Non-Viral Strategies for Nucleic Acid Delivery

Arnaud Éleuthère Felber

Diss. ETH No. 21061

Zürich 2013

DISS. ETH NO. 21061

Non-Viral Strategies for Nucleic Acid Delivery

A dissertation submitted to
ETH Zurich
For the degree of
Doctor of Sciences

presented by

ARNAUD ÉLEUTHÈRE FELBER

MSc in Bioengineering & Biotechnology, EPF Lausanne
born on March 14th, 1985
citizen of Le Locle, NE, Switzerland

accepted on the recommendation of

Prof. Dr. Jean-Christophe Leroux, examiner
Prof. Dr. Jonathan Hall, co-examiner
Dr. Bastien Castagner, co-examiner

2013

TABLE OF CONTENTS

Summary

Modulating gene expression in diseased tissues by silencing the corresponding mRNA has tremendous potential for treating several pathologies, including cancer. Two types of short nucleic acids, antisense oligonucleotides (AONs) and small interfering RNAs (siRNAs), have been extensively investigated for this purpose. However, despite promising initial observations, progress in clinics has been disappointing. As of today, Fomivirsen (Vitravene™) and Mipomersen (Kynamro™) are the only approved nucleic acid drugs with an antisense mechanism which received FDA approval.

A major challenge for the successful clinical use of short nucleic acid therapeutics remains their delivery to target cells. Consequently, most clinical trials with naked nucleic acid drug make use of either local delivery or kidney/liver targeting. Their poor *in vivo* activity can be mainly attributed to their i) enzymatic lability, ii) rapid clearance, and iii) low membrane permeation. Common approaches to improve the performance of nucleic acids drugs consist in using viral or non-viral delivery vehicles. Although viruses offer high transfection efficiency, safety issues regarding the viral envelope have arisen. Non-viral delivery is an elegant alternative to overcome the above limitations. In the last few decades, a plethora of nanoscale constructs and functionalized macromolecules have been developed to mimic the function of viruses and improve the intracellular bioavailability of nucleic acid therapeutics. This Ph.D. thesis investigates two promising non-viral approaches to deliver short nucleic acid drugs (AON and siRNA), and reports their *in vitro* efficiency in a prostate cancer (PC-3) cell line. The first approach relies on their formulation within smart polymeric micelles. The second exploits direct chemical derivatization of the nucleic acid drug with functional molecules.

Chapter 1 deals with the current status and limitations of nucleic acid delivery and highlights the approaches discussed in the later chapters.

Chapter 2 provides an in depth discussion on pH-sensitive vesicles, polymeric micelles, and nanospheres, and their performance as nano-scale drug delivery systems. Titratable polyanions are very common within these constructs and this chapter explains how this class of polymer can be used to trigger the release of a drug in a pH-dependent fashion. It

further discusses their ability to improve endosomal escape, a major limitation to therapeutic efficiency.

Chapter 3 describes an optimized approach to prepare smart pH-sensitive polymeric micelles with the aim to improve the biological effect of nucleic acid drugs. The nanocomplexes prepared were characterized, and were found to release their cargo under mildly acidic conditions. In order to trigger particle uptake by receptor-mediated endocytosis, the micelles were decorated with an antibody fragment (directed against the transferrin receptor) and their cellular uptake as well as their capacity to downregulate the targeted gene were investigated on PC-3 cells. Optimal silencing was achieved with targeted micelles containing chemically modified siRNA (*i.e.*, with expected enhanced stability to serum nucleases and reduced immunostimulatory properties). These data suggest that combining optimized nucleic acid chemistry with an effective delivery system can potentiate the activity of the drug, thereby potentially reducing the total dose of carrier required to achieve a pharmacological effect.

In **chapter 4**, several AON sequences were conjugated to a selection of hydrophobic biomolecules (*e.g.*, fatty acids) to improve their intracellular bioavailability in the absence of a delivery system. The gene silencing activity of these compounds was evaluated *in vitro,* and the most potent conjugates (*i.e.*, AONs functionalized with docosanoic acid) were selected for further experiments. Their activity was, however, decreased in a dose-dependent fashion by adding albumin in the transfection medium. Nevertheless, it was found that supplementing the medium with free fatty acids (*e.g.*, decanoic acid) prevented the interaction of the conjugates with albumin, and restored their silencing activity. The knowledge gained on the role of pendant hydrophobic moieties and their interactions with blood proteins may create new research opportunities to deliver this type of drug.

The general conclusion and outlook (**chapter 5**) provide a discussion on the major achievements of this Ph.D. thesis. This last chapter further addresses the limitations and perspective of the proposed systems, with focus on their *in vivo* translation.

Résumé

La possibilité de moduler l'expression de gènes associés à certaines maladies en inhibant l'ARN messager correspondant offre un potentiel inespéré pour le traitement de nombreuses pathologies, telles le cancer. En particulier, deux types d'acide nucléique connus sous le nom d'oligonucléotides antisens (ONA) et petits ARN interférents (ARNi) ont été largement utilisés dans ce but. Néanmoins, malgré de premiers résultats plutôt convaincants, le développement clinique demeure limité. Actuellement, Fomivirsen (Vitravene™) et Mipomersen (Kynamro™) sont les seuls acides nucléiques à mécanisme antisens ayant reçu l'autorisation sur le marché par la FDA.

Un défi majeur à l'utilisation clinique des acides nucléiques repose sur la difficulté à les acheminer dans les cellules en quantité suffisante. Par conséquent, la plupart des essais cliniques avec ce type de médicament s'effectue actuellement par administration locale ou dans l'objectif de cibler passivement des organes aisément accessibles (*i.e.* reins ou foie). La faible activité des acides nucléiques *in vivo* est principalement due à leur dégradation enzymatique, leur rapide excrétion et leur faible pénétration cellulaire. Afin d'améliorer leurs performances, ces derniers sont communément intégrés dans des vecteurs viraux ou synthétiques. Bien que les vecteurs viraux offrent une grande efficacité de transfection, des problèmes de sécurité concernant l'enveloppe virale (*i.e.* réponse immunitaire) persistent. De ce fait, les vecteurs synthétiques représentent une alternative intéressante pour pallier à ces problèmes. Au cours des dernières décennies, des efforts conséquents ont été faits afin d'imiter certaines fonctions des virus, notamment pour améliorer l'acheminement intracellulaire des acides nucléiques. Dans cette thèse de doctorat, nous avons évalué deux types de stratégie non virale pour le transport d'ONA et d'ARNi et avons démontré leur efficacité sur un modèle de cellules cancéreuses de la prostate (PC-3). La première approche repose sur leur formulation dans des micelles polymères possédant des propriétés favorisant la transfection. La seconde stratégie exploite la dérivation chimique des acides nucléiques avec une molécule hydrophobe afin d'accroître leur affinité pour la membrane cellulaire.

Le **chapitre 1** est une introduction générale qui traite notamment du statut actuel de la recherche sur les acides nucléiques et des contraintes associées à leur acheminement.

Le **chapitre 2** décrit les vésicules, les micelles polymères et les nanoparticules sensibles au pH ainsi que leur performance en tant que vecteurs de médicaments et d'acides nucléiques. Le chapitre est axé sur une classe de polymères anioniques pouvant déclencher la libération du principe actif sous certaines conditions physiologiques. L'habilité de ces derniers à améliorer la fuite des agents thérapeutiques emprisonnés dans les endosomes – une limitation majeure à leur efficacité thérapeutique – est particulièrement abordée.

Le **chapitre 3** traite de la préparation de micelles polymères sensibles au pH dans le but d'améliorer l'effet thérapeutique des acides nucléiques. Ces micelles ont la capacité de libérer leur contenu dans des conditions légèrement acides. Afin d'augmenter la spécificité des nanoparticules envers certains récepteurs cellulaires, des fragments d'anticorps (dirigés contre le récepteur à la transferrine) ont été greffés à leur surface. Leur capacité à se lier avec la membrane cellulaire ainsi que leur aptitude à diminuer l'expression d'un gène ciblé ont été examinées sur les cellules PC-3. Un effet maximal a été observé lorsque les micelles ciblées véhiculant un siRNA chimiquement modifié (*i.e.* censé offrir davantage de résistance à la dégradation enzymatique et moins de stimulation immunitaire) ont été utilisées. Ces données suggèrent que la combinaison d'un acide nucléique de dernière génération avec un vecteur optimal peut améliorer l'activité pharmacologique. De ce fait, la dose de vecteur nécessaire à l'obtention d'un effet thérapeutique pourrait être réduite.

Le **chapitre 4** explore comment la fonctionnalisation chimique de plusieurs séquences d'AON avec une sélection de molécules hydrophobes (*e.g.* acides gras) peut améliorer leur interaction avec la membrane cellulaire. L'effet de ces composés sur l'expression du gène ciblé a été évalué *in vitro* et les conjugués les plus efficaces (typiquement des AONs exhibant un acide docosanoïque) ont été sélectionnés pour des expériences ultérieures. Il a été notamment observé que l'addition d'albumine dans le milieu de transfection inhibait, et cela de manière graduelle, l'activité de ces composés. Néanmoins, cette perte d'efficacité a pu être surmontée en ajoutant des acides gras au milieu de transfection (par ex. acide décanoïque). Les informations tirées de ces observations pourraient ouvrir de nouvelles opportunités de recherche pour ce type de composés.

Le **chapitre 5** est une conclusion générale traitant des principaux résultats de cette thèse de doctorat ainsi que des limitations et des perspectives éventuelles de ces systèmes non viraux. En particuliers, leur éventuelle translation en phase préclinique est abordée.

Chapter 1

Background and Purpose

A simple and universal approach to alter the genetic expression of unhealthy tissues remains a holy grail in biological sciences. While most low molecular weight drugs block protein function, small nucleic acid-based therapeutics aim to inhibit protein expression by knocking down the corresponding mRNA. Thus, short RNA and DNA are considered as promising entities to treat several pathologies such as cancer, viral infections, dominant genetic disorders, and autoimmune diseases [1, 2]. In particular, starting with the pioneering work of Zamecnik and Stephenson in the late 1970's, antisense oligonucleotides (AONs) have been investigated as a means to achieve specific gene silencing [3, 4]. In the post-transcriptional antisense mechanism, AONs hybridize to complementary mRNA of the targeted gene *via* a presumed unassisted process [5, 6]. Once hybridized, AONs can block gene expression by i) sterically obstructing ribosomes (Fig. 1.1A), and ii) forming a DNA-RNA hybrid that can be a substrate for ribonuclease H (RNase H) enzyme (Fig. 1.1B) [7]. It has to be mentioned that another class of AONs, referred to as "splice switching" AONs, can be used to redirect the nuclear splicing of pre-mRNA. Such AONs are designed to be complementary to splice junctions (to include or exclude particular exons, or segments of introns) and to not recruit RNase H [8]. Splice switching AON technology is a promising approach to treat many human diseases involving splicing defects (*e.g.*, Duchenne muscular dystrophy (DMD)), but is out of the scope of the present thesis and will not be further discussed. Post-transcriptional gene silencing can also be caused by another, more complex, mechanism. In 1998, Fire and Mello reported a fundamental pathway in eukaryotic cells called RNA interference (RNAi), in which a long piece of double-stranded RNA (dsRNA) is able to induce the degradation of messenger RNA (mRNA) containing a complementary sequence [9]. This groundbreaking discovery has been hailed as a major breakthrough in biological research and rewarded Fire and Mello with a *Nobel Prize in Physiology or Medicine* in 2006. Briefly, in the endogenous mechanism, an enzyme called Dicer cleaves

dsRNA into small interfering RNAs (siRNAs), which are typically 21–23 nucleotides long. The siRNA is then loaded into a multicomponent machinery called RNA-induced silencing complex (RISC) [7]. The RISC unwinds and releases the sense strand, pairs the antisense (guide) strand with a complementary region in the targeted mRNA, and triggers the cleavage of the latter (Fig. 1.1C). The resulting mRNA fragments are then destroyed by cellular exonucleases [10, 11]. Finally, while protected inside RISC, the antisense strand can then be recycled to seek/degrade additional identical mRNA targets [10]. In order to mimic the Dicer cleavage products that are loaded into RISC, synthetic siRNAs that are 19–23 base pairs have been developed. Such exogenous siRNAs have been used as RNAi inducers in the present thesis (**chapters 3** and **4)** and will, from now on, be referred to as "siRNAs". Other noteworthy inducers of the RNAi mechanism include endogenous microRNA, "vector-based" short hairpin RNA, and synthetic single-stranded siRNA. Particularly, the latter was recently reported to be a valuable alternative to the commonly used double-stranded analog, as these atypical RNAi inducers intrinsically eliminate the possibility of off-target effects due to erroneous strand incorporation [12, 13]. However, despite their high potential, the *in vivo* use of this type of oligonucleotides remains impaired due to their substantial lower silencing activity and instability in serum compared to their double-stranded counterparts [14].

In spite the high initial optimism of leading industrial and academic laboratories, the clinical progress of nucleic acid-based medicines has been difficult. As of today, Fomivirsen (Vitravene™) and Mipomersen (Kynamro™), two chemically modified AONs used for the treatment of cytomegalovirus infections and familial hypercholesterolemia, respectively, are the only United States Food and Drug Administration (FDA) approved nucleic acid drugs with an antisense mechanism (Table 1.1, top). Fomivirsen approval in 1998 was a landmark for the field, but relatively few people have been treated with it and, due to commercial reasons, the drug was withdrawn from the European market in 2002 [7]. In the past several years, a few AONs, including a compound from Genta Inc. (Table 1.1, top), have failed to gain FDA approval after clinical phase III trials, resulting in a general malaise concerning the development of nucleic acid therapeutics [15]. Only recently this year Mipomersen became the second AON to receive FDA approval. As of today, about a dozen mRNA-targeted AONs are in advanced clinical trials (phase II or higher), mostly targeting cancer [1, 15, 16]. Among the more recently discovered RNAi-based therapeutics, siRNAs designed to treat age-related macular degeneration (AMD) and diabetic macular edema (DME) were the first to enter clinical trials in 2004 (Table 1.1, bottom) [17]. These siRNAs were designed against the

vascular endothelial growth factor pathway, but ultimately produced disappointing results. For instance, Bevasiranib, a 21-mer siRNA developed by Opko Health Inc., recently failed phase III trials for AMD due to poor efficacy in reducing vision loss (Table 1.1, bottom) [17]. Furthermore, and despite its successful completion of a phase II trial in the treatment of DME, no phase III trial has yet been announced for Bevasiranib. As of today, only a handful of siRNA therapeutics is in advanced clinical phase II (Table 1.1, bottom) [16, 18]. The slow clinical progress of these drug candidates highlights the difficulties associated with the formulation of clinically viable siRNA therapeutics, and nucleic acid drugs in general.

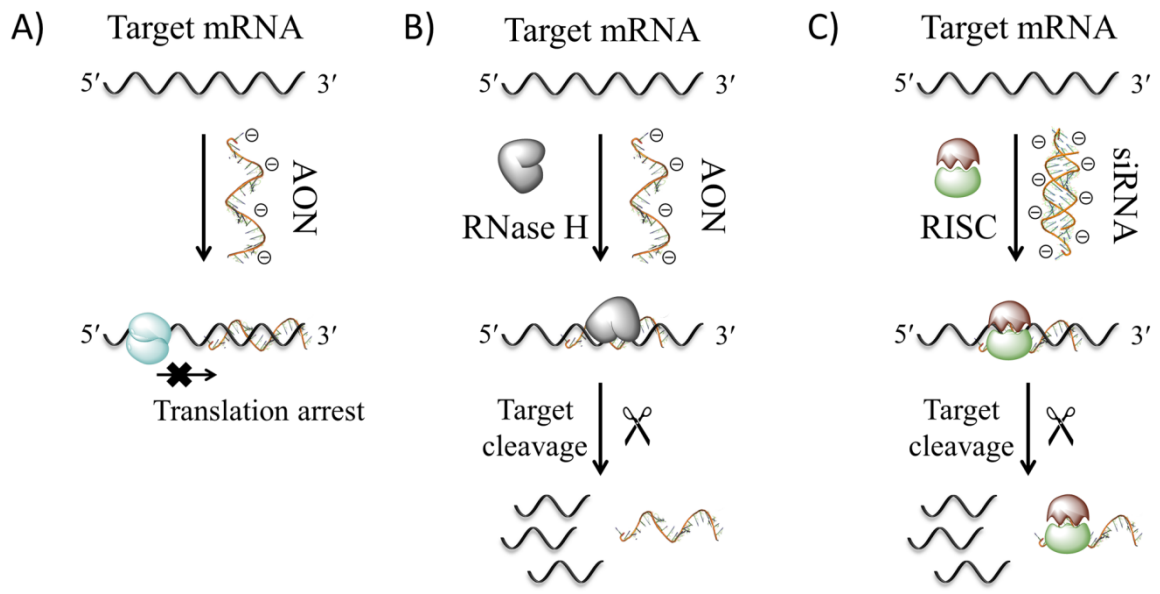


Figure 1.1. mRNA knockdown mechanisms for AONs and siRNA. A) Antisense oligomer binds to target mRNA and sterically obstructs ribosome, leading to translation arrest. B) AON pairs with target mRNA to form a DNA-RNA hybrid, which is subsequently recognized and cleaved by RNase H. C) siRNA duplex is loaded into RISC and target mRNA is cleaved through catalytic-type mechanism. Partially reproduced from Singh *et al.*, with permission from Springer [7].

Table 1.1. Selected AON (top) and siRNA (bottom) therapeutics in advanced clinical trials stage. Information was gathered from a variety of sources including ClinicalTrials.gov, company press releases, and reports in the literature. Updates about the status of selected clinical trials can be found at ClinicalTrials.gov with the NCT number; http://clinicaltrials.gov/show/NCT#.

	Sponsor(s)	Product	Route	Indication	Status
AONs	Novartis/Isis Pharm.	Fomivirsen	Intravitreal	CMV retinitis	Approved [a]
	Genzyme/Isis Pharm.	Mipomersen	Subcutaneous	Cardiovascular	Approved
	Genta Inc.	Oblimersen	Intravenous	Oncology	Phase III (NCT00085124)
	Teva/Isis Pharm./OncoGenex	Custirsen	Intravenous	Oncology	Phase III (NCT01578655)
	Antisense Pharm.	Trabedersen	Intratumoral	Oncology	Phase III (NCT00761280) [b]
	Gene Signal	Aganirsen	Ophthalmic drops	CoNV	Phase III [c]
	Isis Pharm.	Alicaforsen	Intravenous	IBD	Phase III (NCT00048113)
siRNAs	Opko Health Inc.	Bevasiranib	Intravitreal	AMD	Phase III (NCT00499590) [b]
			Intravitreal	DME	Phase II (NCT00306904)
	Alnylam/Cubist/Kyowa Kirin	ALN-RSV01	Nebulization	RSV infections	Phase IIb (NCT01065935)
	Quark/Pfizer/Silence	PF-655	Intravitreal	AMD	Phase II (NCT00713518)
			Intravitreal	DME	Phase II (NCT01445899)
	ZaBeCor	Excellair™	Inhalation	Asthma	Phase II [c]
	Sylentis	SYL-040012	Ophthalmic drops	Glaucoma	Phase II (NCT01739244)
	Quark Pharm.	QPI-1002	Intravenous	DGF	Phase I/II (NCT00802347)

AMD, age-related macular degeneration; CMV, cytomegalovirus; CoNV, corneal neovascularization; DGF, prevention of delayed graft function (kidney); DME, diabetic macular edema; IBD, inflammatory bowel disease; RSV, respiratory syncytial virus infection (lung).
[a] Product withdrawn at the request of marketing authorization holder.
[b] Study was terminated or withdrawn.
[c] Unknown or non-existent NCT number.

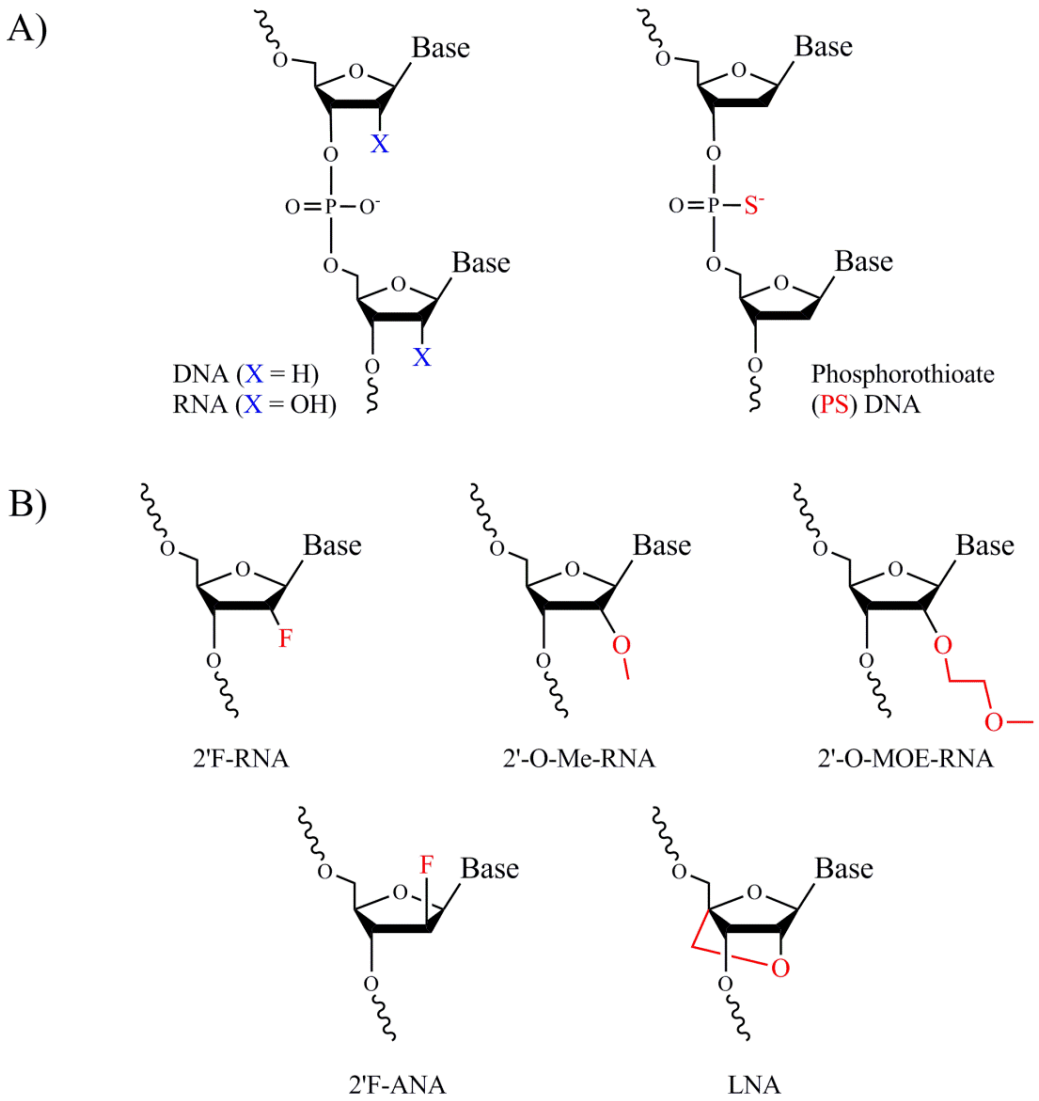


Figure 1.2. Structures of some common chemical modifications on nucleotides. A) Selected internucleotide linkage design: phosphodiester (left) and phosphorothioate (right) linkages. B) Chemical modifications on sugar units. Top row: 2′-fluoro, 2′-O-methyl and 2′-O-methoxyethyl analogs of RNA. Bottom row: 2′-fluoro-arabinonucleic acid (2′F-ANA) and the conformationally constrained modification, locked nucleic acid (LNA).

In an interview to the journal *Science*, John J. Rossi, a pioneer in the field of RNAi therapeutics based at the Beckman Research Institute of Hope of Duarte, California, stated that the three biggest challenges with RNAi therapeutics remain "*delivery, delivery, and delivery*" [19]. In fact, the success of nucleic acid therapeutics depends not only on the intracellular recognition event between the mRNA of the gene to be inhibited and the synthetic nucleic acid drug, but also on many upstream pharmacokinetic processes. For instance, unmodified nucleic acids are rapidly degraded by nucleases, and their anionic nature renders them almost impermeable to the negatively-charged cell membranes. In order to achieve the desired pharmacological effect, a state of the art nucleic acid drug should exhibit

i) a favorable pharmacokinetic profile, ii) low immunogenicity, iii) resistance to nucleases, iv) intracellular bioavailability, and v) high affinity for targeted mRNA [15]. Attempts to imbue nucleic acids with these desirable properties have produced an impressive number of backbone chemical modifications compatible with nucleic acid-mediated gene silencing (for reviews on these modifications, see references [20, 21]).

