unistra.fr (T.F. Vandamme), nanton@unistra.fr (N. Anton).

https://doi.org/10.1016/j.ejpb.2018.11.001
Received 6 June 2018; Received in revised form 20 October 2018; Accepted 2 November 2018
Available online 08 November 2018
0939-6411/

according to their triggering mechanism [16–21]. The most advanced liposomes use lipids with phosphatidyl-ethanolamine (PE) as polar head in their composition. However, pure PE lipids do not form stable liposomes and are thus associated with an additional amphiphilic molecule that stabilizes the liposomes bilayers, such as alpha-tocopheryl succinate (α-TOS), oleic acid, palmitoylhomocysteine, or cholestryl hemisuccinate (CHEMS). These stabilizers are in ionized form (negatively charged) at physiological pH and thus intercalate in between the phosphatidylethanolamine (PE) molecules and favor the lamellar organization, resulting in the formation of liposomes. As these liposomes are exposed to acidic environment, the carboxyl group of the stabilizer is protonated resulting in the reversion of the PE molecules into inverted hexagonal phase, destabilization of the liposomes and thus the release of the contents of the liposomes [12,22–24].

The objective of the present study was to develop and design a novel efficient pH-sensitive formulation to form doxorubicin (DOX) loaded liposomes that should be stable at physiological pH and collapse in slightly acidic media such as the cancerous microenvironment to selectively release their doxorubicin hydrochloride (DOX) within tumor tissues [25].

To this end, in the first part of our study we optimized the formulation of pH-sensitive liposomes, by investigating the impact of formulation parameters, such as the chemical nature and composition of the lipids, on the properties of liposome (size, encapsulation efficiency and pH-sensitivity). A variety of liposomes was prepared using (i) –PE containing lipids (dioleoylphosphatidyl ethanolamine, DOPE, palmitoyloleoylphosphatidyl ethanolamine, POPE, dimyristoylphosphatidyl ethanolamine, DMPE), and (ii) the stabilizing agent, α-TOS at different ratios. The pH-sensitivity was assessed by measuring the release of carboxyfluorescein, CF from liposomes at different pH in the range 5.5–7.4 and for different incubation times, according to a fluorescence methodology based on the self-quenching of CF [26]. CF was encapsulated at millimolar concentration in the internal aqueous phase during liposome preparation, leading to a strong fluorescence quenching and thus a low emission of CF molecules in the liposomes. Once the liposome membrane is disrupted, CF fluorophores are released into the buffer. This leads to a decrease in the concentration and fluorescence quenching of CF molecules in the liposomes, and thus, an increase in their emission.

The second part of the work involves the encapsulation of DOX in pH-sensitive liposomes and study of the effect of nature and concentration of lipid and stabilizer on doxorubicin encapsulation. DOX is a widely used efficient anti-cancer drug [27], but its clinical use is limited by its cardiotoxicity and myelosuppression [28]. Doxorubicin has high anti-tumor activity but specificity is very low, which results in the serious side effects. Interestingly, DOX entrapped in liposomal formulation has shown reduced cardiotoxicity and improved specificity for the tumor area [24,29–33]. However, being a weak base, its solubility in aqueous buffers changes with pH, which makes its encapsulation, in pH-sensitive liposomes, difficult. The neutral form of DOX is membrane permeable at alkaline pH and becomes membrane impermeable when charged at acidic pH. Therefore, the encapsulation of DOX in the liposomes is based on a pH gradient between inner and outer water phase. For example, DOX has been encapsulated in the liposomes using transmembrane sulfate-or phosphate-or citrate-gradient [34–42] with acidic pH inside and physiological pH outside. DOX in its neutral form diffuses into the liposomes and gets protonated, which prevents the leakage of the positively charged DOX once encapsulated [34–40,43]. Moreover, the solubility of DOX increases at low pH [35], while pH-sensitive liposomes become unstable and may disrupt at acidic pH [44]. In this context, encapsulating DOX in pH-sensitive liposomes is a complex problem, addressed by some reports in the literature [44] with formulations composed of DOPE/HSPC/CHEMS/CHOL (respectively dioleoylphosphatidyl ethanolamine/hydrogenated soy phosphatidylcholine/cholesteryl hemisuccinate/cholesterol). The purpose of the present study is more general, exploring the impact of the formulation parameters to understand and optimize the pH sensitivity of liposomes as well as the conditions compatible with the best encapsulation of DOX. The original system chosen here focuses on; i) –PE lipids (DOPE, POPE and DMPE) in association with α-TOS and CHOL, and ii) the active loading of DOX in function of different pH gradients through the liposome bilayer.

2. Materials and methods

2.1. Materials

Dioleoyl phosphatidyl ethanolamine (DOPE), palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) and dimyristoyl phosphatidyl ethanolamine (DMPE) were purchased from Avanti-Polar Lipids, Inc. Alpha-tocopheryl succinate (α-TOS), carboxyfluorescein (CF), triton X-100, phosphate buffered saline (PBS) and chloroform were purchased from Sigma-Aldrich. Doxorubicin hydrochloride was purchased from Alfa Aesar, and Sephadex™ G-25 M PD10 column from GE Heathcare. All other chemicals were of analytical grade.

2.2. Preparation of CF-loaded liposomes

The liposomes were prepared by the polycarbonate membrane extrusion method. Liposomes containing CF were obtained using three different ratios between lipids and α-TOS (90:10, 80:20 and 70:30 respectively). Briefly, specified amounts of lipid and α-TOS were dissolved in 1 mL of chloroform – with the exception for DMPE which was dissolved in chloroform/methanol (2:1) for solubility reasons – in a small round bottom flask to make final lipid concentration of 10 mM. Thereafter, the solvent was evaporated using the rotary evaporator and a dried thin film was formed at the bottom of the flask. The film was further dried under vacuum for 1 h to ensure complete removal of solvent. The lipid film was then rehydrated with 1 mL carboxyfluorescein solution (50 mM) in phosphate buffered saline (pH 7.4), followed by sonication for 5–10 s and then was let 2 h for proper hydration of the film and formation of the suspension of multilamellar vesicles (MLVs). The suspension was then vortexed for 5 min and finally passed through a 100 nm polycarbonate membrane (17 times), using a Liposofast® extruder, to form large unilamellar vesicles (LUVs). The non-encapsulated CF was separated from the liposomes by size exclusion chromatography (PD10 Sephadex® G-25 M column), pre-equilibrated in phosphate buffered saline (pH 7.4).

2.3. Preparation of DOX-loaded liposomes

2.3.1. Passive loading

The liposomes were prepared according to the same protocol as described above, except that the thin film was rehydrated with 1 mL DOX solution in PBS (pH 7.4). Then, non-encapsulated DOX was separated from the liposomes suspension by size exclusion chromatography as described above, with a column equilibrated with PBS (pH 7.4).

2.3.2. Active loading by sodium phosphate and pH gradient

In this method, liposomes were prepared by using sodium phosphate buffer (0.2 M $NaH_2PO_4{\cdot}2H_2O$ + 0.2 M $Na_2HPO_4.12\ H_2O$) at different pH values (7.0, 7.2, 7.4 and 7.8). After formation of liposomes, the external pH was increased, to promote DOX diffusion inside the liposomes, by addition of specified amounts of NaOH 1 M, up to reach pH values of 7.4, 7.8, 8.5 or 9.0. Each pH value corresponds to a single experiment that allowed investigating the impact of pH gradient on the active loading (see below). Then, the DOX-saline solution was added to the liposomes, with DOX concentration at 2 mM, and lipid concentration at 10 mM. The liposomes/DOX were then incubated overnight at room temperature (20–25 °C), and then free DOX was separated by size exclusion chromatography using PBS (pH 7.4) as eluent, as described

above.

2.4. Characterization

2.4.1. Size measurements

Size distribution and polydispersity indices were measured by dynamic light scattering (DLS) with a Malvern apparatus (NanoZS®, Malvern, Orsay, France). Mean particle size was assimilated to z-average hydrodynamic diameter and the width of size distribution to polydispersity index (PDI). DLS measurements were performed using a helium/neon laser, 4 mW, operated at 633 nm, with the scatter angle fixed at 173° and temperature maintained at 25 °C on diluted sample. DLS data were analyzed using a cumulants-based method assuming spherical shape.

2.4.2. Determination of encapsulation efficiency (EE)

The absorbance values of liposomes encapsulating CF or DOX were measured by using Cary 400 and Cary 4000 Scan UV–Visible Spectrophotometers. The liposomes encapsulating the molecules of interest (CF or DOX) were diluted 100x and their absorbance was measured in the range between 300 and 600 nm. The encapsulation efficiency (EE) was obtained from the absorbance values at 492 nm and 500 nm for CF and DOX respectively, according to Eq. (1),

$$EE = \frac{m_x}{m_x^0} \times 100 \quad (1)$$

where x refers to DOX or CF, m_x is the mass encapsulated in the liposomes (measured by visible spectrometry), and m_x^0 is the total mass used.

