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Development of a pH-responsive liposomal drug carrier using poly(phosphazenes)

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Development of a pH-responsive liposomal drug carrier using poly(phosphazenes)

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Résumé

Libérer un principe actif à son site d'action afin d'obtenir une formulation plus efficace et moins toxique ; tel est l'objectif de la vectorisation, en particulier des vecteurs colloïdaux. Par exemple, le relargage dans les compartiments acides de la cellule peut être réalisé grâce à des liposomes rendus sensibles au pH par des polyanions amphiphiles qui induisent une déstabilisation de la membrane à pH acide, et provoquent ainsi une libération contrôlée des agents encapsulés. Dans ce mémoire, des poly(organophosphazenes) (PPZ), polymères biodégradables et polyvalents, ont été modifiés pour obtenir cinq dérivés amphiphiles et ionisables. Différentes proportions de di-éthylène glycol éthyle éther (EEE), d'acide amino-butyrique (ABA) et de polyéthylène glycol octadécyl éther ($C_{18}(EO)_{10}$) ont été utilisées afin d'identifier les proportions optimales pour la formulation des liposomes. La structure et composition des PPZ ont été déterminées par résonance magnétique nucléaire, titration acido-basique et chromatographie par exclusion de taille. Leur sensibilité à la température et au pH a été confirmée par calorimétrie différentielle et par turbidimétrie, respectivement. Il a d'abord été démontré que $C_{18}(EO)_{10}$ permettait un ancrage efficace des polymères dans la bicouche des vésicules. La protonation des ABA, quant à elle, a permis une libération contrôlée d'un marqueur encapsulé dans les PPZ-liposomes. Enfin, l'exposition des PPZ-liposomes au sérum humain a provoqué une diminution de la sensibilité au pH, même lorsque ces vésicules sont PEGylées. Bien que la libération puisse être contrôlée en modifiant la composition et la quantité de PPZ ancrée aux liposomes, une optimisation de la structure des polymères pourrait améliorer la libération en présence de sérum.

Mots-clés : Vectorisation du médicament, polyphosphazene, liposome, sensibilité au pH, LCST, amphiphile, ionisable.

Abstract

Colloidal drug carriers are currently being developed in order to achieve a safer and more efficient drug delivery than classical administration forms. Particularly, pH-responsive liposomes are being designed to specifically release their contents in acidic cellular compartments. Such vesicles can be generated by fixing amphiphilic polyanions to the surface of liposomes in order to induce acid-triggered membrane destabilization and release of encapsulated agents. Amphiphilic ionizable poly(organophosphazenes) (PPZ) have been proposed as a biodegradable polymer that can impart pH-sensitivity to liposomes. In this master's thesis, five PPZ have been synthesized with varying proportions of diethylene oxide ethyl ether (EEE), amino butyric acid (ABA) and polyethylene glycol octadecyl ether ($C_{18}(EO)_{10}$) to identify the requirements for an optimal PPZ-liposome formulation. The structure and composition of the PPZ were determined by nuclear magnetic resonance, acid-base titrations and size exclusion chromatography. Differential scanning calorimetry and turbidimetry assays confirmed the temperature- and pH-sensitivity of the PPZ, respectively. It was shown that $C_{18}(EO)_{10}$ allowed efficient fixation of PPZ to vesicles, while protonation of ABA induced acid-triggered release of an encapsulated marker from the PPZ-liposomes. Exposure to human serum, however, significantly reduced the acid-triggered release of the marker, even when the vesicles were PEGylated. Though release can be tuned by adjusting the composition and the amount of the PPZ anchored to liposomes, further optimization of the PPZ structure may be required to improve the release in the presence of serum.

Keywords: Drug delivery, polyphosphazene, liposome, pH-sensitive, LCST, amphiphilic, ionizable.

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Abbreviations

ABA	Amino butyric acid
$C_{18}(EO)_{10}$	Polyethylene glycol octadecyl ether
CDC	Colloidal drug carriers
Chol	Cholesterol
Chol-BODIPY	Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-dodecanoate
cmc	Critical micelle concentration
DCM	Dichloromethane
ΔH_{LCST}	Enthalpy of lower critical solution temperature transition
DMPE	Dimyristoyl-*N*-[[4-(maleimidomethyl)cyclohexyl]carbonyl] phosphatidyl-ethanolamine
DODA	Dioctadecylamide
DOPE	Dioleoyl phosphatidylethanolamine
Dox	Doxorubicin
DPX	*p*-xylene-bis-pyridinium bromide
DSC	Differential scanning calorimetry
Dtxl	Docetaxel
EAB	Ethyl 4-aminobutyrate
EAB·HCl	Ethyl 4-aminobutyrate hydrochloride
EEE	Diethylene oxide ethyl ether
EPC	Egg phosphatidylcholine
EPR	Enhanced permeation and retention
Et_2O	Diethyl ether

GOV	Giant oligomeric vesicles
Gly	Glycine acrylamide
HBS	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer saline
HCTP	Hexachlorocyclotriphosphazene
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HPTS	8-hydroxypyrene-1,3,6-trisulfonic acid
i.v.	Intravenous
LCST	Lower critical solution temperature
LMWD	Low molecular weight drug
LMWS	Low molecular weight surfactants
LUV	Large unilamellar vesicles
MAA	Methacrylic acid
MCF-7	Human breast adenocarcinoma cell line
MEEP	Poly(bis-methoxyethoxyethoxy-phosphazene)
MES	2-*N*-(morpholino)ethanesulfonic acid
MLV	Multilamellar vesicles
M_n	Number-average molecular weight
MPS	Mononuclear phagocyte system
M_w	Weight-average molecular weight
NCS	Neocarzinostatin
NIPAM	*N*-isopropylacrylamide
NP	Polymeric nanoparticles
ODA	Octadecyl acrylate

P(NIPAM-*co*-MAA)	Poly(*N*-isopropylacrylamide-*co*-methacrylic acid)
P(NIPAM-*co*-MAA-*co*-ODA)	Poly(*N*-isopropylacrylamide-*co*-methacrylic acid-*co*-octadecyl acrylate)
PB	Phosphate buffer
PCL	Poly(ε-caprolactone)
PCPP	Poly[di(carboxylatophenoxy)phosphazene]
PDCP	Poydichlorphosphazene
PEAA	Poly(2-ethylacrylic acid)
PEG	Polyethylene glycol
PEG_{2000}-DSPE	*N*-[methoxy(polyethylene glycol) 2000] carbonyl-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt
PEO	Poly(ethylene oxide)
PFAP	Poly(fluoroalkoxyphosphazenes)
PG	Decylamine-succinylated poly(glycidol)
PGA	Poly(glycolic acid)
PHPMA	Poly-*N*-(2-hydroxypropyl)methacrylamide
PHPMA-Dox	Doxorubicin-conjugated poly-*N*-(2-hydroxypropyl)methacrylamide
PHSM	pH-sensitive mixed micelles
PHSM/f	Folate-conjugated pH-sensitive mixed micelles
PIC	Polyion complex
PK2	Doxorubicin-conjugated poly-*N*-(2-hydroxypropyl)methacrylamide with galactosamine targeting residue
PLA	Poly(D,L-lactic acid)
PLA-*block*-PEG	Poly(lactic acid)-*block*-polyethylene glycol

PLGA	Poly(lactide-*co*-glycolic acid)
PM	Polymeric micelles
PNIPAM	Poly(*N*-isopropylacrylamide)
polyHis	Poly(L-histidine)
polyHis-*block*-PEG	Poly(L-histidine)-*block*-polyethylene glycol
PPZ	Poly(phosphazenes) and poly(organophosphazenes)
PVP	Poly(*N*-vinyl-pyrrolidone)
SEC	Size exclusion chromatography
SMA	Polystyrene-maleic anhydride
SMANCS	Polystyrene-maleic anhydride neocarzinostatin
SUV	Small unilamellar vesicles
TEA	Triethylamine
T_g	Glass transition temperature
THF	Tetrahydrofuran
VP	*N*-vinylpyrrolidone

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CHAPTER I: Colloidal drug carriers

I-1 Introduction

Colloidal drug carriers (CDC) are dispersed systems, typically of nanometric particle size (< 1 μm in diameter), intended to selectively deliver therapeutics to their target. The development of such formulations is particularly important for medicines with poor clinical efficiency due to their physicochemical properties. For instance, several low molecular weight drugs (LMWD) are hydrophobic and need solubilizers to prevent drug precipitation in the bloodstream and ensure adequate bioavailability. Furthermore, many drugs are subject to premature degradation and/or elimination by the system's metabolic pathways. Consequently, LMWD that are intravenously administered (*i.v.*) require high dosages to attain therapeutics levels at the intended site and frequently involve adverse effects at other sites. In order to circumvent these obstacles, CDC have been developed to lengthen the circulation time of drugs and to reach diseased tissues by both passive and active targeting. A variety of CDC has been designed for the delivery of different types of bioactives, that include both water soluble and insoluble LMWD, as well as hydrophilic macromolecules, such as peptides [Bickel *et al.*, 2001; Seong *et al.*, 2006] and genetic material [Masson *et al.*, 2004].

CDC can be designed to achieve targeting of specific tissue, especially solid tumors, using the enhanced permeation and retention (EPR) effect [Maeda *et al.*, 2000]. Compared to healthy tissue, solid tumors are characterized by porous, leaky vasculature (Figure I-1). Thereby, suitable sized vectors (50-200 nm) are able to extravasate through the fenestrations of these blood vessels and reach the tissue interstitium. Moreover, the poor lymphatic drainage observed in the tumors will ensure that the vector remains in the vicinity of the diseased tissue where it might

release its payload. Therefore, the EPR effect not only allows for the drug to be targeted to diseased cells but also ensures local concentration of the active agent.

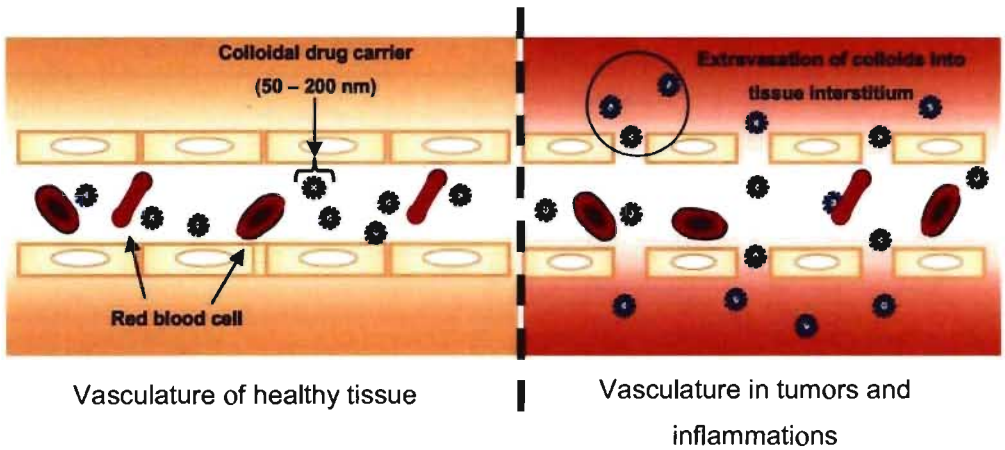


Figure I-1: The EPR effect.

In order to efficiently benefit from the EPR effect, the CDC has to remain in blood circulation for extended periods of time. But free drug administration is often limited by the half-life of the drug in blood circulation. So the role of a vector is to transport the active drug, protect it during its circulation, and prevent it from early elimination, particularly by the mononuclear phagocyte system (MPS). In this aim, a CDC should present specific properties concerning its size and surface properties. For the former, the vector diameter should ideally lie between 50 nm and 200 nm. Smaller CDC are more likely taken up through the fenestrations of the hepatic sinusoidal endothelium [Braet *et al.*, 1995], while colloids above 200 nm are often trapped in the spleen [Moghimi *et al.*, 2001]. To avoid elimination by the MPS, CDC can be coated using biocompatible, flexible, hydrophilic and non-charged polymers, such as polyethylene glycol (PEG). PEG forms a highly hydrated and steric shield against protein adsorption and recognition by cells of the MPS [Allen *et al.*, 2002]. Although PEG remains the most widespread polymer for the preparation of long-circulating colloids [Owens III and Peppas, 2006], several other polymers, such as poly-*N*-(2-

hydroxypropyl)methacrylamide (PHPMA) [Duncan *et al.*, 2001] and poly(N-vinyl-pyrrolidone) (PVP) [Lukyanov and Torchilin, 2004], have been developed for the same purpose. Long-circulating CDC have also been conceptually proposed to serve as drug containing reservoirs in the bloodstream [Moghimi *et al.*, 2001].

Along with the EPR effect, intracellular drug accumulation can be increased by active targeting to specified cells. This can be performed by associating a targeting residue, or ligand, to the vector. The ligand is able to recognize and fix to a distinct receptor on the surface of the target cells, inducing internalization of the CDC. This has been exploited for the targeting of tumors known to over express specific surface receptors, such as folate-receptor [Chung *et al.*, 1993]. Various targeting moieties have been investigated including galactosamine [Seymour *et al.*, 2002; Haag and Kratz, 2006], transferrins [Sahoo *et al.*, 2004], antibodies [Allen *et al.*, 1994; Kocbek *et al.*, 2007] and aptamers [Nutiu and Li, 2005].

Site-specific concentration of the drug can also be promoted by designing "intelligent" CDC that react to an externally applied stimulus, such as heat [Kono, 2001], ultrasound [Kost *et al.*, 1989] or a magnetic field [Vyas and Jain, 1994], to provoke a localized release. Although such techniques were successful, they often require sophisticated equipment. In contrast, some physiological variations within the organism, like changes in pH [Yessine *et al.*, 2003], can be used to drive the discharge of the therapeutic agent without external assistance. pH-sensitive CDC are formulated to retain the therapeutic while in circulation in the blood, which is neutral (pH = 7.4), and release their contents in more acidic compartments, such as tumor interstices and acidic organelles [Schmaljohann, 2006]. Regardless of the stimulus, drug release can be induced by two principal mechanisms: (i) if the drug is covalently bound to the colloid, environmental changes induce hydrolysis of the bond [Seymour *et al.*, 2002]; (ii) if it is physically entrapped within the vector, leakage results from a sudden destabilization of the CDC [Connor *et al.*, 1984; Lee, Shin *et al.*, 2003].

The type of colloidal drug delivery system is chosen according to the drug's physico-chemical properties and the intended mechanism of delivery. The following

chapter presents an overview of various CDC that exploit these concepts culminating with a discussion of liposomes, which have been extensively used in this work.

I-2 Drug-Polymer Conjugates

Drug-polymer conjugates consist of biologically active agents, including peptides and LMWD, covalently bound to a polymer. They have encountered a certain success, since several formulations have reached the market and many more are currently in clinical trial [Duncan, 2006]. While other CDC are usually designed to physically incorporate the therapeutic, conjugation enhances the pharmacokinetics of the drug by creating new "chemical entities" [Duncan, 2003]. Conjugation generally enhances tumor targeting, limits toxicity and increases circulation times of the active agent. Furthermore, the administration of hydrophobic LMWD can be facilitated by associating them to hydrophilic polymers, thus vastly improving their water-solubility.

The first model of drug-polymer conjugate was proposed by Ringsdorf in 1975 (Figure I-2) and consists of three basic elements: the polymer, the linker and the bioactive substance. Optionally, a targeting ligand can be conjugated for cell-specific internalization [Duncan *et al.*, 2001; Seymour *et al.*, 2002]. According to the number and localization of sites for conjugation, several structures could be envisaged (Figure I-2).

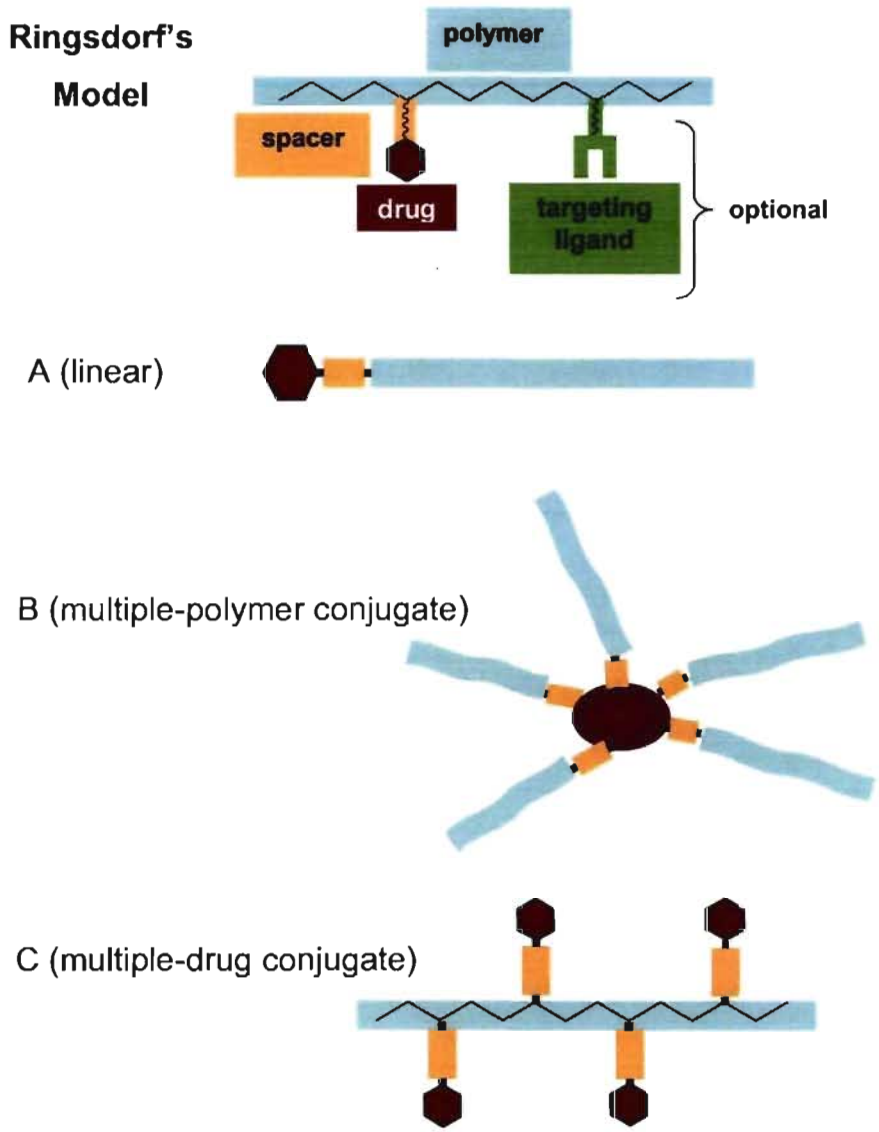


Figure I-2: Schematic representation of Ringsdorf's model of a drug-polymer conjugate and examples of different structures that can arise from conjugation. Haag R and Kratz F.: Polymer therapeutics: Concepts and applications. Angew Chem Int Ed. 2006. 45. 1198-1215. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Polymer properties are crucial to impart appropriate pharmacokinetics to the drug conjugate. First, a neutral, water-soluble and flexible macromolecular structure, like PEG, can create a barrier protecting the complex from immunogenic response. Secondly, the molecular weight of the polymer should be high enough to prolong blood circulation and promote the EPR effect. All the while, depending on the nature of the polymer, the molecular weight should be sufficiently low to allow renal clearance after drug delivery, since most polymers used in conjugation so far are not biodegradable [Duncan, 2006]. As for the drug itself, therapeutics such as peptides, oligonucleotides and LMWD are all potential candidates as long as they possess a functional group permitting conjugation. The linker may be a simple covalent bond or a spacer molecule. Polypeptide drugs are typically conjugated directly to the polymer through amino acid residues bearing nucleophilic groups, such as cysteine and arginine, as well as at the amino and carboxylic termini of the peptidic backbone [Veronese, 2001]. On the other hand, LMWD could be conjugated to the polymer *via* cleavable spacers, such as acid labile peptide sequences [Duncan, 2007] and hydrazone linkages [Lee *et al.*, 2006]. In such cases, the spacer is employed to release the LMWD at the site of action.