The first generation of backbone chemical alterations utilized 2′-deoxyribonucleotide phosphorothioate (PS) modifications (Fig. 1.2A). It entailed AONs with increased stability in biological systems and significantly increased their biological half-life [22]. However, further improvements were still necessary. The second generation of AON constructs typically employed chemically modified sugars, paired with a PS backbone, in order to further enhance stability towards nucleases, and in some cases potency. Common structures of chemically-modified sugars for these applications include 2′-fluoro, 2′-O-methyl and 2′-O-methoxyethyl (2′-O-MOE) analogs of RNA (Fig. 1.2B). A promising example of new emerging AONs is Mipomersen (Kynamro™), a second generation AON developed by ISIS Pharmaceuticals and Genzyme to treat hypercholesterolemia [23]. It consists in a gapmer containing PS-DNA and 2′-O-MOE-RNA (Fig. 1.2) (Table 1.1, top) [23]. Despite some mild toxicity observed, four separate phase III trials were successfully completed and the compound recently received FDA approval (Table 1.1, top) [24]. It is noteworthy that previous experience with AONs is directly relevant to the clinical progress of siRNAs [6]. For example, the poor *in vivo* stability of siRNA could be readily corrected using the rich toolbox of chemical modifications originally developed for AONs [6, 25]. However, chemical modifications of the backbone structure do not generally solve low membrane permeability problems and only modestly improve the pharmacokinetic and/or biodistribution profiles [22, 26].

Today, most clinical trials with nucleic acid drug candidates are performed in the absence of a delivery system and are thus, in general, intended for either local delivery or kidney/liver accumulation (Table 1.1). Indeed, systemic delivery of nucleic acids administered alone (*i.e.,* naked) was rapidly shown to be difficult, thus calling for novel approaches. Strategies aimed at solving these problems can be divided into two categories: (i) the use of particle-based systems (Fig. 1.3A) and (ii) the chemical derivatization of the nucleic acid with functional molecules (Fig. 1.3B). It has to be noted that viral vectors-based particles (*e.g.*, retroviruses and adenoviruses) are beyond the scope of this thesis and will not be addressed. Readers are invited to read the following contributions for more information on this topic [27, 28].

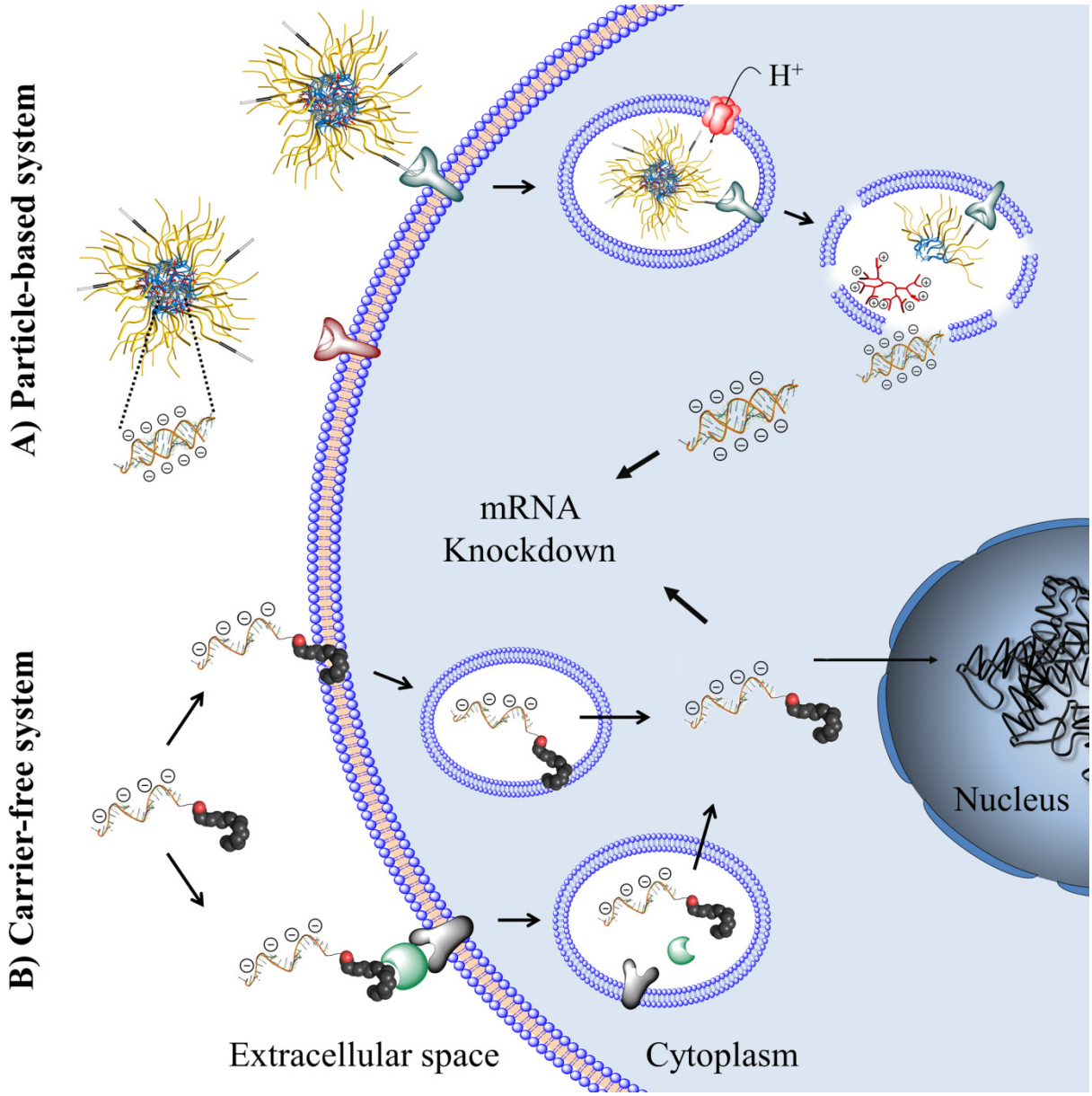


Figure 1.3. Schematic illustrations of A) particle-based and B) carrier-free nucleic acid delivery systems discussed in the present thesis. Once released in the cytoplasm, the antisense drugs can modulate the protein expression through their interactions with complementary mRNA.

1.1. Particle-based delivery systems

Common approaches used to improve the performance of nucleic acids consist in complexing them with either positively-charged lipids (*e.g.*, Lipofectamine™) or polymers (*e.g.*, poly(ethylene imine) (PEI)) to form lipoplexes [29] or polyplexes [30], respectively. Such systems assemble into aggregates, vesicles, or micelles. Cationic lipid- and polymer-based nanoparticles are frequently used in cell culture assays due to their ability to interact with the cell membrane [31]. Usually, excess of positive charges within the assembly is a prerequisite for high transfection efficiency. On the other hand, under *in vivo* conditions, highly charged nanoparticles will lead to opsonization by serum proteins, immunogenicity, and accumulation in the organs of the mononuclear phagocytic system (*i.e.*, liver and spleen) [32]. Such limitations can be partially circumvented by covering the nanoparticle with a hydrophilic polymer, such as poly(ethylene glycol) (PEG), to confer the assembly a neutral surface charge [30]. In the late 1990's, Wagner and co-workers investigated the *in vitro* and *in vivo* properties of DNA/PEI complexes before and after covalent conjugation of PEG [33]. Interestingly, they found that upon incubation with whole blood, the positively charged non-PEGylated DNA complexes were not only binding to plasma proteins such as fibrinogen and fibronectin, but also to erythrocytes [33]. PEGylation of the nanoassembly strongly reduced plasma protein binding and erythrocyte aggregation. Furthermore, they observed that 30 min post-intravenous injection into mice, approximately 0.5% and 30% of the injected DNA dose of DNA/PEI and DNA/PEI/PEG constructs, respectively, was still detectable in plasma. This landmark paper exposed the strong benefits of PEGylation on the *in vivo* characteristics of particles, mainly by reducing their toxicity and prolonging their circulation time. In cancer therapy, the ultimate goal of a nanomedicine is to accumulate at the tumor site. A unique feature of many solid tumors is their fenestrated vasculature, often resulting in an enhanced extravasation of macromolecules from tumor blood vessels and their retention on site, a phenomenon referred to as the enhanced permeation and retention (EPR) effect [34]. Serum-stable colloids with a size ranging from 10–100 nm and a stealth surface (*e.g.*, PEGylated), can, in theory, passively target their payload to specific tissues *via* the EPR effect.

Over the last two decades, the field of nanotechnology has undergone impressive growth with the development of a plethora of nanoscale constructs for the delivery of various types of drugs, including nucleic acids. It should be noted that, as adopted by a recent recommendation of the European Commission, the definition of a nanomaterial is: "*a natural, incidental or manufactured material containing particles, in an unbound state or as an*

aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm […]" [35]. Of particular interest in the wide range of nanosized constructs available for nucleic acid drug delivery are polyion complex micelles (PICMs). These nanocarriers typically result from cooperative electrostatic interactions between the genetic material and a cationic copolymer presenting a water-soluble nonionic segment (*e.g.*, PEG), giving the complexes a core/shell architecture [36]. While conferring the nanocomplexes with a stealth surface was shown to be important for prolonged circulation in blood (and thus accumulation at tumor site), it often reduced cellular uptake. A common strategy to overcome this problem consists of coupling targeting ligands to the outer surface of the carrier, thus facilitating its homing to specific cellular receptors. Ideally, these receptors should be overexpressed by tumor cells compared to those in healthy tissues [37]. Thanks to recent advances in understanding the biology of tumoral tissues, a wide variety of molecules including antibodies [38], peptides [39], aptamers [40], and sugars [41] have been identified for specific cellular delivery of nucleic acid drugs. Some of these actively target complexes and allow them to enter the cells *via* receptor-mediated endocytosis, thus directly delivering the drug in the vicinity of its site of action. In 2008, the first siRNA-containing nanoparticle exploiting receptor-mediated delivery entered clinical trials (NCT00689065). The construct (clinical version denoted as CALAA-01) was developed by Calando Pharmaceuticals (an Arrowhead Research Corp. company) to treat relapsed/refractory cancers and consisted of a cyclodextrin polymer-based particle, complexed with a therapeutic siRNA. It was coated with PEG for stability and bore the human transferrin protein as targeting ligand [42]. Interestingly, this landmark study also showed first mechanistic evidence of RNAi in human [42]. CALAA-01 has recently been enrolled in a phase Ib clinical trial.

Progressive optimization of the nanocarriers has led to the development of finely tuned stimulus-sensitive complexes, which contain triggerable mechanism capable of releasing their payload at the appropriate moment. A large variety of internal and external stimuli have been investigated to destabilize polymeric micelles including pH, temperature, enzymatic reactions, redox processes, ultrasound, light, and their combinations [43, 44]. Following endocytosis, targeted PICMs are immediately transported into endocytic vesicles. Nanocomplexes devoid of endosomolytic properties are often successively processed to early endosomes (pH ~6), late endosomes (pH 5–6), and finally lysosomes (pH ~4.5), where they are eventually degraded [45]. Therefore, it was rapidly observed that the transfection efficiency of nucleic acid delivery systems did not only correlate to the level of cellular uptake but also to their

ability to escape endosomal compartments [46]. Certain cationic polymers, such as poly(amido amine) (PAMAM) dendrimers and PEI, are believed to have intrinsic endosomolytic activity. PAMAM dendrimers have two types of amino groups: terminal primary amines and internal tertiary amines with pK_a's in the range of 9.0 and 6.0, respectively [47]. During the maturation of endosomes, membrane-bound adenosine triphosphatase proton pumps actively transfer protons from the cytosol to the endosomes. At this stage, the amino groups become protonated and the dendrimer is thought to buffer endosomal acidification. Accumulation of protons is followed by passive entry of chloride ions, which increases ion concentration leading to water influx, ultimately causing swelling and rupture of endosomes [45]. This phenomenon is referred to as the proton sponge effect and was first proposed by Boussif *et al*. to explain the high transfection efficiency of PEI/DNA and PAMAM/DNA polyplexes [48]. Although the proton sponge hypothesis was well received, its contribution to endosomal escape is still questioned and a few researchers have provided evidence that some polycationic aggregates could also physically bind to the endosomal/lysosomal membrane and permeabilize it by forming nanoholes [49, 50]. Despite some controversy, it is evident that polyplexes prepared with PEI and PAMAM can escape the endosomal/lysosomal compartment to some extent and increase cytoplasmic availability of the nucleic acid cargo. Similarly, synthetic anionic polymers responding to pH and capable of disrupting lipid membranes have been developed [51, 52]. Titratable polyanions, and particularly amphipathic polymers bearing carboxylate groups, have been used in recent years to produce a variety of pH-sensitive colloids. This is because the transition pH of polycarboxylates (typically around 4–6) is in a physiologically relevant range for drug delivery applications. These different systems are reviewed in depth in **chapter 2** of this thesis. Clearly, we believe that loading modified nucleic acids into a robust carrier system based on some or all of the aforementioned principles is conceptually one of the best approaches to successfully deliver nucleic acid drugs. One aim of this thesis was to design a clinically viable PICM system capable of circumventing the current limitations in nucleic acid delivery. We describe herein (**chapter 3**) an optimized approach for the preparation of smart PICMs for the delivery of nucleic acid drugs. The selected approach relies on the combined efficiency of cationic condensing agents with pH-responsive anionic diblock copolymers (Fig. 1.4). The PICMs were decorated with an antibody fragment (fragment antigen binding (Fab′)) directed against our cellular model (transferrin receptor of prostate cancer cells). However, the delivery vehicle could potentially be tailored on demand to target a large variety of tissues.

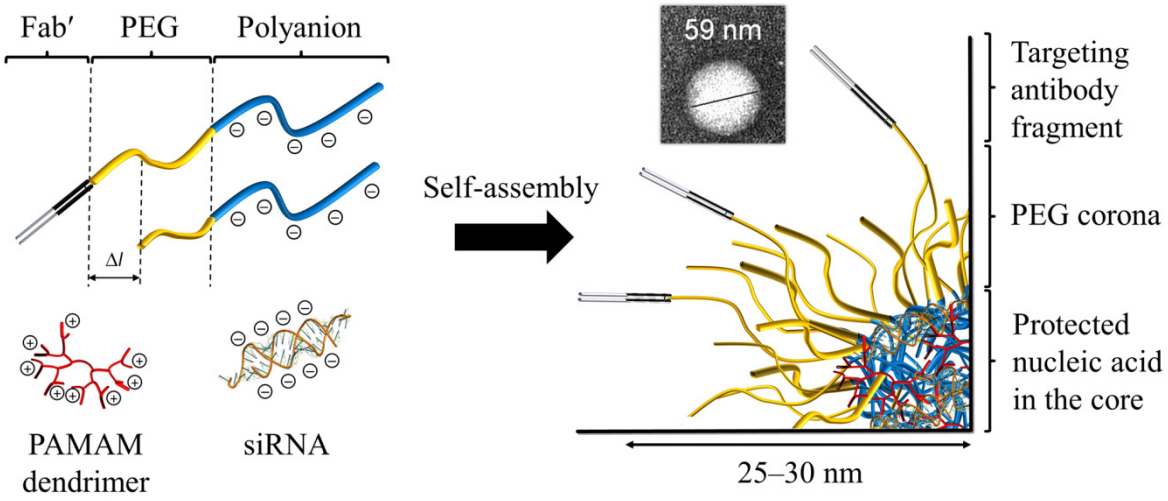


Figure 1.4. Schematic representation of PICM formation through the self-assembly of pH-responsive anionic diblock copolymers with cationic PAMAM dendrimers and selected nucleic acid therapeutic. The resulting nanocomplex has a typical core/shell structure with a size of *ca.* 50 nm and a near-neutral zeta potential. Inset represents a single PICM as observed by transmission electron microscopy (negative stain).

1.2. Carrier-free approaches

The limitations of particle-based nucleic acid delivery systems often reside in the complexity of their design. In fact, the introduction of multiple polymers, necessary to complex the nucleic acid cargo and/or to endow the delivery vehicle with advanced features (*e.g.*, targeting, endosomolytic properties, etc.), can lead to unexpected *in vivo* side-effects. In particular, the presence of large, non-biodegradable, and positively charged polymers has often been associated with severe cellular toxicity [53]. Therefore, an alternative approach used to improve the properties of nucleic acids lies in their direct chemical derivatization with a functional molecule. Such conjugates further differ from particle-based systems, where the nucleic acid of interest is, in the majority of cases, held into the nanostructure by ionic bonds. Strategies based on the chemical derivatization of nucleic acids involve the direct administration of an uncomplexed (*i.e.*, carrier-free) nucleic acid that is covalently linked to a targeting ligand (*e.g.*, antibody, aptamer) [54], cell penetrating peptide (CPP) [55], or hydrophobic moiety (*e.g.*, lipid and fluorophore) [56]. For example, aptamer-siRNA conjugates, also referred to as "*chimeras*", were developed to target prostate specific membrane antigen (PSMA) glycoproteins [54]. A chemically optimized version of the chimeric molecule displayed significant anti-tumor activity against PSMA-expressing tumors in mice following systemic administration [54]. Furthermore, it was found that appending a PEG moiety to the unconjugated-end of the siRNA, thus forming an aptamer-siRNA-PEG macromolecule, further enhanced its biological effect by promoting increased circulation time [54]. The chemical derivatization of nucleic acids not only aims to improve their biodistribution, cell/tissue specificity, or cellular uptake but can also endow them with interesting features, such as fluorescence [57]. For instance, in the mid 2000's Berezhna *et al.* used fluorescently labeled RNAs to unravel the mechanism of RNA silencing by RNAi [58]. It has to be noted that, even though the exact mechanisms involved during the cellular uptake of these systems remain unclear, most of them probably share the same port of entrance: endocytosis. In addition, according to the high concentrations of conjugates usually needed to achieve a significant biological effect, their transit to the cytoplasm seems to be relatively inefficient.

Of particular interest among the vast array of functional molecules that have been conjugated to nucleic acids are CPPs. CPPs are a class of short peptides (generally < 30 amino acids) that have the ability to trigger the cellular uptake of a cargo across the cell membrane, thereby increasing its intracellular availability [59]. These peptides were initially

derived from natural proteins, such as the transcription-transactivating protein of human immunodeficiency virus type-1 or penetratin, a 16-mer peptide present in the homeodomain of *Drosophila antennapedia*. Structurally, most CPPs are polycationic peptides rich in arginine and lysine residues, although some include hydrophobic sequences endowing the peptides with additional membrane interaction features [59]. An important factor to consider for the successful cytosolic delivery of a CPP-cargo is the nature of the cargo itself (*e.g.*, size and charge) as it has been shown to play a key role in the uptake mechanisms [60]. For instance, the delivery of a positively-charged CPP covalently conjugated to an anionic AON or siRNA was rapidly shown to be quite challenging. Indeed, the spontaneous charge neutralization event between the basic residues of the CPP and the anionic backbone of the nucleic acid drastically reduces the cellular membrane affinity of the short peptide [61]. However, this problem can be solved by using uncharged oligonucleotides such as peptide nucleic acid (PNA) and phosphorodiamidate morpholino-oligomer (PMO) [55]. For example, Goyenvalle *et al.* recently reported excellent antisense-mediated exon-skipping activity in cell culture, and promising therapeutic performances in mouse models of DMD following repeated intraperitoneal injection of a CPP-conjugated PMO [62]. Even though the efficiency of CPPs conjugated to uncharged backbone oligonucleotides has been demonstrated in a number of studies, the desired biological effects are, in most cases, only obtained when the conjugates are applied in micromolar concentrations (or correspondingly high *in vivo* concentrations). There is now a consensus that the major port of entrance for cationic CPPs is *via* endocytosis (mainly macropinocytosis) [63]. Consequently, to achieve a significant therapeutic effect, CPP-cargo conjugates must be able to successfully escape the endosomes. An interesting approach to address this consists in preparing CPP-PNA conjugates that also contain a simple lipophilic domain, in the form of a fatty acid, as this was anticipated to promote endosomal escape [64]. It was found that the presence of the fatty acid moiety could increase the *in vitro* biological activity of the conjugate by up to 2 orders of magnitude compared to the control CPP-PNA conjugate [64]. Although the use of "*new generation*" CPPs to enhance the cellular bioavailability of nucleic acid (or analogs) seems quite promising in cell cultures, the *in vivo* applications may be limited due to yet uncharacterized effects on gene expression and potential immune activation. Furthermore, CPPs interact with non-specific tissues or serum proteins [65] in a similar way as with cell membranes, thus probably restricting their clinical potential to local delivery.

Another type of chemical derivatization that has been investigated to enhance the biological effects of nucleic acid drugs consists in conjugating them to a lipophilic moiety. These types of hydrophobized macromolecular structures are often referred to as amphiphilic conjugates. Such modifications have been initially proposed to improve cellular uptake by i) reducing the hydrophilic character of nucleic acid and/or ii) taking advantage of the lipoprotein-mediated endocytic pathway [20]. For this reason, cholesterol-containing conjugates are, to date, among the most studied lipophilic nucleic acid conjugates [66]. In fact, cholesterol has been shown to promote the interaction of the conjugate with serum lipoproteins and subsequently favor their uptake *via* mechanisms involving the hepatic lipoprotein receptors [67]. This particularity of lipophilic conjugates (*i.e.*, the ability to interact with lipoproteins) makes them suitable agents for liver targeting, and they have been mainly exploited in this context [66]. For instance, Nishina *et al.* recently investigated the *in vivo* delivery of siRNA to the liver by conjugation of tocopherol (*i.e.*, vitamin E) [68]. The lipophilic moiety was attached to the 5′-end of the antisense strand of an siRNA targeting the apolipoprotein B (apoB) [68]. After intravenous injection in mice, it was found that the conjugate was using the physiological pathways of vitamin E transport to the liver, and successfully downregulated the endogenous apoB mRNA [68]. Given the success of cholesterol-based lipophilic conjugates, Wolfrum and co-workers investigated the effect of alternative lipid-like molecules to improve siRNA delivery [67]. With the aim of elucidating how lipophilic nucleic acid conjugates interact with lipoproteins for liver delivery, various fatty acids were conjugated to an apoB-targeting siRNA and injected intravenously into mice [67]. Interestingly, it was observed that shorter fatty acids chain lengths ($<C_{18}$) were inefficient, whereas longer chain lengths, such as docosanol (C_{22}), had a potent effect on apoB transcript levels [67]. However, these promising *in vivo* results have to be contrasted with cell culture observations, where serum addition often sequesters the lipophilic conjugate leading to its inactivation. Furthermore, acceptable silencing levels (in serum-free conditions) are usually achieved with high nucleic acid doses (in the 2–10 μM range). Such observations raise important interrogations on the uptake mechanisms of lipophilic conjugates. For instance, Borisenko *et al.* studied the incorporation of fatty acid conjugates onto the outer leaflet of the plasma membrane [69]. In their experiments, they observed that the conjugates were rapidly anchored (through their hydrophobic domain) to the cell membrane in a dose-dependent fashion and were then progressively taken up by the cells, most probably *via* endocytosis [69]. In summary, it seems that the uptake mechanisms of lipophilic nucleic acid conjugates involve different endocytic pathways, with many contradictions needing to be

resolved. In **chapter 4** of the present thesis, we describe the synthesis of amphiphilic AON conjugates and the effect of serum proteins, such as human serum albumin (HSA), which led to the total inhibition of their silencing activity. We further propose a strategy to restore their *in vitro* silencing potential by supplementing the medium with short fatty acids in order to competitively bind/block the protein binding sites (Fig. 1.5).

