Université de Montréal

Study of the Mechanisms of Destabilization of Niosomes and Liposomes by a pH-Sensitive *N*-isopropylacrylamide Copolymer

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Sciences pharmaceutiques
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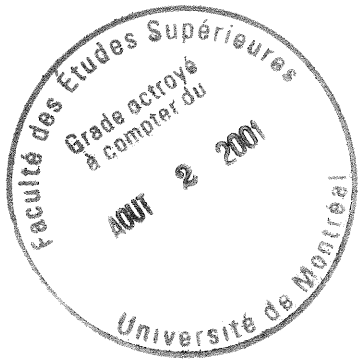
Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de

Maître ès Sciences (M.Sc.)

en sciences pharmaceutiques

Option technologie pharmaceutique



Avril, 2001

Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé :

Study of the Mechanisms of Destabilization of Niosomes and Liposomes by a pH-Sensitive *N*-isopropylacrylamide Copolymer

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Mémoire accepté le : .....

Summary

In this study, we aim to evaluate the destabilization of pH-sensitive non-ionic surfactant-based vesicles (niosomes) under weakly acidic conditions. A formulation of large unilamellar niosome vesicles was prepared using the reverse-phase evaporation method. It consists of a synthetic polyoxyethylene-3-stearyl ether surfactant in a mixture with cholesterol. The vesicles were rendered pH-sensitive by complexation with a hydrophobically-modified pH-responsive copolymer of *N*-isopropylacrylamide, *N*-glycidylacrylamide and *N*-octadecylacrylamide at a copolymer/lipid mass ratio 0.3.

This study reports different parameters affecting the efficiency of pH-sensitive niosomes as drug delivery systems. Areas that are covered include niosome preparation, stability in buffers and in normal human serum, response to pH, fusion, *in vitro* cytotoxicity and cellular uptake studies. Results were reviewed and contrasted with those from a phospholipid liposome formulation of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine and cholesterol examined under the same experimental conditions. At pH 7.2, vesicles were found to be stable over 90 days at 4°C. Under weakly acidic conditions (pH 5.5 – 3.0), the copolymer triggered the destabilization of the lipid bilayer membrane at 37°C, and consequently the release of entrapped drug. However, niosomes were partly destabilized in human serum at 37°C. Premature leakage in serum was attributed to the polymer collapse which is favoured in the presence of multivalent cations. On the cellular level, niosomes were cytotoxic at low concentrations (LD_{50} = 0.1 mM after 2 days) while no appreciable decrease in cell viability was shown for the

liposomes and copolymer. Finally, only liposomes and not niosomes were able to release their content in the cytoplasm after internalization by phagocytosis.

Résumé

Titre **: Étude des mécanismes impliqués dans la déstabilisation des niosomes et des liposomes par un copolymère de *N*-isopropylacrylamide sensible au pH .**

Mots clés : liposomes, niosomes, sensibilité au pH, ciblage des médicaments.

Introduction

Les liposomes sont des vecteurs de médicaments notamment utilisés pour cibler un principe actif vers des tissus spécifiques et diminuer sa localisation dans les tissus sains.

Ils sont constitués d'un volume aqueux contenu à l'intérieur d'une bicouche de lipides naturels ou synthétiques. Cette bicouche peut aussi contenir du cholestérol, ce qui permet d'augmenter la stabilité des vésicules. De plus, des polymères peuvent être incorporés dans la bicouche de façon à conférer aux vésicules certaines caractéristiques spécifiques.

Il a été démontré que les liposomes peuvent servir de vecteurs pour différents types de médicaments hydrophiles ou hydrophobes. Les médicaments hydrophobes sont incorporés dans la bicouche de lipides, tandis que les médicaments hydrophiles sont encapsulés dans les compartiments aqueux des liposomes.

Des liposomes sensibles au pH ont été développés afin de contrôler la libération de leur contenu dans les compartiments cellulaires où le pH est plus bas que le pH physiologique tels que les endosomes où le pH est acide.

Un des moyens utilisés afin de rendre les liposomes sensibles au pH est d'ancrer un polymère sensible au pH à la surface des vésicules.

Dans l'endosome, où le pH est plus acide que dans le milieu extracellulaire, la conformation du polymère change, ce qui entraine la destabilization du liposome qui ainsi libère le principe actif avant de passer vers le lysosome où il y a dégradation enzymatique.

En général, la majorité des études effectuées sur les liposomes concernent les vésicules composées essentiellement de phospholipides naturels. Ceux-ci ont certains désavantages tels que leur instabilité chimique due à une dégradation oxydative ainsi que le prix élevé des phospholipides. Ces désavantages limitent l'utilisation des liposomes phospholipidiques comme vecteurs de médicaments, ce qui a mené au développement des niosomes, vésicules préparées principalement à partir de surfactants non-ioniques synthétiques. Leurs propriétés physiques étant identiques à celles des liposomes, ils peuvent être préparés par les même méthodes et peuvent aussi encapsuler des médicaments hydrophiles et/ou hydrophobes. De plus, ils ont plusieurs avantages comparés aux liposomes phospholipidiques tels que leur stabilité chimique élevée qui permet une meilleure conservation, un faible coût et une pureté élevée.

Objectif

Nous avons préparé une formulation stable de niosomes à partir d'un surfactant non-ionique, d'origine synthétique le polyoxyéthylène-3-stéaryl éther (POE-SE) , et de cholestérol dans un rapport molaire de 3 pour 2. Une formulation similaire de liposomes phospholipidiques de 1-palmitoyl-2-oléoyl-*sn*-glycéro-3-phosphocholine (POPC) et de cholestérol (3:2 mol/mol) a été utilisée en guise de comparaison.

Les vésicules ont été rendues sensibles au pH au moyen d'un copolymère constitué de 3 monomères, soit le *N*-isopropylacrylamide (NIPAM), le *N*-glycidylacrylamide (Gly) qui procure la sensibilité au pH grâce au groupe carboxylique, et le *N*-octadécylacrylamide (C_{18}) qui sert d'ancre hydrophobe.

Méthodolgie et Résultats

1. Caractérisation du polymère

Le copolymère PNIPAM-Gly-C_{18} a été préparé par polymérisation radicalaire, caractérisé par diverses techniques physico-chimiques (GPC, RMN, DSC) et utilisé pour préparer des complexes polymère/niosome sensibles au pH. Le pH auquel ce copolymère précipite a été déterminé par la diffusion de la lumière produite par une solution du copolymère dans des tampons de différents pH à 37°C. Le copolymère précipite à un pH de 4.3. Ainsi, une fois ancré à la surface des vésicules, ce copolymère peut déstabiliser la bicouche externe des vésicules en milieu acide et libérer le principe actif.

2. Incorporation du médicament dans les niosomes

Les vésicules contenant un marqueur fluorescent sont préparées par la méthode dite d'évaporation en phase-inverse. Cette méthode implique une émulsion eau dans l'huile où la phase organique est la solution de lipides dans l'éther et la phase aqueuse est la solution du marqueur fluorescent. Après évaporation du solvent organique, un gel semi-solide est obtenu qui est ensuite agité pour le transformer en suspension homogène de liposomes.

Les vésicules sont ensuite extrudées à travers une membrane de polycarbonate de 200 nm pour réduire leur taille. Ensuite, le polymère est ancré à la surface des vésicules par incubation avec une solution du copolymère pour la nuit à 4°C. Le polymère libre est enfin séparé des vésicules sur un gel de sépharose.

3. Évaluation de la stabilité des niosomes sensibles au pH

La stabilité des niosomes et des liposomes sensibles au pH a été évaluée durant leur conservation dans un tampon neutre à 4°C. La stabilité des formulations contrôles ne contenant pas de copolymère a été évaluée en parallèle. Les niosomes et les liposomes sont stables à pH neutre pendant 90 jours à 4°C. Durant cette période, la libération du contenu des vésicules avec ou sans copolymère n'a pas dépassé 15 %.

4. Libération du médicament en fonction du pH

La déstabilisation de la membrane des vésicules à 37°C en fonction du pH a été étudiée en suivant la libération du marqueur encapsulé dans les vésicules. Le

copolymère déstabilise les vésicules à des pH acides, provoquant une libération d'environ 30 % à pH 4.0 pour les liposomes et 40 % pour les niosomes au même pH. Tandis que les formulations contrôles ne contenant pas de copolymère n'ont libéré que 8 % de leur contenu à tous les pH.

5. Fusion

La fusion entre les vésicules a été investiguée par une technique quantifiant le mélange de lipides. Cette technique permet de déterminer s'il y a une fusion entre les vésicules en suivant le transfert d'énergie de résonance qui dépend de la distance entre 2 lipides fluorescents, soit le *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidyléthanolamine (*N*-NBD-PE) comme donneur d'énergie et le *N*-(lissamine Rhodamine B sulfonyl) dioléoylphosphatidyléthanolamine (*N*-Rh-PE) comme accepteur d'énergie. Deux populations de vésicules sont mélangées: une population marquée avec une concentration finale des lipides fluorescent de 1 % et une non-marquée dans un rapport de 1:10. Ensuite, l'intensité de fluorescence du *N*-NBD-PE est mesurée à 37°C à pH 7.2, 4.3 et 3.0.

En l'absence de fusion, la fluorescence émise par le *N*-NBD-PE est captée par le *N*-Rh-PE en raison de la proximité des 2 sondes. L'intensité de fluorescence du *N*-NBD-PE est faible. En cas de fusion entre les vésicules, il y aura un mélange entre les lipides des membranes des deux populations. Par conséquent, la distance entre le *N*-NBD-PE et le *N*-Rh-PE augmente et le transfert d'énergie diminue, d'où l'augmentation de l'intensité de fluorescence mesurée du *N*-NBD-PE va augmenter.

Pour les vésicules étudiées, niosomes et liposomes de phosphatidylcholine, une fusion négligeable a été détectée à différents pH comparativement à 70 % de fusion observée à pH 3.0 dans le cas de liposomes de dioléoylphosphatidyléthanolamine (DOPE)/acide oléique (OA), qui ont la capacité de fusionner.

6. Stabilité des vésicules sensibles au pH dans le sérum humain

La stabilité des niosomes sensibles au pH a été étudiée pendant 3 heures dans 90 % de sérum humain à 37°C.

Dans de telles conditions, 28 % du contenu des niosomes recouverts de polymère est libéré après 3 heures, alors que les niosomes sans polymère ne libèrent que 5 % de leur contenu. Par contre, les liposomes sont plus stables dans le sérum humain puisqu' après 3 heures d'incubation, seulement 5 % du contenu des liposomes, avec et sans polymère, est libéré.

7. Libération du contenu après incubation dans le sérum humain

L'effet de l'incubation dans le sérum sur la libération du marqueur a aussi été investigué pour vérifier si les vésicules étaient toujours sensibles au pH après avoir été en contact avec le sérum.

Pour ce faire, les vésicules ont été incubées dans 75 % de sérum humain pendant 1 heure à 37°C. Ensuite, la libération du contenu a été évaluée à différents pH.

Aucune libération significative du marqueur à pH acide a été observée après 1 heure d'incubation dans le sérum. Dans le cas des liposomes, la libération du contenu était de 2 % à pH 4.0, alors qu'avant incubation dans le sérum, la libération était de 30 %. Ceci est probablement du aux composants sériques qui extraient le copolymère de la membrane des vésicules résultant en une perte de sensibilité au pH. Pour les niosomes, la libération du contenu était de seulement 4 %, alors qu'avant incubation dans le sérum, la libération était de 40 %. Ceci peut-être du à la destabilisation des niosomes dans le sérum où ils ont déjà libéré 25 % de leur contenu.

8. Étude de la cytotoxicité

La cytotoxicité des différentes concentrations du polymère PNIPAM-Gly-C_{18}, des niosomes et des liposomes a été étudiée sur des macrophages de souris (J774). Les cellules ont été cultivées dans des plaques de 96 puits à une concentration de $1x10^4$ cellules/puit à 37°C sous une atmosphère contrôlée à 5 % CO_2. Les suspensions de niosomes et de liposomes ont été incubées avec les cellules à différentes concentrations (0 – 2 mM). De même, la cytotoxicité du polymère PNIPAM-Gly-C_{18} a été évaluée à des concentrations de 0 à 1.4 mg/mL. Aux différents temps d'incubation (1- 5 jours), la viabilité des cellules a été évaluée par un test de MTT (3-(4,5-diméthylthiazole-2-yl)-2,5-triphényl tetrazolium).

Au niveau cellulaire, la cytotoxicité du polymère était minimale à toutes les concentrations testées (DL_{50} = 1 mg/mL après 5 jours). Quant aux niosomes recouverts de polymère, ils n'ont pas montré d'effet toxique sur les cellules à des concentrations de 0.025 et 0.05 mM (viabilité > 60 % après 5 jours). Par contre,

le pourcentage des cellules viables a commencé à diminuer à partir d'une concentration supérieure à 0.075 mM pour des temps d'incubation supérieurs à 2 jours.

Dans le cas des liposomes recouverts de polymère, une réduction négligeable dans le pourcentage des cellules viables a été observée à différentes concentrations et différents temps d'incubation.

9. Microscopie à fluorescence

La capacité des vésicules sensibles au pH à favoriser la diffusion d'un marqueur fluorescent dans le cytoplasme a été étudiée sur les cellules J774.

Lorsque ces cellules sont traitées avec des vésicules sans polymère pendant 4 h à 37°C et 5% CO_2, on observe des points fluorescents bien définis suggérant que les vésicules sont localisées dans l'endosome et n'ont pas libéré leur contenu dans le cytoplasm malgré le pH acide de l'endosome. Toutefois, quand ces cellules sont traitées avec des liposomes recouverts de polymère, on observe une fluorescence diffuse dans le cytoplasme, ce qui montre que le copolymère ancré à la surface déstabilise la membrane de lipides au pH acide de l'endosome. Une libération rapide et massive du marqueur dans l'endosome peut favoriser une diffusion rapide dans le cytoplasme. Une autre hypothèse serait qu'à pH acide, le polymère déstabilise aussi la membrane endosomale, favorisant le passage du marqueur dans le cytoplasme.

Par contre, dans de telles conditions, les niosomes recouverts de polymère n'ont pas libéré leur contenu dans le cytoplasme.

Conclusions

1- Le copolymère ancré à la surface des vésicules est capable d'induire la déstabilisation des vésicules à pH acide. Pourtant, l'activité est perdue après contact avec le sérum.

2- Au niveau cellulaire, seuls les liposomes, et non les niosomes, sont capables de libérer leur contenu dans le cytoplasme après internalisation par phagocytose.

3- Pour une application clinique, il faudrait essayer d'améliorer la stabilité de l'ancrage du polymère à la surface des vésicules, spécialement pour les niosomes, en présence du sérum. D'autre part, il faudrait mettre au point une formulation capable de se déstabiliser à un pH plus élevé (5.5) pour libérer son contenu dans l'environement acide de l'endosome cellulaire.