Most polymer-peptide conjugates are formed using PEG to increase blood circulation times of therapeutic proteins (Figure I-2A). One of the first proteins conjugated to PEG was the enzyme L-asparaginase. Treatment of lymphoblastic leukemia necessitates frequent intramuscular administration of L-asparaginase in high doses, which causes allergic and toxic reactions. PEGylated L-asparaginase (Pegaspargase, commercialized by Enzon as Oncaspar®) demonstrated drastically improved anti-lymphoma activity over the native enzyme [Graham, 2003]. In pharmacokinetic studies, Pegaspargase demonstrated a half-life of 357 ± 243 h, which was significantly longer than that of L-asparaginase (20 ± 6 h) [Ho *et al.*, 1986]. Since Pegaspargase, several other PEGylated-peptides have been marketed for their capacity to increase plasma residence times of the peptide therapeutics, which include interferon alfa-2a (Pegasys® and Peginterferon®) and 2b (Peg-Intron®), recombinant

methionyl human granulocyte colony stimulating factor (Neulasta®) and adenosine deaminase (Adagen®)[Duncan *et al.*, 2005; Hamidi *et al.*, 2006].

Though peptide conjugation should ideally involve water-soluble polymers, one particular exception must be noted. The anti-tumor protein neocarzinostatin (NCS) was conjugated to the hydrophobic polymer polystyrene-maleic anhydride (SMA) to form SMANCS [Maeda *et al.*, 1984]. Two chains of SMA were bound to the 1st and 20th amino acids of NCS, leading to a double polymer conjugate (Figure I-2B). In aqueous media, SMA presents a globular structure due to the clustering effect of the hydrophobic residues, pushing the carboxylate groups to the surface of the globule. Due to the hydrophobic nature of the complex, SMANCS was formulated with the lipid contrast medium Lipidol® and has been approved for the treatment of hepatocellular carcinoma in Japan [Duncan, 2006].

Since linear PEG possesses only two sites of conjugation, at each terminus, it often carries insufficient payload to meet therapeutic requirements for LMWD. A greater loading capacity can be achieved by using polymers with multiple linking sites along the chain (Figure I-2C) [Duncan, 2006]. Incidentally, it also allows for the conjugation of a targeting moiety [Thatte *et al.*, 2005]. This is the case with PHPMA which displays similar non-toxic and non-immunogenic character as PEG, but additionally possesses several sites of conjugation by substitution of the 2-hydroxypropyl groups. Several anti-cancer drugs have been conjugated to PHPMA and are currently in clinical trials, including paclitaxel, camptothecin, diaminocyclohexane palatinate and doxorubicin (Dox) [Duncan *et al.*, 2001; Duncan, 2006]. The latter was conjugated to the PHPMA backbone via a Gly-Phe-Leu-Gly linker, which is hydrolyzable in the acidic medium of lysosomes [Duncan, 2007]. A liver targeting moiety, galactosamine, was also linked to the terminus of the copolymer. The final complex, called PK2, was then tested for the treatment of hepatocellular carcinomas. In a phase I study, it was determined that 15-20% of injected dose accumulated in the liver of cancer patients, which was significantly higher than the localization of the non-targeted conjugate [Seymour *et al.*, 2002].

Depending on the initial dose, the accumulation of Dox in hepatic tumor was 12 to 50-fold higher for PK2 than for the free drug, thus achieving notable tissue targeting.

Drug-polymer conjugates have formed the framework upon which other CDC might be developed. In their simplicity, they have exploited the beneficial properties of polymers to keep therapeutic agents in circulation long enough to promote passive and active targeting. Their development has thus been adapted to generate advanced CDC with more complex structure and that will potentially allow more efficient formulation, administration and delivery of current and future medicines.

I-3 Polymeric Nanoparticles

Polymeric nanoparticles (NP) are defined as solid dispersions that can be categorized as either nanospheres or nanocapsules [Mohanraj and Chen, 2006]. The first are matrix-like systems within which a drug can be dispersed whereas the second are vesicular structures made of a polymer membrane that confines the drug within an aqueous or oily core [Brigger *et al.*, 2002]. NP have been developed for the delivery of various therapeutics including genetic material [Mao *et al.*, 2001; Yang *et al.*, 2008], proteins [Watnasirichaikul *et al.*, 2000; Sánchez *et al.*, 2003] and LMWD [Gaucher *et al.*, 2007; Haley and Frenkel, 2008].

NP offer several advantages for the delivery of therapeutics. With a wide range of materials and preparation methods, these versatile systems can improve the pharmacokinetics of bioactive compounds [Soppimath *et al.*, 2001]. The release kinetics of the drug can be controlled by the porosity of the polymeric network [Sant *et al.*, 2005] as well as influenced by the biodegradability and erosion of the polymers [Soppimath *et al.*, 2001]. With the help of additives that prevent precipitation, they can form relatively stable dosage forms [Pinto Reis *et al.*, 2006]. Furthermore, NP can be functionalized with ligands for active targeting [Farokhzad *et al.*, 2006; Kocbek *et al.*, 2007]. It is also possible to PEGylate the NP to increase blood circulation and reduce recognition by the MPS, which is especially important since NP are often formed with hydrophobic polymers [Li *et al.*, 2001].

Several methods can be used for the preparation of NP [Pinto Reis *et al.*, 2006]. Nanocapsules are prepared by either interfacial polymerization or by the polymer condensation method. For instance, nanocapsules with aqueous cores have been prepared by the polymerization of alkylcyanoacrylates at the interface of water-in-oil emulsions [Lambert *et al.*, 2000; Watnasirichaikul *et al.*, 2000]. Stable emulsions are first prepared with an aqueous solution of the active agent, an oily phase and stabilizing emulsifiers. Afterwards, a solution of the monomer is added to the mixture which is stirred until complete polymerization, leading to the formation of films around the droplets. The aqueous core makes such systems interesting candidates for the encapsulation of most biological macromolecules [Lambert *et al.*, 2000; Watnasirichaikul *et al.*, 2000]. Similarly, nanocapsules with hydrophobic cores can be prepared by first forming oil-in-water emulsions in order to encapsulate lipophilic molecules [Al Khouri Fallouh *et al.*, 1986].

The preparation of nanospheres is often based on oil-in-water emulsions followed by solvent evaporation. This method is especially used to prepare nanospheres with hydrophobic polymers, such poly(D,L-lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-*co*-glycolic acid) (PLGA) and poly(ε-caprolactone) (PCL) [Soppimath *et al.*, 2001; Pinto Reis *et al.*, 2006]. NP consisting of such polymers have the added advantage of being biodegradable over time in aqueous media [Park, 1994; Sánchez *et al.*, 2003]. The size of the NP can be controlled using emulsifiers, such as surfactants and block copolymers. The latter can additionally be designed to remain attached to the surface to generate long-circulating formulations with well hydrated surfaces [Gaucher *et al.*, 2007]. The NP can be collected by dialysis, filtration or ultra centrifugation, which also helps remove excess emulsifiers and to wash the particles.

Another method to prepare nanospheres is nanoprecipitation of hydrophobic polymers. In one particular study, PEGylated NP were formed with amphiphilic *block* copolymers with large PLGA chains and shorter PEG segments [Farokhzad *et al.*, 2006]. PLGA-*block*-PEG was first dissolved with the drug, Docetaxel (Dtxl), in acetonitrile and added dropwise into water. Without the need of surfactants, the

diffusion of the organic phase allowed the PLGA block to precipitate, thus producing PEGylated nanospheres (d ≈ 153 nm) loaded with Dtxl. Furthermore, each PEG chain possessed a carboxylic acid at the terminus that permitted conjugation of aptamers to the NP for active-targeting. Targeted NP improved tumor regression for mice bearing prostate cancer cells with a 100% survival rate, whereas only 50% survival was observed in mice treated with non-targeted NP and even less when free Dtxl was administered [Farokhzad *et al.*, 2006].

Hydrophilic polymers, such as chitosan [Calvo *et al.*, 1997b; Obara *et al.*, 2005] and dextran [Kim *et al.*, 2000] have been used to prepare nanospheres due to their biocompatibility. They are particularly used for the vectorization of hydrophilic bioactives like peptides [Soppimath *et al.*, 2001]. For instance, protein-loaded NP have been formed with positively charged chitosan by the neutralization of its charges using a counter-ion, tripolyphosphate [Calvo *et al.*, 1997a, b]. Genetic material, which is negatively charged, can be incorporated into chitosan NP by similar electrostatic interactions

There is a wide range of biodegradable and biocompatible materials available for the preparation of NP. Though many NP preparation methods lead to the vectorization different bioactive compounds, the size and polydispersity of the particles are sometimes difficult to control, especially for NP produced by nano-precipitation [Mohanraj and Chen, 2006]. Also, a marked burst release of the drug is noticed when it is located close to the surface of the NP [Mohanraj and Chen, 2006]. Nonetheless, NP often exhibit greater drug loading efficiency and stability than the following CDC, i.e. polymeric micelles.

I-4 Polymeric Micelles

Micelles are colloidal structures formed by the spontaneous self-association of low molecular weight or polymeric amphiphiles in a solvent that is selective for either moiety [Torchilin, 2001]. These molecules exist separately below their critical micelle concentration (cmc), above which they assemble into core-shell structures

[Torchilin, 2001; Torchilin, 2007]. Polymeric micelles (PM) are made of amphiphilic *block* copolymers which possess an inert hydrophilic block to form the hydrated corona and another segment to form the core. Depending on the composition of the core-forming segment, micellization can be driven by hydrophobic or electrostatic cohesive forces. In addition, the length and structure of the segments can influence the size and stability of the micelles. Though PM are generally spherical in shape, the length of the polymer blocks [Zhang and Eisenberg, 1995; Zhang and Eisenberg, 1996] and the solvent conditions [Shen *et al.*, 1999; Choucair and Eisenberg, 2003] may impose other morphological arrangements, such as rods, tubules and lamellae. In non-polar organic solvents, it is also possible to form reverse-micelles, which consist of hydrophobic corona surrounding a hydrophilic core. However, the following discussion will be limited to spherical PM formed in aqueous media as they are the most applied in drug delivery research.

PM offer several advantages, such as their capacity to solubilize or incorporate bioactives, their size that allows for efficient passive targeting, and the biocompatibility of available polymers [Yokoyama, 2005; Torchilin, 2007]. The hydrophilic chains in the corona can also prevent secondary aggregation of the micelles due to their hydration, while also stabilizing core formation. Finally, PM are good candidates for active targeting since they can be formed of stimuli-sensitive polymers and can often be functionalized by chemical conjugation of ligands on the surface of the corona. PM have been successfully designed for the encapsulation of poorly water-soluble drugs [Ramaswamy *et al.*, 1997; Cavallaro *et al.*, 2004; Huh *et al.*, 2005; Elsabahy *et al.*, 2007], genetic material [Kataoka *et al.*, 1999; Itaka *et al.*, 2003; Dufresne *et al.*, 2004] and proteins alike [Harada and Kataoka, 1998, 1999].

Amphiphilic copolymers typically possess cmc values around 10^{-6}-10^{-7} M, whereas those of low molecular weight surfactants (LMWS) usually lie between 10^{-3} and 10^{-4} M [Bae and Kataoka, 2005]. PM are therefore considerably more stable against dissociation than LMWS-micelles upon dilution. Hydration of the hydrophilic block also imparts a steric stabilization that prevents aggregation of the PM.

In the same manner as for NP, organic solvents can be used to introduce hydrophobic LMWD into the core. The poorly water-soluble drug and the amphiphile are first dissolved in an organic phase. The latter is then slowly removed from the medium, either by dialysis against water or by evaporation after addition of the aqueous phase. These methods allow the hydrophobic chains to associate and entrap the drug. Another approach is to covalently conjugate the poorly water-soluble drugs to the core-forming block of the polymers [Yokoyama *et al.*, 1990; Yokoyama *et al.*, 1991]. This method increases loading efficacy, ensures the delivery of high doses and prevents the agent from leaking out. In such instances, it is sometimes preferable that the bonds are cleaved when the vector reaches the target site to improve therapeutic activity.

PM designed for the delivery of charged bioactives are called polyion complex (PIC) micelles and associate *via* electrostatic interactions [Harada and Kataoka, 1998; Kataoka *et al.*, 1999; Itaka *et al.*, 2003; Dufresne *et al.*, 2004]. PIC micelles are formed of copolymers possessing a neutral hydrophilic segment and a polyionic block. The latter can complex with charged bioactives and then self-associate. For instance, negatively charged genetic material can be associated to *block* copolymers with a polycationic segment to form the core of the PM. PIC micelles have been shown to protect therapeutic oligonucleotides from nuclease activity [Katayose and Kataoka, 1998] and improve their pharmacokinetics [Harada-Shiba *et al.*, 2002].

The following example highlights the principal advantages of PM as CDC. Lee *et al.* (2003) prepared Dox-loaded, pH-sensitive mixed micelles (PHSM), targeted or not with folate ligand (PHSM/f) against a malignant breast cancer cell line (MCF-7) [Lee, Na *et al.*, 2003]. The authors synthesized *block* copolymers consisting of poly(L-histidine) (polyHis) and PEG, which micellized under basic pH [Lee, Shin *et al.*, 2003]. The polyHis block that formed the hydrophobic core of the micelles had two roles. First, it provided pH-sensitivity to the PM since protonation of the histidine groups under mildly acidic conditions made the core-forming block water-soluble, thus prompting disassembly of the micelles and the release of Dox [Lee, Shin *et al.*,

2003]. Secondly, polyHis was selected for its endosomolytic properties and was expected to promote intracellular drug delivery by destabilization of the endosome [Lee, Na *et al.*, 2003]. PolyHis-*block*-PEG was formulated with 25 wt% PLA-*block*-PEG to lower the dissociation pH from 7.6 to 7.0 [Lee, Na *et al.*, 2003]. This proportion of PLA-*block*-PEG was necessary to enhance micelle stability at pH 7.4 and yet permit controlled release of the incorporated contents [Lee, Na *et al.*, 2003]. PHSM and PHSM/f were administered by *i.v.* injection into mice grafted with MCF-7, where Dox accumulation was 5 times greater than in the tumors of mice treated with free Dox [Lee *et al.*, 2005]. For mice bearing a drug resistant MCF-7 strain, PHSM/f maintained Dox delivery to the tumor cells, while Dox accumulation was 50% lower after PHSM-treatment and undetectable after administration of the free drug [Lee *et al.*, 2005].

As compared to nanospheres, the size of PM can be more easily controlled due to the way amphiphilic *block* copolymers self-assemble. Second, micelle formation is governed by a dynamic structure that gives PM the potential for improved triggered release. Stimuli-responsive polymers can be employed to control disassembly and delivery of the therapeutic. In the final section of this chapter, we discuss liposomes, which are also formed by self-association of amphiphiles and can likewise be designed to respond to stimuli.

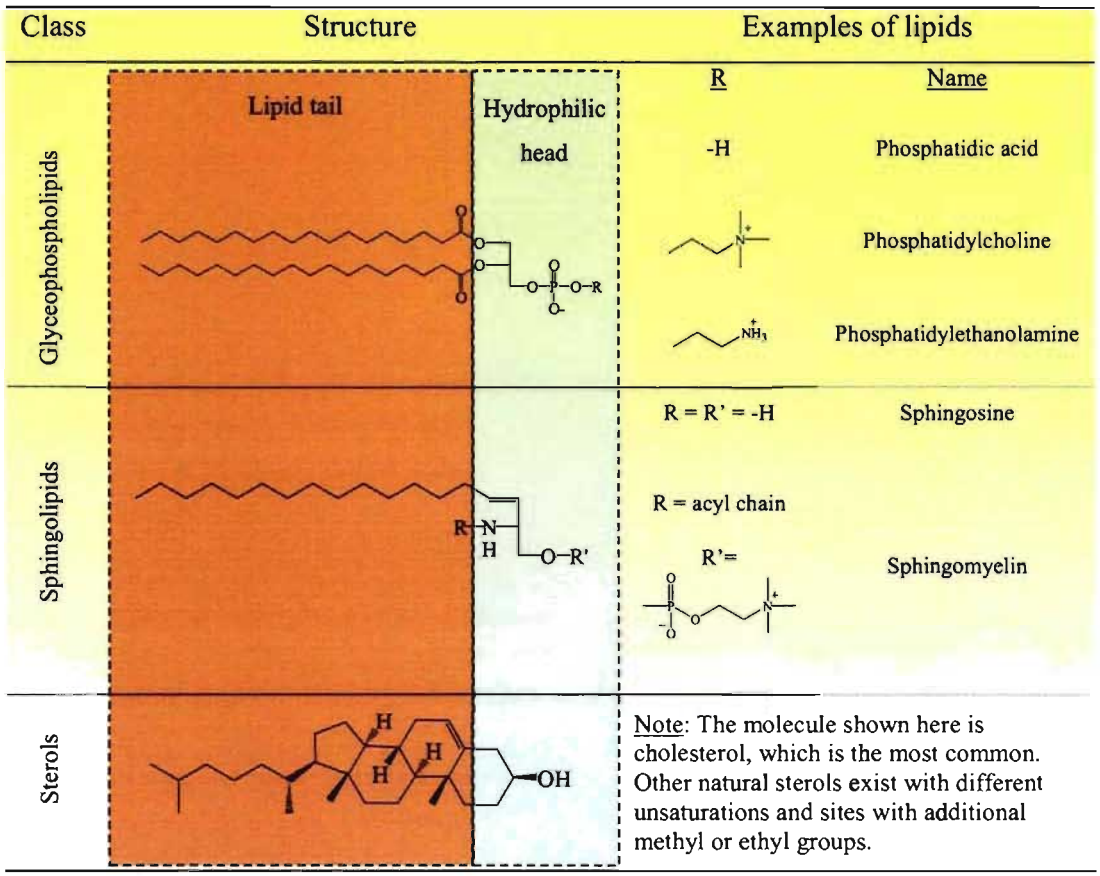
I-5 Liposomes

Liposomes are closed micro- or nanoparticulate vesicles of one or more lamellae that are formed by the self-assembling of phospholipids [Lasic and Templeton, 1996]. As the latter are typically found in the biological membranes of all living organisms, their biocompatibility makes liposomes good CDC candidates. Although liposomes are not composed of synthetic polymers, polymer chemistry has played a role in the design of liposomes for drug delivery. The following sections will begin with a synopsis of the structures and dynamics involved in liposome formation, followed by a brief review of the different types of liposomal vectors.

I-5.1 Liposome composition

One of the principal benefits of liposomes is that they can be prepared using natural amphiphiles. There are three major classes of natural lipids, as described in Table I-1. The first class consists of phospholipids, specifically glycerophospholipids, which are formed of a glycerol molecule bonded to a phosphate group to form the polar head group, and one or two fatty acids *via* ester linkages. The fatty acids generally vary in chain length (12, 14, 16 and 18 carbons) and unsaturation (1, 2 or 3). The second class are the sphingolipids, since they are derivatives of the base structure, sphingosine. Sphingomyelin, which is often used for liposome preparation, may also be categorized as a phospholipid because it, too, possesses a phosphate group. The third type of naturally occurring lipids employed for liposomes is sterols, of which cholesterol (Chol) is the most commonly used. All three classes of lipids have in common a lipidic domain and a hydrophilic head. These two regions account for the attraction of the hydrophobic tails and electrostatic repulsion of the hydrophobic heads. The sum of the attractive and repulsive forces results in the self-assembly of the amphiphiles in aqueous media.