2.4.3. Fluorescence assay

The pH sensitivity of liposomes was evaluated by using the CF quenching assay. All fluorescence measurements were done by using a Fluorolog® spectrofluorimeter (Horiba, France). Excitation and emission wavelengths were set at 480 nm and 517 nm, respectively. CF-loaded liposomes (10 µL) were added to cuvettes containing each 1 mL of PBS at different pH (5.5, 6.0, 6.4 and 7.4), and kept for incubation. Fluorescence intensity was measured after various incubation times (5, 10, 15 and 30 min). Since the quantum yield of CF is pH dependent, the pH was readjusted to 7.4 by addition of specified amount of NaOH 1 M, in order to compare the changes in quenching efficiency, and fluorescence was measured again. Finally, 10 µL of Triton X-100 was added in each cuvette to disrupt all liposomes and fully release CF molecules, and the fluorescence was measured again. The percent of CF release was calculated using:

$$\%\text{CF release} = \frac{F_{pH} - F_0}{F_{TX} - F_0} \times 100 \quad (2)$$

where F_{pH} is the fluorescence intensity following the incubation at a given acidic pH, F_0 is the fluorescence intensity after incubation at $pH = 7.4$, and F_{TX} is the fluorescence intensity after addition of Triton X-100.

3. Results and discussion

3.1. CF-loaded pH sensitive liposomes

3.1.1. Effect of lipid composition on the size of the liposomes

The size of the liposome suspensions encapsulating the CF, made with DOPE, POPE and DMPE, and at different ratio lipid/α-TOS is reported in Fig. 1(a). Liposomes made with DMPE (having the smallest chain length) are the smallest, while those made with DOPE (longest chain) are the largest. Furthermore, these results show that the proportion of α-TOS in the formulation also impacts the liposome size, as increasing the α-TOS content induces a global decrease of the size (except for DMPE:α-TOS 80:20). The size range of the suspensions depends on the nature of the lipid. *i.e.*,varies from 190 nm to 160 nm for DOPE, from 170 nm to 140 nm for POPE, and from 130 nm to 108 nm for DMPE. All samples show a good monodispersity, with PDI values < 0.15. For all compositions, the size range remains compatible with the parenteral administration route.

3.1.2. CF encapsulation efficiency

The absorbance of the CF-loaded liposomes was measured after separation by size exclusion chromatography (PD10 columns). As the concentration of CF loaded in the liposomes was constant (50 mM solution) in all the formulations, the measurements of absorbance before and after the separation of the free dye reflect globally the % of CF inside the liposomes. The results reported in Fig. 1(b) show that the best encapsulation properties were obtained by using POPE, followed by DOPE. Additionally, an increasing α-TOS content increases the encapsulation efficiency (except for DMPE:α-TOS 80:20). This can be explained by the α-TOS impact on the liposome size (Fig. 1(a)), and the increase in the total number of liposomes produced (since lipid concentration is constant and size decreases, thus number of liposomes increases). To prove this point, the number of vesicles of each

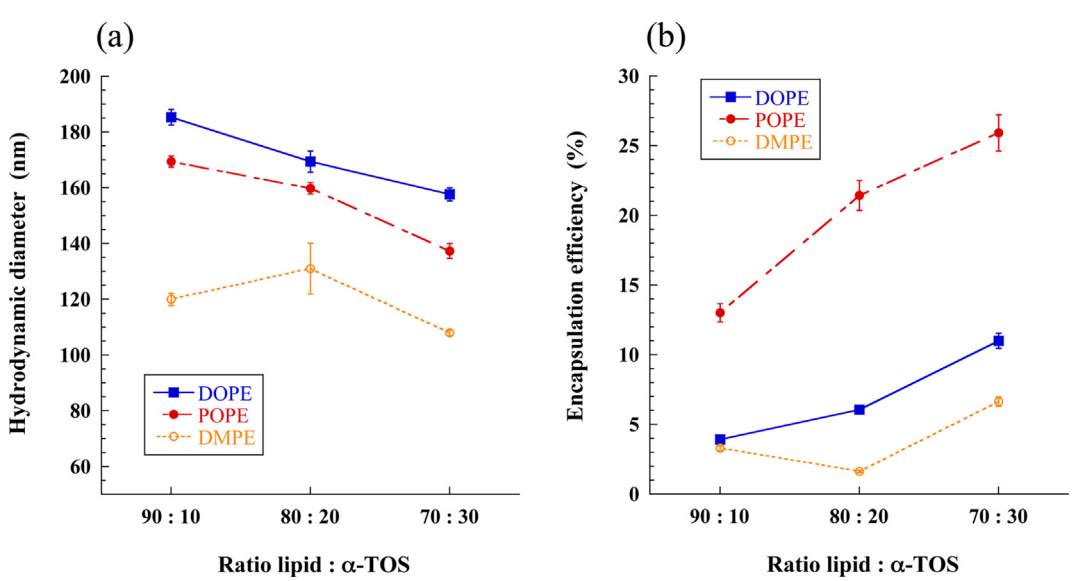


Fig. 1. Effect of the nature of the lipid and the lipid:α-TOS ratio on (a) the average size of liposomes, and (b) the efficiency of CF encapsulation.

formulation was calculated based on the liposome size, values of encapsulation efficiency and concentration of the CF solution inside the vesicles. Results are summarized in Table S1 in supplementary information section. These new results show that, assuming that the dye concentration is similar and constant in all formulations, the encapsulation efficiency is only related to the size and number of vesicles. It is noteworthy that the encapsulation efficiency of these formulations with CF does not exceed 25%, possibly due to the very high CF concentration (50 mM) required for the quenching-based method. It follows therefrom that the percentage of CF encapsulated in liposomes is 10%, 25% and 5% for liposomes made with DOPE, POPE and DMPE, respectively. These differences in values are likely related to variations in the number and size of liposomes for the different formulations as well as to the ability of the lipids to form stable liposomes with α-TOS.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejpb.2018.11.001.

3.1.3. Effect of lipid composition on pH-sensitivity/release of CF:

As described above, the integrity of the lipid bilayer was monitored by a fluorescent assay based on the self-quenching of concentrated CF in the liposome core. Once the bilayer is permeabilized, CF is released into the buffer, which reduces the quenching of the fluorophore and results in a fluorescence intensity increase of the liposome core. It should be noted that the CF fluorescence is pH dependent and decreases in acidic pH. Therefore, all comparison of fluorescence intensities was performed after readjusting pH at 7.4. Since the liposomes are stable at this pH, this readjustment should not influence the liposome permeation measurement. Finally, Triton X-100 was used to completely destroy the remaining intact liposomes, giving rise to the reference signal that corresponds to the complete CF release.

Fig. 2 reports the CF release as a function of the incubation pH and lipid composition for (a) DOPE, (b) POPE, (c) DMPE, containing different fractions of α-TOS. These data highlight the clear sensitivity of these liposomes to acidic pH that results in their gradual destruction as pH is lowered. The nature of the lipid does not strongly impact on this behavior, as all of them were very sensitive to change in pH. But POPE appears less sensitive to slightly acidic pH and more sensitive to pH ≤6. Finally, the stabilizer α-TOS also modifies the pH sensitivity of the liposomes, as the pH-sensitivity decreases when α-TOS concentration increases. This appears fully logical since α-TOS stabilizes the liposomes and thus, decreases the membrane disruption. For all three lipids, among the three different lipid/α-TOS ratios, the 90:10 ratio showed the highest pH-sensitivity. At this ratio, the CF was released very quickly (almost 40–60%) from the liposomes at pH 6.4, that corresponds to the pH of tumor microenvironment [14,15], and was fully released at pH 5.5.

3.1.4. Effect of incubation time on the release of CF from the liposomes

Fig. 3 reports the impact of incubation time on the CF release for DOPE, POPE and DMPE liposomes containing 10% or 30% of α-TOS. The time of incubation shows almost no influence on liposomes containing 10% of α-TOS, where the pH-induced membrane disruption and the release of the liposomes content are almost immediate. In contrast, the incubation time plays an important role in the case of less sensitive DOPE and POPE liposomes (*i.e.*, with 30% α-TOS). In these cases, a prolonged incubation of pH sensitive liposomes in acidic conditions promotes the bilayer permeation and/or liposome destruction, and thus increases the amount of CF release. When incubated at pH = 5.5, the % of released CF increases from 30% (after 5 min) to 55% (after 30 min) for DOPE:α-TOS, from 50% (5 min) to 85% (30 min) for POPE:α-TOS liposomes and finally from 60% (5 min) to 85% (30 min) for DMPE:α-TOS.