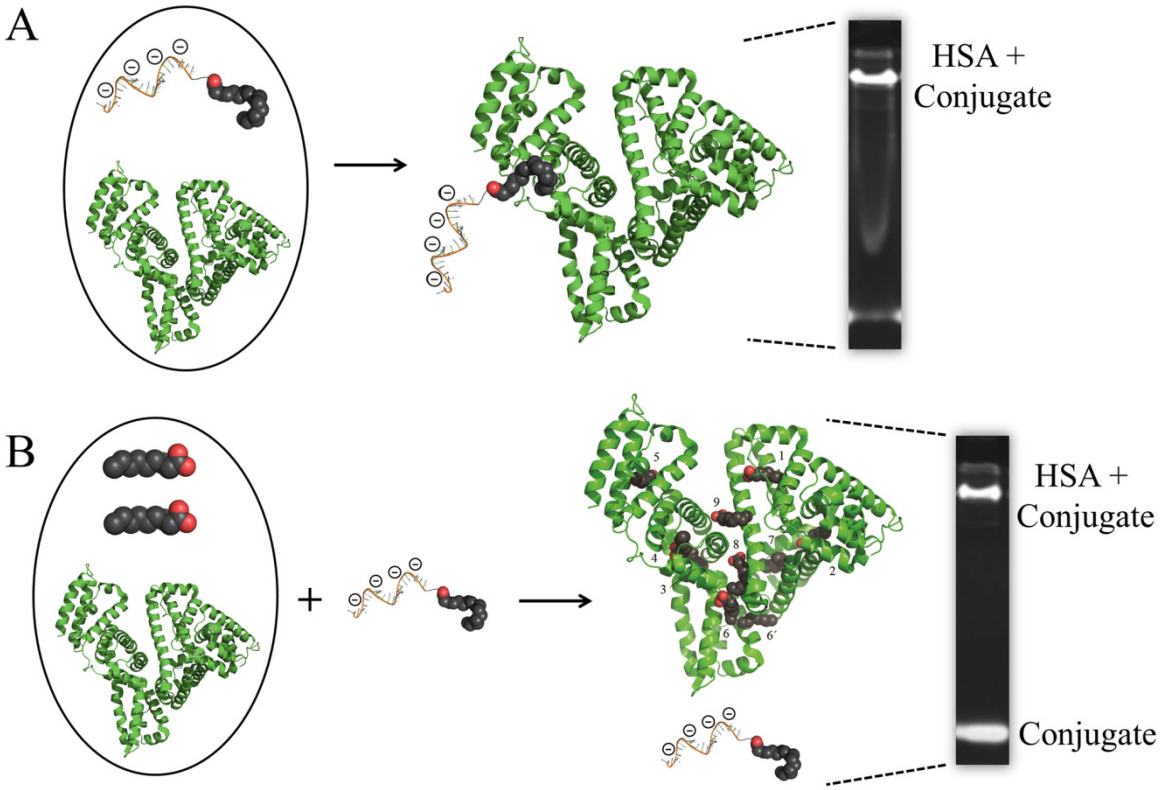


Figure 1.5. Fatty acid displacement assay. (A) Schematic representation of amphiphilic AON conjugate and human serum albumin (HSA) interactions (not on scale). Migration of conjugates in PAGE is significantly reduced (right). (B) Schematic representation of free fatty acids and HSA interactions followed by further incubation with amphiphilic AON conjugate. Part of the migration of conjugates, as a free compound, could be restored (right).

1.3. References

[1] Bennett CF, Swayze EE. RNA targeting therapeutics: Molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu Rev Pharmacol Toxicol. 2010;50:259-93.

[2] Burnett JC, Rossi JJ, Tiemann K. Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol J. 2011;6:1130-46.

[3] Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A. 1978;75:280-4.

[4] Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc Natl Acad Sci U S A. 1978;75:285-8.

[5] Juliano R, Alam MR, Dixit V, Kang H. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Res. 2008;36:4158-71.

[6] Corey DR. RNA learns from antisense. Nat Chem Biol. 2007;3:8-11.

[7] Singh Y, Murat P, Spinelli N, Defrancq E. Oligonucleotide conjugates: Rationale, synthesis, and applications. In: Erdmann VA, Barciszewski J, editors. Nucleic Acids Sequences to Molecular Medicine: Springer Berlin Heidelberg; 2012. p. 85-120.

[8] Sazani P, Kole R. Therapeutic potential of antisense oligonucleotides as modulators of alternative splicing. J Clin Invest. 2003;112:481-6.

[9] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391:806-11.

[10] Whitehead KA, Langer R, Anderson DG. Knocking down barriers: Advances in siRNA delivery. Nat Rev Drug Discov. 2009;8:129-38.

[11] Rand TA, Petersen S, Du F, Wang X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell. 2005;123:621-9.

[12] Yu D, Pendergraff H, Liu J, Kordasiewicz HB, Cleveland Don W, Swayze Eric E, et al. Single-stranded RNAs use RNAi to potently and allele-selectively inhibit mutant huntingtin expression. Cell. 2012;150:895-908.

[13] Lima WF, Prakash TP, Murray HM, Kinberger GA, Li W, Chappell AE, et al. Single-stranded siRNAs activate RNAi in animals. Cell. 2012;150:883-94.

[14] Elsner M. Single-stranded siRNAs for *in vivo* gene silencing. Nat Biotech. 2012;30:1063-.

[15] Watts JK, Corey DR. Silencing disease genes in the laboratory and the clinic. J Pathol. 2012;226:365-79.

[16] Lightfoot HL, Hall J. Target mRNA inhibition by oligonucleotide drugs in man. Nucleic Acids Res. 2012;40:10585-95.

[17] Burnett John C, Rossi John J. RNA-based therapeutics: Current progress and future prospects. Chem Biol. 2012;19:60-71.

[18] Forbes DC, Peppas NA. Oral delivery of small RNA and DNA. J Control Release. 2012;162:438-45.

[19] Perkel J. RNAi therapeutics: A two-year update. Science. 2009;326:454.

[20] Manoharan M. Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. Antisense Nucleic Acid Drug Dev. 2002;12:103-28.

[21] Watts JK, Deleavey GF, Damha MJ. Chemically modified siRNA: Tools and applications. Drug Discov Today. 2008;13:842-55.

[22] Agrawal S, Temsamani J, Tang JY. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. Proc Natl Acad Sci U S A. 1991;88:7595-9.

[23] Raal FJ, Santos RD, Blom DJ, Marais AD, Charng M-J, Cromwell WC, et al. Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: A randomised, double-blind, placebo-controlled trial. Lancet. 2010;375:998-1006.

[24] McGowan MP, Tardif J-C, Ceska R, Burgess LJ, Soran H, Gouni-Berthold I, et al. Randomized, placebo-controlled trial of Mipomersen in patients with severe hypercholesterolemia receiving maximally tolerated lipid-lowering therapy. PLoS One. 2012;7:e49006.

[25] Allerson CR, Sioufi N, Jarres R, Prakash TP, Naik N, Berdeja A, et al. Fully 2'-modified oligonucleotide duplexes with improved *in vitro* potency and stability compared to unmodified small interfering RNA. J Med Chem. 2005;48:901-4.
[26] Sands H, Gorey-Feret LJ, Cocuzza AJ, Hobbs FW, Chidester D, Trainor GL. Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. Mol Pharmacol. 1994;45:932-43.
[27] Waehler R, Russell SJ, Curiel DT. Engineering targeted viral vectors for gene therapy. Nat Rev Genet. 2007;8:573-87.
[28] Ma Y, Nolte RJM, Cornelissen JJLM. Virus-based nanocarriers for drug delivery. Adv Drug Deliv Rev. 2012;64:811-25.
[29] Yokota T, Iijima S, Kubodera T, Ishii K, Katakai Y, Ageyama N, et al. Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C. Biochem Biophys Res Commun. 2007;361:294-300.
[30] Ballarín-González B, Howard KA. Polycation-based nanoparticle delivery of RNAi therapeutics: Adverse effects and solutions. Adv Drug Deliv Rev. 2012;64:1717-29.
[31] Aliabadi HM, Landry B, Sun C, Tang T, Uludağ H. Supramolecular assemblies in functional siRNA delivery: Where do we stand? Biomaterials. 2012;33:2546-69.
[32] Daka A, Peer D. RNAi-based nanomedicines for targeted personalized therapy. Adv Drug Deliv Rev. 2012;64:1508-21.
[33] Ogris M, Brunner S, Schüller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 1999;6:595-605.
[34] Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. J Control Release. 2000;65:271-84.
[35] European Commission Recommendation 2011/696/EU, OJ L 275, 20.10.2011.
[36] Harada A, Togawa H, Kataoka K. Physicochemical properties and nuclease resistance of antisense-oligodeoxynucleotides entrapped in the core of polyion complex micelles composed of poly(ethylene glycol)-poly(L-lysine) block copolymers. Eur J Pharm Sci. 2001;13:35-42.
[37] Sawant RR, Jhaveri AM, Torchilin VP. Immunomicelles for advancing personalized therapy. Adv Drug Deliv Rev. 2012;64:1436-46.
[38] Palanca-Wessels MC, Convertine AJ, Cutler-Strom R, Booth GC, Lee F, Berguig GY, et al. Anti-CD22 antibody targeting of pH-responsive micelles enhances small interfering RNA delivery and gene silencing in lymphoma cells. Mol Ther. 2011;19:1529-37.
[39] Sethuraman V, Lee M, Bae Y. A biodegradable pH-sensitive micelle system for targeting acidic solid tumors. Pharm Res. 2008;25:657-66.
[40] Farokhzad OC, Karp JM, Langer R. Nanoparticle–aptamer bioconjugates for cancer targeting. Expert Opin Drug Deliv. 2006;3:311-24.
[41] Oishi M, Nagasaki Y, Itaka K, Nishiyama N, Kataoka K. Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. J Am Chem Soc. 2005;127:1624-5.
[42] Davis ME, Zuckerman JE, Choi CHJ, Seligson D, Tolcher A, Alabi CA, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature. 2010;464:1067-70.
[43] Sutton D, Nasongkla N, Blanco E, Gao J. Functionalized micellar systems for cancer targeted drug delivery. Pharm Res. 2007;24:1029-46.
[44] Ganta S, Devalapally H, Shahiwala A, Amiji M. A review of stimuli-responsive nanocarriers for drug and gene delivery. J Control Release. 2008;126:187-204.
[45] Liang W, Lam JKW. Endosomal escape pathways for non-viral nucleic acid delivery systems. In: Ceresa B, editor. Molecular Regulation of Endocytosis: InTech; 2012. p. 421-67.
[46] Medina-Kauwe LK, Xie J, Hamm-Alvarez S. Intracellular trafficking of nonviral vectors. Gene Ther. 2005;12:1734-51.
[47] Niu Y, Sun L, Crooks RM. Determination of the intrinsic proton binding constants for poly(amidoamine) dendrimers *via* potentiometric pH titration. Macromolecules. 2003;36:5725-31.

[48] Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. Proc Natl Acad Sci U S A. 1995;92:7297-301.
[49] Bieber T, Meissner W, Kostin S, Niemann A, Elsasser H-P. Intracellular route and transcriptional competence of polyethylenimine–DNA complexes. J Control Release. 2002;82:441-54.
[50] Hong S, Leroueil PR, Janus EK, Peters JL, Kober M-M, Islam MT, et al. Interaction of polycationic polymers with supported lipid bilayers and cells: Nanoscale hole formation and enhanced membrane permeability. Bioconjugate Chem. 2006;17:728-34.
[51] Yessine M-A, Meier C, Petereit H-U, Leroux J-C. On the role of methacrylic acid copolymers in the intracellular delivery of antisense oligonucleotides. Eur J Pharm Biopharm. 2006;63:1-10.
[52] Convertine AJ, Diab C, Prieve M, Paschal A, Hoffman AS, Johnson PH, et al. pH-responsive polymeric micelle carriers for siRNA drugs. Biomacromolecules. 2011;11:2904-11.
[53] Parhamifar L, Larsen AK, Hunter AC, Andresen TL, Moghimi SM. Polycation cytotoxicity: A delicate matter for nucleic acid therapy-focus on polyethylenimine. Soft Matter. 2010;6:4001-9.
[54] Dassie JP, Liu X-y, Thomas GS, Whitaker RM, Thiel KW, Stockdale KR, et al. Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. Nat Biotech. 2009;27:839-46.
[55] Lebleu B, Moulton HM, Abes R, Ivanova GD, Abes S, Stein DA, et al. Cell penetrating peptide conjugates of steric block oligonucleotides. Adv Drug Deliv Rev. 2008;60:517-29.
[56] Petrova NS, Chernikov IV, Meschaninova MI, Dovydenko IS, Venyaminova AG, Zenkova MA, et al. Carrier-free cellular uptake and the gene-silencing activity of the lipophilic siRNAs is strongly affected by the length of the linker between siRNA and lipophilic group. Nucleic Acids Res. 2012;40:2330-44.
[57] Asseline U. Development and applications of fluorescent oligonucleotides. Curr Org Chem. 2006;10:491-518.
[58] Berezhna SY, Supekova L, Supek F, Schultz PG, Deniz AA. siRNA in human cells selectively localizes to target RNA sites. Proc Natl Acad Sci U S A. 2006;103:7682-7.
[59] Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: From molecular mechanisms to therapeutics. Br J Pharmacol. 2009;157:195-206.
[60] Juliano RL, Ming X, Nakagawa O. The chemistry and biology of oligonucleotide conjugates. Acc Chem Res. 2012;45:1067-76.
[61] Fei L, Ren L, Zaro JL, Shen W-C. The influence of net charge and charge distribution on cellular uptake and cytosolic localization of arginine-rich peptides. J Drug Target. 2011;19:675-80.
[62] Goyenvalle A, Babbs A, Powell D, Kole R, Fletcher S, Wilton SD, et al. Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. Mol Ther. 2009;18:198-205.
[63] Juliano RL, Ming X, Nakagawa O. Cellular uptake and intracellular trafficking of antisense and siRNA oligonucleotides. Bioconjugate Chem. 2012;23:147-57.
[64] Koppelhus U, Shiraishi T, Zachar V, Pankratova S, Nielsen PE. Improved cellular activity of antisense peptide nucleic acids by conjugation to a cationic peptide-lipid (CatLip) domain. Bioconjugate Chem. 2008;19:1526-34.
[65] Kosuge M, Takeuchi T, Nakase I, Jones AT, Futaki S. Cellular internalization and distribution of arginine-rich peptides as a function of extracellular peptide concentration, serum, and plasma membrane associated proteoglycans. Bioconjugate Chem. 2008;19:656-64.
[66] Patwa A, Gissot A, Bestel I, Barthelemy P. Hybrid lipid oligonucleotide conjugates: Synthesis, self-assemblies and biomedical applications. Chem Soc Rev. 2011;40:5844-54.
[67] Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, et al. Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. Nat Biotechnol. 2007;25:1149-57.
[68] Nishina K, Unno T, Uno Y, Kubodera T, Kanouchi T, Mizusawa H, et al. Efficient *in vivo* delivery of siRNA to the liver by conjugation of α-tocopherol. Mol Ther. 2008;16:734-40.
[69] Borisenko GG, Zaitseva MA, Chuvilin AN, Pozmogova GE. DNA modification of live cell surface. Nucleic Acids Res. 2009;37:e28.

Chapter 2

pH-Sensitive Vesicles, Polymeric Micelles, and Nanospheres Prepared with Polycarboxylates

Arnaud E. Felber†, Marie-Hélène Dufresne†, Jean-Christophe Leroux

Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland

†AEF and MHD contributed equally to this work.

This chapter is published:

Advanced Drug Delivery Reviews, **2012**;64:979-92.

2.1. Introduction

Drug delivery systems capable of releasing their payload in response to stimuli have received much attention in recent years, whether to target tissues, to reach specific intracellular locations, or to promote drug release. Of the many stimuli that can be exploited, changes in pH are particularly interesting because pH gradients relevant for drug targeting can be found physiologically. For instance, gradients between normal tissues and some pathological sites, between the extracellular environment and some cellular compartments, and along the gastrointestinal (GI) tract are well characterized.

Some pathological states are associated with pH profiles different from that of normal tissues. Examples include ischemia, infection, inflammation, and tumor acquisition, which are often associated with acidosis [1-4]. For instance, compared to the normal blood pH of 7.4, extracellular pH values in cancerous tissues can be as low as 5.7 (though on average 6.8–7.0) [3]. This in part results from the fact that, because tumors proliferate rapidly, their vasculature is often disorganized and may be insufficient to fulfill the nutritional and oxygen needs of the expanding population of tumor cells, leading to hypoxia, production of lactic acid, and hydrolysis of ATP in an energy-deficient manner [2, 4]. Furthermore, whether in a state of deprived oxygen or not, many tumors have high rates of glycolysis, contributing to increased proton production [4]. Together with increased proton production, the poor lymphatic drainage and elevated interstitial pressure of tumors may lead to poor proton clearance, thus participating in the build-up of an acidic microenvironment that can be exploited in the design of pH-sensitive drug delivery systems.

Even greater pH differences can be found at the cellular level between the extracellular environment (pH 7.4) and intracellular compartments such as the endosomes and lysosomes (pH 4.5–6.5) [5]. This pH gradient is of particular importance since several drugs and drug carriers are taken up by endocytosis and found/trapped within endosomes and lysosomes [6-9]. Endocytosis is a process by which cells internalize macromolecules into membrane-bound transport vesicles that form following invagination and pinching off of the plasma membrane [10]. Depending on the exact route of entry and proteins involved, the internalized material will have different fates. For instance, material internalized *via* clathrin-coated vesicles will undergo acidification as the vesicles mature into early and late endosomes (pH 5.0–6.5) [5]. The material will then be trafficked to lysosomes, the terminal degradation compartments of the endocytic pathway, by various processes of content mixing between late endosomes and

lysosomes [11, 12]. Lysosomes not only maintain an internal acidic pH (pH 4.5–5.0) but also contain a great number of hydrolytic enzymes (*e.g.*, nucleases, proteases, phospholipases, esterases, and glycosidases) to degrade the entrapped molecules [13]. It is easily seen how drug delivery systems capable of exploiting the acidic pH of endosomes and lysosomes to evade these organelles and achieve substantial drug release to the cytosol would be valuable.

Alternatively, drugs administered by the oral route experience a pH gradient as they transit from the stomach (pH 1–2, fasted state) to the duodenum (pH of about 6), and along the jejunum and ileum (pH 6–7.5) [14, 15]. The oral route is the route of choice for the delivery of drugs because it is simple to implement and improves patient compliance and quality of life. However, not all drugs possess desirable properties for this route of administration. Notably, peptides, proteins, and nucleic acid drugs administered orally are subject to inactivation in the acidic environment of the stomach and to degradation by digestive enzymes [16, 17]. In addition, poor transport of these highly hydrophilic and large drug molecules across the epithelial membrane limits their absorption and oral bioavailability. Aside from poor permeability, poor water solubility may also greatly restrict the oral bioavailability of drugs [16]. Strategies to prevent GI degradation and/or to promote absorption in the intestine by making use of the pH gradient found along the GI tract appear promising.

In this manuscript, we review drug delivery systems in which protonation (or deprotonation) of free carboxylic acid groups from a polymer triggers drug release. Depending on the intended route of administration and the physical barriers to overcome, three potentially interrelated pH-responsive drug release approaches/strategies can be envisaged. These are based on either i) dissociation or ii) destabilization (*via* collapse or swelling) of drug delivery systems upon changes in pH, or on iii) pH-dependent changes in partition coefficient between the drug and the delivery vehicle (Fig. 2.1). Typically, such systems are stable in their storage conditions but respond quickly when their trigger pH is reached. A prompt response is crucial when the residing time at the target site is short (*e.g.*, endosomes fuse with lysosomes within 30 min of cellular uptake) or to achieve sufficient drug concentrations at the target site. In that respect, titratable drug delivery systems might be advantageous over systems in which drug release is promoted by hydrolysis of a pH-sensitive linkage. Indeed, drug release kinetics from hydrolyzable drug delivery systems is often difficult to adjust, with the systems being either too labile or overly stable. Hydrolyzable drug

delivery systems will not be further addressed in this review and readers are invited to read the following contributions for more information on this topic [18, 19].

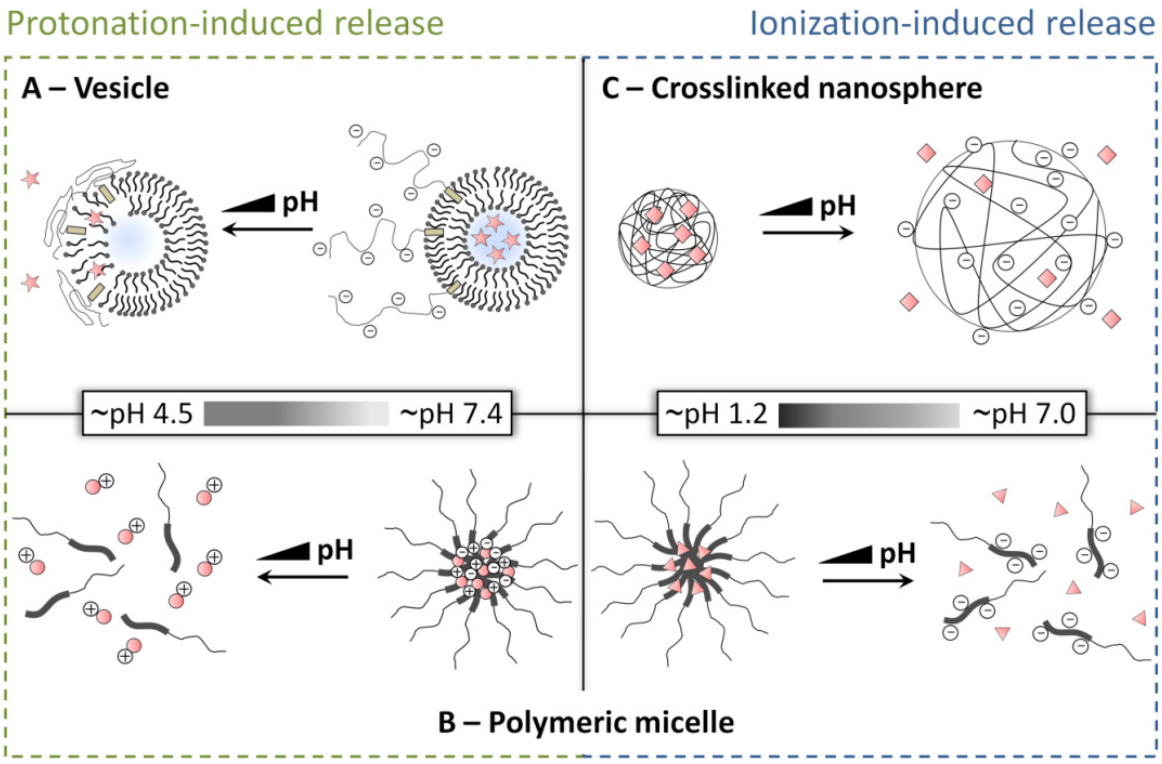


Figure 2.1. Schematic representation of drug release mechanisms from liposomes, multimolecular PMs, and cross-linked polymeric nanospheres prepared with polycarboxylates. (A) Collapse of the polyanion makes the liposomal membrane leaky and promotes efflux of the drug from the liposomes. (B) Protonation-induced (left) and ionization-induced (right) destabilization of multimolecular PMs. (C) Ionization-induced swelling leads to drug release from cross-linked polymeric nanospheres.

Of the available titratable anions, we will focus on carboxylic acids as they are the most commonly used. This is because the transition pH of polycarboxylates (typically around 4–6) is particularly fitting for drug delivery applications. Furthermore, by adjusting the nature of the polymer backbone, the length of the polymer, the nature of co-monomers, etc., it is possible to fine-tune the transition pH and the sharpness of the pH-response. The physico-chemical aspects of the coil-to-globule transition of polymers containing carboxylic acids has been reviewed elsewhere [20].

The following sections will review in turn pH-sensitive vesicles, polymeric micelles (PMs), and nanospheres by providing for each system a description of the drug release mechanism(s) involved and examples illustrating their applicability.