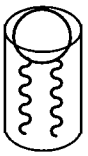
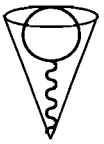
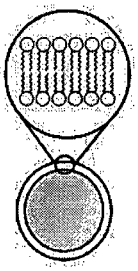
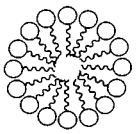
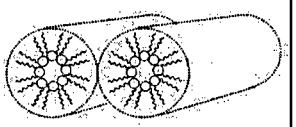
Table I-1: Classification of natural lipids used in liposome preparation



The molecular geometry of these lipids dictates their arrangement in aqueous media (Table I-2). Phospholipids that possess a cylindrical shape associate into lipid bilayers. This arrangement is the most thermodynamically stable conformation since it allows minimal contact of water with the lipophilic chains. Conversely, lipids that possess a single alkyl chain per molecule are said to have an inverted conical shape and preferentially associate to form micelles. Finally, phospholipids with smaller head groups and unsaturated fatty acid chains possess a conical shape and self-associate into a hexagonal phase, which can be illustrated as reverse micelle rods. The physical properties of liposomal membranes are typically controlled by lipid composition and can be influenced by the environmental conditions (temperature, pH,

etc.). Based on the desired membrane properties, synthetic lipids can also be designed to complement or replace naturally existing lipids.

Table I-2: Phospholipid geometry and aggregation morphology. Adapted with permission from [Dowhan and Bogdanov, 2002].

Molecular shape	 Cylinder	 Inverted Cone	 Cone
Phase	Bilayer 	Micellar 	Hexagonal (H_{II}) 

While liposomes are sometimes classified according to the method of preparation [Simard *et al.*, 2007], it may be more intuitive to compare them by size and lamellarity [Sharma and Sharma, 1997; Simard *et al.*, 2007]. When dried lipids are rehydrated, they form a heterogeneous dispersion of multilamellar vesicles (MLV), ranging in size from 0.1 to 10 μm [Ulrich, 2002]. These vesicles generally contain multiple lipid bilayers of concentric spheres [Deamer and Uster, 1983] and can also be called giant oligomeric vesicles (GOV) [Simard *et al.*, 2007]. Large unilamellar vesicles (LUV) are between 100 and 400 nm in diameter and small unilamellar vesicles (SUV) between 40 and 100 nm [Simard *et al.*, 2007]. Sometimes, the term medium sized unilamellar vesicles (MUV) is used for liposomes with a size distribution overlapping LUV and SUV [Simard *et al.*, 2007].

I-5.2 Liposome preparation

Several methods exist to prepare liposomes, depending on the desired size and lamellarity. The first preparation of MLV was described by Bangham *et al.* (1965). The lipids were solubilized in an organic solvent, like chloroform, which was removed by evaporation to obtain a dry lipid film. Then, slow hydration under moderate agitation lead to MLV formation. The size of MLV can be tuned by vigorous vortexing, brief sonication or extrusion [Szoka and Papahadjopoulos, 1981].

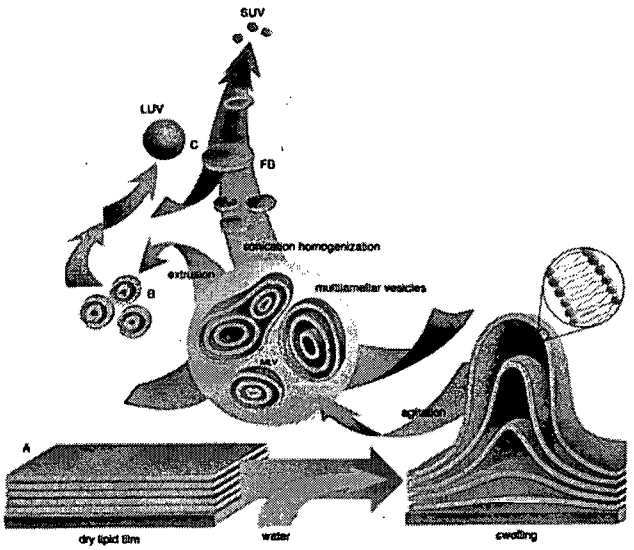


Figure I-3: Schematic representation for the preparation of MLV, LUV and SUV. Reproduced with permission from [Lasic, 1997].

LUV can be generated by several methods. First, extrusion of MLV with multiple passages through polycarbonate filters results in LUV with narrow size distribution [Deamer and Uster, 1983]. The second method is reverse-phase evaporation [Szoka and Papahadjopoulos, 1981], which consists of preparation of inversed micelles by first forming water-in-oil emulsions with lipids as emulsifiers and then sonication to homogenize the droplets. Subsequent evaporation of the organic phase under reduced pressure allows the lipids to coalesce to form LUV [Deamer and Uster, 1983]. A last commonly used method is the detergent removal

method [Deamer and Uster, 1983]. Lipids and detergent are co-suspended in a concentrated aqueous phase. Dilution, often by gel filtration, removes the detergent and allows vesicle formation.

Concerning SUV, there are primarily two methods used for their preparation: sonication and solvent-injection. The former consists of sonicating MLV suspensions either using a probe or bath sonicator. Though the probe can provide more power and produces SUV in a few minutes, it might degrade the lipids and contaminate the vesicles with metal impurities [Szoka and Papahadjopoulos, 1981]. Conversely, bath sonication allows for better control of the temperature and the lipid suspension may be manipulated in an inert atmosphere [Szoka and Papahadjopoulos, 1981]. The solvent-injection method implies that phospholipids, dissolved in a small volume of organic solvent like ethanol, are injected through a narrow syringe into a large aqueous medium where they self-assemble [Szoka and Papahadjopoulos, 1981]. The solvent is then subsequently removed by filtration. A similar method replaces ethanol with diethyl ether. The aqueous medium is heated so that, as the organic solution is injected, the ether is removed by evaporation and the SUV are formed.

In this work, we used the extrusion method to produce LUV smaller than 200 nm in size. Amphiphilic polymers were included in the lipid composition in order to incorporate them into the bilayer.

I-5.3 Liposomes for drug delivery

The application of liposomes in drug delivery first arises from the possibility of encapsulating hydrophilic molecules in the aqueous inner compartment of the vesicles. It has also been established that hydrophobic drugs and amphiphilic molecules can be trapped into the lipid bilayer [Sharma and Sharma, 1997]. Incorporating amphiphilic polymers into the membrane has been particularly useful for the modification of liposome surface properties. Pharmaceutical liposomes can thus be classified on the basis of their composition, which places them in at least one of the following categories (Figure I-4): conventional liposomes, cationic liposomes,

targeted liposomes, long-circulating (stealth) liposomes [Storm and Crommelin, 1998]. Each of these categories has its particularly intended applications [Storm and Crommelin, 1998], yet recent developments are seeking to combine these technologies.

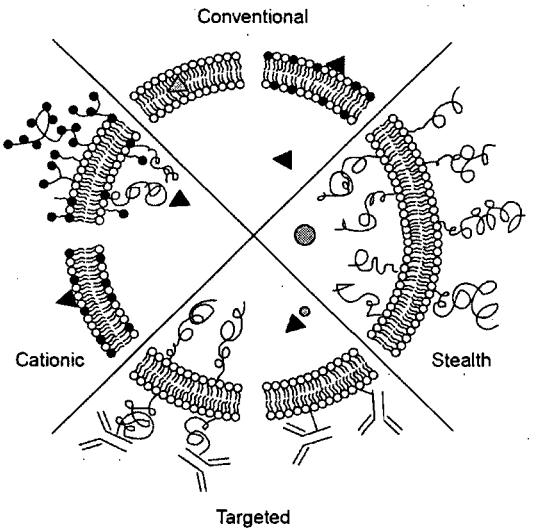


Figure I-4: Schematic representation of the four major categories of liposomes Reproduced with permission from [Storm and Crommelin, 1998].

Conventional liposomes are by far the simplest type, constituted mainly of bilayer-forming phospholipids. These are the basis for the development of all other types of liposomes. Their application in drug delivery has been limited by uptake by the MPS [Storm and Crommelin, 1998], yet the rate of elimination is dependent on the lipids selected for the formulation [Gabizon and D, 1988]. On the other hand, cationic liposomes, or liposomes formed with positively charged lipids, are particularly efficient for the complexation and formulation of genetic material [Lasic and Templeton, 1996; Huang, 2008]. As for all the CDC seen so far, targeted liposomes can be formed by fixing recognition ligands to the phospholipid surface. For instance, antibodies can be grafted to the surface of the liposome, forming *immunoliposomes* [Torchilin, 2006; Khaw *et al.*, 2007]. To overcome premature elimination, long-circulating liposomes can be generated by the addition of PEG-

conjugated lipids to the liposome composition [Ulrich, 2002]. Several long-circulating liposome formulations are on the market or in clinical studies for the passive targeting of cancers [Sharma and Sharma, 1997].

The inherent contradiction with long-circulating liposomes for drug delivery is that the contents are required to be released for therapeutic activity. Stimuli-sensitive liposomes have thus gained noteworthiness for the triggered discharge of encapsulated agents [Kono *et al.*, 1994; Kono *et al.*, 1999; Drummond *et al.*, 2000; Simoes *et al.*, 2004; Ishida *et al.*, 2006; Karanth and Murthy, 2007; Huang, 2008]. pH-sensitive liposomes can use natural physiological changes in pH to release the contents. The first pH-sensitive vesicles were generated using positively charged phospholipids with an inversed conical shape, dioleoyl phosphatidylethanolamine (DOPE), that were stabilized by mildly acidic amphiphiles to form bilayers [Connor *et al.*, 1984]. When the medium was acidified, the amphiphiles were protonated, destabilizing the hydrophobic interactions within the bilayer. The liposomes thus became fusogenic and the encapsulated material was released [Ellens *et al.*, 1984, 1985]. Other pH-responsive liposomes have since been developed, employing various mechanisms, such as fusogenic peptides [Subbarao *et al.*, 1987], pH-sensitive polymers [Yong-Hee *et al.*, 1994] and acid-labile bonds [Guo and Szoka, 2001; Boomer *et al.*, 2003]. These are discussed in more depth in the fourth chapter of this thesis.

More recently, advanced liposomal CDC have been developed, combining the different types of liposomes. For instance, it was possible to formulate vesicles with both pH-sensitive polymers and PEG-lipids in order to produce pH-responsive, long-circulating liposomes [Roux *et al.*, 2004]. Others prepared vesicles by stabilizing DOPE with lipids conjugated to PEG using acid labile bonds [Hong *et al.*, 2002]. Cleavage of PEG induced liposomal fusion and leakage of the contents. Long-circulating immunoliposomes have also been developed by decorating the surface of the vesicles with both PEG and monoclonal antibody conjugates [Allen *et al.*, 1995; Maruyama *et al.*, 1997]. Currently, liposomal delivery systems are being developed bearing all three features: long-circulation, target-mediated and stimuli-induced

release [Mastrobattista *et al.*, 1999]. In addition to this, numerous polymers are being investigated for either their capability to shield liposomes or their response to stimuli. Indeed, we propose poly(phosphazenes) for the pH-induced drug release from liposomes. In the following chapter, we review the history and principles of phosphazene chemistry, as well as the various applications of poly(phosphazenes), including drug delivery.

CHAPTER II: Poly(phosphazenes) - Polyvalent polymers

II-1 Introduction to poly(phosphazenes)

The term phosphazene refers to molecules possessing a phosphorus and nitrogen atom linked by a double bond [Allcock, 2002]. Therefore, poly(phosphazenes) (PPZ) are linear or cyclic chains of alternating phosphorus and nitrogen atoms, as depicted in Figure II-1.

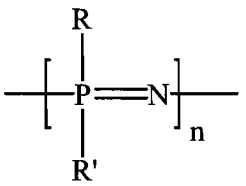


Figure II-1: The general structure of PPZ

In fact, the backbone of PPZ resembles a conjugated system since there is an observed contraction of the length of the σ-bonds in comparison to normal P-N bonds [Allcock, 1972]. Note that, in a phosphazene repeat unit, two other functional groups are linked to the phosphorus atom. The nature of these pendant groups alters the physicochemical properties of the polymers. Hence, phosphazene chemistry has been especially dedicated to the synthesis and modification of PPZ. Numerous side groups, organic, inorganic and organometallic, have been used to generate PPZ with a wide range of properties [Mark *et al.*, 1992]. The present chapter will give a brief overview of the history of phosphazene chemistry and the versatility of PPZ through the different synthetic approaches and some of their applications.

II-2 Poly(phosphazene) synthesis

The history of phosphazene chemistry dates back to the 19th century, before Lewis proposed his theory on bonding [Lewis, 1916], and well before the term polymer was universally accepted [Staudinger, 1920]. In 1850, Laurent proposed that the white precipitate formed by heating phosphorus pentachloride (PCl_5) with ammonia (NH_3) should simply be Cl_2PN [Laurent, 1850]. Over ten years later, Gladstone confirmed that the product more likely possessed the empirical formula $P_3N_3Cl_6$ [Gladstone and Holmes, 1864], or three times the formula proposed by Laurent. Near the end of the century, Stokes obtained some groundbreaking results, isolating and characterizing a series of four *phosphonitrilic chlorides*, $[Cl_2PN]_{3\text{-}7}$, also multiples of Laurent's formula [Stokes, 1897]. Stokes suggested that these were cyclic polymers, and the trimer was the primary solid product [Stokes, 1895]. The structure of the trimer, hexachlorocyclotriphosphazene (HCTP, Figure II-2), was properly defined several decades later [Jaeger and Beintema, 1932].

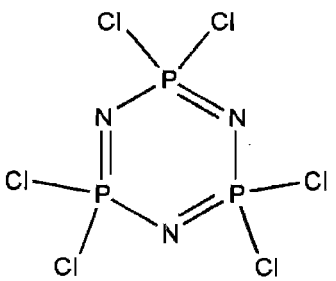


Figure II-2: Hexachlorocyclotriphosphazene

In addition to this, Stokes noticed that, upon further heating, these cyclic molecules were transformed into a highly elastic *inorganic rubber* that was insoluble in neutral solvents, yet swelled in benzene [Stokes, 1897]. This substance had a complex structure and high molecular weight but he failed to analyze it precisely as it broke down to a mixture of smaller molecules. It was later determined that the *inorganic rubber* swelled in benzene because it was highly cross-linked [Meyer *et al.*, 1936]. In 1962, Shaw *et al.* rightly proposed to replace the term *phosphonitrilic*

chloride for *phosphazene* because of the double bond between phosphorus and nitrogen [Shaw *et al.*, 1962]. In the mid-1960's, Allcock *et al.* finally fine-tuned Stokes' polymerization procedure to obtain high molecular single chains of polydichlorophosphazene (PDCP), where the side groups are chlorides. Today, PDCP is a precursor to most functional PPZ by the substitution of the chlorine side-groups. In the following section, we shall first discuss the conclusions of Allcock's initial work which has led to the vastly employed thermal ring-opening polymerization. We will then examine the evolution of the alternative condensation polymerization procedures as well as the post-polymerization modifications that impart stability and functionality to these polymers.

II-2.1 Thermal ring-opening polymerization

The major problem with Stokes's polymer was its hydrolytic instability, due to the reactivity of the phosphorous-chlorine bonds. Stabilization of the polymer required the synthesis of a linear PDCP that would be soluble in a suitable solvent for side-group substitution. Allcock *et al.* (1964) first noted that, unlike condensation reactions, there was no leaving group in this polymerization as the empirical formula essentially remained the same. Conductivity data obtained during polymerization suggested that heating induced dissociation of a chloride ion, which permitted nucleophilic attack by the nitrogen from a neighboring HCTP [Allcock and Best, 1964]. Hence, the mechanism for the ring-opening polymerization was elucidated, as depicted in Figure II-3. Later, careful control of the reaction time, temperature and the purity of HCTP yielded linear PDCP that was soluble in several aprotic solvents (*e.g.* THF) and could subsequently be substituted with various nucleophiles [Allcock and Kugel, 1965, 1966; Allcock *et al.*, 1966]. These reactions will be further discussed with other post-polymerization modifications.

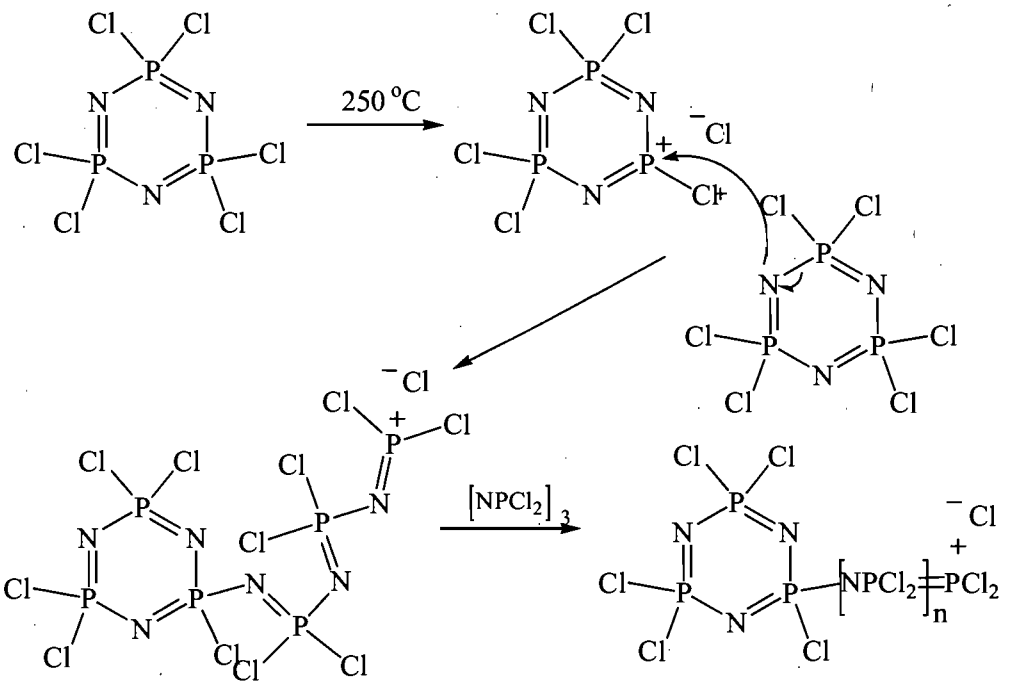


Figure II-3: Mechanism of thermal ring-opening polymerization of HCTP.

Thermal ring-opening polymerization is usually performed in the bulk state in closed tubes. Trace impurities such as water [Allcock *et al.*, 1975] and Lewis acids [Hergenrother *et al.*, 1986; Sohn *et al.*, 1995] can catalyze the reactions. Cyclotriphosphazenes bearing good leaving groups, such as other halogens, can be polymerized in the same way. However, the temperature of the reaction should be adjusted accordingly. For example, hexafluorocyclotriphosphazene polymerizes at 350 °C instead of 250 °C because of the strength of the phosphorus-fluorine bond [Seel and Langer, 1956].

The thermal ring-opening polymerization of HCTP possesses several advantages. First, polymers of very high molecular weight, over 100,000 g mol^{-1}, can be generated by this method. Regularly alternating repeat units can also be formed by partially substituting some of the chlorines on HCTP prior to polymerization [Allcock *et al.*, 1978; Allcock *et al.*, 1990]. To this end, no more than 2 or 3 chlorides should be replaced to prevent over-stabilization of the trimeric unit, unless trans-annular organic or ferrocenyl species are substituted on the ring [Allcock *et al.*, 1991]. It is

believed that the strain imposed by these substituents on the cyclic monomer promotes ring-opening upon heating. The lower reactivity of substituted-cyclotriphosphazenes can generally help limit the rate of chain elongation when desired. The principal disadvantages of the ring-opening procedure are the high polydispersity of the resulting PPZ (> 2) and the difficulty to control the molecular weight.

II-2.2 Condensation polymerization

Although the ring-opening polymerization is the most popular synthesis pathway for the preparation of PDCP, condensation polymerization offers several advantages that are particularly interesting for the preparation of CDC. Some of the methods could be simply transposed in industrial settings while others give access to polymers with controlled chain length and low polydispersity. Furthermore, they may lead to PPZ with different and precise structures. The following will describe the synthesis and polymerization of three types of condensation monomers, presenting for each their synthetic advantages and disadvantages.