3.2. DOX-loaded pH-sensitive liposomes

Based on the results obtained above, we adapted the formulation processes to the encapsulation of DOX, which has a pH-dependent solubility. All the assays described with CF were performed with liposomes prepared by a passive loading method, where the lipid multilayers were rehydrated with an aqueous buffer already containing CF. Then after extrusion, the non-encapsulated CF molecules were removed by size exclusion chromatography.

In order to formulate DOX-loaded liposomes, we first tried to use the same passive loading method, using liposomes containing different stabilizers (α-TOS, CHEMS, CHOL and their combination). However, when rehydrating the lipid film with a DOX solution at neutral or alkaline pH, the liposomes could not be formed, likely due to electrostatic repulsive interactions between the liposome components and the DOX. We then used an active loading method to encapsulate DOX. This technique is based on the pH gradient between the external and internal aqueous phase. The most commonly reported buffer solutions for hydrating the lipid film are citrate buffer at pH 4.0 (300 mM), ammonium sulfate buffer at pH 5.5 (120 mM) and magnesium sulfate buffer at pH 3.5 (300 mM) [41]. Herein, we hydrated the lipid film at neutral to alkaline pH using phosphate buffer.

Different pH gradients of phosphate buffer were tested to optimize the encapsulation of DOX (Table 1). Part I of Table 1 shows the assays performed with passive loading, which were not working as liposomes did not form. Part II of Table 1 describes the assays with the active loading technique using POPE and DOPE/α-TOS system at different lipid:α-TOS ratios, and different values of internal/external pH. The formulations associated with "+" result represent the best formulations with efficient formation of liposomes and significant encapsulation of

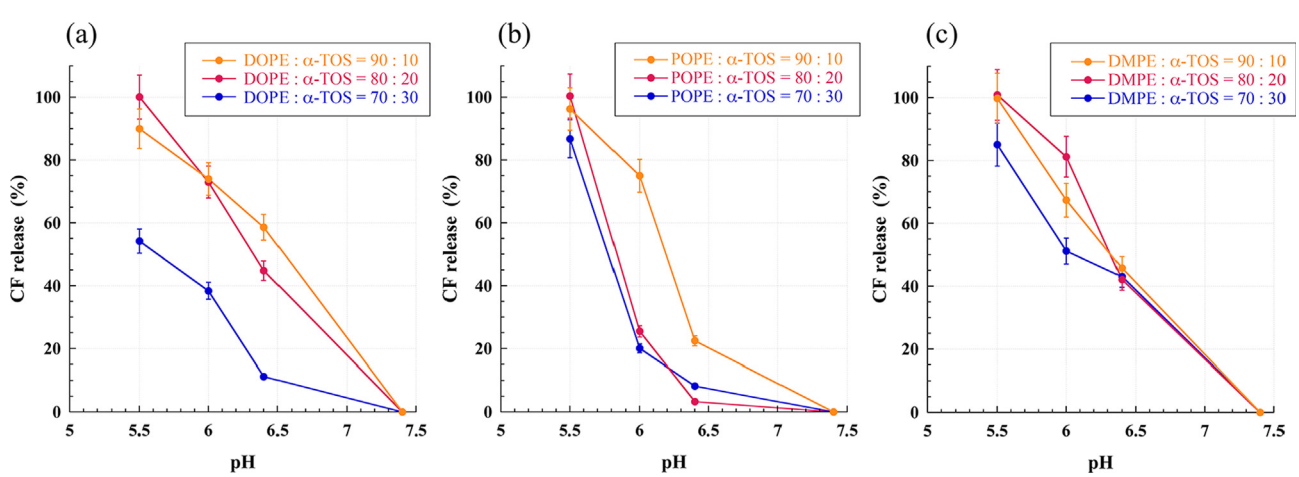


Fig. 2. Evaluation of the pH sensitivity of CF-loaded liposomes, after 30 min incubation in PBS at different pH, as a function of the nature of lipid (a) DOPE, (b) POPE, (c) DMPE, and as a function of the lipid:α-TOS ratio.

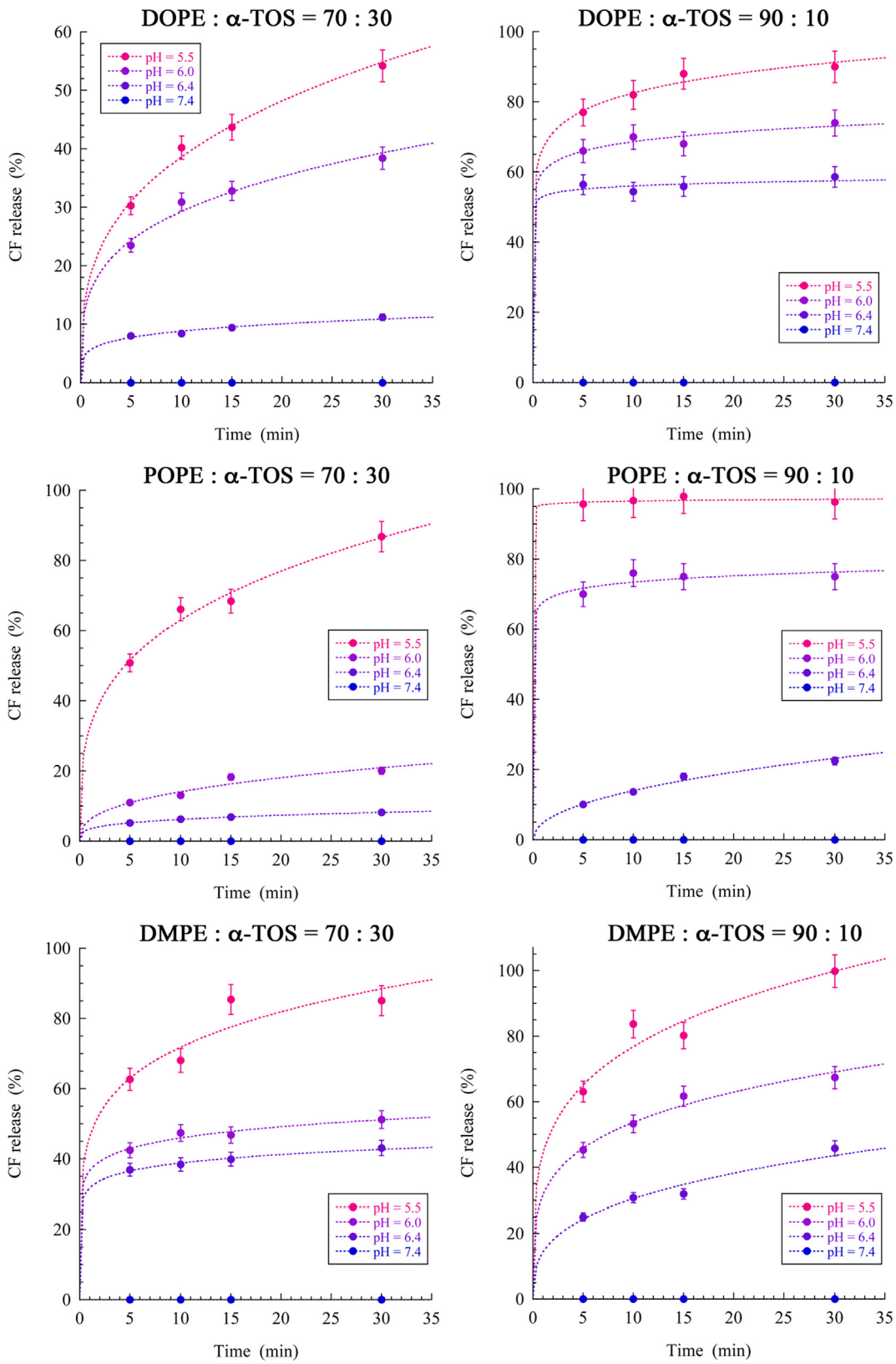


Fig. 3. Effect of incubation time on the release of CF from liposomes at different pH, for different lipids (DOPE, POPE and DMPE) and two different lipid:α-TOS ratios, 70:30 and 90:10.

DOX (20–100%). The formation of liposomes was verified by DLS measurements and the DOX encapsulation was checked by the absorbance measurements of all the formulations. The highest load of DOX was obtained with "POPE:α-TOS 80:20" and "POPE/DOPE:α-TOS 70:30" using pH 7.4/9.0 gradient. Finally, the part III of Table 1 shows additional assays performed using different stabilizing agents and also by using a combination of stabilizing agent and CHOL. Our data show successful formation of liposome and encapsulation of DOX using α-

Table 1
Optimization of conditions for getting efficient loading of DOX in different formulations of liposomes. Hydrating and external buffers were PBS in both cases.

