UNIVERSITÉ DE SHERBROOKE
Faculté de génie
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Physico-chemical Characterization of Layers of Intact Liposomes for Drug Release Applications

Caractérisation physico-chimique de couches de liposomes intacts pour des applications en libération d'agents actifs

Thèse soumise pour l'obtention du diplôme de
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Résumé

Les vésicules lipidiques, communément appelées liposomes, retiennent beaucoup d'attention dans les travaux visant le développement de systèmes de libération contrôlée permettant une libération d'agents actifs efficace tout en limitant les réactions systémiques chez l'hôte. Toutefois, dans la plupart des cas, les liposomes injectés dans la circulation sanguine sont rapidement éliminés par le système immunitaire et seulement une infime fraction rejoint la cible même lorsque les liposomes stabilisés avec du poly(éthylène glycol) (PEG) sont utilisés.

La littérature scientifique est abondante dans le développement, la caractérisation et la validation de suspensions de liposomes, particulièrement dans le domaine biomédical. Toutefois, l'immobilisation de couches de liposomes intacts, pouvant trouver des application dans les domaines de la chromatographie, des modèles cellulaires et de la libération localisée d'agents actifs, reste pourtant beaucoup moins étudiée. En fait, une surface garnie de liposomes immobilisés a été validée comme système de libération localisée par Vermette et ses collègues. Bien que quelques articles scientifiques soient disponibles sur la caractérisation de liposomes immobilisés sur des surfaces, plusieurs propriétés physico-chimiques de ces couches complexes, telles que les comportements élastique/viscoélastique, l'adsorption de protéines, la multi-libération et le module de Young, ont besoin d'être étudiées afin d'être mieux comprises. Cette étude vise donc à apporter de nouvelles informations fondamentales sur ces couches de liposomes intacts afin d'identifer les critères de design afin de développer un système de libération localisée et contrôlée "sur mesure" pour une application donnée.

Le premier chapitre de cette thèse revoit les phénomènes fondamentaux d'interactions entre les liposomes et les surfaces solides. Les techniques utilisées afin d'immobiliser des liposomes à l'intérieur et/ou à la surface de différents substrats sont discutées. Finalement, les propriétés des liposomes utilisés pour la libération d'agents actifs sont brièvement revues.

Le deuxième chapitre présente l'immobilisation de couches de liposomes sur des surface solides par le lien NeutrAvidin-biotin. La construction des ces couches a été suivie

par spectroscopie des photoélectrons de rayons-X (XPS) et par la microbalance à cristal de quartz avec mesure de la dissipation d'énergie (QCM). Le QCM a aussi été utilisé afin d'étudier l'adsorption dynamique de protéines sur ces couches de liposomes. Le relarguage silmultané de deux molécules fluorescentes encapsulées dans les liposomes a aussi été étudié dans le but de montrer que les couches de liposomes immobilisés peuvent agir comme un système de libération séquencée.

Le troisième chapitre présente l'extraction du module de Young de ces couches de liposomes intacts à l'aide de mesures de forces faites par microscopie à force atomique (AFM). Des courbes de forces obtenues par AFM, une estimation du module de Young de ces couches hydratées est obtenue en utilisant une théorie de mécanique de contact basée sur le modèle de Hertz.

La combinaison de plusieurs couches de liposomes et de différents agents actifs puis une meilleure compréhension des propriétés physico-chimiques de ces couches permettront de contrôler les profils de libération de plusieurs composantes encapsulées dans ces liposomes. Toutefois, il est clair que chaque système de libération d'agents actifs doit être conçu de façon à répondre au cahier des charges dicté par une application spécifique.

Abstract

Interest in lipid vesicles, commonly named liposomes, as drug carriers has increased over the last 20 years. Because of the simplicity of their preparation, there has been considerable interest to find a way to fabricate drug delivery systems which sustained a good release without inducing any systemic reactions into the human body. However, in most cases, liposomes injected into the blood stream are rapidly cleared from the system and only a fraction reaches the target site even when poly(ethylene glycol) (PEG)-coated "Stealth" liposomes are used.

The scientific literature is abundant on the development, characterization and validation of liposome suspensions, particularly in the biomedical fields. However, much less is known on the surface immobilization of layers of intact liposomes, which can find applications in several fields including drug-partitioning chromatography, cell structure mimicking, and localised drug release. In fact, surface-bound liposomes have been validated as localized drug release systems by Vermette and colleagues. Although some papers are available on the characterization of surface-immobilized layers of intact liposomes, many physicochemical properties of these complex layers as elastic/viscoelastic behaviour, proteins adsorption, multi-delivery capacity and Young's modulus still need to be elucidated. This study aims to bring some new fundamental information on these layers of intact liposomes in order to identify design criteria to develop tailor-made localized drug release systems for given applications.

The first chapter of this thesis reviews the fundamental phenomena of the interactions between liposomes and solid substrates. Various techniques that have been used to immobilize intact liposomes onto and into different substrates are addressed. Finally, properties of liposomes used as drug delivery systems are also briefly reviewed.

The second chapter presents the immobilization of liposome layers on solid surfaces by the NeutrAvidin-biotin link. The construction of these layers have been followed-up by X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance (QCM) with energy dissipation monitoring. QCM is also used to study the dynamics of protein adsorption on these surface-bound liposomes. The simultaneous release of two fluorescent

probes from these liposome layers has been investigated with the aim to validate surface-bound liposomes as multi-release delivery system.

The third chapter aims to calculate Young's moduli of layers of intact liposomes from atomic force microscopy (AFM) force measurements. From AFM force measurements, estimations of Young's moduli of these well hydrated layers are obtained using the mechanical contact theory based on the Hertz model.

Combining multiple layers of liposomes with different molecules and understanding some of the physicochemical properties of the layers bring possibility to modulate release kinetic profiles of multi-components encapsulated into surface-bound liposomes. However, it is clear that each drug delivery system needs to be customized to meet the requirements dictated by a specific application.

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Introduction générale

Mise en contexte

Les vésicules lipidiques, plus connues sous le nom de liposomes, consistent en une organisation de lipides dans un milieu aqueux. Selon la concentration des lipides, leur nature et la solution dans laquelle ils se trouvent, ils adoptent une structure suivant la loi de l'énergie libre minimum.[1]

Cette structure lipidique est semblable à la plus petite unité vivante du corps humain, soit la cellule. La membrane d'un liposome est formée d'une double couche lipidique où les chaînes hydrophobes des lipides se retrouvent à l'intérieur de la membrane et leurs têtes hydrophiles à l'extérieur exposés au milieu aqueux. Cette structure permet l'encapsulation de molécules. Les molécules encapsulables peuvent être hydrophiles ou lipophiles et se logeront dans le cœur de la vésicule (hydrophile) ou dans la bicouche lipidique (lipophiles).[2]

Selon les lipides utilisés, leur proportion et le processus de fabrication, il est possible d'obtenir différentes structures, multi ou unilamellaire, et différentes tailles de vésicules allant de quelques nanomètres à plusieurs microns. De plus, par une sélection de lipides appropriés, il est possible de contrôler la charge de la membrane pouvant ainsi favoriser des interactions électrostatiques. Il est également possible de décorer la surface de la membrane des liposomes par des molécules actives comme des anticorps, des protéines ou des polymères pouvant ainsi aider à cibler l'action ou la destination des liposomes.[2]

Depuis quelques années, les liposomes sont utilisés comme véhicules de principes actifs à des fins médicales. Ils peuvent relarguer leur contenu de façons localisée et contrôlée permettant de limiter l'utilisation de principes actifs diminuant ainsi les effets secondaires reliés aux traitements.[3]

Il est possible de munir la surface des vésicules de poly(éthylène glycol) (PEG) afin de prolonger le temps de circulation dans le corps,[4] améliorant ainsi la capacité de ces liposomes à atteindre la zone à traiter comme dans le cas d'une tumeur. La présence des

PEG permet une certaine protection contre le système immunitaire du fait qu'ils diminuent l'adsorption de protéines à la surface de la membrane liposomale. L'adhésion de protéines est responsable de l'activation du complément.[5] Une adsorption moindre diminue donc l'attaque de l'organisme hôte envers les corps étrangers que sont les liposomes.

L'immobilisation de liposomes sur une surface est une méthode étudiée pour la fabrication de systèmes de libération d'agents actifs de façons contrôlée et localisée. Pour obtenir un système efficace, il est primordial de construire un système stable qui permettra une libération lente et continue dans le temps. L'encapsulation de différents agents actifs ainsi qu'une organisation structurée de liposomes lors de l'immobilisation pourraient permettre une libération séquencée de ces agents dans leur milieu.[6] De cette façon, il serait possible de produire des implants induisant une réponse biologique spécifique à leur surface.

La modification de surfaces, par l'immobilisation de liposomes dans une perspective de surface biomédicale est un concept qui a déjà été proposé.[6-8] Il a été démontré que l'immobilisation de liposomes contenant de l'orthovanadate de sodium, un agent ciblant l'angiogenèse, sur une membrane d'éthylène-propylène fluoré (FEP), a inhibé la formation de micro-vaisseaux sanguins à partir d'une paroi aortique de rat lors d'essais d'angiogenèse *in vitro*.[9] Dans une autre étude, l'immobilisation de liposomes contenant un antibiotique, la levofloxacine, à la surface de lentilles cornéennes a démontré la capacité de limiter l'infection bactérienne *in vitro*.[10]

Dans une autre optique, ces surfaces rendent possible l'utilisation des liposomes pour créer des substrats fonctionnalisés en incluant des molécules dans les membranes liposomales. Par exemple, des surfaces créées par lithographie exhibant des liposomes immobilisés ont été fabriquées dans le but de proposer des surfaces biologiquement actives.[11] D'autres substrats, ayant comme base des films lipidiques, ont aussi servi de base à l'immobilisation de liposomes.[12,13]

L'utilisation de surfaces exposant des liposomes stables est envisagée pour l'étude des interactions entre des protéines membranaires et des membranes dans des conditions se rapprochant de celles *in vivo*. Comme l'activité et la mobilité des protéines sont réduites

lorsqu'elles sont mises dans un film lipidique,[14,15] l'immobilisation de liposomes intacts avec ces protéines devient tout à fait appropriée. Des surfaces ont donc été produites avec des liposomes contenant ces protéines membranaires permettant ainsi de conserver leur activité protéique.[16] De plus, l'organisation des liposomes immobilisés peut être contrôlée par l'ancrage de liposomes munis de brins d'ADN avec des brins d'ADN complémentaires présents sur la surface ciblée.[17,18]

Des puces (*micro-array*) exposant différents types de liposomes pourraient aussi être utilisées pour étudier, avec un criblage à haut rendement (*high-throughput screening*), les interactions entre des molécules pharmaceutiques et des protéines membranaires contenues dans les liposomes immobilisés.[19-21] Le « *microcontact printing* », une technique pour produire des interfaces biomimétiques pour des applications de biosenseurs, peut être utilisé pour fabriquer des surfaces avec des liposomes immobilisés permettant aussi des analyses à haut rendement de composantes interagissant avec les membranes cellulaires ou pour sonder les interactions entre cellules vivantes et membranes synthétiques.[22]

Dans une perspective de travail avec des surfaces dédiées à la culture cellulaire ou à la production de surfaces bioactives par l'encapsulation de facteurs de croissances dans des liposomes immobilisés, le contrôle des propriétés mécaniques de ces couches minces dynamiques est essentiel. Sachant que la flexibilité d'un substrat peut agir sur la croissance et la mort cellulaire,[23,24] les caractéristiques mécaniques d'une telle surface deviennent des éléments de base dans la conception d'un substrat dédié à l'étude des interactions avec le Vivant.

Les principales techniques de caractérisation pour l'étude des liposomes immobilisés sont :

- l'encapsulation de molécules fluorescentes et le suivi de leur libération dans le temps par spectrophotométrie ;
- l'addition de lipides avec sondes fluorescentes dans la membrane liposomale et leur observation par microscopie optique ;

- l'imagerie par microscopie à force atomique (AFM) permettant de caractériser la topographie de la surface ;

- la quantification d'espèces chimiques par spectroscopie des photoélectrons de rayons X (XPS).

Ces techniques confirment la présence des liposomes sur la surface et leur intégrité mais fournissent peu d'information relative aux propriétés mécaniques des surfaces.

Des techniques relativement récentes proposent des moyens d'analyses permettant une caractérisation plus approfondie des propriétés mécaniques des surfaces. Par exemple, la microbalance à cristal de quartz avec mesure de la dissipation (QCM)[25] et l'analyse de la résonance des plasmons de surface (SPR)[26] permettent l'observation indirecte de modifications d'un film déposé sur une surface en temps réel. Ces techniques, par un modèle mathématique, permettent de calculer la masse déposée et l'épaisseur du film, respectivement. De plus, le QCM permet d'observer s'il y a une variation d'énergie lors de la modification de surface, signe d'une variation de la viscosité au niveau du film.

L'adhésion de liposomes, comme modèle cellulaire, sur un cristal de QCM a permis d'étudier l'adhésion de vésicules sur une surface et ainsi d'observer que les vésicules réagissent comme des corps viscoélastiques.[27] L'immobilisation de liposomes à l'aide de brins d'ADN et leur observation par QCM ont aussi permis d'observer un comportement viscoélastique par la mesure de la dissipation d'énergie à travers les couches de liposomes.[18,28]

L'AFM en mode de mesure de forces d'interactions est depuis longtemps utilisée pour sonder des échantillons et en déterminer le module de Young par la théorie de Hertz ; la théorie de contact entre deux corps élastiques.[29] De la même façon, des mesures faites sur des liposomes adsorbés ont permis d'estimer le module de Young de vésicules lipidiques par l'application de la théorie de la mécanique du contact de Hertz.[30,31]

Objectifs des travaux

L'analyse de ces travaux antérieurs a inspiré la réalisation de cette thèse doctorale afin de mieux comprendre les propriétés physico-chimiques de couches de liposomes intacts immobilisés sur des substrats solides et ce, pour arriver à contrôler ces propriétés en fonction d'une application visée.

Les objectifs spécifiques de cette thèse étaient :

- d'étudier de façons physico-chimique et mécanique les étapes d'immobilisation des différents éléments requis pour la construction de couches de liposomes ;
- d'étudier l'adsorption non-spécifique de protéines sur les couches de liposomes intacts immobilisés ;
- de moduler la séquence de libération d'agents actifs par une organisation spécifique de liposomes immobilisés et intacts ;
- d'extraire le module de Young des couches de liposomes immobilisés.

Cette étude qui porte sur la caractérisation de liposomes immobilisés sur des substrats solides devrait apporter de nouvelles informations fondamentales concernant la compréhension de ce système complexe. De meilleures connaissances permettront la fabrication et l'amélioration de systèmes de libération contrôlée en fonction du cahier des charges imposé.

Dans cette thèse, le lecteur est invité à lire un premier chapitre présentant une revue de littérature dédiée à l'étude des différents systèmes de libération de médicament utilisant des liposomes immobilisés ou ensachés. L'interaction des vésicules lipidiques avec les surfaces, les techniques d'immobilisation des liposomes ainsi que les propriétés des liposomes sont revues. Dans ce chapitre, les liens sont établies entre la technique d'immobilisation, le type de liposomes choisis et le but recherché à savoir, la production d'un système de libération contrôlée possiblement utilisée dans un milieu *in vivo*. Ce chapitre a été publié dans la revue *Current Drug Delivery* (2004, vol. 3, p. 299).

Dans un deuxième chapitre, le lecteur est amené à observer les résultats de la caractérisation physico-chimique de substrats solides recouverts de couches de liposomes intacts. Chacune des étapes de construction est suivie par deux techniques : le XPS et le QCM afin de démontrer la présence des composantes ajoutées lors de chacune des étapes de la fabrication des surfaces. Par QCM, la capacité des liposomes immobilisés à minimiser l'adsorption non-spécifique de protéines venant d'une solution de sérum de veau fœtal est étudiée. De plus, à l'aide d'une technique de fluorescence, la capacité de libération du système dans le temps et la possibilité d'agir sur la séquence de libération d'agents actifs par l'encapsulation de deux molécules ont été démontrées. Ce chapitre a été publié dans la revue *Langmuir* (2007, vol. 23, p. 7679).

Dans un troisième et dernier chapitre, les propriétés mécaniques des liposomes immobilisés sont étudiées à l'aide de mesure de forces faites par AFM. Dans cette étude, le comportement viscoélastique des liposomes immobilisés sur une surface est confirmé puis le module de Young du système est estimé à l'aide de modèles de mécanique du contact suivant la théorie de Hertz.[29] Ce chapitre a été accepté pour publication dans la revue *Langmuir* (sous presse, 2008).

Finalement, la thèse se termine par une conclusion générale et des annexes présentent les courbes étalons utilisées en fluorescence ainsi que la logique de calcul du programme informatique utilisé pour l'extraction du module de Young.

Points d'originalité du travail

- Immobilisation de liposomes sur un film de PEG lié en utilisant une solution de PEG en conditions de « cloud-point » ;
- caractérisation de ce système par XPS, QCM et AFM ;
- démonstration de la capacité du système d'empêcher l'adsorption non-spécifique de protéines venant d'une solution de sérum de veau fœtal ;
- démonstration de la capacité de libération d'agents actifs sur une période de 300 heures ;

- démonstration de la possibilité de varier la cinétique de libération de différentes molécules par l'organisation des couches de liposomes ;

- étude du module de Young (module élastique) des couches de liposomes immobilisés en utilisant l'AFM.

Contributions

Tous les résultats d'expériences mentionnés dans cette thèse ont été obtenus par l'étudiante. Le plan expérimental ainsi que l'analyse et l'interprétation des résultats ont été réalisés par l'étudiante avec l'aide de son Directeur de thèse, le Professeur Patrick Vermette. Seules les analyses de XPS ont été effectuées par le personnel de l'Institut des matériaux et systèmes intelligents (IMSI) de l'Université de Sherbrooke.

Le programme informatique utilisé pour le traitement des données brutes de l'AFM a été programmé par Alain Gervais selon les directives de l'étudiante et de son Directeur de thèse. La vérification du bon fonctionnement de ce programme a été faite par l'étudiante.

Références

(1) Israelachvili, J. *Intermolecular and Surface Forces*; Academic Press: New York, **1992**.

(2) Lasic, D. *Liposomes: From Physics To Applications*; Elsevier: New York, **1995**.

(3) Allen, T. M.; Cullis, P. R. *Science* **2004**, *303*, 1818-1822.

(4) Papahadjopoulos, D.; Allen, T. M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K. D.; Woodle, M. C.; Lasic, D. D.; Redemann, C. *Proc .Natl. Acad. Sci. U.S.A* **1991**, *88*, 11460-11464.

(5) Anderson J. M.; Gristina A. G.; Hanson, S.; Harker L. A.; Johnson R. J.; Merritt K.; Naylor P. T.; Schoen, F. *Host reactions to biomaterials and their evaluation;* Academic Press: New York, **1996**; pp 165-214.

(6) Vermette, P.; Griesser, H. J.; Kambouris, P.; Meagher, L. *Biomacromolecules* **2004**, *5*, 1496-1502.

(7) Lunelli, L.; Pasquardini, L.; Pederzolli, C.; Vanzetti, L.; Anderle, M. *Langmuir* **2005**, *21*, 8338-8343.

(8) Danion, A.; Brochu, H.; Martin, Y.; Vermette, P. *J. Biomed. Mater. Res., Part A* **2007**, *82A*, 41-51.

(9) Vermette, P.; Meagher, L.; Gagnon, E.; Griesser, H. J.; Doillon, C. J. *J. Controlled Release* **2002**, *80*, 179-195.

(10) Danion, A.; Arsenault, I.; Vermette, P. *J. Pharm. Sci.* **2007**, 96, 2350-2563.

(11) Michel, R.; Reviakine, I.; Sutherland, D.; Fokas, C.; Csucs, G.; Danuser, G.; Spencer, N. D.; Textor, M. *Langmuir* **2002**, *18*, 8580-8586.

(12) Yoshina-Ishii, C.; Boxer, S. G. *J. Am. Chem. Soc.* **2003**, *125*, 3696-3697.

(13) Yoshina-Ishii, C.; Miller, G. P.; Kraft, M. L.; Kool, E. T.; Boxer, S. G. *J. Am. Chem. Soc.* **2005**, *127*, 1356-1357.

(14) Brian, A. A.; McConnell, H. *Biochemistry* **1984**, *81*, 6159-6163.

(15) Salafsky, J.; Groves, J. T.; Boxer, S. G. *Biochemistry* **1996**, *35*, 14773-14781.

(16) Knoll, W.; Frank, C. W.; Heibel, C.; Naumann, R.; Offenhäusser, A.; Rühe, J.; Schmidt, E. K.; Shen, W. W.; Sinner, A. *Reviews Mol. Biotechol.* **2000**, *74*, 137-158.

(17) Pfeiffer, I.; Hook, F. *J. Am. Chem. Soc.* **2004**, *126*, 10224-10225.

(18) Stadler, B.; Falconnet, D.; Pfeiffer, I.; Hook, F.; Voros, J. *Langmuir* **2004**, *20*, 11348-11354.

(19) Ramanujan, C. S.; Sumitomo, K.; de Planque, M. R. R.; Hibino, H.; Torimitsu, K.; Ryan, J. F. *App. Phys. Lett.* **2007**, *90*, 033901.

(20) Stamou, D.; Duschl, C.; Delamarche, E.; Vogel, H. *Angew. Chem., Int. Ed.* **2003**, *42*, 5580-5583.

(21) Kalyankar, N. D.; Sharma, M. K.; Vaidya, S. V.; Calhoun, D.; Maldarelli, C.; Couzis, A.; Gilchrist, L. *Langmuir* **2006**, *22*, 5403-5411.

(22) Kohli, N.; Vaidya, S.; Ofoli, R. Y.; Worden, R. M.; Lee, I. *J. Colloid Interface Sci.* **2006**, *301*, 461-469.

(23) Lo, C. M.; Wang, H. B.; Dembo, M.; Wang, Y. L. *Biophys. J.* **2000**, *79*, 144-152.

(24) Wang, H. B.; Dembo, M.; Wang, Y. L. *Am. J. Physiol Cell Physiol.* **2000**, *279*, C1345-C1350.

(25) Rodahl, M.; Hook, F.; Fredriksson, C.; Keller, C. A.; Krozer, A.; Brzezinski, P.; Voinova, M.; Kasemo, B. *Faraday Discuss.* **1997**, 229-246.

(26) Lofas, S.; Malmqvist, M.; Ronnberg, I.; Stenberg, E.; Liedberg, B.; Lundstrom, I. *Sens. Actuators,B* **1991**, *5*, 79-84.

(27) Luthgens, E.; Herrig, A.; Kastl, K.; Steinem, C.; Reiss, B.; Wegener, J.; Pignataro, B.; Janshoff, A. *Meas. Sci. Technol.* **2003**, *14*, 1865-1875.

(28) Graneli, A.; Edvardsson, M.; Hook, F. *ChemPhysChem* **2004**, *5*, 729-733.

(29) Hertz, H. *J. Reine Angew. Math.* **1881**, *92*, 156-171.

(30) Liang, X. M.; Mao, G. Z.; Ng, K. Y. S. *Colloids Surf., B* **2004**, *34*, 41-51.

(31) Liang, X. M.; Mao, G. Z.; Ng, K. Y. S. *J. Colloid Interface Sci.* **2004**, *278*, 53-62.

Chapitre 1

Drug delivery systems using intact immobilized liposomes: A comparative and critical review

Systèmes de libération contrôlée d'agents actifs utilisant des liposomes immobilisés et intacts: Une revue comparative et critique

Chapitre adapté d'une publication:

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1.1 Abstract

Liposomes sustain considerable interest to develop a way to fabricate drug delivery systems to provide a good release without inducing any systemic reactions into the host. However, in many cases, liposomes injected into the blood stream are rapidly cleared from the system and only a fraction reaches the target site even when poly(ethylene glycol) (PEG)-coated liposomes are used. Composite drug delivery systems with liposomes i.e., liposomes linked to other substrates can be good candidates for certain type of drug release to achieve a localised treatment.

This paper reviews the fundamental phenomena of the interactions between liposomes and solid substrates. Then, we address various techniques that have been used to immobilize intact liposomes onto and into different substrates. Finally, properties of liposomes used as drug delivery systems are briefly reviewed.

1.2 Résumé

Les vésicules lipidiques, communément appelées liposomes, retiennent beaucoup d'attention dans les travaux visant le développement de systèmes de libération contrôlée permettant une libération d'agents actifs efficace tout en limitant les réactions systémiques chez l'hôte. Toutefois, dans la plupart des cas, les liposomes injectés dans la circulation sanguines sont rapidement éliminés par le système immunitaire et seulement une infime fraction rejoint la cible même lorsque les liposomes stabilisés avec du poly(éthylène glycol) (PEG) sont utilisés. Par contre, un système de libération contrôlée « composite » incluant des liposomes, c'est-à-dire l'utilisation de liposomes liés à un autre substrat, pourrait être une bonne alternative afin d'obtenir une libération contrôlée tout en ayant un traitement localisé.

Dans cette perspective, ce travail revoit les principes fondamentaux lors d'interactions entre les liposomes et les surfaces solides. Par la suite, une revue de littérature mentionne les différentes techniques d'immobilisation de liposomes utilisées sur des surfaces ou à l'intérieur de diverses matrices. Finalement, une synthèse des différentes propriétés des liposomes utilisés dans les systèmes de libération contrôlée est faite.