2.2. Vesicles

2.2.1. Liposomes

2.2.1.1. Description

Liposomes are vesicles composed mainly of phospholipids arranged in a bilayer membrane structure. They are commonly used as drug carriers because of their capability to either encapsulate water-soluble drugs in their cavity or to solubilize lipophilic drugs in their bilayer. Different classes of pH-sensitive liposomes have been described [19, 21-23]. A first class comprises liposomes that release their cargo following a pH-triggered change in the long-range order of their lipids. Other studies describe the use of either pH-sensitive (hydrolyzable) lipids or fusogenic peptides/proteins to trigger drug release. The latter act by promoting fusion between liposomes and the endosomal membrane. Finally, liposomes can be made pH-sensitive by anchoring polymers capable of rendering phospholipid bilayers responsive to a drop in pH (Fig. 2.1A) [19]. In most cases, the polymers present alkyl chains for association with the lipid bilayer, carboxylic acid groups for pH-sensitivity, and an amphiphilic character for achieving membranolytic properties [24, 25]. Examples of membrane-active pH-sensitive polymers that have been anchored to liposomes include copolymers of *N*-isopropylacrylamide (NIPAM) copolymers, poly(alkyl acrylic acid)s, modified poly(glycidol)s (PGs), polyphosphazenes, and poly(malic acid)s. The exact mechanism of release from pH-sensitive liposomes depends on the polymer used, with some polymers simply destabilizing the bilayer (to promote drug efflux to the endosome) and others leading to fusion between the liposome and endosome/lysosome membranes (to promote drug efflux to the cytosol) [19, 26].

NIPAM-based copolymers are among the most widely used to prepare pH-sensitive liposomes. NIPAM homopolymers are characterized by a lower critical solution temperature (LCST) of approximately 32 °C in water [27]. By randomly introducing titratable monomers such as acrylic acid (AA), methacrylic acid (MAA), propylacrylic acid, and *N*-glycidylacrylamide into the polymer chain, the LCST rises above 37 °C and the polymer also becomes pH-responsive. In the bloodstream, at neutral pH, the carboxylic acid groups of the copolymer are ionized and the polymer chain adopts an extended conformation (Fig. 2.1A). In the endosome, the pH drops and protonation of the carboxylic acid units reduces the solubility of the polymer, thus lowering the temperature at which coil-to-globule phase transition occurs

[28]. At this point, hydrophobic interactions dominate, enabling the NIPAM copolymers to interact with and destabilize the lipid bilayer (Fig. 2.1A). Liposomes bearing NIPAM copolymers do not fuse at acidic pH in the absence of fusogenic lipids [29, 30]. Rather, studies indicate that the globular and insoluble NIPAM copolymer chains interact with the lipid bilayer and introduce curvature in the bilayer plane, thus making the liposomes leaky and promoting drug release (Fig. 2.1A) [28, 31]. Recently, the formation of transient hydrophilic pores with a diameter of a few nanometers has been proposed to explain the permeabilization of vesicles by pH-sensitive NIPAM and related copolymers [32, 33].

Numerous studies have been conducted to elucidate the interaction of poly(alkyl acrylic acid)s with liposomes and membranes following the pioneering work of Tirrell *et al.* [34] (for reviews, see [20, 35]). Fewer studies, however, have described the use of liposomes anchored with poly(alkyl acrylic acid)s as pH-responsive drug delivery systems. Poly(alkyl acrylic acid)-modified liposomes release their contents following membrane-destabilization and possibly fusion [36, 37]. Poly(alkyl acrylic acid)s are able to destabilize membranes at low pH values because protonation of the carboxylate ions increases the hydrophobicity of the polymers, allowing the hydrophobic segments to penetrate the lipid bilayer and to introduce defects in the membrane. Fusion, on the other hand, would result from the insertion of the hydrophobic segments of the polymer into the membrane of neighboring liposomes and/or endosomes. This would lead to close vesicle-vesicle contacts, facilitating local dehydration at the contact site, causing defects in the packing of the membrane lipids, and eventually promoting fusion [37]. Egg phosphatidylcholine liposomes covalently conjugated to poly(ethyl acrylic acid) have indeed been found to fuse with erythrocyte ghosts at pH 5.0 [37]. The pH at which the polymers destabilize and/or fuse membranes is related to but not strictly dictated by the pK_a of the carboxylic acid groups. Rather, the hydrophilicity/hydrophobicity balance of the polymers at a given pH, influenced by the extent of ionization but also by factors such as the nature of the monomers, promotes the transition.

Similarly to poly(alkyl acrylic acid)s, anchoring of PG copolymers also imparts fusogenic properties to liposomes [38-43]. While the exact mechanism by which pH-sensitive PGs destabilize membranes has not been directly studied, it is assumed that protonation of the carboxylic acid groups and subsequent H-bond formation with the phosphate groups of the phospholipids are responsible for bringing the polymer in contact with the bilayer in a pH-

dependent fashion. Once in close contact with the liposome, the oxyethylene units of the PG backbone would be responsible for dehydration of the membrane and fusion *per se* [38].

Polyphosphazenes were recently introduced as a new class of pH-sensitive polymers for the design of stimuli-responsive vesicles [44]. Polyphosphazenes are inorganic polymers with a backbone consisting of alternating nitrogen and phosphorus atoms. Each of the phosphorus atoms can be modified with two side groups, whether organic or organometallic, to tune the properties of the copolymer. As for pH-sensitive NIPAM copolymers, pH-sensitive polyphosphazenes have been synthesized to present a LCST that rises as pH is increased. In this case, ethoxy groups are responsible for the thermoresponsive behavior while amino butyric acid units confer pH-sensitivity [44]. To date, no information is available on the mechanism of release from pH-sensitive liposomes modified with polyphosphazenes. While much work still needs to be done with this class of polymer, a potential advantage is that polyphosphazenes can be rendered biodegradable by introducing substituents such as amino acid esters on their backbone [45]. Biodegradability can also be achieved with hydrophobized poly(*β*-malic acid), which has been used to produce fusogenic pH-responsive vesicles [46, 47].

2.2.1.2. Applications

pH-Sensitive liposomes prepared with membrane-anchored polyanions have found applications in the delivery of membrane impermeable drugs (*e.g.*, DNA, proteins, etc.) that are labile under the conditions encountered in the lysosomes. In addition to addressing issues of chemical stability, pH-sensitive liposomes may advantageously influence the release rate of drugs, helping for instance in the treatment of drug resistant tumors. In this case, rapid release of an anticancer agent may overwhelm the capacity of drug transporters located at the surface of cells. The release mechanism afforded by the polymer will closely influence the choice of drug to be encapsulated in the pH-sensitive liposomes and the appropriate application. For instance, small molecules such as 1-*β*-D-arabinofuranosylcytosine, which reach the cytoplasm *via* nucleoside transporters located in the endosomal membrane, may benefit from simple destabilization of the liposome and endosomal release. In contrast, fusion with or disruption of the endosomal membrane is essential for large molecules such as proteins or DNA to reach the cytosol. Numerous *in vitro* studies have been conducted in view of optimizing pH-sensitive liposomes for the intracellular delivery of drugs. Fewer studies, however, actually evaluate their *in vivo* performance. The following lines will first describe the *in vitro* results

that have shaped and guided the design of optimized pH-sensitive liposomes. An evaluation of the results obtained *in vivo* will then be presented.

Polymer composition and pH-responsiveness

The extent of polymer anchoring affects the performance of pH-sensitive liposomes. This is seen from the fact that content leakage from polymer-modified liposomes can be enhanced by increasing the copolymer to lipid ratio in the liposome [25, 28, 30, 38, 48]. Such enhanced content leakage may be explained by the work of Cho *et al.* who found that lipid-lipid cohesion was weakened as more polymer was anchored in the liposome bilayer [49]. Likewise, randomly-alkylated copolymers, which are better anchored to the lipid bilayer than copolymers of equivalent molecular weight but that are alkylated at one chain end, destabilize liposomes more efficiently [25]. The anchoring of pH-sensitive copolymers can be improved by carefully adjusting the proportion of the alkyl chain of the copolymer, whether by decreasing the molecular weight of terminally-alkylated polymers or by incorporating more alkyl groups along longer polymer chains [30, 36, 45, 50]. However, as shown for pH-sensitive liposomes modified with polyphosphazenes, the alkyl chain content should be kept low enough to ensure solubility of the polymer during liposome preparation in order to favor interaction with liposomes over self-association *via* hydrophobic interactions [44, 45]. Also, care should be taken when modifying the molecular weight of polymers as this parameter also affects their biological fate. When polymers are non-degradable, the molecular weight should be kept low enough (*e.g.*, < 32 kDa in case of NIPAM copolymers) to ensure excretion by the renal route [51].

In addition to the alkyl chain content and the molecular weight, the hydrophobicity of the polymer main chain also affects the responsiveness of pH-sensitive liposomes. Increasing the hydrophobicity of the acidic moiety of PGs and of NIPAM copolymers shifted the precipitation pH to higher values [52, 53]. Liposomes prepared with these polymers could respond to pH changes occurring earlier in the endocytic process and could even target tumor acidosis. Recently, it was shown that the architecture of the pH-sensitive copolymer also affected pH-responsiveness, with liposomes modified with hyperbranched PGs promoting increased fusion with the endosomal membrane *in vitro* compared to liposomes modified with their linear equivalents (Fig. 2.2) [42].

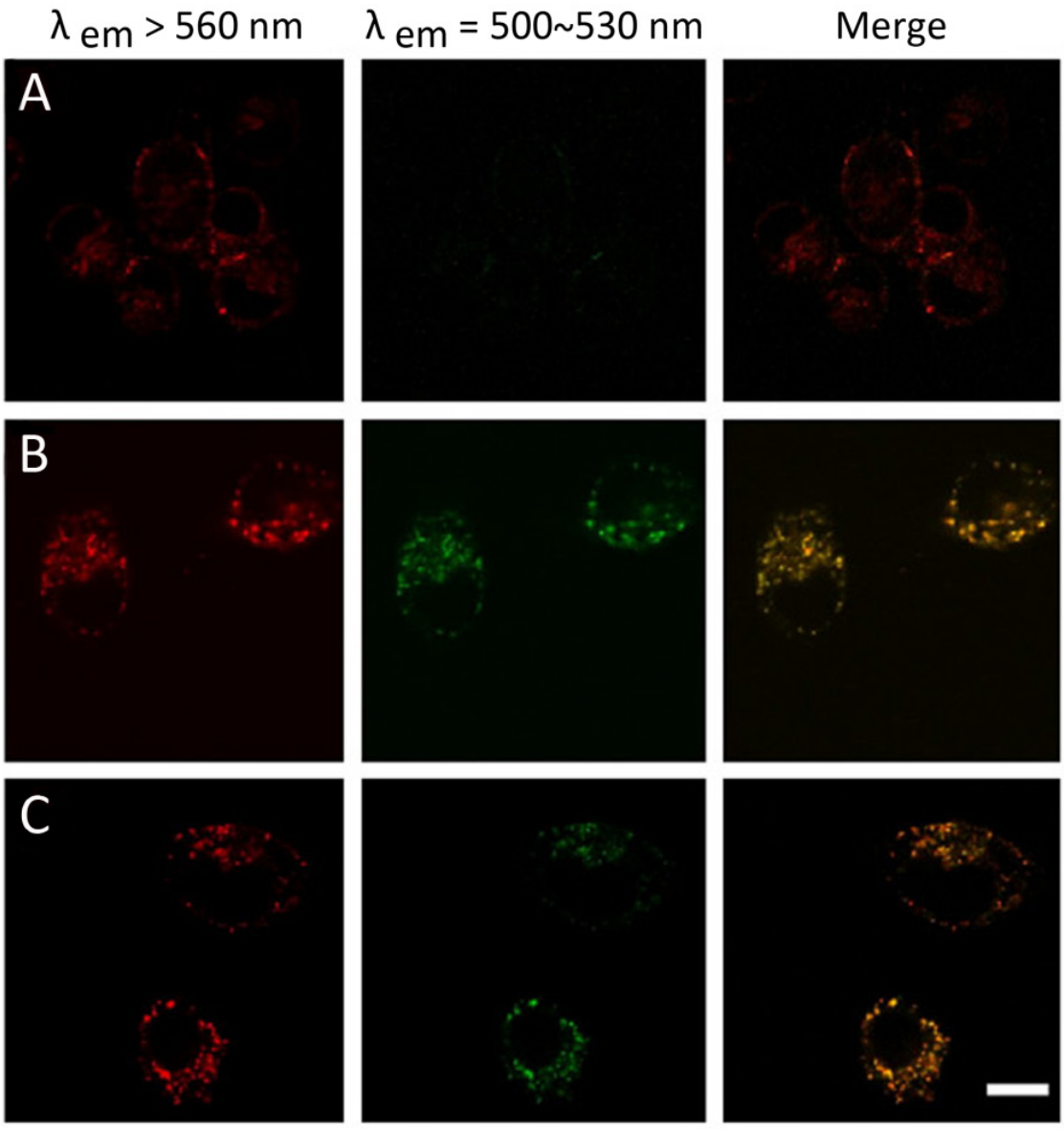


Figure 2.2. Confocal laser scanning microscopic images of immature murine dendritic cells (DC2.4) treated with egg yolk phosphatidylcholine/DOPE (1/1, mol/mol) liposomes. (A) Plain liposomes, (B) liposomes anchored with an hyperbranched PG, and (C) liposomes anchored with a linear PG. All liposomes were doped with two fluorescent lipids. Fusion of the labeled liposomes with endosomal membranes causes dilution of the fluorescent lipids and results in a decrease of the energy transfer efficiency between the fluorescent probes. Intact liposomes are detected at λ_{em} = 560 nm while fusion is observed at λ_{em} = 500–530 nm. Scale bar represents 10 μm. Reproduced from Yuba *et al.*, with permission from Elsevier [42].

Lipid composition and pH-responsiveness

One of the advantages of using polymers to prepare pH-sensitive liposomes is the possibility to render almost any liposomal composition sensitive to pH. Polycarboxylates can trigger content leakage from neutral as well as from charged liposomes, from liposomes composed of fluid phase lipids, and from liposomes made of lipids presenting high phase

transition temperatures [19, 26, 54]. When the lipids are positively charged, electrostatic interactions between the lipid membrane and acid groups can enhance the binding strength of the polymer to the liposome [54, 55]. pH-Sensitive copolymers can even stabilize lipids such as dioleoylphosphatidylethanolamine (DOPE), which alone form a hexagonal phase (H_{II}), into a lamellar vesicle at physiological pH while inducing content leakage at acidic pH [48]. A potential advantage of introducing DOPE in the membrane composition is to confer and/or improve the fusogenic properties of polymer-based pH-sensitive liposomes [42].

Formulation method and pH-responsiveness

The preparation method of the pH-sensitive liposomes, *i.e.*, whether the pH-sensitive copolymer is simply incubated with pre-formed vesicles or incorporated during the liposome preparation procedure, affects polymer binding and content release from pH-sensitive liposomes [45, 54]. Formulations in which the polymer was added during liposome preparation incorporated more polymer and triggered more contents release at acidic pH [45, 54]. It is to be kept in mind, however, that this method may not be compatible with liposomal technologies using pH or ammonium sulfate gradients for drug loading and with formulations containing lipids with high phase transition, as premature precipitation of the polymer might occur.

PEGylation and pH-responsiveness

Poly(ethylene glycol) (PEG) is a hydrophilic polymer that is often coupled at the surface of liposomes to increase their circulation time. This is because PEG forms a steric barrier that decreases and/or slows down protein adsorption, thus decreasing clearance by the mononuclear phagocyte system [56]. The corresponding downside, however, is that PEG generally reduces the fusogenicity and pH-responsiveness of the liposomes. This is evident from the literature on pH-sensitive liposomes composed of polymorphic lipids such as DOPE, which shows that PEGylation significantly decreases the pH-dependent release of calcein *in vitro* [21].

Even for pH-sensitive liposomes that do not fuse, incorporation of PEG in the formulation may be deleterious as PEG hinders the anchoring of the pH-sensitive polymer, increases the stability of the bilayer, and interferes with the aggregation of the copolymer, thereby reducing the extent of destabilization of the liposomal membrane. Many studies have shown that pH-sensitive liposomes anchored with NIPAM copolymers lost their pH-sensitivity when PEG was included in the liposomal formulation [25, 28, 30]. When the NIPAM copolymer was inserted by simple incubation with pre-formed PEGylated vesicles,

the loss of pH-responsiveness was in part attributed to poor anchoring of the polymer into the lipid bilayer [25, 30]. In those cases, decreasing the molecular weight of the polymer and increasing the alkyl chain content permitted the recovery of some pH-sensitivity. Interestingly, it was found that including the NIPAM copolymers in the hydration buffer during liposome preparation, as opposed to simple incubation with pre-formed vesicles, greatly improved binding of the polymer to the sterically-stabilized liposomes and helped maintain pH-responsiveness [30]. Using optimal preparation conditions, good pH-sensitivity can be achieved with PEGylated formulations [57, 58].

Stability in serum

A key aspect in the development of pH-sensitive liposomes is their stability in biological fluids. The formulations should, on the one hand, present minimal leakage while circulating and, on the other hand, maintain their pH-responsiveness. Anchoring of alkylated NIPAM copolymers was reported to stabilize the lipid bilayer and reduce drug leakage from liposomes in the presence of serum [25]. This stabilizing effect, however, was compromised when a targeting antibody was added to the formulation, with drug leakage from immuno pH-sensitive liposomes bearing a whole monoclonal antibody being greater *in vivo* compared to non-targeted pH-sensitive liposomes [59]. In turn, the pH-sensitivity of the vesicles can be lost in biological fluids [26]. Extraction of the polymer from the bilayer by serum components and/or a shift in transition pH due to protein adsorption have been put forth to explain this loss of sensitivity [26]. Examination of the existing body of literature on NIPAM copolymer-based pH-sensitive liposomes indicated that loss of pH-sensitivity in presence of serum was mainly observed when the pH-sensitive copolymer was simply incubated with pre-formed vesicles (as in [26]) as opposed to when the polymer was incorporated during the liposome preparation procedure (as in [28, 54, 57, 60]). This might be because the polymer anchored during liposome preparation is located in both the internal and external leaflets of the bilayer, with only the external chains being subject to desorption. This correlation, however, is only tentative since liposomes in reference [26] were prepared with a high transition temperature lipid while all others were prepared with egg phosphatidylcholine. Strategies aiming at increasing the affinity of the polymer for the lipid bilayer have also been tested to help maintain the pH-sensitivity of liposomes in serum. Polymers with either a high proportion of alkyl chain anchors distributed along the polymer chain or with an increased number of terminal alkyl chains (two *vs.* one) and low molecular weight all afforded minimal loss of pH-sensitivity in serum [28, 54]. However, from lack of systematic studies in the literature

establishing relationships between pH-sensitivity in serum and polymer composition, the importance of those parameters remains unclear. Recently, serum-stable pH-responsive liposomes were prepared by cross-linking poly(acrylic acid) (PAA) chains anchored in the lipid bilayer *via* a terminal cholesterol [61]. While this approach was highly efficient in stabilizing the vesicles, it remains unknown whether such a non-biodegradable cross-linked coating can be readily eliminated from the body after parenteral administration.

Efficacy on cells

In numerous studies, pH-sensitive liposomes prepared with anionic polymers have been found to maintain their activity in cells. This was shown either by monitoring lipid mixing between liposomes and endosomal/lysosomal membranes (Fig. 2.2) [39, 42, 43] or by imaging the delivery of fluorescent molecules to the cytosol [26, 36, 39, 41, 42, 50, 52]. When 1-*β*-D-arabinofuranosylcytosine was used as anticancer drug, the increased cytosolic delivery achieved with pH-sensitive liposomes translated into an increase in the cytoxicity compared to plain liposomes in both macrophage-like and leukemia cell lines [26, 50, 54]. Efficient delivery of α-ketoglutaric acid was also achieved by pH-sensitive liposomes, and promoted procollagen production in human dermal fibroblasts [62]. More notably, the administration of pH-sensitive liposomes loaded with ovalbumin (a large molecule, *ca.* 45 kDa) promoted antigen presentation on bone marrow-derived dendritic cells *via* major histocompatibility complex class I molecules compared to unmodified liposomes. In this case, only the most hydrophobic (and consequently most pH-responsive) polymer was able to efficiently transfer the ovalbumin to the cytosol [41].

Pharmacokinetics and *in vivo* efficacy of pH-sensitive liposomes

Liposomes not only need to be stable in biological fluids but should exhibit long circulation times when administered intravenously in order to reach target cells and mediate cytoplasmic delivery. Therefore, any premature uptake by the mononuclear phagocyte system could compromise their therapeutic value. Indeed, it has been found that negatively charged PAA-and PG-conjugated liposomes were taken up by phagocytic cells, probably *via* the scavenger receptor [63, 64]. Yamazaki *et al.* reported that the anchoring of terminally-alkylated NIPAM copolymers could reduce the adsorption of plasma proteins on liposomes below the LCST [65]. Since low protein adsorption is typically correlated with increased circulation times *in vivo* and accumulation of liposomal formulations at tumor sites, the pharmacokinetic profiles of liposomes coated with randomly- and terminally-alkylated pH-sensitive NIPAM copolymers were evaluated [28, 57, 60]. It was shown that the pH-sensitive

NIPAM copolymers conferred some steric protection *in vivo*, increasing the area under the blood concentration *vs.* time curve (AUC) by 1.2- to 1.9-fold compared to naked liposomes. This protection, however, was marginal compared to what can be achieved with PEG. A recent study by Bertrand *et al.* indicated that there was minimal polymer desorption of a terminally-alkylated NIPAM copolymer occurring from pH-sensitive liposomes *in vivo*, ruling out this phenomenon as a possible explanation for the limited steric protection offered by NIPAM copolymers [51]. Rather, the weak steric protection may be explained by the fact that the copolymers were actually not in a fully random coil conformation at neutral pH [28]. The addition of PEG to pH-sensitive liposomes permitted to prolong their AUCs by an additional 2- to 3.4-fold [28, 57]. Finally, the influence of the presence of an antibody, whether whole or fragmented (fragment antigen-binding (Fab')), on the pharmacokinetic profile and biodistribution of PEGylated pH-sensitive liposomes was evaluated [59]. Compared to the non-targeted pH-sensitive PEGylated formulation, the presence of a whole antibody resulted in a substantial decrease of liposomal blood levels while the Fab' fragment had a lesser impact on clearance and AUC values.

An *in vivo* efficacy study conducted by Simard *et al.* completed the picture for pH-sensitive liposomes based on NIPAM copolymers [59]. The capability of PEGylated pH-sensitive liposomes decorated with the anti-CD33 Fab' fragment at improving the survival of leukemic immunodepressed mice treated with 1-β-D-arabinofuranosylcytosine was tested. Unfortunately, while Fab'-PEGylated liposomes were able to prolong the survival of leukemic mice, the addition of the pH-sensitive polymer did not have any further benefit. Overall, it appears that the marginal advantage of the pH-sensitive liposomes seen *in vitro* was offset by the less favorable pharmacokinetics of the formulation *in vivo* [50]. More promising *in vivo* results have been obtained using ovalbumin-loaded pH-sensitive PG liposomes to immunize mice [41]. In this case, pH-sensitive liposomes administered *via* the nasal cavity induced a stronger cellular immune response than plain liposomes. The immune response achieved with the liposomes was comparable to that of Freund's complete adjuvant. Therefore, it appears that future studies on pH-sensitive liposomes should focus on systems capable of fusion so as to ensure efficient drug delivery to the cytosol. Furthermore, the applicability of systems capable of fusion is extendable to large molecules such as peptides and nucleic acids.

2.2.2. Niosomes

Niosomes are vesicles composed of non-ionic surfactants. They have been investigated as alternatives to liposomes to obtain pH-sensitive vesicles that better retain the anchored pH-sensitive polymers in presence of serum. This is because the binding of pH-sensitive polymers to niosomes is thought to be stronger as it involves cooperative hydrogen bonds in addition to hydrophobic and/or electrostatic interactions [55]. The results, however, showed that polymer-coated vesicles were leaky in presence of serum at neutral pH, and lost pH-sensitivity after incubation in serum [29, 54]. Furthermore, pH-sensitive niosomes failed to deliver calcein to the cytoplasm of macrophage-like cells [29, 54]. Accordingly, despite the pH-sensitive polymer binding strongly to niosomes, the overall stability of the formulation remains inadequate in physiological fluids for *in vivo* applications.