	Composition of liposomes	Loading technique	pH of hydrating buffer	pH of external buffer	Result
I	POPE/DOPE:αTOS (90:10)	Passive	7.4	–	(−)
	POPE/DOPE:αTOS (70:30)	Passive	7.4	–	(−)
	POPE/DOPE:αTOS:CHOL (65:20:15)	Passive	7.4	–	(−)
II	POPE/DOPE:αTOS (80:20)	Active	7.0	7.4	(−)
	POPE/DOPE:αTOS (80:20)	Active	7.4	8.0	(−)
	POPE/DOPE:αTOS (80:20)	Active	7.4	8.5	(−)
	POPE/DOPE:αTOS (80:20)	Active	7.8	8.5	(−)
	POPE/DOPE:αTOS (80:20)	Active	7.0	9.0	(−)
	DOPE:αTOS (80:20)	Active	7.4	9.0	(+/−)
	POPE:αTOS (80:20)	Active	7.4	9.0	(+)
	POPE/DOPE:αTOS (90:10)	Active	7.4	9.0	(−)
	POPE/DOPE:αTOS (70:30)	Active	7.4	9.0	(+)
III	POPE/DOPE:CHEMS (70:30)	Active	7.4	9.0	(+)
	DOPE:αTOS:CHOL (65:20:15)	Active	7.0	9.0	(−)
	DOPE:αTOS:CHOL (65:20:15)	Active	7.4	9.0	(+/−)
	POPE:αTOS:CHOL (65:20:15)	Active	7.0	9.0	(+)
	POPE:αTOS:CHOL (65:20:15)	Active	7.4	9.0	(+)
	POPE/DOPE:CHEMS:CHOL (65:20:15)	Active	7.4	9.0	(+/−)

The notation POPE/DOPE:α-TOS means that two experiments were performed, one being with POPE:α-TOS and the other one with DOPE:α-TOS, but results were identical.
(−) = Not working: either DOX precipitated or no liposome was formed
(+) = Liposome formed and DOX was encapsulated
(+/−) = Liposomes were formed and DOX was encapsulated but quickly released within 1–2 min

TOS in association with POPE and CHOL, as well as for the combination of POPE/DOPE with CHEMS.

The formulations in Table 1 associated with "+/−" results provide efficient liposome formation and DOX encapsulation, but lead to DOX leaking after size exclusion chromatography using PBS 7.4 as an eluent. This might be related to the inadequate strength of the membrane to retain the DOX in the liposomes. This phenomenon was observed by the precipitation of the DOX within few minutes after the separation of the liposomes encapsulating DOX from the free DOX by size exclusion chromatography.

The general overview emphasized in Table 1 was further investigated through the measurement of encapsulation efficiencies for the best formulations, reported in Fig. 4(a). The encapsulation efficiency of DOX in pH sensitive liposomes depends on the nature of the –PE lipid in general higher with POPE, but also on the nature of the stabilizer. In addition, Table 1 referred to the conditions for which stable liposome containing DOX formed, then, among these, Fig. 4(a) compared quantitatively the values of the encapsulation efficiency. For example, in the case of POPE/DOPE:CHEMS (70:30) stable liposomes effectively formed (+), however with a lower EE values than for other systems. And in general, higher encapsulation efficiencies are resulted with α-TOS and α-TOS/CHOL combination as compared to CHEMS and CHEMS/CHOL combination. The graphs represent the results for two different pH gradients inside and outside the liposomes. In case of pH gradient with pH 7 inside and 9 outside the liposomes, the DOPE did not from liposomes at pH 7. But there was liposome formation in case of POPE at this pH. That's why the results have been shown only for POPE in case of the pH gradient with pH 7 inside and pH 9 outside the liposomes. In the other half of the graph the results have been shown for the experiments conducted with pH 7.4 inside and pH 9 outside the liposomes. Importantly, the two most interesting formulations are "POPE:α-TOS 80:20" and "POPE:α-TOS:CHOL 65:20:15", which provide encapsulation efficiencies around 100%. This result is likely due to the presence of lipid POPE, as we have got higher encapsulation efficiencies (25%) using POPE in case of CF as well (Fig. 1b). The reason could be the better affinity of the POPE to form liposomes and encapsulate CF/DOX under these conditions as compared to other lipids used. Interestingly, compared to the encapsulation efficiencies obtained with CF (Fig. 1(b)), the values with DOX are much higher, due to the active loading methodology. The presence of cholesterol is also very important because cholesterol considerably decreases the leakage of the encapsulated drugs in the extracellular environment or throughout the circulation [22] and helps to achieve immediate release of the encapsulated drugs when used for triggered release applications due to its non-bilayer structure forming properties [45].

DOX being neutral at alkaline pH migrates into the liposomes through the bilayer. Two phenomena are likely involved in an efficient encapsulation and trapping of DOX in the liposomes (Fig. 4a). The first one is the protonation of the drug inside the liposomes. The resulting charged DOX is unable to cross the bilayer and stays thus inside the liposomes [43,46,47]. The second phenomenon involved in the active loading of DOX is its precipitation due to an increase of its concentration in the liposome above the saturation threshold. Li et al. [47] showed that DOX starts aggregating at 0.5–1.5 mM concentrations in citrate and sulfate buffers even if its concentration is about 100 times lower than its normal aqueous solubility threshold and the DOX release from the liposomes containing DOX fibers was relatively slow. Cullis and coworkers [46,48] proposed that DOX is predominantly bound to the inner monolayer, leading to invaginations of the membrane. Both these phenomena contribute towards retention of DOX inside the liposomes. Literature also reported that, due to its amphiphilic nature, part of the DOX can be entrapped in the lipid bilayer of the liposome, thus, further increasing the DOX loading [49].

The size of the liposomes was measured after extrusion both before and after active DOX loading. The data in Fig. 4(b) shows that "empty" liposomes present a relatively constant size whatever their composition. After drug loading, the majority of samples showed only a slight increase in their size. A significant increase in size was only observed in CHEMS-containing liposomes (DOPE:CHEMS 70:30 and POPE:CHEMS 70:30 liposomes), likely as a result of the sensitivity of CHEMS to the prolonged exposure to alkaline pH, resulting in the aggregation and increase in the average size of the liposomes.

The presence of CHOL seems to be important for stability of the liposomes and for the retention of the DOX inside liposomes at physiological pH. In order to confirm the pH sensitivity for this new formulation containing CHOL (*i.e.* the system "POPE:α-TOS:CHOL 65:20:15"), we performed the experiments to form liposomes with the same composition but using passive loading technique. We used the CF solution in PBS (pH 7.4) to load CF into the liposomes and then measured the release of CF by a fluorescent assay explained above in

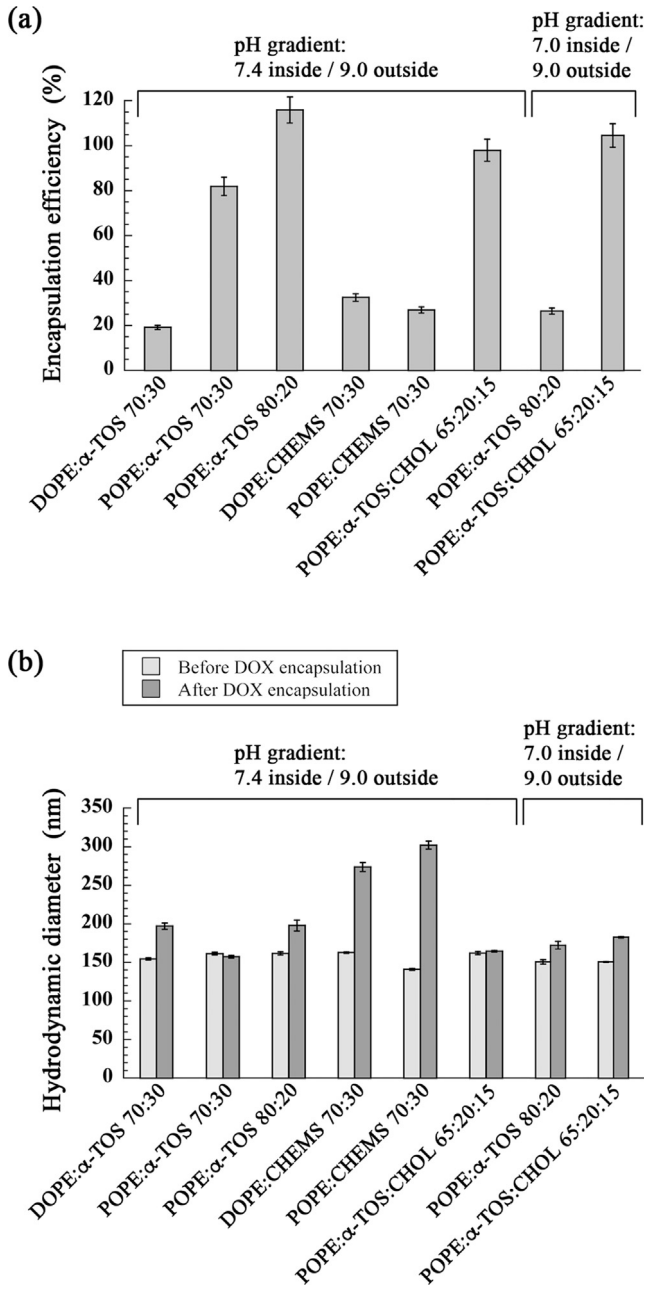


Fig. 4. Characterization of the formulations of pH sensitive liposomes encapsulating DOX. (a) Encapsulation efficiency and (b) hydrodynamic diameter (before and after DOX encapsulation) as a function of the nature of the lipid and stabilizing agent, and their respective proportions.