1.3 Abbreviations

AFM: Atomic Force Microscopy
aGM1: Gangliotetraosyl ceramide
Biotin-X-DHPE: *N*-((6-biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine
CHOL: Cholesterol
CL: Cardiolipin
CMC: Critical Micelle Concentration
Cryo-TEM: Cryofracture observations by Transmission Electron Microscopy
DCP: Diacetylphosphate
DMPC: 1,2-Dimyristoyl-*sn*-glycero-3-phosphoCholine
DMPE: 1,2-Dimyristoyl-*sn*-glycero-3-phosphoEthanolamine
DMPG: 1,2-Dimyristol-*sn*-glycero-3-phosphoGlycerol
DOPC: 1,2-Dioleoyl-*sn*-glycero-3-phosphoCholine
DOPE: 1,2-Dioleoyl-*sn*-glycero-3-phosphoEthanolamine
DPGS: 1,2-Dipalmitoyl-*sn*-glycerol-3-succinate
DPPC: 1,2-Dipalmitoyl-*sn*-glycero-phosphoCholine
DPPE: 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoEthanolamine
DSC: Differential Scanning Calorimetry
DSPC: 1,2-Distearoyl-*sn*-glycero-3-phosphoCholine
DSPE: 1,2-Distearoyl-*sn*-glycero-3-phosphoEthanolamine
DSPE-PEG: 1,2-Distearoyl-*sn*-glycero-3-phosphoEthanolamine-N-[Poly(ethylene glycol)]
EDTA: EthyleneDiamineTetraacetic Acid
ELISA: Enzyme-Linked Immunosorbent Assay
EM: Electron Microscopy
EPC: Egg PhosphatidylCholine
ESR: Electron Spin Resonance
GD_{1b}: Disialoganglioside
GM_1: Monosialoganglioside
GT_{1b}: Trisialoganglioside
GV: Giant vesicle
LISA: Liposome ImmunoSorbent Assays
LUV: Large Unilamellar Vesicle
MLV: Multilamellar Vesicle
MPS: Mononuclear Phagocyte System
NMR: Nuclear Magnetic Resonance
PA: Phosphatidic Acid

PC: PhosphatidylCholine
PCS: Photon Correlation Spectroscopy
PE: PhosphatidylEthanolamine
PEG: Poly(ethylene glycol)
PG: PhosphatidylGlycerol
PI: PhosphatidylInositol
PLA_2: Cobra Venom Phospholipase A_2
PS: PhosphatidylSerine
Pt: Platinum
QCM: Quartz Crystal Microbalance
QCM-D: Quartz Crystal Microbalance with Dissipation monitoring
RES: Reticulo-Endothelial System
RICM: Reflection Interference Contrast Spectroscopy
SAM: Self Assembled Monolayer
SFA: Surface Force Apparatus
SiO_2: Silicon dioxide
Si_3N_4: Silicon nitride
SPR: Surface Plasmon Resonance
SUV: Small Unilamellar Vesicle
T_m: Gel-to-liquid-crystalline phase transition temperature
TiO_2: Titanium oxide
ULV: Unilamellar Vesicle
UV: Ultraviolet

1.4 Introduction

Interest in lipid vesicles, commonly named liposomes, as drug carriers has increased over the last 20 years. Because of the simplicity of their preparation, there has been considerable interest to find a way to fabricate drug delivery systems which sustained a good delivery without inducing any systemic reactions into the human body. However, in most cases, liposomes injected into the blood stream are rapidly cleared from the system and only a fraction reaches the target site even when poly(ethylene glycol) (PEG) coated "Stealth" liposomes are used.

Composite drug delivery systems with liposomes i.e., liposomes linked to other substrates can be considered for certain type of drug delivery. However, interactions of liposomes with solid surfaces and with other materials or substrates have not been well addressed in the literature, mainly because this concept has been only recently proposed as possible drug delivery systems.

This paper reviews the fundamental phenomena of the interactions between lipid vesicles and solid substrates. First, the interactions between "naked" lipid vesicles and solid surfaces are reviewed. Then, we address various techniques that have been used to immobilize lipid vesicles onto and into different substrates such as collagen and chitosan. Finally, properties of liposomes used as drug delivery systems are briefly reviewed.

The literature relative to liposomes is often confusing and there can be found apparent contradictions. Some of the discrepancies in the literature may perhaps be attributable to variability in the liposome formulation and/or in the method of preparation used to fabricate liposomes and/or in the *in vitro* and animal models used to test drug delivery by liposomes. Space and information available does not permit us to discuss possible reasons for all the major discrepancies. Nor do we aim to cite comprehensively; there is a fair degree of duplication in concepts guiding some of the studies, and at times even the execution of studies, which has led us to omit some reports. To limit the scope of this manuscript, we have omitted to review studies on the physico-chemical properties of lipid vesicles and their methods of preparation. For a review, the reader is referred to Part 1 of Lasic's book

about liposomes,[1] as this subject, fundamental to drug delivery by liposome technology, requires a thorough and lengthy treatment. We trust the reader to make appropriate connections to other relevant literature when necessary.

1.5 "Naked" lipid vesicles and their interactions with solid surfaces

Interest in the interactions between lipid vesicles and solid surfaces has increased over the last few years. Because of the simplicity of preparation, there has been considerable interest in the manufacture of planar bilayer systems via the exposure of "suitable" surfaces to unilamellar lipid vesicles. Planar supported phospholipid bilayers provide simple models of biological membranes since constituents of the vesicles can be transferred on the surface-restrained bilayers[2] and can be studied using a wide variety of spectroscopic and microscopic techniques.[3]

Most papers involving the adsorption of lipid vesicles onto solid surfaces from a suspension report some possible interactions between the lipid vesicles and the surfaces (e.g., hydrophobic, van der Waals, and electrostatic forces). These interactions can induce important stresses on the membranes resulting in deformation, flattening and even rupture.[4] Any modification of the curvature will induce a variation in the dynamic behaviour of the membrane, which can even lead to fusion and/or rupture of the membrane as often seen in vesicle adsorption experiments[2,4,5] because the driving force of the deformation is a competition between the bending and the adhesion energies.[6] Many groups have studied the mechanisms of adsorption, fusion and rupture onto solid surfaces as a means to produce supported lipid bilayers.[2,5,7,8] It is widely believed that lipid vesicles rupture upon contact with surfaces, forming a more or less uniform "flat" bilayer.

The formation (e.g. kinetics) of lipid bilayers onto solid surfaces and their properties (e.g., thickness, roughness, stability, defects, etc.) are believed to depend on the properties of the liposomes (including the type of lipids used),[7,9,10] the experimental conditions during the immobilization procedure (e.g., pH, temperature, chelating agents)[7,10] and the properties of the solid surfaces (e.g., charges, hydrophobicity, structure).[2,7,10] In spite of the practical importance of vesicle fusion on solid surfaces, very little is known about the mechanisms and the kinetics of formation of supported bilayers from a lipid vesicle suspension. There

are at least two potential mechanisms by which a bilayer could form. The addition of a layer of lipids to a hydrophobic monolayer can occur via (i) a vesicle-dependent process, and/or (ii) formation can occur by individual monomer phospholipids transferring from the vesicles to the aqueous phase and from there to the surface.[2] However, at high concentrations, the process of lipid bilayer formation is most likely vesicle-dependent,[2] because the critical micelle concentration (CMC) of most lipids used to produce liposomes is very low (from 10^{-8} to 10^{-6} M).

The mechanism of lipid spreading, following vesicle adsorption, has been studied by Rädler et al.[8] In their study, swelling of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was observed by reflection interference contrast spectroscopy onto different coated glass surfaces and mica samples. Their study showed that the phenomena of lipid film spreading onto a hydrophobic surface can be explained by two mechanisms: (i) the sliding of a single bilayer on a water film or (ii) the rolling of two superposed bilayers as a "tank track motion".[8]

Even if planar bilayer membranes are relatively well defined, depending on the fabrication method used, there may be problems when using them as model membrane systems, primarily due to the decreased fluidity of the phospholipid monolayer caused by the constraint imposed by the underlying solid-like alkyl monolayer which is often used in the construction of such membranes.[11,12] Thus, layers of intact immobilized liposomes have been proposed as an alternative approach to study biological membranes. Such usage hinges, however, on the ability to bind liposomes intact onto carrier surfaces, which runs counter the general notion that lipid vesicles are prone to destabilization when in contact with a surface.

Kasemo's group observed by quartz crystal microbalance with dissipation monitoring (QCM-D) intact vesicles on different surfaces. QCM-D experiments measure changes in quartz frequency as a sign of mass adsorption and change in the dissipation energy, which is an indication of the mechanical properties of the adsorbed mass. Small unilamellar vesicles (SUV) (25 nm mean diameter obtained by photon correlation spectroscopy (PCS) with very narrow distribution, according to the authors) made from egg

phosphatidylcholine (EPC) by sonication were observed intact onto oxidized gold layers.[13] The authors combined QCM measurement and dissipation monitoring to study lipid membranes and demonstrated the capacity of the technique to distinguish different types of surface-specific adsorption kinetics and to determine the properties of the membrane. Lipid vesicle adsorption was also observed by QCM-D assays onto silicon dioxide (SiO_2), onto self assembled monolayer (SAM) made of methyl-terminated thiols on gold and onto oxidized gold surfaces. The results obtained by frequency and dissipation shifts analysis showed (i) the formation of a lipid monolayer on methyl terminated SAM surface, (ii) the formation of a lipid bilayer on the SiO_2 surface and (iii) adsorption of intact vesicles on oxidized gold surface.[13] However, when comparing adsorption on SiO_2 and on oxidized gold surfaces, the authors proposed that the adsorption onto SiO_2 began with intact vesicles adsorption followed by the rupture of vesicles leading to the formation of the observed bilayers.[13] When considering adsorption as a competition between adhesive and bending energies as proposed by Seifert et al.,[6] the authors observed that the high polarizability of the oxidized gold surface maximizes the attractive potential that resulted in vesicle adsorption.[13] Although the QCM-D is a surface-sensitive technique to carry out surface adsorption experiments, it is in our opinion that conclusive information on the stability of surface-immobilized liposomes cannot be obtained using this technique alone. It would be warranted to perform independent stability experiments (e.g., by measuring the release of a fluorescent probe from the surface-adsorbed liposomes) to support the statement that lipid vesicles adsorbed intact on oxidized gold surfaces. This experiment would have been essential to validate the usefulness of the QCM-D technique to probe the stability of surface-bound liposomes.

Similar experiments concerning adsorption of lipid vesicles were also made by Reimhult et al.[14,15] onto different substrates including SiO_2, silicon nitride (Si_3N_4), titanium oxide (TiO_2), oxidized gold and oxidized platinum (Pt). However, EPC vesicles were from different diameters ranging from 25 to 200 nm. As observed by Keller et al.,[13] vesicles adsorbed intact onto oxidized gold layers but also onto oxidized Pt and TiO_2 surfaces and vesicles ruptured on SiO_2 and Si_3N_4.[15] When comparing adsorption of vesicles of different sizes on SiO_2 and TiO_2 surfaces, the authors observed that the adsorption behaviour was not dependent of the vesicle size and that deformation of the vesicles was larger on SiO_2

surfaces when compared to TiO_2 surfaces.[14] A two-step bilayer formation was proposed by the authors onto SiO_2 and Si_3N_4 surfaces; vesicles adsorbed intact up to a critical coverage after then, membranes were found to rupture and bilayer formation occurred for all vesicles used in this study.[14,15] On TiO_2, oxidized gold and oxidized platinum surfaces, vesicles adsorbed intact at all densities.[15] The authors proposed that the different observed adsorption behaviour depended on the strength of the interaction between the vesicles and the surface.[14,15]

1.6 Immobilization of lipid vesicles

When considering liposomes as drug delivery systems, their integrity is one of the principal concerns in order to obtain sustained delivery of a therapeutic agent. Ways to immobilize liposomes onto solid surfaces or into solid or gel matrices have been proposed by some groups as it is exemplified by some papers found in the literature. However, immobilization of intact liposomes can be done with "naked" vesicles only with very specific types of surfaces and coverage density.[13-15] Attachment of liposomes needs some specific strategy to avoid the rupture of the vesicles and the rapid loss of the therapeutics. Burst release is often not desired since it can induce local and even systemic toxicity in the host organism and the rapid end of a sustained treatment.

Some strategies have been investigated to fix intact liposomes onto solid surfaces and into 3-D matrices. These strategies are (i) steric entrapment,[16-19] (ii) attachment by hydrophobic interactions,[20] (iii) covalent linkage,[21,22] and (iv) specific binding.[23,24]

Steric entrapment has been used in different kinds of natural and synthetic gels and in natural matrices such as collagen sponges. Liposomes are often mixed with the gel, which is, at first, in the liquid state. Following the mixing, depending on the kind of gel used, changes in the experimental conditions such as temperature induces phase transition from a liquid to a gel-solid state. Liposomes are thus trapped inside the gel-matrix during this transition step. For collagen, the sponge is simply saturated with a liposome-loaded suspension and the liposomes are then trapped into the matrix during the impregnation process.

As it will be discussed below, the combination of the lipid vesicles and the matrix seems to provide better stability to the liposomes.[16-19,25,26] Less rupture and slower diffusion are often observed resulting in sustained release of the encapsulated product.

Immobilization of lipid vesicles using hydrophobic interactions needs a substrate with hydrophobic moieties. When soaked in a liposome suspension, it is believed that surface-immobilized hydrophobic ligands penetrate the vesicle membranes to get to the hydrophobic region of the liposomes. In this way, it is believed that liposomes can be anchored intact to the surface. However, as proposed by Hara et al., it is fair to postulate that the hydrophobic moieties can either stabilize or destabilize the vesicles when penetrating the lipid bilayers.[20] Membrane rupturing can be observed leading to a continuous bilayer.

Covalent immobilization of liposomes to a reactive substrate has been used in gel column to obtain more stable fixation of the coated liposomes.[21,22] Very recently, Khaleque et al. used disulfide linkages to fixe liposomes on a modified Sephacryl gel.[21] Both liposomes and gel particles were bearing mercapto moities. Conversion of mercapto groups to pyridinedithio groups by a reaction with 2,2'- dipyridyl disulfide lead to immobilization of liposomes on polymer gel. Furthermore, under reduction conditions the vesicles were detached and the gel could be re-used.[21] Covalent immobilization was also studied by Mao et al.[22] using 4-nitrophenyl chloroformate in presence of dimethylaminopyridine for activated silica gel particles. In this way, vesicles were covalently fixed by the phosphorus head of the lipids into the membrane to the activated gel leading to vesicle immobilization.

Specific binding involves strong interactions between two complimentary molecules. It can be interaction as avidin-biotin as often seen or immunological interactions such as human IgG and anti-human IgG. When using specific binding, the liposomes and the substrate have to possess the complementary ligands.

Among the immobilization methods that have been used, some work has been done for liposome immobilization on silica-gel or gel beads.[21-23,27,28] The ultimate goal in these cases was to obtain a mimetic membrane to do drug membrane partitioning for chromatography experiments. The immobilization was required for partitioning and to

obtain stability. Objectives as slow release and localized delivery as it is desired in drug delivery systems was not required.

1.6.1 Specific immobilization of intact liposomes on solid surfaces

In this section, we will first examine some examples reporting that liposomes can remain "intact" upon contact with solid surfaces. Several studies have reported the binding of different proteins to model membranes by surface plasmon resonance (SPR) using immobilized lipid vesicles.[29,30] Biotinylated liposomes were immobilized on carboxylated dextran matrix sensor chips (CM5, Pharmacia) containing streptavidin. However, none of these studies demonstrated that the probe lipid vesicles were attached intact onto the hydrogel coatings.

In a series of studies, Yang et al. investigated the specific avidin-biotin immobilization of unilamellar liposomes in gel beads[23] and in fused-silica capillaries[31] for chromatographic analysis of drug-membrane partitioning. Stable and high-yield immobilization of the liposomes in the gel beads was reported by avidin-biotin multiple-site binding.[23,31] Covalent immobilization of unilamellar liposomes in gel beads for chromatography was also reported by the same research group.[32] Although Yang et al. reported that liposomes were specifically immobilized and remained stable in the gel beads or in the fused-silica capillaries,[23,31,32] insufficient and only macroscopic observations of the surface-bound liposomes were reported in support of this claim.

Specific interaction of lipid vesicles with surfaces has been also explored by Rongen et al.[33] These authors developed liposome immunosorbent assays (LISA) for the detection of the cytokines interferon-γ and interleukin-2.[33] In their study, biotinylated liposomes were successfully used to detect cytokines immobilised onto micro-titre plates. However, no surface characterization of the surface-bound liposomes was carried out and integrity of the bound liposomes was not demonstrated.

Shibata-Seki et al. imaged in liquid, using the atomic force microscopy (AFM) technique, human IgG-containing liposomes adsorbed onto anti-human IgG-coated substrates.[34] AFM imaging of the liposomes showed "balloon-like" structures. It was also found that the quality (contrast and/or reproducibility) of the AFM images depended both

on the type of cantilever tips used and on the load forces at which AFM tips were scanned over the sample.[34] While specific binding via IgG/anti-IgG interaction would be capable of binding liposomes onto surfaces, it should, however, also be investigated whether liposomes might be attracted to the substrates via non-specific (physisorptive) interaction forces. In addition, the method by which the lipid vesicles were "decorated" with human IgG was not described, making it difficult to draw firm conclusions.

QCM measurements were used to characterize immunoliposome-hapten interactions.[35] QCM immuno-sensors often suffer from low sensitivity since the mass change by antigen binding to the crystal surface can be small. Liposomes containing lipid-tagged antibodies on their surfaces were therefore successfully used as signal-enhancing reagents. The binding of immunoliposomes to the hapten-coated quartz crystal was decreased but not completely inhibited by the pre-incubation of immunoliposomes with "free" hapten.[35] This finding suggests the presence, although small, of non-specific interactions between the hapten-coated liposomes and the solid surface. Liposome stability upon binding to the solid surface was not reported.

Liebau et al.[36] investigated the adhesion of receptor-coupled liposomes using a QCM. Briefly, a supported planar bilayer containing glycolipid ligands was transferred onto a quartz surface and subsequently exposed to lectin Concanavalin A-bearing liposomes.[36] An important finding from their study was that specific interactions could be differentiated from non-specific liposome attachment, but could not be eliminated.

In a study, a method was proposed to measure weak point forces exerted on giant vesicles adhering to solid surfaces by sub-micron pinning centres.[37] The method was based on the analysis of the shape of the contact line between lipid vesicles and substrates using reflection interference contrast microscopy (RICM). It was shown that the formation of domains of tight adhesion (adhesion plaques) between biotinylated liposomes and streptavidin-coated surfaces was a function of time.[37] Three regimes were observed: (i) a spontaneous initial formation of adhesion plaques, (ii) a regime of fast growth, and (iii) a slowing down behaviour of the patch growth, which resulted in saturation of the tight contact area.[37]

More recently, a method based on intact lipid vesicles immobilized on a SPR sensor was developed to obtain dissociation equilibrium constants, *Kd*, between phosphatidylcholine vesicles and cobra venom phospholipase A_2 (PLA_2).[12] Briefly, the foundation of the substrate was a gold surface functionalised with a mixed monolayer of alkylthiols end-capped with a biotin moiety and short-chain poly(ethylene glycol)-terminated alkylthiols. The surface was then used to immobilize streptavidin with high coverage, specificity and activity. Lipid vesicles were finally immobilized intact onto the streptavidin layer and successfully used to quantify the binding of PLA_2.[12]

The specific adhesion of unilamellar vesicles with an average diameter of 100 nm on functionalised surfaces mediated by molecular recognition has been investigated in detail by Pignataro et al.[38] Two complementary techniques, AFM and QCM, were used to study the adhesion of liposomes made of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and varying concentrations of *N*-((6-biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Biotin-X-DHPE) to avidin-coated gold surfaces.[38] Monitoring the adhesion of the biotin-doped vesicles to avidin-coated gold surfaces by QCM revealed an increased shift in resonance frequency with increasing biotin concentration up to 10 mol % Biotin-X-DHPE indicating that the liposomes were "docking" onto the avidin-functionalised surface. The authors also found that, with increasing biotin-lipid concentration (up to 30 mol %) the height of the surface immobilized liposomes decreased considerably, up to the point where vesicle rupture occurred.[38]

In an attempt to identify specific factors that promote binding of targeted liposomes to defined target surfaces (e.g., cells), liposomes containing biotinylated phosphatidyl-ethanolamine were used.[39] It was demonstrated that the avidity of a targeted biotinylated liposome for streptavidin-coated enzyme-linked immunosorbent assay (ELISA) plates and cells was influenced by liposomal lipid composition, the amount of targeting molecule present per liposome, the nature of the targeting ligand and the target surface.[39] The apparent affinity of biotinylated liposomes for surface-associated streptavidin was found to increase with increasing biotin content.[39] The presence of a hydrocarbon spacer composed of at least 6 carbons between biotin and the amino group of phosphatidylethanolamine

significantly increased the apparent affinity of the liposomes for surface-associated streptavidin as opposed to a biotinylated lipid containing no "spacer" arm.[39] In addition, removal of cholesterol from the liposomes resulted in a 2 to 6 fold, depending upon lipid concentration, decrease in the amount of liposome bound to the streptavidin-coated plates.

Liposome immobilization using specific interaction between NeutrAvidin™ and biotin and strategies of surface engineering has been proposed by Vermette et al. in order to get an application to stop angiogenesis in cancer treatment.[24] Briefly, an aldehyde surface was created by plasma polymerization and polyethylenimine was covalently bound to aldehyde groups present on the surface. Then, NHS-PEG-Biotin was covalently bound to the amine group. By strong affinity, NeutrAvidin™ molecules were fixed to PEG-Biotin present on the surface and finally, liposome containing PEG-biotinylated lipids were docked on free binding sites of NeutrAvidin™ molecules.[24] AFM images showed intact liposomes but their surface density was below "monolayer" packing. A low leakage rate was observed, probably caused by the PEGylated lipids in the membrane of the lipid vesicles providing better stability.[24] An *in vitro* experiment showed that angiogenesis was inhibited by liposome release of orthovanadate, even though a small amount of the drug was available.[24] The localized nature of this application has great advantages in ensuring effective delivery adjacent to the carrier and could result in considerable cost saving for expensive drugs as well as reduction of adverse side effects remote to the target site.[24]

From the examples cited above, it appears that lipid vesicles can be immobilized intact on solid substrates by specific interactions. If loaded with a therapeutic agent and remaining intact, layers of liposomes immobilized on solid substrates could make good candidates for local drug delivery applications from biomedical devices.

1.6.2 Three-dimensional matrices containing liposomes

Some studies report the combination of liposomes and a 3-D matrix to obtain (i) better stability of liposome by entrapment, (ii) slower diffusion of a therapeutic agent and (iii) a localized effect.[17,19,26,40] Collagen,[16,19,41] and different polymer gels or hydrogels,[18,40] chitosan,[17,42] have been considered to entrap liposomes. In this section, we will examine

some of these examples reporting 3-D matrices containing liposomes that could potentially be used as drug delivery systems.

1.6.2.1 Collagen-liposome systems

Weiner et al. sequestered insulin and labelled-growth hormone loaded liposomes in collagen-based gel matrix in order to get a drug delivery system providing a stable and non-toxic drug release.[16] Loaded-EPC stable multilamellar vesicles bearing or not fibronectin were mixed in rat tail collagen solution (0.3 or 0.9 % w/v). Studies with fibronectin bearing liposomes were carried out to observe if the protein could enhance sequestration of liposomes within collagen gel. Fibronectin was enzymatically linked to the lipid vesicles. Temperature-induced gelation was done at 37°C for 5 to 15 minutes. Electron microscopy (EM) showed lipid vesicles sequestered in a complex organisation of collagen fibres. From these EM observations, the authors proposed that there was no specific interaction between the unmodified liposomes (with no fibronectin) and the collagen gel.[16] But, in our opinion, insufficient characterizations have been carried out to support this claim. *In vivo* experiments performed in rats suffering of diabetes showed slower changes in the insulin and serum glucose levels in rats that had been injected with collagen-gel containing insulin loaded-vesicles preparation, than for rats that have been treated with insulin loaded-vesicles alone. Intramuscular injection in mice showed better retention when treated with labelled-growth hormone loaded-liposomes entrapped in collagen-gel than with labelled-growth hormone loaded-liposomes or free hormone alone. The authors also reported that fibronectin bearing liposomes loaded with the growth hormone significantly enhanced the retention of the hormone at the injection site.[16] The authors demonstrated that the liposome-collagen system provided a suitable release profile even if the total dosage provided in the collagen-liposome system corresponded to a lethal dosage. Fibronectin bearing liposomes entrapped in the collagen gel showed a slow release of the therapeutics.[16] The authors proposed that this system could be used (i) for slow release of bioactive drugs, (ii) has a topical application in the treatment of surgical or nonsurgical wounds and burns and (iii) as a local implant for sustained release of active agents.[16]

Drug delivery systems using porcine collagen shields have been studied for ophthalmic applications.[19] Cyclosporine A, an endecapeptide metabolite of the fungus *Tolypocladium*, has been loaded in large unilamellar vesicles (mean diameter of 340 nm) made of a 7:3 molar ratio of phosphatidylcholine (PC) and phosphatidylserine (PS) mixed with the detergent β-D glucopyranoside (0.2 mg/mol lipid). Instead of the conventional cyclosporine A used in drop formulation or dispersed "free" in collagen sponges, collagen shields containing cyclosporine A-loaded vesicles were investigated because of the liposome property to protect the encapsulated drug from metabolic enzymes present in tear fluids and corneal epithelium and for increasing intra-ocular drug delivery.[19] The collagen shields were simply soaked in a solution of liposomes loaded with cyclosporine A and tested for release in an *in vitro* experiment and for ocular penetration in rabbit eyes. The authors claimed that liposomes bind reversibly to the collagen shields and were released in an intact form.[19] This latter statement was based on unpublished results, so it is difficult to verify the basis of this claim. But, it can be postulated that the liposomes released from the collagen shields would be washed away by the tearing action.

Collagen sponges saturated with liposomes loaded with polymycin B, an antibacterian, were tested *in vivo* and *in vitro* as a drug delivery system.[41] Multilamellar liposomes were made with lecithin and cholesterol (CHOL) in a 2:1 ratio. Non-entrapped polymycin was removed by dialysis. The liposome-loaded sponges applied onto the infected wound by *P. aeruginosa* significantly reduced, after 8 days, the bacterial cell number below the critical value of invasive infection fixed at $1x10^5$ colony forming units per 1g resulting in effective treatment of pseudomonal infection in mice.[41] *In vivo* experiments showed that drug release was slower for encapsulated drug in liposomes than for the drug freely dispersed in collagen.[41] This observation demonstrated a better sustained release for encapsulated drugs which is desired in most drug delivery systems. The author proposed that this kind of antibiotic delivery system could be used for local infection.[41] But we submit that multilamellar liposomes do not provide an adjustable drug release in term of kinetics and drug loading capacity.

1.6.2.2 Chitosan-liposome systems

Chitosan is a natural polysaccharide prepared from deacetylation of chitin from shells of Crustaceans and it is known to be biodegradable and biocompatible.[43] Amelu et al. used chitosan gel to entrap liposomes because liposomes alone in blood plasma are often rapidly cleared.[42] PC liposomes containing either oleic or steric acid in a molar ratio of 10:1 were used to encapsulate both dapsone and bromothymol blue. Dapsone was used as a model lipophilic drug and bromothymol blue as a lipophilic marker for spectrophotometric analysis. The sonicated unilamellar vesicles were dialysed to remove the non-entrapped drug. Loaded liposomes and chitosan were mixed and jellified at 37°C for 30 minutes. In some samples, liposomes bearing carboxyl groups were attached to the amino groups of the chitosan gel by carbodiimide chemistry. By spectrophotometric assays, the authors obtained slower diffusion for liposomes coupled to the gel. The study showed that the drug release rate can be modulated by appropriate cross-linking degree and liposome composition.[42] The authors observed better stability in 1% mice plasma solution for the liposome-chitosan system in which liposomes were covalently attached than for the liposome-chitosan in which liposomes were not linked to the chitosan matrix and for the "free" liposome suspension.