2.2.3. Lipoplexes

Lipoplexes result from the interaction of cationic lipids with nucleic acids to form either nucleic acid-coated vesicles or aggregates. The exact morphology of lipoplexes is often ill-characterized and both systems will therefore be discussed jointly in this section on vesicles. To date, two approaches have been tested to confer pH-sensitivity to lipoplexes using polyanions. In the first approach, pH-sensitive membrane-active polyanions are directly incorporated in the preparation of lipoplexes, leading to so-called ternary complexes. Ternary complexes prepared with either poly(propylacrylic acid) or copolymers of MAA/ethyl acrylate (EA)/butyl methacrylate (BMA) have both been able to increase transfection efficiency compared to the parent, polymer-free complexes [66-68]. The role of the polymer is several folds and includes transfer of the nucleic acid from late endosomes to the cytoplasm in a pH-dependent manner [66, 68]. Upon the decrease in pH met in the endosomes, the carboxylate ions of the polymer became protonated, promoting dissociation of the polymer from the complex, interaction with the endosomal membrane, and membrane destabilization. In this process, the MAA/EA/BMA polymer remained trapped/bound to the endosomal vesicles, suggesting that the organelles are not destroyed but rather that defects in the membrane allow for permeation of the nucleic acids to the cytosol [66]. The polymers also appear to improve transfection by stabilizing the ternary complexes towards dissociation in presence of serum components [69]. Finally, the presence of poly(propylacrylic acid) in ternary complexes was shown to enhance cellular uptake [67, 69]. This beneficial effect, however, was not seen by Yessine *et al.* [66]. On the contrary, they observed a decrease in cellular uptake of the ternary complexes compared to plain lipoplexes. Without additional

information on the physicochemical properties (*i.e.*, size, zeta potential) of the poly(propylacrylic acid) complexes, it is difficult to explain this inconsistency. Poly(propylacrylic acid)-based ternary complexes have been tested *in vivo* in a mouse wound healing model and compared to polymer-free complexes [70]. While quantification of the regulation of the target protein in tissues was found to be difficult, biological changes in the healing response were seen and indicated successful transfection by the ternary complexes only [70].

The second strategy undertaken to prepare pH-sensitive lipoplexes consists in adding pH-sensitive liposomes to lipoplexes. Such assemblies have been prepared with PG-based pH-sensitive liposomes. In this case, a decrease in pH not only promoted fusion between liposomes and endosomes/lysosomes (as described in section 2.2.1), but also between the liposomes and the lipoplexes, potentially detaching the nucleic acid from the assemblies [71]. The approach led to increased transfection efficacy, which was correlated to the fusion ability of the parent pH-sensitive liposomes [40, 64, 71, 72].

2.2.4. Polymersomes

Polymersomes are vesicular structures composed of a polymeric membrane surrounding an aqueous internal compartment. The membrane generally comprises an entangled hydrophobic layer with two hydrophilic polymer brushes [73]. Owing to their thicker membranes, they are often viewed as being more stable than lipid-based vesicles, and are therefore receiving increased interest among the drug delivery community [74]. While important for storage and pharmacokinetic considerations, the robustness of the membrane can be questionable if the latter impedes drug release at the target site. Moreover, depending on the dimensions and hydrophobicity of the amphiphilic polymer, the hydration of the latter and subsequent formation of polymersomes in aqueous media can be problematic, often requiring the use of organic solvents, sonication or high temperature. For the above reasons, the possibility of controlling the assembly and disassembly of polymeric vesicles, and thereby the encapsulation and release of drugs, following changes in pH is particularly attractive [75]. In the area of drug formulation, pH-sensitive polymersomes based on polyanions have been less studied than those relying on cationic polymers [75]. Polyanionic polymersomes can be obtained from diblock copolymers consisting of a hydrophobic block forming the inner leaflet of the membrane and an ionized block polyacid such as PAA or poly(L-glutamic acid) (PGA)

forming the outer hydrophilic brushes [76-78]. At low pH values, the polyanionic block can, however, also form the inner leaflet when the other block is hydrophilic [79].

As previously reported for polycationic systems, the ability of polyanionic block copolymers to spontaneously self-assemble in water could, in principle, be exploited to load sensitive drugs (*e.g.*, proteins, nucleic acids) in the absence of organic solvent by simply adjusting the pH to the value where vesicle formation occurs [80]. In the case of biodegradable polymersomes prepared from poly(trimethyl carbonate)-*b*-PGA, it was shown that the release rate of doxorubicin increased upon lowering the pH from 7.4 to 5.5 [81]. Although the faster release was attributed to the increase in the drug's hydrophilicity following its protonation, it is possible that the coil to α-helical transition of PGA may have facilitated drug escape by transiently affecting the membrane's permeability and/or increasing the inner pressure. Indeed, at acidic pH, the vesicular structure remained but size decreased [82]. The same phenomenon occurred with polymersomes composed of poly(butadiene)-*b*-PGA, where it was shown that the protonation of the PGA block induced a strong decrease in vesicle size and membrane thickness [83]. PGA-based polymersomes loaded with doxorubicin have been recently tested *in vivo* and found to be more efficient than the free drug on a murine tumor model [84].

Apart from block copolymers, polyanion-based pH-responsive polymersomes have been prepared from hyperbranched and graft polymers (*e.g.*, hydroxyethylcellulose-*g*-PAA) [85]. The introduction of chemical cross-links in such systems has also been investigated as a means to prevent disassembly and control vesicle swelling upon the ionization of the cross-linked polyacid [86, 87]. Recently, Koide *et al*. have described the preparation of pH-sensitive polyion complex vesicles [88]. These vesicles formed at neutral pH upon the self-assembly of oppositely-charged PEG block polyanions (*i.e.*, PEG-*b*-poly(L-aspartic acid) (PEG-*b*-PAsp)) and homo or PEG block polycations, and encapsulated water-soluble compounds. After the acidification of the external medium to pH values corresponding to that of the endosomes, the membrane permeability increased as a result of the neutralization of the PAsp block and, eventually, fragmentation of the vesicles into smaller particles [89]. Pharmacokinetic studies demonstrated that such vesicles exhibited long circulation times and tumor deposition, but only after cross-linking of the vesicle membrane [90].

Finally, polymersomes with pH-responsive transmembrane channels have been described by Chiu *et al.* [91]. These vesicles consisted of PAA partially esterified with distearin. Upon raising the pH from 5 to 8, the globule-to-coil phase transition of PAA domains resulted in the formation of permeable channels, through which the fluorescent probe calcein could diffuse. Such vesicles may find practical applications in oral drug delivery by protecting drugs from the harsh acidic environment in the stomach and allowing release in the intestine. For systems exhibiting a strong negative zeta potential and which are intended to be administered by intravenous injection, it will be important to determine the impact of surface charge on the opsonization of vesicles and clearance by the mononuclear phagocyte system [92, 93].

2.3. Polymeric micelles

As classically defined, PMs are nanoscopic constructs that possess a core/shell architecture. They are obtained from the self-assembly of amphiphilic block copolymers in aqueous media above the critical micelle concentration (CMC). The core, consisting of the hydrophobic domain, acts as a reservoir and protects the drug payload whereas the hydrophilic shell mainly confers aqueous solubility and steric stability to the ensemble [94, 95]. The self-association of polymeric chains can involve other forces than hydrophobic interactions. For example, the cooperative electrostatic interactions between oppositely-charged polymers were shown to produce a subclass of PMs known as polyion complex micelles (PICMs). Both PMs and PICMs typically exhibit a narrow size distribution, with diameters ranging from 10 to 100 nm. In the following section, PMs responding to a change in pH will be discussed according to their intended administration route (and, by extension, their drug release mechanism) (Fig. 2.1B). pH-Responsive micellar structures prepared with polyanions that were derivatized with acid-labile linkages such as cyclic acetals [96], hydrazones [97, 98], or *β*–thiopropionate [99] will not be covered in this review.

2.3.1. Parenteral drug delivery

As seen for pH-sensitive vesicles, the mildly acidic pH encountered in tumors, inflammatory tissues, as well as in the endosomal and lysosomal compartments of the cells offers an opportunity to trigger the disassembly and/or destabilization of pH-sensitive PMs. PMs prepared for parenteral administration have to be stable at pH 7.4. A first approach to prepare pH-sensitive PMs is to combine positively-charged drugs (such as doxorubicin) with

an oppositely charged block copolymer to form a polyion complex core (Table 2.1) (Fig. 2.1B, left). At neutral pH (*e.g.*, bloodstream), above pK_a of the polymer, the carboxylic acid units are negatively charged hence allowing cooperative electrostatic interactions with the encapsulated compounds. Once in a mildly acidic environment, protonation of the carboxylic acid groups results in a net decrease in electrostatic interactions and dissociation of the drug-containing core (Fig. 2.1B, left). Another approach to prepare pH-sensitive PMs is to incorporate a drug in the uncharged hydrophobic core of a micelle presenting an ionized polyanion shell [100]. In this case, protonation of the shell induced a perturbation of the core-shell structure and ultimately affected the intracellular distribution of the drug [100].

The principal drawbacks of PMs are their relative instability upon dilution in body fluids and sensitivity to increased ionic strength, both rapidly leading to premature drug release [18, 101]. Bronich *et al*. were able to produce stable nanoscale ionic gels by cross-linking the micelle core of PEG-*b*-poly(methacrylic acid) (PEG-*b*-PMAA) micelles with divalent metal cations (*e.g.*, Ca^{2+}) [102]. The resulting PMs were loaded with chemotherapeutic agents such as doxorubicin and cisplatin [103, 104]. Similarly, Yuan *et al.* were able to entrap and stabilize lysozyme in chemically cross-linked micelles of PEG-*b*-PAsp [105].

Table 2.1. pH-Sensitive polyanions typically used to prepare PMs for parenteral drug delivery.

Polyanion	Drug/compound	Size (nm)[a]	Ref.
PEG-*b*-PMAA	Doxorubicin	150	[106]
	Cisplatin	100–200	[103, 104]
PEG-*b*-PAsp	Lysozyme	50–100	[105, 107]
	Zinc porphyrin dendrimer	55	[108]
P(NIPAM-*co*-MAA-*co*-ODA)	AlClPc	13–35	[100, 109]
P(NIPAM-*co*-MAA-*co*-ODA-*co*-VP)	AlClPc	20–34	[110]
P(NIPAM-*co*-MAA)-*g*-PLA	Doxorubicin	120–200	[111]
P(NIPAM-*co*-DMAA-*co*-UA)	Doxorubicin	250–300	[112]
P(NIPAM-*co*-DMAA-*co*-UA)-*g*-cholesterol	Doxorubicin	200	[113]
P(NIPAM)-*b*-PUA	Prednisone acetate	160	[114]
P(NIPAM)-*b*-PAA	Doxorubicin	160	[115]
P(NIPAM-*co*-AA-*co*-HEMA)-*g*-PCL	Doxorubicin	100–120	[116]
PSMA-alkylamide derivative	Doxorubicin/ siRNA	30–100	[117]
P(MAA-*co*-EA-*co*-BMA)	AON	30	[118]
PEG-*b*-P(PrMA-*co*-MAA)	AON	50–60	[119]
	siRNA	50–60	[119, 120]
PDMAEMA-*b*-P(BMA-*co*-DMAEMA-*co*-PropylAA)	siRNA	45	[121]

ODA, octadecyl acrylate; VP, *N*-vinyl-2-pyrrolidone; PLA, poly(D,L-lactide); DMAA, dimethylacrylamide; UA, 10-undecenoic acid; PUA, poly(10-undecenoic acid); HEMA, 2-hydroxyethyl methacrylate; PCL, poly(ε-caprolactone); PSMA, poly(styrene-*alt*-maleic anhydride); AlClPc, Aluminum chloride phthalocyanine; PDMAEMA, poly(*N*,*N*-dimethylaminoethyl methacrylate).

[a]Determined by dynamic light scattering with drug-loaded PMs.

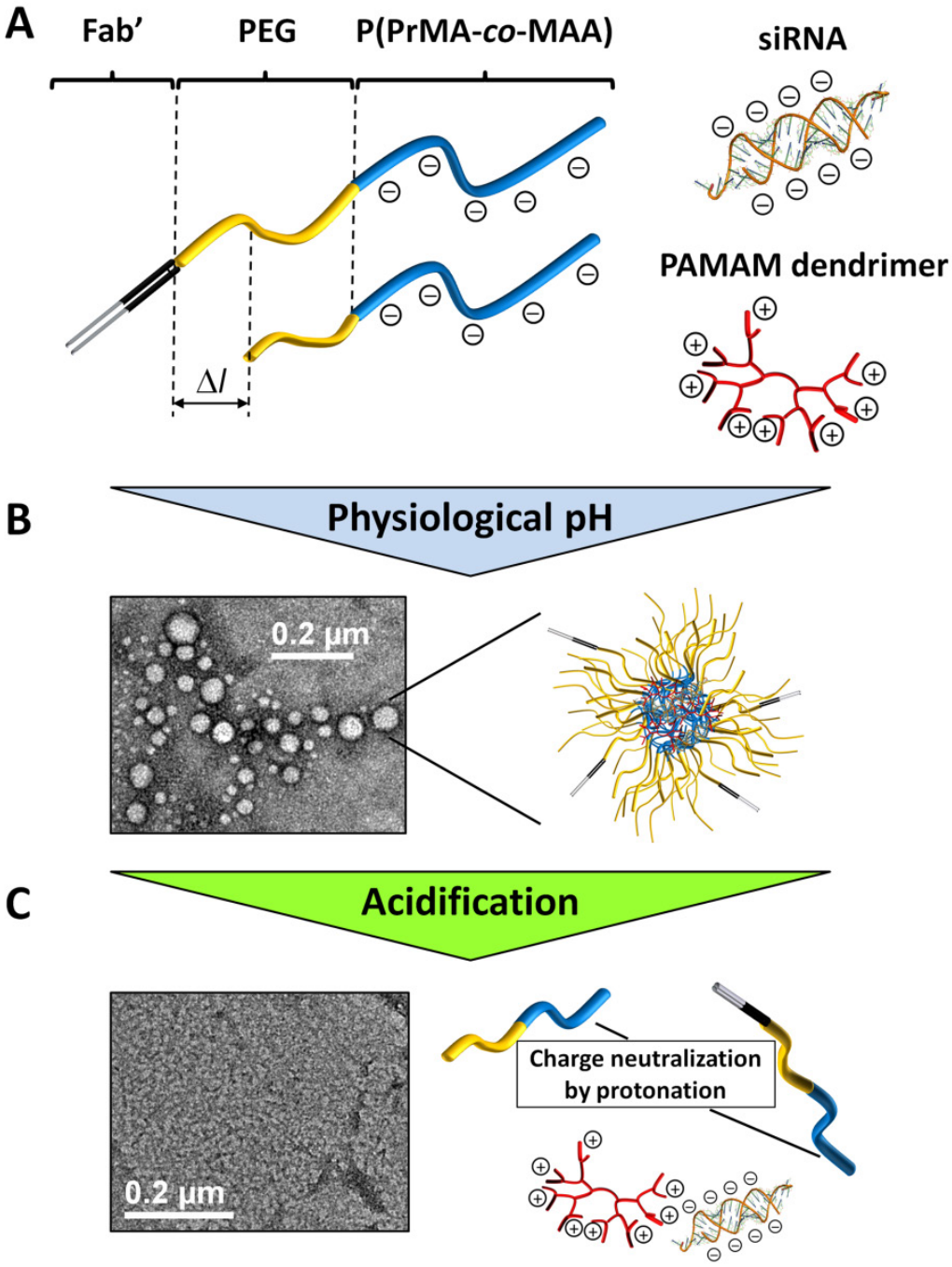


Figure 2.3. (A) Schematic representation of the different components needed to prepare targeted ternary PICMs. (B) At physiological pH, these components self-assemble to form PICMs. (C) Following acidification of the milieu, the PEG-*b*-polyanion is displaced from the core resulting in the dissociation of the PICM. Insets represent PICMs as observed by transmission electron microscopy at pH 7.4 (B) and 5.0 (C), respectively. For transmission electron microscopy, samples were adsorbed to glow discharged carbon-coated copper grids for 2 min and negatively stained with 2 % (w/v) uranyl acetate solution for 30 s. The specimens were examined with a Philips CM12 (FEI, Hillsboro, OR) electron microscope operating at 100 kV and images were recorded with a Gatan CCD 794 camera (Gatan Inc. Pleasanton, CA).

Nucleic acids have also been loaded into polyanion-based PMs (Table 2.1). Incorporation of genetic material into PMs prepared with a pH-sensitive polyanion necessitates the introduction of a polycationic molecule to allow bridging of the negatively-charged macromolecules. The optimal cationic polymer should be non-toxic, efficient in condensing the polyanions, and eventually exhibit endosomolytic properties to maximize transfection [122, 123]. Such assemblies have been referred to as ternary PICMs and have, for instance, been prepared by combining an endosomolytic copolymer of MAA and PEG-*b*-poly(aminoethyl methacrylate) or PEG-*b*-poly(propyl methacrylate-*co*-methacrylic acid) (PEG-*b*-P(PrMA-*co*-MAA)) and poly(amido amine) (PAMAM) dendrimers [118-120, 124]. In the presence of a PAMAM dendrimer, PEG-*b*-P(PrMA-*co*-MAA) formed discrete 50–60 nm core-shell type PICMs at physiological pH (Fig. 2.3) [119]. These nanocomplexes could accommodate antisense oligonucleotides (AONs) and small interfering RNAs (siRNAs) in their core [119]. The ternary PICMs are designed to disassemble and release the nucleic acid/polycation core in the mildly acidic milieu of the endosomes after protonation of the carboxylic acid groups of the polyanion, leaving excess positive charges available to interact with the endosomal membrane [119]. The extent of this disassembly was investigated by tracking the siRNA release from PICMs conditioned at pH 5.0 or 7.4 (Fig. 2.4). At pH 5.0 and after a 24-h period, *ca.* 85 % of the initially loaded siRNA was available to diffuse through a size-restrictive membrane whereas PICMs conditioned at pH 7.4 remained stable, hence preventing siRNA diffusion through the membrane.

Such micellar constructs exhibited good stability in the presence of serum and efficiently protected their nucleic acid cargo against enzymatic degradation [119, 120]. In order to trigger PICM uptake by receptor-mediated endocytosis, the micelles were decorated with an antibody fragment directed against the transferrin receptor (CD71), *via* either disulfide or thioether linkages [119, 120]. It was found that the targeted PICMs could efficiently downregulate *in vitro* the oncoprotein Bcl-2 in human prostate adenocarcinoma (PC-3) cells, especially when using a 2'F-modified siRNA [120].

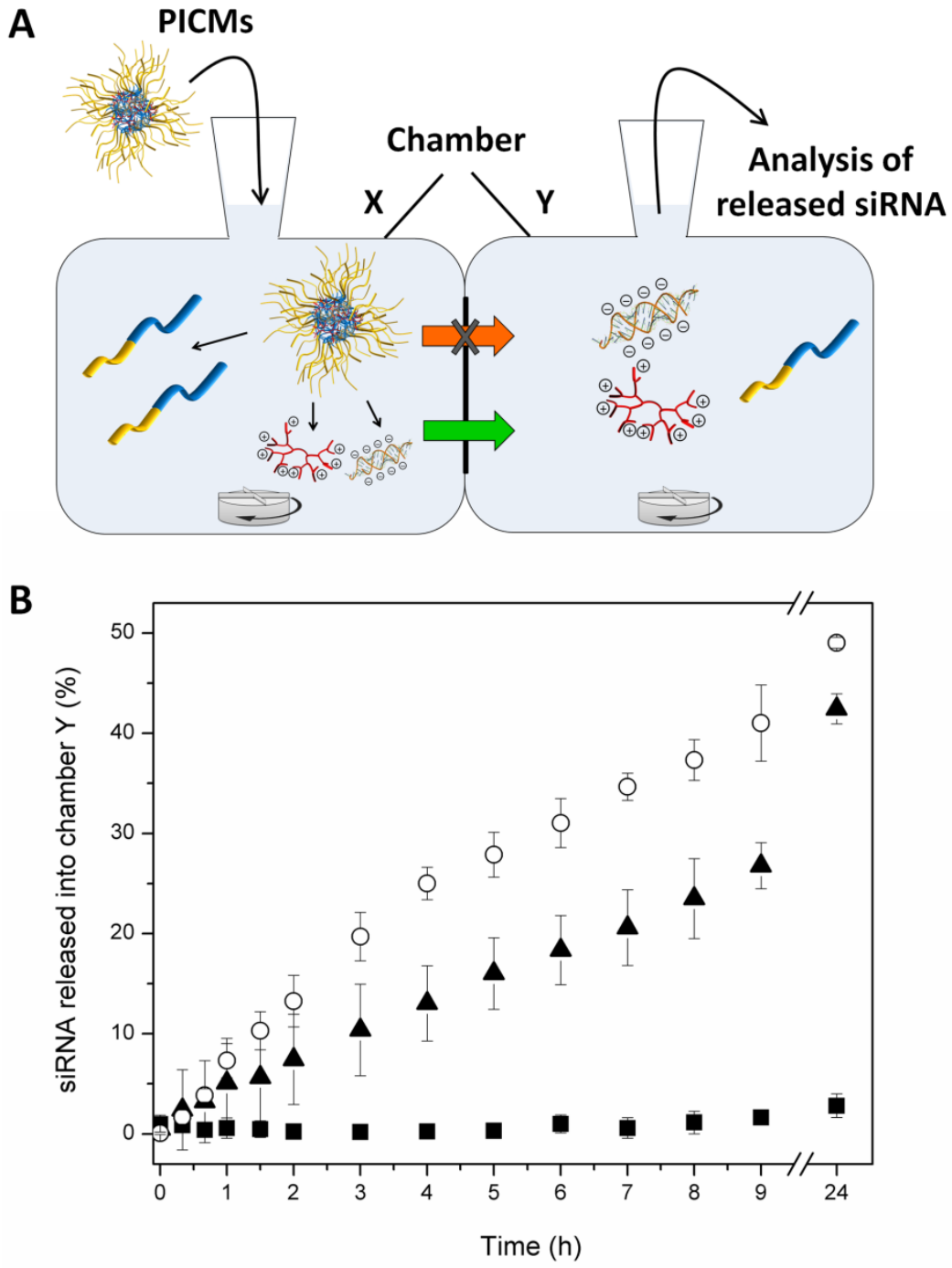


Figure 2.4. In vitro siRNA release from siRNA-loaded PICM at different pH values. (A) Schematic representation of the experimental set-up. The PICM formulation was loaded into the donor chamber X and diffusion of dissociated PICM components was monitored in acceptor chamber Y by spectrophotometry at 260 nm. Both chambers were conditioned at pH 7.4 or 5.0 (10 mM Tris buffer). Membrane was size restrictive and only allowed the diffusion of free-siRNA and siRNA/PAMAM G5 dendrimer complexes. (B) siRNA/PAMAM release from PICMs at pH 5.0 (▲) and 7.4 (■). Diffusion of control free-siRNA is also represented (○). Results are expressed as mean ± SD (n = 3).