Section 2.4.3. The results are reported in Fig. 5, after 5 min, 10 min, 15 min and 30 min of incubation at different pH. It clearly appears that, in that case (*i.e.* POPE:α-TOS:CHOL 65:20:15), liposome formulation containing CHOL remain significantly sensitive to pH like the formulations without CHOL described above.

Finally, in contrast to the liposomes passively loaded with CF in the absence of CHOL (Fig. 3), the incubation time has no impact on the disruption of the CHOL containing liposomes. For a given pH, the dye is almost completely released after 5 min, thus ensuring immediate release of dye/drug at the desired pH. Therefore, this last, cholesterol-containing formulation suits perfectly for the aimed applications of selective pH-sensitive nanocarrier.

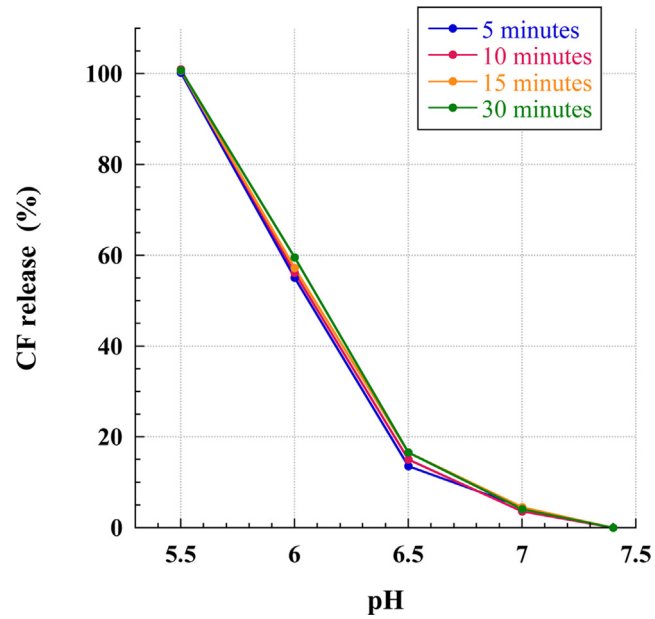


Fig. 5. pH sensitivity of CF-loaded liposomes, after different incubation times (5 min, 10 min, 15 min and 30 min) in PBS at different pH, for the system "POPE:α-TOS:CHOL 65:20:15".

4. Conclusion

This study investigated the formulation and optimization of pH-sensitive liposomes as a function of the chemical nature of –PE lipids and stabilizing agents and the lipid: stabilizer ratio. The ultimate objective was to find the most efficient system to encapsulate doxorubicin in pH-sensitive liposomes. In the first part of the study, we have evaluated the pH-sensitivity of the nanocarriers with a fluorescent method based on the encapsulation of CF as a model dye. Different lipids (DOPE, POPE and DMPE) were used, associated with α-TOS as stabilizing agent at different lipid:α-TOS ratios. These combinations were found to form stable liposomes at pH 7.4, with the higher encapsulation efficiency obtained with POPE. The integrity of liposomes was significantly pH-dependent with a possible modulation as a function of the concentration of α-TOS. As the concentration of α-TOS increases, the breakdown of liposomes and release of CF are slowed down. Incubation time also has an impact on the release of the dye from the liposomes because incubation for longer periods increases the extent of the liposome disruption in acidic pH. The second part of the study focused on the encapsulation of DOX in pH sensitive liposomes. We showed that DOX was not compatible with the passive loading approach, and showed good encapsulation tendencies by active loading technique (but only in specific pH ranges). Different pH gradients were studied, along with the variation of the nature and amounts of lipids and stabilizing agents. Values of the pH gradient was shown to be a crucial parameter in the DOX loading. The encapsulation efficiency of DOX in pH sensitive liposomes depends on the nature of the –PE lipid (higher with POPE) (Fig. 4a). And among different liposomal formulations of POPE, higher encapsulation efficiencies are resulted with α-TOS and α-TOS/CHOL combination as compared to CHEMS and CHEMS/CHOL combination. In function of the composition, the presence of cholesterol also increases the encapsulation of DOX and also helps in the retention of the DOX inside the liposomes.

Acknowledgment

The authors would like to acknowledge the financial support provided by King Abdulaziz City for Science and Technology (KACST), Grant no. 14-MED1472-10 and the Higher Education Commission

(HEC) of Pakistan. The authors are grateful to Nicolas Humbert for his experimental support.