More recently, Ruel-Gariépy et al. also used a combination of thermosensitive chitosan-based hydrogels and carboxyfluorescein loaded liposomes as an eventual formulation for tissue repair and drug delivery.[17] The thermosensitive gel was a mixture of chitosan solution and β-glycerophosphate; this mixture is liquid at room temperature but jellified as the temperature increases.[17] *In vitro* experiments were done to study the carboxyfluorescein release from the vesicles. Large unilamellar and multilamellar liposomes were made from different composition including EPC, CHOL, 1,2-dimyristol-*sn*-glycero-3-phosphoglycerol (DMPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) lipids. The study showed that the system combining liposomes and chitosan gel decreased the release rate of the encapsulated component compared to the liposomes or chitosan gel used separately.[17] As demonstrated by Weiner et al.,[16] the physical barrier provided by the vesicle membrane and the slower diffusion of the liposomes in the gel influence the release rate. Entrapment of lipid vesicles within chitosan gel matrices can

maintain vesicles at the delivery site and avoid rapid clearance by the body resulting in a better sustained delivery.[17] The authors observed that the release rate was dependent of the composition of the liposomes, the size of the liposomes and the presence of phospholipase in the release medium; increasing size of liposomes from 100 nm to 280 nm drastically decreased the released kinetics as well as the burst release.[17]

Even if these two studies addressed above showed better stability of the liposomes and slower diffusion rate of the drug, how would chitosan provide that stabilizing effect in the real body environment? Can it be hypothesized that harsh conditions of the biological fluid could destroy the matrix resulting in an attack towards the liposomes? Is 1% mice plasma representative of the human body conditions to assess the *in vivo* liposome stability?

1.6.2.3 Other hydrogel-liposome systems

Kim et al. combined hydrocortisone-loaded EPC and CHOL liposomes with Carbopol 934 hydrogel, a carboxyvinyl polymer, in order to get a targeted and sustained delivery to the skin.[40] Liposomes were made by sonication and were 224 nm (mean diameter). Non-entrapped hydrocortisone was removed by centrifugation. Liposome suspension was blended in the Carbopol gel until the homogeneity was confirmed by the distribution of radioactive labelled hydrocortisone. *In vivo* experiments during an eight-hour period on either normal or stratum corneum-removed skin showed that the gel-liposomes system provided higher and sustained concentration of hydrocortisone to the skin than did the conventional ointment.[40] This observation was proposed to be due to delayed diffusion of the adsorbed drug into the skin towards the systemic blood stream. To describe this phenomenon, the authors proposed that liposomes reduced percutaneous penetration and then, reduced systemic absorption.[40]

Bochot et al. prepared different kinds of calcein-loaded liposomes bearing negatively, positively, neutrally-charged and PEGylated lipids for mixing within thermosensitive Poloxamer 407 to develop a delivery system for ocular treatment.[25,26] Poloxamer 407 is a copolymer of ethylene glycol and propylene glycol that displays reverse thermal gel characteristics: it is liquid at room temperature and solid at body temperature. Lipid

vesicles made by extrusion had a mean diameter of 200 nm. Non-entrapped calcein was removed by exclusion-diffusion gel chromatography. ζ-potential measurements showed that Poloxamer interacted strongly with and adsorbed onto both negatively and positively-charged liposomes.[26] This latter observation was confirmed by size measurements which showed an increase in the diameter of the liposomes, up to 85 %, as a function of the Poloxamer concentration (from 0.9 to 27 % w/v).It would have been important to report the osmolarity of these solutions. Lipid vesicles close to neutrality did not show significant modification.[26] Fluorescent measurements showed a slower calcein release in sterically stabilized liposomes, i.e. containing PEGylated lipids, than non-sterically stabilized liposomes. The authors proposed that the calcein release could be due to the intrusion of the diblock polymer into the membrane of the vesicles.[26] The authors also suggested, that 1,2-distearoyl-*sn*-glycero-3-phosphatidyletanolamine-N-(poly(ethylene glycol)-2000) (DSPE-PEG(2000)) did not induce complete repulsion of the copolymer but provided a steric barrier reducing the possibility of insertion from the diblock within the membrane resulting in a better permeability of the vesicle.[25,26]

More recently, Glavas-Dodov et al. mixed lidocaine HCl-loaded lipid vesicles made of soy lecitin and CHOL (9:1 ratio) with Carbopol 940 hydrogel concentration of 1.5, 1.75 and 2 %.[18] Lidocaine HCl is an anesthetic agent. *In vitro* results showed a slower drug release for the hydrogel containing the drug-loaded liposomes than for the hydrogel containing the "free" drug alone. Also, Carbopol concentration did not show significant difference in terms of the drug entrapment.[18]

1.7 Properties of liposomes used in drug delivery systems and the relationships with their drug release characteristics

The interactions between drug delivery systems using layers of intact immobilized liposomes and living organisms have been poorly studied so far. Although interactions between "free" liposomes and living organisms or biological fluids certainly differ from those encountered with drug delivery systems using layers of intact immobilized liposomes, we submit that some observations observed between "free" liposomes and living organisms can, in part, be applied to drug delivery systems using layers of intact immobilized

liposome systems, as liposome composition can be similar. For instance, it is fair to postulate that complement activation could be involved with surface immobilized liposomes exposed to biological fluids as it is observed when some biomaterials are exposed to blood fluids or implanted in living organisms.[44] It is clear, however, that the nature of interactions with the cellular components would differ simply because these liposomes, if firmly attached to a solid substrate, would not be allowed to circulate within the living organisms. In this section, we believe, by reviewing the interactions between "free" liposomes and biological fluids or living organisms, along with their relationships with liposome composition and liposome size to meet the requirements of a functional drug delivery system, we will convey useful information that will highlight design criteria to construct functional drug delivery systems using layers of intact immobilized liposomes.

1.7.1 Effect of liposomes size on drug delivery

It is essential to examine the importance of modulating vesicle size for drug delivery applications. It has been found that the rate of clearance of particulate matter is proportional to particle diameter.[45-47] In fact, vesicle size is one of the most important factors determining the disposition of liposomes in the body. In general, larger liposomes are eliminated from the blood circulation more rapidly than smaller ones.[45-47] The overall amount of adsorbed proteins is markedly different. For example, small unilamellar vesicles (SUVs) adsorb approximately 40-times as much protein mass per mg of phospholipids as do large unilamellar vesicle (LUVs).[48] The amounts of liposomes remaining in the blood after 20 hrs were approximately the same for 85 and 130 nm liposomes, both for "naked" (EPC:CHOL; 2:1) and for PEGylated vesicles (EPC:CHOL:DSPE-PEG; 2:1:4 mol %). There were, however, substantial differences in spleen/liver distribution as well as in intra-hepatic distribution as well as in intra-hepatic distribution. Based on information collected to date, a liposome diameter of about 100 nm is likely to be an optimal size, not only for the more effective blood-to-tumour transfer of liposomes, but also for their longer retention in tumour tissues.[49] In a more recent study, however, it has been suggested that the pore size of tumour vessels varies depending on the type of tumour, the site of the tumour growth, and the degrees of tumour growth and regression.[50] In another study, Fisher et al. have shown that giant vesicles (GVs) formed from 1-palmitoyl-2-oleoyl-sn-glycero-3-

phosphocholine by electroporation were permeable to certain low molecular weight molecules such as the nucleic acid dye YO-PRO-1 and fluorescein diphosphate whereas conventional liposomes (large or small unilamellar liposomes) were not.[51] In addition, it was shown that non-membrane proteins, such as DNases or RNases, added to the selected GVs from the outside, were able to convert their substrate, which was strictly localized on the internal side of the membrane. This effect was only seen in GVs and was absent in conventional liposomes. The fact that these effects were only present in GVs obtained by electro-formation and not in conventional small liposomes is taken as in indication that certain physico-chemical properties of the bilayer are affected by the membrane curvature, although the mechanisms underlying such differences have not been established.[51] On the other hand, it was reported that phospholipid chain ordering and dynamics were very similar in small and large unilamellar vesicles.[52]

1.7.2 PEGylated-liposomes

The use of PEGylated-lipids in the formulation of liposomes seems to increase their circulation time in the blood stream.[53-64] In a study, in the absence of DSPC-PEG in DSPC:CHOL extruded liposomes, it was shown that liposomes frequently aggregated into large clusters of deformed liposomes.[65] Upon inclusion of small amounts of PEG-lipids, the liposomes become spherical and appear to be well separated in the micrographs.[65] The same general appearance was observed for all samples containing up to 10 mol % PEG-lipid.[65] Above this concentration, however, the cryo-TEM revealed the formation of a new aggregate of bilayered discs. This study showed that incorporation of more than 10 mol % PEG-lipid into DSPC and EPC lipid mixtures for liposome preparation is meaningless. At higher PEG-lipid concentrations the preparation will contain a large fraction of open bilayer discs,[65] structures that are of no use as vehicles for the delivery of water-soluble drugs.

It was also shown that the incorporation of >10 mol % DSPE-PEG(5000) or DSPE-PEG(2000) increased the transition temperature of DSPC vesicles by up to 10°C.[66] For concentrations of 10-20 mol % DSPE-PEG(2000) or DSPE-PEG(5000) there was evidence for two endothermic transitions.[66] At 100% PEG-lipid, only DSPE-PEG(350) exhibited a main endothermic transition; all other PEG-lipids did not exhibit a transition between 30 and 90°C (DSPC suspension exhibited a main transition temperature at 54.5°C and a small

pre-transition event at 52.0°C). For all PEG-lipids, the pre-transition peak temperature remained nearly constant with increasing PEG-lipid up to approximately 10 mol %, and disappeared at higher PEG-lipid concentrations.[66] By using wide-angle and low-angle diffraction, nuclear magnetic resonance (NMR), and differential scanning calorimetry (DSC), it was also shown that the incorporation of PEG-lipid changed the lipid phases. In a separate experiment, at low concentrations up to 7 mol % of DPPE-PEG(1000) and DPPE-PEG(3000) incorporated into DPPC liposomes, a single phase transition peak was observed suggesting some miscibility of the short chain PE-PEG with the PC bilayer. Beyond 7 mol %, a sharp particle size reduction coupled with complete loss of turbidity by 17 mol % suggested that the observed shoulder signified a transition from a multilamellar state to a mixed micellar phase.[67] In the case of long chain PE-PEG(12 000), the DSC thermograms indicated immiscibility of the DPPE-PEG(12 000) with the DPPC bilayer resulting in phase separation in the gel state. At concentrations of PE-PEG(5000) < 7mol %, there appeared to be some miscibility of the PE-PEG with DPPC. Beyond this concentration phase separation occurred, and beyond 11 mol %, transition to the micellar state was observed in addition to phase separation.[67] In addition, long chain PEG (> 5000 in molecular weight) appears unsuitable for preparing liposomes with long circulation times, and the observed formation of phase separated lamellae could be a possible explanation.[67]

Nicholas et al. demonstrated that PEG chains on the inside of vesicle bilayers can restrict encapsulation of water-soluble molecules.[68] The addition of up to 8 mol % of DSPE-PEG(2000) into liposomes made of DSPC:CHOL and EPC:CHOL reduces the permeability of carboxyfluorescein in buffer solution.[69] In contrast, the leakage of the more amphiphilic dye ethidium was not to any measurable extent affected by PEG-lipid inclusion. More importantly, it was found that liposomes composed of EPC or EPC with 5mol % DSPE-PEG(2000), displayed a dramatic increase in permeability when subjected to a medium composed of 20 mol % human serum in buffer.[69] Addition of 40 mol % cholesterol to the EPC bilayers reduced the observed release rate in human serum substantially, whereas no stabilizing effect was observed upon PEG-lipid inclusion.

Although DOPE when used to anchor short and intermediate chain length PEG prevents the formation of distinct phase separated lamellae, it was reported that bilayer

stability is reduced due to increased fluidity caused by the kinked DOPE.[67] In addition, they reported that DSPE-PEG(5000):DSPC mixtures have very similar trend to that observed with DPPE-PEG(5000):DPPC.[67] Incorporation of cholesterol resulted in more homogenous and stable lamellar structures. The formation of phase separated lamellae and mixed micellar states was completely inhibited by cholesterol concentrations of 30 mol % or greater.[67] It has been proposed that formation of phase separated lamellae can be caused by different factors. It can be influenced by the interaction of the long PEG chains leading to PEG chain-chain entanglement resulting in a segregation of PE-PEG in portions of the bilayer.[67] It is also possible that dehydration of the bilayer surface by longer chain PEG causing a reduction in the effective size of the PC polar headgroup, leading to reduced acyl chain spacing and segregation of the PE-PEG. Lehtonen and Kinnunen reported that transmembrane osmotic gradient can be induced by PEG in solution, which caused the shrinkage of liposomes.[70,71] Moreover, the presence of cholesterol abolished PEG-induced phase separation.

1.7.3 Effect on lifetime in bloodstream of the steric stabilization of liposomes

Typically, intravenously administered liposomes are taken up by cells of the mononuclear phagocytic system within 12 minutes. The biological stability of liposomes by modification with polymers such as poly(ethylene glycol) (PEG) is increased several folds as a result of the overlap of the PEG layers, i.e. steric stabilization by PEG.[55-59] Alza Corporation (Mountain View, CA, USA) has developed STEALTH® liposome technology, commonly PEGylated liposomes, to target drugs such as anticancer agents to a specific site in the body, reducing their toxicity. It is believed that STEALTH® liposomes can evade recognition by the immune system because of their PEG coating. The major role of surface modifications of lipid vesicles is to help overcome the rapid detection and uptake by the host defense system.[53-62,72,73] Thereby, the blood circulation of PEG-liposomes is prolonged and the uptake of the drug carrier by lymph node macrophages is decreased. A long circulation time increases the likelihood that the liposomes and their pharmaceutical contents will reach the sites of disease, thereby potentially increasing the efficacy and reducing the toxicity of potent medications. Addition of PEG-lipid anchor conjugates onto a liposome can also reduce aggregation during protein coupling.[74] Length and quantity of

PEG can be modulated to control the level of aggregation while preserving the binding ability of liposomes.

1.7.3.1 Biological response towards liposomes

The main site of clearance of particles, such as lipid-based drug carriers, from the circulation is the mononuclear phagocyte system (MPS), also referred to by the older term reticuloendothelial system (RES).[45] The MPS consists of circulating monocytes and of fixed monocytes of liver (Kupffer cells), spleen, lymph nodes, and bone marrow.[47,75,76] One of the main functions of the MPS is the removal of foreign particulate matter from circulation.[47,75,76] Other functions include defence against micro-organisms, parasites, and neoplastic cells and involvement in host responses to endotoxin, haemorrhagic shock, drug response, and response to circulating immune complexes.[47,75,76] MPS activity is often measured by colloid clearance methods.[47,75] Essentially, the rate at which test particles are removed from circulation is assumed to be proportional to the phagocytic capacity of the MPS. However, as suggested by Allen,[75] accurate measurement of phagocytic activity of the MPS depends on the dose of test particles being below saturating doses for the MPS, and below doses that deplete blood opsonins, which are postulated to be necessary for phagocytosis to occur.[75]

It is postulated that the reason why naturally occurring phospholipids are treated as foreign material, following *in vivo* administration is due to the opsonization of "naked" phospholipid surfaces by plasma proteins leading to recognition, binding, and uptake by the MPS.[62] The route of administration of liposomes appears to have an important effect on the rate of uptake by liver and spleen. Multilamellar vesicles (MLVs) regardless of composition caused greater MPS impairment than SUVs.[77] Depression of MPS activities could be due either to saturation of binding sites and uptake mechanisms with liposomes or to depletion of plasma opsonins by liposomes. In fact, it has been a matter of debate for some time whether the increased circulation times that are typically observed with higher lipid doses are a result of saturation of MPS uptake or depletion of blood opsonins.[78] It appears, however, that depletion of plasma opsonins has been rejected as playing a role in depression of MPS activity by some workers.[79,80]

To examine whether liver uptake of liposomes involves serum components or so-called opsonins, liposomes with different lipid compositions were incubated with freshly collected mouse serum and the mixture was then perfused through pre-washed mouse liver via portal vein.[81] Liposomes pre-incubated with buffer were used as a control. All of these liposomes contained PC:CHOL (10:5; molar ratio). In the absence of serum about 10 % of perfused liposomes composed of PC:CHOL were taken up by the perfused liver. Inclusion into liposome bilayer with 6.25 mol % of PE, PG, PI, monosialoganglioside (GM_1), gangliotetraosyl ceramide (aGM_1), disialoganglioside (GD_{1b}), trisialoganglioside (GT_{1b}) or PE-PEG(2000) with final lipid ratio of 10:5:1 (PC:CHOL:additional lipid; molar ratio) decreased liposome uptake by the liver.[81] Conversely, inclusion of the same molar percentage of PS, diacetylphosphate (DCP), phosphatidic acid (PA), cardiolipin (CL), or 1,2-dipalmitoyl-sn-glycerol-3-succinate (DPGS) into liposomes increased the liposome uptake by the perfused liver. Compared to the level of liver uptake in the absence of serum, serum enhanced the liver uptake of liposomes containing PE, PG, aGM_1 and GD_{1b}. A negative effect of serum on the level of liposome uptake by the liver was observed with liposomes containing PA and CL.[81] However, serum did not affect the level of liposome uptake by the perfused liver containing PI, GM_1, GT_{1b}, PE-PEG, PS, DCP, DPGS. Liposomes composed only of PC:CHOL also belong to this group. It should be pointed out that over 90% of serum-mediated liposome uptake by the perfused liver was blocked by pre-treatment of serum with either EDTA, EGTA/Mg^{+2} or high temperature (56°C for 30 minutes). These observations are suggesting that complement activation was responsible, at least in part, for serum-enhanced liposome uptake by the liver. In conclusion, liposomes can be taken up by the liver through a serum-independent clearance pathway or a pathway that required complement activation. It was also suggested that non-Kupffer cells were responsible in taking up liposomes containing CL and DCP. The Kupffer cells, however, were reported to be solely responsible for liver uptake of PS, PE, and aGM_1-containing liposomes.[81]

The difference in liposome pharmacokinetics and tissue distribution in different animal species were also examined by Liu et al.[82] For example, in mice, liposomes containing 10 % of either GM_1 or PE-PEG(5000) exhibited relatively long circulation time. Inclusion of the same molar percentage of PS into liposomes resulted in a rapid blood

clearance with a half-life of less than 10 minutes. The order of longevity of blood circulation time in mice for the various liposome types was PC:CHOL:GM_1 > PC:CHOL:PE-PEG(5000) > PC:CHOL > PC:CHOL:PS. In contrast to what it was observed in mice, GM_1-containing liposomes showed a very short circulation time in rats with an estimated half-life in blood of less than 10 minutes; over 100-fold less that that observed in mice.[82] Liposomes with or without PE-PEG displayed almost identical clearance kinetics in rats. Four hours after injection, approximately 57 % of the injected dose of liposomes composed of PC:CHOL remained in the blood compared to approximately 52 % for PEG-containing liposomes.[82] The effect of PE-PEG(5000) in prolonging liposome circulation time as observed in mice was not obvious in rats within the 4 hour time period. These observations therefore pointed the importance of choosing the appropriate animal model for the development of liposome-based drug delivery systems.[82]

The use of polymers (e.g., GM_1, PEGs) can increase the lifetime of lipid-based drug carriers. While ganglioside GM_1 substantially increased circulation time of liposomes in mice, it had the opposite effects in rats.[83] On the other hand, typically, PEGylated liposomes exhibit a circulation half-life of 12-20 hrs in rats or mice and 40-60 hrs in humans.[1,56,58,59] PEGylated liposomes exhibit significant accumulation into several important pathologies including solid tumours, infections, and inflammation. Although the grafted polymer approach clearly decreases protein adsorption (e.g., complement activation) and a significant improvement in circulation residency time, PEG coating of vesicles may not be optimal for every application as PEGs have been shown to interfere with the release of encapsulated materials and may interfere with effective targeting.

It is clear from the above discussions that proteins (e.g., opsonins) play an important role in liposome clearance. Then, it is important to appreciate the importance that PEGs play in "stabilizing" liposomes and also their interaction with proteins.

1.7.3.2 Repulsion mechanisms

It is clear that poly(ethylene glycol) (PEG) is by far the most widely used polymer to impart steric stabilization in liposome formulations. In addition, it should now be clear from the examples listed in this section that PEGs are capable of reducing protein

adsorption. But what are the mechanisms involved in the protein repellency of PEGs? What makes one PEG coating more resistant to protein adsorption than another? We believe that it is important for the reader to answer these questions and also to appreciate the proposed mechanisms (at least what are believed to be) of protein repellency offered for PEG materials. The protein resistance of PEG coatings has been associated with two main mechanisms. These are "steric repulsion" and/or "hydration/water structuring". These two mechanisms are associated directly with the interactions between protein molecules and the PEG surface. The reader is referred to a review paper written by Vermette and Meagher for further details.[84]

1.8 Conclusions

Drug delivery systems represent a major field of research. Many techniques have been elaborated to obtain controlled and localized drug release characteristics. Despite all the effort to produce highly stable vesicles, their clinical uses are still limited due to their rapid clearance from the host and/or uncontrolled release properties.

Composite drug delivery systems can become very good candidates to provide a controlled and localized release of a therapeutics. Combination of the advantages of the drug carriers, the lipid vesicle, and the substrate and/or the matrix, such as collagen or chitosan, for example, could lead to finely adjustable drug release properties at the target site and then limiting the clearance of the vesicles by the host.

In our viewpoint, the utilization of "naked" vesicles in composite drug delivery systems is not very promising since the possible stress imposed on the membrane during the immobilization or entrapment procedures can lead to the rupture of the vesicles. The utilization of PEGylated-liposomes can provide better stability and a parallel can be made with circulating sterically-stabilized liposomes, which appear in most cases to have a longer circulation time in most animal models than "naked" liposomes.

In this article, we have reviewed several papers on the surface immobilization and matrix entrapment of liposomes. The presence, and presumably the magnitude, of the non-specific interaction forces involved in the interaction process between liposomes and

solid/gel materials (e.g., solid surfaces and gel or solid matrices) can be influenced by different factors. For example, the use of PEG lipids, or other stabilising layers, in the liposome formulations could have a direct influence on the non-specific interactions. In our viewpoint, "naked" phospholipid vesicles exposed to biological fluids will be covered rapidly by proteins and this may disrupt surface-immobilised or matrix-entrapped liposomes. It appears reasonable to conjecture that the use of "naked" phospholipid vesicles would result in more pronounced non-specific interactions between the lipid vesicles and the "host" materials (e.g., solid surfaces and gel or solid matrices) than the use of PEGylated liposomes. Direct measurement of forces between different lipid bilayers showed a short-range attraction between lipid bilayers.[85-87] It is believed that the well-hydrated PEG gel layers generate a more pronounced steric repulsion at the collision surface than "naked" phospholipid headgroups.[84]

An important difference noted between the different studies reporting specific liposome immobilization on solid surfaces or within gel/solid materials was the nature of the "spacer arm" between the ligands and the phospholipid headgroups. For example, it has been reported by several authors that the presence of a hydrocarbon-based "spacer" between biotin and the amino group of phosphatidylethanolamine was sufficient to specifically attach the liposomes to streptavidin-covered surfaces. We submit that this should be viewed with caution. These hydrocarbon-based "spacer arms" are hydrophobic and thus are unlikely to extend into aqueous solution, as opposed to the more hydrophilic PEG spacer arm used by Vermette and colleagues. The length and hydrophobicity of the spacer arms could have a direct influence on liposome immobilization. But, unless proven, this statement will remain speculative.

Differences in experimental protocols among the various studies could explain, at least in part, the discrepancies reported in the literature. The time-scale of the experiment, the temperature used to attach the liposomes, the liposome formulation, the type of interlayers used to immobilize receptor molecules (e.g., avidin-like proteins) used in the specific immobilization of liposomes, the surface density of the receptor, etc., can all affect the presence or extent of non-specific interactions between the liposomes and the receptor-bearing coatings.

1.9 References

(1) Lasic, D. *Liposomes: From Physics To Applications*; Elsevier: New York **1995**.

(2) Plant, A. *Langmuir* **1999**, *15*, 5128-5135.

(3) Knobler, C. *Adv. Chem. Phys.* **1990**, *77*, 397-449.

(4) Lipowsky, R.; Seifert, U. *Mol. Cryst. Liq. Cryst.* **1991**, *202*, 17-25.

(5) Reviakine, I.; Brisson, A. *Langmuir* **2000**, *16*, 1806-1815.

(6) Seifert, U.; Lipowsky, R. *Phys. Rev. A* **1990**, *42*, 4768-4772.

(7) Steinem, C.; Janshoff, A.; Ulrich, W. P.; Sieber, M.; Galla, H. J. *Biochim. Biophys. Acta* **1996**, *1279*, 169-180.

(8) Radler, J.; Strey, H.; Sackmann, E. *Langmuir* **1995**, *11*, 4539-4548.

(9) Kumar, S.; Hoh, J. *Langmuir* **2000**, *16*, 9936-9940.

(10) Egawa, H.; Furusawa, K. *Langmuir* **1999**, *15*, 1660-1666.

(11) Dahmen-Levison, U.; Brezesinski, G.; Mohwald, H. *Thin Solid Films* **1998**, *329*, 616-620.

(12) Jung, L. S.; Shumaker-Parry, J. S.; Campbell, C. T.; Yee, S. S.; Gelb, M. H. *J. Am. Chem. Soc.* **2000**, *122*, 4177-4184.

(13) Keller, C.; Kasemo, B. *Biophys. J.* **1998**, *75*, 1397-1402.

(14) Reimhult, E.; Höök, F.; Kasemo, B. *J. Chem. Phys.* **2002**, *117*, 7401-7404.

(15) Reimhult, E.; Höök, F.; Kasemo, B. *Langmuir* **2003**, *19*, 1681-1691.

(16) Weiner, A. L.; Carpenter-Green, S. S.; Soehngen, E. C.; Lenk, R. P.; Popescu, M. C. *J. Pharm. Sci.* **1985**, *74*, 922-925.

(17) Ruel-Gariepy, E.; Leclair, G.; Hildgen, P.; Gupta, A.; Leroux, J. C. *J. Controlled Release* **2002**, *82*, 373-383.

(18) Glavas-Dodov, M.; Goracinova, K.; Mladenovska, K.; Fredro-Kumbaradzi, E. *Int. J. Pharm.* **2002**, *242*, 381-384.