II-2.2.1 Condensation of phosphorus pentachloride and ammonia

The first condensation polymerization is based on the same starting blocks as for the preparation of HCTP. However, Hornbaker *et al.* (1980) developed an industrial process for the synthesis of short oligomers. Since manipulation of PCl_5 is delicate on large scales, it was prepared *in situ* prior to the addition of ammonia. Liquid PCl_3 was first fed along with gaseous chlorine (Cl_2) into a chlorobenzene filled reactor set between 100 and 140 °C, followed by addition of ammonia or ammonium chloride (Figure II-4). Controlling the feed to have a slight excess in PCl_3 yielded linear PPZ with 2 to 9 repeat units [Hornbaker and Li, 1980].

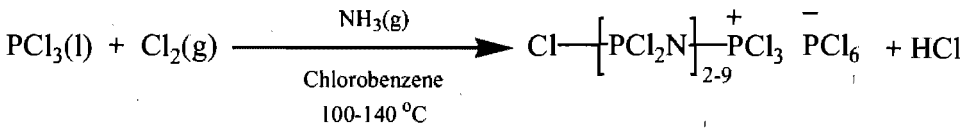


Figure II-4: Preparation of short PPZ oligomers

After oligomerization, the temperature can be increased to 140-160 °C and NH_4Cl added to the reactor to link the short chains together, forming PPZ up to 6900 g mol^{-1} [Pettigrew *et al.*, 1983]. This method offers the advantages of using affordable reactants and modest polymerization temperatures (160 *vs.* 250 °C for ring-opening). Nevertheless, this technique leads to considerable losses as cyclic side-products and short polymer chains.

II-2.2.2 Synthesis and polymerization of $Cl_3P=NP(O)Cl_2$

$Cl_3P=NP(O)Cl_2$ is a moisture sensitive, inorganic solid that could be polymerized to form PPZ with higher molecular weight than the previous method. It was most efficiently obtained by heating PCl_5 with ammonium sulfate (Figure II-5).

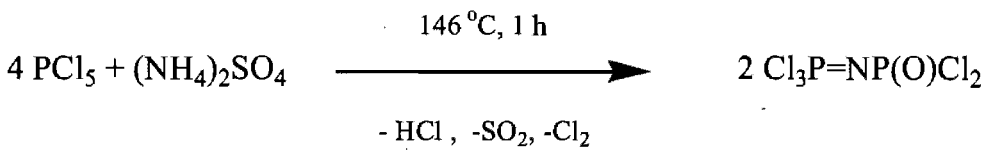


Figure II-5: Synthesis of $Cl_3P=NP(O)Cl_2$

This reaction achieved a 100%, high purity yield since all of the side products are gaseous and readily removed from the reaction medium [Emsley *et al.*, 1971]. The monomer polymerizes by heating above 240 °C and is monitored by the removal of gaseous $OPCl_3$ from the reaction medium [D'Halliun and De Jaeger, 1989]. When $Cl_3P=NP(O)Cl_2$ was heated to 245 °C, $OPCl_3$ production was no longer detected in the vessel after 7.7 h and 95% completion of the reaction [D'Halliun *et al.*, 1992]. Further heating for two additional hours at 276 °C improved the conversion to almost

98%. This approach presents the advantages of cheap starting blocks and simplicity of the reaction conditions. Conversely, the molecular weight of the PPZ cannot be controlled nor reach the chain lengths obtained by ring-opening polymerization. The polymerization of $Cl_3P{=}NP(O)Cl_2$ also displays a broader weight distribution than the next method using (trimethylsilyl)phosphoranimines.

II-2.2.3 Synthesis and polymerization of (trimethylsilyl)phosphoranimine

Most recently, PDCP has been synthesized by "living" cationic polymerization of trichloro(trimethylsilyl)phosphoranimine ($Cl_3P{=}NSiMe_3$). The application of the $Cl_3P{=}NSiMe_3$ was at first strongly disadvantaged by typically low synthesis yields, around 20% [Niecke and Bitter, 1973]. This procedure consisted of reacting PCl_5 with $LiN(SiMe_3)_2$ at 10 °C. Subsequent attempts to improve the synthesis of $Cl_3P{=}NSiMe_3$ were limited to 60% yields [Honeyman *et al.*, 1994; Allcock, Crane *et al.*, 1999]. It was later proposed to replace PCl_5 with the less reactive PCl_3 [Wang *et al.*, 2002] when it was discovered that the former can initiate polymerization [Honeyman *et al.*, 1995; Allcock *et al.*, 1996]. The optimized reaction scheme was as follows:

$$PCl_3 + LiN(Si(CH_3)_3)_2 \xrightarrow[0\,^{\circ}C]{Et_2O} Cl_2PN(Si(CH_3)_3)_2 + LiCl$$

$$Cl_2PN(Si(CH_3)_3)_2 + SO_2Cl_2 \xrightarrow[-\,SO_2]{Et_2O,\ 0\,^{\circ}C} Cl_3P{=}NSi(CH_3)_3 + ClSi(CH_3)_3$$

Figure II-6: The optimized synthesis pathway for $Cl_3P{=}NSiMe_3$.

As shown in Figure II-6, the second step consists in oxidation of the intermediate product, $Cl_2PN(SiMe_3)_2$, by sulfuryl chloride (SO_2Cl_2) to produce the phosphoranimine. More than 80% yields were consistently obtained with over 98%

purity [Wang *et al.*, 2002]. $Cl_3P{=}NSiMe_3$ is a clear, oily and reactive liquid that can polymerize or cyclize at room temperature [Allcock, Crane *et al.*, 1999] and, therefore, must be stored carefully under dry conditions at -20°C. Also, Me_3SiCl must be removed as it was found to inhibit chain propagation during polymerization.

Two groups collaborated and studied the polymerization of $Cl_3P{=}NSiMe_3$ catalyzed by several Lewis acids, including PCl_5 [Honeyman *et al.*, 1995; Allcock *et al.*, 1996]. The synthesis can be carried out in the bulk phase as well as in several solvents. Methylene chloride and hexanes allowed the synthesis of polymers with the lowest polydispersity. The initiation and chain propagation steps are shown in Figure II-7.

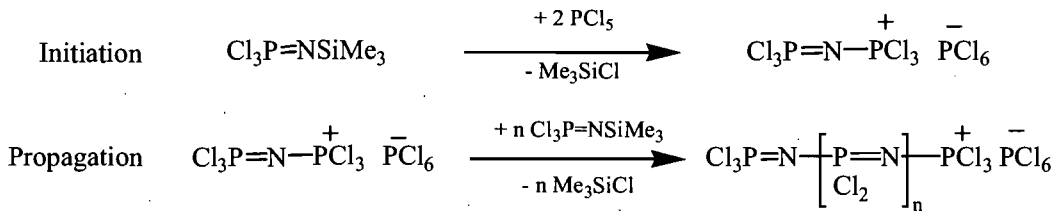


Figure II-7: PDCP synthesis by the "living" cationic polymerization of $Cl_3P{=}NSiMe_3$.

Indeed, this procedure was a breakthrough in the preparation of PDCP for several reasons. Firstly, most methods required considerable heating, whereas the cationic polymerization procedure could be carried out at room temperature, which is especially convenient for large scale synthesis. Secondly, "living" cationic polymerization allows good control of the chain length, since monomeric units could be added to the ionized termini, which remain active until the phosphorus-chloride bonds are substituted after polymerization. The desired polymer chain length could be obtained with narrow polydispersity by varying the monomer:initiator ratio. Furthermore, PCl_5 as well as "living" polymer chains can initiate the polymerization of mono and bis(organo)phosphoranimines to produce phosphazene *block* copolymers [Allcock, Nelson *et al.*, 1997; Allcock, Reeves *et al.*, 1997; Allcock *et al.*, 2000; Allcock *et al.*, 2001]. Various initiators have been designed for the synthesis of PPZ

dendrimers [Cho and Allcock, 2007], star-shaped polymers [Nelson and Allcock, 1997], telechelic [Allcock, Nelson *et al.*, 1999] and *block* copolymers [Nelson *et al.*, 1998; Prange *et al.*, 2000; Chang, Bender *et al.*, 2002; Chang, Prange *et al.*, 2002]. Polymerization of tri(organo)phosphoranimine has also been developed, but one of the substituents must be a good leaving group, such as trifluoroethanolate [Wisian-Neilson and Neilson, 1980; Neilson *et al.*, 1987; Matyjaszewski *et al.*, 1992; Matyjaszewski *et al.*, 1993]. Since the phosphorus atoms were already substituted, the resulting PPZ did not require post-polymerization modifications. However, this polymerization reaction required heating over 190 °C and produced polymer batches with broad size distributions.

II-2.3 Post-polymerization modifications

Traditional polymers are generally limited by the different types of available monomers. Furthermore, the reactivity of these monomers influences their even distribution along the backbone of random copolymers. However, macromolecular substitution of PDCP overcomes these challenges with the availability of a large variety of potential side-groups and the reactivity of the P-Cl bonds. It is thus an indispensable route to the preparation of a wide range of PPZ with customized physical and chemical properties.

The macromolecular substitution process consists of a nucleophilic attack on the labile phosphorus-halide bond to graft new side-groups along the PPZ backbone. A multitude of nucleophiles can be generated from alcohols [Allcock *et al.*, 1986], primary or secondary amines [Allcock *et al.*, 1972; Allcock *et al.*, 1977] and organometallic compounds [Diaz and Valenzuela, 2006]. Hence, researchers have access to an innumerable possibility of polymers with tailored properties and functionality. The cyclic monomer, HCTP, can be used as model for PDCP substitution, especially since it is commercially available in a pure form.

Secondary reactions can also be carried out on the organic side-groups. For instance, there are many ways to cross-link PPZ depending on the nature of the side-groups. PPZ with alkoxy substituents can be covalently cross-linked by irradiating

with gamma [Allcock *et al.*, 1988] or UV rays [Nelson *et al.*, 1991]. Similarly, hydrolysis of side-groups bearing ethyl-ester groups have lead to PPZ that form networks through ionic intermolecular bonding [Allcock and Kwon, 1989; Cohen *et al.*, 1990]. In addition to cross-linking, other secondary reactions include numerous protection, deprotection and functionalization chemistry [Allcock, 2006]. These primary and secondary transformations have helped design PPZ for various applications. Some examples are described in the following section.

II-3 Applications of poly(phosphazenes)

II-3.1 Industrial applications of poly(phosphazene) materials

Synthetic polymers have become more prevalent in everyday settings. PPZ are finding their niche in a wide range of applications as a result of developments in material science. In this field of research, PPZ are characterized by their hydrophobic/hydrophilic properties, which dictate the behaviour of the polymer in different solvents. They are also described by their physical properties in the solid state. Since solid PPZ are generally amorphous, the parameter most often evaluated is the glass transition temperature (T_g). High T_g values indicate brittle solids, while polymeric materials with low T_g are more malleable.

Since Stokes first described the polymer as an *inorganic rubber,* PPZ have logically been used as elastomeric materials. The elastic properties of PPZ are due to the flexibility of the phosphazene backbone [Allcock, 2002]. In the case of PDCP, the small size of the chlorine atoms also allow for increased mobility of the chains as expressed by sub-zero T_g values. Substituting the P-Cl bonds with small, flexible organic side groups, such as alkyloxy [Reynard and Rose, 1974], aryloxy [Futamura *et al.*, 1980] and organosilicons side groups [Allcock and Brennan, 1988], can produce stable elastomeric PPZ. By using combinations of different, randomly substituted side-groups, it is possible to avoid the formation of crystalline domains and to lower the T_g [Reynard and Rose, 1974]. In contrast, cross-linking and the

addition of fillers, such as iron oxide, help in reducing the elasticity of the material [Mitchell and Obester, 1980; Mueller and Landry, 1989]. Particular characteristics of PPZ elastomers include improved flexibility at low temperatures, resistance to hydrocarbons, flame retardation, and insulation to heat, sound and electrical currents. They can therefore be used in fuel lines, seals and junctions for aeronautic and automotive applications, or blended with other polymeric fibers, such as polypropylene [Zhang and Horrocks, 2003] to form flame-retardant textiles.

PPZ elastomers are also useful as *solid solvents* for metal cations to form polymer electrolytes. Normally, polyethylene oxides (PEO), which are semi-crystalline in the solid state can coordinate lithium salts in their amorphous regions, and so are used as solid conducting matrices for rechargeable batteries [Armand and Duclot, 1981; Armand, 1986]. In contrast, poly(bis-methoxyethoxyethoxy-phosphazene) (MEEP) can form a completely amorphous solid that exhibits a conductivity 2.5 orders of magnitude greater than PEO [Blonsky *et al.*, 1986]. The flexibility of MEEP's side-groups helps coordination of the lithium ions while improving the migration of the salts through the polymer matrix [Blonsky *et al.*, 1984; Allcock *et al.*, 1986].

PPZ have also been proposed for the design of optical devices. Phenyl-, biphenyl- or naphthyl-substituted PPZ are able to form thin films with refractive indices on average 0.1 higher than liquid phenol, biphenol and naphthalene by increasing π-electron density [Olshavsky and Allcock, 1995]. These films were essentially transparent in the entire visible spectrum, except for naphthyl-substituted PPZ, which cut-off near UV light. Such PPZ usually form crystalline or liquid crystalline structures when decorated with a single substituent, while PPZ with different side-groups co-substituted are generally amorphous. PPZ with chiral biphenyl groups formed liquid crystals with high T_g values [Allcock and Klingenberg, 1995] whereas PPZ with combinations of these side-groups formed refractive films that were both transparent and amorphous at room temperature [Allcock *et al.*, 1998].

II-3.2 Biomedical applications of poly(phosphazenes)

Significant research advances have made PPZ promising candidates for numerous biomedical applications. Careful selection of side-groups for macromolecular substitution has lead to the synthesis of a variety safe, non-toxic PPZ with biological functionality. The choice is first evaluated by the desired physical properties, namely: elastomers to glasses, hydrophilic to hydrophobic, bioinert to bioactive materials, and electrical conductors to insulators [Honarkar and Rahimi, 2007]. However, for biological considerations, the polymers are required to induce minimal immune response and resist fungal and bacterial colonization, which can also be regulated by careful selection of the side-groups [Laurencin *et al.*, 2003]. It is impossible to give an account of all the potential biomedical applications of PPZ in very few pages. Therefore, the final part of this chapter is dedicated to highlighting the biologically relevant properties that can be imparted to the phosphazene backbone through examples of PPZ as solid biomaterials, and hydrophilic pharmaceutical systems, including CDC.

II-3.2.1 Biomedical poly(phosphazene) materials

As previously mentioned, PPZ have been extensively used for their elastomeric properties. Poly(fluoroalkoxyphosphazenes) (PFAP) have been particularly promising for the coating of implants and prosthetics. For example, denture liners have been developed with PFAP to improve durability, comfort and stress relief by strategically controlling the softness of the elastomer throughout the lining [Gettleman and Gebert, 1987]. In another application, they are proposed to coat surgical implants [Grunze and Gries, 2007]. For instance, metallic coronary stents have been coated to aid arterial healing [Verweire *et al.*, 2000].

PPZ have also been widely investigated as scaffolds for tissue engineering. The concept is based on implanting three-dimensional polymeric networks where cells can adhere and proliferate to restore, regenerate or improve tissue functions [Langer and Vacanti, 1993]. Different PPZ scaffolds have been designed to guide

nerve regeneration [Aldini *et al.*, 1997], hepatocyte adherence and proliferation [Heyde *et al.*, 2007], as well as bone repair [Nukavarapu *et al.*, 2008]. The PPZ in question are generally of variable porosity and possess T_g values above physiological temperatures.

II-3.2.2 Pharmaceutical applications of poly(phosphazenes)

Polymers with various biologically relevant properties have been developed for *in vivo* applications, particularly where water-solubility is required. For this reason, the design of PPZ with hydrophilic character represents a rapidly expanding subfield of research. A vast array of water-soluble side-groups is available for the synthesis of PPZ that can be applied in pharmaceutical settings. For instance, it was noticed that alkyl ether-substituted PPZ in aqueous solution possess a lower critical solution temperature (LCST). This phenomenon consists of a coil-to-globule phase transition that occurs upon heating. By varying the nature of the substituents, the LCST can be adjusted to physiological temperatures [Allcock *et al.*, 1992; Allcock and Dudley, 1996]. Cross-linked, temperature-sensitive PPZ can form hydrogels that expand and swell upon cooling below the LCST and collapse upon heating. Biodegradable PPZ can also be synthesized by grafting amino acid esters [Crommen *et al.*, 1992a, b] or imidazole [Allcock *et al.*, 1982; Andrianov *et al.*, 2005] side-groups. These substituents can be cleaved from the polymer by intramolecular or intermolecular catalysis [Allcock *et al.*, 1994]. The phosphazene backbone might then be exposed to nucleophilic attack by water and the subsequent rupturing of phosphorus-nitrogen bonds [Schacht *et al.*, 1996; Andrianov, 2006]. The final product of complete degradation is a mixture of phosphate, ammonia and the free side-groups. Furthermore, it is possible to combine thermo-sensitivity and biodegradability through co-substitution of PDCP with appropriate side-groups [Lee *et al.*, 1999]. Recently, biodegradable hydrogel implants have been developed with PPZ for tissue engineering [Sethuraman *et al.*, 2006] and drug delivery [Kang *et al.*, 2006] applications.

One of the major application of PPZ consists of an adjuvant for vaccines [Andrianov, 2006]. It is understood that when antigens are physically or chemically bound to polymers, the immune response is improved over the free antigen as a result of aggregation of the antigens [Cairo *et al.*, 2002; Kabanov, 2004]. The first and most studied PPZ immunoadjuvant is poly[di(carboxylatophenoxy)phosphazene] (PCPP), which has been shown to considerably increase immune response for numerous infections, such as herpes and hepatitis B, by physically complexing antigens [Andrianov, 2006]. Many derivatives of PCPP have been investigated as improved immunoadjuvants, including poly[di(carboxylatoethylphenoxy)phosphazene] [Mutwiri *et al.*, 2007]. The latter has been shown to increase immune response up to 10 times more than PCPP [Andrianov *et al.*, 2006].

PPZ have also become significant candidates for the development of CDC. Spacer molecules can be bound to the backbone through macromolecular substitution and used to conjugate anti-cancer drugs, such as Dox [Song *et al.*, 1999] and platinum(II) derivatives [Sohn *et al.*, 1997; Song *et al.*, 2005]. Co-substitution with short PEG molecules enhances tumor accumulation by the EPR effect [Jun *et al.*, 2005]. Colloidal micro and nanoparticles can be formed from dispersed PPZ hydrogels. As they consist of polymeric matrices entrapping water, they are suitable vectors for water-soluble therapeutics, such as peptides [Veronese *et al.*, 1998; Caliceti *et al.*, 2000] and DNA [Yang *et al.*, 2008]. Furthermore, the rate of release of encapsulated agents can be regulated if the matrix is biodegradable [Laurencin *et al.*, 1987]. PPZ-based nanoparticles can also be surface modified to increase *in vivo* circulation times [Vandorpe *et al.*, 1996; Vandorpe *et al.*, 1997].

Zhang *et al.* published a series of articles that focused on synthesizing thermosensitive amphiphiles that can be used to generate micelles by randomly co-grafting oligomeric poly(*N*-isopropylacrylamide) (PNIPAM) and different amino acid esters [Zhang *et al.*, 2004; Zhang, Li *et al.*, 2006; Zhang, Qiu, Jin *et al.*, 2006b, a; Zhang, Qiu, Wu *et al.*, 2006]. PNIPAM, which possesses a LCST below 37 °C, forms the corona of the micellar structures with relatively broad size distributions. When solution temperature exceeded the LCST, PPZ co-substituted with PNIPAM and ethyl

glycinate aggregated with a narrow unimodal size distribution [Zhang, Qiu, Wu *et al.*, 2006]. It was then found that the actual morphology of PPZ aggregation depended on the proportion of the substituents on the backbone and the organic solvent used in preparation [Zhang, Qiu, Jin *et al.*, 2006a]. When ethyl tryptophan was co-substituted with PNIPAM, PPZ micelles were able to encapsulate several structurally different hydrophobic LMWD, such as indomethacin [Zhang, Li *et al.*, 2006]. Indomethacin-loaded PPZ micelles, tested *in vivo* in a rat model, showed increased plasma retention over the free drug and seemed to be best suited for local sustained release for treatment of arthritis [Zhang, Li *et al.*, 2006].