References

[1] D.B. Longley, P.G. Johnston, Molecular mechanisms of drug resistance, J. Pathol. 205 (2005) 275–292.
[2] O. Warburg, On the origin of cancer cells, Science (80-.). 123 (1956) 309–314.
[3] M. Walsh, S. Fais, E.P. Spugnini, S. Harguindey, T. Abu Izneid, L. Scacco, P. Williams, C. Allegrucci, C. Rauch, Z. Omran, Proton pump inhibitors for the treatment of cancer in companion animals, J. Exp. Clin. Cancer Res. 34 (2015) 93.
[4] Z. Omran, P. Scaife, S. Stewart, C. Rauch, Physical and biological characteristics of multi drug resistance (MDR): an integral approach considering pH and drug resistance in cancer, Semin. Cancer Biol. 43 (2017) 42–48.
[5] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1965) 238–252.
[6] P. Sapra, T.M. Allen, Ligand-targeted liposomal anticancer drugs. Progress in lipid research, Prog. Lipid Res. 42 (2003) 439–462.
[7] J.O. Eloy, M. Claro de Souza, R. Petrilli, J.P. Barcellos, R.J. Lee, J.M. Marchetti, Liposomes as carriers of hydrophilic small molecule drugs: strategies to enhance encapsulation and delivery, Colloids Surf. B Biointerface 123 (2014) 345–363.
[8] O.V. Gerasimov, Y. Rui, D.H. Thompson, Triggered release from liposomes mediated by physically and chemically induced phase transitions, in: M. Rosoff (Ed.), Vesicles, Marcel Dekker, New York, 1996, pp. 679–746.
[9] G. Shi, W. Guo, S.M. Stephenson, R.J. Lee, Efficient intracellular drug and gene delivery using folate receptor-targeted pH-sensitive liposomes composed of cationic/anionic lipid combinations, J. Control. Release 80 (2002) 309–319.
[10] H. Bi, S. Ma, Q. Li, X. Han, Magnetically triggered drug release from biocompatible microcapsules for potential cancer therapeutics, J. Mater. Chem. B 4 (2016).
[11] H. Bi, X. Han, Magnetic field triggered drug release from lipid microcapsule containing lipid-coated magnetic nanoparticles, Chem. Phys. Lett. 706 (2018) 455–460.
[12] S. Simoes, J.N. Moreira, C. Fonseca, N. Duzgunes, M.C. de Lima, On the formulation of pH-sensitive liposomes with long circulation times, Adv. Drug Deliv. Rev. 56 (2004) 947–965.
[13] I.F. Tannock, D. Rotin, Acid pH in tumors and its potential for therapeutic exploitation, Cancer Res. 49 (1989) 4373–4384.
[14] A.J. Thistlethwaite, D.B. Leeper, D.J. Moylan, R.E. Nerlinger, pH distribution in human tumors, Int. J. Radiat. Oncol. Biol. Phys. 11 (1985) 1647–1652.
[15] L.E. Gerweck, The pH difference between tumor and normal tissue offers a tumor specific target for the treatment of cancer, Drug Resist. Updat. 3 (2000) 49–50.
[16] D.C. Drummond, M. Zignani, J. Leroux, Current status of pH-sensitive liposomes in drug delivery, Prog. Lipid Res. 39 (2000) 409–460.
[17] V.P. Torchilin, F. Zhou, L. Huang, pH-sensitive liposomes, J. Liposome Res. 3 (1993).
[18] P. Venugopalan, S. Jain, S. Sankar, P. Singh, A. Rawat, S.P. Vyas, pH-sensitive liposomes: mechanism of triggered release to drug and gene delivery prospects, Pharmazie 57 (2002).
[19] J.J. Sudimack, W. Guo, W. Tjarks, R.J. Lee, A novel pH-sensitive liposome formulation containing oleoyl alcohol, Biochim. Biophys. Acta. 1564 (2002) 31–37.
[20] X. Zhang, W. Zong, Y. Hu, N. Luo, W. Cheng, X. Han, A pH-responsive asymmetric lipid vesicle as drug carrier, J. Microencapsul. 33 (2016) 663–668.
[21] X. Zhang, W. Zong, H. Bi, K. Zhao, T. Fuhs, Y. Hu, W. Cheng, X. Han, Hierarchical drug release of pH-sensitive liposomes encapsulating aqueous two phase system, Eur. J. Pharm. Biopharm. 127 (2018) 177–182.
[22] H. Karanth, R.S.R. Murthy, pH-sensitive liposomes-principle and application in cancer therapy, J. Pharm. Pharmacol. 59 (2007) 469–483.
[23] S. Koudelka, P. Turanek Knotigova, J. Masek, L. Prochazka, R. Lukac, A.D. Miller, J. Neuzil, J. Turanek, Liposomal delivery systems for anti-cancer analogues of vitamin E, J. Control. Release 207 (2015) 59–69.
[24] J.C. de Oliveira Silva, S.E.M. Miranda, E.A. Leite, A. de Paula Sabino, K.B.G. Borges, V.N. Cardoso, G.D. Cassali, A.G. Guimarães, M.C. Oliveira, A.L.B. de Barros, Toxicological study of a new doxorubicin-loaded pH-sensitive liposome: a preclinical approach, Toxicol. Appl. Pharmacol. 352 (2018) 162–169.
[25] Z. Omran, C. Rauch, Acid-mediated Lipinski's second rule: application to drug design and targeting in cancer, Eur. Biophys. J. Biophys. Lett. 43 (2014) 199–206.
[26] H. Mojzisova, B. Bonneau, P. Maillard, K. Berg, D. Brault, Photosensitizing properties of chlorins in solution and in membrane-mimicking systems, Photochem. Photobiol. Sci. 8 (2009) 778–787.
[27] R.C. Young, R.F. Ozols, C.E. Myers, The anthracycline antineoplastic drugs, N. Engl. J. Med. 305 (1981) 139–153.
[28] J. Bouma, J.H. Beijnen, A. Bult, W.J. Underberg, Anthracycline antitumour agents. A review of physicochemical, analytical and stability properties, Pharm. Weekbl. Sci. 8 (1986) 109–133.
[29] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, F. Martin, A. Huang, Y. Barenholz, Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes, Cancer Res. 54 (1994) 987–992.
[30] A. Gabizon, A. Dagan, D. Goren, Y. Barenholz, Z. Fuks, Liposomes as in vivo carriers of adriamycin: reduced cardiac uptake and preserved antitumor activity in mice, Cancer Res. 42 (1982) 4734–4739.
[31] J. O'Shaughnessy, Liposomal anthracyclines for breast cancer: overview, Oncologist 8 (2003) 1–2.
[32] J.C. de Olivera Silva, R.S. Fernandes, S.C. Lopes, V.N. Cardoso, E.A. Leite, G.D. Cassali, M.C. Marzola, D. Rubello, M.C. Oliveira, A.L. de Barros, pH-sensitive, long-circulating liposomes as an alternative tool to deliver doxorubicin into tumors: a feasibility animal study, Mol. Imag. Biol. 18 (2016) 898–904.
[33] X. Zhang, W. Zong, W. Cheng, X. Han, Codelivery of doxorubicin and sodium tanshinone IIA sulfonate using multicompartmentalized vesosomes to enhance synergism and prevent doxorubicin-induced cardiomyocyte apoptosis, J. Mater. Chem. B 6 (2018) 5243–5247.
[34] E.M. Bolotin, R. Cohen, D.D. Lasic, Y. Barenholz, Ammoniumsulfate gradient for efficient and stable remote loading of amphipathic weak base into liposomes and ligandoliposomes, J. Liposome Res. 4 (1994) 455–479.
[35] A. Fritze, F. Hens, A. Kimpfler, R. Schubert, R. Peschka-Suss, Remote loading of doxorubicin into liposomes driven by a transmembrane phosphate gradient, Biochim. Biophys. Acta. 1758 (2006) 1633–1640.
[36] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim. Biophys. Acta 1151 (1993) 201–215.
[37] T. Ishida, M.J. Kirchmeier, E.H. Moase, S. Zalipsky, T.M. Allen, Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells, Biochim. Biophys. Acta. 1515 (2001) 144–158.
[38] X. Li, D. Cabral-lilly, A.S. Janoff, W.R. Perkins, Complexation of internalized doxorubicin into fiber bundles affects its release rate from liposomes, J. Liposome Res. 10 (2000) 15–27.
[39] N. Maurer, D.B. Fenske, P.R. Cullis, Developments in liposomal drug delivery systems, Expert Opin. Biol. Ther. 1 (2001) 923–947.
[40] L.D. Mayer, L.C. Tai, M.B. Bally, G.N. Mitilenes, R.S. Ginsberg, P.R. Cullis, Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients, Biochim. Biophys. Acta. 1025 (1990) 143–151.
[41] S.A. Abraham, D.N. Waterhouse, L.D. Mayer, P.R. Cullis, T.D. Madden, M.B. Bally, Liposomal formulation of doxorubicin, Methods Enzymol. 391 (2005) 71–97.
[42] S.R. Paliwal, R. Paliwal, H.C. Pal, A.K. Saxena, P.R. Sharma, P.N. Gupta, G.P. Agrawal, S.P. Vyas, Estrogen-anchored pH-sensitive liposomes as nanomodule designed for site-specific delivery of doxorubicin in breast cancer therapy, Mol. Pharm. 9 (2012) 176–186.
[43] L.D. Mayer, P.R. Cullis, M.B. Bally, Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient, Biochim. Biophys. Acta. 857 (1986) 123–126.
[44] T. Ishida, Y. Okada, T. Kobayashi, H. Kiwada, Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood, Int. J. Pharm. 309 (2006) 94–100.
[45] I.V. Zhigaltsev, N. Maurer, K.F. Wong, P.R. Cullis, Triggered release of doxorubicin following mixing of cationic and anionic liposomes, Biochim. Biophys. Acta-Biomembr. 1565 (2002) 129–135.
[46] P.R. Harrigan, K.F. Wong, T.E. Redelmeier, J.J. Wheeler, P.R. Cullis, Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients, Biochim. Biophys. Acta. 1149 (1993) 329–338.
[47] X. Li, D.J. Hirsh, D. Cabral-Lilly, A. Zirkel, S.M. Gruner, A.S. Janoff, W.R. Perkins, Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient, Biochim. Biophys. Acta. 1415 (1998) 23–40.
[48] P.R. Cullis, M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer, D.B. Fenske, Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles, Biochim. Biophys. Acta. 1331 (1997) 187–211.
[49] L. Dupou-Cezanne, A.M. Sautereau, J.F. Tocanne, Localization of adriamycin in model and natural membranes. Influence of lipid molecular packing, Eur. J. Biochem. 181 (1989) 695–702.

CHAPTER FOUR

CONCLUSION AND PERSPECTIVES

The low-aqueous solubility of drugs has always been a serious concern because it creates many problems in developing a suitable dosage form to deliver lipophilic drugs to the human body. Different kinds of nano-carriers have been developed but the lipid based nano-carriers have gained much importance for the delivery of the lipophilic drugs because of their special features, like high drug loading capacity, high biocompatibility (non-toxic, stable in physiological media, inert to tissue), tailorable surface to apply any kind of targeting strategy and an improved biodistribution and pharmacokinetic profile. In this research project, we have developed new strategies to formulate surface functionalized nano-carriers and have shown that they are the best candidates for the imaging and targeted drug delivery applications.

Project 1: Nano-emulsions as carrier of lipophilic molecules for biomedical applications

The nano-emulsion droplets constitute a high-potential nano-carrier for hundreds of applications owing to their reservoir capability, with main drawbacks being the limitation of their surface modification and stability. The solution herein presented is an illustration that simple solutions can exist, can be easily developed and transposed to large-scale production. We have developed and optimized the novel techniques to formulate and functionalize the nano-emulsions and have shown successfully that these functionalized nano-emulsions provide a platform for a large variety of contrast agents, ligands, and antibodies. The ligands can be covalently attached with these nano-emulsions and the surface functionalized nano-carriers can be used as imaging, therapeutic and targeted drug delivery systems.

Objective 1: Development of a new strategy to formulate nano-emulsions in the absence of the surfactant as well as organic solvent

An amphiphilic polymer was designed, specially tailored to exhibit an important surface-active property, followed by the evaluation of its ability to generate nano-emulsions through the spontaneous emulsification method. The synthesis of amphiphilic polymer was based on the modification of a polymer poly(maleic anhydride-alt-1-octadecene) (PMAO), by performing a systematic reaction of Jeffamine® polyetheramine (polyethylene glycol / polypropylene glycol copolymer) on each anhydride function, in order to generate an octadecene / Jeffamine® pattern on each monomeric element. The results showed that the amphiphilic polymer successfully formed the stable nano-emulsions and among all the conditions explored, the best results were obtained, (in terms of average size and PDI), with PMAO amphiphile when used with longer Jeffamine® compound (*i.e.*, J-2000). It was further

observed that the size of the formed droplets could significantly vary with the change in the nature of the oil. Furthermore, it was observed that the spontaneous emulsification process is related to the relative affinities of the amphiphilic polymer for oil and water. The originality comes with the non-dispersion of the polymer in water and precipitation in the interfacial region, forming a spherical shell shape, confirmed by TEM. The novelty of this work lies in the fact that spontaneous emulsification has never been reported with polymer alone in the absence of low molecular weight surfactants.