(19) Pleyer, U.; Elkins, B.; Ruckert, D.; Lutz, S.; Grammer, J.; Chou, J.; Schmidt, K. H.; Mondino, B. J. *Curr. Eye Res.* **1994**, *13*, 177-181.

(20) Hara, M.; Yuan, H. Q.; Miyake, M.; Iijima, S.; Yang, Q.; Miyake, J. *Mater. Sci. Eng., C.* **2000**, *13*, 117-121.

(21) Khaleque, M. A.; Okumura, Y.; Yabushita, S.; Mitani, M. *Chem. Lett.* **2003**, *32*, 416-417.

(22) Mao, X. Q.; Kong, L.; Li, X.; Guo, B. C.; Zou, H. F. *Anal. Bioanal. Chem.* **2003**, *375*, 550-555.

(23) Yang, Q.; Liu, X. Y.; Ajiki, S.; Hara, M.; Lundahl, P.; Miyake, J. *J. Chromatogr., A* **1998**, *707*, 131-141.

(24) Vermette, P.; Meagher, L.; Gagnon, E.; Griesser, H. J.; Doillon, C. J. *J. Controlled Release* **2002**, *80*, 179-195.

(25) Bochot, A.; Fattal, E.; Gulik, A.; Couarraze, G.; Couvreur, P. *Pharm. Res.* **1998**, *15*, 1364-1369.

(26) Bochot, A.; Fattal, E.; Grossiord, J. L.; Puisieux, F.; Couvreur, P. *Int. J. Pharm.* **1998**, *162*, 119-127.

(27) Lunqvist, A.; Ocklind, G.; Haneskog, L.; Lundahl, P. *J. Mol. Recognit.* **1998**, *11*, 52-57.

(28) Lundahl, P.; Beigi, F. *Adv. Drug Delivery Rev.* **1997**, *23*, 221-227.

(29) Lange, C.; Koch, K. W. *Biochemistry* **1997**, *36*, 12019-12026.

(30) Stachowiak, O.; Dolder, M.; Wallimann, T. *Biochemistry* **1996**, *35*, 15522-15528.

(31) Yang, Q.; Liu, X. Y.; Miyake, J.; Toyotama, H. *Supramol. Sci.* **1998**, *5*, 769-772.

(32) Yang, Q.; Liu, X. Y.; Yoshimoto, M.; Kuboi, R.; Miyake, J. *Anal. Biochem.* **1999**, *268*, 354-362.

(33) Rongen, H. A. H.; Vannierop, T.; Vanderhorst, H. M.; Rombouts, R. F. M.; Vandermeide, P. H.; Bult, A.; Vanbennekom, W. P. *Anal. Chim. Acta* **1995**, *306*, 333-341.

(34) Shibata-Seki, T.; Masai, J.; Tagawa, T.; Sorin, T.; Kondo, S. *Thin Solid Films* **1996**, *273*, 297-303.

(35) Yun, K.; Kobatake, E.; Haruyama, T.; Laukkanen, M. L.; Keinanen, K.; Aizawa, M. *Anal. Chem.* **1998**, *70*, 260-264.

(36) Liebau, M.; Bendas, G.; Rothe, U.; Neubert, R. H. H. *Sens. Actuators, B* **1998**, *47*, 239-245.

(37) Albersdorfer, A.; Feder, T.; Sackmann, E. *Biophys. J.* **1997**, *73*, 245-257.

(38) Pignataro, B.; Steinem, C.; Galla, H. J.; Fuchs, H.; Janshoff, A. *Biophys. J.* **2000**, *78*, 487-498.

(39) Redelmeier, T. E.; Guillet, J.; Bally, M. B. *Drug Delivery* **1995**, *2*, 98-109.

(40) Kim, M. K.; Chung, S. J.; Lee, M. H.; Cho, A. R.; Shim, C. K. *J.Controlled Release* **1997**, *46*, 243-251.

(41) Trafny, E.; Grzybowski, J.; Olszowska-Golec, M.; Antos, M.; Struzyna, M. *Pharmacol. Res.* **1996**, *33*, 63-65.

(42) Alamelu, S.; Panduranga Rao, K. *Carbohydr. Polym.* **1994**, *24*, 215-221.

(43) Chandy, T.; Sharma, C. P. *Biomaterials* **1996**, *17*, 61-66.

(44) Anderson J. M.; Gristina A. G.; Hanson, S.; Harker L. A.; Johnson R. J.; Merritt K.; Naylor P. T.; Schoen, F. Host reactions to biomaterials and their evaluation. In *Biomaterials science: An introduction to materials in medecine*, Ratner, B. D.; Hoffman, A.; Shoen, F.; Lemons, J., Eds.; Academic Press: San Diego, CA, **1996**; pp. 165-214.

(45) Allen, T.; Everest, J. *J. Pharmacol. Exp. Ther.* **1983**, *226*, 539-544.

(46) Juliano, R.; Stamp, D. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 651-658.

(47) Saba, T. *Arch. Intern. Med.* **1970**, *126*, 1031-1052.

(48) Rodrigueza, W.; Mazany, K.; Essenburg, A.; Pape, M.; Rea, T.; Bisgaier, C.; Williams, K. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 2132-2139.

(49) Nagayasu A.; Uchiyama K.; Kiwada H. *Adv. Drug Delivery Rev.* **1999**, *40*, 75-87.

(50) Hobbs, S.; Monsky, W.; Yuan, F.; Roberts, W.; Griffith, L.; Torchilin, V.; Jain, R. *Proc. Natl. Acad. Sci.USA* **1998**, *95*, 4607-4612.

(51) Fischer, A.; Oberholzer, T.; Luisi, P. *Biochim. Biophys. Acta* **2000**, *1467*, 177-188.

(52) Lepore, L.; Ellena, J.; Cafiso, D. *Biophys. J.* **1992**, *61*, 767-775.

(53) Ceh, B.; Winterhalter, M.; Frederik, P.; Vallner, J.; Lasic, D. *Adv. Drug Delivery Rev.* **1997**, *24*, 165-177.

(54) Allen, T. *Curr. Opin. Colloid Interface Sci.* **1996**, *1*, 645-651.

(55) Woodle, M.; Martin, F.; Yan-Young, A.; Redemann, C., **1991**, USPTO 5013556.

(56) Woodle, M.; Collins, L.; Sponsler, E.; Kossovski, N.; Papahadjopoulos, D.; Martin, F. *Biophys. J.* **1992**, *61*, 902-910.

(57) Woodle, M.; Engbers, C.; Zalipsky, S. *Bioconjugate Chem.* **1994**, *5*, 493-496.

(58) Woodle, M. *Adv.Drug Delivery Rev.* **1995**, *16*, 249-265.

(59) Woodle, M. *Adv.Drug Deliv.Rev.* **1998**, *32*, 139-152.

(60) Allen, T.; Hansen, C.; Lopez de Menezes, D. E. *Adv. Drug Delivery Rev.* **1995**, *16*, 267-284.

(61) Marjan, J.; Allen, T. *Biotechnol. Adv.* **1996**, *14*, 151-175.

(62) Allen, T.; Hansen, C.; Rutledge, J. *Biochim. Biophys. Acta* **1989**, *981*, 27-35.

(63) Torchilin, V.; Trubetskoy, V. *Adv. Drug Delivery Rev.* **1995**, *16*, 141-155.

(64) Gref, R.; Luck, M.; Quellec, P.; Marchand, M.; Dellacherie, E.; Harnisch, S.; Blunk, T.; Muller, R. *Colloids Surf., B* **2000**, *18*, 301-313.

(65) Edwards, K.; Johnsson, M.; Karlsson, G.; Silvander, M. *Biophys. J.* **1997**, *73*, 258-266.

(66) Kenworthy, A. K.; Simon, S. A.; Mcintosh, T. J. *Biophys. J.* **1995**, *68*, 1903-1920.

(67) Bedu-Addo, F.; Huang, L. *Adv. Drug Delivery Rev.* **1995**, *16*, 235-247.

(68) Nicholas, A.; Scott, M.; Kennedy, N.; Jones, M. *Biochim. Biophys. Acta* **2000**, *1463*, 167-178.

(69) Silvander, M.; Hansson, P.; Edwards, K. *Langmuir* **2000**, *16*, 3696-3702.

(70) Lehtonen, J.; Kinnunen, K. *Biophys. J.* **1994**, *66*, 1981-1990.

(71) Lehtonen, J.; Kinnunen, K. *Biophys. J.* **1995**, *68*, 525-535.

(72) Allen, T. **1989**, USPTO 4837028.

(73) Allen, T.; Chonn, A. *FEBS Lett.* **1987**, *223*, 42-46.

(74) Harasym, T.; Tardi, P.; Longman, S.; Ansell, S.; Bally, M.; Cullis, P.; Choi, L. *Bioconjug. Chem.* **1995**, *6*, 187-194.

(75) Allen, T. Toxicity and systemic effects of phospholipids. In *Phospholipids handbook*; Cevc, G. Ed.; Marcel Dekker Inc.: New York, **1993**; pp. 801-816.

(76) Altura, B. Pharmacology of the microcirculation. In *Microcirculation*; Effros, R. M.; Schmid- Schoenbein, H.; Ditzel, J. Eds.; Academic Press: New York, **1981**; pp.51-105.

(77) Allen, T.; Murray, S.; MacKeigan, S.; Shah, M. *J. Pharmacol. Exp. Ther.* **1984**, *229*, 267-275.

(78) Semple, S.; Chonn, A.; Cullis, P. *Adv. Drug Delivery Rev.* **1998**, *32*, 3-17.

(79) Abra, R.; Hunt, C. *Biochim. Biophys. Acta* **1981**, *666*, 493-503.

(80) Ellens, H.; Mayhew, E.; Rustum, Y. *Biochim. Biophys. Acta* **1982**, *714*, 479-485.

(81) Liu, D. *Adv. Drug Delivery Rev* **1997**, *24*, 201-213.

(82) Liu, D.; Huang, J.; Song, Y. *Biochim. Biophys. Acta* **1995**, *1240*, 277-284.

(83) Liu, D.; Song, Y.; Liu, F. *Pharm. Res.* **1995**, *12*, 1775-1780.

(84) Vermette, P.; Meagher, L. *Colloids Surf., B* **2003**, *28*, 153-198.

(85) Marra, J.; Israelachvili, J. *Biochemistry* **1985**, *24*, 4608-4618.

(86) Afshar-Rad, T.; Bailey, A.; Luckham, P.; MacNaughton, W.; Chapman, D. *Faraday Discuss. Chem. Soc.* **1986**, *81*, 239-248.

(87) Lis, L.; McAlister, M.; Fuller, N.; Rand, R.; Parsegian, V. *Biophys. J.* **1982**, *37*, 657-66.

Chapitre 2

Liposomes layers characterized by quartz crystal microbalance and multi-release delivery

Caractérisation de couches de liposomes par microbalance à cristal de quartz et multi-libération

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2.1 Abstract

Intact liposomes have been immobilized onto solid surfaces by NeutrAvidin-biotin link. The construction of these layers has been followed-up by X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance (QCM) with energy dissipation monitoring. Also, the simultaneous release of two fluorescent probes from these liposome layers has been investigated with the aim to validate this method as multi-release delivery systems. XPS showed the successful immobilization of the different layers. XPS results also point out to the importance of the deactivation method used to reveal the presence of the specific NeutrAvidin-biotin attachment. QCM measurements allowed to follow the build-up of the different layers in real-time and in situ, and suggest that biotinylated-liposomes stay intact upon surface attachment on NeutrAvidin-covered surfaces and had viscoelastic behaviour. QCM experiments also demonstrated that surface-immobilized liposomes were able to resist adsorption from foetal bovine serum (FBS). Release kinetic profiles were studied by monitoring the release of two different fluorescent probes, namely carboxyfluorescein and levofloxacin, from these liposome layers. These studies showed that it was possible to modulate to some extent the release rates of the two molecules by using different configurations of liposome layers.

2.2 Résumé

Des liposomes ont été immobilisés sur une surface solide à l'aide du lien spécifique NeutrAvidin-biotin. Toutes les étapes de fabrication ont été suivies par la spectroscopie à photoélectron de rayons-X (XPS) et par microbalance à cristal de quartz avec mesure de la dissipation d'énergie (QCM). De plus, la libération simultanée de deux molécules fluorescentes a été étudiée afin de valider le principe de libération de plusieurs agents actifs de façon séquencée. Les résultats de XPS ont montré que les étapes de fabrication ont été réussies. Ces résultats ont aussi montré l'importance de la technique de désactivation afin de révéler la présence spécifique du lien NeutrAvidin-biotin. Les mesures par QCM ont permis de suivre les étapes de fabrication des différentes couches en temps réel et suggèrent que les liposomes restent intacts à la suite de leur immobilisation sur une surface exposant des molécules de NeutrAvidin et ont un comportement viscoélastique. Les mesures par QCM ont aussi démontré que les liposomes immobilisés en surface ont la capacité de résister à l'adsorption irréversible de protéines provenant d'une solution de sérum de veau fœtal. Les profils de libération ont été étudiés en suivant deux molécules fluorescentes, la carboxyfluoresceïne et la levofloxacine, encapsulées dans les liposomes. Ces études ont montré qu'il était possible de moduler le profil de libération en utilisant une certaine configuration des couches de liposomes.

2.3 Introduction

Composite drug delivery systems with liposomes i.e., liposomes combined to other substrates can be considered for certain types of drug delivery.[1] Various techniques have been used to immobilize lipid vesicles onto and into different substrates such as collagen[2-4] and chitosan.[5,6] Vermette et al. have developed a method to immobilize intact pegylated-liposomes onto surfaces by using the strong NeutrAvidin-biotin specific interaction.[7,8] If loaded with a therapeutic agent and kept intact, layers of liposomes immobilized on solid substrates could make good candidates for local drug delivery applications from biomedical devices. For example, Danion et al.[9] have reported the detailed surface immobilization of layers of intact liposomes on contact lens surfaces. Also, the antibacterial activity of contact lenses bearing surface-immobilized layers of intact liposomes loaded with levofloxacin was successfully demonstrated.[10] However, some properties of these surface-bound liposomes are still not well understood, mainly because this system has been only recently proposed as a possible drug delivery system.

Therefore, the aim of the present investigation was to further study the immobilization steps of the interlayers used in the immobilization of surface-bound liposomes. The level of biofouling on these liposome layers was investigated, as this property often will dictate the applicability of a biomedical device including drug carriers.[11] The formation of surface-bound liposomes assembly was characterized by X-ray photoelectron spectroscopy (XPS) and by quartz crystal microbalance (QCM) with energy dissipation monitoring. Fluorescent release measurements were carried out to study the capacity to modulate release rate profiles of two loaded molecules.

2.4 Experimental Section

2.4.1 Materials

N-heptylamine monomer (no.126802), N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES, no.H-3375, 99.5%), t-octylphenoxypolyethoxyethanol (Triton X-100, no.T-9284), levofloxacin (98% purity, no.28266) and foetal bovine serum (FBS, no.F2442) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sodium chloride (NaCl, ACS grade), hydrochloric acid (HCl, ACS grade), sodium sulfate (Na_2SO_4,

ACS grade), Sparkleen™ (no.04-320-04), hydrogen peroxide 30% (H_2O_2, ACS grade), sulphuric acid (H_2SO_4, ACS grade), and chloroform (HPLC grade) were purchased from Fisher Scientific (Ottawa, ON, Canada). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, >99%, no.850365-P), cholesterol (CHOL, >99%, no.700000-P), N-[ω-(biotinoylamino)poly(ethylene glycol) 2000]-1,2-distearoyl-sn-glycero-3-phospho-ethanolamine (DSPE-PEG_{2000}-Biotin, >96%, no.890129-P) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Biotin-PEG(3400)-NHS (NHS-PEG-Biotin, no.0H4M0F02) was purchased from Nektar Therapeutics (Huntsville, AL, USA). 5-(and-6)-carboxyfluorescein (CF, mixed isomers, 99%, no.C194) was obtained from Molecular Probes (Eugene, OR, USA).

ImmunoPure® NeutrAvidin™ biotin-binding protein (no.P31000) and ImmunoPure® D-Biotin (biotin, no.29129) were obtained from Pierce (Rockford, IL, USA).

Tissue culture polystyrene (TCPS) 6-well and 12-well plates (Costar, sterile) were obtained from Corning (Corning, NY, USA) and 96-well black plates for fluorescence-based assays (FluoroNunc™ Plate) were purchased from Nalge Nunc International (Rochester, NY, USA). They were used as received.

Borosilicate plates (12 mm x 12 mm) were purchased from Carolina Biological Supply Company (Burlington, NC, USA, no.63-3069). Borosilicate substrates were cleaned in Sparkleen™ solution overnight, immersed in ethanol (ACS grade), rinsed in water and finally blown dry using a high-velocity stream of 0.2-μm filtered air (Millex® GP, Millipore, Cork, Ireland). Samples were only used once.

2.4.2 Preparation of liposomes

Detailed procedures used to produce liposomes[12-15] and to immobilize these intact liposomes on surfaces[7-10] were reported elsewhere. Briefly, unilamellar vesicles were prepared using DSPC, CHOL in 2:1 molar ratio and DSPE-PEG_{2000}-Biotin (5 % (mol/mol) of the total lipid content) by extrusion through 100-nm pore polycarbonate Avestin® track-etch membranes using the Avestin Liposofast (Avestin Inc., Ottawa, ON, Canada). The total lipid concentration of the liposome suspension was adjusted using the HEPES buffer to the desired concentration, usually 1 mg total lipids/ml. The HEPES buffer contained

10mM HEPES at pH 7.4 and a NaCl concentration that was adjusted to an osmolarity of 290 mOsm, using The Advanced Osmometer, model 3250 (Advanced Instruments Inc., Norwood, MA). Milli-Q® grade water (Millipore Canada, Nepean, Canada) with a resistivity of 18.2 MΩ·cm was used to prepare buffer solutions.

2.4.3 Surface immobilization of liposomes

The complete surface modification strategy is schematically illustrated in Figure 2.1. Liposome immobilization using a low-fouling PEG interlayer[16] and the specific NeutrAvidin-biotin linkage have been well described by Vermette and colleagues.[7-9,17]

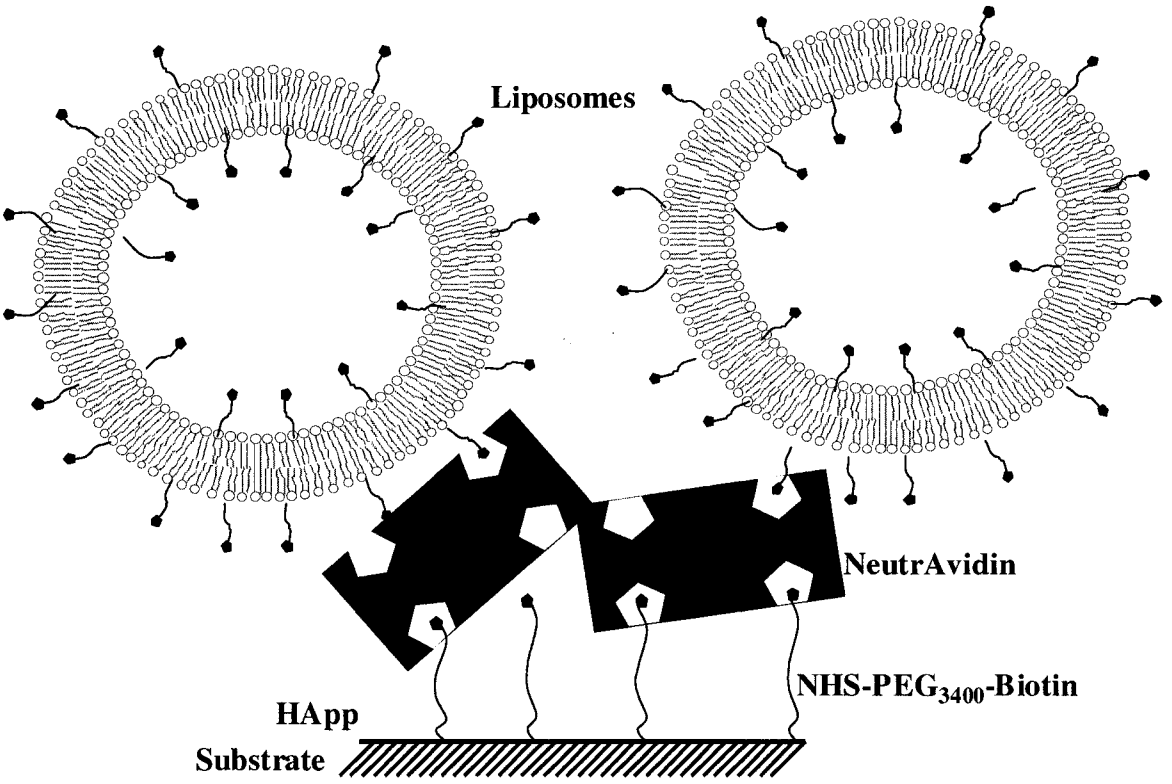

Figure 2.1 Scheme, not to scale, of the multi-layer strategy to immobilize layers of stable liposomes.

Deposition of thin plasma polymer films was carried out from the vapor of *n*-heptylamine monomer in a custom-built reactor described elsewhere[18] onto borosilicate surfaces and onto quartz crystals that are used for QCM measurements. Before utilization, these

substrates were cleaned using a UV/ozone treatment (PSD-UV, Novascan, IA, USA) for 50 minutes and then immersed in ethanol, rinsed in water, and blown dry using a high-velocity stream of 0.2-μm filtered air. Borosilicate substrates were used once but quartz crystals were reused for few experiments following an additional cleaning step in piranha solution (3:1 ratio H_2SO_4/H_2O_2) for 2 minutes after UV/ozone cleaning. CAUTION: Piranha solution is a strong oxidant and should be used with extreme caution.

NHS-PEG-Biotin solution was prepared under cloud-point conditions.[16] Briefly, 1 mg/ml of NHS-PEG Biotin was solubilised in a 170 mg/ml Na_2SO_4 solution to form aggregates of PEG in the solution and thus, to increase the density of PEG on the surfaces. Solution was made just before needed for the subsequent experiments in order to prevent hydrolysis of NHS functionalised groups. NHS-PEG-Biotin was grafted onto *n*-heptylamine plasma polymer (HApp) layers using water-soluble carbodiimide chemistry.[19] After plasma deposition, substrates were deposited in multi-wells and the NHS-PEG-Biotin solution under cloud point was added. The reaction was allowed to proceed overnight at room temperature under vigorous shaking. To remove any non-covalently attached NHS-PEG-Biotin, the substrates were rinsed overnight under vigorous shaking in water with the solution changed three times.

Next, PEG-Biotin-coated surfaces were immersed in a 50 μg/ml solution of NeutrAvidin in HEPES buffer.[17] The reaction was allowed to proceed overnight at room temperature under vigorous shaking. To remove any proteins not linked to PEG-Biotin (i.e., NeutrAvidin molecules that can be loosely adsorbed onto the PEG layer), samples were rinsed first, in HEPES buffer and then, in water. Solutions were changed three times.

Immobilization of liposomes was performed by adding suspension of biotinylated-liposomes made of DSPC:CHOL:DSPE-PEG_{2000}-Biotin (1 mg/ml total lipid concentration). The incubation was allowed to proceed overnight at room temperature under vigorous shaking. To remove loosely adsorbed liposomes, the samples were rinsed overnight at room temperature in HEPES buffer. The buffer solution was changed three times. It is important to note that liposomes should be exposed only to iso-osmolar solutions.[20]

Further liposome layers can be built up by adding, after the attachment of the first liposome layer, more NeutrAvidin protein, which can bind to free biotins from the first liposome layer, followed by addition of more liposome suspension. Thus, NeutrAvidin molecules act as a bridge between the different liposome layers.

To compare specific and non-specific liposome attachment, some samples were prepared by using NeutrAvidin pre-blocked with excess biotin. The biotin solution contained 10mM of biotin in HEPES buffer adjusted to pH 7.4. NeutrAvidin-coated samples were immersed in a biotin solution for one hour and then rinsed with HEPES buffer for another hour. The substrates were then covered by the liposome suspension described above. To compare with the first deactivation method, immobilization of liposomes was carried out using liposomes made in a solution of excess biotin. In this case, the concentration of the liposomes was adjusted with the biotin solution and NeutrAvidin-coated samples were immersed in this solution and then rinsed as describe above.

2.4.4 Surface chemical composition by X-ray photoelectron spectroscopy (XPS) analyses

XPS analyses were performed using an AXIS HSi spectrometer from Kratos Analytical Ltd. (Manchester, UK). XPS measurements were conducted with a monochromated aluminium Kα source at a power of 120 Watts and samples were placed at an angle of 90° from the detector. Pressure of the XPS chamber during measurements was lower than 5 x 10^{-8} mbar and high-resolution spectra were collected at 40 eV pass energy. Borosilicate substrates were fixed on XPS holder by a silver glue to prevent charge accumulation during measurements. To remove salts from the HEPES buffer solution, samples were rinsed in water before XPS analyses and blown dry using a high-velocity stream of 0.2-μm filtered air. The HApp oxygen sensitive layers were kept under argon atmosphere until analysis. High-resolution C 1s spectra along with survey spectra were taken for each sample. Each condition was represented by three samples and results averaged.

2.4.5 In situ follow-up of the build-up of layers of intact liposomes, their viscoelastic responses, and level of biofouling by quartz crystal microbalance (QCM) measurements

QCM measurements with energy dissipation monitoring were performed using an apparatus from Resonant Probes GmbH (Goslar, Germany). The gold-coated quartz resonators (Maxtek Inc, Cypress,CA, no.149273-1) were placed into a commercial Teflon holder (Maxtek Inc, Cypress, CA, no.CHT-100). Data from the network analyzer (Agilent, Palo Alto, CA, HP4396A) were analyzed using the software from Resonant Probes.

Surface fabrication steps (i.e., NeutrAvidin, deactivation of NeutrAvidin surfaces with biotin and layers of intact liposomes) were followed *in situ* and in real time using QCM measurements. The crystal holder mounted with a fluid cell made of Kynar (Maxtek Inc, Cypress, CA, no.FC-550) was connected to a fluidic system that allows controlled injection of solution. Injections were done with a programmable injection system (KDS100, KD Scientific Inc. Holliston, MA) that allows utilization of different syringe sizes and injection speeds. All QCM experiments were done at room temperature.

For each QCM analysis, the rear electrode of the surface-modified quartz was rinsed, dried, and placed into the holder before HEPES buffer was injected. Then, the frequencies of harmonics vibrations were located. Upon stability, harmonics were redefined and 2 ml of the solution containing one of the layer components was injected at a rate of 0.5 ml/min. The frequency and the half-band-half-width (HBHW) shifts were recorded to monitor changes in the layer mass and in the layer viscoelasticity, respectively.