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Abreviations

AIBN	2,2'-azobisisobutyronitrile
CHEMS	cholesterol hemisuccinate
Chol	cholesterol
CF	5(6)-carboxyfluorescein
DOPC	1,2-dioleoyl-*sn*-3-phosphatidylcholine
DOPE	dioleoylphosphatidyl ethanolamine
DPSG	double-chain amphiphile 1,2-palmitoyl-3-succinylglycerol
DPX	*p*-xylene-*bis*-pyrimidinium bromide
DTA	A chain of diptheria toxin
FBS	fetal bovine serum
F_x	fluorescence intensity at time x
F_0	fluorescence intensity at time zero
F_t	fluorescence intensity recorded after the rupture of vesicles with Triton X-100
Gly	*N*-glycidylacrylamide
HEPES	*N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethane sulfonic acid)
HM	hydrophobically-modified
HPTS	Tri-sodium salt of 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine)

LCST	lower critical solution temperature
LUV	large unilamellar vesicles
MES	2-*N*-(morpholino) ethane-sulfonic acid
MTT	3-(4,5 dimethylthiazole-2yl)-2,5 triphenyl tetrazolium bromide
NAGEE	*N*-acryloyl-glycine ethyl ester
NaH_2PO_4	monobasic sodium phosphate
Na_2HPO_4	dibasic sodium phosphate
N-DSC	NANO differential scanning calorimeter
NIPAM	*N*-isopropylacrylamide
N-NBD-PE	*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine
N-Rh-PE	*N*-(lissamine rhodamine B sulfonyl) dioleoylphosphatidylethanolamine
NSV	non-ionic surfactant vesicles
OA	oleic acid
ODA	*N*-octadecylacrylamide
PBS	phosphate-buffered saline
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PNIPAM	poly(*N*-isopropylacrylamide)
POE-SE	polyoxyethylene-3-stearyl ether ($C_{18}EO_3$)

PNIPAM-Gly-C_{18}	poly[(*N*-isopropylacrylamide)-*co*-(*N*-glycidylacrylamide)-*co*-(*N*-octadecyl-acrylamide)]
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SSL	sterically stabilized liposomes
SUV	small unilamellar vesicles
TEA	triethylamine

Acknowledgements

This master's research would not have been possible without the contribution of many people.

First, I would like to express my sincere appreciation and gratitude to my supervisor Dr. Jean-Christophe Leroux. His support and suggestions made this work realizable. I particularly recognize his availability, a quality not easily found in a competitive domain such as pharmaceutical research.

Thanks are also due to my co-supervisor Dr. Françoise Winnik for her guidance throughout the course of this work. I feel privileged to work with a person of her great integrity and to benefit from her vast knowledge and experience.

Special thanks to Dr. Daryl Drummond, in the Research Institute of California Pacific Medical Center, for answering my numerous questions. His suggestions and discussions were very helpful.

I would like to extend my thanks to all my colleagues in the U-334 and the V-333. their friendship and encouragement are simply invaluable. I feel lucky to be a member of such friendly groups.

The financial support of the Canadian International Development Agency, in the form of a postgraduate scholarship, is gratefully acknowledged.

And finally, a heartfelt thanks goes to my beloved parents Marvel and Fathy, and my precious sisters Viviane and Flora, for their perpetual and unconditional love, support and encouragement. I hereby dedicate this thesis to them.

CHAPTER 1

INTRODUCTION

1. Liposomes

1.1. Introduction

Recent advances in biomedical science have resulted in the design and synthesis of new therapeutic agents with potential activity *in vitro* (e.g. antisense oligonucleotides, plasmid DNA, etc.)[1]. However, these drugs showed crucial problems such as their stability *in vitro* and *in vivo*, their low therapeutic index together with their non-specific cytotoxicity to critical normal tissues had to be solved before such drugs can be used in the clinic. Among the strategies developed to deliver drugs to target organs and modify drug disposition, the association with carriers such as liposomes has been considered.

1.2. Liposome structure

Liposomes are microparticulate phospholipid vesicles, usually 0.05 – 5.0 μm in diameter, which form spontaneously when certain lipids are hydrated in aqueous media[2]. Due to their biocompatibility, biodegradability and colloidal properties, liposomes are one of the most studied drug delivery systems. They consist of an aqueous volume entrapped by one or more bilayers of lipids. Drugs with widely varying lipophilicities can be encapsulated in liposomes. Hydrophobic drugs are incorporated in the lipid bilayer while hydrophilic drugs are entrapped in the aqueous volume (Fig. 1).

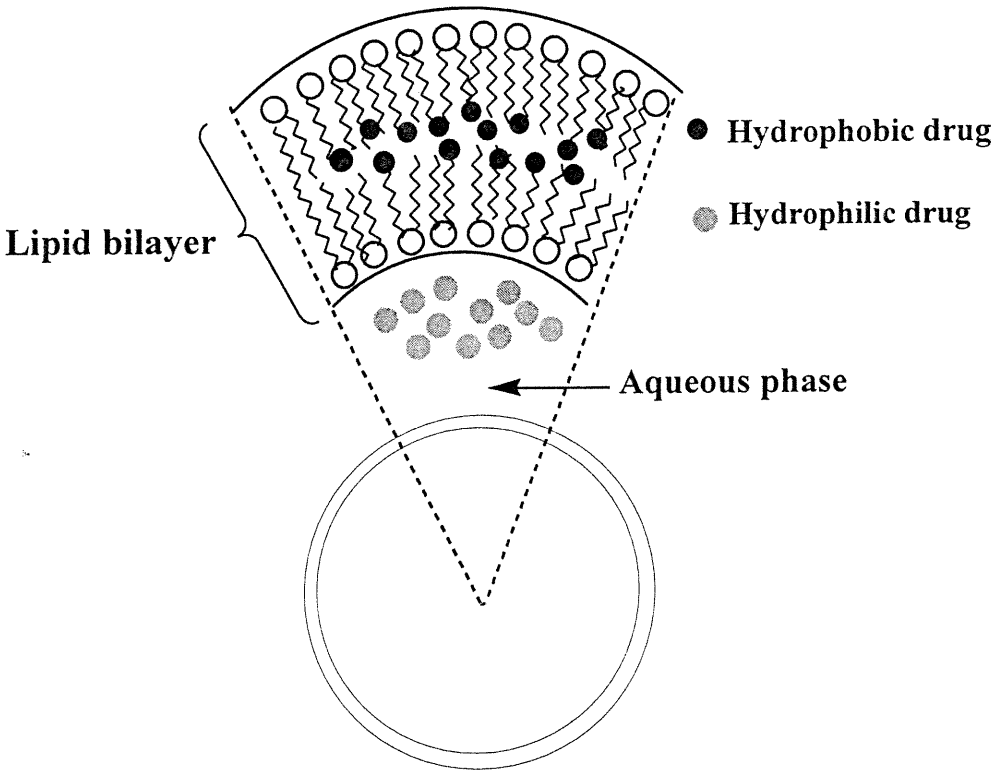

Figure 1.

Schematic representation of a liposome vesicle

1.3. Applications of liposomes in the clinic

Liposomes are extensively studied as carrier systems for the effective delivery of therapeutic agents such as antineoplastic and antimicrobial drugs, steroids, vaccines and genetic material[3-5]. Due to new developments in liposome technology, several liposome-based drug formulations are currently in clinical trial, and recently a number of drugs, ranging from water-soluble molecules (doxorubicin: Doxil®, Daunoxome®) to water-insoluble compounds (amphotericin B: AmBisome®) have been approved for clinical use. However, the number of liposome products available as marketed pharmaceuticals is still limited.

1.4. Limitations of liposomes

One of the major obstacles that prevent many promising liposomal drug candidates moving from the experimental scale to the pharmaceutical market is their chemical and physical instability during manufacturing and storage. It has been shown that the stability of liposomes is sensitive to several formulation parameters, which include pH[6,7], ionic strength of the buffer[8] and dissolved oxygen content within the formulation[9]. Another problem is the cellular uptake of the liposomes through a phagocytic pathway[10-12] where the liposomal lipids and contents are enzymatically degraded in the lysosomes. Such a profile of intracellular distribution prevents the delivery of liposomal content directly to the cytoplasm.

2. pH-sensitive liposomes

Therefore, there is a need for effective and stable delivery systems that alter the biodistribution of drugs in such a way that a greater fraction of the dose reaches the target site to achieve therapeutic concentrations especially for low therapeutic index drugs. In the past few years, the use of pH-sensitive liposomes[13-16] was suggested as a convenient way of enhancing the delivery of encapsulated content to the cytosol, where they can diffuse to their cytosolic or nuclear targets, prior to reaching the lysosomes where the liposome and its contents may be degraded by various hydrolases and peptidases[1,17].

2.1. Mechanistic rationale for pH-induced vesicle destabilization

Delivery is based on acid-triggered destabilization of the liposome membrane within the acidic environment of cellular endosomes (average pH of approximately 5.0[18,19] but can be as low as 4.5 in macrophages[20,21]) since the primary mechanism of liposome uptake by cells is via phagocytosis[22-24].

Following cellular internalization, pH-sensitive liposomes release their contents into the cytosol by one, or a combination, of several mechanisms. These liposomes can be induced to undergo a pH-induced fusion of liposomal membranes with endosomal membranes, directly releasing liposomal contents into the cytosol. Alternatively, liposomes can become destabilized and cause the destabilization of endosomal membranes, resulting in leakage of liposomal content into the cytosol. In the case of certain encapsulated molecules, pH-sensitive liposomes may also become destabilized and release their contents inside

the endosome, where amphipathic molecules may diffuse across endosomal membranes[25]. In addition to low pH, such liposomes usually have also been shown to fuse or leak in response to increasing Ca^{+2}[26].

2.2. Composition of pH-sensitive liposomes

The general strategy used in the preparation of such pH-sensitive liposomes has been the incorporation of a major lipid component containing pH-sensitive groups such as phosphatidylethanolamine (PE)[27-30]. In fact, PE assembles spontaneously in non-bilayered hexagonal (H_{II}) phase when dispersed in water. However, in the cellular membrane, PE is stabilized in a lamellar phase[30-32]. At neutral pH, the phospholipid is stabilized under a lamellar phase. By decreasing the pH, the amino groups of PE become protonated, which in return provides less repulsion between the head groups, thus allowing the organization under a H_{II} phase[33] since the transition of PE from lamellar phase to the hexagonal phase is influenced by the surrounding pH. The obtention of stable PE liposomes necessitates the use of a second component containing titrable acidic groups able to provide electrostatic repulsions, which will prevent the formation of the non-desired hexagonal phase at physiological pH. Practically, the most common PE liposomes stabilizers are fatty acids like oleic acid (OA)[15,34], cholesterol hemisuccinate (CHEMS)[35] or palmitoylhomocystein[36].

2.3. pH-sensitive fusion and leakage of stabilized PE liposomes

Of the various formulations examined, DOPE:CHEMS and DOPE:OA liposomes have been the most extensively studied for their pH-sensitive fusion and leakage properties. DOPE:CHEMS (7:3) liposomes were found to be sensitive to content leakage below pH 5.5[26,29] while DOPE:OA liposomes were found to be considerably more sensitive to pH, releasing their contents below pH 6.5[37]. On the other hand, mixing of the lipid component of apposing membranes is an important criteria for membrane fusion[38]. With both formulations, an increase in lipid mixing was observed as the pH was decreased[14,26]. Furthermore, the role of aggregation in the pH-induced destabilization of these membranes has been investigated since aggregation, or close apposition of membranes, is the first step in the fusion process. In many of these experiments, lipid mixing and content leakage were shown to be preceded by aggregation[33]. This suggests that the destabilization of PE liposomes is dependent on bilayer contact.

2.4. Main applications of pH-sensitive PE liposomes

pH-sensitive liposomes have been used to deliver cytotoxic agents[25,39] and toxins[40,41] to the cytosol of cultured cells. Chu et *al.*[42] delivered the A chain of diphtheria toxin (DTA) to a macrophage-like cell-line, P388D1. Inhibition of protein synthesis was observed only when DTA was delivered to the cytoplasm *via* the DOPE:CHEMS formulation. Cytoplasmic delivery was blocked in the presence of ammonium chloride, indicating that DTA is delivered to the cytosol through a pH-sensitive pathway as the process requires a low-pH environment.

Moreover, the pH-sensitive formulation (DOPE:CHEMS) was calculated to be at least one hundred times as effective as a pH-insensitive formulation (DOPC:CHEMS) at delivering DTA to the cytoplasm of P388D1 cells.

2.5. Problems associated with pH-sensitive liposomes

Although such liposome formulations have been shown to be efficient systems for cytoplasmic delivery in cultured cells[40], their leakage in presence of human blood[43], loss of sensitivity to pH after being in contact with human serum as well as their rapid blood clearance[44] have hampered their *in vivo* use.

The insability of PE-based liposomes to retain entrapped substances when incubated with blood has been known for about a decade. It has been found that serum destabilization effect occurs as a result of the insertion of serum proteins into the phospholipid bilayer or the extraction of the stabilizing fatty acid by serum proteins[34,45]. Furthermore, due to the opsonization process (complement activation and interaction with other seric proteins), these liposomes are rapidly cleared from the blood stream and accumulate in the liver and spleen[46]. Unfortunately, because of such limitations, these liposomes fail to remain pH-sensitive *in vivo*.

On the other hand, other pH-sensitive liposomes formulations have been shown to be stabilized by human plasma. Small unilamellar vesicles (SUV) composed of OA:DOPE or OA:DOPE:cholesterol were stabilized to pH-induced leakage by pre-incubation in the presence of human plasma[15,47]. pH-sensitive liposome formulations containing DOPE and the double-chain amphiphile 1,2-

palmitoyl-3-succinylglycerol (DPSG) were significantly more stable in the presence of plasma than OA-containing liposomes, but showed a similar decrease in acid sensitivity following pre-incubation in the presence of plasma[14].

Thus, in order to effectively develop a liposomal drug delivery system, the effects of serum on liposomal stability require careful investigation.

2.6. Sterically-stabilized liposomes

To improve the colloidal stabilization of pH-sensitive PE-based liposomes, inclusion of poly(ethylene glycol)-derivatized lipids (PEG-PE) has been suggested[48]. These so-called sterically stabilized liposomes (SSL) or Stealth® liposomes have shown high stability and long circulation time in blood but simultaneously a decrease in their pH-sensitivity was observed[5,49].

3. pH-sensitive polymer/liposome complexes

In recent years, acid-triggered liposome destabilization was achieved extrinsically by using peptidic[50] and non-peptidic titratable synthetic polymers. One interesting feature of polymers is that they can undergo marked temperature-dependent[51,52] and pH-dependent[53,54] water solubility changes.

3.1. pH-sensitive polymers

in recent years, synthetic polymers have received growing scientific attention in the formulation of liposomal preparations. The first polymers that were identified to have pH-dependent fusogenic properties were synthetic polypeptides such as poly(L-lysine)[55,56] or poly(L-histidine)[57]. At high pH-

values, these polymers are neutral but aquire a positive charge as the pH decreases. In solution, the ionized polymer can interact with negatively charged membranes, perturb lipid packing and promote aggregation and fusion of liposomes[58]. Weak acid electrolytes differ from polycations in that they can trigger contents leakage from neutral as well as from charged liposomes. One common characteristic of all ph-sensitive polyanions is that they bear carboxylic acid groups which state of ionization determines the polymer stability to destabilize lipid bilayers. Three major systems were found in the literature, poly(acrylic acid) derivatives[59], succinylated poly(glycidol)s[48] and copolymers of N-isopropylacrylamide (NIPA)[60].

3.2. Advantage of pH-sensitive polymer/liposome complexes over PE-based liposomes

The advantage of this approach is the possibility to render different lipid-based formulations, of almost any composition, sensitive to pH without the limitations associated with PE-based liposomes. This approach appears as a promising alternative to PE-based formulations and fusogenic peptides for the preparation of pH-sensitive liposomes.