2.3.2. Oral drug delivery

The oral route is the most convenient and economical drug administration pathway. Class II drug molecules (*i.e.*, highly permeable and poorly water-soluble) often exhibit a low oral bioavailability due to incomplete dissolution in the GI tract. If the poor solubility behavior of Class II drugs in GI fluids remains the main hurdle to their efficient oral absorption, drug expulsion by the intestinal permeability glycoprotein is also of concern. The intestinal permeability glycoprotein, a membrane-associated protein present on the intestinal epithelium, acts as a pump modulating the outward (efflux) transport of drugs [125, 126]. Over the past decade, PMs have been extensively studied as potential oral delivery systems for Class II drugs [1, 16, 127-129]. Unfortunately, vehicles designed to increase the oral bioavailability of these drugs often exhibit release times that exceed the transit time in the small intestine [130, 131]. Camilleri *et al.* studied the stomach emptying and the small bowel transit times in healthy human volunteers by monitoring the migration of a radiolabeled marker previously mixed in their meal [132]. They observed half times of *ca.* 177 min and *ca.* 168 min for stomach emptying and small bowel transit times, respectively [132]. Hence, state of the art oral PM formulations should exhibit adequate drug release behaviors in order to avoid i) precipitation upon administration and ii) sequestration within the micellar phase, both leading to incomplete absorption. A promising strategy to circumvent these problems is the encapsulation of drugs in PMs responding to a change in pH. Upon an increase in pH, pH-sensitive PMs ionize/dissociate to release the loaded drug in a molecularly dispersed form (Fig. 2.1B, right). Such assemblies are stable at acidic pH and can efficiently dissolve hydrophobic drugs, thereby minimizing the burst release and possible drug precipitation in the stomach. The use of this type of PMs for oral drug delivery is still fairly recent, and only few systems have been investigated (Table 2.2).

pH-Sensitive PMs can be either unimolecular or multimolecular [133-135]. Upon pH increase, the core of the unimolecular micelles became more polar hence promoting the release of the hydrophobically incorporated drug [133]. As these micelles do not possess a CMC, they have the advantage of being intrinsically stable upon dilution. Conversely to unimolecular micelles that maintain their integrity upon a change in pH, pH-sensitive multimolecular PMs based on ionizable polyanions disassemble following an increase in environmental pH. For instance, Kim *et al.* developed a hydrotropic polymer, PEG-*b*-(4-(2-vinylbenzyloxy)-*N*,*N*-(diethylnicotinamide)) (PEG-*b*-VBODENA), doped with AA units (≤ 50 mol%) to confer pH-sensitivity to PMs (Table 2.2) [135]. They observed that the loading

content and efficiency of paclitaxel was governed by the pH of the loading medium, with both maxima at pH ≤ 4 [135]. Increasing the pH above the pK_a of the polymers provoked a rapid dissociation of the complexes [135]. Alternatively, PEG-*b*-poly(alkyl(meth)acrylate-*co*-methacrylic acid)s (PEG-*b*-P(Al(M)A-*co*-MAA)s) are diblock copolymers displaying a pH-dependent micellization behavior in aqueous media (Table 2.2). The self-association into well-defined micellar structure is facilitated by the hydrophobic non-ionizable Al(M)A units, whereas the pH-sensitivity is conferred by the carboxylic acid groups of the MAA moieties [134, 136, 137]. It has been observed that diblock copolymers devoid of Al(M)A led to the formation of large aggregates, most likely resulting from extensive hydrogen bonding between the MAA groups and the PEG chains (Table 2.2) [136, 138-141]. Furthermore, the nature and abundance of Al(M)A moieties played a critical role on the particle size, CMC, and stability. Small alkyl side groups such as EA yielded larger particles that associated at higher concentration compared to more hydrophobic units such as *n*-butyl acrylate (*n*BA), *iso*-butyl acrylate (*i*BA), or PrMA (Table 2.2) [134, 137, 138]. Depending on their composition, these copolymers self-assembled in PMs at pH values lower than 4.5 to 5.5 and dissociated into unimers and/or smaller aggregates upon an increase in pH [134].

Table 2.2. pH-Sensitive polyanions typically used to prepare PMs for oral drug delivery.

Polyanion[a]	Drug	Size (nm)[b]	Ref.
Star-P(EMA-*co*-MAA)-*b*-P(PEGMA)	Progesterone	10–16	[133]
PEG-*b*-P(VBODENA-*co*-AA)	Paclitaxel	85–90	[135]
PEG-*b*-P(Al(M)A-*co*-MAA)	Indomethacin	180–380	[134]
	Fenofibrate	30–380	[134]
	Candesartan cilexetil	40–50	[137]
P(MMA-*co*-MAA)-*b*-P(PEGMA)	Ibuprofen	120–250	[142]
PAA-*b*-PLA	Prednisone acetate	200	[143]
PEG-*b*-PMAA	Naproxen	230	[140]
	BMS-A	150	[141]

EMA, ethyl methacrylate; MMA, methyl methacrylate; BMS-A, Proprietary compound from Brystol-Myers Squibb Company.
[a]For PEG-*b*-P(Al(M)A-*co*-MAA), the Al(M)A unit is either EA, *n*BA, *i*BA or PrMA.
[b]Determined by dynamic light scattering with drug-loaded PMs.

PEG-*b*-P(Al(M)A-*co*-MAA)-based PMs have been investigated notably for the solubilization of candesartan cilexetil, an ionizable poorly water-soluble drug used for the treatment of hypertension [137]. The release profiles of candesartan cilexetil from pH-

sensitive PEG-*b*-P(*i*BA-*co*-MAA) and pH-insensitive PEG-*b*-P(*i*BA-*co*-*tert*-butyl methacrylate) micelles were studied *in vitro* [137]. The PMs were first immersed in simulated gastric fluid for 2 h and then exposed to pH 7.2 for an additional 7 h. Both formulations showed relatively low drug leakage at acidic pH. However, sudden increase in the release rate occurred when raising the pH to 7.2 for the PEG-*b*-P(*i*BA-*co*-MAA), eventually leading to the complete release of the loaded drug after 9 h [137]. *In vivo* testing on rats showed that such micelles yielded *ca.* 25% greater drug exposure than both their pH-insensitive counterpart and a commercial formulation [16]. Finally, these PMs mainly addressed the solubility problems of candesartan cilexetil, as PEG-*b*-P(*i*BA-*co*-MAA) was reported to have almost no effect on the activity of the permeability glycoprotein and on transepithelial permeability at intestinal pH [16].

Recently, novel pH-responsive polymers composed of an anionic polypeptide and a low molecular weight nonionic surfactant (Brij®) have been found to form reversible nanoscopic assemblies in acidic media [144, 145]. Further experiments are needed to determine if such systems would be adequate to efficiently encapsulate drugs and deliver them in the GI tract.

2.4. Polymeric nanospheres

2.4.1. Oral drug delivery

Of all the pH-sensitive drug delivery systems described so far, polymeric nanospheres intended for oral applications have the greatest chance of success on a short-term basis. Indeed, such particles are easy to formulate and can be prepared from polymers already commonly used in drug formulation. Furthermore, the nanospheres are not absorbed by the GI tract, diminishing toxicity issues.

Polymeric nanospheres are generally defined as insoluble colloidal systems having sizes ranging from about 10 to 1000 nm and a solid polymeric core [146]. pH-Sensitive polymeric nanospheres were initially developed by Gurny and co-workers in the mid/late 90's to improve the bioavailability of peptidic and peptidomimetic drugs [147-151]. They were produced with MAA copolymers of the Eudragit® (L100–55, L100, and S100) family, which were originally marketed as gastro-resistant coating agents. Particles prepared with these polymers had a size in the range of 250–300 nm, and were obtained by an emulsification-

diffusion procedure [148, 151]. It was demonstrated that the dissolution pH of the particles could be finely tuned by selecting the appropriate Eudragit® polymer [147-149]. In rodents and dogs, these pH-sensitive nanospheres were shown to improve the oral absorption of human immunodeficiency virus (HIV) type 1 protease inhibitors compared to the crude suspensions [147, 148, 150]. Discrepancies between the studies with respect to the impact of food on the extent of absorption were observed. However, they could be related to the nature of protease inhibitor used.

More recently, the oral delivery of different molecules using various Eudragit®-based nanospheres was examined [152-157]. It was found that the formulation of cyclosporine A into Eudragit® S100 nanospheres improved its bioavailability compared to the Neoral® microemulsion system [154]. This increase in absorption was attributed to a shorter transit time in the stomach, greater bioadhesiveness, and better protection against degradation [154]. In addition to the dissolution pH of the particles, it was demonstrated using Rhodamine 6G as a model compound that the nature of polymer used influenced the release rate, as well as adhesion to the GI mucosa [155].

Apart from Eudragit® copolymers, nanospheres have been obtained from mixtures of chitosan (a positively-charged absorption enhancer capable of opening the tight junctions) and pH-sensitive polyanions. Among these carriers, insulin-loaded nanospheres made from chitosan and γ-PGA, and cross-linked with sodium tripolyphosphate and magnesium sulfate, have been well-characterized for oral delivery [158-161]. The pK_a values of chitosan and γ-PGA are 6.5 and 2.9, respectively [162]. At pH values comprised between 2.5 and 6.5, both macromolecules are ionized and polyelectrolyte complexes with a spherical structure were obtained [158]. At pH 7.0–7.4, the chitosan is deprotonated, resulting in the disintegration of the nanospheres and expected drug release in the intestine. However, at pH 1.2–2.0, which would correspond to the pH of the stomach in the fasted state, most of the carboxyl groups of γ-PGA are protonated, and nanospheres were found to be unstable due to reduced electrostatic interactions. To circumvent this problem, the nanospheres were freeze-dried and filled in an enteric-coated capsule [160, 163]. As shown in Figure 2.5, the coated-capsule filled with insulin-loaded pH-sensitive nanospheres exhibited increased plasmatic drug levels after oral gavage to rats compared to the free form of insulin encapsulated in the coated capsule [160].

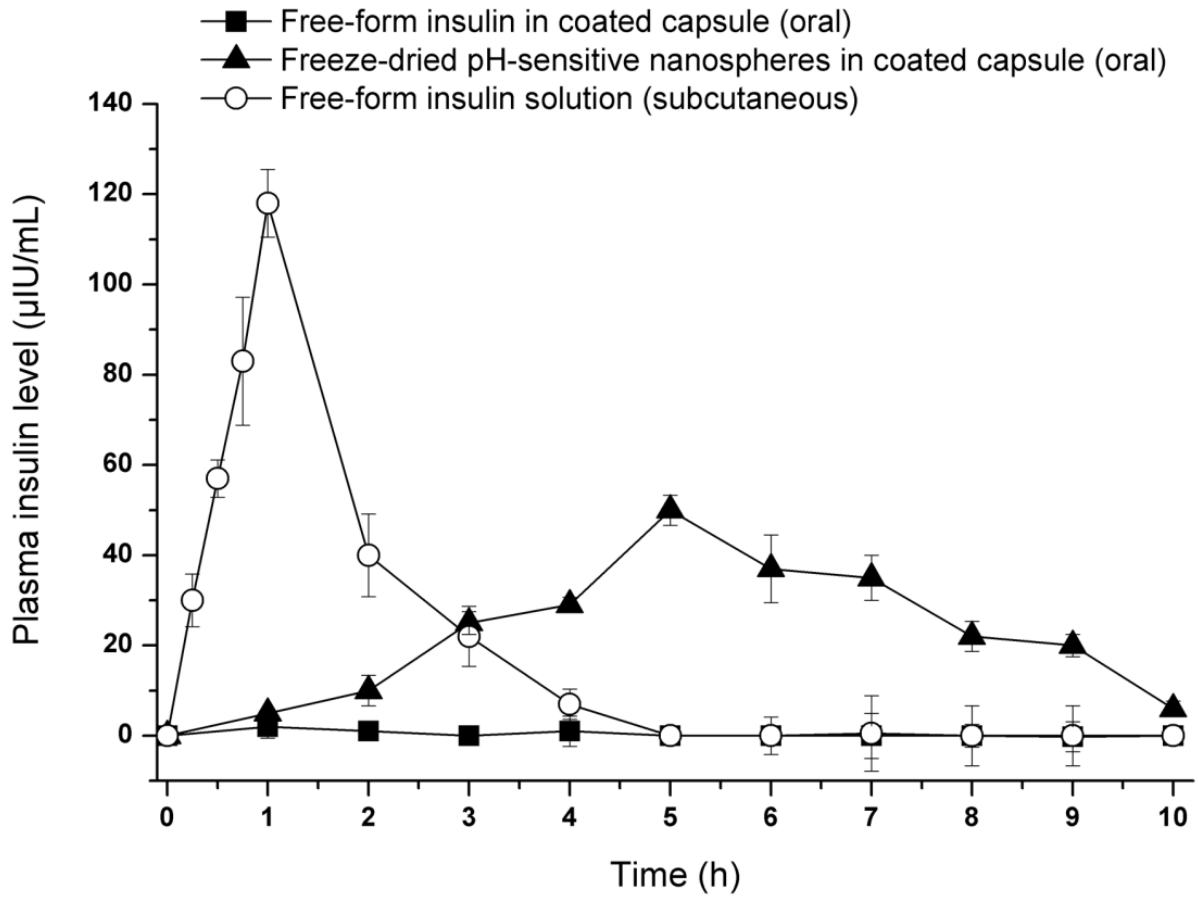


Figure 2.5. Plasma insulin level vs. time profiles of diabetic rats following the administration of different insulin formulations. The dose for oral and subcutaneous administration was 30 and 5 IU/kg, respectively. Results are expressed as mean ± SD (n = 5). Reproduced from Sonaje *et al.*, with permission from Elsevier [160].

Peppas and co-workers also described pH-responsive nanospheres capable of augmenting the bioavailability of peptides and proteins [164-166]. Their system consisted of cross-linked nanosized (200–400 nm) hydrogels of PEG methacrylate (PEGMA) and either AA or MAA. Under acidic conditions, these gels formed collapsed networks as a result of hydrogen bonding between the carboxylic acid groups and the grafted PEG chain, but ionized and swelled upon raising the pH, thereby allowing the release of the entrapped drug (Fig. 2.1C) [165]. Previous work conducted on similar but larger hydrogels showed that these systems could inhibit proteolytic enzymes in the GI tract and open the tight junctions of intestinal epithelium [167, 168]. After oral gavage in rats, the pH-responsive nanospheres were found to significantly reduce the serum glucose levels with respect to that of a control animal [165]. Recent *in vitro* data suggested that the permeability of insulin through the intestinal epithelial barrier could be further increased by conjugating insulin to a targeting ligand [169, 170]. These findings illustrate the potential of orally delivered labile drugs using

smart pH-sensitive nanospheres. Nevertheless, more work using these nanoparticles is needed to assess the safety and potential risk of letting other potentially immunogenic peptides benefit from the loosened junctions and enter the systemic circulation.

2.4.2. Vaginal drug delivery

Microbicides are drug delivery systems for the prevention of HIV and other sexually transmitted diseases. pH-Sensitive microbicides, exploiting the pH difference between human vagina (pH 4–5) [171] and human semen (pH ~7.5) [172] have been developed for semen-triggered vaginal drug delivery [173, 174]. Polymeric nanospheres composed of a blend of Eudragit® and biodegradable poly(lactic-*co*-glycolic acid) were prepared for the vaginal delivery of HIV reverse transcriptase inhibitors [174]. An *in vitro* release study demonstrated that over 40 % of the loaded drug was released within the first 24 h after contact with simulated semen fluid, whereas *ca.* 10 % was released in simulated vaginal fluid after the same period [174]. Even if the observed release time of such pH-sensitive nanospheres may, at first, appear unsuitable for clinical applications, it has been proven that human semen can be detected in the vaginal tract up to 48 h after sexual intercourse, and that the cervicovaginal pH remains at a relatively high level during this period [175]. However, these Eudragit®/poly(lactic-*co*-glycolic acid) nanospheres showed relatively low encapsulation efficiencies and further studies are needed to characterize conditions for optimal formulation stability, as well as their *in vivo* safety and efficacy.

2.5. Concluding remarks

In the field of drug delivery, nanosized systems efficiently responding to changes in the external pH have a wide range of applications. Compared to formulations that rely on the chemical cleavage of a hydrolyzable bond, systems based on titratable polyanions have the advantage of being able to undergo a quick and controllable change in conformation upon a change in pH, while exhibiting a good chemical stability. They are, however, sensitive to the ionic strength of the environment, which ultimately can impact on their pH-responsiveness. Furthermore, depending on the sharpness of their transition pH, systems based on polycarboxylates may not be sensitive enough to respond to the small pH gradient typically found between tumoral and normal tissues. Sulfonamide-based drug delivery systems, which sharply respond to pH changes around the physiological pH, might be able to address this issue [176-178]. Finally, polyanions can also impart the colloidal carrier with a strong

negative zeta potential, which in the case of parenteral dosing carries the risk of altering the pharmacokinetic profile and biodistribution of the transported drug. As of today, most pH-sensitive polyanion-based colloids have only been studied *in vitro* and there is a critical lack of solid *in vivo* data supporting their potential viability in a clinical context. In the future, it will be of prime importance to study in more systematic fashion their interaction with the biological milieu in order to develop formulations which will demonstrate a substantial improvement of the drug's activity, while maintaining a good shelf-life, simple manufacturing process, and adequate safety profile.

2.6. Acknowledgements

This work was financially supported by an ETH Research Grant to J.-C. Leroux (ID ETH-0209-3). The authors acknowledge the support from the Electron Microscopy Center of ETH Zurich (EMEZ).

2.7. References

[1] Gaucher G, Dufresne M-H, Sant VP, Kang N, Maysinger D, Leroux J-C. Block copolymer micelles: Preparation, characterization and application in drug delivery. J Control Release. 2005;109:169-88.

[2] Ganta S, Devalapally H, Shahiwala A, Amiji M. A review of stimuli-responsive nanocarriers for drug and gene delivery. J Control Release. 2008;126:187-204.

[3] Engin K, Leeper DB, Cater JR, Thistlethwaite AJ, Tupchong L, McFarlane JD. Extracellular pH distribution in human tumours. Int J Hyperthermia. 1995;11:211-6.

[4] Stubbs M, McSheehy PMJ, Griffiths JR, Bashford CL. Causes and consequences of tumour acidity and implications for treatment. Mol Med Today. 2000;6:15-9.

[5] Mellman I, Fuchs R, Helenius A. Acidification of the endocytic and exocytic pathways. Annu Rev Biochem. 1986;55:663-700.

[6] Iversen T-G, Skotland T, Sandvig K. Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies. Nano Today. 2011;6:176-85.

[7] Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. J Control Release. 2010;145:182-95.

[8] Zaki NM, Tirelli N. Gateways for the intracellular access of nanocarriers: A review of receptor-mediated endocytosis mechanisms and of strategies in receptor targeting. Expert Opin Drug Deliv. 2010;7:895-913.

[9] Bareford LM, Swaan PW. Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev. 2007;59:748-58.

[10] Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature. 2003;422:37-44.

[11] Storrie B, Desjardins M. The biogenesis of lysosomes: Is it a kiss and run, continuous fusion and fission process? Bioessays. 1996;18:895-903.

[12] Luzio JP, Rous BA, Bright NA, Pryor PR, Mullock BM, Piper RC. Lysosome-endosome fusion and lysosome biogenesis. J Cell Sci. 2000;113:1515-24.

[13] Kornfeld S, Mellman I. The biogenesis of lysosomes. Annu Rev Cell Biol. 1989;5:483-525.

[14] Daugherty AL, Mrsny RJ. Transcellular uptake mechanisms of the intestinal epithelial barrier Part one. Pharmaceut Sci Tech Today. 1999;2:144-51.

[15] Fallingborg J. Intraluminal pH of the human gastrointestinal tract. Dan Med Bull. 1999;46:183-96.

[16] Gaucher G, Satturwar P, Jones M-C, Furtos A, Leroux J-C. Polymeric micelles for oral drug delivery. Eur J Pharm Biopharm. 2010;76:147-58.

[17] Fuhrmann G, Leroux J-C. In vivo fluorescence imaging of exogenous enzyme activity in the gastrointestinal tract. Proc Natl Acad Sci U S A. 2011;108:9032-7.

[18] Miyata K, Christie RJ, Kataoka K. Polymeric micelles for nano-scale drug delivery. React Funct Polym. 2011;71:227-34.

[19] Drummond DC, Zignani M, Leroux J-C. Current status of pH-sensitive liposomes in drug delivery. Prog Lipid Res. 2000;39:409-60.

[20] Yessine M-A, Leroux J-C. Membrane-destabilizing polyanions: Interaction with lipid bilayers and endosomal escape of biomacromolecules. Adv Drug Deliv Rev. 2004;56:999-1021.

[21] Simões S, Moreira JN, Fonseca C, Düzgünes N, Pedroso de Lima MC. On the formulation of pH-sensitive liposomes with long circulation times. Adv Drug Deliv Rev. 2004;56:947-65.

[22] Karanth H, Murthy RSR. pH-Sensitive liposomes-principle and application in cancer therapy. J Pharm Pharmacol. 2007;59:469-83.

[23] Sawant RR, Torchilin VP. Liposomes as 'smart' pharmaceutical nanocarriers. Soft Matter. 2010;6:4026-44.

[24] Meyer O, Papahadjopoulos D, Leroux J-C. Copolymers of N-isopropylacrylamide can trigger pH sensitivity to stable liposomes. FEBS Lett. 1998;421:61-4.

[25] Leroux J-C, Roux E, Le Garrec D, Hong K, Drummond DC. N-isopropylacrylamide copolymers for the preparation of pH-sensitive liposomes and polymeric micelles. J Control Release. 2001;72:71-84.

[26] Roux E, Francis M, Winnik FM, Leroux J-C. Stimuli-responsive liposome-polymer complexes. In: Svenson S, editor. Carrier-based drug delivery. Washington DC: American Chemical Society; 2004. p. 26-39.
[27] Heskins M, Guillet JE. Solution properties of poly(*N*-isopropylacrylamide). J Macromol Sci Chem Part A 2. 1968;2:1441 - 55.
[28] Roux E, Lafleur M, Lataste É, Moreau P, Leroux JC. On the characterization of pH-sensitive liposome/polymer complexes. Biomacromolecules. 2003;4:240-8.
[29] Francis MF, Dhara G, Winnik FM, Leroux JC. *In vitro* evaluation of pH-sensitive polymer/niosome complexes. Biomacromolecules. 2001;2:741-9.
[30] Zignani M, Drummond DC, Meyer O, Hong K, Leroux J-C. *In vitro* characterization of a novel polymeric-based pH-sensitive liposome system. Biochim Biophys Acta - Biomembr. 2000;1463:383-94.
[31] Pétriat F, Roux E, Leroux JC, Giasson S. Study of molecular interactions between a phospholipidic layer and a pH-sensitive polymer using the langmuir balance technique. Langmuir. 2004;20:1393-400.
[32] Vial F, Oukhaled AG, Auvray L, Tribet C. Long-living channels of well defined radius opened in lipid bilayers by polydisperse, hydrophobically-modified polyacrylic acids. Soft Matter. 2007;3:75-8.
[33] Vial F, Cousin F, Bouteiller L, Tribet C. Rate of permeabilization of giant vesicles by amphiphilic polyacrylates compared to the adsorption of these polymers onto large vesicles and tethered lipid bilayers. Langmuir. 2009;25:7506-13.
[34] Tirrell DA, Takigawa DY, Seki K. pH sensitization of phospholipid vesicles *via* complexation with synthetic poly(carboxylic acid)s. Ann NY Acad Sci. 1985;446:237-48.
[35] Bulmus V. Biomembrane-active molecular switches as tools for intracellular drug delivery. Aust J Chem. 2005;58:411-22.
[36] Chen T, Mcintosh D, He Y, Kim J, Tirrell DA, Scherrer P, et al. Alkylated derivatives of poly(ethylacrylic acid) can be inserted into preformed liposomes and trigger pH-dependent intracellular delivery of liposomal contents. Mol Membr Biol. 2004;21:385-93.
[37] Chen T, Choi LS, Einstein S, Klippenstein MA, Scherrer P, Cullis PR. Proton-induced permeability and fusion of large unilamellar vesicles by covalently conjugated poly(2-ethylacrylic acid). J Liposome Res. 1999;9:387-405.
[38] Kono K, Zenitani K, Takagishi T. Novel pH-sensitive liposomes: Liposomes bearing poly(ethylene glycol) derivative with carboxyl groups. Biochim Biophys Acta. 1994;1193:1-9.
[39] Kono K, Igawa T, Takagishi T. Cytoplasmic delivery of calcein mediated by liposomes modified with a pH-sensitive poly(ethylene glycol) derivative. Biochim Biophys Acta - Biomembr. 1997;1325:143-54.
[40] Sakaguchi N, Kojima C, Harada A, Koiwai K, Kono K. The correlation between fusion capability and transfection activity in hybrid complexes of lipoplexes and pH-sensitive liposomes. Biomaterials. 2008;29:4029-36.
[41] Yuba E, Kojima C, Harada A, Tana, Watarai S, Kono K. pH-Sensitive fusogenic polymer-modified liposomes as a carrier of antigenic proteins for activation of cellular immunity. Biomaterials. 2010;31:943-51.
[42] Yuba E, Harada A, Sakanishi Y, Kono K. Carboxylated hyperbranched poly(glycidol)s for preparation of pH-sensitive liposomes. J Control Release. 2011;149:72-80.
[43] Mizoue T, Horibe T, Maruyama K, Takizawa T, Iwatsuru M, Kono K, et al. Targetability and intracellular delivery of anti-BCG antibody-modified, pH-sensitive fusogenic immunoliposomes to tumor cells. Int J Pharm. 2002;237:129-37.
[44] Couffin-Hoarau AC, Leroux JC. Report on the use of poly(organophosphazenes) for the design of stimuli-responsive vesicles. Biomacromolecules. 2004;5:2082-7.
[45] Ghattas D, Leroux J-C. Amphiphilic ionizable polyphosphazenes for the preparation of pH-responsive liposomes. In: Andrianov AK, editor. Polyphosphazenes for biomedical applications. Hoboken: John Wiley & Sons, Inc.; 2008. p. 225-47.
[46] Seki K, Tirrell DA, Braud C, Vert M. pH-Dependent structural modification of dipalmitoylphosphatidylcholine vesicle membranes by a degradable poly(carboxylic acid) of pharmacological importance. Makromolekulare Chemie Rapid Communications. 1984;5:187-90.