Foetal bovine serum (FBS) was heat de-complemented, aliquoted and stored at -80°C. When needed, FBS aliquots were thawed and diluted in HEPES buffer at a concentration of 10% v/v. The choice of the protein medium used for the biofouling tests was dictated by the wide range of proteins contained in FBS, so that the versatility of protein repellency of the layers could be assessed. Rear electrodes of liposome-coated quartzes were rinsed, dried and placed in the QCM holder. Immediately, HEPES buffer was injected into the QCM chamber. Upon stability of the signal, 2 ml of FBS solution was injected into the chamber at a rate of 0.5 ml/min. As for the other QCM assays, the

frequency and half-band-half-width shifts were recorded by the software to monitor changes in layer mass and in the layer viscoelasticity, respectively.

All QCM experiments were carried out on at least three different samples per condition. For all QCM measurements, data were taken at least for harmonics located at 5 MHz, 15 MHz and 25 MHz (the fundamental frequency being 5 MHz). Harmonic number 3 (i.e., 15 MHz) is the harmonic for which the results are reported in this paper.

2.4.6 Stability of liposome layers and kinetic of multi-release of two fluorescent probes

Measurements of the stability of the surface-bound liposomes were performed at room temperature using a technique adapted from the fluorescent self-quenching method.[21,22] For these stability experiments, liposomes were produced using the same technique as mentioned in *Section 2.4.2*, except that the lipids were hydrated in the dark using a solution containing 85mM caboxyfluorescein (CF) in HEPES buffer at pH 7.4. The osmolarity of this CF solution was adjusted to be approximately 290 mOsm.

The total fluorescence is determined after disrupting the remaining liposomes with the detergent Triton X-100. Pure CF has an excitation maximum at 487 nm at pH 7.5 and an emission maximum at ~520 nm; these spectral positions are not affected by the addition of Triton X-100.[21,22]

To investigate the kinetics of the simultaneous release of two liposome-entrapped molecules from different layers of intact liposomes, a second fluorescent molecule with a different emission spectrum than that of CF was needed. For that purpose, levofloxacin was selected as it has an excitation at 310 nm and an emission at 460 nm. The solution of levofloxacin used to make liposome suspensions contained 100 mg/ml (270mM) of levofloxacin in Milli-Q water. The pH was adjusted to 6.8. Subsequently, NaCl was added to obtain a levofloxacin solution with an osmolarity of 290 mOsm. The solubility of levofloxacin is strongly pH dependent and is maximal at pH 6.7. Before the release experiments, calibration curves were done to correlate the levofloxacin and the CF concentration to the fluorescence readings. The addition of Triton X-100 to a levofloxacin solution was found to affect levofloxacin emission (data not shown). Therefore, a

calibration curve was made to compare levofloxacin emission in HEPES buffer to that in Triton X-100, the latter being used to measure the 100% release.

Separation of the CF- and levofloxacin-containing vesicles from non-entrapped fluorescent molecules was achieved by gel chromatography, which involved passage through a 2.5×25 cm column of Sephadex™ G-50 Fine from Amersham Pharmacia Biotech Inc. (Baie d'Urfé, QC, Canada). The column was eluted at room temperature with HEPES buffer. The total lipid concentration of the liposome suspension collected at the column outlet was adjusted to the desired concentration (i.e., 1 mg/ml) using HEPES buffer.

To study the simultaneous release of CF and levofloxacin from layers of intact liposomes, 3 layers of liposomes containing levofloxacin were first immobilized onto a NeutrAvidin-coated surface followed by the subsequent addition of 3 layers of CF-containing liposomes. The release of levofloxacin and CF from these surface-bound liposomes was monitored over time using a Bio-Tek Synergy HT well-plate reader from Bio-Tek Instruments (Winooski, VE, USA). Briefly, 1 ml of the appropriate medium (either 0.5% (v/v) Triton X-100 solution made with Milli-Q water or HEPES buffer) was added to each well containing a liposome-bearing surface and incubated at room temperature. At low concentration and neutral pH, Triton X-100 instantaneously disrupts the lipid vesicles and liberates their contents without significant interference with the intrinsic fluorescence of the CF dye.[21,22]

At periodic intervals, 200 µl of the incubating solution was withdrawn from the wells containing the surfaces and transferred into 96-well plates for fluorescence measurement. The fluorescent emission signal was monitored either at 460 nm (levofloxacin signal) or at 528 nm (CF signal). Each experiment was done in triplicate. The levofloxacin or CF release over time was calculated by applying Equation 2.1:

$$\text{Fraction of molecules left in vesicles} = 1 - F/F_T \qquad (2.1)$$

Where F is the fluorescence at emission wavelength measured at any time during the experiment, and F_T is the total fluorescence at 460 nm or at 528 nm obtained after disruption of the liposomes with a 0.5% (v/v) Triton X-100 solution.

2.5 Results and Discussion

2.5.1 Surface chemical composition by XPS analyses

The steps in the progressive construction of the multilayer coating (Fig. 2.1) were assessed by XPS analyses. The elemental compositions determined by XPS survey spectra are listed in Table 2.1.

Table 2.1 Elemental composition determined by XPS of samples at various stages of the multistep coating on borosilicate.

Samples	%C	%O	%N	%P	O/C	N/C
HApp	89.6 ± 0.2	2.5 ± 0.3	7.9 ± 0.2	-	0.03	0.09
HApp+PEG-Biotin	77.7 ± 1.2	18.0 ± 1.3	4.3 ± 0.7	-	0.23	0.06
HApp+PEG+NeutrAvidin (NA)	73.8 ± 1.3	18.4 ± 0.6	7.9 ± 0.7	-	0.25	0.11
HApp+PEG+NA+ Liposomes	81.6 ± 1.1	13.4 ± 0.4	4.0 ± 0.7	0.9 ± 0.2	0.16	0.05
HApp+PEG+NA+Biotin +Liposomes	79.7 ± 1.1	14.4 ± 1.1	5.2 ± 0.5	0.7 ± 0.2	0.18	0.07
HApp+PEG+NA+ (Biotin+Liposomes)	72.8 ± 1.2	19.2 ± 0.6	7.9 ± 0.5	-	0.26	0.11

HApp: n-heptylamine plasma polymer; Biotin+Liposomes: addition of excess biotin on NeutrAvidin surfaces with the subsequent addition of liposomes; (Biotin+Liposomes): addition of a liposomes suspension made in a solution of biotin in excess.

The elemental composition of HApp layers is in agreement with earlier results.[18] The absence of a signal from the element Si from the borosilicate substrate indicates that the coating thickness is more than 10 nm.

Figure 2.2 and Table 2.1 show the surface composition obtained by XPS of PEG-Biotin films attached on HApp layers. The presence of PEG-Biotin is indicated by the increase in the C–O contribution at 286.5 eV (Fig. 2.2). The theoretical oxygen content for a pure PEG coating of >10 nm thickness is 33%. The obtained 18% oxygen content can be explained by the fact that the PEG layer is not as thick as 10 nm. Since XPS spectra are recorded in a high-vacuum environment, it is possible that this vacuum causes the hydrated

PEG coatings to collapse resulting in an apparent thinner layer when looking at XPS analyses. Also, AFM compression force measurements done on similar PEG surfaces revealed an "apparent" hydrated thickness of layers between 4 and 8 nm[16] which is under the 10 nm analysed by XPS.

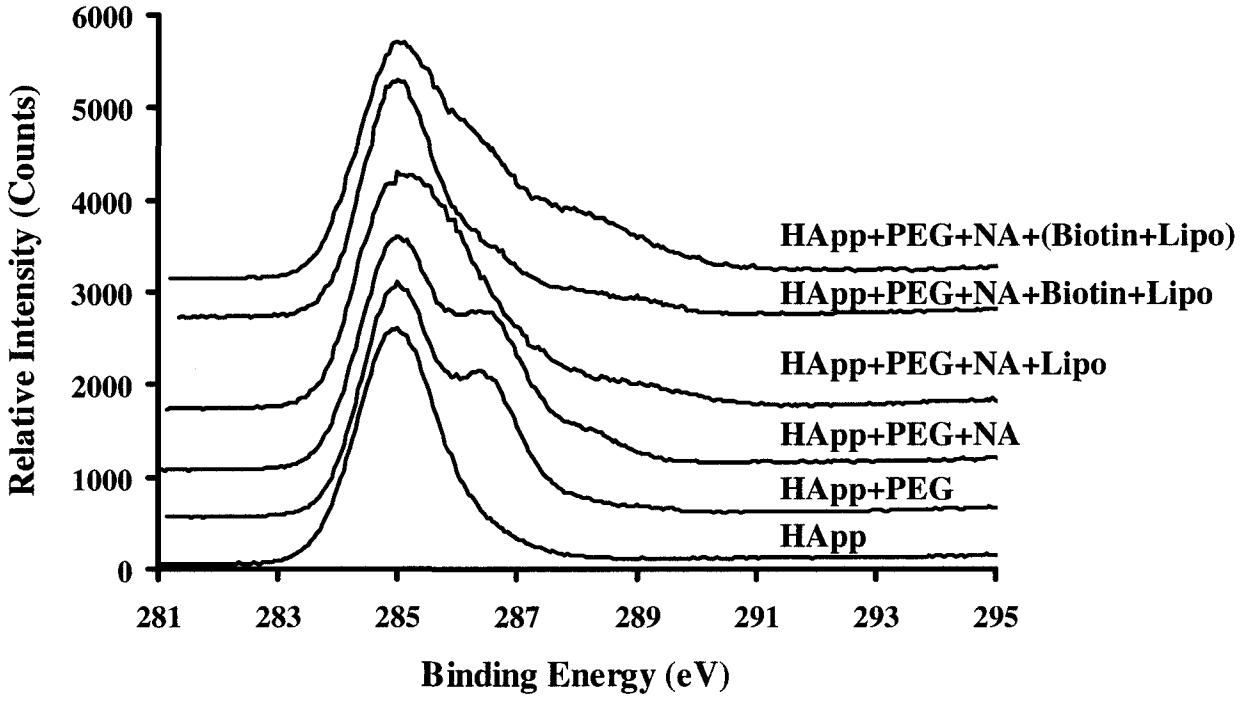

Figure 2.2 High-resolution XPS C 1s spectra recorded at different stages of the multi-layer construction.

Following the addition of NeutrAvidin, a substantial amount of proteins was detected. High-resolution XPS C 1s spectra showed an increase in the component at the spectral position indicative of amide groups (288.5 eV). The increase in the percentage of nitrogen content detected on NeutrAvidin-coated surfaces over those of PEG-Biotin-coated samples (Table 2.1) also indicated the presence of proteins.

After immobilizing biotinylated liposomes onto NeutrAvidin-coated samples and washing to remove loosely adsorbed lipid vesicles, lipids were detected by XPS. Contributions assignable to the lipids, as C(=O)-C at 289.2 eV, were observed on the spectra of substrates bearing surface-bound liposomes (Fig. 2.2).[23] The presence of a

phosphorus signal (Table 2.1) also confirms the presence of phospholipids. This result is in good agreement with previous results obtained on fluorinated surfaces.[7]

Further liposome layers can be built up by adding, after the attachment of the first liposome layer, more NeutrAvidin protein, which can bind to free biotins from the first liposome layer, followed by addition of more liposome suspension. Thus, NeutrAvidin molecules act as a bridge between the different liposome layers.

Pre-blocked NeutrAvidin layers have been made to investigate whether or not the immobilization of liposomes on PEG-NeutrAvidin layers was specific. Firstly, NeutrAvidin proteins were deactivated using a solution of excess biotin followed by a washing with HEPES prior to the addition of the liposome suspension. As seen in Table 2.1, the presence of lipids was observed as indicated by the phosphorus signal that originates from the phospholipids. It is possible that each biotin "pocket" of the NeutrAvidin molecules can accommodate several biotin molecules at the same time resulting in a strong enough attraction to also dock biotinylated liposomes onto these deactivated NeutrAvidin coatings. In other words, when the liposome suspension is added onto the pre-deactivated NeutrAvidin surfaces, the biotin binding sites of the NeutrAvidin could partly accommodate the biotinylated-PEG arm of the liposomes, resulting in a strong enough bond that can retain liposomes on the surfaces even following a thorough rinsing.

To investigate this hypothesis, samples have been made with the addition of a liposome suspension already containing excess biotin; the lipid concentration was adjusted to 1 mg/ml in a 10mM biotin solution. From Table 2.1, no phosphorus signal was detected and the atomic O/C and N/C ratios of these deactivated NeutrAvidin surfaces exposed to liposome suspension were similar to those of the bare NeutrAvidin coatings indicating the absence of liposomes. This finding reinforces our hypothesis. There seems to be a competition between the free biotins available in excess and the biotinylated-liposomes for the surface-attached NeurAvidin proteins. Given the size of the biotin molecules (244 Da), the diffusion coefficient of the free biotin is larger than that of the liposomes allowing the free biotins to reach NeutrAvidin faster than the biotinylated liposomes. Free biotin molecules added in excess during liposome attachment would completely saturate the

biotin binding sites of the proteins and would compete with the biotinylated liposomes for binding sites thereby hindering the biotinylated liposomes from docking strongly enough on the NeutrAvidin surfaces so liposomes are flushed away during the rinsing step before XPS analyses.

2.5.2 In situ follow-up of the build-up of layers of intact liposomes

The shift of the resonance frequency (Δf) of the quartz resonator, as described by Sauerbrey,[24] is linearly proportional to the mass of the deposited film as long as the film is uniform, thin compared to the quartz thickness, and as rigid as the quartz itself so it can be treated as an extension of the quartz crystal. Using the Sauerbrey equation, the deposited mass can be approximated by Equation 2.1:

$$\Delta m = C \cdot \Delta f / n \qquad (2.2)$$

Where C is the mass sensitivity constant and is equal to 17.7ng/(cm^2·Hz) at 5 MHz and n is the overtone order.[25] For thin and rigid films in solution, the frequency shifts due to the liquid and to the mass are additive and the film mass can be calculated using Sauerbrey equation. However, for surface-bound liposomes and the underlayers (apart from HApp layer), which are expected to be viscoelastic, Sauerbrey mass has been calculated only as an approximation of the real mass adsorbed on the crystal. Because liposome shape and size can change upon adsorption on the composite layers and also because surface-bound liposomes can deform under crystal vibration no modelling was attempted. To our knowledge, no viscoelastic model can be used to describe the hydrated multi-layered system fabricated here.

QCM measurements of layers bearing NeutrAvidin and intact liposomes are summarized in Table 2.2. Each layer was rinsed in situ with HEPES buffer to make sure that only signals generated from the intended surface modification step were recorded and analyzed. The attachment procedures were not done in the QCM chamber except for the coating deposition of interest that was monitored in situ by QCM. The injection was carried out over 4 minutes (2 ml at 0.5 ml/min). This injection rate was chosen to limit potential perturbation of the surface-bound liposomes by the fluid flow that would also result in QCM signal perturbation. Although great care has been taken to limit the injection

perturbation, as depicted in Figure 2.3, sharp signal shift was observed upon injection. These pulse shifts can be attributed to the change in stagnation pressure imposed on crystal when flow starts and stops.

Table 2.2 Frequency (*f*) and half-band-half-width (HBHW) shifts following injection of the different components in the build-up of the multi-layers.

Component	$\Delta f/n$ (Hz)	Sauerbrey mass ($\mu g/cm^2$)	ΔHBHW/n (Hz)
NeutrAvidin	-18 ± 1	0.11	-2.0 ± 0.5
+Biotin	-3 ± 2	0.02	2.0 ± 0.3
+ 1st liposome layer	-200 ± 50	1.18	85 ± 20
+ 2nd liposome layer	-220 ± 50	1.30	113 ± 16
NeutrAvidin +(Liposomes + Biotin)	-155 ± 15	0.91	60 ± 4

Data presented were taken at the third harmonics (15 MHz i.e., overtone order (n) of 3). Liposomes + Biotin means addition of a liposomes suspension made in a solution of biotin in excess. Data represent means ± standard deviations.

NeutrAvidin attachment on PEG-Biotin layer reached stability within 20 minutes following NeutrAvidin injection (Fig. 2.3A). At the third harmonics, resonant frequency and HBHW shifts decreased by 18±1 Hz and 2.0±0.5 Hz, respectively, following NeutrAvidin injection. Rinsing with buffer did not affect both frequency and HBHW pointing out that only a specific adsorption took place as expected by the use of the low-fouling PEG layers produced under cloud point conditions[16] and the specific PEG-Biotin:NeutrAvidin link. The observed frequency shift of -18±1 Hz for NeutrAvidin attachment is close to frequency shifts measured for avidin protein adsorbed by electrostatic interactions on surfaces, which were on the order of -30 to -40 Hz.[26] The difference can be explained by the fact that the PEG layers used in the present study limit the non-specific protein adsorption to almost zero as measured by previous QCM measurements on similar PEG layers.[16] Also, the surface arrangement of the avidin proteins between the two studies might differ. The small HBHW shift confirms that the attachment of the NeutrAvidin on the PEG-Biotin layers produces no significant viscoelastic changes given the sensitivity of the QCM apparatus.

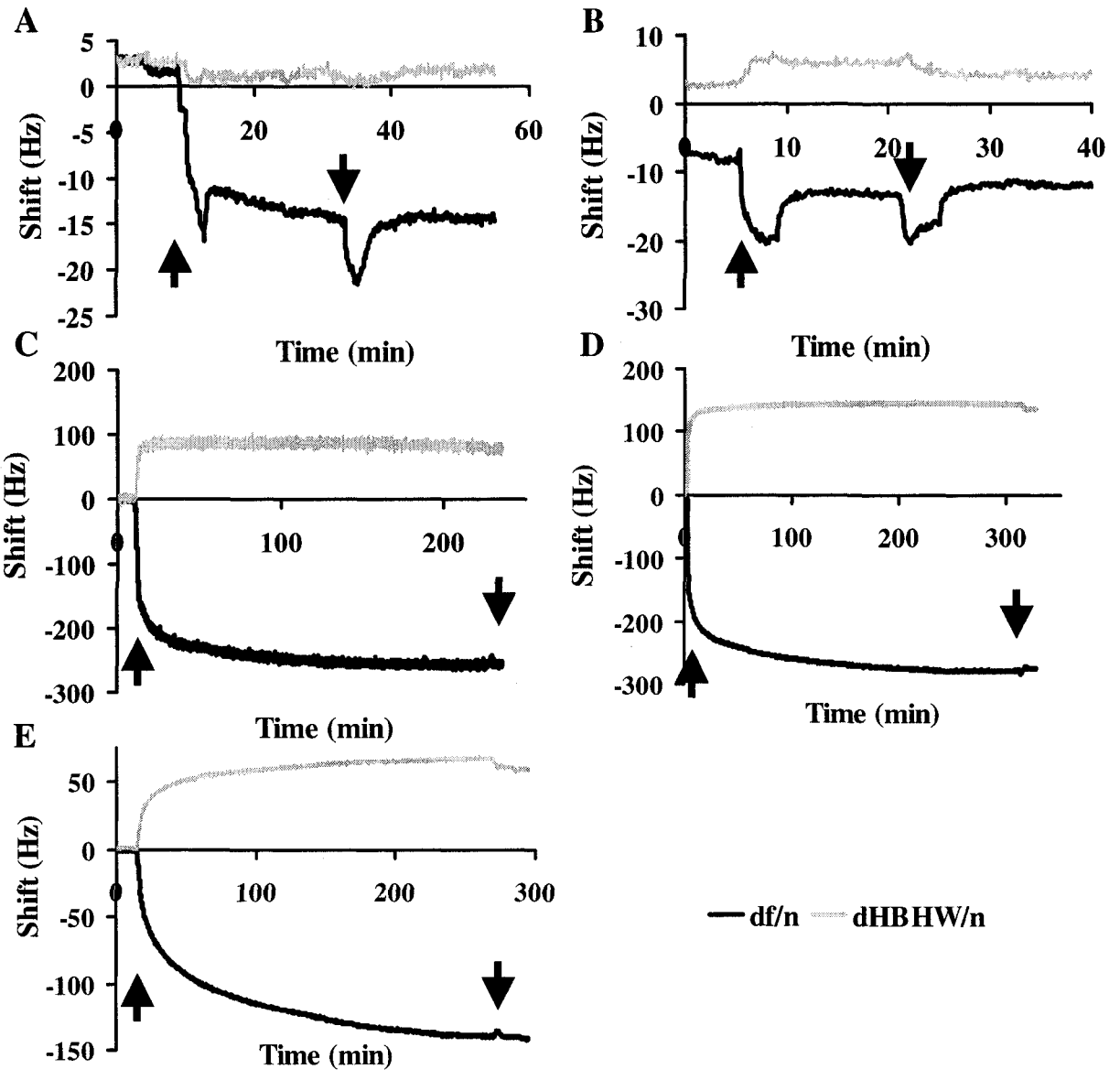

Figure 2.3 Frequency (*f*) and half-band-half-width (HBHW) graphs showing the injection of **A**: NeutrAvidin on PEG-Biotin layers, **B**: Biotin on NeutrAvidin surfaces, **C**: 1st liposome layer on active NeutrAvidin layers, **D**: 2nd liposome layer on the first liposome layer exposed to NeutrAvidin, and **E**: 1st liposome layer made in a solution of biotin in excess on active NeutrAvidin layers. Each injection was followed by a rinsing step. Each graph is representative of the observed tendency. First arrows indicate injection and second ones indicate rinsing steps.

Solution of excess biotin was injected over layers bearing surface-immobilized NeutrAvidin to investigate whether or not NeutrAvidin "blocking" could be detected by QCM. Biotin injection resulted in small frequency (-3±2 Hz) and HBHW (2.0±0.3 Hz) shifts following subsequent rinsing with buffer (Fig. 2.3B). The system reached equilibrium rapidly. When looking at frequency and HBHW shifts graphs over time, after biotin injection there was a moderate change both for frequency and HBHW but it almost came back to baseline after rinsing with buffer. Since biotin is a small molecule compared to the components of the under layers, it is not so surprising that it resulted in such small frequency and HBHW shifts. Because of the instrument detection limit of ca. ±2Hz, it is difficult to draw firm conclusion about the detection of the biotin on the surface-immobilized NeutrAvidin layers. NeutrAvidin protein layer, in another study,[17] seemed to detach in presence of excess biotin. The only difference between these two studies is the PEG layer i.e., the use of cloud-point vs non cloud-point conditions. PEG conformation might affect the (PEG-Biotin)-NeutrAvidin link strength since binding properties are related to receptors density, orientation, and flexibility.[27]

Injection of liposomes containing PEG-Biotin lipids over NeutrAvidin-modified surfaces caused major changes over frequency and HBHW (Fig. 2.3C). Frequency and HBHW shifts of -200±50 Hz and +85±20 Hz, respectively, were measured revealing that liposomes bound to the surface and have a viscoelastic behaviour indicated by the significant energy dissipation. The injection graph shows that the attachment is quite fast at first and then slow down to reach stability (between 200 and 400 minutes). Rinsing over the surface did not affect frequency and HBHW. The observed frequency shift for surface-bound liposomes is correlated to the total mass adsorbed on the quartz crystal - in this case only surface-bound liposomes because baselines were obtained from the NeutrAvidin-coated surfaces - and therefore includes the water located inside the lipid vesicles. These results obtained with the specific attachment of lipid vesicles are in good agreement with those reported with similar structures: liposome adsorption[25,28-31] and cell adhesion[26] (ranging from -90 to -400Hz depending on liposome sizes, adhesion process and on cell types). Also, it is worthy to notice that surface-bound liposomes stay intact upon and following surface attachment, because no significant frequency or HBHW shifts were

observed following the immobilization for a period of at least 200 minutes, suggesting that the lipid vesicles were stable over time.

The injection of a biotinylated liposome suspension over the first layer of intact liposomes exposed to a NeutrAvidin solution resulted in frequency and HBHW shifts of -220±50 Hz and +113±16 Hz (Fig. 2.3D). The slightly higher HBHW shift obtained for 2 layers of intact liposomes compared to that measured for the first liposome layer could be interpreted as the second layer of liposomes being more free to deform upon the crystal shearing action and more softer than the first liposome layer since vesicles in the first layer are probably more flattened due to a more constrained immobilization as suggested in another study.[31]

The large standard deviations calculated on the frequency and HBHW shifts for the attachment of layers of intact liposomes deserve more discussion. Comparison of the mean frequency shift between one layer of intact liposomes and the second liposome layer revealed that the difference was only 20 Hz and with the standard deviation, ±50 Hz, the difference between the 2 conditions is not significant, *t* test 42%, and for the HBHW shifts, *t* test 88%. Several possible hypotheses might support the large calculated standard deviations. Firstly, although the multi-layered strategy used in the surface immobilization of liposomes produces bottom layers (i.e., HApp and PEG coatings) having reproducible surface properties (composition, thickness and structure),[9,16,18] a small difference in surface characteristics of one or more of the interlayers can be amplified and resulted in batch-to-batch variations in QCM measurements owing to the high sensitivity of the QCM method. If comparisons between the QCM signals of the build-up of the different layers is only done on one "representative" sample, significant shifts between the different layers could be obtained (see Fig. 2.3). Often, only representative QCM data have been reported in the literature and perhaps compared with over enthusiasm. In fact, many QCM studies only report one set of representative data. To obtain meaningful QCM results and interpretation, frequency and HBHW shifts from in situ measurements on the same sample can be compared but it should be combined with a thorough statistical analysis to compare QCM results obtained from different samples. Secondly, it is possible that the acoustic wave did not propagate into the entire thickness of the second layer of intact liposomes. In fact,

although it is difficult to know the exact film thickness in which the acoustic wave propagates in such a complex multi-layers system, the acoustic wave is only 250 nm in water with 5 MHz quartz crystal[32] which may be near the thickness of the 2 liposome layers construction. In summary, the present results point out to the necessity to use a multi-technique assessment in order to draw firm conclusions.