3.3. Copolymers of *N*-isopropylacrylamide (PNIPAM)

The most common approach to achieving anchoring of the polymer to liposomes is to use hydrophobically-modified water-soluble polymers. These polymers carry along their backbone a small number of long alkyl chains, which act as anchors into the lipid bilayer[61]. Several recent studies explored the use of hydrophobically-modified poly-(*N*-isopropylacrylamide) copolymer (HM-

PNIPAM) as liposome coating[54,62]. The copolymer consists of a neutral *N*-isopropylacrylamide hydrophilic chain to which a few octadecyl groups are attached at random serving as anchor for the polymer on to the liposomes. A distinctive property of the aqueous solutions of HM-PNIPAM is that they undergo phase separation when heated above their lower critical solution temperature (LCST), which is around 32°C. At the LCST, PNIPAM chains undergo a collapse from hydrated extended chains to hydrophobic globules which aggregate and form a separate phase[63]. Thus, the contraction of polymer chains anchored to the lipid bilayer results in liposomes with temperature-responsive properties[51,64,65]. It was further demonstrated that, by randomly introducing a small proportion of a titratable comonomer in the structure of PNIPAM, it is possible to increase the LCST above 37°C and make the polymer pH-responsive[66,67]. Recently, Spafford et *al.*[62] synthesized HM-PNIPAM copolymer bearing glycine as pH-sensitive moiety. Moreover, such polymer was shown to interact strongly with non-phospholipids primarily via hydrogen bonding[68]. Thus, by taking advantage of the pH-phase transitions of modified PNIPAM and the ability of this polymer to destabilize lipid bilayers,

new pH-sensitive liposomes were designed presenting preliminary evidence of pH-triggered release[53,69].

3.4. Mechanistic rationale for pH-induced polymer destabilization

The mechanism of cell delivery by polymer/liposome complexes is summarized in Figure 2. It involves:

1- Incorporation of pH-responsive copolymer on the surface of the liposome vesicle.

2- Internalization in endosome compartment where destabilization takes place due to the surrounding acidic pH.

3- Leakage of the pH-sensitive liposomal content into the cytoplasm.

4- Finally, enzymatic degradation of lipids and their contents takes place in the lysosomes of the cell.

3.5. Limitations of pH-sensitive polymer/liposome systems

Recently, intense research has been directed towards pH-sensitive polymer/liposome systems composed mainly of natural phospholipids[16,29,53,70]. However, despite extensive studies and substantial progress, several liposome-based drug formulations showed certain limitations[71] which restricted their therapeutic utility, such as being chemically unstable as they are exposed to oxidative degradation. Thereby, they must be stored and handled under an inert atmosphere. This limitation, in combination with the high cost and variable purity of natural phospholipids restricted somehow the use of phospholipid liposomes as drug delivery vehicles. Consequently, such considerations lead to the exploitation of alternatives to phospholipid liposomes.

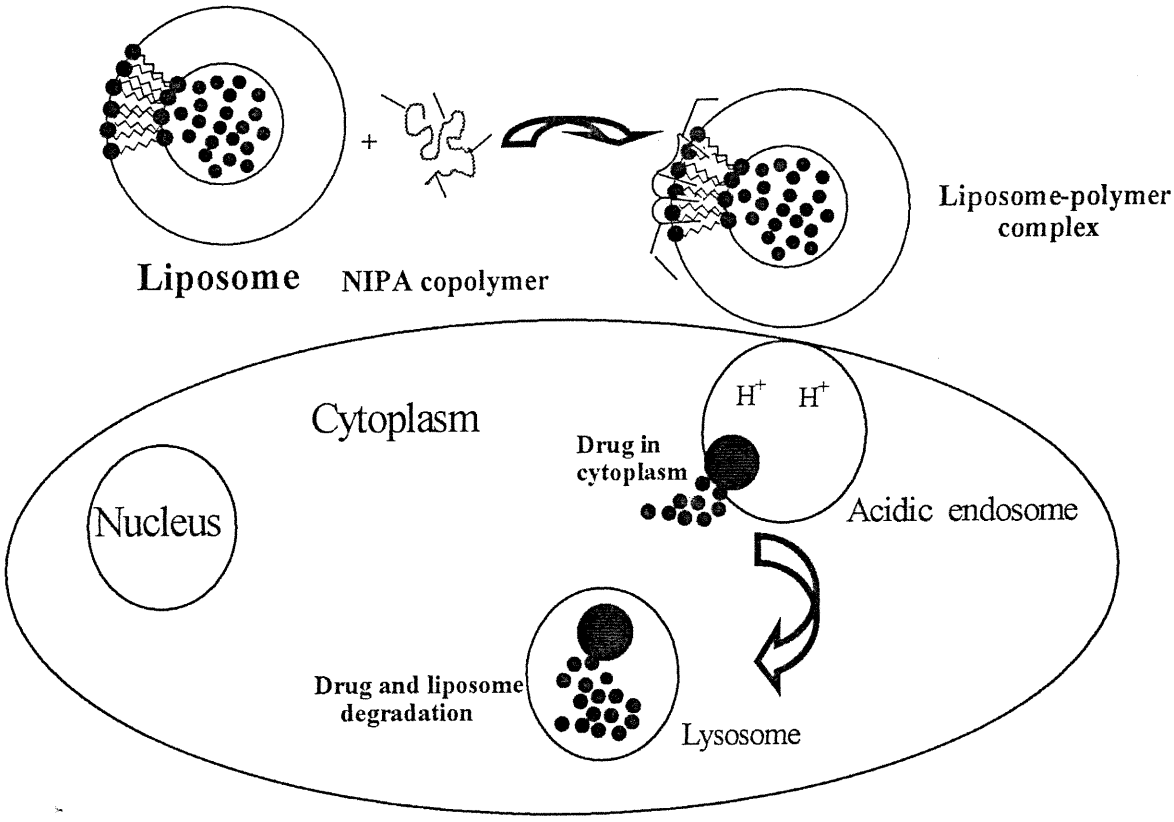

Figure 2.

Predominant mechanism of intracellular drug delivery by pH-sensitive polymer/liposome system

4. Non-ionic surfactant-based vesicles (Niosomes)

Recently, significant interest has been given to study synthetic non-ionic surfactant-based vesicles (NSV) or niosomes[72-74] as potential drug delivery systems for different routes of administration (e.g. intravenous[75], oral[76], topical[77]) as well as in cosmetics[78].

4.1. Structure of niosome vesicles

Niosomes are formed from the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed bilayer structures. Such process is governed not only by the nature of the surfactant but also by the presence of membrane additives, the actual method of preparation and the nature of the encapsulated drug. Advantageously, niosomes may be constructed from a diverse array of synthetic amphiphiles[79-81] bearing sugar, polyoxyethylene, polyglycerol, crown ether and amino acid hydrophilic head groups. These amphiphiles typically possess one to two hydrophobic alkyl[82], perfluoroalkyl[83] or in certain cases steroidal groups[84]. The two parts of the molecule may be linked via ether, amide or ester bonds[79].

4.2. Advantages and drawbacks of niosomes over liposomes

Following extensive investigations, niosome vesicles were found to have similar physical properties to liposomes[85]. Thus, they are analogous to phospholipid vesicles (liposomes) and are capable of entrapping hydrophilic and hydrophobic solutes[85]. In addition, it should be noted that, in most cases, handling

and storage of the surfactants require no special conditions[71] and consequently, niosomes take the advantage of being highly chemically stable in addition to their low cost and high purity[81]. On the other hand, it has been shown that the properties of niosome vesicles are remarkably dependent on the technique of preparation[71]. Furthermore, it should be noted that the amount and molecular structure of the ionic surfactant can affect the behavior of the vesicles. Finally, vesicle stability can be remarkably different according to the type and steric hindrance of the ionic surfactant used in the formulation[86].

4.3. Applications of niosomes in drug delivery

Niosomes were first reported by Handjani-Vila et *al.* as a feature of the cosmetic industry[78]. Actually, hundreds of products exist, ranging from skin creams, to body lotions, to sunscreens.

In the pharmaceutical field, although niosome formulations have yet to be commercially exploited, a number of studies have demonstrated the potential of niosomes in drug delivery.

Indeed, these systems have been evaluated as immunological adjuvants, anti-cancer/anti-infective drug targeting agents[73] and carriers of anti-inflammatory drugs. For example, the tumoricidal activity of the doxorubicin sorbitan monostearate formulation was found to be superior to the free drug in solution[73,87]. Also, a 23-fold increase in the area under the plasma level time curve was observed when span methotrexate niosomes were administered by the intravenous route to tumor bearing mice[88,89]. On the other hand, one of the

earliest diseases for which niosomal formulations proved particular benefit was from the antiparasitic class, specifically in the treatment of experimental leishmaniasis[90]. Moreover, diclofenac niosomes reportedly prepared mainly from polysorbate 60 were found to reduce the inflammation in rats with carageenan induced paw oedema on intraperitoneal administration to a greater extent than the free drug[91]. Also, the intravenous administration of sodium stibogluconate niosomes containing 30% cholesterol resulted in higher liver levels of antimony when compared with the administration of the drug in solution[90].

Apart from the use of niosomes as various drug carriers, one report in the literature details the evaluation of these systems as diagnostic agents. Stibogluconate niosomes containing cholesterol and stearylamine encapsulating the radiopaque agent, iopromide, were found to concentrate in the kidneys on intravenous administration[92]. This kidney targeting was attributed to the presence of the positive charge on the niosome surface.

A number of surfactants have shown immunostimulatory properties[93] and have been used in emulsion vaccine adjuvants.

The oral delivery of drugs using niosomal formulations was first demonstrated by a study involving methotrexate stiboglucanate niosomes[88]. Significantly higher levels of methotrexate were found in the serum, liver and brain of PKW mice orally administered a nioosmal formulation when compared with the administration of the free drug. It thus appears that there is enhanced drug absorption with these nioosmal formulations.

Although the emergence of niosomes into the pharmaceutical area was the result of activity in the cosmetic industry, it was only recently that the transdermal delivery of drugs with niosomes was seriously considered. The enhanced delivery through the stratum corneum of niosome encapsulated drugs has been observed[77] and it therefore remains to elucidate the mechanism of this delivery, especially as the stratum corneum is considered to be a particularly impermeable barrier[94].

A single study reports on the biological evaluation of a niosomal drug delivery system for ophthalmic delivery[95]. Cyclopentolate was encapsulated within niosomes prepared from polysorbate 20 and cholesterol and found to penetrate the cornea in a pH-dependant manner within these niosomes.

4.4. Factors governing the stability of niosome formulations

It has been found that, in general, the incorporation of non-ionic surfactant into liposome formulation is governed by various factors affecting its stability such as the choice of the main surfactant, nature of membrane additives, lipids molar ratio, nature of encapsulated drug, method of preparation and interaction of the lipid bilayer with the destabilizing copolymer.

4.4.1. Choice of the main surfactant

It is evident that the choice of membrane surfactant affects the stability of the system. In previous studies, stable niosome formulations were prepared using non-ionic polyglycerol alkyl ethers[71,96], sorbitan esters (Spans)[73,85,87] as well as polyoxyethylene ethers (Brijs)[97,98]. Some of these surfactants, such as sorbitan

monostearates (Span) and polyoxyethylene alkyl ethers (Brij), are already established pharmaceutical excipients[76,78,85].

4.4.2. Nature of membrane additives

The stability of niosomes is also governed by the presence of membrane additives[80]. The most common additives found in niosomal systems are cholesterol[85,99] and Solulan C24 (a cholesteryl poly-24-oxyethylene ether)[73], which prevent vesicle aggregation by repulsive steric effects especially in the case of Solulan C24[100]. On the other hand, cholesterol is known to abolish the gel to liquid phase transition of liposomal[101] and niosomal systems resulting in vesicles that are less leaky[96]. For example, Span 60 niosomes prepared without cholesterol formed a gel and only on the addition of cholesterol, a homogeneous niosome dispersion was obtained[85]. Accordingly, cholesterol is usually included in most formulations. However, even after the addition of cholesterol, the intrinsic phase transition behaviour of vesicle-forming surfactants still influence the properties of the dispersion such as the membrane permeability, encapsulation efficiency which increased with increasing cholesterol content[85], bilayer rigidity, ease of rehydration, toxicity, etc[80].

4.4.3. Lipid concentration

The concentration of lipids used to prepare niosome dispersions is generally 10 – 30 mM[71,102,103]. Altering the lipid/water ratio during the hydration step may affect the system microstructure[104] and hence the system properties.

However, increasing the lipids concentration also increases the total amount of encapsulated drug, although highly viscous systems are obtained if the concentration of lipids is too high.

4.4.4. Nature of encapsulated drug

Another important factor often overlooked is the influence of an amphiphilic drug on the vesicle formation.

For instance, while Span 60 niosomes containing dicetyl phosphate formed homogeneous dispersions when encapsulating 5(6)-carboxyfluorescein (CF)[85], this system formed an aggregated dispersion when encapsulating the amphipathic doxorubicin drug[73]. For this reason, a steric stabilizer Solulan C24 was added to the formulation to ensure homogeneous formulation devoid of aggregates[73].

4.4.5. Method of preparation

The physical characteristics of the vesicles were found to be dependent on the method of production[71]. The most commonly used laboratory methods of niosome preparation are:

1- The "Hydration method" where a surfactant/lipid film is formed by the evaporation of an organic solution of surfactant/lipids. The film is then hydrated with an aqueous solution of the drug to be encapsulated[105].

2- The "Reverse-phase evaporation vesicle (REV) method" which involves a water-in-oil emulsion[106], the oil phase being the lipids solution in organic solvent and the water phase is the aqueous solution of the drug. The organic solvent is then evaporated. The emulsion dries down to a semi-solid gel, which is then subjected to sonication until the gel becomes a homogeneous free-flowing suspension of vesicles.

The niosome vesicles are capable of entrapping and retaining water-soluble solutes. It should be noted that both methods were previously described for the preparation of liposomes. Moreover, the hydrating temperatures used in the preparation of niosomes should be above the gel to liquid phase transition temperature of the system.

Often, a size reduction step must be included in the niosome production procedure to obtain a homogeneous colloidal dispersion with vesicle size in the nanometer size-range. This is usually achieved by probe sonication[88] or extrusion through membrane filters of different mesh sizes[107]. In some instances, the combination of sonication and filtration has been used to achieve doxorubicin-loaded Span 60 niosomes in the 200 nm size range[73].

It is usually a requirement that unencapsulated drug be removed either by centrifugation[96], gel filtration[99] or dialysis[71].

4.4.6. Interaction of the lipid bilayer with the destabilizing copolymer

One interesting feature of polymers complexation to niosomes is that they can be tailored to participate actively in the release of drugs by sensitizing the bilayer membrane to a variety of environmental stimuli such as pH and temperature[68]. In contrast, regular niosomes lacking the copolymer complexation do not show any stimuli-dependent release of the encapsulated drug.

For instance, in case of pH-sensitive polymer/niosome systems, acid-triggered niosome destabilization is achieved due to the pH-responsive polymers fixed on the lipid bilayer. Acid titration of the polymer is usually accompanied by a modification of the polymer conformation and/or association with the vesicle bilayer, which results in its destabilization.