[47] Osanai S, Nakamura K. Effects of complexation between liposome and poly(malic acid) on aggregation and leakage behaviour. Biomaterials. 2000;21:867-76.
[48] Jo SM, Kim JC. pH sensitivities of egg phosphatidylcholine liposomes and dioleoylphosphatidylethanolamine liposomes triggered by poly(*N*-isopropylacrylamide-*co*-methacrylic acid-*co*-octadecylacrylate). Colloid Polym Sci. 2009;287:1065-70.
[49] Cho EC, Lim HJ, Shim J, Park JY, Dan N, Kim J, et al. Effect of polymer characteristics on structure of polymer-liposome complexes. J Colloid Interface Sci. 2007;311:243-52.
[50] Simard P, Leroux J-C. pH-sensitive immunoliposomes specific to the CD33 cell surface antigen of leukemic cells. Int J Pharm. 2009;381:86-96.
[51] Bertrand N, Fleischer JG, Wasan KM, Leroux J-C. Pharmacokinetics and biodistribution of *N*-isopropylacrylamide copolymers for the design of pH-sensitive liposomes. Biomaterials. 2009;30:2598-605.
[52] Sakaguchi N, Kojima C, Harada A, Kono K. Preparation of pH-sensitive poly(glycidol) derivatives with varying hydrophobicities: Their ability to sensitize stable liposomes to pH. Bioconjugate Chem. 2008;19:1040-8.
[53] Cherkaoui S, Bettinger T, Hauwel M, Navetat S, Allémann E, Schneider M. Tracking of antibody reduction fragments by capillary gel electrophoresis during the coupling to microparticles surface. J Pharm Biomed Anal. 2010;53:172-8.
[54] Roux E, Francis M, Winnik FM, Leroux J-C. Polymer based pH-sensitive carriers as a means to improve the cytoplasmic delivery of drugs. Int J Pharm. 2002;242:25-36.
[55] Polozova A, Winnik FM. Contribution of hydrogen bonding to the association of liposomes and an anionic hydrophobically modified poly(*N*-isopropylacrylamide). Langmuir. 1999;15:4222-9.
[56] Drummond DC, Meyer O, Hong K, Kirpotin DB, Papahadjopoulos D. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. Pharmacol Rev. 1999;51:691-744.
[57] Roux E, Passirani C, Scheffold S, Benoit J-P, Leroux J-C. Serum-stable and long-circulating, PEGylated, pH-sensitive liposomes. J Control Release. 2004;94:447-51.
[58] Bertrand N, Simard P, Leroux J-C. Serum-stable, long-circulating, pH-sensitive PEGylated liposomes. Methods Mol Biol. 2010;605:545-58.
[59] Simard P, Leroux J-C. *In vivo* evaluation of pH-sensitive polymer-based immunoliposomes targeting the CD33 antigen. Mol Pharm. 2010;7:1098-107.
[60] Roux E, Stomp R, Giasson S, Pezolet M, Moreau P, Leroux JC. Steric stabilization of liposomes by pH-responsive *N*-isopropylacrylamide copolymer. J Pharm Sci. 2002;91:1795-802.
[61] Lee SM, Chen H, Dettmer CM, O'Halloran TV, Nguyen ST. Polymer-caged lipsomes: A pH-responsive delivery system with high stability. J Am Chem Soc. 2007;129:15096-7.
[62] Cho EC, Lim HJ, Kim HJ, Son ED, Choi HJ, Park JH, et al. Role of pH-sensitive polymer-liposome complex in enhancing cellular uptake of biologically active drugs. Mater Sci Eng, C. 2009;29:774-8.
[63] Fujiwara M, Baldeschwieler JD, Grubbs RH. Receptor-mediated endocytosis of poly(acrylic acid)-conjugated liposomes by macrophages. Biochim Biophys Acta - Biomembr. 1996;1278:59-67.
[64] Yuba E, Kojima C, Sakaguchi N, Harada A, Koiwai K, Kono K. Gene delivery to dendritic cells mediated by complexes of lipoplexes and pH-sensitive fusogenic polymer-modified liposomes. J Control Release. 2008;130:77-83.
[65] Yamazaki A, Winnik FM, Cornelius RM, Brash JL. Modification of liposomes with N-substituted polyacrylamides: Identification of proteins adsorbed from plasma. Biochim Biophys Acta - Biomembr. 1999;1421:103-15.
[66] Yessine M-A, Meier C, Petereit H-U, Leroux J-C. On the role of methacrylic acid copolymers in the intracellular delivery of antisense oligonucleotides. Eur J Pharm Biopharm. 2006;63:1-10.
[67] Cheung CY, Murthy N, Stayton PS, Hoffman AS. A pH-sensitive polymer that enhances cationic lipid-mediated gene transfer. Bioconjugate Chem. 2001;12:906-10.
[68] Jones RA, Cheung CY, Black FE, Zia JK, Stayton PS, Hoffman AS, et al. Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by pH-sensitive disruption of endosomal vesicles. Biochem J. 2003;372:65-75.

[69] Cheung C, Stayton P, Hoffman A. Poly(propylacrylic acid)-mediated serum stabilization of cationic lipoplexes. J Biomater Sci Polym Ed. 2005;16:163-79.
[70] Kyriakides TR, Cheung CY, Murthy N, Bornstein P, Stayton PS, Hoffman AS. pH-Sensitive polymers that enhance intracellular drug delivery *in vivo*. J Control Release. 2002;78:295-303.
[71] Kono K, Torikoshi Y, Mitsutomi M, Itoh T, Emi N, Yanagie H, et al. Novel gene delivery systems: Complexes of fusigenic polymer-modified liposomes and lipoplexes. Gene Ther. 2001;8:5-12.
[72] Sakaguchi N, Kojima C, Harada A, Koiwai K, Shimizu K, Emi N, et al. Enhancement of transfection activity of lipoplexes by complexation with transferrin-bearing fusogenic polymer-modified liposomes. Int J Pharm. 2006;325:186-90.
[73] Massignani M, Lomas H, Battaglia G. Polymersomes: A synthetic biological approach to encapsulation and delivery. Adv Polym Sci. 2010;229:115-54.
[74] Discher DE, Ortiz V, Srinivas G, Klein ML, Kim Y, Christian D, et al. Emerging applications of polymersomes in delivery: From molecular dynamics to shrinkage of tumors. Prog Polym Sci. 2007;32:838-57.
[75] Meng F, Zhong Z, Feijen J. Stimuli-responsive polymersomes for programmed drug delivery. Biomacromolecules. 2009;10:197-209.
[76] Wu J, Eisenberg A. Proton diffusion across membranes of vesicles of poly(styrene-*b*-acrylic Acid) diblock copolymers. J Am Chem Soc. 2006;128:2880-4.
[77] Chécot F, Lecommandoux S, Gnanou Y, Klok H-A. Water-soluble stimuli-responsive vesicles from peptide-based diblock copolymers. Angew Chem-Int Edit. 2002;41:1339-43.
[78] Holowka EP, Pochan DJ, Deming TJ. Charged polypeptide vesicles with controllable diameter. J Am Chem Soc. 2005;127:12423-8.
[79] Rodríguez-Hernández J, Lecommandoux S. Reversible inside-out micellization of pH-responsive and water-soluble vesicles based on polypeptide diblock copolymers. J Am Chem Soc. 2005;127:2026-7.
[80] Lomas H, Canton I, MacNeil S, Du J, Armes SP, Ryan AJ, et al. Biomimetic pH sensitive polymersomes for efficient DNA encapsulation and delivery. Adv Mater. 2007;19:4238-43.
[81] Sanson C, Schatz C, Le Meins J-F, Soum A, Thévenot J, Garanger E, et al. A simple method to achieve high doxorubicin loading in biodegradable polymersomes. J Control Release. 2010;147:428-35.
[82] Sanson C, Schatz C, Le Meins J-F, Brûlet A, Soum A, Lecommandoux S. Biocompatible and biodegradable poly(trimethylene carbonate)-*b*-poly(L-glutamic acid) polymersomes: Size control and stability. Langmuir. 2009;26:2751-60.
[83] Chécot F, Brûlet A, Oberdisse J, Gnanou Y, Mondain-Monval O, Lecommandoux S. Structure of polypeptide-based diblock copolymers in solution: Stimuli-responsive vesicles and micelles. Langmuir. 2005;21:4308-15.
[84] Upadhyay KK, Mishra AK, Chuttani K, Kaul A, Schatz C, Le Meins J-F, et al. The *in vivo* behavior and antitumor activity of doxorubicin-loaded poly(γ-benzyl L-glutamate)-block-hyaluronan polymersomes in Ehrlich ascites tumor-bearing BalB/c mice. Nanomedicine: NBM. 2012;8:71-80.
[85] Shi Z, Zhou Y, Yan D. Facile fabrication of pH-responsive and size-controllable polymer vesicles from a commercially available hyperbranched polyester. Macromol Rapid Commun. 2008;29:412-8.
[86] Dou H, Jiang M, Peng H, Chen D, Hong Y. pH-dependent self-assembly: Micellization and micelle–hollow-sphere transition of cellulose-based copolymers. Angew Chem-Int Edit. 2003;42:1516-9.
[87] Chiang W-H, Hsu Y-H, Tang F-F, Chern C-S, Chiu H-C. Temperature/pH-induced morphological regulations of shell cross-linked graft copolymer assemblies. Polymer. 2010;51:6248-57.
[88] Koide A, Kishimura A, Osada K, Jang W-D, Yamasaki Y, Kataoka K. Semipermeable polymer vesicle (PICsome) self-assembled in aqueous medium from a pair of oppositely charged block copolymers: Physiologically stable micro-/nanocontainers of water-soluble macromolecules. J Am Chem Soc. 2006;128:5988-9.

[89] Kishimura A, Liamsuwan S, Matsuda H, Dong W-F, Osada K, Yamasaki Y, et al. pH-dependent permeability change and reversible structural transition of PEGylated polyion complex vesicles (PICsomes) in aqueous media. Soft Matter. 2009;5:529-32.
[90] Anraku Y, Kishimura A, Oba M, Yamasaki Y, Kataoka K. Spontaneous formation of nanosized unilamellar polyion complex vesicles with tunable size and properties. J Am Chem Soc. 2010;132:1631-6.
[91] Chiu H-C, Lin Y-W, Huang Y-F, Chuang C-K, Chern C-S. Polymer vesicles containing small vesicles within interior aqueous compartments and pH-responsive transmembrane channels. Angew Chem-Int Edit. 2008;47:1875-8.
[92] Sheng Y, Liu C, Yuan Y, Tao X, Yang F, Shan X, et al. Long-circulating polymeric nanoparticles bearing a combinatorial coating of PEG and water-soluble chitosan. Biomaterials. 2009;30:2340-8.
[93] Gabizon A, Papahadjopoulos D. The role of surface charge and hydrophilic groups on liposome clearance *in vivo*. Biochim Biophys Acta - Biomembr. 1992;1103:94-100.
[94] Kwon GS, Kataoka K. Block copolymer micelles as long-circulating drug vehicles. Adv Drug Deliv Rev. 1995;16:295-309.
[95] Jones M-C, Leroux J-C. Polymeric micelles - a new generation of colloidal drug carriers. Eur J Pharm Biopharm. 1999;48:101-11.
[96] Gillies ER, Frechet JMJ. A new approach towards acid sensitive copolymer micelles for drug delivery. Chem Commun. 2003:1640-1.
[97] Bae Y, Nishiyama N, Fukushima S, Koyama H, Yasuhiro M, Kataoka K. Preparation and biological characterization of polymeric micelle drug carriers with intracellular pH-triggered drug release property: Tumor permeability, controlled subcellular drug distribution, and enhanced *in vivo* antitumor efficacy. Bioconjugate Chem. 2005;16:122-30.
[98] Bae Y, Fukushima S, Harada A, Kataoka K. Design of environment-sensitive supramolecular assemblies for intracellular drug delivery: Polymeric micelles that are responsive to intracellular pH change. Angew Chem-Int Edit. 2003;42:4640-3.
[99] Oishi M, Nagasaki Y, Itaka K, Nishiyama N, Kataoka K. Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. J Am Chem Soc. 2005;127:1624-5.
[100] Taillefer J, Brasseur N, van Lier JE, Lenaerts V, Garrec DL, Leroux JC. *In-vitro* and *in-vivo* evaluation of pH-responsive polymeric micelles in a photodynamic cancer therapy model. J Pharm Pharmacol. 2001;53:155-66.
[101] Solomatin SV, Bronich TK, Bargar TW, Eisenberg A, Kabanov VA, Kabanov AV. Environmentally responsive nanoparticles from block ionomer complexes: Effects of pH and ionic strength. Langmuir. 2003;19:8069-76.
[102] Bronich TK, Keifer PA, Shlyakhtenko LS, Kabanov AV. Polymer micelle with cross-linked ionic core. J Am Chem Soc. 2005;127:8236-7.
[103] Bontha S, Kabanov AV, Bronich TK. Polymer micelles with cross-linked ionic cores for delivery of anticancer drugs. J Control Release. 2006;114:163-74.
[104] Oberoi HS, Laquer FC, Marky LA, Kabanov AV, Bronich TK. Core cross-linked block ionomer micelles as pH-responsive carriers for cis-diamminedichloroplatinum(II). J Control Release. 2011;153:64-72.
[105] Yuan X, Yamasaki Y, Harada A, Kataoka K. Characterization of stable lysozyme-entrapped polyion complex (PIC) micelles with crosslinked core by glutaraldehyde. Polymer. 2005;46:7749-58.
[106] Kim JO, Kabanov AV, Bronich TK. Polymer micelles with cross-linked polyanion core for delivery of a cationic drug doxorubicin. J Control Release. 2009;138:197-204.
[107] Harada A, Kataoka K. Novel polyion complex micelles entrapping enzyme molecules in the core: Preparation of narrowly-distributed micelles from lysozyme and poly(ethylene glycol)-poly(aspartic acid) block copolymer in aqueous medium. Macromolecules. 1998;31:288-94.
[108] Zhang G-D, Nishiyama N, Harada A, Jiang D-L, Aida T, Kataoka K. pH-sensitive assembly of light-Harvesting dendrimer zinc porphyrin bearing peripheral groups of primary amine with poly(ethylene glycol)-*b*-poly(aspartic acid) in aqueous solution. Macromolecules. 2003;36:1304-9.

[109] Taillefer J, Jones MC, Brasseur N, van Lier JE, Leroux JC. Preparation and characterization of pH-responsive polymeric micelles for the delivery of photosensitizing anticancer drugs. J Pharm Sci. 2000;89:52-62.
[110] Le Garrec D, Taillefer J, Van Lier JE, Lenaerts V, Leroux J-C. Optimizing pH-responsive polymeric micelles for drug delivery in a cancer photodynamic therapy model. J Drug Target. 2002;10:429-37.
[111] Lo C-L, Huang C-K, Lin K-M, Hsiue G-H. Mixed micelles formed from graft and diblock copolymers for application in intracellular drug delivery. Biomaterials. 2007;28:1225-35.
[112] Soppimath KS, Tan DC-W, Yang YY. pH-triggered thermally responsive polymer core–shell nanoparticles for drug delivery. Adv Mater. 2005;17:318-23.
[113] Soppimath KS, Liu LH, Seow WY, Liu SQ, Powell R, Chan P, et al. Multifunctional core/shell nanoparticles self-assembled from pH-induced thermosensitive polymers for targeted intracellular anticancer drug delivery. Adv Funct Mater. 2007;17:355-62.
[114] Wei H, Zhang X-Z, Cheng H, Chen W-Q, Cheng S-X, Zhuo R-X. Self-assembled thermo- and pH responsive micelles of poly(10-undecenoic acid-*b*-*N*-isopropylacrylamide) for drug delivery. J Control Release. 2006;116:266-74.
[115] Li G, Song S, Guo L, Ma S. Self-assembly of thermo- and pH-responsive poly(acrylic acid)-*b*-poly(N-isopropylacrylamide) micelles for drug delivery. J Polym Sci, Part A: Polym Chem. 2008;46:5028-35.
[116] Dai W, Zhang Y, Du Z, Ru M, Lang M. The pH-induced thermosensitive poly(NIPAAm-*co*-AAc-*co*-HEMA)-*g*-PCL micelles used as a drug carrier. J Mater Sci Mater Med. 2010;21:1881-90.
[117] Benoit DSW, Henry SM, Shubin AD, Hoffman AS, Stayton PS. pH-responsive polymeric siRNA carriers sensitize multidrug resistant ovarian cancer cells to doxorubicin via knockdown of polo-like kinase 1. Mol Pharm. 2010;7:442-55.
[118] Yessine M-A, Dufresne M-H, Meier C, Petereit H-U, Leroux J-C. Proton-actuated membrane-destabilizing polyion complex micelles. Bioconjugate Chem. 2007;18:1010-4.
[119] Elsabahy M, Wazen N, Bayó-Puxan N, Deleavey G, Servant M, Damha MJ, et al. Delivery of nucleic acids through the controlled disassembly of multifunctional nanocomplexes. Adv Funct Mater. 2009;19:3862-7.
[120] Felber AE, Castagner B, Elsabahy M, Deleavey GF, Damha MJ, Leroux J-C. siRNA nanocarriers based on methacrylic acid copolymers. J Control Release. 2011;152:159-67.
[121] Convertine AJ, Diab C, Prieve M, Paschal A, Hoffman AS, Johnson PH, et al. pH-responsive polymeric micelle carriers for siRNA drugs. Biomacromolecules. 2011;11:2904-11.
[122] Stiriba SE, Frey H, Haag R. Dendritic polymers in biomedical applications: From potential to clinical use in diagnostics and therapy. Angew Chem-Int Edit. 2002;41:1329-34.
[123] Thomas M, Klibanov AM. Non-viral gene therapy: Polycation-mediated DNA delivery. Appl Microbiol Biotechnol. 2003;62:27-34.
[124] Bayó-Puxan N, Dufresne M-H, Felber AE, Castagner B, Leroux J-C. Preparation of polyion complex micelles from poly(ethylene glycol)-*block*-polyions. J Control Release. 2011:118-27.
[125] Hunter J, Hirst BH. Intestinal secretion of drugs. The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. Adv Drug Deliv Rev. 1997;25:129-57.
[126] Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: Role of ATP-dependent transporters. Nat Rev Cancer. 2002;2:48-58.
[127] Croy SR, Kwon GS. Polymeric micelles for drug delivery. Curr Pharm Des. 2006;12:4669-84.
[128] Nishiyama N, Kataoka K. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. Pharmacol Ther. 2006;112:630-48.
[129] Le Garrec D, Ranger M, Leroux J-C. Micelles in anticancer drug delivery. Am J Drug Deliv. 2004;2:15-42.
[130] Pierri E, Avgoustakis K. Poly(lactide)-poly(ethylene glycol) micelles as a carrier for griseofulvin. J Biomed Mater Res Part A. 2005;75A:639-47.
[131] Ould-Ouali L, Noppe M, Langlois X, Willems B, Te Riele P, Timmerman P, et al. Self-assembling PEG-P(CL-*co*-TMC) copolymers for oral delivery of poorly water-soluble drugs: A case study with risperidone. J Control Release. 2005;102:657-68.

[132] Camilleri M, Colemont LJ, Phillips SF, Brown ML, Thomforde GM, Chapman N, et al. Human gastric emptying and colonic filling of solids characterized by a new method. Am J Physiol Gastrointest Liver Physiol. 1989;257:G284-G90.
[133] Jones M-C, Ranger M, Leroux J-C. pH-sensitive unimolecular polymeric micelles: Synthesis of a novel drug carrier. Bioconjugate Chem. 2003;14:774-81.
[134] Sant VP, Smith D, Leroux J-C. Novel pH-sensitive supramolecular assemblies for oral delivery of poorly water soluble drugs: Preparation and characterization. J Control Release. 2004;97:301-12.
[135] Kim S, Kim JY, Huh KM, Acharya G, Park K. Hydrotropic polymer micelles containing acrylic acid moieties for oral delivery of paclitaxel. J Control Release. 2008;132:222-9.
[136] Ranger M, Jones M-C, Yessine M-A, Leroux J-C. From well-defined diblock copolymers prepared by a versatile atom transfer radical polymerization method to supramolecular assemblies. J Polym Sci, Part A: Polym Chem. 2001;39:3861-74.
[137] Satturwar P, Eddine MN, Ravenelle F, Leroux J-C. pH-responsive polymeric micelles of poly(ethylene glycol)-*b*-poly(alkyl(meth)acrylate-*co*-methacrylic acid): Influence of the copolymer composition on self-assembling properties and release of candesartan cilexetil. Eur J Pharm Biopharm. 2007;65:379-87.
[138] Sant VP, Smith D, Leroux J-C. Enhancement of oral bioavailability of poorly water-soluble drugs by poly(ethylene glycol)-*block*-poly(alkyl acrylate-*co*-methacrylic acid) self-assemblies. J Control Release. 2005;104:289-300.
[139] Holappa S, Kantonen L, Winnik FM, Tenhu H. Self-complexation of poly(ethylene oxide)-*block*-poly(methacrylic acid) studied by fluorescence spectroscopy. Macromolecules. 2004;37:7008-18.
[140] Jin G-W, Ji XY, Choi J-S. Preparation of naproxen-loaded poly(ethylene oxide-*b*-methacrylic acid) micelle and its pH-dependent drug release behavior. Bull Korean Chem Soc. 2009;30:931-4.
[141] Adams ML, Rao VM, Thakur A, Hussain MA. Colloidal phase behavior of pH-responsive, amphiphilic PEGylated poly(carboxylic acid)s and effect on kinetic solubility under acidic conditions. Pharm Dev Technol. 2010;15:266-75.
[142] Yang YQ, Zheng LS, Guo XD, Qian Y, Zhang LJ. pH-sensitive micelles self-assembled from amphiphilic copolymer brush for delivery of poorly water-soluble drugs. Biomacromolecules. 2011;12:116-22.
[143] Wang X-L, Xu R, Wu X, Gillespie D, Jensen R, Lu Z-R. Targeted systemic delivery of a therapeutic siRNA with a multifunctional carrier controls tumor proliferation in mice. Mol Pharm. 2009;6:738-46.
[144] Filippov S, Hruby M, Konak C, Mackova H, Spirkova M, Stepanek P. Novel pH-responsive nanoparticles. Langmuir. 2008;24:9295-301.
[145] Filippov SK, Starovoytova L, Konak C, Hruby M, Mackova H, Karlsson G, et al. pH sensitive polymer nanoparticles: Effect of hydrophobicity on self-assembly. Langmuir. 2010;26:14450-7.
[146] Soppimath KS, Aminabhavi TM, Kulkarni AR, Rudzinski WE. Biodegradable polymeric nanoparticles as drug delivery devices. J Control Release. 2001;70:1-20.
[147] Leroux J-C, Doelker E, Gurny R, Cozens R, Roesel JL, Galli B, et al. Pharmacokinetics of a novel HIV-1 protease inhibitor incorporated into biodegradable or enteric nanoparticles following intravenous and oral administration to mice. J Pharm Sci. 1995;84:1387-91.
[148] Leroux J-C, Cozens RM, Roesel JL, Galli B, Doelker E, Gurny R. pH-sensitive nanoparticles: An effective means to improve the oral delivery of HIV-1 protease inhibitors in dogs. Pharm Res. 1996;13:485-7.
[149] Allémann E, Leroux J-C, Gurny R. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. Adv Drug Deliv Rev. 1998;34:171-89.
[150] De Jaeghere F, Allémann E, Kubel F, Galli B, Cozens R, Doelker E, et al. Oral bioavailability of a poorly water soluble HIV-1 protease inhibitor incorporated into pH-sensitive particles: Effect of the particle size and nutritional state. J Control Release. 2000;68:291-8.
[151] De Jaeghere F, Allémann E, Doelker E, Gurny R, Cerny R, Galli B, et al. pH-dependent dissolving nano- and microparticles for improved peroral delivery of a highly lipophilic compound in dogs. AAPS Pharmsci. 2001;3:92-9.