Injection of a liposome suspension made in a solution of excess biotin was carried out to compare with the results obtained by XPS analysis (Table 2.1) revealing that no lipid was detected when liposomes were made in a solution of excess biotin. Surprisingly, frequency and HBHW shifts of -155±15 Hz and +60±4 Hz were observed, respectively, as a result of liposome injection onto NeutrAvidin surfaces even if biotin molecules were present during the immobilization step (Fig. 2.3E). There is no significant difference for frequency and HBHW shifts when liposome are injected in presence or not of excess biotin, *t* tests for *f* 77% and HBHW 89%. This is not in agreement with the XPS results presented in Section 2.5.1. One possible hypothesis to support this finding would be that NeutrAvidin proteins can accommodate more than one biotin molecule in each binding site, as previously stated. It is therefore possible that each binding site can accommodate some free biotin molecules along with a DSPE-PEG-Biotin lipid contained in liposome membrane. The rinsing step (20 minutes) with buffer following liposome attachment would not be efficient and long enough, in the QCM chamber, to remove these loosely bounded lipid vesicles as compared to the specific liposome attachment observed by XPS on samples that were rinsed overnight in HEPES buffer (under vigorous agitation and buffer replenished three times). Furthermore, there are some attractive forces including electrostatic and van der Waals[33] forces between biotin and avidin-like proteins and those forces are very strong since this biotin-avidin link is one of the strongest non-covalent interaction known.[34] These attractive forces may extend to separations greater than 100 Å between two surfaces[35] and may support the hypothesis that biotin molecules not completely engaged within the binding "pocket" of the protein could not be completely washed away following the short rinsing step used in QCM measurements. Rinsing was perhaps not sufficient in QCM experiments to remove these liposomes still immobilized strongly enough on NeutrAvidin layers through weaker but longer range forces.

2.5.3 Biofouling from serum by in situ QCM measurements

The utilization of PEG molecules in the making of liposomes has been shown to lower the level of non-specific protein adsorption.[36,37] Since this proposed drug delivery system would be in contact with complex biological fluids such as blood components and culture media (*in vitro* applications), it is essential to study the level of non-specific protein adsorption on surface-bound liposomes to estimate how "viable" this system could be in an *in vivo* environment. The dynamic biofouling from foetal bovine serum (FBS) on layers of intact liposomes was monitored *in situ* by QCM (Δf and $\Delta HBHW$) and results are summarized in Table 2.3.

Table 2.3 Frequency (f) and half-band-half-width (HBHW) shifts following FBS injection over layers of intact liposomes.

Component	Δf/n (Hz)	Sauerbrey mass ($\mu g/cm^2$)	ΔHBHW/n (Hz)
10% FBS (on 1 liposome layer)	-4.0 ± 0.2	0.02	2.0 ± 0.8

Data were taken at the third harmonics (15 MHz i.e., overtone order (n) of 3). Data represent means ± standard deviations.

Mean frequency and HBHW shifts of -4.0±0.2 Hz and +2.0±0.8 Hz were observed following the injection of FBS (10%v/v) over 1 layer of immobilized liposomes. FBS interaction was immediately observed after solution injection and was quickly stable. When looking at frequency and HBHW shifts graph (Fig. 2.4) over time, it is worthy to notice that after FBS injection there was a moderate change both for frequency and HBHW but signals almost came back to baseline after rinsing. These initial shifts upon FBS injection can be explained by either adsorption and/or even penetration of some FBS components (e.g., proteins, amino acids) within the liposome layers (within the liposome structures themselves and/or between the surface-bound liposomes). Also, it is possible that these shifts were influenced by a change in viscosity (water → FBS solution). Since the frequency and HBHW shifts can be considered as very small, it can be assumed that surface-bound liposomes suffer from very small permanent adsorption following FBS exposure, or if there is some irreversible fouling, QCM is not sensitive enough to detect it

or adsorption would result after longer period of time. This repellence capacity is probably induced by both the presence of PEGylated lipids in the liposome membrane creating steric repulsion[1,11,38] and by the phosphatidylcholine.[39,40]

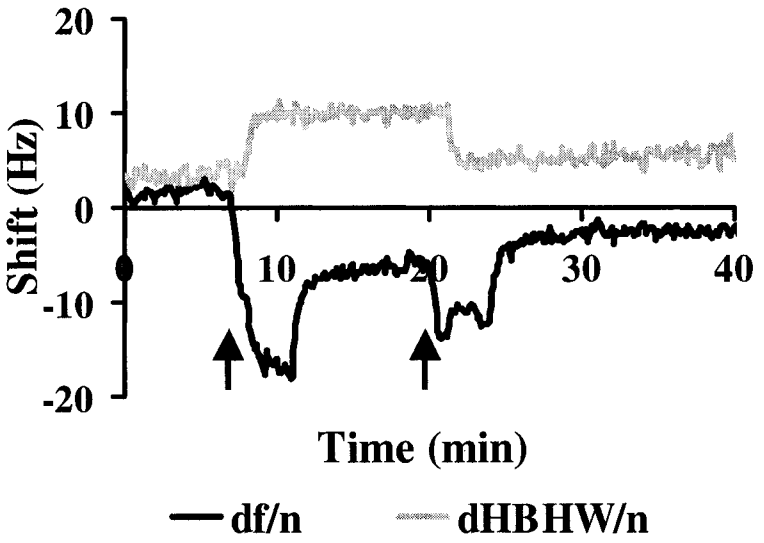

Figure 2.4 Frequency (*f*) and half-band-half-width (HBHW) graphs showing the injection of FBS 10% v/v. First arrows indicate injection and second ones indicate rinsing steps.

2.5.4 Stability of liposome layers and kinetic of multi-release of two fluorescent probes

Stability measurements and release kinetic studies of the surface-bound liposomes were carried out by using two different fluorescent probes namely carboxyfluorescein (CF) and levofloxacin. Two systems were compared: 1) Independent systems which consist of 3 layers of intact liposomes containing either one of the fluorescent probes and 2) A combined system composed of a first set of 3 layers of intact liposomes containing levofloxacin covered by 3 subsequent layers of intact liposomes loaded with CF. These systems were used to investigate whether or not it was possible to modulate the release of more than one molecule. The release kinetics at room temperature of levofloxacin and CF are shown in Figure 2.5.

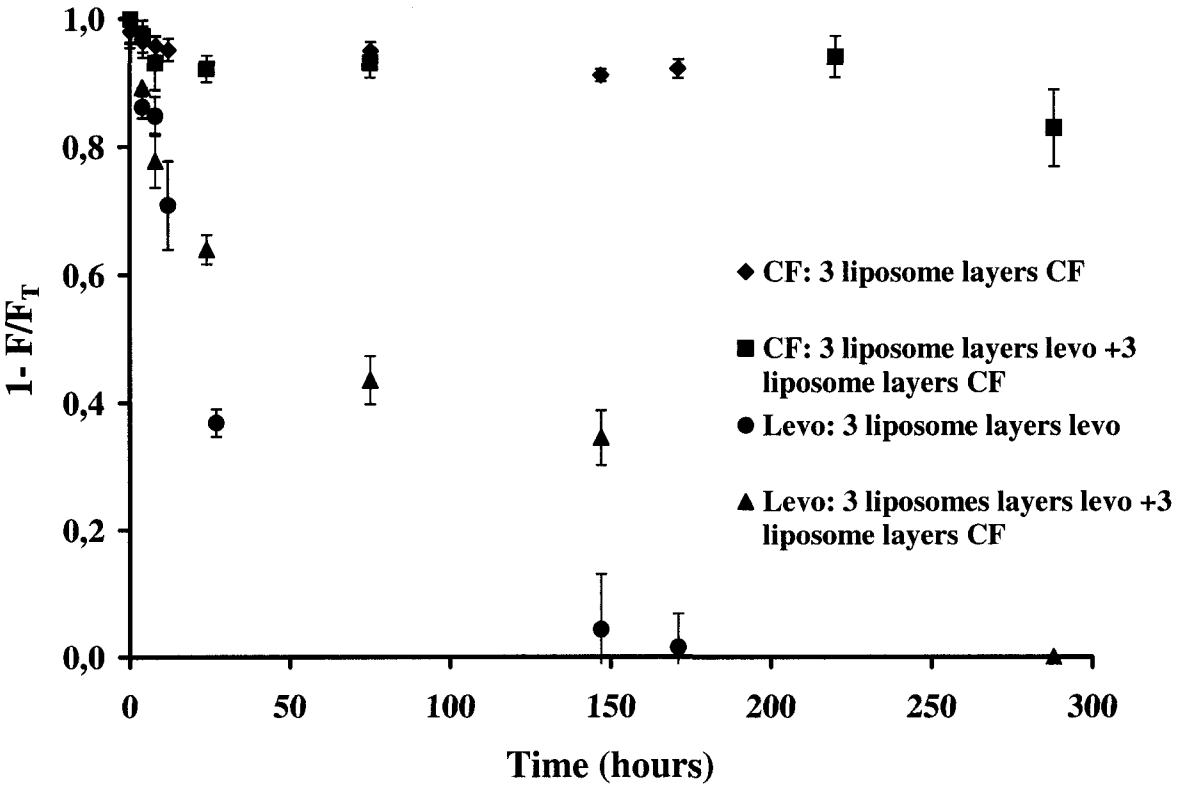

Figure 2.5 Fraction of levofloxacin (levo) or carboxyfluorescein (CF) remaining in surface-bound liposomes on borosilicate at room temperature. Error bars correspond to standard deviation.

As it can be observed, surface-bound liposomes containing CF are very stable and do not release much of their content over 300 hours (i.e. 12 days). Measurements could not be carried out for longer incubation time because following 300 hours incubation, solution evaporation from the multi-wells containing the samples started to be a concern. There was no significant difference between the release kinetics of 3 layers of CF-loaded surface-bound liposomes either if they were surface immobilized alone or if they were combined with 3 layers of intact liposomes containing levofloxacin. This finding was not surprising given the fact that both sets of layers were in direct contact with the incubating media.

For layers of liposomes loaded with levofloxacin, there was a significant difference between the release kinetics of the independent system and that combined with layers of liposomes containing CF. This result was expected given the fact that the 3 layers of intact

liposomes loaded with levofloxacin were covered by layers of liposomes loaded with CF. Indeed, levofloxacin release observed in the combined system was slower than that involving only 3 layers of surface-bound liposomes loaded with levofloxacin. This observation could be explained by mass transfer principles. These additive layers of liposomes became a *de facto* filter medium for the diffusing levofloxacin coming from the bottom of the structure. The top liposome layers created an additional resistance to the molecule diffusion. The liposome layers can be seen as a bulky mass of vesicles, among which appeared to run small channels that allowed a restrictive molecular movement. Upon addition of more liposome layers, the macromolecular mobility across the liposome layers became increasingly limited inducing a slower diffusion of levofloxacin in this situation contrasting with the situation where there were only 3 layers of levofloxacin-loaded vesicles on the surface.

2.6 Conclusions

X-ray photoelectron spectroscopy (XPS) showed the immobilization of liposomes onto surfaces by using the specific NeutrAvidin-biotin link. XPS results also reveal the importance of the deactivation method used to demonstrate the presence of the specific NeutrAvidin-biotin linkage for liposome immobilization. Comparison of the present results with those of other studies points out to the necessity to use low-fouling interlayer (in this case biotinylated-PEG) to immobilize NeutrAvidin molecules in a specific manner.

Quartz crystal microbalance (QCM) with energy dissipation monitoring allowed to follow the build-up of the layers in real-time and *in situ*, revealing that biotinylated-liposomes had viscoelastic behaviour.

QCM experiments also demonstrated that surface-immobilized liposomes were able to resist irreversible adsorption of proteins from foetal bovine serum (FBS, 10% v/v). Fouling resistance can be explained by the capacity of PEG on the liposome membrane to create a steric barrier.

Stability measurements and release kinetic studies of the surface-bound liposomes were carried out by monitoring the release of two different fluorescent probes, namely

carboxyfluorescein and levofloxacin, from these surface-bound liposomes. These studies showed that it was possible to modulate to some extent the release rates of the two molecules by using different configurations of layers of intact liposomes. These findings also revealed that these surface-bound liposomes can be very stable over time but that the release rate of the loaded molecules depends on the selected molecules and probably on the liposome composition (but not tested in the present study).

This study on layers of intact liposomes brings some new fundamental information as the viscoelasticity behaviour of the system and the resistance towards proteins adsoprtion that could help to design more efficient tailor-made localized drug delivery systems. Combining multiple layers of liposomes with different molecules bring possibility to modulate release kinetic profiles of multi-components encapsulated into surface-bound liposomes. However, it is clear from this model study that every drug delivery system needs to be customized to meet the requirements dictated by a specific application.

2.7 Acknowledgments

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2.8 References

(1) Brochu, H.; Polidori, A.; Pucci, B.; Vermette, P. *Curr. Drug Delivery* **2004**, *1*, 299-312.

(2) Weiner, A. L.; Carpenter-Green, S. S.; Soehngen, E. C.; Lenk, R. P.; Popescu, M. C. *J. Pharm. Sci.* **1985**, *74*, 922-925.

(3) Pleyer, U.; Elkins, B.; Ruckert, D.; Lutz, S.; Grammer, J.; Chou, J.; Schmidt, K. H.; Mondino, B. J. *Curr. Eye Res.* **1994**, *13*, 177-181.

(4) Trafny, E.; Grzybowski, J.; Olszowska-Golec, M.; Antos, M.; Struzyna, M. *Pharmacol. Res.* **1996**, *33*, 63-65.

(5) Alamelu, S.; Panduranga Rao, K. *Carbohydr. Polym.* **1994**, *24*, 215-221.

(6) Ruel-Gariepy, E.; Leclair, G.; Hildgen, P.; Gupta, A.; Leroux, J. C. *J. Controlled Release* **2002**, *82*, 373-383.

(7) Vermette, P.; Meagher, L.; Gagnon, E.; Griesser, H. J.; Doillon, C. J. *J. Controlled Release* **2002**, *80*, 179-195.

(8) Vermette, P.; Griesser, H. J.; Kambouris, P.; Meagher, L. *Biomacromolecules* **2004**, *5*, 1496-1502.

(9) Danion, A.; Brochu, H.; Martin, Y.; Vermette, P. *J. Biomed. Mater. Res., Part A* **2007**, 82A, 41-51.

(10) Danion, A.; Arsenault, I.; Vermette, P. *J. Pharm. Sci.* **2007**, 96, 2350-2563.

(11) Vermette, P.; Meagher, L. *Colloids Surf., B* **2003**, *28*, 153-198.

(12) Hope, M.; Bally, M.; Mayer, L.; Janoff, A.; Cullis, P. *Chem. Phys. Lipids* **1986**, *40*, 89-107.

(13) Mayer, L.; Hope, M.; Cullis, P. *Biochim. Biophys. Acta* **1986**, *858*, 161-168.

(14) Hope, M.; Wong, K.; Cullis, P. *Journal Electron Microsc. Tech.* **1989**, *13*, 277-287.

(15) Lasic, D. *Liposomes: from physics to applications*; Elsevier: 1995.

(16) Martin, Y.; Vermette, P. *Macromolecules* **2006**, *39*, 8083-8091.

(17) Vermette, P.; Gengenbach, T.; Divisekera, U.; Kambouris, P. A.; Griesser, H. J.; Meagher, L. *J. Colloid Interface Sci.* **2003**, *259*, 13-26.

(18) Martin, Y.; Boutin, D.; Vermette, P. *Thin Solid Films* **2007**, 515, 6844-6852.

(19) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: New York 1996.

(20) Allen, T.; Cleland, L. *Biochim. Biophys. Acta* **1980**, *597*, 418-426.

(21) Weinstein, J.; Ralston, E.; Leserman, L.; Klausner, R.; Dragsten, P.; Henkart, P.; Blumenthal, R. Self-quenching of carboxyfluorescein fluorescence: uses in studying liposome stability and liposome-cell interactions. In *Liposome Technology. Volume III. Targeted drug delivery and biological interaction*; Gregoriadis G, Ed.; CRC Press Inc.: Boca Raton, FL, **1984**; pp. 183-204.

(22) Lelkes, P. Methodological aspects dealing with stability measurements of liposomes in vitro using the carboxyfluorescein-assay. In *Liposome Technology. Volume III. Targeted drug delivery and biological interaction*; Gregoriadis G, Ed.; CRC Press Inc.: Boca Raton, FL, **1984**; pp. 225-246.

(23) Beamson, G.; Briggs, D. *High resolution XPS of organic polymers*; John Wiley & Sons: Chichester, **1992**.

(24) Sauerbrey, G. Z. *Phys* **1959**, 155, 206-210.

(25) Reimhult, E.; Höök, F.; Kasemo, B. *Langmuir* **2003**, 19, 1681-1691.

(26) Reiss, B.; Janshoff, A.; Steinem, C.; Seebach, J.; Wegener, J. *Langmuir* **2003**, 19, 1816-1823.

(27) Schmitt, F. J.; Haussling, L.; Ringsdorf, H.; Knoll, W. *Thin Solid Films* **1992**, 210, 815-817.

(28) Luthgens, E.; Herrig, A.; Kastl, K.; Steinem, C.; Reiss, B.; Wegener, J.; Pignataro, B.; Janshoff, A. Meas. Sci. Technol. **2003**, 14, 1865-1875.

(29) Reimhult, E.; Höök, F.; Kasemo, B. J. Chem. Phys. **2002**, 117, 7401-7404.

(30) Patel, A. R.; Frank, C. W. *Langmuir* **2006**, *22*, 7587-7599.

(31) Graneli, A.; Edvardsson, M.; Hook, F. *ChemPhysChem* **2004**, *5*, 729-733.

(32) Rodahl, M.; Hook, F.; Fredriksson, C.; Keller, C. A.; Krozer, A.; Brzezinski, P.; Voinova, M.; Kasemo, B. *Faraday Discuss.* **1997**, 229-246.

(33) Leckband, D. E.; Schmitt, F.-J.; Israelachvili, J. N.; Knoll, W. *Biochemistry* **1994**, *33*, 4611-4624.

(34) Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85-133.

(35) Israelachvili, J. *Intermolecular and Surface Forces*; Academic Press: New York, **1992**.

(36) Allen, T. *Curr. Opin. Colloid Interface Sci.* **1996**, *1*, 645-651.

(37) Marjan, J.; Allen, T. *Biotechnol. Adv.* **1996**, *14*, 151-175.

(38) Lasic, D. *Liposomes: From Physics to Applications*; Elsevier: New York, **1995**.

(39) Malmsten, M. *J. Colloid Interface Sci.* **1995**, 172, 106-115.

(40) Malmsten, M. *Colloids Surf., A* **1999**, 159, 77-87.

Chapitre 3

Young Moduli of Surface-Bound Liposomes by Atomic Force Microscopy Force Measurements

Extraction du module de Young de liposomes immobilisés sur une surface par mesures de forces à l'aide de la microscopie à force atomique

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3.1 Abstract

Mechanical properties of layers of stable liposomes attached by specific interactions on solid surfaces were studied by atomic force microscopy (AFM) force measurements. Force-distance measurements using colloidal probe tips were obtained over liposome layers and used to calculate Young's moduli by using the Hertz contact theory. A classical Hertz model and a modified Hertz one have been used to extract Young's moduli from AFM force curves. The modified model, proposed by Dimitriadis, is correcting for the finite sample thickness since Hertz classical model is assuming that the sample is infinitely thick. Values for Young's moduli of 40 kPa and 8 kPa have been obtained using the Hertz model for 1 and 3 layers of intact liposomes, respectively. Young's moduli of approximately 3 kPa have been obtained using the corrected Hertz model for both 1 and 3 layers of surface-bound liposomes. Compression work performed by the colloidal probe to compress these liposome layers has also been calculated.

3.2 Résumé

Les propriétés mécaniques de couches de liposomes intacts immobilisés par liaisons spécifiques ont été étudiées à l'aide de mesures de forces faites par microscopie à force atomique (AFM). Utilisant une sonde colloïdale, des courbes de la force appliquée en fonction de la distance de séparation ont été obtenues sur des échantillons ayant 1 ou 3 couches de liposomes. Ces résultats ont servi à extraire le module de Young (module élastique) des échantillons à l'aide de la théorie de contact de Hertz. Le modèle classique de Hertz ainsi qu'un modèle amélioré ont été utilisés pour extraire le module des courbes obtenues par AFM. Le modèle amélioré, proposé par Dimitriadis, corrige le fait que l'échantillon à une épaisseur finie comparativement à la théorie de Hertz qui considère une épaisseur d'échantillon infinie. Des valeurs de modules de Young de 38 kPa et 8 kPa ont été obtenues pour 1 et 3 couches de liposomes, respectivement, selon le modèle de Hertz. Selon le modèle amélioré, un module de Young d'environ 3 kPa a été obtenu pour 1 et 3 couches de liposomes. Le travail de compression exercé par la sonde colloïdale sur les couches de liposomes a aussi été calculé pour les 2 conditions étudiées soient 1 et 3 couches de liposomes.

3.3 Introduction

The scientific literature is abundant on the development, characterization and validation of liposome suspensions. However, much less is known on the surface immobilization of layers of intact liposomes, which can find applications in several fields including drug chromatography,[1-7] cell mimicking system,[8,9] and localized drug release.[10-12] Various techniques have been used to attach vesicles onto and into different substrates such as collagen[13-15] and chitosan.[16,17]

Vermette and colleagues have developed a method to immobilize intact pegylated-liposomes onto surfaces by using the strong NeutrAvidin-biotin specific interaction.[10,18] If loaded with a therapeutic agent and remaining intact, layers of liposomes immobilized on solid substrates could make good candidates for local drug delivery applications from biomedical devices. For example, Danion et al. have reported the detailed surface immobilization of layers of intact liposomes on contact lens surfaces.[11] Also, the antibacterial activity of contact lenses bearing surface-immobilized layers of intact liposomes loaded with levofloxacin was successfully demonstrated.[12] Liposomes were also immobilized using different systems on quartz crystal microbalance crystals by Lüthgens et al. to study liposomes adhesion with the aim to model cell adhesion by using liposomes bearing receptor.[9] Arrays of intact liposomes have been successfully immobilized into chemically functionalized micro-wells using also the NeutrAvidin-biotin link.[19] The goal of this application was to use liposomes arrays as a platform for sequestering and displaying cell surface membrane proteins in the liposomes in order to form micro-arrays exposing membrane proteins for subsequent screening purposes.[19]

Although some papers are available on the characterization and applications of surface-immobilized layers of intact liposomes, many physicochemical properties of these complex and dynamic hydrated layers still need to be elucidated. With available surface characterization instruments such as atomic force microscopy (AFM), quartz crystal microbalance (QCM) with energy dissipation monitoring, and surface plasmon resonance (SPR), physical characterization of these surface-bound liposomes is becoming more and more accessible.

AFM force measurements, combined with an appropriate model of contact theory, have been used almost since the AFM introduction to obtain Young's moduli (E) of different materials including rubber,[20] gels[21,22] and biological samples such as living cells.[20,23-25] By knowing Young's moduli of liposome layers, some basic properties such as shear stress resistance and mechanical stability could be correlated to other layer properties. For example, knowing these moduli could allow developing contact lenses bearing layers of surface-bound liposomes with mechanical properties that can match those of the mucous layer of the cornea, and therefore optimizing mechanical interaction between the contact lens and the ocular surface as well as the eyelid to mimic, to some extent, physiological conditions. This could potentially improve contact lens wear comfort.

Young's moduli can be obtained from AFM force curves by fitting data with an adequate model of contact to extract moduli. Most often used theories are Hertz,[26] Johnson-Rendall-Roberts (JKR),[27] and Derjaguin-Müller-Toporov (DMT).[28-30] The JKR and DMT models are based on the Hertz theory but they take into account adhesive forces between the probe and the sample during contact; when no adhesive forces are involved, these models return to the Hertz model. Since the original Hertz model[26] involves the contact between a sphere and a plane surface, variations of the Hertz theory have been proposed for systems involving other geometries of contact.[31] More recently, a modified Hertz model has been developed by Dimitriadis et al.,[32] adding a correction factor for the effect of the finite sample thickness. The Dimitriadis model can be applied either to bonded or to not bonded (i.e., free to slip on the surface) samples. Colloidal probe measurements using a micro-sphere attached to AFM cantilevers are often used to probe material properties with the aim to get a defined geometry and also to minimize sample damage due to the high pressure involved with the use of bare cantilevers.[24,32-35]

The aim of this study was to extract Young's moduli of surface-bound liposomes from AFM colloidal probe force measurements. A modified Hertz model derived from Dimitriadis et al. was used since liposomes layers can be considered as thin samples. Young's modulus values obtained with the corrected model are compared with those from the basic Hertz model and with other results where lipid vesicles were investigated using AFM force measurements fitted with the Hertz model.[36-39]

3.4 Experimental Section

3.4.1 Materials

N-heptylamine monomer (no.126802) and N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES, no.H-3375, 99.5%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sodium chloride (NaCl, ACS grade), sodium sulfate (Na_2SO_4, ACS grade), Sparkleen™ (no.04-320-04), ethanol (EtOH, ACS grade), chloroform (ACS grade), and Petri dishes were purchased from Fisher Scientific (Ottawa, ON, Canada). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, >99%, no.850365-P), cholesterol (CHOL, >99%, no.700000-P), N-[ω-(biotinoylamino)poly(ethylene glycol) 2000]-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG_{2000}-Biotin, >96%, no.890129-P) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Biotin-PEG(3400)-NHS, (NHS-PEG-Biotin, no.0H4M0F02) was purchased from Nektar Therapeutics (Huntsville, AL).

Borosilicate plates (12 mm x 12 mm) were purchased from Carolina Biological Supply Company (Burlington, NC, no.63-3069). Borosilicate substrates were cleaned in Sparkleen™ solution overnight, immersed in ethanol, rinsed in water, and finally blown dry using a high-velocity stream of 0.2-μm filtered air (Millex® GP, Millipore, Cork, Ireland). Samples were only used once.

Silica spheres (no.SS05N, mean diameter of 4.12 μm) were purchased from Bangs Laboratories, Inc. (Fishers, IN). Model DNP-S cantilevers with integrated pyramidal tips were purchased from Veeco (Camarillo, CA), cleaned under a UV/ozone atmosphere system (185 and 254 nm, PSD-UV, Novascan, IA) before utilization.