PPZ are proven to be a promising family of polymers. We have reviewed the evolution, improvements and synthetic versatility that have made PPZ candidates for numerous biomedical applications, including for the development of CDC. The focus of the following chapters is the application of tri-substituted PPZ for the development of pH-responsive liposomes.

II-4 Research hypothesis and objectives

"Intelligent" polymers that respond to environmental stimuli have been used to trigger the release of liposome-encapsulated drugs. Polyanions have received particular attention for their property of conferring pH-sensitivity to liposomes. As first reported by Couffin-Hoarau and Leroux (2004), pH-responsive vesicles can be generated using customized amphiphilic and polyanionic PPZ. These PPZ were synthesized with three pendant groups, in particular an amino acid substituent, which was intended to provide both pH-sensitivity and potential biodegradability. However, PPZ-liposomes that were first reported were not stable at pH 7.4 and 37 °C, normal physiological conditions in the blood, and leakage of encapsulated marker was observed. Based on these results, it was necessary to determine the requirements for an optimized PPZ-liposome formulation, especially in the composition of the PPZ.

In order to do so, we established the following objectives:

1. Synthesize a set of pH-sensitive PPZ, varying the ratios of the three substituents.

2. Characterize the PPZ for composition, as well as pH- and temperature-dependent phase transitions.
3. Evaluate the biodegradability of the PPZ. This feature was not considered in the previous report and it appeared important to determine whether this aspect was truly advantageous.
4. Determine the efficiency of PPZ fixation to the surface of liposomes.
5. Evaluate the release kinetics of PPZ-liposomes at different pH, before and after serum exposure.

These objectives were completed through the series of experiments described in the following chapter, which is a copy of the manuscript submitted for future publication in the book *Biomedical Applications of Polyphosphazenes*, edited by Alexander K. Andrianov (Publisher, John-Whiley and Sons).

CHAPTER III: Amphiphilic ionizable poly(phosphazenes) for the preparation of pH-responsive liposomes

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III-1 Abstract

Amphiphilic ionizable poly(organophosphazenes) (PPZ) were investigated for the preparation of pH-sensitive liposomes, which are designed to deliver drugs from the intracellular acidic organelles to the cytoplasm. Randomly grafted PPZ were synthesized with different ratios of diethylene glycol ethyl ether (EEE), polyethylene glycol octadecyl ether ($C_{18}(EO)_{10}$) and amino butyric acid (ABA) by sequential macromolecular substitution of poly(dichlorophosphazene). Differential scanning calorimetry and turbidimetry analysis of aqueous solutions of PPZ (M_w = 15,100 - 19,600, PDI $\leq$ 1.06) revealed that the polymers displayed both temperature- and pH-sensitivity. Stable pH-sensitive vesicles (120 - 180 nm) were prepared at pH 7.4 by the fixation of PPZ during vesicle formation or by incubation of the polymers with preformed liposomes. The latter method was preferred for PPZ containing the highest proportions of the anchoring moiety ($C_{18}(EO)_{10}$) as liposomes otherwise aggregated. *In vitro* release kinetic assays performed at physiological temperature (37°C) showed that the PPZ-liposome systems released 33-82% of their content within ~30 min upon lowering the external pH to 5. The extent of pH-triggered release was dependent on PPZ nature and composition of the phospholipid bilayer. Upon incubation with human serum, a substantial loss of pH-sensitivity was observed, suggesting a possible extraction of PPZ by the serum components. While these data clearly show that PPZ can impart pH-responsive properties to liposomes, they indicate that the polymer composition should be fine-tuned to resist vesicle inactivation in the blood.

III-2 Introduction

A key challenge in the field of drug delivery has been improving targeting of the active agent in order to maximize efficacy and reduce toxicity. A promising approach is to provoke site-specific drug release from a vector in response to stimuli that can be either applied externally or be physiologically produced. Several means can thus be exploited for this purpose, such as ultrasound, enzymatic cleavage, temperature and pH. The latter has peaked interest of researchers as variations in acidity are observed in certain pathologies as well as in normal intracellular activity.

Differences in pH that exist between normal vasculature (pH 7.4) and the tissue interstices of tumors, infections and inflammations (~pH 6.5) pushed for the design of a delivery system targeting such extracellular compartments [Schmaljohann, 2006]. Yet it has been technically challenging to construct a vector that could respond to such a narrow variation. In contrast, pH-responsive formulations have shown to improve the cytoplasmic delivery of therapeutic agents rather than simply in the vicinity of the target cells [Drummond *et al.*, 2000; Simoes *et al.*, 2004; Yessine and Leroux, 2004]. Upon receptor-mediated internalization, the pH-gradient established between the endosomal/lysosomal compartments and the cytoplasm is used to induce discharge of the encapsulated material.

Of the vectors explored, pH-sensitive liposomes have received distinctive attention as controlled release can be easily prompted by destabilization of phospholipid bilayers. There are three mechanisms proposed for the delivery of a liposome-encapsulated agent from the endosomal compartment to the cytoplasm [Karanth and Murthy, 2007] (Figure III-1). The first mechanism presumes that pH-sensitive liposomes can induce pore formation in liposomal and eventually endosomal membranes. The second involves passive diffusion of the drug through the endosomal membrane once liberated from the destabilized vector. This process is limited by the nature of the therapeutic in question. The final pathway suggests fusion between the liposome and endosome for direct release into the cytoplasm. The delivery of the drug

is ultimately dependent on the composition of the liposome, the destabilization mechanism and the interaction of the formulation with the endosomal membrane.

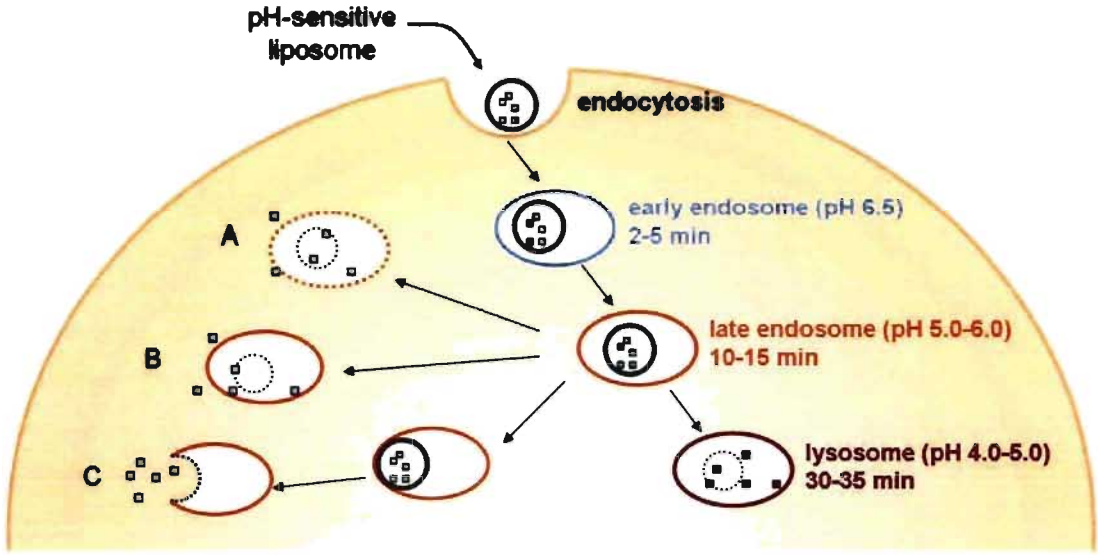

Figure III-1: Mechanisms of intracellular targeting. Upon endocytosis, the acidification of the endosomal lumen induces one of three possible release mechanisms: destabilization and pore formation of both liposome and endosome (A), destabilization of the liposome and passive diffusion of the active agent (B) or fusion between liposomal and endosomal lamella (C). Adapted with permission from [Simoes *et al.*, 2004].

The first generation of pH-sensitive liposomes were prepared by the combination of unsaturated phosphatidylethanolamine (PE) and mildly acidic lipids [Connor *et al.*, 1984], such as oleic acid and cholesterylhemisuccinate. PE alone cannot form liposomes due to its molecular geometry and requires the presence of the charged amphiphiles to construct bilayers at neutral pHs. Following endocytosis and acidification of the endosomal lumen, the charged lipids are neutralized by protonation resulting in transition from lamellar to hexagonal (H_{II}) phase, which leads to liposome destabilization and, eventually, fusion with the endosome membrane. Such liposomes have been found to efficiently deliver encapsulated agents to the cytosol when tested *in vitro* [Drummond *et al.*, 2000]. However, moderate stability in

the blood and rapid elimination have hampered their efficiency when administered systemically. These problems can be resolved in part by using lipid-conjugated hydrophilic polymers inserted within the bilayer to form a steric barrier, stabilizing the liposomes [Hong *et al.*, 2002; Ishida *et al.*, 2006]. In similar fashion, such polymers have been linked to hydrophobic anchors *via* acid-labile bonds which can be cleaved in the endosome from the surface of the vesicle, in order to allow fusion after endocytosis [Guo and Szoka, 2001; Boomer *et al.*, 2003].

Peptides and proteins inspired from nature have also been used to improve cytoplasmic delivery of liposomal content. For instance, the pore forming protein, listeriolysin O (LLO), was co-encapsulated with an active agent into PE-based pH-sensitive liposomes [Provoda *et al.*, 2003]. Upon release of the liposomal contents, LLO created pores in the endosome membrane releasing the therapeutic into the cytosol. Similarly, association of derivatives of the influenza virus fusion protein, hemagglutinin, to cationic liposomes has been proven to increase transfection efficiency several fold [Kamata *et al.*, 1994; Kichler *et al.*, 1997]. Many other pH-sensitive fusion peptides have been studied for the destabilization of liposomal and endosomal membranes [Drummond *et al.*, 2000; Li *et al.*, 2004], yet their use poses some challenges. Employing proteins in a drug delivery system incurs the possibility of immunogenicity. Moreover, co-encapsulation of drug and pore-forming elements within the vector may not solve *in vivo* stability and circulation time issues [Karanth and Murthy, 2007].

An alternative method consists of using synthetic polymers tailored to induce pH-triggered drug release. pH-responsive liposomes have been generated by anchoring polyanions into the lipidic bilayer. Such polymers undergo a coil-to-globule phase transition below a critical pH that elicits destabilization of lipid membranes [Yessine and Leroux, 2004]. Table III-1 summarizes some of the research employing polyanions for the preparation of pH-sensitive liposomes. It should be noted that copolymers of *N*-isopropylacrylamide (NIPAM) have been the most investigated so far and that pH-triggered release has been predominantly tested *in vitro* with fluorescent probes.

Table III-1: Summary of pH-sensitive copolymers investigated for liposomes.

Polymer	Terminal (T) or random (R) anchor	Anchoring element	Lipids	Marker or drug encapsulated	Reference
PEAA	T	DMPE	EPC/DMPE	Calcein	[Maeda *et al.*, 1988]
PG	T	Decylamine	EPC/PG	Calcein	[Kono *et al.*, 1997]
P(NIPAM-*co*-Gly-*co*-ODA)	R	ODA	POE-SE/Chol or POCP/Chol	HPTS/DPX	[Francis *et al.*, 2001]
P(NIPAM-*co*-MAA-*co*-VP-*co*-ODA)	R	ODA	EPC/Chol or EPC/Chol/PEG-DSPE	HPTS/DPX	[Roux, Francis *et al.*, 2002; Roux *et al.*, 2003]
P(NIPAM-*co*-MAA-*co*-ODA)	R	ODA	EPC/Chol	HPTS/DPX	[Leroux *et al.*, 2001]
			DOPC/Chol	DOX	[Leroux *et al.*, 2001]
DODAm-P(NIPAM-*co*-MAA)	T	DODA	EPC/Chol	HPTS/DPX	[Leroux *et al.*, 2001]
			EPC/Chol/PEG-DSPE	HPTS/DPX	[Leroux *et al.*, 2001; Roux *et al.*, 2004]
PPZ (EEE, ABA, $C_{18}(EO)_{10}$)	R	$C_{18}(EO)_{10}$	EPC/Chol	HPTS/DPX	[Couffin-Hoarau and Leroux, 2004]

PEAA: poly(2-ethylacrylic acid); **DMPE**: dimyristoyl-*N*-[[4-(maleimidomethyl)cyclohexyl]carbonyl] phosphatidyl-ethanolamine; **EPC**: egg phosphatidylcholine; **PG**: decylamine-succinylated poly(glycidol); **NIPAM**: *N*- isopropylacrylamide; **MAA**: methacrylic acid; **VP**: *N*-vinylpyrrolidone; **Gly**: glycine acrylamide; **ODA**: octadecyl acrylate; **DODA**: dioctadecylamide; **HPTS**: trisodium 8-hydroxypyrene trisulfonate; **DPX**: *p*-xylene-bis-pyridinium; **PEG-DSPE:** *N*-[methoxy(polyethylene glycol) 2000] carbonyl-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; **PPZ (EEE, ABA, $C_{18}(EO)_{10}$)**: ethylene oxide diethyl ether-aminobutyric acid-polyethylene glycol octadecyl ether-grafted poly(organophosphazenes); **$C_{18}(EO)_{10}$:** polyethylene glycol octadecyl ether.

NIPAM derivatives have been proposed early for the design of stimuli-responsive liposomes. Original interest was spurred by PNIPAM's sharp lower critical solution temperature (LCST) at 32 °C [Heskins and Guillet, 1968; Winnik, 1990]. This transition can be tuned to temperatures relevant to physiological applications by introducing a weakly acidic monomer such as methacrylic acid (MAA), which also renders the polymer pH-responsive [Chen and Hoffman, 1995; Brazel and Peppas, 1996]. Liposomes formulated with alkylated NIPAM/MAA copolymers rapidly released their contents in an acid environment [Meyer *et al.*, 1998; Zignani *et al.*, 2000; Leroux *et al.*, 2001; Roux, Stomp *et al.*, 2002]. It was shown that upon collapse, the interaction area between the phospholipids and the copolymers increased [Petriat *et al.*, 2004]. The latter introduced a curvature in the bilayer plane, inducing membrane defects [Roux *et al.*, 2003] and release of the entrapped content [Francis *et al.*, 2001]. Although no acute toxicity has been observed for NIPAM copolymers [Taillefer *et al.*, 2000; Li *et al.*, 2005; Malonne *et al.*, 2005], their safety following long-term exposure has thus far not been demonstrated as they are not biodegradable.

Poly(organophosphazenes) (PPZ) have previously been introduced as biodegradable alternatives to NIPAM copolymers [Couffin-Hoarau and Leroux, 2004]. It was shown that the properties of PPZ can be tailored by incorporating three critical moieties into the polymer composition, namely polyethylene glycol octadecyl ether ($C_{18}(EO)_{10}$), amino butyric acid (ABA) and ethylene oxide ethyl ether (EEE) (Figure III-2). These units provide for liposome-anchoring capabilities, pH- and temperature-responsiveness, respectively. EEE was selected over other alkoxy side groups since EEE-substituted PPZ possessed an LCST close to physiological temperature [Allcock and Dudley, 1996]. ABA helps modulate the LCST with respect to environmental pH. Furthermore, it can confer biodegradability by mediating intramolecular catalysis of phosphorus-nitrogen bonds [Allcock *et al.*, 1982; Allen *et al.*, 2002]. Liposomes prepared with the tri-substituted PPZ displayed pH-dependent release but were unstable under physiological temperature (37 °C) at pH 7.4 [Couffin-Hoarau and Leroux, 2004]. In the present work, we investigated whether the stability

of the formulation at neutral pH could be improved by varying the content of the ionizable ABA moiety and lowering the molecular weight of the polymer. An advantage of a lower molecular weight polymer would be faster excretion after administration. We also examined the degradation of the PPZ under physiological conditions and studied the impact of human serum on the pH-sensitivity of the formulations.

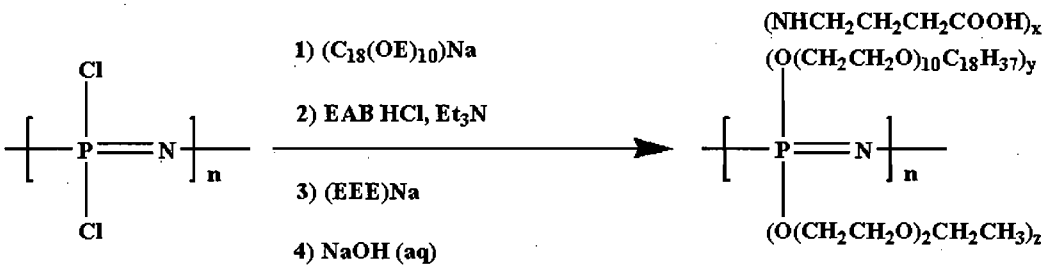

Figure III-2: Synthesis of tri-substituted amphiphilic, pH-sensitive PPZ.

III-3 Materials and Methods

III-3.1 Materials

Cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-dodecanoate (Chol-BODIPY), 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) and *p*-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes (Burlington, ON, Canada). Egg phosphatidylcholine (EPC) and N-[methoxy(polyethylene glycol) 2000] carbonyl-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (PEG_{2000}-DSPE) were purchased from Northern Lipids (Vancouver, BC, Canada). All other chemicals were obtained from Sigma (Oakville, ON, Canada) and used as received, except for the following: diethyl ether (Et_2O), dichloromethane (DCM) and tetrahydrofuran (THF) were run through PureSolv™ drying columns (Innovative

Technologies, Newburyport, MA); triethylamine (TEA) was distilled over calcium hydride; phosphorus trichloride (PCl_3) and sulfuryl chloride (SO_2Cl_2) were distilled under argon; phosphorus pentachloride (PCl_5) was sublimed under vacuum; PEG octadecyl ether (Brij®76, $C_{18}(EO)_{10}$) and ethyl 4-aminobutyrate hydrochloride (EAB·HCl) were dried overnight under vacuum over phosphorus pentoxide.

III-3.2 Synthesis and characterization

III-3.2.1 Phosphoranimine synthesis

All solid products were weighed in a glove box under inert argon atmosphere while reactions were performed using standard Schlenk techniques. ^1H (400 MHz) and ^{31}P (162 MHz) NMR spectra were recorded on a Bruker ARX 400 spectrometer (Milton, ON, Canada) in deuterated chloroform ($CDCl_3$). Chemical shifts for ^{31}P spectra were recorded with respect to an 85% phosphoric acid standard. Trichloro(trimethylsilyl)phosphoranimine ($Cl_3P{=}NSiMe_3$) was synthesized as reported by Wang *et al.* (2002). Briefly, lithium bis(trimethylsilyl)amide (10 g, 0.058 mol) was suspended in 200 mL dry Et_2O and cooled to 0 °C before the dropwise addition of distilled PCl_3 (5.06 mL, 0.058 mol). Completion of the reaction (~1 h) was monitored by ^{31}P NMR from the disappearance of the PCl_3 peak (δ = 220 ppm) and appearance of a new species ($Cl_2PN(SiMe_3)_2$, δ = 186 ppm). Distilled SO_2Cl_2 (4.7 mL, 0.058 mol) was then added dropwise at 0 °C and allowed to react for 1 h. Complete conversion was evidenced by the appearance of a single peak at δ = -54 ppm in the ^{31}P NMR spectra. The reaction mixture was then filtered through dry celite. Et_2O and trimethylsilyl chloride, a reaction side product, were sequentially evaporated at 0 °C from the filtrate under reduced atmosphere (200 and 50 mmHg, respectively). Crude $Cl_3P{=}NSiMe_3$, a colorless liquid, was purified by distillation (25 °C, 0.1 mmHg of static vacuum) into a liquid nitrogen-cooled trap to collect the final product (10.6 g, 81 % yield).