<u>Objective 2:</u> Preparation and characterization of tunable surface functionalized oil-in-water nano-emulsions

We used the same amphiphilic polymer with a slight modification in the structure, *i.e.*, a proportion (20 %) of the Jeffamine® herein used was replaced with another polyethylene glycol (PEG)-containing compound terminated by a reactive azide group (*i.e.*, N_3-PEG-NH_2) which has the affinity to link with ligands. The nano-emulsions were prepared, using this newly synthesized polymer, by ultrasonication method with a contrast agent (NR668/CY5.5TPB) being encapsulated inside the nano droplets. These nano-emulsions were then decorated with a ligand, namely DBCO-PEG4-biotin, by the reaction between the active azide groups and the alkyne group of the ligand. Different techniques were used to characterize these nano-emulsion formulations namely differential light scattering (DLS), UV-visible spectroscopy, transmission electron microscopy (TEM), nuclear magnetic resonance (NMR) and gel electrophoresis. The results have shown that these nano-emulsion systems have the tendency to covalently link with such ligands (biotinylated) and can be used as an efficient carrier of contrast agents and drugs for the imaging and targeted drug delivery applications.

<u>Objective 3:</u> Development of a novel theranostic approach to target endothelial senescence: Nano-emulsions as targeted theranostic nano-carriers

The third objective of this study was to explore the potential of nano-emulsions as targeted theranostic nano-carriers for imaging and biomedical applications. To achieve this objective, we took advantage of the unique features of the cell membrane of senescent endothelial cells (ECs) for the *in vivo* early assessment of arterial sites at risk, and for the selective delivery of agents for preventive and therapeutic purposes to restore optimal vascular protection using a nanomedicine theranostic approach. The amphiphilic polymer was designed in the similar pattern as to achieve second objective with a slight modification in the structure, *i.e.*, the linker "N_3-PEG-NH_2" used in the project was replaced with another polyethylene glycol (PEG)-containing compound terminated by a reactive maleimide group which has the affinity

to react with the cysteine function of the antibody (anti-VCAM). The formulations were optimized in terms of the lipid/coating polymer ratio, the PEG/PEG-antibody ratio, the optimization of functional sites, the nano-carrier size and the temperature stability. The formulations were also optimized in close relation with the biological evaluation, performed with antibody targeting membrane proteins (VCAM-1) of senescent ECs. The nano-emulsions were loaded with a fluorescent probe (CY5.5 TPB and NR668) to allow detection of senescent ECs covering athero-susceptible sites at risk for diagnosis and monitoring purposes using fluorescence imaging, and a therapeutic vasoprotective agent for delivery and regeneration of the protective endothelial function. The results obtained with *in vitro* and *ex vivo* studies have shown that the developed theranostic nano-carriers specifically target (within 1 hr at 4°C and 37°C) the cultured and native senescent ECs induced by pro-senescent inducer (Angiotensin II) and with minimal off-target cell surface interactions and that the theranostic nano-carriers accumulate preferentially at well-defined sites characterized by disturbed flow and low shear, thereby providing important information, based on the fluorescent signal, regarding the localization and the extent of the area at risk. The novelty of the project is the targeting of the atherogenesis process at a very early step characterized by the induction of endothelial senescence and dysfunction by taking advantage of distinct surface features appearing at the senescent ECs surface such as expression of the adhesion molecules VCAM-1 and ICAM-1. The *in vivo* studies of the theranostic nano-emulsions are still in process and we are expecting that the administration of theranostic nano-carriers will affect minimally hemodynamic parameters, and that they will remain in the circulation to accumulate preferentially at arterial sites exposed to disturbed flow. Furthermore, subsequent to the local accumulation, nano-carriers will deliver on-site the therapeutic agent to help restore the endothelial function and vascular homeostasis by the combined action involving normalization of the level of oxidative stress and the redox-sensitive genes, and the restoration of an efficient endothelial formation of NO.

Project 2: Liposomes as carrier of drug molecules for biomedical applications

In parallel with the above-mentioned study, our objective was to develop and characterize pH-sensitive liposomes as nano-carriers for anticancer drug, doxorubicin (DOX). The most important requirement for these liposomes is that, they should be stable at physiological pH, and disrupt in slightly acidic media, such as the tumor microenvironment, to release their DOX load. The major challenge for encapsulating DOX in pH sensitive liposomes lies in the

fact that this drug is soluble at low pH (when the pH-sensitive liposomes are not stable), but the DOX aqueous solubility decreases in the pH conditions corresponding to the stability of the pH-sensitive liposomes. The study of pH-sensitivity of liposomes was conducted using carboxyfluorescein (CF) encapsulated in high concentration, i.e. quenched, and following the dye dequenching as sensor of the liposome integrity. Different lipids (dioleoyl phosphatidyl ethanolamine (DOPE), palmitoyl-oleoyl phosphatidyl ethanolamine (POPE) and dimyristoyl phosphatidyl ethanolamine (DMPE)) were used, associated with alpha-tocopheryl succinate (α-TOS) as stabilizing agent to prepare liposomes by polycarbonate membrane extrusion method. The impact of (i) the chemical nature of lipids and (ii) the lipid/stabilizing agent ratio on the pH sensitivity of the liposomes was studied. These lipid:α-TOS combinations were found to form stable liposomes at pH 7.4, with the higher encapsulation efficiency obtained with the lipid POPE (27 %). The integrity of membrane of the liposomes was significantly pH-dependent with a possible modulation as a function of the concentration of α-TOS. As the concentration of α-TOS increased, the breakdown of liposomes and release of CF were slowed down. Incubation time also had an impact on the release of the dye from the liposomes because incubation of liposomes at acidic pH for longer period of time increased the extent of the liposome disruption in acidic pH. Optimized liposome formulations were then selected for the encapsulation of DOX by an active loading procedure, i.e. driven by a difference in pH inside and outside the liposomes. Numerous experimental conditions were explored, in function of the pH gradient and liposome composition, which allowed identifying critical parameters for the efficient DOX encapsulation in pH-sensitive liposomes. Values of the pH gradient were shown to be a crucial parameter in the DOX loading. It was further observed that the encapsulation efficiency of DOX in pH sensitive liposomes depends on the nature of the –PE lipid (higher with POPE). And among different liposomal formulations of POPE, higher encapsulation efficiencies (95-100 %) were resulted with α-TOS and α-TOS/CHOL combination as compared to CHEMS and CHEMS/CHOL combination. Moreover, in function of the composition, the presence of cholesterol also increased the encapsulation of DOX and helps in the retention of the DOX inside the liposomes.

Currently, we are working on a project to develop a new strategy for surface modification of the liposomes by using the amphiphilic polymer derivative, in order to achieve better control of the surface modification. This will facilitate the grafting of a range of ligands, proteins or antibodies, at the surface of the liposomes with slight modification in the polymer structure, keeping the liposome composition, method of preparation and all the rest of the parameters constant. The liposomes were prepared by polycarbonate membrane extrusion technique

using dioleoyl phosphatidyl choline (DOPC) as lipid, with addition of the amphiphilic polymer (PMAO:N_3PEGNH_2:J-2000) during the preparation of liposomes. Then a model dye (JS-050) was attached covalently with the azide groups available at the surface of the liposomes. So far, we have got good initial results, in terms of functionalization and attachment of the dye, and we are moving towards grafting of ligands at the surface of the liposomes for targeted drug delivery applications.