3.4.2 Liposomes Preparation and Surface Immobilization of Liposomes

Procedures to extrude liposomes have been previously reported by other workers.[40-43] Briefly, unilamellar vesicles were made of DSPC, CHOL in 2:1 molar ratio and DSPE-PEG_{2000}-Biotin (5 %(mol/mol) from total lipids). Unilamellar vesicles were produced by extrusion through 100-nm pore polycarbonate membranes using the LiposoFast-Basic extruder both from Avestin Inc. (Ottawa, ON, Canada) operated at 75°C. This temperature is higher than the main transition temperature which is approximately 55°C.[44] The total

lipid concentration of the liposome suspension collected at the end of the extrusion was adjusted using the HEPES buffer to the desired concentration, usually 1 mg total lipids/mL. The HEPES buffer contained 10mM HEPES at pH 7.4 and a NaCl concentration (approximately 150 mM) that was adjusted to an osmolarity of 290 mOsm, using The Advanced Osmometer, model 3250 (Advanced Instruments Inc., Norwood, MA). Milli-Q® gradient water (Millipore Canada, Nepean, Canada) with a resistivity of 18.2 MΩ·cm was used to prepare buffer solutions. Liposome immobilization using a low-fouling PEG interlayer[45] and the specific NeutrAvidin-biotin linkage have been well described by Vermette and colleagues.[10,11,18,46] For a detailed description of the immobilization procedures, readers should refer to a previous article.[47]

3.4.3 AFM Colloidal Probe Force Measurements over Surface-bound Liposomes

A Bioscope (Digital Instruments, Santa Barbara, CA) linked to a Nanoscope IIIa controller and coupled to an inverted microscope (Zeiss) was used to carry out AFM force measurements. Force measurements using the colloidal probe method developed by Ducker et al.[48] was selected to limit sample damage.[24] Also, the use of the colloidal-probe approach allows probing much larger surface area of the liposome layers when compared to bare cantilever tip, which would probe a much reduced surface area. Silica micro-spheres were attached to Si_3N_4 cantilevers using the piezo to dip the AFM tip into a UV-curable glue and then to hook the sphere. The spring constant of the cantilevers (DNP-S model) was measured to be 0.14 N/m using the resonance method proposed by Cleveland et al.[49]

For AFM force measurements, samples were placed in a Petri dish containing HEPES buffer in order to cover sample surfaces. Samples were firmly fixed to the bottom by a custom-made Teflon o-ring to prevent sample movement or floating upon measurements. Before probing, cantilever was approached near the sample surface, immersed into the buffer, and left for at least one hour to reach thermal equilibrium. Experiments were carried out at room temperature in a custom-made acoustic box, which protect the AFM from external acoustic vibration. For each condition, three different samples were studied and three different spots were probed on each sample. At least three force curves were recorded for each spot. Approach and retraction force curves were obtained at a constant speed of 40

nm/s. This slow probing speed was selected to limit time-dependent viscous contribution, which can contribute to hysteresis between approach and retraction force curves.[25]

The reference substrate used to calculate the indentation was bare borosilicate glass, which is the same substrate on which the surface-bound liposomes were attached. Reference force curves obtained before and following each sample analysis were compared and no difference was observed indicating that lipids (or other molecules) adsorption on the silica sphere was not observed (data not shown). Also, the AFM tip was visually inspected before and after each sample analysis to make sure that the micro-sphere was always present during the measurements. Tips were used more than once to limit contact geometry variation between the samples but were always thoroughly cleaned to remove lipids, if any, between each experiment.

Raw cantilever deflection curves of the different layers were used for Young's moduli calculation or were transformed into force-versus-distance curves to obtain the work done to compress the layer. Young's moduli calculations were done using the classical Hertz model and a modified Hertz model.[32] Force-versus-distance curves were used to obtain the work done by the colloidal probe to compress the layers by calculating the area under the curve using the trapezoidal method. Transformation of deflection-vs-z-position curves, based on the developments by Ducker et al.,[50] and the Young's modulus extraction were done by a custom-made software. The software is freely available upon request to P. Vermette.

3.4.4 Young's Moduli Calculation from Hertz and Modified Hertz Models

Young's modulus calculations, based on the basic Hertz model and the corrected-model of Dimitriadis,[32] were done on samples composed of 1 and 3 layers of liposomes. Given that it takes considerable time to produce multi-layers of surface-bound liposomes, a system composed of 3 layers of intact liposomes was selected in order to limit the effect of storage on the liposome structures and to allow more accurate comparison with the one-layer liposome system. The system composed of 3 layers of intact liposomes can be prepared within 24 hours prior to the AFM analyses.

The Hertz model of contact is based on two assumptions: (1) linear elasticity and (2) infinite sample thickness. Owing to the slow piezo element speed, only elastic deformation of the samples was assumed. Considering that liposome layers do not have infinite thickness, a corrected Hertz model was used to take into account the stiffening effect observed as sample becomes thinner.[32] The Dimitriadis model was developed to allow reliable determination of elastic moduli on soft samples, irrespective of the thickness and the way that they are supported on rigid surfaces (bonded or not bonded).[32] Not bonded status means that the sample is free to slip over the surface i.e., that the boundary condition of sample velocity is not equal to zero.[51] Calculations were done using both hypotheses i.e., bonded or not bonded, to be able to compare the impact of this hypothesis on the calculated moduli. These models show the relationship between the applied force F and the indentation δ, as shown in the following equations:

Hertz model:

$$F = \frac{4}{3}\frac{ER^{1/2}\delta^{3/2}}{(1-\upsilon^2)} \tag{3.1}$$

Dimitriadis model for not bonded samples:

$$F = \frac{16}{9}\left(ER^{1/2}\delta^{3/2}\right)\left[1+0.884\chi+0.781\chi^2+0.386\chi^3+0.0048\chi^4\right] \tag{3.2}$$

Dimitriadis model for bonded samples:

$$F = \frac{16}{9}\left(ER^{1/2}\delta^{3/2}\right)\left[1+1.133\chi+1.283\chi^2+0.769\chi^3+0.0975\chi^4\right] \tag{3.3}$$

where E [Pa] is the Young's modulus, R the probe-sphere radius,υ the Poisson ratio, and $\chi = (R\delta)^{1/2}/h$ where h is the sample thickness. A Poisson ratio of 0.5 was assumed for liposome layers as the samples were considered incompressible as it is for most biological samples.[32] The sample indentation, δ, is defined as the difference between the piezo translation and the cantilever deflection, since there is an indentation in the sample caused by its softness. This indentation is calculated from the difference between the piezo position

considering a same deflection of the cantilever on a hard sample and on a soft sample, here borosilicate and liposome layers, respectively. The indentations were obtained from the approaching curves since no force (i.e., no jump-to-contact) interfered between the micro-sphere and the sample. For this indentation, the contact point has to be known and since it is difficult to determine this contact point, the contact point was estimated along with the Young's modulus during the calculations in order to obtain the best fit to the model considering the least squares method as proposed by Dimitriadis et al.[32] It is important to mention that an indentation of only 10% of the total sample thickness was considered for the calculation to limit the influence of the underlying substrate on the result as also proposed by Dimitriadiset al.[32]

To calculate Young's moduli using the corrected Hertz model, the thickness of the sample needs to be specified. As it is difficult to obtain the exact thickness of liposome layers, approximations have been done. N-heptylamine plasma polymer (HApp) layer thickness over the borosilicate substrate has been neglected, since it is a very flat and rigid surface that do not swell in aqueous media as reported by our group.[52] PEG layer thickness has been estimated to 8 nm as reported by AFM force measurements.[45] The thickness of the immobilized NeutrAvidin has been estimated to 4 nm, as reported by a study with a similar protein, streptavidin, with SFA measurements.[53] This is in good agreement with other studies carried out by Vermette et al.[18,46] For the liposomes thickness, since they have a diameter of approximately 110 nm in suspension,[54] overall sample thickness for 1 layer has been estimated to 100 nm and to 300 nm for 3 layers. The effect of liposome layers thickness on the calculated Young's moduli of 3 liposome layers using the Dimitriadis' model has been evaluated and the results are presented in Table 2.

3.5 AFM Imaging of Surface-bound Liposomes

Atomic Force Microscopy (AFM) imaging was performed using a Digital Nanoscope IIIa Bioscope. All imaging was performed via Tapping mode with oxide-sharpened silicon nitride cantilevers with integrated pyramidal tips (Model DNP-S, Veeco NanoProbe Tips). Tapping mode imaging greatly reduces the magnitude of lateral forces applied to samples and appears more appropriate for imaging liposomes. The drive frequencies were chosen

between 7.8 and 8.1 kHz. The RMS amplitude was fixed at 0.3 V. Cantilevers used in this study have a spring constant of 0.32 N/m. As for the force measurements, the imaging was done in HEPES buffer. Imaging of 1 layer of intact liposomes is presented in Figure 1.

3.6 Results and Discussion

3.6.1 AFM Colloidal Probe Force Measurements of Surface-bound Liposomes

Figure 3.1 clearly illustrates the presence of spherical particles, wich represent intact liposomes.

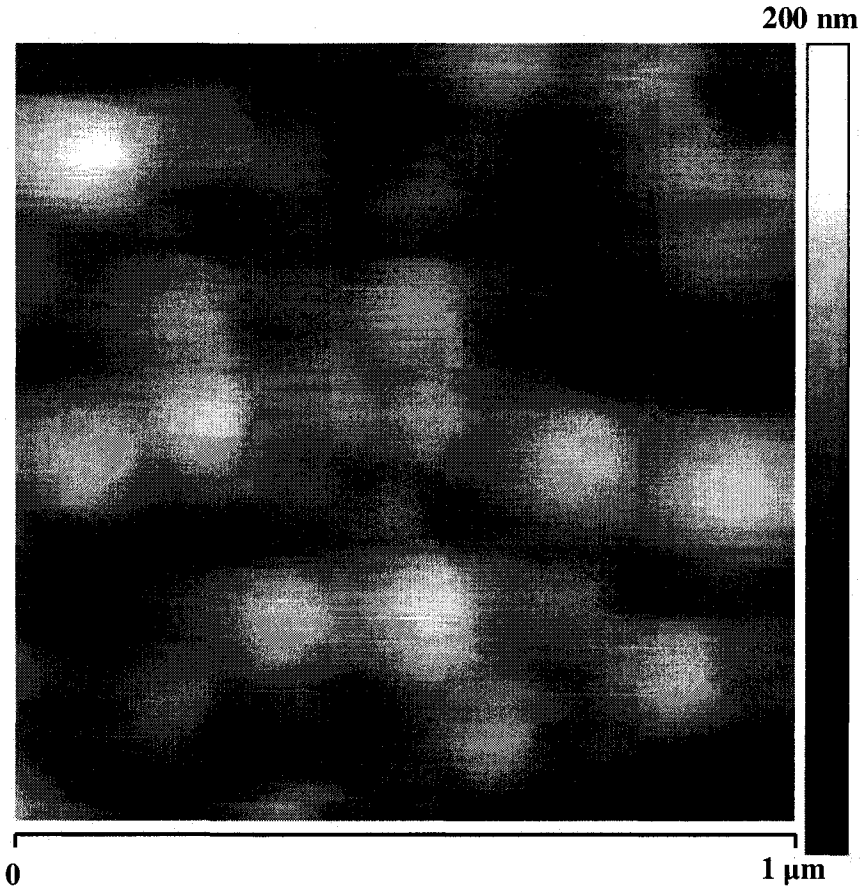

Figure 3.1.AFM Taping mode image of 1 layer of intact liposomes immobilized on HApp: PEG-Biotin: NeutrAvidin layers on borosilicate glass in HEPES buffer.

Many force curves were obtained at the same spot on each sample without any change in the curve profile, confirming that layers of stable liposomes were not damaged by the AFM colloidal probe and that no plastic (i.e., permanent) deformation was induced.

However, liposomes were probably reorganizing after probing even if the probing speed was quite slow, since the contact point between two measurements was not always at the same distance i.e. the zero-force separation distance was slightly changing.

Even at a speed of 40 nm/s, hystereses were observed between approach and retraction curves, as depicted in Figure 3.2. These hystereses may be caused by liposomes reorganization between the approach and retraction. Also, it is possible that this reorganization could involve the flow of water molecules (i.e., a viscous loss), contained within and between lipid vesicles, in and out from the liposome layers. Figure 3.2 also suggests that when the cantilever relieves the pressure over the vesicles, it can take more time before the liposome layers return to their original thicknesses, again supporting the hypothesis that some water might be expulsed from the liposome layers upon compression. QCM with energy dissipation monitoring revealed that layers of intact liposomes were shown to have viscoelastic behaviour.[47] Also, the approach and retraction curves do not join at "0 distance" (i.e., the hard wall). Perhaps the reorganization of the structure is causing this difference in the "force" felt by the cantilever. More investigation will be warranted to study this complex phenomenon.

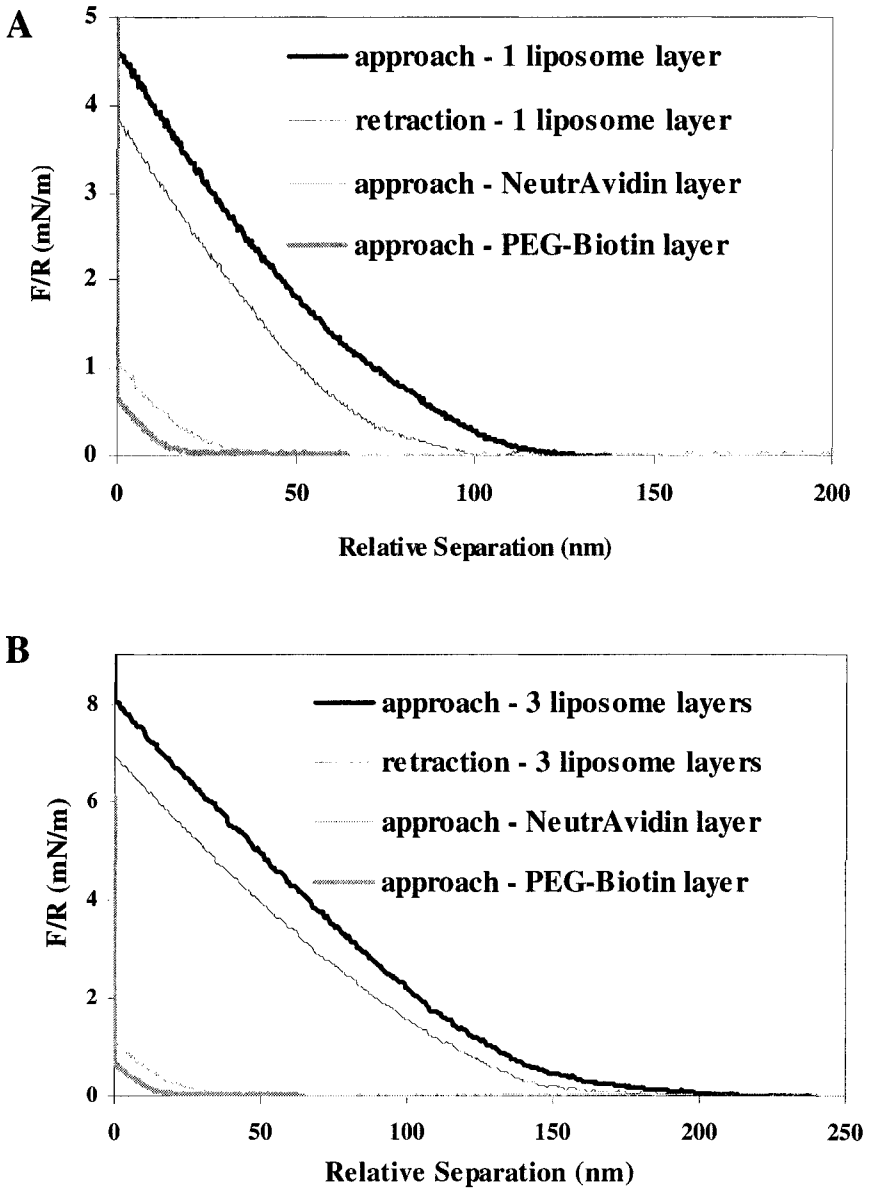

Figure 3.2 Representative approach and retraction force-vs-distance curves of samples having **A**) 1 layer of intact liposomes and **B**) 3 layers of intact liposomes. PEG-Biotin and NeutrAvidin approach curves are also presented.

The work done to compress the liposome layers from zero-force separation up to the hard-wall was obtained by calculating the area under force-vs-distance curves. As it was intuitively thought, significantly (*t*-test: $p<0.001$) more work was necessary to compress 3 liposome layers than 1 layer of liposomes with 700±300 aJ and 290±50 aJ, respectively. Also, it was observed that the compression was higher for 3 liposome layers than for 1

layer. In fact, mean compressions of 170±20 nm and 110±20 nm were obtained for 3 and 1 layers of liposomes, respectively. The compression capacity was not proportional to the number of layers of liposomes immobilized on the surface. This finding is in good agreement with a previous study.[10,18] The term layers should be used loosely here since a regular flat monolayer-like structure over the previous one is not envisaged when adding NeutrAvidin and biotinylated liposomes to the first liposome layer. Given that liposomes are compressible structures, some liposomes can fill some small, but large enough gaps available between previously bounded liposomes. It may explain why the compression capacity is not proportional to the number of layers.

3.6.2 Young's moduli of Surface-bound Liposomes

Young's moduli (*E*) were extracted from probed samples having 1 and 3 layers of liposomes using the Hertz model and the corrected modified Hertz model of Dimitriadis. Figure 3.3 shows the steps done by the software to extract the Young's modulus from the AFM raw data. Young's modulus extraction using the Dimitriadis model has been done considering that samples could either be "bonded" or "not bonded" to the substrate. Results are summarized in Table 3.1.

Table 3.1 Young's Moduli of Layers of Intact Liposomes.

Model/Sample	Young's modulus (kPa)
Hertz model	
1 layer of liposomes	40 ± 20
3 layers of liposomes	8 ± 2
Dimitriadis model	
1 layer of liposomes - *Not Bonded*	4 ± 2
1 layer of liposomes - *Bonded*	3 ± 2
3 layers of liposomes - *Not Bonded*	2.2 ± 0.6
3 layers of liposomes - *Bonded*	1.5 ± 0.4

The data represent means ± standard deviations. In this Table, thicknesses of 1 and 3 layers of liposomes were estimated to 100 nm and 300 nm, respectively.

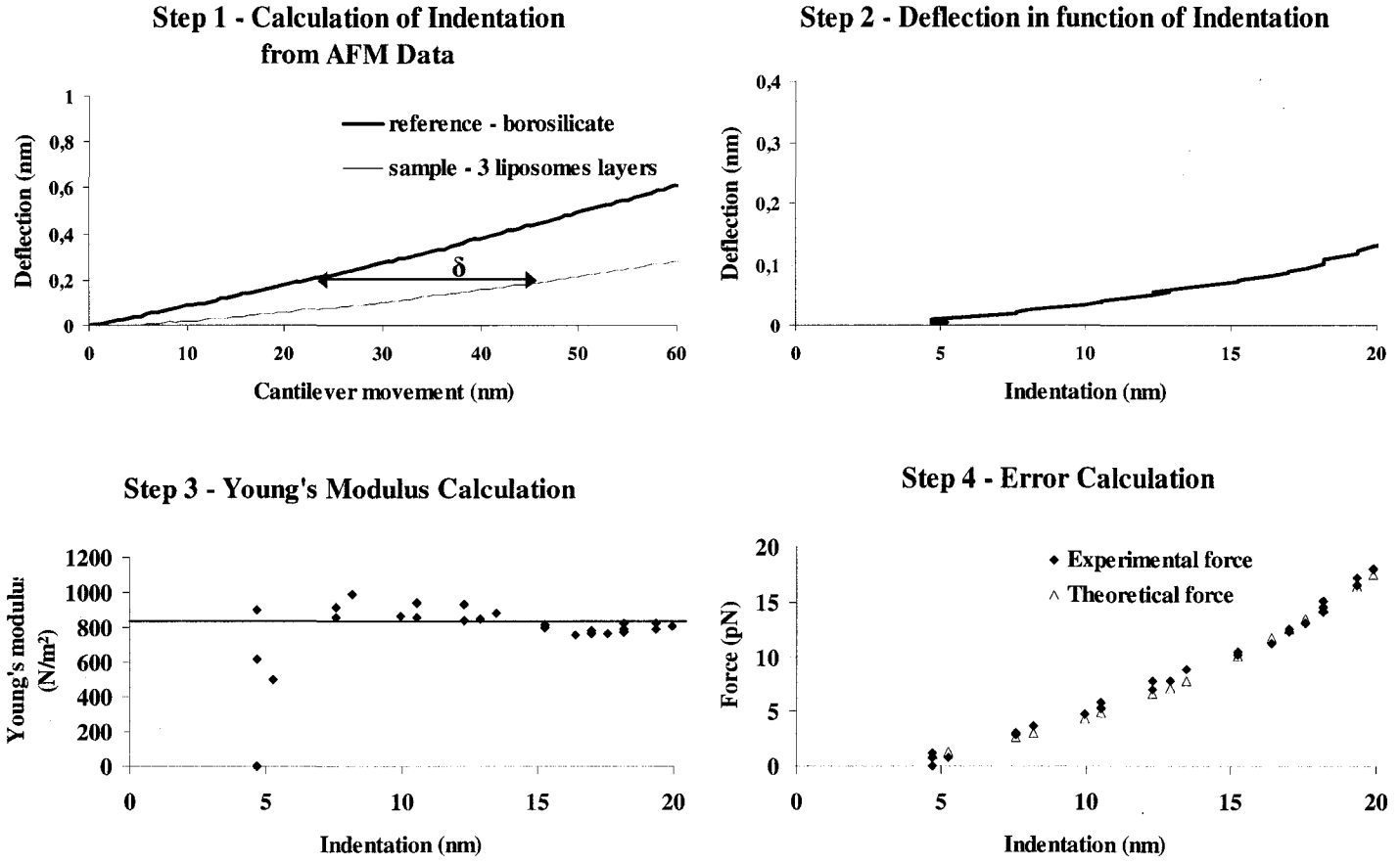

Figure 3.3 Summary of the steps involved in Young's modulus calculation from raw AFM data. **Step 1:** From an estimated contact point, the indentation (δ) is calculated. **Step 2:** A relationship is obtained between the deflection, which is related to the force, and the indentation. This relationship is used with the developed equations of the corresponding models. **Step 3:** Young's moduli are calculated in function of the indentation. An experimental modulus is therefore extracted from the averaged plateau values. **Step 4:** Errors between the experimental and theoretical forces are calculated. The theoretical forces are obtained from the modulus calculated from Step 3. These errors are compared for each assumed contact point and the smallest error dictates the Young's modulus, for a given sample considering one model and one estimated sample thickness. The representative calculation steps illustrated here correspond to a sample bearing 3 layers of intact liposomes using the Dimitriadis model (not bonded) for an estimated sample thickness of 200 nm.

Hertz model gives significantly larger Young's moduli compared to the ones calculated with the Dimitriadis model. It is known that the Hertz model may overestimate E for samples having finite thickness[32] as it is the case here. Also, Young's moduli calculated

for 1 layer of liposomes are much larger than those of 3 liposome layers. This difference may be an example of Hertz model overestimation of E when sample the becomes thinner.

The Dimitriadis model gives similar values for samples having 1 and 3 layers of liposomes, as observed in Table 3.1. Surprisingly, the hypothesis of samples "bonded" or "not bonded" to the underlying substrate did not reveal any difference in the results. The differences are not significant (t-tests have all $p>0.2$). Since those values are not significantly different, we may presume that we obtain a bulk value for the immobilized liposome structures i.e., approximately 3 kPa.

If we consider that the anchors of NeutrAvidin on liposomes are DSPE-PEG-Biotin molecules, a liposome in the two first layers of the 3-layers system may be involved in many connections with neighbors of the same layer and/or of the preceding/next layer. Indeed, when added to construct the second layer (for example), NeutrAvidin (approximately 4 to 5 nm diameter) may be able to diffuse between intact liposomes and reach the first layer to laterally connect the liposomes. These interconnections in the multilayer system could cause a rigidification (by NeutrAvidin-induced reticulation) of the system and as a consequence, this may affect the Young moduli obtained for the multilayers. However, this potential NeutrAvidin-induced reticulation does not seem to contribute to the stiffening of the 3-layers system since the Young's moduli of 1 and 3 layers of intact liposomes calculated from the Dimitriadis model are not significantly different.

Calculations were made to observe the effect of the input sample thickness over the calculated Young's modulus. Different sample thicknesses were tested for the 3 liposomes layers samples. Sample thicknesses ranging from 160 nm to 340 nm were selected, with 20 nm increments. The results are summarized in Table 3.2. As the estimated thickness increases, Young's modulus increases (significant differences with $p< 0.05$).It can be seen that the values are higher for the model considering the "not bonded" hypothesis, but the results are not significantly different as observed earlier. The similar results obtained from both models considering either "bonded" or "not bonded" samples may be explained by the fact that the liposomes layers are probably just in between these two scenarios. The vesicles

are bound to the underlying substrate, but can surely exert some lateral movement over the substrate because of the hydrated nature of these layers.

Table 3.2. Young's Moduli calculated from the Dimitriadis Model for 3 Layers of Intact Liposomes as a Function of the Estimated Thickness.

Estimated thickness	Young's modulus (kPa)
Not Bonded	
160 nm	0.7 ± 0.2
180 nm	0.8 ± 0.2
200 nm	1.1 ± 0.3
220 nm	1.3 ± 0.4
240 nm	1.5 ± 0.4
260 nm	1.7 ± 0.5
280 nm	2.0 ± 0.6
300 nm	2.2 ± 0.6
320 nm	2.4 ± 0.5
340 nm	2.6 ± 0.7
Bonded	
160 nm	0.4 ± 0.1
180 nm	0.6 ± 0.2
200 nm	0.7 ± 0.2
220 nm	0.9 ± 0.2
240 nm	1.0 ± 0.3
260 nm	1.2 ± 0.3
280 nm	1.4 ± 0.4
300 nm	1.5 ± 0.4
320 nm	1.7 ± 0.5
340 nm	1.8 ± 0.6

The data represent means ± standard deviations.

For comparison, few research teams have measured Young's moduli of similar surface-bound liposomes using the Hertz model. In other studies, liposomes were simply physisorbed on surfaces and force measurements were carried out with sharp cantilever tip.

Liang et al. obtained Young's moduli ranging between 1.97 and 13 MPa, depending on the cholesterol/lipid ratio used to make the liposomes.[36,37] Ramachandran et al. obtained Young's modulus of 800 kPa for lipid vesicles containing cisplatin and 450 kPa for those without cisplatin.[38] Another group, Delorme et al., used a shell deformation theory model and found Young's moduli of 110 MPa for liposomes physisorbed on silicon substrate.[39]

As outlined above, discrepancies of Young's moduli of intact liposomes attached to solid surfaces are observed in the scientific literature and these can be explained by different methods used to produce these complex layers, by the different liposome composition, and/or by the lack of a detailed surface characterization to verify that these liposomes remain, indeed, intact upon surface attachment.

It can be tempting to compare our results with those obtained on living cells. When compared to living cells, which have been more studied than surface-immobilized lipid vesicles with respect of their mechanical properties, Young's moduli fluctuate from one study to another; different cell types will give different values. Moreover, a different localization on the same cell gives different moduli. For example, the Hertz model was used to fit AFM colloidal probe force measurements carried out over human epithelial cells to obtain Young's moduli. Values of 14 and 33 kPa over the nucleus for young and old cells, respectively, were obtained and, over cytoplasm, values of 37 and 110 kPa were obtained for young and old cells, respectively.[34] Young's moduli of surface-immobilized liposomes are smaller than those of human epithelial cells and those of most cells. Considering the much more complex organisation of cell membranes and the number of organelles found inside cells, it is not surprising that a cell may be stiffer than bare liposome layers.