III-3.2.2 Synthesis of poly(dichlorophosphazene)

Poly(dichlorophosphazene) (PDCP) was obtained by cationic polymerization using PCl_5 as the initiator [Allcock *et al.*, 1996]. A concentrated solution of $Cl_3P{=}NSiMe_3$ (6.1 g, 0.027mol) in dry DCM (5 mL) was cannulated to a solution of PCl_5 (0.16 g, 7.8 x 10^{-4} mol, $Cl_3P{=}NSiMe_3$:PCl_5 molar ratio of 35:1) under inert argon atmosphere to reach a final initiator concentration of 0.035 mol/L. The polymerization reaction was carried out at room temperature and monitored by ^{31}P NMR by following the disappearance of the $Cl_3P{=}NSiMe_3$ peak and the appearance of the PDCP backbone peak (δ = -17 ppm). After 2 h, DCM was evaporated and the crude product stored under inert conditions at -20 °C.

III-3.2.3 Synthesis of poly(organophosphazenes)

pH-sensitive PPZ were prepared as described before [Couffin-Hoarau and Leroux, 2004]. Synthesized polymers are named A_x-P_y, with x and y representing the ratios of the ABA and $C_{18}(OE)_{10}$ moieties, respectively. The following is the typical procedure as performed for the synthesis of PPZ A_7-P_6 (Table III-2). Under inert argon atmosphere, a solution stirred overnight of $C_{18}(EO)_{10}$ (0.72 g, 1.0 mmol) and NaH (0.026 g, 1.0 mmol) was added dropwise to a PDCP solution (obtained from 1.0 mmol of $Cl_3P{=}NSiMe_3$) dissolved in 10 mL dry THF. After 6 h at room temperature, a solution of EAB·HCl (0.35 g, 2.0 mmol) treated with 2.8 eq. distilled TEA (0.8 mL, 5.7 mmol) was added and the mixture was heated 48 h at 50 °C before being cooled to room temperature. Finally, an excess solution of EEE (4.8 mL, 3.5 mmol), treated overnight by NaH (0.88 g, 3.5 mmol), was added dropwise and the reaction was stirred overnight at room temperature. The progression of each substitution reactions was tracked in ^{31}P NMR by the appearance of a peak at δ = -8 ppm corresponding to the substituted phosphazene. After completion of the last reaction, the final solution was filtered from excess salts, concentrated and dialyzed against deionized water for 48 h (molecular weight cut-off 12,000-14,000). The resulting aqueous polymer solution was treated by 5 mL of 1 N NaOH for 4 h at room temperature to complete

hydrolysis of EAB to ABA. The final PPZ was dialyzed against water for 24 h and lyophilized to obtain 3 g of a yellow colored oil (75% yield).

III-3.3 Physical characterization of pH-responsive polymers

The degree of substitution was estimated using ^{1}H NMR by calculating the ratios between the methyl protons of $C_{18}(OE)_{10}$ and EEE (δ = 0.9 ppm and 1.2 ppm, respectively) and a CH_2 of ABA (δ = 1.7 ppm). The percentage of ABA was also confirmed by potentiometric titration using an Accumet AP61 pH-meter (Fisher Scientific, Montreal, QC, Canada), according to the following procedure: an aqueous solution of the polymer (5 mL, 1 mg/mL) was treated with excess NaOH (3 mL, 0.01 N) to ensure dissolution of the PPZ and complete ionization of the acid functions. Titrations were performed by adding increments of 0.01 N HCl and measuring aqueous pH. During this process, both the amine and carboxylic acid of the ABA molecules were titrated and considered in calculations [Couffin-Hoarau and Leroux, 2004].

The absolute number- (M_n) and weight- (M_w) average molecular weights of the polymer samples were determined by size exclusion chromatography (SEC) using a Breeze system (Waters, Milford, MA) equipped with a Waters 2410 refractometer and PD2000 light-scattering detector (Precision Detectors, Bellingham, MA). Measurements were performed in *N,N*-dimethylformamide containing 10 mM lithium bromide at a flow rate of 1 mL/min at 40°C. Molecular weight separation was achieved using three Waters Styragel columns (HT2, HT3 and HT4) in series and the instrument calibrated with monodisperse polystyrene standards.

The pH-dependent precipitation of PPZ in aqueous solution was investigated by turbidimetry. PPZ were dissolved in 200 mL phosphate buffer (PB) saline (53 mM Na_2HPO_4, 13 mM NaH_2PO_4, 75 mM NaCl) at a concentration of 0.2 mg/mL. The pH of the solution was adjusted to pre-determined values and the turbidity of aliquots

was measured at 480 nm (37 °C) using a Series 2 Aminco Bowman fluorometer (Spectronics Instruments Inc., Rochester, NY) [Roux, Stomp *et al.*, 2002].

The lower critical solution temperature (LCST) and enthalpy of transition (ΔH_{LCST}) of the PPZ were determined in triplicate on three distinct samples by differential scanning calorimetry on a MicroCal VP-DSC (MicroCal, Northampton, MA). Polymer samples were dissolved in saline 2-*N*-(morpholino)ethanesulfonic acid (MES) (100 mM, 110 mM NaCl, pH 5.0) at a concentration of 10 mg/mL. Scans were performed on samples of 0.509 mL at a rate of 20 °C/h from 7 to 65 °C.

The degradability of the PPZ was tested by incubating 5 mL aliquots of polymer solutions (1.2 mg/mL in 10 mM PB, pH 7.4), which were filtered under sterile conditions, and then incubated at 37 °C for 21 weeks. The samples were lyophilized and changes in M_w were measured by SEC.

III-3.4 Analysis of pH-sensitive liposomes

III-3.4.1 Incorporation of poly(organophosphazenes) into liposomes

The pH-sensitive liposomes were prepared as described before [Zignani *et al.*, 2000, DG205; Leroux *et al.*, 2001]. Briefly, a lipid film was obtained by evaporating chloroform solutions of EPC, cholesterol and PPZ with a respective molar ratio of 59:40:1. The polymer/lipid mass ratio was approximately 0.2. In the case of PEGylated liposomes, 5.5 mol% PEG_{2000}-DSPE was included in the lipid bilayer as reported elsewhere [Yang *et al.*, 2003; Roux *et al.*, 2004]. The film was then hydrated overnight in an isotonic 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffered saline solution (HBS, 20 mM HEPES, 144 mM NaCl, pH 7.4) to obtain a lipid concentration of 40 mM. Finally, the mixture was extruded through 400-, 200- and 100-nm polycarbonate membranes (Avanti, Alabaster, AL) 21 times each. For some formulations, the polymer was post-inserted by incubation with preformed extruded vesicles overnight at 4 °C in HBS (PPZ molar ratio of 1%). In both cases, unbound polymer was removed by SEC using a Sepharose 2B column.

Liposome size was measured by dynamic light scattering on a Malvern Zetasizer ZS (Malvern, Worcestershire, UK) with a fixed angle of 173° at 25 °C. Final vesicle sizes were between 120 and 180 nm, with narrow polydispersity (< 0.12).

A procedure adapted from G.R. Bartlett (1959) was used to measure the total amount of phosphorus in the formulations from which the efficiency of PPZ incorporation was calculated. Chol-BODIPY (0.2 mol% of lipids) was added during the preparation of liposomes as an internal standard to normalize for phospholipid concentrations. The percent PPZ incorporated could then be obtained by subtracting the phosphorus content of bare liposomes from PPZ-liposomes.

III-3.4.2 In vitro release kinetics

In vitro release kinetics were monitored for EPC/Chol/PPZ liposomes incorporating the fluorescent markers HPTS (35 mM) and quencher DPX (50 mM) in HEPES buffer (20 mM) before and after 1 h incubation with 50% *(v/v)* human serum [Han *et al.*, 2006]. SEC was performed to remove non-encapsulated marker/quencher as well as excess serum components. The release profiles of the various formulations were measured by adjusting the external pH with either HBS (pH 7.4) or MES adjusted to pH 5.0 or 6.0. HPTS release was monitored by fluorescence assay using a Tecan Safire plate reader (Tecan, Durham, NC) (λ_{ex}= 412 nm and λ_{em}= 513 nm) at 37 °C. The percent release at each time point was obtained from the relative fluorescence intensity with respect to the intensity obtained after sample lysis with 0.5 % (*v/v*) Triton X-100.

III-4 Results and Discussion

III-4.1 Synthesis and characterization of pH-sensitive poly(organophosphazenes)

III-4.1.1 Synthesis

PDCP was synthesized by cationic polymerization of the phosphoranimine monomer as described previously [Allcock *et al.*, 1996; Couffin-Hoarau and Leroux, 2004]. Five different pH-sensitive PPZ (Table III-2) were generated by performing three sequential substitution reactions of P-Cl bonds in PDCP backbone (Figure III-2). Due to increasing reactivities, $C_{18}(EO)_{10}$ was added first, followed by EAB and then EEE. Final substitution ratios of 7-14% and 5-16 mol% were obtained for ABA and $C_{18}(OE)_{10}$, respectively (Table III-2). The slightly lower than theoretical ratios of ABA may result from metathetical exchange during substitution of some EAB by the stronger nucleophile, EEE [Allcock, 1977]. Moreover, the basic conditions used for hydrolysis of EAB to ABA might induce cleavage of the aminophosphazene bond [Allcock *et al.*, 1982], also decreasing the ABA molar ratio. It was previously reported that PPZ (M_W = 38,000, 9% ABA, 5 mol% $C_{18}(EO)_{10}$, respectively) can provide pH-responsive properties to liposomes [Couffin-Hoarau and Leroux, 2004], but no further studies were carried out to determine the relation between structure and properties. The PPZ synthesized here possessed lower M_w (15,000-20,000) and various ABA/$C_{18}(OE)_{10}$ molar ratios, which allowed examination into the impact of PPZ composition on the release kinetics.

Table III-2: Characteristics of synthesized poly(organophosphazenes)

PPZ	Composition (ABA : $C_{18}(EO)_{10}$: EEE) (mol%)		M_w	M_w/M_n	LCST[c] (°C)	ΔH_{LCST}[c] (J/g)	% liposome fixation[d]
	Theoretical[a]	Experimental[b]					
A_7-P_6	10 : 5 : 85	7 : 6 : 87	16,300	1.01	33.5 ± 0.1	17.6 ± 0.8	81.3 ± 7.9[e]
A_9-$P_{5.5}$	15 : 5: 80	9 : 5.5 : 85.5	19,300	1.01	35.5 ± 0.7	10.1 ± 0.8	97.3 ± 6.18[e]
$A_{9.5}$-$P_{7.5}$	10 : 7 : 83	9.5 : 7.5 : 83	15,100	1.06	34.0 ± 0.3	12.6 ± 0.4	92.3 ± 16.7[e]
A_{11}-P_{10}	10 : 10 : 80	11 : 10 : 79	18,300	1.03	31.7 ± 0.6	6.3 ± 2.5	33.8 ±18.6[f]
A_{14}-P_{16}	15 : 10 : 75	14 : 16 : 70	19,600	1.03	33.0 ± 1.1	4.6 ± 1.3	52.8 ± 14.3[f]

a) Theoretical values are calculated from the proportions of the reagents used for the substitution of the polymers.
b) Experimental values are based on ^{1}H NMR and acid-base titration results.
c) DSC results LCST and ΔH_{LCST} were obtained at pH 5.0 and performed in triplicate.
d) Efficiency of PPZ fixation to EPC/Chol (3:2 mol/mol) liposomes prepared with 1 mol% PPZ as determined by phosphorus content.
e) PPZ added to lipids before the extrusion process.
f) PPZ fixed to liposomes after overnight incubation with preformed vesicles at 4 °C.

III-4.1.2 Physical characterization

Figure III-3 shows the typical pH-dependent phase transition of 3 representative PPZ at 37 °C. Turbidimetry was used to detect the polymers' phase separation from the buffered medium under dilute conditions. With the exception of polymer A_7-P_6, the PPZ were fully soluble at pH 7.4, while the turbidity markedly increased upon lowering the pH below 6.0. The change in solubility around this pH is what is sought to destabilize the phospholipid membrane after endocytosis and release the liposomal content. As shown in Figure III-3, sample A_7-P_6, displayed some turbidity near pH 7, reflecting the incomplete dissolution of the polymer. This might be attributed to its lower ABA content, which renders the polymer less hydrophilic. We indeed previously reported that a PPZ with comparable composition (9 mol% ABA, 5 mol% $C_{18}(EO)_{10}$), but higher molecular weight (M_w = 38,000) possessed a LCST of 32.4 °C at pH 7.4 [Couffin-Hoarau and Leroux, 2004]. It has been shown that fully EEE-substituted PPZ have a LCST of 32°C at pH 7.4 [Couffin-Hoarau and Leroux, 2004] while the introduction of a sufficient amount of ionizable moiety, such as ABA, can raise the LCST at this pH [Hirotsu *et al.*, 1987; Chen and Hoffman, 1995]. Therefore, owing to their better solubility at physiological temperature and neutral pH, PPZ A_{14}-P_{16} and A_9-$P_{5.5}$ are expected to be better candidates than A_7-P_6 for the design of pH-responsive vesicles that would be stable at pH 7.4 and destabilized under mildly acidic conditions.

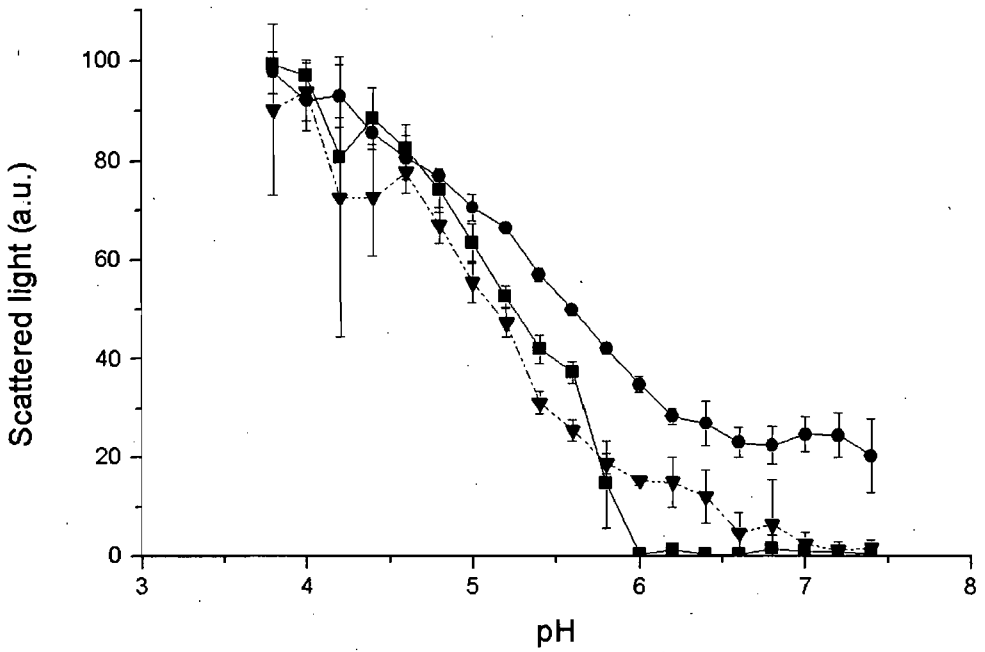

Figure III-3: pH-dependent phase transition of PPZ A_7-P_6 (circles), A_9-$P_{5.5}$ (triangles) and A_{14}-P_{16} (squares) as determined by turbidimetry in PBS at 37 °C. Mean ± SD (n=3).

DSC thermograms were recorded for the different PPZ at pH 5.0. LCST values obtained were taken at the maxima of the endotherms and ranged between 32 and 35.5 °C, with transition enthalpies varying from 4.6 to 17.6 J/g (1.1 to 4.2 cal/g). As shown in Table III-2, all LCSTs were in the same range under acidic conditions. For previously synthesized pH-sensitive PPZ, acidification to pH 5.0 decreased the LCST below 30 °C [Couffin-Hoarau and Leroux, 2004], which is lower than for the PPZ presented here. Feil *et al.*(1993) have noted that the LCST of NIPAM copolymers was strongly influenced by their overall hydrophilicity and the structuring of water around hydrophobic groups. In the present case, it is difficult to predict the precise variations the substituents impose on the LCST of the PPZ as there are three side groups involved. Moreover, the $C_{18}(EO)_{10}$ side group is by itself amphiphilic due to the contribution of the $(EO)_{10}$ and C18 segments. While, the $(EO)_{10}$ chain may

raise the LCST as the additional oxygen atoms can increase hydration [Allcock and Dudley, 1996], the alkyl chain may decrease the LCST depending on whether they self-assemble (*i.e.* exclusion from the solvent) or not in water.

Interestingly, the changes in enthalpy associated to the phase transition were lower than previously observed for other pH-responsive PPZ [Couffin-Hoarau and Leroux, 2004]. It could be hypothesized that the decreased ΔH_{LCST} is a result of the generally higher proportions of $C_{18}(EO)_{10}$ and protonated ABA moieties, which may reduce of the interactions between the polymer and the water molecules and/or increase interactions of the polymer with itself. This tendency was also observed for PPZ A_{14}-P_{16} which had the highest level of ABA and $C_{18}(EO)_{10}$ while exhibiting the lowest ΔH_{LCST} at pH 5. Indeed, a similar dependence was observed by Laukkenen *et al.* (2005) for a thermosensitive polymer modified by increasing proportions of an amphiphilic graft.

III-4.1.3 Biodegradation study

Poly(aminophosphazenes) have been extensively explored as degradable alternatives to other synthetic polymers [Allcock *et al.*, 1977; Crommen *et al.*, 1992a; Allcock *et al.*, 1994]. The degradation of two PPZ, $A_{9.5}$-$P_{5.5}$ and A_{14}-P_{16}, was compared after a period of 21 weeks at pH 7.4 and 37 °C. Only 20% decrease in M_w was observed for both polymers, showing that the degradation was partial. It is known that the degradation of PPZ involves the cleavage of the aminophosphazene bond [Allcock *et al.*, 1982; Lee *et al.*, 1999] catalyzed by the free acid of ABA. However, the extent of degradation is dependent on the nature of the amino acid [Allcock *et al.*, 1982] and its molar ratio [Crommen *et al.*, 1992b; Lemmouchi *et al.*, 1998]. It is thus likely that the low ABA content along the PPZ backbone could not promote complete degradation.

III-4.2 Characterization of pH-responsive liposomes

III-4.2.1 Incorporation of poly(organophosphazenes) into liposomes

pH-responsive liposomes were prepared by either of two methods. PPZ with lower anchor content were incorporated by the inclusion of 1 mol% PPZ in the lipid film. However, for A_{11}-P_{10} or A_{14}-P_{16}, this method failed to produce monodisperse vesicles. For these two polymers, bridging between vesicles may have resulted from the relatively high PPZ/lipid ratio (0.2 *w/w*) and elevated $C_{18}(OE)_{10}$ content, thus forming a complex network [Meier et al., 1996]. The increased viscosity thus could have made it also mechanically difficult to extrude. As a consequence, A_{11}-P_{10} and A_{14}-P_{16} were associated to the lipid membrane by incubating a PPZ solution with preformed extruded vesicles overnight at 4 °C. The post-incorporation method involved the addition of PPZ to the vesicle suspension resulting in a more dilute mixture. Therefore, it permitted the formation of stable liposomes with PPZ inserted solely on the external leaflet of the bilayer.