APPENDICES

Oral communications:

- Asad Ur Rehman, Mayeul Collot, Thierry F. Vandamme, Nicolas Anton: **Development of a novel strategy for formulation and functionalization of nano-emulsions for targeted drug delivery**, *26th International Conference on Bioencapsulation*, **Strasbourg, France** August 27-29, **2019.**

- Asad Ur Rehman, Thierry F. Vandamme, Nicolas Anton: **pH-sensitive liposomes**. *Journée de l'UMR*, **château de Liebfrauenberg, France** December 18-19, **2017.**

Poster communications:

- Asad Ur Rehman, Ziad Omran, Halina Anton, Yves Mély, Salman Akram, Thierry F. Vandamme, Nicolas Anton: Investigation on the impact of chemical nature of lipids and composition of liposomes on the pH-sensitivity of doxorubicin loaded liposomes. *The 46th Annual meeting and exposition of the Control Release Society*, **Valencia, Spain** July 21-24, **2019.**

- Asad Ur Rehman, Mayeul Collot, Andrey S. Klymchenko, Salman Akram, Bilal Mustafa, Thierry F. Vandamme, Nicolas Anton: A novel approach for the development of surfactant-free polymeric nano-emulsions, *Journées du campus d'Illkirch (JCI)*, **Strasbourg, France** April 1-2, **2019.**

- Asad Ur Rehman, Ziad Omran, Halina Anton, Yves Mély, Salman Akram, Thierry F. Vandamme, Nicolas Anton: Formulation, optimization and characterization of doxorubicin loaded pH-sensitive liposomes, *European Materials Research Society (E-MRS) 2018 Spring Meeting*, **Strasbourg, France** June 18-22, **2018.**

Original research articles:

- Asad Ur Rehman, Mayeul Collot, Andrey S. Klymchenko, Salman Akram, Thierry Vandamme, Nicolas Anton: **A novel strategy to formulate tunable functionalized oil-in-water nano-emulsions**. (In progress)

- Asad Ur Rehman, Eugenia Belcastro, Mayeul Collot, Andrey S. Klymchenko, Schini-Kerth Valerie, Thierry Vandamme, Nicolas Anton: **Development of targeting theranostic nanocarriers for early detection and treatment of endothelial senescence**. (In progress)

- Asad Ur Rehman, Mayeul Collot, Andrey S. Klymchenko, Salman Akram, Bilal Mustafa, Thierry Vandamme, Nicolas Anton: **Spontaneous nano-emulsification with tailor-made amphiphilic polymers and related monomers**, European Journal of Pharmaceutical Research 1(1) 27-36 (2019)

- Rehman AU, Omran Z, Anton H, Mély Y, Akram S, Vandamme TF, Anton N: **Development of doxorubicin hydrochloride loaded pH-sensitive liposomes: Investigation on the impact of chemical nature of lipids and liposome composition on pH-sensitivity**, European Journal of Pharmaceutics and Biopharmaceutics 133, 331–338 (2018)

As co-author:

- Akram S, Wang X, Vandamme TF, Collot M, Rehman AU, Messaddeq N, Mély Y, Anton N: Toward the Formulation of Stable Micro and Nano Double Emulsions through a Silica Coating on Internal Water Droplets. Langmuir, 12;35(6) 2313-2325 (2019)

Book chapters:

- Rehman Asad Ur, Akram Salman, Seralin Aidar, Vandamme Thierry and Anton Nicolas: **Lipid nanocarriers: Formulation, properties, and applications**, in "Smart Nanocontainers: Fundamentals and Emerging Applications", (2019) Series: Micro and Nano Technologies, ISBN 978-0-12-816770-0

UNIVERSITÉ DE STRASBOURG

Asad Ur REHMAN

EDSC
École Doctorale des Sciences Chimiques

Development of new strategies for formulation and functionalization of lipid nano-carriers for biomedical applications

Abstract

The lipid nano-carriers are considered as mainstream delivery systems for lipophilic drugs because of their biocompatibility, high drug loading capacity and good biodistribution and pharmacokinetic profile. Nano-emulsions (NEs) constitute high-potential nano-carriers (NCs) for hundreds of applications owing to their reservoir capability, with main drawbacks being the limitations of their surface modification and stability. This thesis focuses on the development of strategies to overcome these limitations and also on the applications of the lipid NCs for imaging and targeted drug delivery. The study begins with the synthesis of an amphiphilic polymer, specially designed to exhibit an important tunable surface-active property. The azide-decorated NEs were first developed, treated with a model dye (JS050) and optimized to obtain maximum grafting, followed by replacement of the dye by a biotin-containing ligand. Ligand-decorated NEs were treated with streptavidin and investigated for the biotin-streptavidin reaction by gel electrophoresis. In the second strategy, maleimide-decorated NEs were prepared and loaded with a fluorescent probe, to allow detection of senescent endothelial cells (ECs) covering athero-susceptible sites at risk, for diagnosis and monitoring purposes, and/or a therapeutic agent for regeneration of the protective endothelial function. The maleimide decorated NCs were treated with antibodies (anti-VCAM1) and theranostic potential of these NCs was evaluated *in vitro* and *ex vivo*. The results have shown that the theranostic NCs specifically target (within 1 hr at 4°C/37°C) cultured and native senescent ECs and accumulate preferentially at well-defined sites characterized by disturbed flow and low shear. The novelty of the project lies in the fact that these theranostic NCs target endothelial senescence at very early stages, therefore reducing the risk of development of a subsequent advanced calcified atherosclerotic plaque. In parallel, pH-sensitive liposomes encapsulating doxorubicin (DOX)/dye, were developed and investigated for their pH-sensitivity. The resulting liposomes were found to be very sensitive to pH and maximum release of the dye/drug was obtained at pH 5.5. The loading of DOX was mainly dependent on the values of the pH gradient and the nature of lipid and stabilizer. Moreover, the breakdown of liposomes as well as release of dye/drug from the liposomes was inversely related to the amount of the stabilizer, while addition of cholesterol helped in the prolonged retention of DOX inside the liposomes. To conclude, the developed strategies overcome, very efficiently, the limitations associated with the surface functionalization and stability of the lipid nano-carriers, making them the ideal candidates for the biomedical imaging and targeted drug delivery applications.
Keywords: Lipid nano-carriers, polymer, nano-emulsions, targeted drug delivery, surface functionalization, imaging, endothelial senescence, theranostic, antibody, ligands, pH-sensitive liposomes, doxorubicin, cholesterol

Résumé

Les nano-transporteurs lipidiques sont considérés comme des systèmes d'administration classiques pour les médicaments lipophiles en raison de leur biocompatibilité, de leur capacité de charge médicamenteuse élevée, de leur bonne biodistribution et de leur profil pharmacocinétique. Les nano-émulsions (NE) constituent des nano-transporteurs (NT) à potentiel élevé pour des centaines d'applications en raison de leur capacité de réservoir, avec les principaux inconvénients étant les limites de leur modification de surface et de leur stabilité. Cette thèse porte sur le développement de stratégies visant à surmonter ces limitations, ainsi que sur les applications des NT lipidiques pour l'imagerie et l'administration ciblée de médicaments. L'étude commence par la synthèse d'un polymère amphiphile spécialement conçu pour présenter une propriété tensioactive accordable importante. Les NE décorés d'azides ont d'abord été développés, traités avec un colorant modèle (JS050) et optimisés pour obtenir un greffage maximal, puis remplacés par un ligand contenant de la biotine. Les NE décorés de ligands ont été traités avec de la streptavidine et analysés pour déterminer la réaction biotine-streptavidine par électrophorèse sur gel. Dans la deuxième stratégie, des NE décorés au maléimide ont été préparés et chargés avec une sonde fluorescente afin de permettre la détection de cellules endothéliales sénescentes (CE) couvrant des sites athéro-sensibles à risque, à des fins de diagnostic et de surveillance, et / ou un agent thérapeutique pour la régénération de la fonction endothéliale protectrice. Les NT décorées avec maléimide ont été traitées avec des anticorps (anti-VCAM1) et le potentiel théranostique de ces NT a été évalué *in vitro* et *ex vivo*. Les résultats ont montré que les NT théranostiques ciblent spécifiquement (en moins d'une heure à 4 ° C / 37 ° C) des CE sénescentes cultivées et indigènes et s'accumulent de préférence à des sites bien définis caractérisés par un écoulement perturbé et un faible cisaillement. La nouveauté du projet réside dans le fait que ces NT théranostiques ciblent la sénescence endothéliale à des stades très précoces, réduisant ainsi le risque de développement d'une plaque d'athérosclérose calcifiée avancée ultérieure. En parallèle, des liposomes sensibles au pH encapsulant la doxorubicine (DOX) / colorant ont été développés et étudiés pour déterminer leur sensibilité au pH. Les liposomes obtenus se sont révélés très sensibles au pH et une libération maximale du colorant / médicament a été obtenue à pH 5,5. La charge de DOX dépendait principalement des valeurs du gradient de pH et de la nature des lipides et des stabilisants. De plus, la dégradation des liposomes ainsi que la libération de colorant / médicament à partir des liposomes étaient inversement proportionnelles à la quantité de stabilisant, tandis que l'ajout de cholestérol facilitait la rétention prolongée de DOX dans les liposomes. En conclusion, les stratégies développées surmontent très efficacement les limitations associées à la fonctionnalisation de surface et à la stabilité des nano-transporteurs lipidiques, ce qui en fait le candidat idéal pour l'imagerie biomédicale et les applications ciblées d'administration de médicaments.
Keywords: Nano-transporteurs lipidiques, polymère, nano-émulsions, administration ciblée de médicaments, fonctionnalisation de surface, imagerie, sénescence endothéliale, théranostic, anticorps, ligands, liposomes sensibles au pH, doxorubicine, cholestérol