5. Aim of this study

The aim of the present work is to evaluate the efficiency of pH-sensitive polymer/niosome complexes as delivery systems. The niosome formulation was prepared from a synthetic polyoxyethylene-3-stearyl ether (POE-SE) surfactant ($C_{18}EO_3$) in a mixture with cholesterol at a molar ratio of 3 to 2. Special attention has been directed towards the comparison of such system with a typical liposome system, which has been previously shown to be effective delivery system. In this respect, results were reviewed and contrasted with those from a phospholipid

liposome formulation of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol (3:2) examined under the same experimental conditions.

The studied vesicles were rendered pH-sensitive by complexation with a hydrophobically-modified pH-responsive copolymer of *N*-isopropylacrylamide (NIPAM), *N*-glycidylacrylamide (Gly) and *N*-octadecylacrylamide (ODA) at concentrations of 93, 5 and 2 mol %, respectively.

For comparison, polymer-free control formulations were prepared in parallel for both niosomes and liposomes.

On the basis of these criteria, this study analyses different parameters governing the efficiency of such polymer/niosome vesicles as drug delivery system compared to polymer/liposome vesicles.

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CHAPTER 2

Presentation of the article

In vitro Evaluation of pH-Sensitive Polymer/Niosome Complexes

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Biomacromolecules (2001) (in press).

1. Abstract

Large unilamellar niosome and control liposome vesicles were rendered pH-sensitive by complexation with a hydrophobically-modified pH-responsive copolymer of *N*-isopropylacrylamide, *N*-glycidylacrylamide and *N*-octadecylacrylamide at a copolymer/lipid mass ratio 0.3. The vesicles were characterized and tested for their stability and pH-sensitivity in buffer and human serum. Their *in vitro* cytotoxicity was evaluated as well as their ability to mediate cytoplasmic delivery of encapsulated fluorescent probe using J774 murine macrophage-like cells. At pH 7.2, vesicles were found to be stable over 90 days at 4°C. At 37°C, the polymer destabilized the vesicles under weakly acidic conditions. However, niosomes but not liposomes were partly destabilized in human serum at 37°C. Premature leakage of niosomal contents in serum was attributed to the polymer collapse which is favoured in the presence of multivalent cations. On the cellular level, niosomes were cytotoxic above 0.075 mM while no appreciable decrease in cell viability was shown for the liposomes and copolymer alone at short incubation times (< 2 days). Finally, only liposomes and not niosomes were able to release their contents in the cytoplasm after internalization by phagocytosis.

2. Introduction

Over the last decade, liposome-mediated delivery of therapeutic agents has made considerable progress in the transition from the laboratory to the clinic[1]. Nonetheless, a number of key issues remain to be addressed in order to overcome practical limitations of liposome delivery systems. One of the problems

encountered with targeted-liposomes is related to the fact that, after cell internalization, they are delivered to lysosomes where their contents may be degraded by various hydrolases and peptidases. This often results in the poor cytoplasmic transfer of labile compounds that do not readily cross cell membranes (*e.g.* antisense oligonucleotides, plasmid DNA, etc.)[2]. It is possible to circumvent this problem with liposomes designed to release their contents only under mild acidic conditions, such as those found in cellular endosomes[3]. Such pH-sensitive liposomes may also destabilize or fuse with the endosomal/lysosomal membrane, thereby facilitating the delivery of their contents (*e.g.* DNA, cytotoxic drugs) to the cytoplasm[4-6]. Typical pH-sensitive liposome formulations consist of polymorphic lipids, such as unsaturated phosphatidylethanolamine (PE) used in combination with mildly acidic amphiphiles which act as membrane stabilizers at neutral pH. Upon acidification, the amphiphile headgroups are protonated, resulting in charge neutralization and destabilization of the vesicles due to the conversion of the PE component to the inverted hexagonal phase[7]. Although this class of pH-sensitive liposomes has been under intense scrutiny, it still suffers from important limitations, such as instability[8] or loss of pH-sensitivity[9] in serum, and rapid blood clearance[10,11].

Another approach to pH-sensitive liposomes consists in anchoring onto the liposome membrane a pH-responsive hydrophobically-modified polymer[12-14]. The polymer structure is such that, once adsorbed on the liposome surface, it promotes the disruption of the lipid bilayer when brought to acidic pH conditions.

Examples of such pH-responsive polymers which have shown promising results include poly(alkyl acrylic acid)s[13,15,16], succinilated poly(glycidol)s[17,18] and copolymers of *N*-isopropylacrylamide (NIPAM)[12,19]. The main advantage of this strategy is that it should prove possible to impart pH-sensitivity to liposome formulations with proven serum stability. Liposomes coated with hydrophobically-modified (HM) copolymers of NIPAM exhibit suitable stability at neutral pH. Moreover, copolymers of NIPAM and acrylic acid have been shown to destabilize lipid bilayers under physiological acidic conditions[12,20]. Recently, Winnik and co-workers[21] have shown that copolymers of NIPAM, *N*-glycidylacrylamide and *N*-octadecylacrylamide have a strong affinity to polyoxyethylene alkyl ether vesicles (niosomes), as a result of hydrogen bond formation between the hydroxyl groups of the surfactant and the amide groups of the NIPAM residues. They also demonstrated that complexes between this copolymer and phospholipid liposomes are significantly weaker, compared to niosome-based systems. This observation suggests that it may be possible to design effective drug delivery systems based on polymer/niosome complexes. There would be several advantages in using therapeutic formulations based on niosomes rather than phospholipid liposomes. Natural phospholipids are relatively expensive and susceptible to oxidative degradation. Therefore, they must be stored and handled under inert atmosphere. In contrast, niosomes can be prepared from mixtures of a wide variety of synthetic amphiphiles consisting of a hydrophobic tail linked to a hydrophilic head group via ether, amide or ester bonds and additives, such as cholesterol (Chol), which enhance the stability of the

niosome bilayer[22-24]. Handling and storage of these components require no special care[25]. Thus, niosomes form a versatile class stable and low cost vesicles[22]. Moreover, several suitable surfactants, such as polyoxyethylene alkyl ethers, are already established pharmaceutical excipients[26,27].

These considerations prompted the comparative study of the two types of polymer-coated vesicles presented in this report. Two pH-sensitive polymer/vesicle formulations were prepared from a copolymer of NIPAM, glycine acrylamide and octadecylacrylamide (PNIPAM-Gly-C_{18}) and either niosomes consisting of Chol and polyoxyethylene-3-stearyl ether (POE-SE) surfactant or phospholipid liposomes. The complexes were evaluated with regard to their pH-sensitivity, stability in serum, *in vitro* cell delivery and toxicity. The study revealed limitations of polymer/niosome complexes which were traced to the specific chemical entities selected. Suggestions are made to overcome the defficiencies uncovered.

3. Experimental Section

3.1. Reagents and Materials

3.1.1. Materials

POE-SE, calcein, *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethane sulfonic acid) (HEPES), 2-*N*-(morpholino) ethane-sulfonic acid (MES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), Sepharose 2B gel, human serum and polyoxyethylene-8-lauryl ether were purchased from Sigma Chemical. Oleic acid (OA) and 2-propanol (99.5+ %) were obtained from Aldrich. Chol, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl) dioleoylphosphatidylethanolamine (*N*-Rh-PE) were supplied by Avanti Polar-Lipids Inc. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Northern Lipids Inc. Tri-sodium salt of 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) (HPTS) and *p*-xylene-*bis*-pyridinium bromide (DPX) were purchased from Molecular Probes. Sephadex G-100 resin was purchased from Pharmacia Biotech AB. Anhydrous dextrose and calcium chloride dihydrate were purchased from Fischer scientific Ltd. RPMI 1640 with L-glutamine, penicillin-streptomycin (10,000 U/mL penicillin G and 10,000 µg/mL streptomycin) and fetal bovine serum (FBS) were supplied from Gibco laboratories. J774 macrophage-like cell lines were a kind gift from professor Alain Petit (Lady Davis Institute for Medical Research, Montreal PQ, Canada).

Preparation of PNIPAM-Gly-C_{18}: The hydrophobically-modified PNIPAM-Gly-C_{18} copolymer was prepared by free radical polymerization as previously described by Principi et *al.*[28]. The polymerization proceeded in homogeneous solution, yielding polymer with a random distribution of the comonomers at a concentration of 93, 5 and 2 mol % for NIPAM, Gly and C_{18} respectively. M_V = 260,000.

3.2. Experimental Techniques

3.2.1. Characterization of the random copolymer

Infra red spectra were recorded on a BioRad FTS-40 spectrometer. UV spectra were measured with a Hewlett-Packard 8452A photodiode array spectrophotometer equipped with a Hewlett-Packard 89090A temperature controller. Calorimetric measurements were performed on a NANO differential scanning calorimeter N-DSC (Calorimetry Sciences Corp) with an external pressure of 3.0 atm. The cell volume was 0.368 mL. The heating rate was 1.0°C min^{-1}, unless otherwise specified. Cloud points were determined by spectrophotometric detection of the changes in turbidity (λ = 600 nm) of aqueous polymer solutions (1.0 g L^{-1}) in citric acid buffer (150 mM, NaCl 0.1 M). The value reported is the temperature corresponding to a decrease of 20 % of the solution transmittance. To determine the pH-dependence of the polymer phase transition, solutions of PNIPAM-Gly-C_{18} (2.0 mL, 0.025 g L^{-1}) in MES buffered saline (20 mmol MES, 144 mmol NaCl) were prepared and their pH was adjusted from 3.0 to 7.2. Each solution was heated at 37°C for 5 min. The turbidity of the

sample was immediately measured using a series 2 Aminco Bowman (Spectronic Instruments Inc.) fluorescence spectrometer (λ_{ex} = 480 nm, λ_{em} = 480 nm).

3.2.2. Vesicle preparation

Large unilamellar vesicles (LUVs) of niosomes and liposomes were prepared using the reverse-phase evaporation procedure[29] as follows. Lipid mixtures of POE-SE/Chol or POPC/Chol (3:2 mol/mol) were dissolved in chloroform. The solvent was evaporated under reduced pressure at 35°C. The dry lipid/surfactant film was dissolved in anhydrous diethyl ether followed by the addition of 1 mL of a buffered solution of HPTS-DPX (20 mM HEPES, 35 mM HPTS and 50 mM DPX, pH 7.2). The vesicles were extruded through 0.2 μm pore-size polycarbonate membranes using LiposoFast extrusion syringes (Avestin, Inc.). Free fluorescent marker was separated by gel filtration on a Sephadex G-100 gel column equilibrated with an HEPES/dextrose buffer (5 mM HEPES buffer containing 5% dextrose, pH 7.2). The phospholipid molar concentration in the liposome vesicles was determined by the method of Bartlett[30].

3.2.3. Incorporation of the copolymer into the vesicles

A solution of PNIPAM-Gly-C_{18} in HEPES/dextrose buffer (2 mg/mL) was added to vesicle suspensions in the same buffer in amounts such that the final copolymer/lipid mass ratio was 0.3. The vesicles were kept at 4°C overnight under an inert atmosphere. Free unbound polymer was separated from the vesicles over a Sepharose 2B gel column equilibrated at pH 7.2 with HEPES/dextrose buffer.

3.2.4. In vitro stability study

Dispersions of niosomes and liposomes, prepared as described above, were kept at 4°C during 90 days. At different time points, aliquots of 20 μL were assayed for content leakage by monitoring HPTS fluorescence intensity after 3 min incubation in 2 mL HEPES-buffered saline solution (20 mM HEPES, 144 mM NaCl buffer, pH 7.2) at room temperature. Excitation and monitoring wavelengths were set at 413 nm and 512 nm, respectively. We considered 100 % release as the fluorescence intensity recorded after the addition of detergent (Triton X-100, 100 μL of 10 % w/v solution, final concentration 0.5 % w/v). The extent of content release was expressed as a percentage calculated from *Eq.1.*

$$\% \text{ Released } = 100 \text{ X } (F_x - F_0) / (F_t - F_0) \qquad \textit{Eq. 1.}$$

where F_x is the fluorescence intensity recorded after 3 min incubation in neutral buffer, F_T is the total fluorescence intensity recorded after the rupture of vesicles with Triton X-100 and F_0 is the initial fluorescence intensity. Vesicle average diameters were determined by dynamic laser light scattering (N4 Plus, Coulter Electronics) at 25°C, with a scattering angle of 90°.

3.2.5. In vitro study of the release of encapsulated fluorescent probe

Twenty microliters of each of the different vesicle suspensions were incubated at 37°C in 2 mL MES-buffered saline solutions of varying pH values ranging from 7.2 to 3.0. The intensity of fluorescence resulting from the release of the encapsulated fluorescent probe from the vesicles was recorded followed by

vesicle lysis with Triton X-100 as detergent. The extent of release was expressed as a percentage calculated from *Eq. 1* where F_x represents the fluorescence intensity recorded after 3 min incubation in buffers of different pH values. In order to investigate the effect of Ca^{2+} ions on the vesicle stability, content leakage was also evaluated in HEPES/dextrose buffer at pH 7.2 in the presence of increasing concentrations of $CaCl_2$. For each formulation, the percentage of content release at different pH values was measured 3 times for the same preparation and each experiment was repeated 3 times.

3.2.6. Lipid mixing assay

An assay for vesicle-vesicle fusion based on the non-radiative resonance energy transfer (NRET) between two fluorescent lipid analogues *N*-NBD-PE (energy donor), and *N*-Rh-PE (energy acceptor) was used. This technique provides a means by which lipid mixing during vesicle fusion can be followed[31] since fusion of the vesicles will result in intermixing of the membrane lipids and probe dilution. This leads to an increased distance between the NBD-PE and Rh-PE, thereby decreasing the RET efficiency and increasing NBD-fluorescence intensity. In contrast, in absence of fusion, the two probes come into close proximity so that the fluorescence emitted by *N*-NBD-PE is quenched by the *N*-Rh-PE.

Niosomes incorporating the two fluorophores were prepared by the reverse-phase evaporation method as 20 mM POE-SE/Chol/*N*-NBD-PE/*N*-Rh-PE (3:2:0.05:0.05 molar ratio) and were mixed in a cuvette with probe-free vesicles at

a 1:10 (v/v) ratio. The extent of vesicle fusion was calculated by means of the quantitative evaluation of changes in *N*-NBD-PE fluorescence intensity over 60 s at pH values 7.2, 4.3 and 3.0 at 37°C. Samples were excited at 470 nm and fluorescence emission intensity was monitored spectrofluorophotometrically at 520 nm. Following each measurement, vesicles were disrupted with polyoxyethylene-8-lauryl ether as detergent (1 % w/v final concentration) to eliminate the energy transfer. The extent of vesicle fusion was calculated as:

$$\% \text{ Lipid mixing} = (F_x / F_T) \times 100 \qquad \textit{Eq. 2.}$$

Where F_x is the intensity of fluorescence emitted from *N*-NBD-PE at 10 s intervals during 60 s, and F_T is the intensity of fluorescence after the addition of the detergent. On the other hand, kinetics and quantitative measurements of the fusion process were investigated, under the same experimental conditions, for DOPE/OA liposomes, known to become fusion competent at weakly acidic pH[32]. These fusogenic liposomes were prepared using the hydration method where dry film of 20 mM lipid mixture of DOPE/OA (2:1 molar ratio) was hydrated using HEPES buffer (50 mM HEPES, 130 mM NaCl, pH 9.5), followed by extrusion as described above.