The extent of polymer incorporation for the different formulations was calculated from the phosphorous content (Table III-2). PPZ fixation was significantly higher when included in vesicle preparation, as over 80% PPZ incorporation (0.16 g PPZ/g lipid) was obtained. For PPZ A_{11}-P_{10} and A_{14}-P_{16}, which were incorporated by incubation, anchoring efficiencies of 35 and 50% were achieved, respectively. These findings can be compared to EPC/Chol liposomes prepared with NIPAM/MAA copolymers containing 2% octadecyl acrylate (ODA) for fixation. A 2-fold increase in binding efficiency was obtained for P(NIPAM-*co*-MAA-*co*-ODA) when included in vesicle preparation rather than post-incorporated [Zignani *et al.*, 2000]. This can be explained by the increased surface area available for incorporation on both sides of the bilayer and lipid mixing. Furthermore, the post incorporation of P(NIPAM-*co*-MAA-*co*-ODA) yielded a maximum of 0.038 g copolymer/g lipid, which corresponded to a plateau with an efficiency of 30% when prepared with an initial mass ratio of 0.12 g copolymer/g lipid. This is somewhat lower than what was seen with PPZ A_{11}-P_{10} and A_{14}-P_{16} (0.07 and 0.1 g PPZ/g lipid, respectively). Increasing

the content of anchoring moiety seems to have improved copolymer fixation. Kono *et al.* (1999) also observed increased liposome binding for polymers of larger molecular weight while maintaining the proportion of the anchor, suggesting that the binding efficiency improves with an increasing number of anchoring moieties per polymer chain. This general trend is also observed for PPZ with increasing proportions of $C_{18}(OE)_{10}$.

III-4.2.2 In vitro release kinetics of pH-responsive liposomes

pH-sensitive PPZ are required to promote maximal discharge under acidic conditions while permitting complete retention as long as the vector remains in circulation. To test for this character, the release of the encapsulated probe HPTS from pH-responsive liposomes was measured at pHs 5.0, 6.0 and 7.4, and at a temperature of 37 °C. Figure III-4 shows the *in vitro* release kinetics of formulations prepared with PPZ A_7-P_6 (A), $A_{9.5}$-$P_{7.5}$ (B) and A_{14}-P_{16} (C). It can be seen that PPZ induced a marked increase in the release rate of the encapsulated dye as the pH was acidified. Liposomes prepared with PPZ A_7-P_6 released a substantial amount of HPTS at neutral pH (27% within 35 min, Figure III-4). As discussed above, this polymer is partially dehydrated at pH 7.4 and 37°C, and thus can destabilize the lipid membrane. In our previous report, pH-sensitive liposomes prepared with PPZ having an LCST of 32 °C at pH 7.4 showed similar profiles under the same experimental conditions [Couffin-Hoarau and Leroux, 2004]. In contrast, the other two formulations were significantly more stable with less than 5% dye released after 35 min at neutral pH. $A_{9.5}$-$P_{7.5}$ (Figure III-4B) demonstrated the best triggered release profile (75 and 47% HPTS released at pHs 5.0 and 6.0, respectively). As depicted in Figure III-1, release should ideally occur within the transit time of the endocytosed material to mature lysosomes (< 35 min). A rapid response to the decrease in pH would also improve discharge of the content and delivery to the cytoplasm. PPZ $A_{9.5}$-$P_{7.5}$ not only exhibited high marker release over 35 min, yet also showed a triggered discharge within the first 5 min, which was not seen for the other PPZ reported here. A_{14}-P_{16} was less efficient in destabilizing the liposomes at acidic pH. After 35 min, about

45% leaked from the vesicles at pH 5.0. The lower performance of A_{14}-P_{16} can be explained by the presence of PPZ only on the outer leaflet of the liposomes due to the incorporation method. We and others previously reported that pH-responsive liposomes were more readily destabilized when polymers were fixed on both sides of the bilayer [Hayashi *et al.*, 1999; Zignani *et al.*, 2000; Roux, Francis *et al.*, 2002; Couffin-Hoarau and Leroux, 2004]

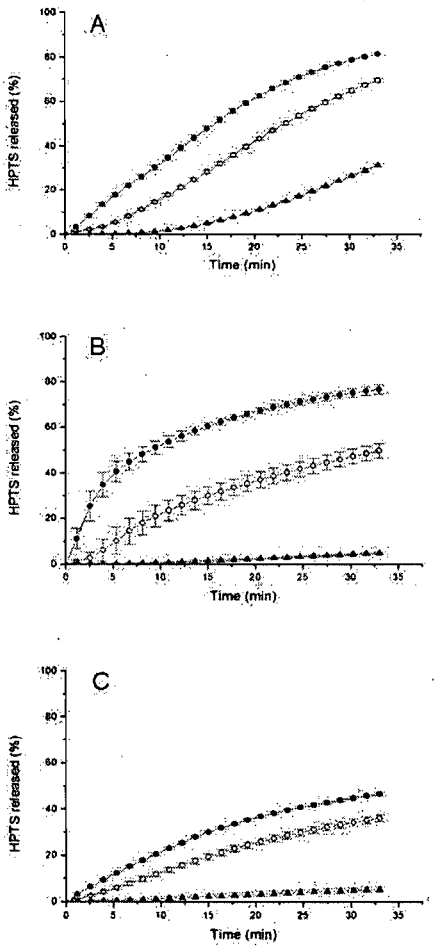

Figure III-4: Percent HPTS released from EPC/Chol (3:2 mol/mol) liposomes (120-180 nm) prepared with 1mol% PPZ A_7-P_6 (A), $A_{9.5}$-$P_{7.5}$ (B) and A_{14}-P_{16} (C) at 37 °C and pH 7.4 (solid triangles), 6.0 (open circles) and 5.0 (solid circles). Mean ± SD (n=3).

pH-sensitive liposomes, injected intravenously, must circulate for a sufficiently long period to attain target cells. However, EPC/Chol liposomes typically do not survive in the blood stream as they are quickly opsonized and eliminated by the mononuclear phagocyte system (MPS). Pharmacokinetic studies revealed that their biological half life ($t_{1/2}$) is less than 35 min in rats after *i.v.* injection [Roux *et al.*, 2003]. PEGylation is well known for providing liposomes with a steric barrier from opsonins and other serum proteins, as well as considerably extending circulation times in the blood stream [Klibanov *et al.*, 1990; Simoes *et al.*, 2004]. pH-sensitive liposomes can additionally be PEGylated to improve their circulation half life [Roux, Stomp *et al.*, 2002; Roux *et al.*, 2003].

The effect of PEG_{2000}-DSPE was therefore evaluated on $A_{9.5}$-$P_{7.5}$-liposomes. This PPZ was chosen as it showed to the best release kinetics of HPTS. They were both incorporated into the bilayer during vesicle preparation, in the same manner as for the non-PEGylated forms. In spite of this, only 32% PPZ fixation was achieved, which is a decline of 50% in $A_{9.5}$-$P_{7.5}$ binding efficiency. Steric hindrance caused by the PEG chains may have impaired the anchoring of the PPZ into the bilayer. The HPTS release kinetics of PEGylated pH-sensitive liposomes is reported in Figure III-5. In comparison to the unmodified formulation, the amount of dye liberated decreased from 75 to 55% after 35 min at pH 5.0. Also, a lag time was seen for the onset of the release. Roux *et al.* (2003) had previously shown that PEG_{2000}-DSPE contributed to a significant stabilization of pH-sensitive liposomes. The loss in pH-responsiveness could therefore be attributed to both the reduced fixation of the PPZ and the stabilizing effect of PEG_{2000}-DSPE on the bilayer.

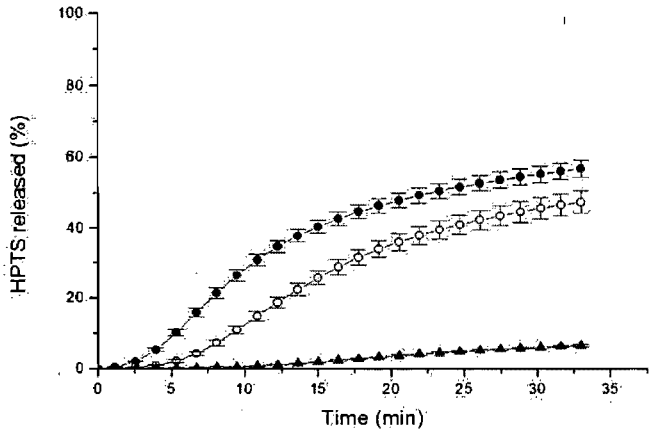

Figure III-5: Percent HPTS released from EPC/Chol (3:2 mol/mol) liposomes (*ca.* 120 nm) at 37 °C prepared with PPZ $A_{9.5}$-$P_{7.5}$ and 5.5 mol% PEG_{2000}-DSPE. Release performed at pH 7.4 (solid triangles), 6.0 (open circles) and 5.0 (solid circles). Mean ± SD (n=3).

For a formulation to be clinically viable, it is crucial that it remains stable in the presence of serum. Figure III-6 compares the amount of HPTS released after 30 min for $A_{9.5}$-$P_{7.5}$-liposomes with and without PEG, before and after serum incubation. Decreased release at acidic pH was observed when PPZ-liposomes were pre-incubated with 50% (*v/v*) human serum for 1 h. In other studies, exposure to serum reduced pH-sensitivity of PEGylated vesicles bearing randomly alkylated P(NIPAM-*co*-MAA) [Roux *et al.*, 2003] whereas no significant desensitization was observed when the anchor was present on the terminus of the polymer chain [Roux *et al.*, 2004]. The reduced response may be a result of polymer extraction and/or a shift in transition pH due to protein adsorption [Harvie *et al.*, 1996]. Randomly alkylated polymers may affect the formation of an adequate protective PEG barrier around the liposome, thus allowing protein adsorption. In contrast, terminally alkylated copolymers may facilitate resistance to serum inactivation by allowing uniform polymer distribution on the vesicle surface.

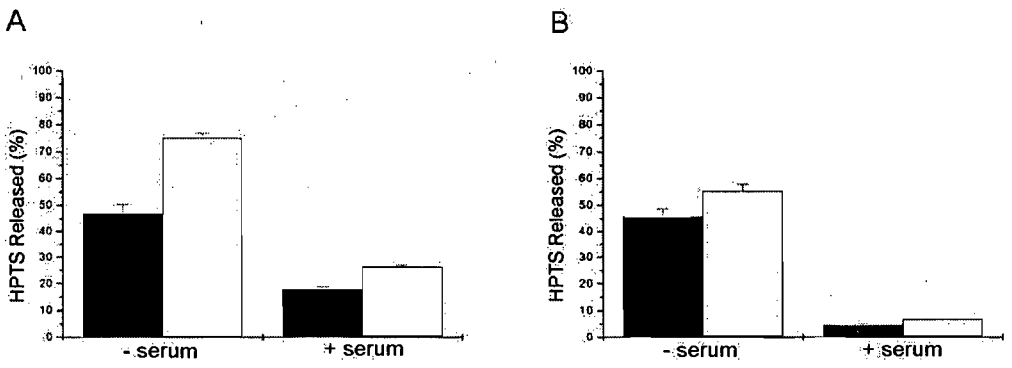

Figure III-6: Percent HPTS released after 30 min at 37 °C from pH-sensitive EPC/Chol (3:2 mol/mol) $A_{9.5}$-$P_{7.5}$-liposomes (*ca.* 120 nm) prepared without (A) and with (B) 5.5 mol% PEG-DSPE. pH-sensitivity was evaluated before and after 1-h incubation with 50:50 (*v/v*) human serum at pH 6.0 (solid bars) and 5.0 (open bars). Mean ± SD (n=3).

III-5 Conclusion

Amphiphilic polyelectrolyte PPZ are candidates to regulate the targeted release of liposome-encapsulated agents. The LCST of EEE-substituted PPZ was modified as a function of pH by co-substitution of the acidic moiety ABA. The relatively small proportion of this amino acid grafted seems to have limited the degradability of the PPZ, thus making it preferable at this time to keep the molecular weight low enough to favor renal excretion after administration. Adding $C_{18}(EO)_{10}$ randomly along the backbone permitted efficient anchoring of the pH-responsive PPZ into EPC/Chol liposomes, both during or after the preparation of the vesicles. Liposomes formulated with PEG_{2000}-DSPE maintained some pH-sensitivity in spite of a significant reduction of polymer anchoring. However, exposure to serum reduced the pH-responsiveness for both PEGylated and non-PEGylated forms. Additional investigation is, thus, required to determine the cause of this partial deactivation. In conclusion, the potential of PPZ has been further demonstrated for the development

of stimuli-responsive liposomal drug carriers. Steps have been taken in order to define the parameters required to implement such polymers in an efficient and viable drug delivery system. Consequently, improved systems can possibly be formulated by further fine-tuning the PPZ structure to allow the preparation of serum-stable pH-sensitive liposomes.

III-6 Acknowledgments

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CHAPTER IV: Discussion

IV-1 Synthesis and characterization of pH-sensitive poly(organophosphazenes)

IV-1.1 Synthesis

Five pH-sensitive PPZ were synthesized according to the procedure developed by Couffin-Hoarau and Leroux (2004) to evaluate the parameters required for the preparation of pH-responsive liposomes. They were prepared by the sequential substitution of PDCP with $C_{18}(OE)_{10}$, EAB and EEE, then hydrolysis of the ethyl ester to convert EAB to ABA (Figure IV-1). PPZ were named A_x-P_y according to the molar percentage, x and y, of ABA and $C_{18}(OE)_{10}$, respectively. Although the synthesis of PPZ has been well established over the years (as described in Chapter II), a few challenges were encountered at the onset for the synthesis of $Cl_3P{=}NSiMe_3$. The best yields were 10%, significantly lower than reported in the literature [Wang *et al.*, 2002], thus producing insufficient monomer for the preparation of an adequate amount of PPZ for physical characterization and liposome studies. In consequence, the experimental procedures were fine-tuned, raising the yields between 65 and 90%. Critical improvement resulted from removing the slightly volatile Me_3SiCl by distillation, before final purification of $Cl_3P{=}NSiMe_3$. As can been seen in Figure IV-1, the removal of Me_3SiCl is crucial since it is a by-product of both the monomer synthesis and cationic polymerization. Its presence during the latter can inhibit chain propagation causing cyclization [Allcock, Crane *et al.*, 1999]. Table III-2 lists the PPZ that were synthesized with 7-14% and 5-16% of ABA and $C_{18}(OE)_{10}$, respectively, and completed using an excess of EEE. Grafting ratios were determined by NMR and acid-base titrations. The proportion of ABA was generally slightly lower than the theoretical feed of the substituent. The replacement of ABA by EEE, which is a stronger nucleophile [Allcock, 1977], and cleavage of ABA from the

backbone during hydrolysis of the ethyl ester [Allcock *et al.*, 1982] are potential explanations for the lower ABA portions than expected. The molecular weight of the PPZ was limited to 20,000 g mol^{-1}, or half the molecular weight of the PPZ previously reported [Couffin-Hoarau and Leroux, 2004]. The biodegradability of such PPZ had not yet been assessed and it was deemed important to consider the need for renal clearance in eventual *in vivo* applications. Therefore, we set out to determine whether pH-responsiveness can be maintained while reducing the molecular weight of the polymers.

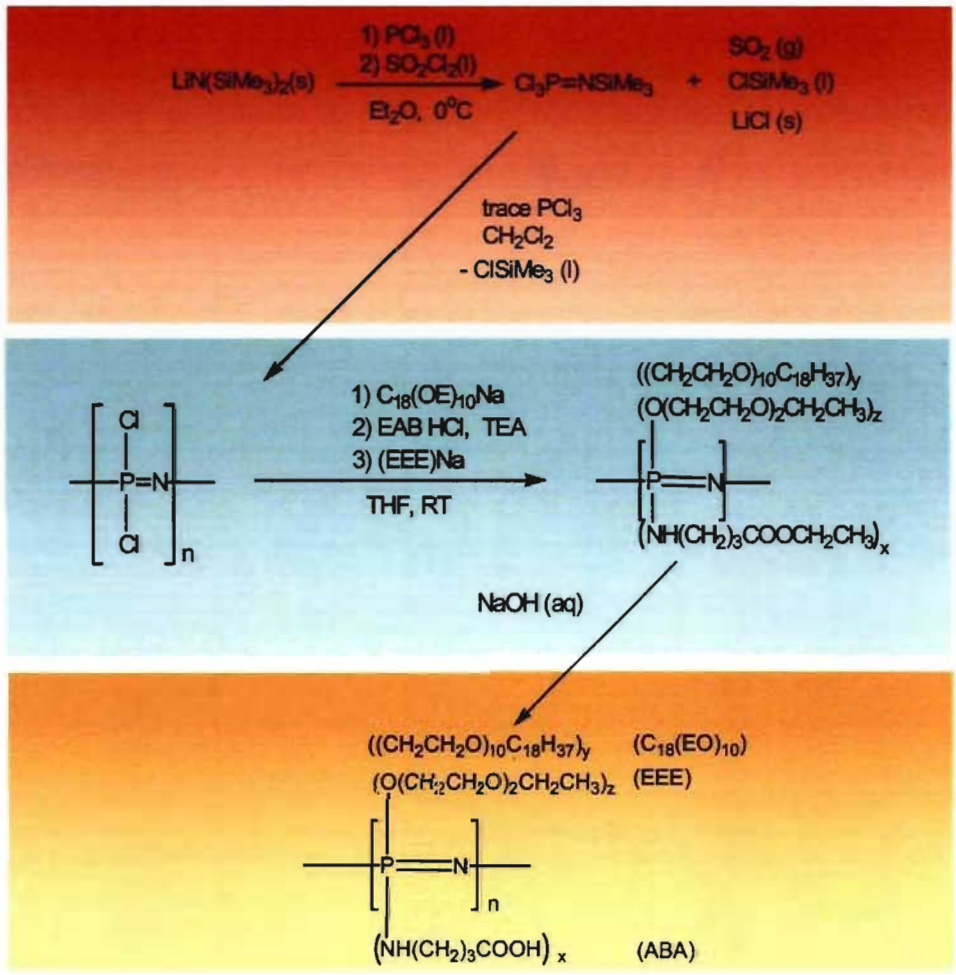

Figure IV-1: Complete synthesis of amphiphilic ionizable PPZ.

IV-1.2 Physical characterization

The physical properties of the PPZ were then evaluated. It is necessary that the polymers be soluble under physiological conditions (pH 7.4, 37 °C) in order to coat the surface of the bilayers. Yet in order to efficiently target cytoplasmic delivery, they should also become more hydrophobic upon acidification to destabilize the liposomes and to release the vesicle's contents. For this reason, the pH-dependent phase transition was determined for each PPZ by turbidimetry at 37 °C (Figure III-3). It was noticed that increasing the proportion of ABA-grafts along the PPZ backbone improved the solubility of the polymer at pH 7.4. Solutions of PPZ with greater ABA portions began phase transitioning at lower pH. This effect is indicative of how increasing the proportion of ionizable moieties improves the water solubility of the polymer [Hirotsu *et al.*, 1987; Chen and Hoffman, 1995]. A greater ABA ratio also provided a sharper coil-to-globule transition upon acidification by increasing polymer solubility at pHs close to 7.4 (Figure III-3). Increasing the solubility of the PPZ at neutral pH and the sharpness of the transition could therefore prevent premature leakage of agents encapsulated in PPZ-liposome and improve the acid-triggered release thereof.

The LCST represents the temperature at which a polymer in solution evolves from a free coil to a globular state. This phenomenon principally results from dehydration of the polymer, thus favoring intramolecular interactions, especially hydrophobic ones. Hence, microcalorimetry by DSC is a good technique to measure the amount of energy absorbed leading to polymer dehydration, which can be expressed by the ΔH_{LCST}. Experimentally, the LCST can be measured at the maximum of the endothermic peak, as described elsewhere [Schild, 1992; Lessard *et al.*, 2001; Kujawa *et al.*, 2006]. The experiment was conducted in pH 5 buffered solutions, since transition is expected to take place in acidic compartments. The results are listed in Table III-2. The measured LCST of the PPZ were close to each other, between 32 and 35.5 °C, and the ΔH_{LCST} ranged from 4.6 to 17.6 J/g. Several parameters may influence the LCST; particularly, increasing the hydrophobic

character of the copolymer should decrease it [Feil *et al.*, 1993]. But in this case, the hydrophobicity supplied by the alkyl chains of an increased $C_{18}(EO)_{10}$ ratio could be counter-balanced by the hydrophilicity of the ethylene oxide units. More thorough analysis could be given by synthesizing PPZ with a wider range of grafting levels, independently varying the side-group proportions.