For comparison purposes, Young's moduli of gelatin in water, at different pH, range between 1 and 9 kPa.[55] Polyacrylamide gels with various bis-acrylamide cross-linker content showed E ranging between 1 and 8 kPa.[56]

3.7 Conclusions

Young's moduli of layers of intact liposomes have been obtained using AFM force measurements by fitting two models of contact mechanics. Given the finite thickness of the liposomes layers, the corrected Hertz model from Dimitriadis revealed more realistic values than the classical Hertz model, as expected. Young's moduli around 3 kPa were obtained. Knowing in more detail physicochemical properties of layers of intact liposomes could pave the way to create tailor-made liposome layers for specific applications. Lipid composition and vesicle size could be adapted to modulate liposome layers properties.

3.8 Acknowledgments

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3.9 References

(1) Lundahl, P.; Beigi, F. *Adv. Drug Delivery Rev.* **1997**, *23*, 221-227.

(2) Lunqvist, A.; Ocklind, G.; Haneskog, L.; Lundahl, P. *J. Mol. Recognit.* **1998**, *11*, 52-57.

(3) Yang, Q.; Liu, X. Y.; Ajiki, S.; Hara, M.; Lundahl, P.; Miyake, J. *J. Chromatogr., A* **1998**, *707*, 131-141.

(4) Yang, Q.; Liu, X. Y.; Miyake, J.; Toyotama, H. *Supramol. Sci.* **1998**, *5*, 769-772.

(5) Yang, Q.; Liu, X. Y.; Yoshimoto, M.; Kuboi, R.; Miyake, J. *Anal. Biochem.* **1999**, *268*, 354-362.

(6) Khaleque, M. A.; Okumura, Y.; Yabushita, S.; Mitani, M. *Chem. Lett.* **2003**, *32*, 416-417.

(7) Mao, X. Q.; Kong, L.; Li, X.; Guo, B. C.; Zou, H. F. *Anal. Bioanal. Chem.* **2003**, *375*, 550-555.

(8) Liebau, M.; Bendas, G.; Rothe, U.; Neubert, R. H. H. *Sens. Actuators, B.* **1998**, *47*, 239-245.

(9) Luthgens, E.; Herrig, A.; Kastl, K.; Steinem, C.; Reiss, B.; Wegener, J.; Pignataro, B.; Janshoff, A. *Meas. Sci. Technol.* **2003**, *14*, 1865-1875.

(10) Vermette, P.; Meagher, L.; Gagnon, E.; Griesser, H. J.; Doillon, C. J. *J. Controlled Release* **2002**, *80*, 179-195.

(11) Danion, A.; Brochu, H.; Martin, Y.; Vermette, P. *J. Biomed Mater Res., Part A* **2007**, *82A*, 41-51.

(12) Danion, A.; Arsenault, I.; Vermette, P. *J. Pharm. Sci.* **2007**, 96, 2350-2563.

(13) Weiner, A. L.; Carpenter-Green, S. S.; Soehngen, E. C.; Lenk, R. P.; Popescu, M. C. *J. Pharm. Sci.* **1985**, *74*, 922-925.

(14) Pleyer, U.; Elkins, B.; Ruckert, D.; Lutz, S.; Grammer, J.; Chou, J.; Schmidt, K. H.; Mondino, B. J. *Curr. Eye Res.* **1994**, *13*, 177-181.

(15) Trafny, E.; Grzybowski, J.; Olszowska-Golec, M.; Antos, M.; Struzyna, M. *Pharmacol. Res.* **1996**, *33*, 63-65.

(16) Alamelu, S.; Panduranga Rao, K. *Carbohyd. Polym.* **1994**, *24*, 215-221.

(17) Ruel-Gariepy, E.; Leclair, G.; Hildgen, P.; Gupta, A.; Leroux, J. C. *J.Controlled Release* **2002**, *82*, 373-383.

(18) Vermette, P.; Griesser, H. J.; Kambouris, P.; Meagher, L. *Biomacromolecules* **2004**, *5*, 1496-1502.

(19) Kalyankar, N. D.; Sharma, M. K.; Vaidya, S. V.; Calhoun, D.; Maldarelli, C.; Couzis, A.; Gilchrist, L. *Langmuir* **2006**, *22*, 5403-5411.

(20) Weisenhorn, A.; Khorsandi, M.; Kasas, S.; Gotzos, V.; Butt, H.-J. *Nanotechnology* **1993**, *4*, 106-113.

(21) Radmacher, M.; Fritz, M.; Hansma, P. *Biophys. J.* **1995**, *69*, 264-270.

(22) Domke, J.; Radmacher, M. *Langmuir* **1998**, *14*, 3320-3325.

(23) Tao, N. J.; Lindsay, S. M.; Lees, S. *Biophys. J.* **1992**, *63*, 1165-1169.

(24) Mahaffy, R. E.; Shih, C. K.; MacKintosh, F. C.; Kas, J. *Phys. Rev. Lett.* **2000**, *85*, 880-883.

(25) Mathur, A. B.; Collinsworth, A. M.; Reichert, W. M.; Kraus, W. E.; Truskey, G. A. *J. Biomech.* **2001**, *34*, 1545-1553.

(26) Hertz, H. *J. Reine Angew. Math.* **1881**, *92*, 156-171.

(27) Johnson, K. L.; Kendall, K.; Roberts, A. D. *Proc. Royal Soc. London. A, Math. Phy. Sci.* **1971**, *324*, 301-313.

(28) Derjaguin, B. V.; Muller, V. M.; Toporov, Y. P. *J. Colloid Interface Sci.* **1980**, *73*, 293-294.

(29) Muller, V. M.; Yushchenko, V. S.; Derjaguin, B. V. *Colloid Interface Sci.* **1980**, *77*, 91-101.

(30) Muller, V. M.; Derjaguin, B. V.; Toporov, Y. P. *Colloids Surf.* **1983**, *7*, 251-259.

(31) Sneddon I. N. *Int. J. Engr. Sci.* **1965**, *3*, 47-57.

(32) Dimitriadis, E. K.; Horkay, F.; Maresca, J.; Kachar, B.; Chadwick, R. S. *Biophys. J.* **2002**, *82*, 2798-2810.

(33) Richert, L.; Engler, A. J.; Discher, D. E.; Picart, C. *Biomacromolecules* **2004**, *5*, 1908-1916.

(34) Berdyyeva, T. K.; Woodworth, C. D.; Sokolov, I. *Phys. Med. Bio.* **2005**, *50*, 81-92.

(35) Lulevich, V.; Zink, T.; Chen, H. Y.; Liu, F. T.; Liu, G. Y. *Langmuir* **2006**, *22*, 8151-8155.

(36) Liang, X. M.; Mao, G. Z.; Ng, K. Y. S. *Colloids Surf., B* **2004**, *34*, 41-51.

(37) Liang, X. M.; Mao, G. Z.; Ng, K. Y. S. *J. Colloid Interface Sci.* **2004**, *278*, 53-62.

(38) Ramachandran, S.; Quist, A. P.; Kumar, S.; Lal, R. *Langmuir* **2006**, *22*, 8156-8162.

(39) Delorme, N.; Fery, A. *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.* **2006**, *74*, 030901.

(40) Hope, M.; Bally, M.; Mayer, L.; Janoff, A.; Cullis, P. *Chem. Phys. Lipids* **1986**, *40*, 89-107.

(41) Mayer, L.; Hope, M.; Cullis, P. *Biochim. Biophys. Acta* **1986**, *858*, 161-168.

(42) Hope, M.; Wong, K.; Cullis, P. *J. Electron Microsc Tech.* **1989**, *13*, 277-287.

(43) Lasic, D. *Liposomes: from physics to applications*; Elsevier: New York, **1995**.

(44) Kenworthy, A.; Simon, S.; McIntosh, T. *Biophys. J.* **1995**, *68*, 1903-1920.

(45) Martin, Y.; Vermette, P. *Macromolecules* **2006**, *39*, 8083-8091.

(46) Vermette, P.; Gengenbach, T.; Divisekera, U.; Kambouris, P. A.; Griesser, H. J.; Meagher, L. *J. Colloid Interface Sci.* **2003**, *259*, 13-26.

(47) Brochu, H.; Vermette, P. *Langmuir* **2007**, *23*, 7679-7686.

(48) Ducker, W. A.; Senden, T. J.; Pashley, R. M. *Nature* **1991**, *353*, 2239-2241.

(49) Cleveland, J. P.; Manne, S.; Bocek, D.; Hansma, P. K. *Rev. Sci. Instrum.* **1993**, *64*, 403-405.

(50) Ducker, W. A.; Senden, T. J.; Pashley, R. M. *Langmuir* **1992**, *8*, 1831-1836.

(51) Chadwick, R. S. *SIAM J. App. Math.* **2002**, *62*, 1520-1530.

(52) Martin, Y.; Boutin, D.; Vermette, P. *Thin Solid Films* **2007**, *515*, 6844-6852.

(53) Leckband, D. E.; Schmitt, F.-J.; Israelachvili, J. N.; Knoll, W. *Biochemistry* **1994**, *33*, 4611-4624.

(54) Vermette, P.; Taylor, S.; Dunstan, D.; Meagher, L. *Langmuir* **2002**, *18*, 505-511.

(55) Benmouna, F.; Johannsmann, D. *Langmuir* **2004**, *20*, 188-193.

(56) Engler, A. J.; Richert, L.; Wong, J. Y.; Picart, C.; Discher, D. E. *Surf. Sci.* **2004**, *570*, 142-154.

Conclusions générales

La libération contrôlée d'agents actifs dans le corps humain est un sujet de recherche majeur. Depuis longtemps, les liposomes sont étudiés afin de produire des systèmes de libération efficaces. Toutefois, afin de répondre aux besoins d'une application de libération contrôlée d'agents actifs en termes de stabilité et de biocompatibilité, les propriétés physico-chimiques et mécaniques doivent être spécifiques. Une caractérisation appropriée des systèmes de libération produits fournie les réponses quant aux différentes propriétés de ces systèmes.

L'objectif général de ce travail a été atteint. Ce travail a permis de déterminer des propriétés physico-chimiques et mécaniques de couches de liposomes intacts immobilisés sur des substrats solides en utilisant diverses techniques de caractérisation. La connaissance de ces propriétés permettra de les contrôler lors de la fabrication d'une application de libération contrôlée choisie.

Les objectifs spécifiques de ce travail ont aussi été atteints. Tel que démontré dans le second chapitre, les étapes de fabrication lors de la construction de couches de liposomes ont été observées de façon physico-chimique par spectroscopie des photoélectrons de rayons-X (XPS) et d'une façon mécanique, par microbalance à cristal de quartz avec mesure de la dissipation d'énergie (QCM). Les études par XPS et QCM montrent la présence succesive de chacune des espèces nécessaires à la construction des couches et une efficacité d'immobilisation par lien spécifique entre la NeutrAvidin et la biotin. Par QCM, il est aussi observé que les vésicules immobilisées sont intacts et ce, même après l'ajout d'une 2e couche de vésicules.

L'interaction entre les liposomes immobilisés et des protéines d'une solution de sérum veau fœtal a aussi été étudiée par QCM. Il a été observé que cette surface est efficace contre l'adsorption de protéines probablement dû à l'encombrement stérique créé par les poly(éthylène glycol) (PEG) à la surface des liposomes comme mentionné dans le premier chapitre.

En suivant la libération de deux molécules fluorescentes, la levofloxacine et la carboxyfluoresceïne, par spectrophotométrie, il est démontré au chapitre 2 que le système à la capacité de libérer plus d'un agent actif et ce, d'une façon séquencée grâce à une organisation spécifique des liposomes en surface. De plus, une libération prolongée des molécules dans le temps est observée.

Au troisième chapitre, il est démontré qu'il est possible d'extraire le module de Young de couches de liposomes à l'aide de la microscopie à force atomique (AFM). Utilisant un modèle de théorie du contact basé sur le modèle de Hertz, un module de Young d'environ 3 kPa est obtenu pour les liposomes immobilisés, un module semblable à celui de la gélatine.

De plus, les analyses par microbalance à cristal de quartz ont révélé que les liposomes immobilisés ont un comportement viscoélastique. Cette information a aussi été confirmée par microscopie à force atomique.

Ces études de caractérisation de liposomes immobilisés sur des substrats solides apportent de nouvelles informations fondamentales concernant la compréhension de ce système complexe. Toutefois, il serait intéressant d'obtenir encore plus d'informations sur ce système comme, par exemple, l'organisation des liposomes en surface.

L'AFM permet d'imager les surfaces à une échelle nanométrique. Toutefois l'imagerie de liposomes immobilisés s'avère difficile de par l'organisation 3D des vésicules et de leur comportement dynamique lors de l'imagerie. Des techniques de cryo-fracture et de cryo-microscopie pourraient permettre une imagerie de liposomes de façon statique. Des fractures succesives pourraient permettre de comprendre l'organisation de chacune des vésicules dans la structure de la couche. Quelle est la densité de recouvrement d'une couche? Voyons-nous vraiment une superposition des vésicules d'une couche à l'autre ou observons nous une imbrication des vésicules de la première et de la deuxième couche? Quelle est la taille des vésicules lorsqu'elles sont immobilisées? Ces réponses ne sont pas encores connues mais pourraient amener des éléments pertinents pour l'optimisation d'un tel système de libération.

En plus des applications *in vivo*, plusieurs applications *in vitro* pourraient être produites à l'aide des liposomes pour obtenir une libération particulière. Comme mentionné dans l'introduction, la fabrication de surfaces spécifiques aux cellules, par exemple, pourraient être faites avec des liposomes. Il pourrait être possible de produire des surfaces dédiées à la culture cellulaire. Ces surfaces seraient recouvertes de liposomes contenant des molécules spécifiques et arborant des protéines membranaires pour favoriser la différenciation ou la prolifération de certains types cellulaires. Toutefois, ce type de surface doit impérativement convenir aux cellules afin d'obtenir l'effet voulu. Une surface trop flexible, par exemple, pourrait provoquer l'apoptose des cellules plutôt que leur prolifération.

Dans la même optique, la surface des matériaux devient un sujet d'étude de plus en plus important dans le domaine des implants. Les réactions de l'organisme par rapport à une nouvelle surface ou un nouveau matériau dans le corps est un aspect très importatnt dans le processus d'acceptation de l'implant. En effet, lors de l'implantation d'un matériau dans le corps, il est connu que des protéines ainsi que des composantes de matrices cellulaires s'adsorbent à la surface de l'implant. Cette adsorption est possiblement la cause du rejet par l'organisme. Dans la plupart des cas, le rejet s'exprime par l'apparition d'une « capsule » autour de l'implant afin d'isoler le matériau de la circulation sanguine provoquant ainsi des complications au niveau de la guérison et du fonctionnement de l'implant.

Une surface fonctionalisée par des liposomes pourrait permettre une meilleure implantation de matériaux dans le corps. S'il était possible d'induire une organisation cellulaire « normale » autour de l'implant, l'organisme accepterait mieux la présence de ce corps étranger. L'encapsulation d'agents actifs appropriés, la présence de protéines membranaires spécifiques à la surface des liposomes et des propriétés mécaniques favorables à l'environnement du corps pourraient favoriser une prolifération cellulaire à la surface de l'implant pourraient limiter les risques de rejet.

La caractérisation des systèmes de libération produits et la versatilité de leurs propriétés permettra la création d'une infinité d'applications utilisant des liposomes en libération contrôlée d'agents actifs.

Annexes

Annexe 1: Courbes étalons de fluorescence obtenues par spectrophotométrie

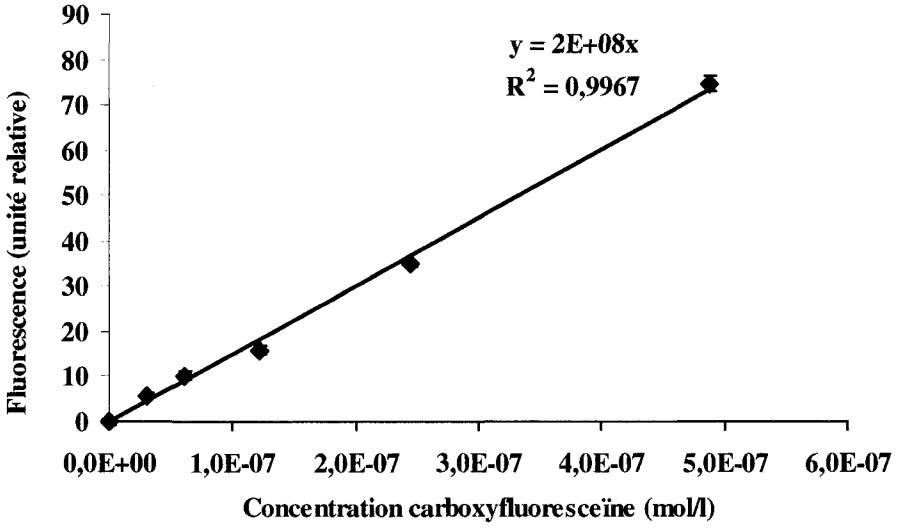


Figure A.1.1 Courbe étalon de la carboxyfluoresceïne dans une solution tampon de HEPES (10 mM).

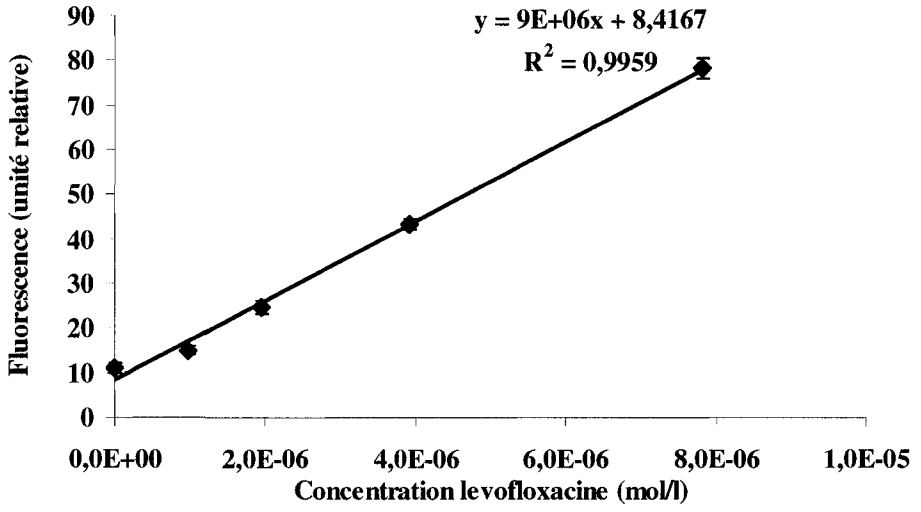


Figure A.1.2 Courbe étalon de la levofloxacine dans une solution tampon de HEPES (10 mM).

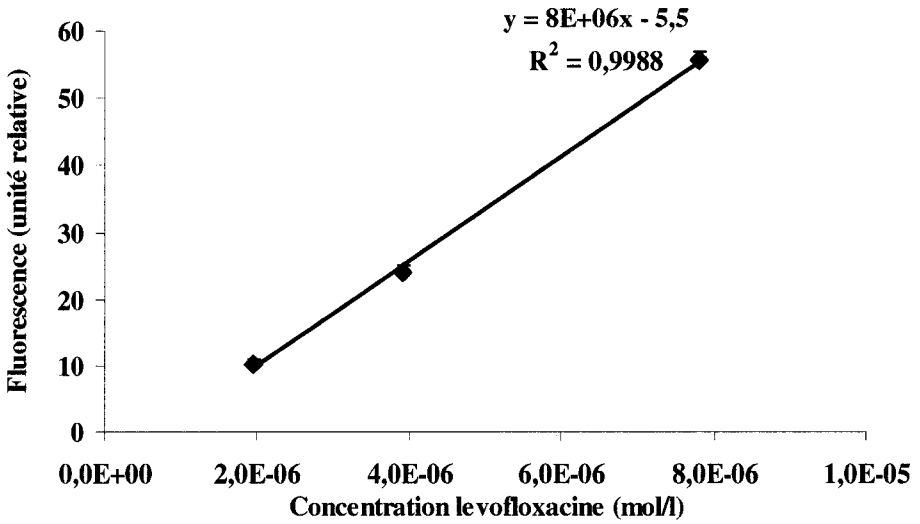


Figure A.1.3 Courbe étalon de la levofloxacine dans une solution de Triton (0.5% v/v).

Levofloxacine : excitation : 310 nm, émission : 460 nm.
Carboxyfuoresceïne : excitation : 487 nm, émission : 520 nm.

Annexe 2: Logique de l'algorithme utilisé pour l'extraction du module de Young

L'extraction du module de Young (E) a été faite par un programme informatique. Ce programme permet de prendre les courbes brutes fournies par la miscroscopie à force atomique (AFM), soient les courbes références et les courbes échantillons, puis d'en extraire le module de Young suivant le modèle de contact, Hertz ou Hertz-modifié (Dimitriadis), choisi par l'utilisateur. Les courbes références désignent ici les courbes obtenues sur le substrat de borosilicate et les courbes échantillons désignent celles obtenues sur une ou trois couches de liposomes.

Voici, de façon résumé, les étapes d'extraction du module de Young:

- Plusieurs mesures de forces étaients effectuées sur les différentes surfaces étudiées. De ces mesures, 3 courbes représentatives étaient choisies afin de procéder au calcul du module de Young.

- Pour un point analysé, 3 courbes références et 3 courbes échantillons sont fournies au logiciel de calcul puis une courbe moyenne est générée pour la condition référence de même que pour la condition échantillon. Pour générer cette courbe, un point arbitraire sur chacune des courbes sert de point d'alignement afin de faire la moyenne des courbes; ce point se situe à 10% de déflection par rapport à l'amplitude totale de la déflection observée.

- Ensuite, le calcul d'itérations est enclanché afin d'obtenir le point de contact (d_0) et le module de Young permettant d'obtenir la meilleure correspondance (la plus petite erreur) entre la théorie du modèle choisi et les résultats expérimentaux. Pour ce faire, à chacun des points de contact proposé:

    - l'indentation est obtenue par la soustraction des positions du piezo sur la référence et sur l'échantillon pour une même force appliquée, une relation est donc obtenue entre la force et l'indentation et un E expérimental est déterminé pour chacun des points d'indentation;

- une valeur globale de E est estimée pour ce d_0 suivant la méthode des moindres carrés;
- de ce E estimé, la force théorique est calculée pour tous les points d'indentation;
- puis, de la sommation des écarts entre les points de force expérimentale et théorique obtenues, l'erreur pour ce d_0 est obtenue.

- Les valeurs finales de d_0 et du module de Young (E) sont celles où l'écart entre la théorie et la pratique produit la plus petite erreur, la plus petite sommation.

Voici un exemple de calculs où l'on peut observer les 30 premières valeurs de module de Young expérimental obtenues.

Échantillon: 3 couches de liposomes

Modèle: Dimitriadis, not bonded

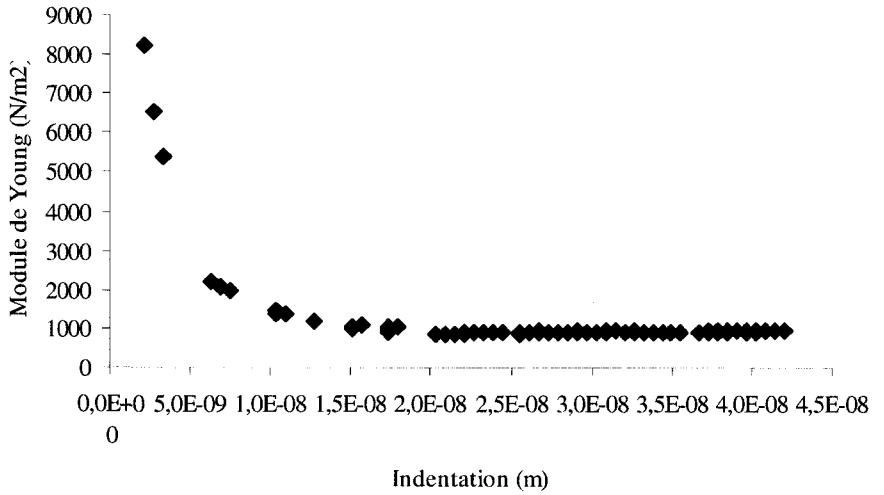
$$F = \frac{16}{9}\left(ER^{1/2}\delta^{3/2}\right)\left[1 + 0.884\chi + 0.781\chi^2 + 0.386\chi^3 + 0.0048\chi^4\right]$$

où R est le rayon de la sphère colloïdale (2,06 µm), $\chi = (R\delta)^{1/2}/h$ et h est la hauteur de l'échantillon ici estimée à 240 nm.

δ (m)	**χ**	**Correction**	**Module de Young (N/m^2)**
2,14846E-09	0,277195068	1,31	8236,5843
2,73438E-09	0,312717129	1,36	6521,7671
3,32037E-09	0,344600353	1,41	5387,60044
6,24998E-09	0,472782752	1,63	2205,90293
6,83596E-09	0,494449889	1,67	2098,8782
7,42188E-09	0,515203953	1,72	2004,41313
1,03516E-08	0,60845028	1,91	1367,36564
1,03516E-08	0,608450966	1,91	1455,92798
1,09375E-08	0,625435164	1,95	1373,72554
1,26953E-08	0,673820328	2,07	1193,5796

1,50391E-08	0,733386615	2,22	986,055428
1,50391E-08	0,733386046	2,22	1032,36401
1,50391E-08	0,733386452	2,22	1059,60445
1,5625E-08	0,747536902	2,26	1079,85292
1,73828E-08	0,788464929	2,37	913,165177
1,73828E-08	0,788465156	2,37	954,205603
1,73828E-08	0,788465534	2,37	992,164078
1,73828E-08	0,788465081	2,37	1022,94864
1,79688E-08	0,801643883	2,41	1032,06233
2,03125E-08	0,8523226	2,56	873,563379
2,08984E-08	0,86452858	2,60	867,571601
2,14844E-08	0,87656461	2,64	850,656302
2,20703E-08	0,888436928	2,68	834,120337
2,20703E-08	0,888437129	2,68	862,113178
2,20703E-08	0,888437464	2,68	878,017764
2,26563E-08	0,900153135	2,71	882,507087
2,32422E-08	0,911719056	2,75	876,626967
2,32422E-08	0,911718664	2,75	893,805558
2,38281E-08	0,923139563	2,79	884,205105
2,38281E-08	0,923139822	2,79	909,233456
2,44141E-08	0,934420563	2,83	903,285372

Valeurs des *E* obtenues et obtention d'un *E* global avec la valeur de 927 N/m².



Avec cette valeur globale la force théorique est calculée pour chacun des points d'indentation et comparée avec les valeurs expérimentales obtenues lors de l'expérimentation. La sommation des erreurs au carré sera ensuite faite.