3.2.7. In vitro stability study of the pH-sensitive vesicles in human serum

Following the separation of unbound polymer by gel filtration on Sepharose 2B column, the vesicle suspension was diluted 10 folds in normal human serum prewarmed to 37°C. The mixture was incubated at 37°C throughout

the assay (3 h). The content released from 100 μL aliquots of the mixture was determined as a function of time by spectrofluorophotometric measurements in a cell containing 2 mL HEPES-buffered saline solution (pH 7.2) at room temperature. The percent leakage of liposome contents was determined using *Eq. 1.*

3.2.8. Acid-induced leakage after incubation in serum

The preformed vesicles were incubated with 75 % (v/v) normal human serum for 1 h at 37°C. Free polymer and excess serum components were then separated over Sepharose 2B gel column. Aliquots of 20 μL were assayed for acid-induced HPTS release by spectrofluorophotometric determination of the resulting fluorescence intensity in 2 mL MES-buffered saline solutions of varying pH values ranging from 7.2 down to 3.0 at 37°C.

3.2.9. Cell culture

The macrophage-like cell lines J774 were maintained in a monolayer culture in RPMI 1640 medium containing L-glutamine. The medium was supplemented with 5 % heat-inactivated FBS and 1 % penicillin-streptomycin antibiotics solution (100 U/mL penicillin G, 100 μg/mL streptomycin). Cultured cells were incubated at 37°C in a humidified atmosphere containing 5 % CO_2.

3.2.10. In vitro cytotoxicity studies

J774 murine macrophage cells were seeded in triplicate in 96-well plates at $1x10^4$ cells/well. Cells were allowed to adhere for 4 h at 37°C in a humidified atmosphere of 5 % CO_2 in air. Vesicle suspensions were prepared in HEPES/dextrose buffer by the reverse-phase evaporation method as previously described. Increasing concentrations of PNIPAM-Gly-C_{18} (0 - 1.4 mg/mL) as well as polymer-coated and polymer-free niosome and liposome suspensions (0 - 2 mM total lipids/surfactant) were added to the cells. Following different periods of incubation (1 - 5 days), cell viability was determined by a MTT test according to the procedure described by Mosmann[33]. The test is based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. Ten microliters of 5 mg/mL MTT solution in phosphate-buffered saline (PBS) were added to each well. After 4 h of additional incubation at 37°C, 100 µL of acid-isopropanol (0.4 N HCl in isopropanol) were added to each well to ensure solubilization of formazan crystals. The optical density values were determined using a multiwell-scanning spectrophotometer (Bio-Tek Instruments Inc.) at 570 nm.

3.2.11. Fluorescence microscopy

Cells were plated at a concentration of $1x10^6$ cells per 35 mm dish in a 6-well plastic culture plate 24 h prior to use. The cultures were incubated at 37°C in a humidified atmosphere of 5 % CO_2 in air. Cells were then rinsed twice with 2 mL PBS (75 mM NaCl, 53 mM Na_2HPO4, 13 mM NaH_2PO4, pH 7.2) and were incubated for 1 h in serum-free culture medium. Vesicles loaded with 120 mM

calcein were prepared by the reverse-phase evaporation method as described above. Free calcein was removed by gel filtration on a Sephadex G-100 column equilibrated with HEPES/dextrose buffer followed by incorporation of the copolymer at a 0.3 mass polymer/lipid ratio. The unbound polymer was removed by gel filtration over a Sepharose 2B column. Cells were incubated with calcein-containing vesicles at a final concentration of 0.5 mM lipids at 37°C in a humidified incubator. Following 4 h of incubation, cells were washed twice with 2 mL cold PBS to remove free, non-endocytosed vesicles and non-viable macrophages. Cells were then viewed with an Axiovert inverted microscope equipped with a fluorescence illuminator (Zeiss) and were photographed using a 1310C DVC digital camera (DVC Company Inc.). Cells were viewed by phase contrast or epifluorescence with a 40X objective and a filter set that produces excitation in the range 435 - 485 nm, and allows observation of fluorescence emission in the range 515 - 555 nm with a long wave path dichroic mirror and barrier filter.

3. Results and Discussion

3.1. Properties of the polymer in solution

The hydrophobically-modified copolymer PNIPAM-Gly-C_{18} was obtained by copolymerization of NIPAM, *N*-glycine acrylamide ethyl ester and *N*-n-octadecylacrylamide, followed by deprotection of the glycine acrylamide carboxylic acid moieties[28]. The polymerization proceeded in homogeneous solution, yielding a polymer with random distribution of the comonomers, since NIPAM, NAGEE and ODA have similar reactivity ratios. The monomer feed ratio was chosen to yield a copolymer containing approximately 2 mol % of ODA and 5 mol % of glycine. The copolymer composition was confirmed by ^{1}H NMR spectroscopy and potentiometric titration of the carboxylic acid residues[28].

The copolymer was soluble in aqueous buffers at or below room temperature, independently of the pH of the solutions. However, the solutions became turbid upon heating indicating precipitation of the polymer. The temperature of phase transition (cloud point), which depends on pH, was determined by simple spectrophotometric methods based on the detection of changes in either the solution transmittance at 600 nm or in the intensity of light scattered by a sample illuminated at 480 nm. In Figure 2A we present a series of plots of the changes in turbidity of PNIPAM-Gly-C_{18} solutions ranging in pH from 3.3 to 4.4. The drop in transmittance as a function of temperature was very sharp for solutions of low pH, when the glycine carboxylic groups are fully protonated. As the pH of the copolymer solutions increases, and consequently the

glycine groups are progressively deprotonated, the decrease in transmittance takes place over an increasingly wider temperature range, until a pH is reached for which the solution remains clear over the entire temperature range scanned (20 to 60°C). In solutions of this pH value (~ 4.5 or higher) the polymer is fully ionized. A second set of measurements was performed aimed at determining the pH of phase transition for solutions of PNIPAM-Gly-C_{18} kept at constant temperature. At 37°C, the phase transition of polymer solutions in MES buffers takes place for pH values ranging from 4.0 to 4.4 (Fig. 2B).

A microcalorimetric study of the heat-induced phase transition of PNIPAM-Gly-C_{18} in buffers of varying pH was conducted in parallel to gain further insight into the thermodynamics of the phenomenon. The phase transition of solutions was endothermic independently of the pH (ΔH 0.9 $\pm$ 0.1 kcal/mol NIPAM at pH 3.5), with temperatures of maximum heat capacity (T_m) identical, within experimental error, to the cloud point determined spectrophotometrically, at least for pH < 4.0. A plot of the changes in T_m as a function of pH (glycine buffers) is presented in Figure 3. From the data, it is possible to estimate the pH of phase transition at 37°C (see graph). This value (~ 4.4) is in good agreement with the pH of clouding at 37°C obtained by light scattering (Fig. 2B). On a molecular level, conditions of pH 4.5 and T = 37°C correspond to the coil/globule transition of the polymer.

3.2. In vitro release studies of PNIPAM-Gly-C_{18}/liposome systems

A series of polymer/vesicle systems incorporating a fluorescent dye in the aqueous pool were prepared in order to assess their storage stability. The reverse-phase evaporation method was employed to prepare both niosomes composed of POE-SE and Chol (3:2 mol/mol) and liposomes consisting of POPC and Chol (3:2 mol/mol). The vesicles were treated with PNIPAM-Gly-C_{18} in amounts such that the final polymer/lipid weight ratio was 0.3 and the pH of the suspension was 7.2. The stability over time of vesicles kept at 4°C for up to 90 days was evaluated by monitoring the time-dependent leakage of encapsulated fluorescent marker from vesicles incubated in the presence and/or absence of the copolymer. In all cases, the amount of dye leaching out of the vesicles was small. For example, after 90 days, the % dye leakage was less than 13 % in the case of naked and polymer-coated POE-SE/Chol niosomes after 90 days (results not shown).

The storage stability of the polymer/vesicles complexes was assessed also by dynamic light scattering measurements carried out after several time intervals (Table 1). A small, but significant increase in mean particle size was detected, but large aggregates were absent, confirming that the vesicle systems kept their integrity over 90 days. Interestingly, the mean diameters of niosomes and liposomes incubated with the copolymer were found to be smaller than those of the corresponding naked vesicles, a possible indication that the polymer acts as a stabilization against aggregation. Similar effects were reported already by Meyer et *al.*[12] in their study of phospholipid vesicles coated with hydrophobically-modified NIPAM copolymers. The PNIPAM chains in their hydrated extended

conformation may act as a steric barrier preventing close interactions with other vesicles, in a mechanism similar to that describing the interactions of poly(ethylene glycol)-modified liposomes[34]. In addition, the carboxylate groups of the copolymer may act as steric stabilizers, thus contributing to the stabilization effect as well.

As it has been shown previously that ionic species affect the cloud point of PNIPAM and its copolymers[35,36], it was important to assess the stability of polymer/vesicles in the presence of salts at physiological pH. Polymer collapse triggered by ions at neutral pH would result in premature leakage of the vesicle content in physiological fluids. In our study, vesicles were brought in contact with a Ca^{2+} solution of pH 7.2, a cation selected as it is present in significant amounts in human serum (2 - 2.5 mM). The amount of dye leakage was monitored as a function of Ca^{2+} concentration (Fig. 4). As anticipated, naked phospholipid liposomes and naked niosomes were not affected by increasing amounts of cation. However, Ca^{2+} triggered significant leakage of dye encapsulated in polymer-coated niosomes. The % dye released reached 18% in the case of polymer/niosome complexes in 4 mM Ca^{+2} aqueous solution. In contrast, polymer/liposome complexes were stable under these conditions. This macroscopic difference in stability against cations is taken as an indication of the occurrence of different polymer/bilayer binding mechanisms in polymer/liposome and in polymer/niosome systems.

The polymer/vesicle complexes were subjected to changes in pH, and their stability was assessed by determination of the extent of dye release following acidification. Results are summarized in Figure 5, where we plot the pH-dependent percent dye release from polymer/vesicle complexes as well as from the corresponding naked vesicles. In the case of polymer/liposome complexes, a significant amount of dye was released from samples brought from pH 7.2 to pH < 4.5, the pH corresponding to the collapse of the polymer at 37°C. In contrast, in the case of polymer/niosome systems, dye release was detected upon acidification from pH 7.2 to a pH value (4.8) less acidic than the pH of PNIPAM collapse at 37°C. The range of pH over which release took place is broader than in the case of polymer/liposome systems. Maximum release (40 %) was attained at pH 4.0. It is worth noting (Fig. 5) that the naked niosomes and liposomes were stable over the entire pH range scanned, confirming that, indeed, it is the polymer that triggers the leakage of encapsulated dye through the vesicle membrane. The difference in the release properties of polymer-coated liposomes and niosomes can be understood if one assumes the occurrence of specific interactions between the NIPAM groups of the polymer chain and the non-ionic surfactant head groups. Polozova et *al.*[21] showed that the binding of the copolymer to the niosomes is controlled primarily through hydrogen bonding formation between the hydroxyl functional groups of the surfactant and the amide groups of the copolymer units, and, a lesser extent, by hydrophobic forces. From our results, it appears that the interactions of the copolymer to the niosomes affect the temperature of polymer collapse, causing a lowering of its cloud point. This assumption is supported

further by previous observations that the binding of hydrophobically-modified PNIPAM to a phospholipid membrane, via the insertion of the hydrophobic anchors into the bilayer, does not significantly modify its cloud point[37]. At present, we do not have any hypothesis to explain why hydrogen bonding with niosomes triggers a decrease in the cloud point. Further experimental work is currently underway to clarify this issue.

To gain further insight into the differences between polymer/niosome and polymer/liposome systems, we carried out a study of the fusogenicity of polymer/vesicles by a lipid mixing assay. In this test, vesicle population labeled with two fluorescent lipids is prepared in parallel with probe-free vesicle population. The two populations are brought into contact as the extent of NRET is monitored under various conditions. An increase in the fluorescence intensity of *N*-NBD-PE (energy donor) indicates that membrane intermixing takes place between the two vesicle populations[38]. Distinctly labeled niosomes and liposomes suspensions in buffers of various pH values were mixed, either naked or in the presence of polymer. In no case were we able to detect any significant increase in the efficiency of NRET (Fig. 6). To ascertain the validity of our experimental protocol, we performed the same test using control liposomes consisting of DOPE and OA, a formulation known to undergo fusion. Data gathered for these liposomes are plotted in Figure 6, together with those of polymer-coated niosomes and liposomes. Mixing of two populations of DOPE/OA liposomes in a buffer of pH 3 lead to an extent of NRET corresponding to more than 70 % fusion. Overall,

these results provide strong evidence that the pH-triggered dye release from PNIPAM-Gly-C_{18}/vesicles does not involve intervesicle fusion[19].

3.3. Stability studies in human serum

Both naked liposomes and niosomes exhibited excellent stability upon incubation in 90 % normal human serum at 37°C and neutral pH. After a 3 h treatment, the % dye release was approximately 5 %. The same level of dye release was detected upon incubation of polymer-coated liposomes under identical conditions. However, polymer coated niosomes did not possess the same stability: the level of dye release reached 28 % when these vesicles were treated with human serum at 37°C and neutral pH. The leakage of dye through the bilayer of polymer-coated niosomes signals a change of the interactions between niosome head groups and the polymer chain by serum components. It may be that, as described above (Fig. 4), serum cations, such as Ca^{2+}, trigger polymer collapse and dye release from niosomes at neutral pH. Interactions between serum proteins and the polymer-coated niosomes could also destabilize the membrane, even though such an effect was not observed when polymer-coated liposomes were treated with human serum. With regard to liposomal formulations, the loss in pH-sensitivity following incubation in serum possibly results from the extraction of the copolymers by the serum components. This has been previously described for other acidic amphiphiles and can be generally overcome by using double chain anchor[39] or increasing the proportion (Roux and Leroux, unpublished data) of the

anchor. Work is in progress to gain further insight into this important difference in serum stability between the two types of vesicles.

5.7. *In vitro* cell studies

J774 murine derived mononuclear macrophage-like cells were selected since they are highly phagocytic, and, therefore, are appropriate to study endocytosis and intracellular processing of endocytosed material[40]. The cytotoxicity of polymer, vesicles, and polymer-coated vesicles was examined using a common MTT assay. The results of this study are presented in Figure 7, where we report the time- and dose-dependent viability of cells treated by the various synthetic components. At short incubation times (< 2 days), the polymer alone showed minimal cytotoxicity (Fig. 7A) at all polymer concentrations employed (0 - 1.4 mg/mL). Similarly, the PNIPAM-Gly-C_{18}/liposome systems exhibited no significant cytotoxicity even at high lipid concentration (2 mM), reflecting the relative inertness of the phospholipids (Fig. 7B). It turned out, however, that the polymer/niosome systems inhibited cell growth upon prolonged exposure (> 2 days) and at high surfactant concentrations (> 0.075 mM), even though no significant cytotoxicity was detected at lower surfactant concentrations (0.025 - 0.05 mM) after a 1-day incubation period (Fig. 7C). The toxicity of polymer/niosome systems can be traced to the surfactant itself, since naked niosomes presented a similar toxicity pattern. The deleterious effect of niosomes may be due to the propensity of polyoxyethylene alkyl ether surfactants to affect membrane integrity[41].