In order to study the influence of pH on LCST, results were compared at pH 5 and 7.4 for A_7-P_6 (raw data on Figure IV-2). As noticed on curve A, thermodynamic data was impossible to determine at pH 7.4 because the baseline and transition could not be clearly defined on the thermogram. However, the peak of the transition was approximately 36 °C. This is slightly higher than under acidic conditions (33.5 °C), when the polymer was protonated and more hydrophobic [Hirotsu *et al.*, 1987; Chen and Hoffman, 1995]. It had previously been shown for a pH-sensitive PPZ that the ΔH_{LCST} decreased from about 39 to 27 J/g and the LCST increased from 29 to 31 °C when the pH was raised from 5 to 7.4 [Couffin-Hoarau and Leroux, 2004]. The magnitude of these variations is relatively close to those observed in Figure IV-2.

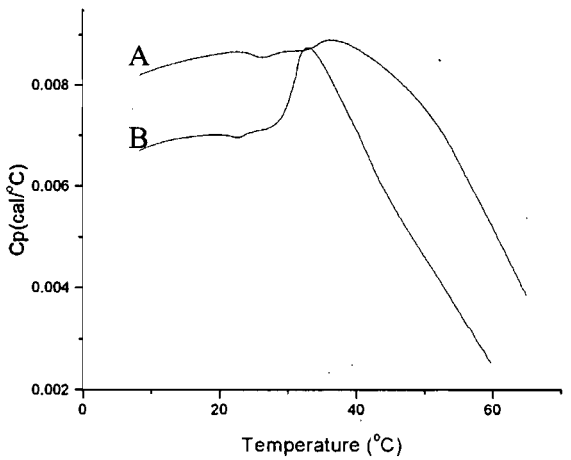

Figure IV-2: Comparison of raw DSC thermograms for A_7-P_6 at pH 7.4 (A) and 5 (B)

IV-1.3 Biodegradation study

PPZ have the particular advantage of being stable against hydrolysis when desired or biodegradable by simply grafting cleavable side-groups. Aminophosphazenes have been extensively studied for their hydrolytic properties [Allcock *et al.*, 1982], especially PPZ bearing amino acid esters [Allcock *et al.*, 1977; Crommen *et al.*, 1992a]. Allcock, *et al.* (1994) proposed three possible mechanisms for PPZ hydrolysis (Figure IV- 3).

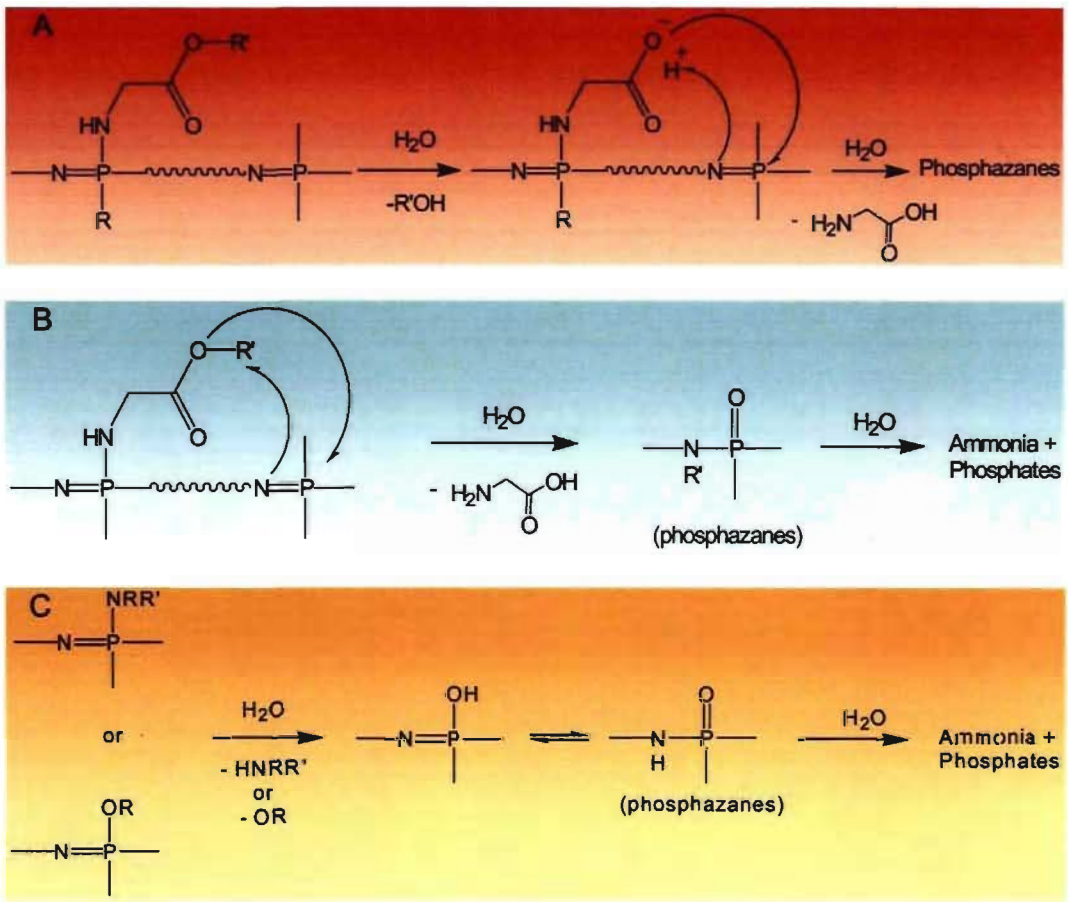

Figure IV- 3: Possible mechanisms for PPZ hydrolysis, adapted with permission from [Allcock *et al.*, 1994].

In all mechanisms, it is believed that a *phosphazane,* a species possessing an oxidized phosphorous and a single bond to nitrogen, is the intermediate to backbone hydrolysis, which finally results in substituted phosphates and ammonia. However, the first steps may differ: in mechanism A, hydrolysis of an ester function would precede nucleophilic attack of the free acid on the phosphorous backbone, whereas in mechanism B, nucleophile substitution might be conducted directly by the ester, releasing the amino acid from the backbone in one step. Intramolecular catalysis by mechanism A may be preferred over mechanism B since poly(amino acid ester)phosphazenes degrade at slower rates than the free acid polymers. Hydrolysis of the ester could be the rate-limiting step for the hydrolysis of poly(amino acid ester) phosphazenes [Schacht *et al.*, 1996]. Others have also noticed that the extent of degradation can be dependent on the substitution ratio of the side-group [Lee *et al.*, 1999]. However, it cannot be neglected that a water molecule can catalyze hydrolysis, as shown in mechanism C. This is particularly true for other side groups, such as imidazole [Allcock *et al.*, 1982; Andrianov *et al.*, 2005] and *N*-ethylpyrrolidone [Andrianov *et al.*, 2005], that have also been used to produce hydrolysable PPZ. Nonetheless, most PPZ that have shown complete degradation are substituted with relatively high portions of the hydrolysable side-group.

The biodegradability of the synthesized PPZ was assayed by incubating polymers in buffered solutions at pH 7.4 and 37 °C. Samples were collected at different time intervals over 21 weeks, lyophilized and re-suspended for molecular weight analysis by SEC. The degradation test showed that the M_w of the PPZ decreased by only 20% within the first four weeks and did not vary much over the following 17 weeks of the assay. This result could be related to the relatively low ABA substitution ratio, since it is primarily responsible for degradation. Once all the ABA was consumed, the PPZ backbone may no longer be cleaved, assuming that alkoxy groups are relatively stable to hydrolysis. Moreover, hydrolysis can also be limited by the increased hydrophobicity of the PPZ, once ionized ABA is cleaved from the backbone. One study compared the biodegradability of PPZ co-substituted with different ratios of glycine ethyl ester and ω-methylpoly(ethylene oxide) (PEO)

of various molecular weight (750, 2000 and 5000 g mol^{-1}) [Vandorpe and Schacht, 1996]. The authors reported that increasing the portion of high molecular weight PEO (PEO-2000 and PEO-5000) accelerated the rate of degradation. They suggested that these polymers were more hydrated and allowed greater access of water to the backbone. Moreover, increasing the amount of PEO-750 along the backbone did not affect the rate of hydrolysis [Vandorpe and Schacht, 1996]. In comparison, the EEE substituent, which is smaller (134.0 g mol^{-1}) and more hydrophobic than PEO-750, might slow down degradation, especially as its ratio increased while ABA was cleaved from the backbone. The remaining PPZ resulting from the hydrolysis may rather behave like EEE-PPZ. Couffin-Hoarau and Leroux (2004) previously reported that the LCST of EEE-PPZ was around 32 °C. It was also demonstrated by turbidimetry that A_7-P_6, with 7% ABA, was not completely soluble at 37 °C and pH 7.4. An LCST-type transition of the remaining chains could therefore limit hydrolysis, as confirmed by a slight turbidity in the sample tubes prior to lyophilization.

IV-2 Characterization of pH-responsive liposomes

IV-2.1 Incorporation of poly(organophosphazenes) into liposomes

Physical evaluation of PPZ showed their pH-sensitivity and potential to induce liposome destabilization. Consequently, the next step of this work was to prepare EPC/Chol liposomes and evaluate the fixation efficiency of the PPZ onto the surface. A 1 mol% PPZ/lipid ratio was used to prepare stable vesicles. It was initially intended to include PPZ in the lipid film, since this method was shown to incorporate amphiphilic polymers with greater efficiency than by post-incorporation [Zignani *et al.*, 2000]. However, lipid mixtures containing the PPZ A_{11}-P_{10} or A_{14}-P_{16} revealed difficult to extrude due to increased viscosity after only a few passages, which suggests bridging of the lipid vesicles [Meier *et al.*, 1996]. Therefore, these PPZ were

formulated with pre-formed vesicles using the same molar percentage in order to compare with previously obtained data.

In spite of the different fixation methods, it was possible to extract and compare some valuable information from the experimental data. First, over 80% fixation was obtained for PPZ A_7-P_6, A_9-$P_{5.5}$ and $A_{9.5}$-$P_{7.5}$ (incorporated in lipid film), while 35 and 50% fixation where obtained for A_{11}-P_{10} and A_{14}-P_{16} (post-incorporated in liposomes). The decreased fixation by the second method might correlate with the reduced area exposed for polymer incorporation, since only the outer surface of the liposome is available.

IV-2.2 In vitro release kinetics of pH-responsive liposomes

In order to ensure maximal drug delivery, pH-responsive liposomes should efficiently retain their payload at neutral pH and then release their contents upon acidification of their environment. In principle, complete discharge should occur before maturation of the endosome to lysosomes (Figure III-1). A fluorescent marker, HPTS, was encapsulated within PPZ-liposomes and the release was followed at 37 °C in pH 5.0, 6.0 and 7.4. The *in vitro* release kinetics was measured before and after 1 h exposure to 50% *v/v* of human serum, in order to evaluate the influence of serum components on the pH-triggered release of the vesicles.

Figure III-4 shows the acid triggered release of HPTS from PPZ-liposomes. At pH 7.4, $A_{9.5}$-$P_{7.5}$ and A_{14}-P_{16} retained well their contents (only 5% of HPTS released after 30 min), whereas A_7-P_6 released more HPTS, likely because of its partial dehydration (Figure III-3). The pH-triggered release was observed for each PPZ, with $A_{9.5}$-$P_{7.5}$ performing the best (75% HPTS released in 30 min). The slower release from A_{14}-P_{16} can be explained by the fact that incorporating pH-responsive polymers only on the outer surface of the vesicles reduces their capacity to destabilize the bilayer [Hayashi *et al.*, 1999; Zignani *et al.*, 2000; Roux, Francis *et al.*, 2002; Couffin-Hoarau and Leroux, 2004]. Furthermore, it was previously shown that the

extent of release increases with greater PPZ:lipid ratios [Couffin-Hoarau and Leroux, 2004].

All PPZ-liposomes were tested for resistance to serum proteins and showed a significant loss in pH-sensitivity after 1 h incubation with 50% (*w/w*) human serum (shown in Figure III-6A for $A_{9.5}$-$P_{7.5}$-liposomes). The loss in sensitivity can possibly be attributed to the serum proteins that are either extracting the PPZ or binding to the bilayer decreasing the transition pH [Harvie *et al.*, 1996]. Interestingly, the acid-induced release was not completely eliminated for PPZ solely incorporated on the outer surface of the bilayer. $A_{9.5}$-$P_{7.5}$-liposomes demonstrated the best retention at pH 7.4 and release under acidic conditions, before and after serum exposure.

In order to reduce serum-induced desensitization and obtain long circulating liposomes, 5.5 mol% PEG-DSPE was added in the formulation of PPZ-liposomes. For PEGylated liposomes to be stable in serum conditions, it is important that the surface of the liposomes be coated by PEG in what is called a *brush regime* [de Gennes, 1980], which forms a steric barrier against serum proteins. This regime consists of PEG chains in the extended coil conformation, evenly distributed on the surface of the vesicles, and occurs when the surface of the lipid bilayer is nearly saturated with polymers [de Gennes, 1980; Hristova and Needham, 1995]. PEG should also be of sufficiently high molecular weight (ca. 2000 g mol^{-1}) since short PEG-lipids cannot form a significant barrier nor provide long-circulation properties [Allen *et al.*, 2002].

Characterization of these liposomes showed that PEG-DSPE reduced the PPZ incorporation efficiency (32% *vs.* 92%), because of a possible adsorption competition effect. Secondly, DSPE-PEG incorporation also reduced the efficiency of pH-triggered release (Figure III-5 and Figure III-6). This can be caused by a combination of the reduced fixation of the PPZ when formulated with PEG and from the stabilizing effect of PEG corona on the lipid-bilayer [Roux *et al.*, 2003]. PEGylated $A_{9.5}$-$P_{7.5}$-liposomes were then tested in the event that PEG could help maintain pH-sensitivity after exposure to serum. Figure III-6 compares the pH-induced release of HPTS from PPZ-liposomes before and after serum incubation. Exposure to serum

considerably reduced the sensitivity of the liposomes, even when PPZ-liposomes were formulated with PEG. Others have also observed that PEGylation of pH-responsive liposomes prepared with randomly-alkylated P(NIPAM-MAA) could not limit interaction with serum components nor prevent potential polymer extraction [Roux *et al.*, 2003]. On the other hand, liposomes prepared with both PEG and terminally-alkylated P(NIPAM-*co*-MAA) were not desensitized by serum exposure [Roux *et al.*, 2004]. Terminally-alkylated PPZ may therefore be better candidates for the preparation of serum-stable pH-responsive vesicles.

It was noticed that the profile of the kinetic release studies had an overall different shape in comparison to other profiles reported in the literature [Roux, Stomp *et al.*, 2002; Boomer *et al.*, 2003]. Since the analytical methods differed, we compared use of the multiple well plate reader with that of a conventional fluorometer for kinetic measurements. Indeed, a faster release was observed with the latter device (data not shown). Though the plate reader allowed comparison of several samples in a single assay, this technique has two principal drawbacks: (i) the samples are prepared at room temperature; (ii) it was impossible to maintain constant stirring. These limitations account for an uneven heat distribution, resulting in a delayed release of the marker. Conversely, the conventional fluorometer allowed the conditions in the cuvette to remain constant, since it is equipped with a heating block and stirrer.

The influence of serum on the pH-sensitivity was then reassessed with the conventional fluorometer. Figure IV-4 compares the amount of HPTS released at 30 min from non-PEGylated (A) and PEGylated (B) A_9-$P_{5.5}$-liposomes before and after 1 h incubation with 50% (*w/w*) human serum. The primary observation is that PEGylated liposomes retained their contents more efficiently than the non-PEGylated ones at pH 7.4, demonstrating the stabilization effect of the PEG corona. Although PEG could change the acid-induced kinetics (as previously shown in Figure III-5 and Figure III-6), the release levels here were comparable after 30 min, even post-serum exposure. In the latter case, it was confirmed that PEGylation could not prevent

serum-induced desensitization to acidic conditions, in concordance to the previously obtained data.

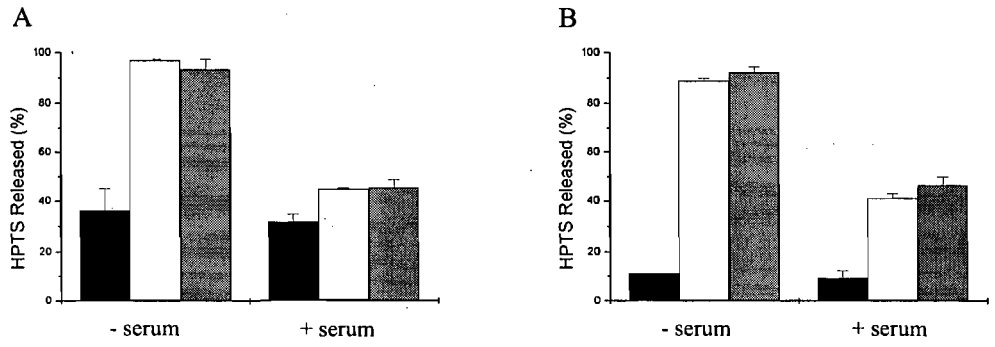

Figure IV-4: Percent HPTS released after 30 min at 37 °C from pH-sensitive EPC/Chol (3:2 mol/mol) A_9-$P_{5.5}$-liposomes (*ca.* 120 nm) prepared without (A) and with (B) 5.5 mol% PEG-DSPE. Percent released is relative to complete HPTS released from lysed liposomes. pH-sensitivity was evaluated before and after 1-h incubation with 50:50 (*v/v*) human serum at pH 7.4 (solid bars), 6.0 (open bars) and 5.0 (grey bars). Mean ± SD (n=3).

In summary, though PEGylation did not provide sufficient protection towards serum exposure, it prevented some leakage at pH 7.4. Also, $A_{9.5}$-$P_{7.5}$-liposomes showed the overall best pH-responsive triggered release with good retention under neutral conditions.

Chapter V: Conclusion and research perspectives

Polyanionic amphiphilic polymers are "intelligent" polymers that are capable of promoting the acid-triggered release of liposome-encapsulated bioactives. Ionizable PPZ have been proposed as biodegradable pH-sensitive polymers, which can be used to coat the surface of liposomal CDC in order to achieve targeted delivery of therapeutics [Couffin-Hoarau and Leroux, 2004]. In this master's thesis, we have described the latest developments in defining the required parameters for the implementation of PPZ for pH-responsive liposomes.

It was determined that increasing the content of the ionizable moiety, ABA, increases the water-solubility of the PPZ at neutral pH. Though difficult to predict, the LCST can be adjusted by varying the proportion of the three substituents. In a biodegradation assay, the PPZ were only subject to partial hydrolysis. This was attributed to the PPZ's relatively low ABA content, which may have limited intramolecular catalysis and the possibility for water to access the phosphazene backbone. Randomly alkylated PPZ showed efficient liposome fixation, especially when included during vesicle formation, and mediated acid-induced release of an encapsulated marker. Finally, exposure to serum proteins reduced the pH-responsiveness of the PPZ-liposomes. Although PEGylation reduced leakage of the marker from the PPZ-liposomes at pH 7.4, it was unable to protect them against serum components.

There is evidence that randomly-alkylated polymers may not be ideal for the preparation long-circulating pH-responsive liposomes and that terminally alkylated PPZ may allow more efficient coating of membrane surfaces. We therefore propose that future research investigates the synthesis of hydrophobically-modified telechelic PPZ in order to increase serum resistance. Furthermore, limiting alkylation to the terminus will allow easier identification of the proportions ABA and EEE required to eliminate leakage under neutral conditions and maximize acid-triggered release.

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