In spite of the long-term cytotoxicity of niosomes, we set about to detect the ability of J774 murine macrophages to internalize polymer-coated vesicles. Cells were treated at 37°C with calcein-containing vesicles, naked or coated with polymer. An incubation time of 4 h was selected in these experiments, to minimize the cytotoxic effect of niosomes. Treated cells were imaged in a fluorescence microscope. Significant micrographs are presented in Figure 8. In micrographs of cells incubated with naked vesicles, liposomes or niosomes, a vacuolar fluorescence pattern was observed (Fig. 8A, liposome treatment), indicating that the vesicles remained intact in the cell endosomes/lysosomes and they did not deliver the calcein to the cytoplasm. To our disappointment, micrographs of cells treated with polymer-coated niosomes (Fig. 8B) presented a similar vacuolar fluorescence pattern, indicating that the polymer did not facilitate the transfer of dye to the cytoplasm. This might be explained by the presence of cationic species in the RPMI 1640 culture medium (2.5 mM Ca^{2+}) which trigger polymer collapse at physiological pH, resulting in partial content leakage (see Figure 4). Under these conditions, niosome vesicles would release their contents in the medium before they end up in the endosomes of the macrophages. Micrographs of cells treated with polymer/liposome complexes (Fig. 8C), in contrast, displayed a strong and diffuse fluorescence, a clear indication that dye molecules were, at least partially, delivered to the cytoplasm. As a control experiment, cells were treated with the fusogenic DOPE/OA liposomes loaded with calcein. Micrographs of these cells (Figure 8D) also presented a diffuse fluorescence, albeit of lower intensity. This qualitative observation is an

indication that POPC/Chol liposome/polymer complexes may be more effective carriers of dye to the cytoplasm than DOPE/OA liposomes. One possible interpretation of these results may be that, upon cell internalization, the polymer/liposome complexes rapidly release part or all of their content in the endosomes/lysosomes, and that free dye diffuses in the cytoplasm. Alternatively, in the more acidic intracellular environment, the collapsed polymer may destabilize the endosomal/lysosomal membrane, thereby facilitating the escape of calcein from the polymer/liposome complexes.

4. Conclusion

Non-ionic surfactant based vesicles are now widely studied as alternative to liposomes[23]. To our knowledge this study is the first report describing the preparation and characterization of a pH-sensitive niosomal formulation. Such a formulation is prepared by the complexation of a pH-responsive NIPAM copolymer to non-ionic surfactant vesicles as previously described for liposomes[19,42]. Those copolymers were found to be relatively non-toxic to cells and capable of facilitiating the transfer of vesicle contents when associated to conventional liposomal formulation. Unfortunately, the poor stability of PNIPAM-Gly-C_{18}/niosome complexes in serum constitutes an important limitation that will need to be solved before considering any pharmaceutical applications. The serum instability seems to be a consequence of the particular binding of the polymer to the surfactant which upon interaction with multivalent cations triggers polymer collapse and content leakage. This problem could, in

theory, be overcome by preparing copolymers with a higher LCST. Also, since it was found that the selected surfactant (i.e. POE-SE) is potentially harmful to cells a particular attention should be given on the total dose of niosomes that will be used in future investigations.

5. Acknowledgments

Financial support from the Canadian Institutes of Health Research is acknowledged. MF acknowledges a scholarship from the Canadian International Development Agency. Dr. D. Drummond, Mrs E. Roux and Mrs E. Ruel-Gariépy are acknowledged for their helpful scientific advices.

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Table 1. Influence of storage time on the mean diameter of vesicles incubated for 90 days at 4°C and neutral pH.

Time (days)	Diameter (nm) ± SD			
	Niosomes		Liposomes	
	Polymer-coated	Polymer-free	Polymer-coated	Polymer-free
1	185 ± 6	210 ±13	145 ± 6	155 ± 7
14	230 ± 10	260 ± 7	145 ± 4	165 ± 8
28	240 ± 7	270 ± 9	150 ± 6	170 ± 8
42	255 ± 12	275 ± 7	160 ± 7	170 ± 5
56	265 ± 9	285 ± 9	160 ± 7	185 ± 7
90	270 ± 8	300 ± 11	170 ± 7	200 ± 10

Figure captions.

Figure 1. Chemical structure of PNIPAM-Gly-C_{18} (93:5:2 mol%).

Figure 2 Plots of the changes in transmittance as a function of temperature for solutions of PNIPAM-Gly-C_{18} in glycine buffers of various pH (polymer concentration: 1.0 g L^{-1}) (**A**) pH-dependent phase-transition of PNIPAM-Gly-C_{18} determined by light-scattering incubated at 37°C in MES buffers of varying pH values (3.0-7.2) (**B**). Each value is the mean of two independent measurements.

Figure 3. Plot of the changes with pH of the transition maximum temperatures of PNIPAM-Gly-C_{18} measured by high sensitivity differential scanning calorimetry (polymer concentration : 1.0 g L^{-1}, 150 mmol glycine buffers). As shown by the arrow, at 37°C, the pH at which phase transition occurs is approximately 4.4.

Figure 4. Effect of Ca^{2+} concentration on the content release from POE-SE/Chol niosomes (circles) and POPC/Chol liposomes (triangles) in presence (closed symbols) or in absence (open symbols) of PNIPAM-Gly-C_{18}, at 37°C and pH 7.2. Mean ± S.D.(n = 3).

Figure 5. Release of HPTS from POE-SE/Chol niosomes (circles) and POPC/Chol liposomes (triangles) incubated in presence (closed symbols) or in absence (open symbols) of PNIPAM-Gly-C_{18} as a function of pH (3.0 - 7.2) after 3 min incubation at 37°C. Mean ± S.D. (n = 3)

Figure 6. Mixing of membrane lipid components. Labeled vesicles (1% *N*-NBD-PE, 1% *N*-Rh-PE) were incubated with unlabeled vesicles as 1:10 molar ratio respectively, in buffers of different pH values. Lipid mixing was determined at 37°C by monitoring the increase in *N*-NBD-PE fluorescence as a function of time over 60 s. Results are shown for PNIPAM-Gly-C_{18}/niosomes (POE-SE/Chol) (squares), PNIPAM-Gly-C_{18}/liposomes (POPC/Chol) (triangles) and DOPE/OA liposomes (circles) at pH 4.3 (closed symbols) and 3.0 (open symbols). Data are expressed as percentage of lipid mixing.

Figure 7. Effect of vesicle/polymer concentration on J774 cell viability measured by MTT assay during 5 days: PNIPAM-Gly-C_{18} (0-1.4 mg/mL) (**A**), PNIPAM-Gly-C_{18}-coated liposomes (0-1.2 mM total lipids) (**B**) and PNIPAM-Gly-C_{18}-coated niosomes (0-1.2 mM total sufactant/Chol) (**C**). Mean ± S.D. ($n = 3$).

Figure 8. Fluorescence micrographs of J774 macrophage cells treated with vesicles containing 120 mM calcein. The concentration of lipids/surfactant in the incubation was 0.5 mM. Cells were washed with cold PBS and examined using 40X objective with illumination at λ_{ex} 435-485 nm. Vacuolar fluorescence resulting from incubation with naked POPC/Chol liposomes (**A**) or PNIPAM-Gly-C_{18}-coated POE-SE/Chol niosomes (**B**), and diffuse (cytoplasmic) fluorescence obtained by incubation with PNIPAM-Gly-C_{18}-coated POPC/Chol (**C**) or DOPE/OA (**D**) liposomes.

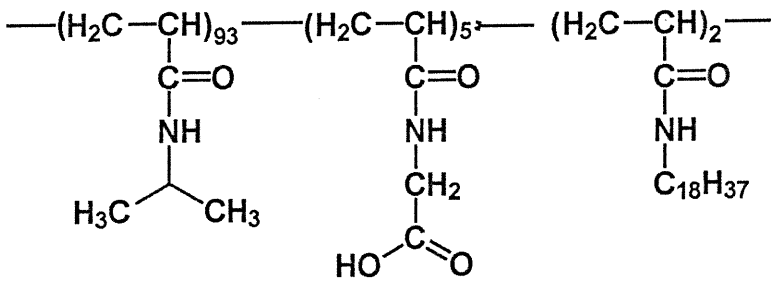
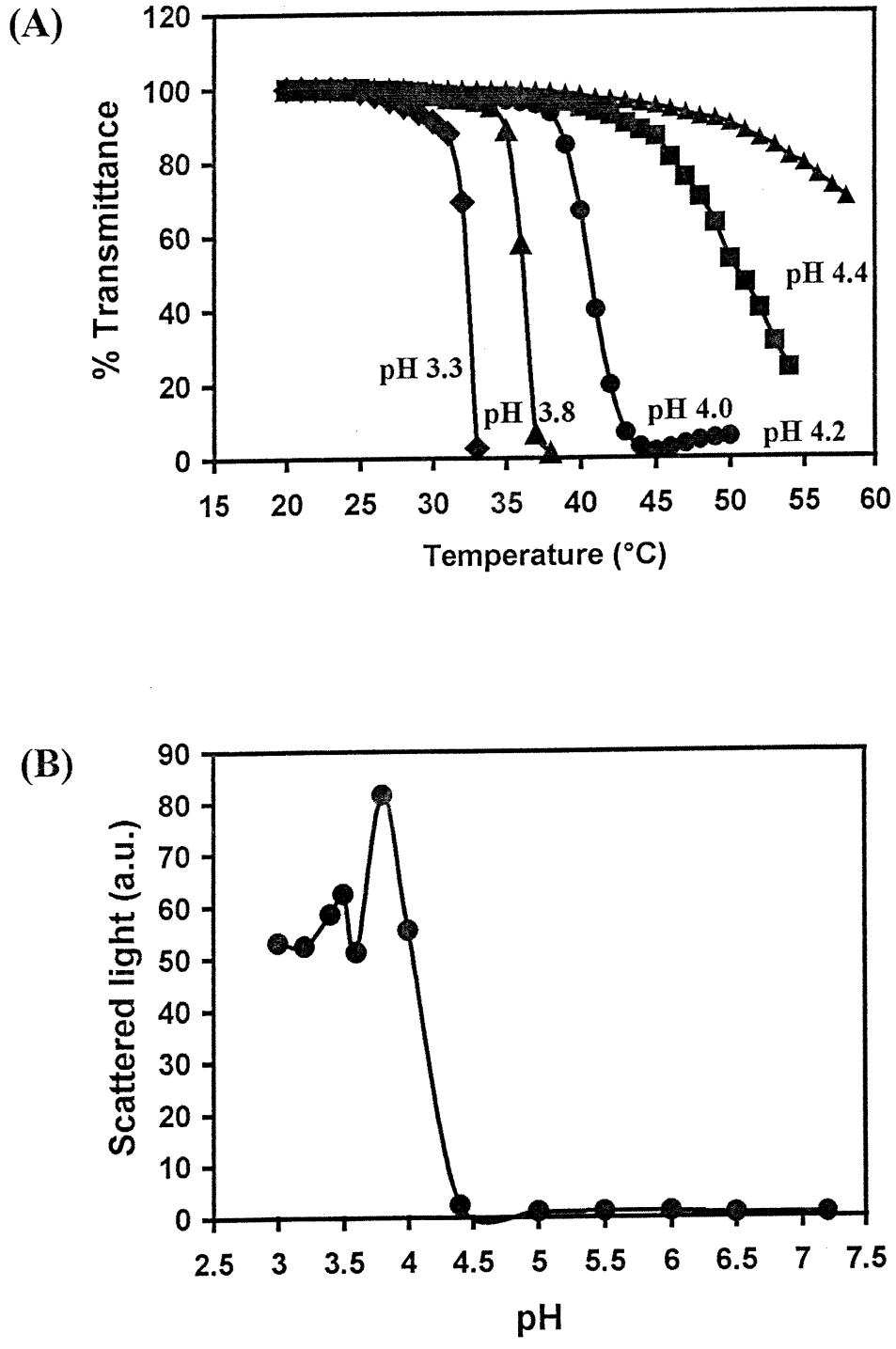


Fig. 1. (Francis et al.)



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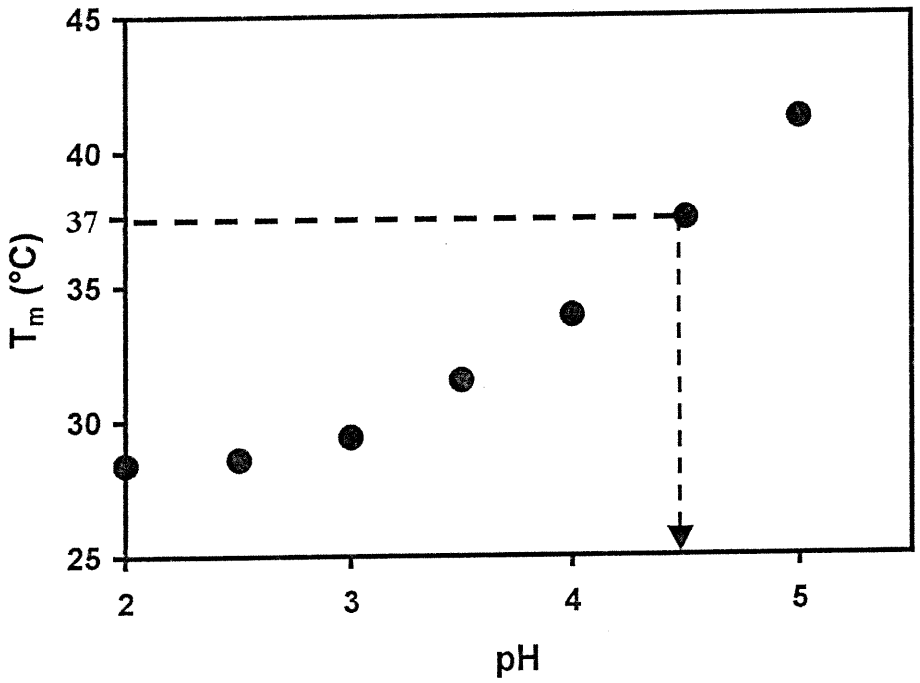

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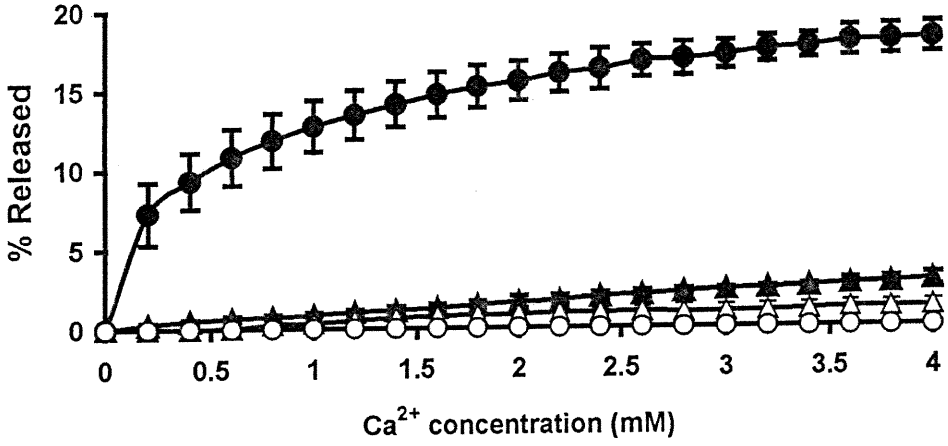

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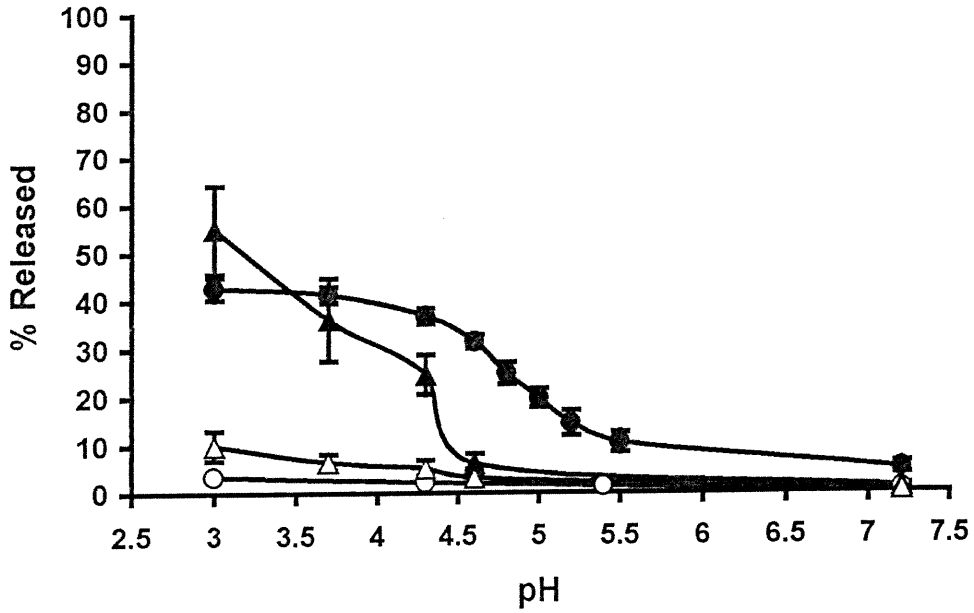

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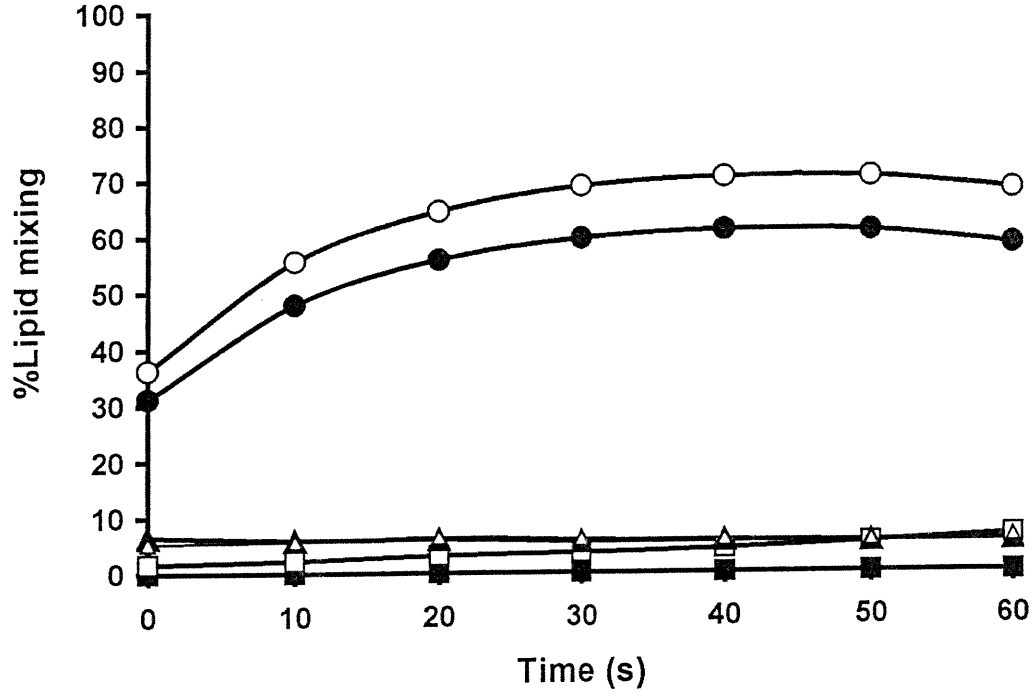

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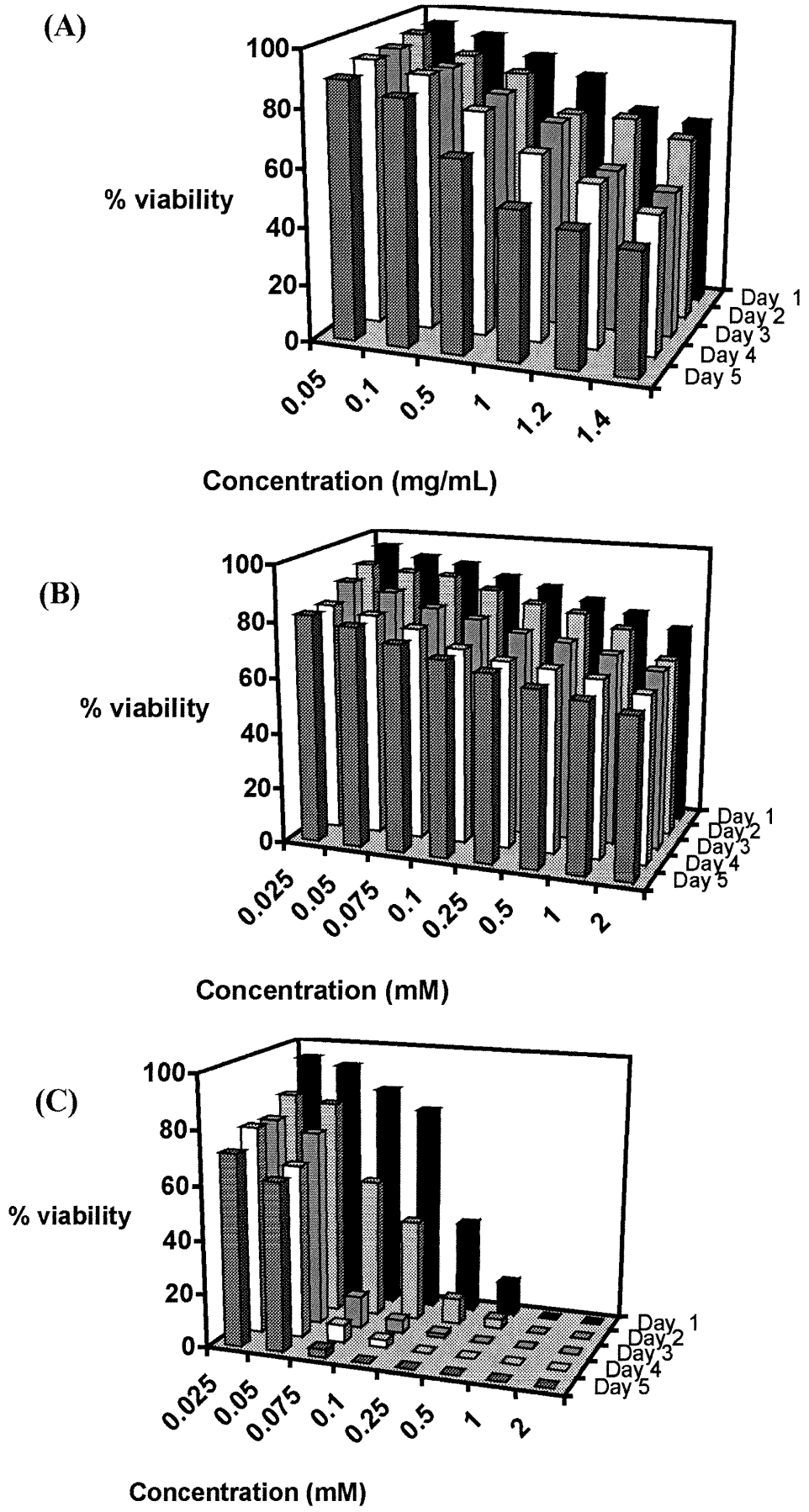

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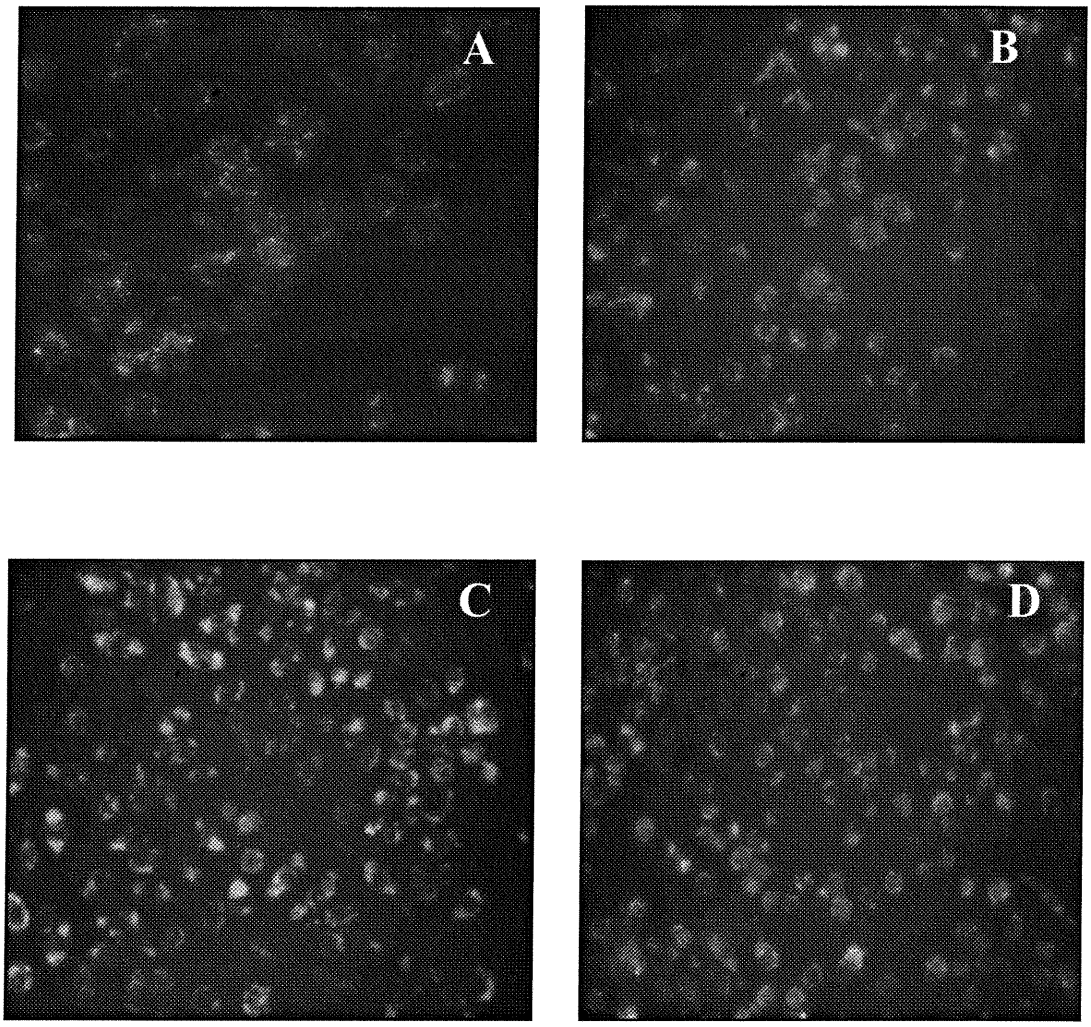

Fig. 8. (Francis et al.)

CHAPTER 3

DISCUSSION

1. Introduction

The therapeutic future of liposomes as a drug delivery system is quite promising. One must bear in mind that only in the last 20 years, real advances have been made in translating the progress from university laboratories into clinical applications.

Recently, more attention has been given to niosomes as carrier systems physically similar to liposomes. They are regarded as inexpensive alternatives, of non-biological origin, to liposomes.

In the present study, significant interest has been given to the characterization of pH-sensitive niosome vesicles as they could represent a low cost promising strategy for drug delivery. Vesicles were characterized and tested for their stability, pH-sensitivity as well as for their ability to mediate cytoplasmic delivery of encapsulated content to cultured cells.

In our experiments, large unilamellar niosome vesicles were prepared mainly from a chemically stable non-ionic surfactant of polyoxyethylene stearyl ether (POE-SE) using the reverse-phase evaporation technique. The inclusion of 40 mol % of cholesterol in the lipid bilayer was used due to its known stabilizing effect on vesicle membrane[1-3] without affecting the pH-sensitivity.

2. *In vitro* stability studies

Since the design of a drug delivery system should include stability testing, we have demonstrated that polymer-coated as well as polymer-free niosomes and/or liposomes were relatively stable at pH 7.2 during 90 days of incubation at

4°C (Fig. 1). By monitoring the change in vesicle dimensions, we have observed that phospholipidic vesicles were smaller in size than non-ionic surfactant based vesicles.

3. pH-dependent content release

Vesicles were then assayed for acid-induced leakage of encapsulated content at 37°C. Accordingly, we started by determining the pH at which the (PNIPAM-Gly-C_{18}) copolymer collapses, which was found to be around 4.3. Thus, for pH-sensitive niosome and/or liposome dispersion, the copolymer should trigger significant release of the encapsulated fluorescent probe from the vesicles under weakly acidic conditions as a result of the phase separation of the copolymer solution. Actually, the copolymer was found to induce the destabilization of pH-sensitive liposomes at the pH of the copolymer phase transition. Surprisingly, the polymer-coated niosomes released their content at a less acidic pH than the value of the copolymer phase transition pH. This can be understood on the basis of complex formation between the copolymer and the non-ionic surfactant head groups. Polozova et *al.* showed that the binding of the copolymer to the niosomes is controlled primarily through hydrogen bonding formation between the hydroxyl functional groups of the polyoxyethylene non-ionic surfactant and the amide groups of the PNIPAM copolymer units, and, a lesser extent, by hydrophobic forces[4]. From this observation, we hypothesized that a decrease in the copolymer lower critical solution temperature (LCST)

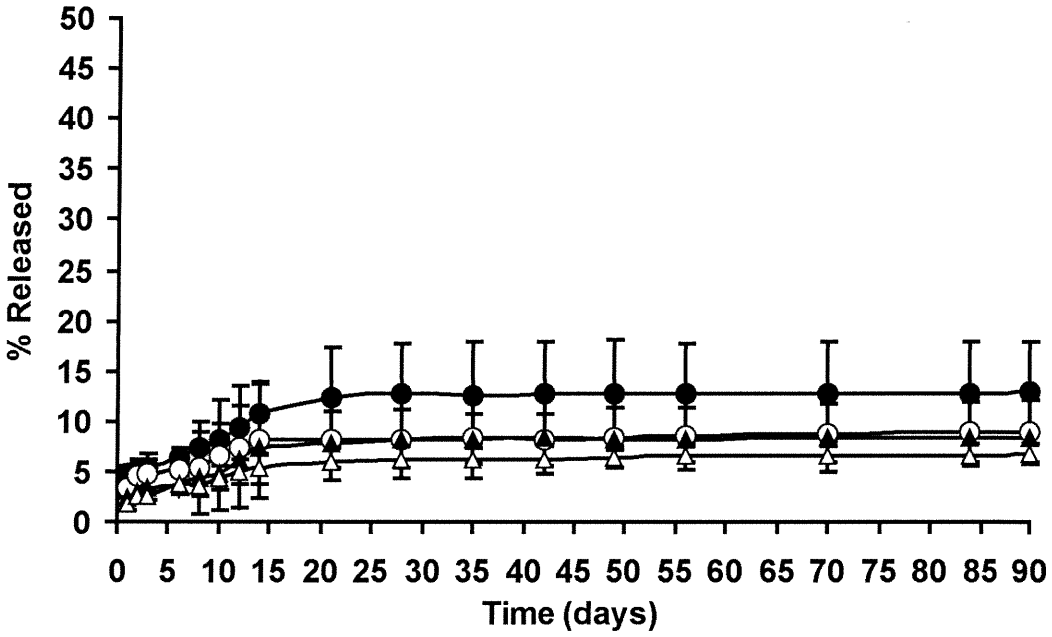

Figure 1. Influence of the storage time on the stability of Brij/Chol (3:2 mol/mol) niosomes stored at 4°C and neutral pH. Vesicles were prepared by the REV method in 5 mM HEPES buffer containing 5% dextrose, in presence (●) and absence (○) of the PNIPAM-Gly-C_{18} copolymer (93:5:2 mol%) with a polymer/lipid mass ratio of 0.3. HPTS release was measured at room temperature (22°C) as an increase in the fluorescence intensity (λ_{ex} = 413 nm, λ_{em} = 512 nm) recorded at different times at neutral pH. Results obtained for POPC/Chol (3:2 mol/mol) liposomes in presence (▲) and in absence (Δ) of the copolymer are also shown. Mean ± S.D. (n = 2).

promotes the niosome destabilization at pH different than the free copolymer phase transition pH value.

4. Study of the fusogenicity of polymer/vesicle systems

We have also explored the influence of the pH on the intervesicle fusion in order to investigate the ability of vesicles to fuse in the acidic endosome and transfer a significant amount of their content into the cell cytoplasm before reaching the lysosomes.

Theoretically, fusion between two vesicle bilayers can occur in three different ways:

- Mixing of aqueous content *and* mixing of lipid bilayer components (complete fusion).
- Mixing of bilayer components without leakage of the aqueous content *or* mixing of aqueous content (incomplete fusion).
- Mixing of bilayer components without mixing of the aqueous content *but* with leakage of the aqueous content (lysis).

In particular, the ideal mechanism for drug delivery to the cell cytoplasm is the first one. In fact, the mechanism of fusion greatly depends on the composition of the pH-sensitive vesicles. For instance, in case of PE/OA liposomes, both mixing of lipids and mixing of aqueous content were clearly observed in acidic conditions[5].

In our study, significant fusion between membrane bilayers did not occur for the polymer-modified liposomes and/or niosomes compared to the fusogenic

DOPE/OA liposomes suggesting that the vesicle destabilization at acidic pH is not due to the interbilayer contact. Consequently, the destabilization of the bilayered structure at acidic pH was not induced by the leaky membrane fusion.

However, liposome-endosome fusion is not the only event involved in the endosome-cytoplasm translocation. It was proposed that rupturing of the endosomal membrane could also occur as an alternative pathway with subsequent release into the cytoplasm[6].

5. Stability of vesicles in normal human serum

Generally, the efficiency of niosomes and liposomes as drug carriers is governed somehow by their stability in blood since they should be administered by intravenous injection into the circulation[7-9].

Thereby, for niosomal and liposomal formulations that exhibited pH sensitivity in buffer, we also evaluated their stability in normal human serum at neutral pH together with their capacity to maintain the pH-sensitivity response following incubation in serum. The obtained results demonstrated that polymer-modified niosomes were partly destabilized at neutral pH during 3 h incubation in normal human serum (Fig. 2). We hypothesized that this was due to the polyvalent cations present in serum. To provide further evidence, content leakage was determined in buffer solution (pH 7.2) where increasing concentrations of Ca^{+2} ions were added to the niosome dispersion in the presence of the copolymer. As expected, a calcium-dependent content leakage was observed under such conditions. This was caused predominantly by the binding of Ca^{+2} ions to the

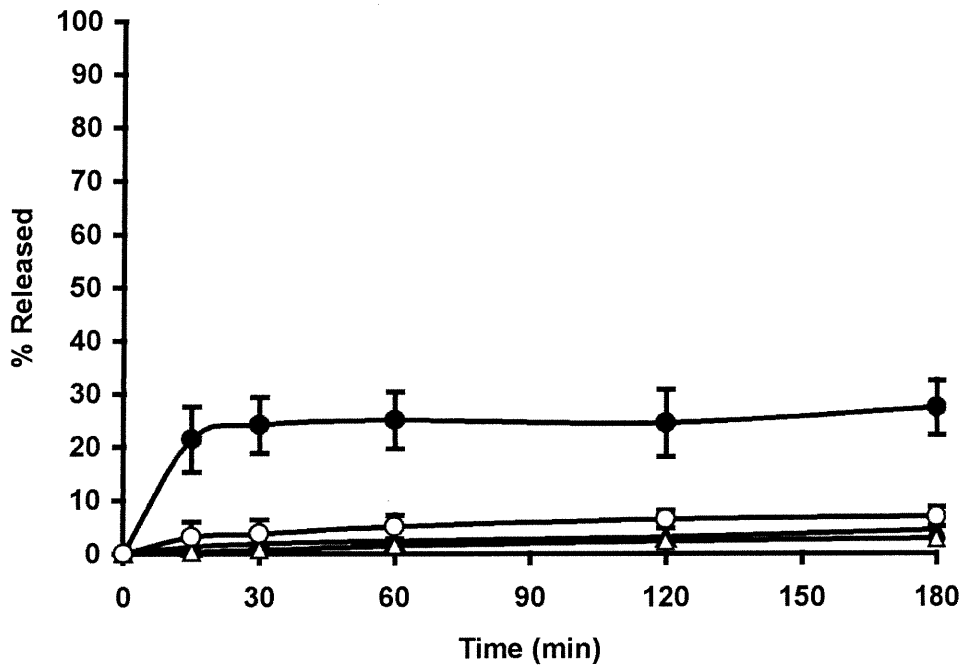

Figure 2. Time-dependent release kinetics for 20 mM POE-SE/Chol (3:2 mol/mol) niosomes prepared by the REV method in 5 mM HEPES buffer containing 5% dextrose and incubated overnight at 4°C in presence (●) and absence (○) of the PNIPAM-Gly-C_{18} copolymer (93:5:2 mol%) with a polymer/lipid mass ratio of 0.3. All measurements were taken after passage of both; niosomes and control formulations, over Sepharose 2B column. Niosomes were diluted 10 fold into normal human serum prewarmed to 37°C. The mixture was then incubated at 37°C for 3 h. HPTS release was measured as an increase in the fluorescence intensity (λ_{ex} = 413 nm, λ_{em} = 512 nm) as a function of time at neutral pH. Results obtained for POPC/Chol (3:2 mol/mol) liposomes in presence (▲) and absence (Δ) of the copolymer are also shown. Mean ± S.D. (n = 3).

surface of the niosome/polymer complexes. Accordingly, a decrease in the LCST of the copolymer occurs at neutral pH resulting in an increase in content release to higher values.

Quite obviously, the pH-sensitivity was lost following incubation in human serum since most of vesicle contents were lost during incubation in serum (Fig. 3). For instance, in the case of polymer/niosome complexes, only 4% content release were recorded for polymer-modified niosomes at pH 4 after incubation in serum (Fig. 3), compared with 40% release at the same pH for the vesicles without the serum treatment. This was due, presumably, to the serum factor(s), lipid and/or protein, which extracted the copolymer from the vesicle membrane[10] resulting in loss of the pH-sensitive leakage of vesicle content. Recent studies from our laboratory suggest that pH-sensitivity after incubation in serum can be preserved by increasing the concentration of the alkyl chain (anchor) in the copolymer structure.

6. *In vitro* cellular studies

The biocompatibility of pH-sensitive niosomes is not well described in the literature. This study represents the first attempt to elucidate their cytotoxicity as well as interrelated cellular uptake. These phenomena were investigated using J774 cell line, a murine macrophage-like cell line, as an *in vitro* model since macrophages are highly phagocytic cells and are ideal for studies of phagocytosis and intracellular processing of phagocytosed material.

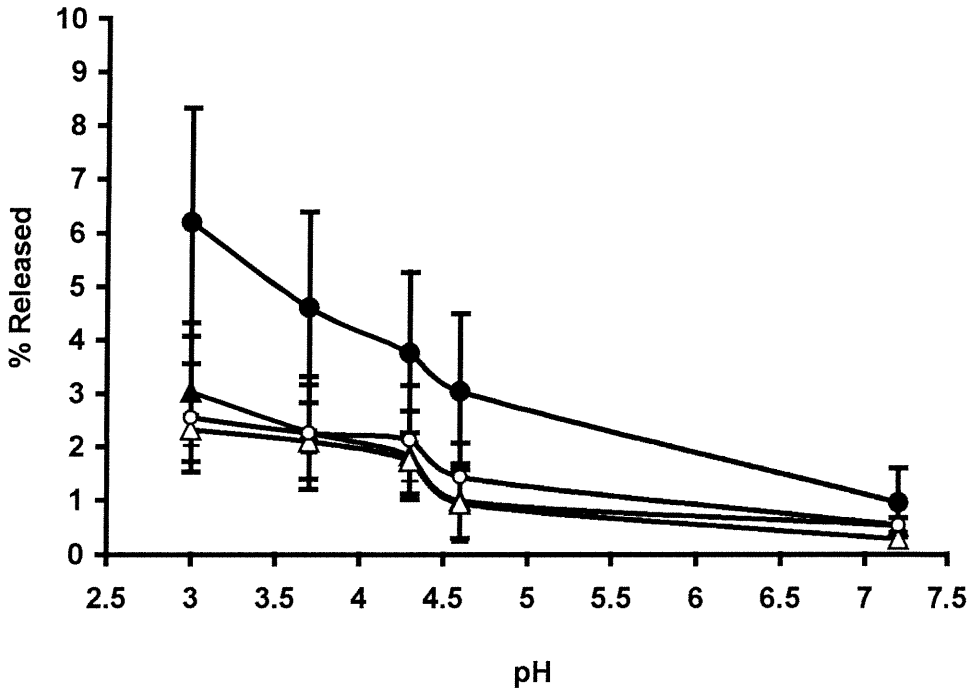

Figure 3. Release kinetics for 20 mM POE-SE/Chol (3:2 mol/mol) niosomes prepared by the REV method in 5 mM HEPES buffer containing 5% dextrose and incubated overnight at 4°C in presence (●) and absence (○) of the PNIPAM-Gly-C_{18} copolymer (93:5:2 mol%) with a polymer/lipid mass ratio of 0.3. All measurements were taken after passage of niosomes over Sepharose 2B column. Niosomes were incubated in normal human serum at 37°C for 1 h. HPTS release was measured as an increase in the fluorescence intensity (λ_{ex} = 413 nm, λ_{em} = 512 nm) recorded over a pH range (7.2-3.0). Results obtained for POPC/Chol (3:2 mol/mol) liposomes in presence (▲) and absence (Δ) of the copolymer are also shown. Mean ± S.D. (n = 3).

The evaluation of niosomes and/or liposomes cytotoxicity was carried out using a 3-(4,5 dimethylthiazole-2yl)-2,5 triphenyl tetrazolium (MTT) test on J774 murine macrophages. The test is based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. We also examined the effect of vesicle composition, concentration and incubation time on their uptake by these macrophages.

PNIPAM-Gly-C_{18} exhibited no significant toxicity toward the cells during the assay. On the other hand, at different incubation periods, the liposomes resulted in a less toxic effect as compared to NSVs which showed high toxicity almost at all the tested concentrations. This feature implies that the cytotoxic effect of niosomes is most likely due to the non-ionic surfactant incorporated in the lipid bilayer.

Hofland et *al.* showed that, for alkyl polyoxyethylene surfactants, the more hydrophobic the compound, the less toxic it will. This feature was found to be related to the alkyl chain length since an increase in alkyl chain length is accompanied by a decrease in toxicity. on the other hand, they found that an increase in polyoxyethylene chain length caused an increase in toxicity[11]. Moreover, it is important to note that detergents of non-ionic polyoxyethylated lauryl ether surfactants were reported to alter the membrane structure in some way resulting in a disruption and destabilization of cellular membranes regardless the membrane type, thus enhancing the cytotoxicity of incorporated cytotoxic drugs (e.g. adriamycin) in tumor cell culture systems[12-14].

On the cellular level, it is well established that macrophages internalize the vesicles and accumulate them in low pH compartments[15]. Previous work has shown that the primary mechanism of delivery involves vesicles adsorption onto the cell surface and subsequent endocytosis[15-17]. Consequently, vesicles come in contact with low pH compartments within the cell, presumably endosomes and lysosomes[18,19]. Once exposed to the mildly acidic pH of these compartments, pH-sensitive vesicles would eventually fuse with or destabilize the endosomes membrane and release their contents into the cell cytoplasm[20-22].

When J774 cells were incubated with polymer-coated niosome vesicles at 37°C, only weak and vesicular fluorescence of calcein was observed indicating that polymer/niosome complexes were present mostly in the endosomes and did not deliver their content into the cytoplasm despite the acidic pH of the endosomal compartments (pH<6). In contrast, the cells treated with the polymer-modified POPC/Chol liposomes displayed more intensive and diffuse cytoplasmic fluorescence, indicating that the cellular uptake of liposomes was enhanced by fixation of the pH-responsive copolymer on the surface of the vesicles. Thus, at the low local pH within the endosomal compartments, the surface copolymer destabilized the vesicle bilayer and, perhaps, the endosomal membrane resulting in content release into the cell cytoplasm.

Furthermore, when the efficiency of cytoplasmic delivery by polymer-modified POPC/Chol liposomes was compared with that of pH-sensitive DOPE/OA (2:1) liposomes, the POPC/Chol vesicles were found to deliver more calcein to the cytoplasm.

Although niosomes represent an interesting vehicle for pH-sensitive drug delivery, still a significant amount of work needs to be completed to improve the compatibility of these systems with the biological medium and to increase the cytoplasmic delivery of their content.

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CHAPTER 4

CONCLUSION

The future of specialized polymer/niosome systems for the delivery of cytotoxic drugs is quite promising. The research work presented in this study provides a foundation to suggest that this system can be used for further developments. However, to achieve the final goal of producing niosome vesicles acting as effective drug carriers, several different aspects must be examined. For instance, quantitative studies aimed at optimizing the stability and reactivity of pH-sensitive polymer/niosome systems are needed before starting the maturation process from *in vitro* investigations to *in vivo* studies. Regarding this point, two specific areas appear to be very important for the production of targeted delivery systems able to overcome the current limited success in this area: that is, the optimization of vesicle stability in normal human serum for *in vivo* applications and the evaluation of biological fate of niosomal carriers. These basic studies will undoubtedly result in an improved understanding of some principal features such as the administration route and the formulation parameters, which will effectively facilitate the development of therapeutically useful targeted pH-sensitive polymer/niosome systems.

It is hoped that this work will introduce new researchers to this topic and more importantly offers the industrial community an idea of the potential utility of pH-sensitive polymer/niosome systems as drug carriers.