[152] Jelvehgari M, Zakeri-Milani P, Siahi-Shadbad M, Loveymi B, Nokhodchi A, Azari Z, et al. Development of pH-sensitive insulin nanoparticles using Eudragit L100-55 and chitosan with different molecular weights. AAPS PharmSciTech. 2011;11:1237-42.
[153] Li M-G, Lu W-L, Wang J-C, Zhang X, Zhang H, Wang X-Q, et al. Preparation and characterization of insulin nanoparticles employing chitosan and poly(methylmethacrylate/methylmethacrylic acid) copolymer. J Nanosci Nanotechnol. 2006;6:2874-86.
[154] Wang X-Q, Dai J-D, Zhang H, Zhang X, Wang J-C, Zhang Q. Absorption mechanism of cyclosporine A loaded pH-sensitive nanoparticles in rats. J Nanosci Nanotechnol. 2008;8:2422-31.
[155] Wu C-s, Wang X-q, Meng M, Li M-g, Zhang H, Zhang X, et al. Effects of pH-sensitive nanoparticles prepared with different polymers on the distribution, adhesion and transition of Rhodamine 6G in the gut of rats. J Microencapsulation. 2010;27:205-17.
[156] Jia Z, Lin P, Xiang Y, Wang X, Wang J, Zhang X, et al. A novel nanomatrix system consisted of colloidal silica and pH-sensitive polymethylacrylate improves the oral bioavailability of fenofibrate. Eur J Pharm Biopharm. 2011;71:126-34.
[157] Cetin M, Atila A, Kadioglu Y. Formulation and in vitro characterization of Eudragit® L100 and Eudragit® L100-PLGA nanoparticles containing diclofenac sodium. AAPS PharmSciTech. 2010;11:1250-6.
[158] Lin Y-H, Sonaje K, Lin KM, Juang J-H, Mi F-L, Yang H-W, et al. Multi-ion-crosslinked nanoparticles with pH-responsive characteristics for oral delivery of protein drugs. J Control Release. 2008;132:141-9.
[159] Sonaje K, Lin Y-H, Juang J-H, Wey S-P, Chen C-T, Sung H-W. *In vivo* evaluation of safety and efficacy of self-assembled nanoparticles for oral insulin delivery. Biomaterials. 2009;30:2329-39.
[160] Sonaje K, Chen Y-J, Chen H-L, Wey S-P, Juang J-H, Nguyen H-N, et al. Enteric-coated capsules filled with freeze-dried chitosan/poly(γ-glutamic acid) nanoparticles for oral insulin delivery. Biomaterials. 2010;31:3384-94.
[161] Sonaje K, Lin K-J, Wang J-J, Mi F-L, Chen C-T, Juang J-H, et al. Self-assembled pH-sensitive nanoparticles: A platform for oral delivery of protein drugs. Adv Funct Mater. 2010;20:3695-700.
[162] Lin Y-H, Chung C-K, Chen C-T, Liang H-F, Chen S-C, Sung H-W. Preparation of nanoparticles composed of chitosan/poly-γ-glutamic acid and evaluation of their permeability through Caco-2 cells. Biomacromolecules. 2005;6:1104-12.
[163] Nguyen H-N, Wey S-P, Juang J-H, Sonaje K, Ho Y-C, Chuang E-Y, et al. The glucose-lowering potential of exendin-4 orally delivered *via* a pH-sensitive nanoparticle vehicle and effects on subsequent insulin secretion *in vivo*. Biomaterials. 2011;32:2673-82.
[164] Robinson DN, Peppas NA. Preparation and characterization of pH-responsive poly(methacrylic acid-*g*-ethylene glycol) nanospheres. Macromolecules. 2002;35:3668-74.
[165] Foss AC, Goto T, Morishita M, Peppas NA. Development of acrylic-based copolymers for oral insulin delivery. Eur J Pharm Biopharm. 2004;57:163-9.
[166] Donini C, Robinson DN, Colombo P, Giordano F, Peppas NA. Preparation of poly(methacrylic acid-*g*-poly(ethylene glycol)) nanospheres from methacrylic monomers for pharmaceutical applications. Int J Pharm. 2002;245:83-91.
[167] Kavimandan NJ, Peppas NA. Confocal microscopic analysis of transport mechanisms of insulin across the cell monolayer. Int J Pharm. 2008;354:143-8.
[168] Morishita M, Peppas NA. Is the oral route possible for peptide and protein drug delivery? Drug Discov Today. 2006;11:905-10.
[169] Kavimandan NJ, Losi E, Peppas NA. Novel delivery system based on complexation hydrogels as delivery vehicles for insulin-transferrin conjugates. Biomaterials. 2006;27:3846-54.
[170] Wood KM, Stone GM, Peppas NA. The effect of complexation hydrogels on insulin transport in intestinal epithelial cell models. Acta Biomater. 2010;6:48-56.
[171] Owen DH, Katz DF. A vaginal fluid simulant. Contraception. 1999;59:91-5.
[172] Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. J Androl. 2005;26:459-69.

[173] Gupta KM, Barnes SR, Tangaro RA, Roberts MC, Owen DH, Katz DF, et al. Temperature and pH sensitive hydrogels: An approach towards smart semen-triggered vaginal microbicidal vehicles. J Pharm Sci. 2007;96:670-81.
[174] Zhang T, Sturgis TF, Youan B-BC. pH-responsive nanoparticles releasing tenofovir for the prevention of HIV transmission. Eur J Pharm Biopharm. 2011;79:526-36.
[175] Tevi-Benissan C, Belec L, Levy M, Schneider-Fauveau V, Si Mohamed A, Hallouin M, et al. In vivo semen-associated pH neutralization of cervicovaginal secretions. Clin Diagn Lab Immunol. 1997;4:367-74.
[176] Han SK, Na K, Bae YH. Sulfonamide based pH-sensitive polymeric micelles: Physicochemical characteristics and pH-dependent aggregation. Colloids Surf Physicochem Eng Aspects. 2003;214:49-59.
[177] Na K, Seong Lee E, Bae YH. Adriamycin loaded pullulan acetate/sulfonamide conjugate nanoparticles responding to tumor pH: pH-Dependent cell interaction, internalization and cytotoxicity *in vitro*. J Control Release. 2003;87:3-13.
[178] Sethuraman V, Lee M, Bae Y. A biodegradable pH-sensitive micelle system for targeting acidic solid tumors. Pharm Res. 2008;25:657-66.

Chapter 3

siRNA Nanocarriers Based on Methacrylic Acid Copolymers

Arnaud E. Felber[1], Bastien Castagner[1], Mahmoud Elsabahy[2], Glen F. Deleavey[3], Masad J. Damha[3], Jean-Christophe Leroux[1]

[1] Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland

[2] School of Pharmacy, University of Waterloo, Kitchener, ON N2G 1C5, Canada

[3] Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC H3A 2K6, Canada

This chapter is published:

Journal of Controlled Release, **2011**; 152:159-67.

3.1. Introduction

The use of nucleic acids, such as antisense oligonucleotides and small interfering RNAs (siRNAs), represents an elegant strategy to tackle several pathological processes such as cancer, viral infections, dominant genetic disorders, and autoimmune diseases [1]. In particular, since its first description by Fire and Mello in 1998 and their experiments on the nematode *Caenorhabditis elegans* [2], RNA interference (RNAi) has drawn considerable attention for its potential to silence almost all endogenous genes [3-5]. Nevertheless, the clinical applications of RNAi-based therapeutics are hampered by a number of undesirable physicochemical and biopharmaceutical characteristics. These include i) enzymatic lability, ii) lack of tissue or organ specificity [6, 7], iii) rapid renal clearance [8], iv) poor cellular uptake, and v) limited access to intracellular targets [9]. The intracellular bioavailability of siRNAs is indeed largely compromised by their sequestration in the endosomal/lysosomal compartments [10].

In the past 10 years, tremendous efforts have been made to improve the *in vivo* delivery of siRNAs to eukaryotic cells. Among the strategies currently under investigation, one can cite the extensive chemical modifications of the oligonucleotide backbone [11], and the development of nanosized polymeric [12] and lipidic [13] non-viral vectors. siRNA duplexes have been modified in many ways to permit their use in clinical studies [14, 15]. Most of the reported modifications typically enhance parameters such as thermal stability, binding affinity to target mRNA and/or increased resistance to digestion by nucleases [16]. Deleavey *et al.* [17] recently reported that siRNA duplexes heavily modified with a combination of a DNA analog containing a fluorine substitute at the 2'-position of the sugar (2'F-ANA) and rigid RNA analogs [2'F-RNA and/or locked nucleic acid (LNA)] could produce potent gene silencing agents, in particular through enhanced stability to serum nucleases and reduced immunostimulatory properties, relative to native duplexes. However, despite these attractive properties, the intracellular bioavailability of modified nucleic acids remains low due to their polyanionic nature and large molecular weight. During siRNA delivery, most of the few internalized siRNAs are, in the end, degraded or trapped in the endosomal/lysosomal compartments [18, 19]. Loading modified oligonucleotides into a robust carrier system capable of increasing circulation time, allowing targeted cellular uptake, and facilitating endosomal escape is conceptually one of the best approaches to the successful delivery of siRNA drugs.

Recent advances in the field of nanotechnology have led to the design of highly tuned nanoscale constructs and state of the art supramolecular assemblies derived from synthetic macromolecules [20]. Of particular interest in nucleic acid delivery are polyion complex micelles (PICMs) [21-23]. These nanocarriers typically result from cooperative electrostatic interactions between the genetic material and a cationic diblock copolymer presenting a water-soluble nonionic segment. Upon complexation, the charge-compensated nucleic acid/cationic chains self-assemble into a micellar core while the hydrophilic segments form a protective corona [24]. The corona not only confers solubility and colloidal stability to the system but also shields excess cationic charges [25]. Recently, our group reported that diblock copolymers, namely poly(ethylene glycol) (PEG)-*b*-poly(alkyl(meth)acrylate-*co*-methacrylic acid)s can self-assemble in aqueous media in a pH-dependent manner to form polymeric micellar structures that can be exploited for the oral delivery of drugs [26]. These micelles were initially designed to gradually but completely release their contents in a pH-dependent fashion as they transit from the stomach to the small intestine. Such micellar systems were shown to substantially increase the aqueous solubility and oral absorption of class II drugs [27]. Interestingly, we discovered that a member of this family of polymers, *i.e.*, PEG-*b*-poly(propyl methacrylate-*co*-methacrylic acid) (PEG-*b*-P(PrMA-*co*-MAA)), could interact in a reversible fashion with conventional poly(amido amine) (PAMAM) dendrimers to form pH-responsive discrete 50–60 nm core-shell type PICMs [28]. The resulting nanocomplexes could accommodate antisense oligonucleotides and siRNAs in their core (Fig. 3.1A) [28]. It is thought that upon cellular uptake *via* receptor-mediated endocytosis, the acidic pH in the endosomal compartment protonates the carboxylate groups of the MAA, thus causing the displacement of PEG-*b*-P(PrMA-*co*-MAA) from the PICM. The endosomolytic-active protonated MAA copolymer [29] and the remaining unshielded PAMAM-nucleic acid core could then promote endosomal escape by interaction with the endosomal membrane and/or *via* the proton sponge effect [30, 31] (Fig. 3.1B). Although a potent silencing effect was obtained with fragment antigen binding (Fab')-decorated PICMs [28], the coupling procedure (disulfide linkage) employed to attach the targeting ligand (anti-transferrin receptor Fab', *i.e.*, anti-CD71 Fab') to PEG-*b*-P(PrMA-*co*-MAA) was relatively inefficient and potentially subject to cleavage in the blood. In the present work, the anti-CD71 Fab' was conjugated to a modified amino-PEG-*b*-P(PrMA-*co*-MAA) *via* a maleimide/activated ester bifunctional linker, thus forming a more stable thioether bond. The cellular uptake of the targeted PICMs was then studied by flow cytometry. The micelles were loaded with unmodified and 2'-modified (2'F-RNA and 2'F-ANA) siRNAs (Fig. 3.1C) and their ability to knockdown the B-

cell lymphoma 2 (Bcl-2) oncoprotein and mRNA was evaluated on PC-3 cells, using different PAMAM derivatives as condensing agents and endosomal escape promoters. This study showed that maximal silencing effect could be achieved by combining the targeted ternary PICMs with 2'-modified siRNA.

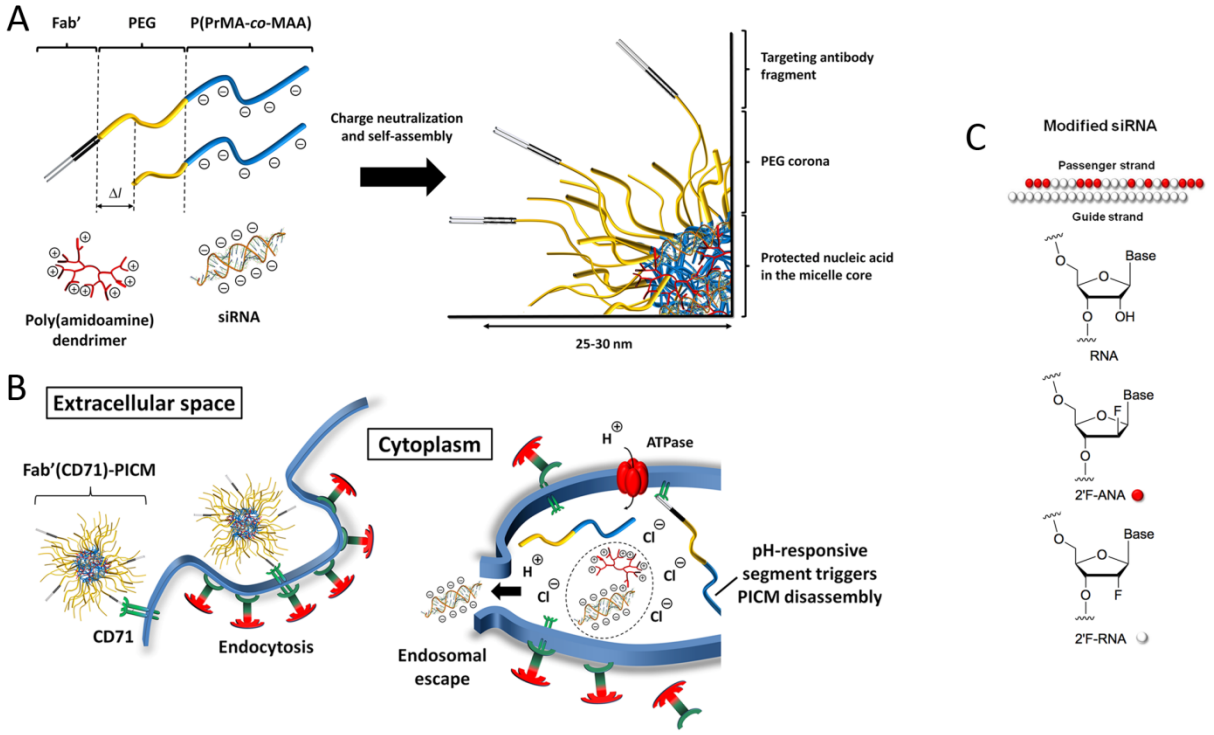


Figure 3.1. Schematic illustrations of (A) PICM formation, (B) proposed mechanism of PICM entry through receptor-mediated endocytosis, and (C) chemical structure representation of modified siRNA sequence.

3.2. Material and Methods

3.2.1. Materials

Heterobifunctional PEG ($HO\text{-}PEG_{169}\text{-}NH_2$, M_n = 7500) was obtained from Jenkem Technology (Beijing, China). Methoxy-PEG_{115}-OH (M_n = 5000), PAMAM dendrimers (Table 3.1) and unspecific mouse IgG1-κ (MOPC-21) were purchased from Sigma-Aldrich (Buchs, Switzerland). Propyl methacrylate (PrMA), *tert*-butyl methacrylate (*t*BMA), and triethylamine were from ABCR-Chemicals (Karlsruhe, Germany) and were distilled before use. Monoclonal anti-human CD71 (transferrin receptor) antibody was ordered from Ancell

(Bayport, MN). RPMI medium, Opti-MEM I medium, fetal bovine serum (FBS), trypsin, lipofectamine 2000 (used according to the supplier's instructions), and phosphate-buffered saline (PBS) were obtained from Invitrogen (Carlsbad, CA). Unmodified (labeled or unlabeled) siRNA sequences (Table 3.2) were designed and synthesized by Dharmacon (Chicago, IL). All other products, unless otherwise specified, were purchased from Fisher Scientific AG (Wohlen, Switzerland).

Table 3.1. List of tested PAMAM dendrimers and characteristics of the PICMs after complexation with PEG_{115}-*b*-$P(PrMA_{21}$-*co*-$MAA_{45})$.

PAMAM	M_n (k)	# -NH_2	[a] N/(P+COOH)	[b] PICM size (nm)	[c] PI	ζ-potential (mV) [S.D.]
G4	14.2	64	2.0	78	0.23	5.8 [0.5]
$G4_{C12}$	17.2	48	2.0	97	0.16	4.6 [0.2]
G5	28.8	128	2.0	48	0.24	4.5 [0.2]
$G5_{S-S}$	28.9	128	2.0	69	0.27	4.8 [1.3]

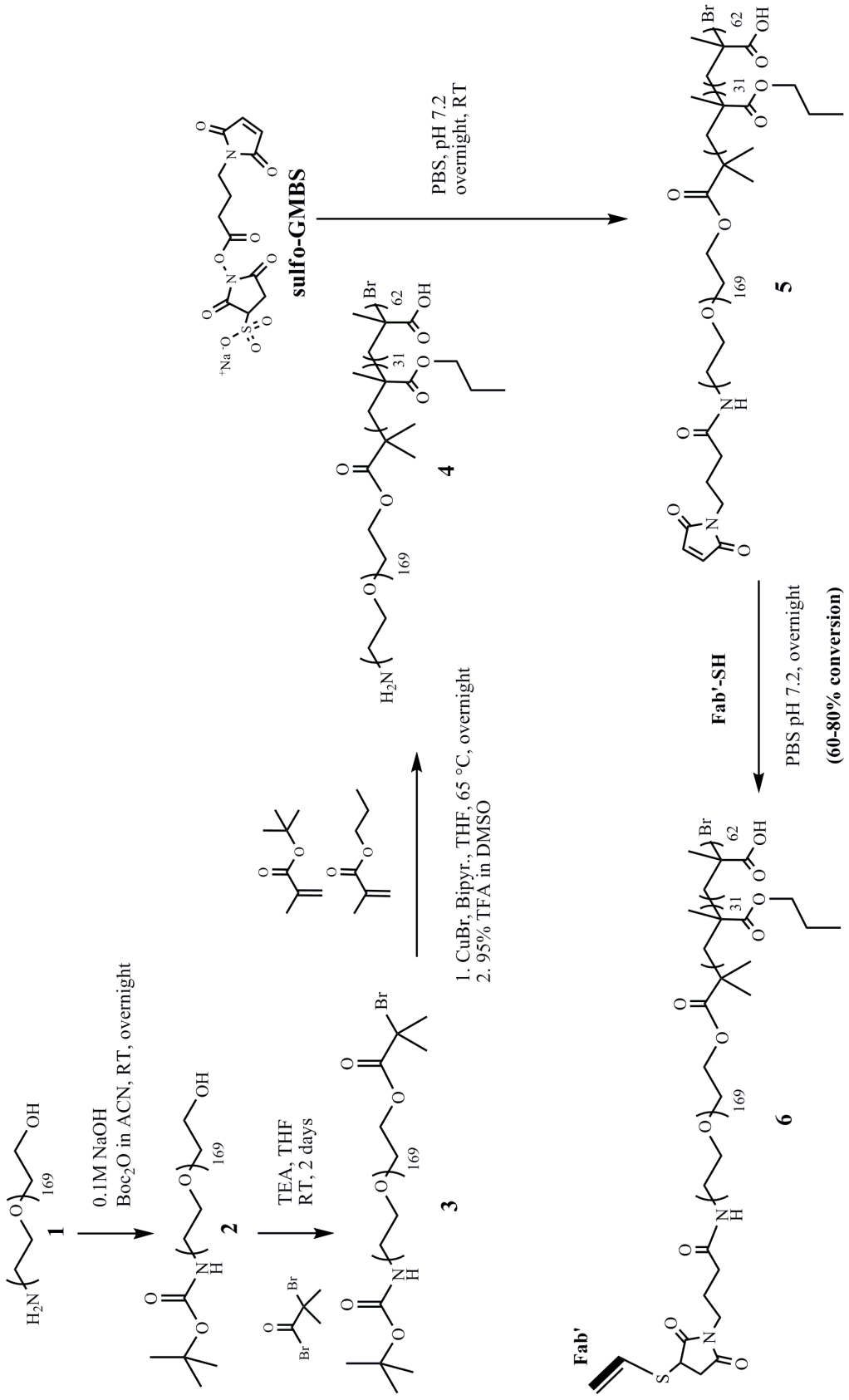
[a] Optimal N/(P+COOH) ratio for micelle formation.
[b] PICM mean hydrodynamic diameter at optimal N/(P+COOH) ratio.
[c] Polydispersity index.

Table 3.2. Sequences of small interfering RNAs.

Label	Sequence
Bcl2	5'- GCAUGCGGCCUCUGUUUGAUU-3' 3'-UUCGUACGCCGGAGACAAACU -5'
Scrambled	5'- UAGCGACUAAACACAUCAAUU-3' 3'-UUAUCGCUGAUUUGUGUAGUU -5'
DY547-Bcl2	5'- (DY547) GCAUGCGGCCUCUGUUUGAUU-3' 3'- UUCGUACGCCGGAGACAAACU -5'
DY547-Bcl2-Fl	5'- (DY547) GCAUGCGGCCUCUGUUUGAUU-3' 3'- (Fl) UUCGUACGCCGGAGACAAACU -5'
[a]Modified Bcl2	5'- **GCA***UGC***GGC***CUC***U***G***U***U***G***AUU**-3' 3'-*UUCGUACGCCGGAGACAAACU*p -5'

[a] Description of chemical modifications: **2'F-ANA**, *2'F-RNA*, p = phosphorylation.

3.2.2. Diblock copolymer synthesis



Scheme 3.1. Synthesis of Fab'-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}). Full IgG was fragmented and freshly reduced prior to maleimide conjugation.

Non-targeted MeO-PEG$_{115}$-*b*-P(PrMA-*co*-MAA) was synthesized by atom transfer radical polymerization (ATRP) as previously described using a methoxy-PEG$_{115}$ (M_n = 5000) [32]. Fab'-PEG$_{169}$-*b*-P(PrMA$_{31}$-*co*-MAA$_{62}$) copolymer **6** (scheme 3.1) was synthesized starting from H_2N-PEG$_{169}$-OH **1** (M_n = 7500). Briefly, H_2N-PEG$_{169}$-OH **1** was protected using di-*tert*-butyl dicarbonate (2.5 eq., dissolved in acetonitrile (ACN)) and NaOH (3 eq., 0.1 M) for 2 h. The resulting product **2** was acylated with 2-bromoisobutyryl bromide using a previously reported procedure to afford the macroinitiator **3** [33]. The polymerization reactions were carried out by ATRP, using PrMA and *t*BMA, under conditions reported previously [32]. H_2N-PEG$_{169}$-*b*-P(PrMA$_{31}$-*co*-MAA$_{62}$) **4** was obtained by deprotection of the *tert*-butyl carbamate and *tert*-butyl ester with 95% trifluoroacetic acid in dimethyl sulfoxide, followed by purification through a dialysis membrane against water for 2 days. The purified polymer was recovered by lyophilization of the aqueous solution (Table 3.3). The yield of polymerization/deprotection steps was approximately 50%. The maleimide bifunctional linker, *N*-[γ-maleimidobutyryloxy]-sulfosuccinimide ester (sulfo-GMBS, 10 eq), was reacted with polymer **4** overnight in PBS pH 7.2. Excess linker was removed using a 3-kDa filtration membrane (Millipore, Billerica, MA), and the product **5** was recovered by lyophilization. The final active maleimide content of the polymer was approximately 60% as determined by ^{1}H NMR spectroscopy. Selected ^{1}H-NMR resonance (D_2O, 400 MHz) δ: 6.85 (s, maleimide), 3.96 (br m, OCH_2 in PrMA), 3.75 (m, OCH_2CH_2 in PEG chain), 2.20–1.60 (br, CH_2 in the PrMA and polymer backbone), 1.15–0.80 (br, CH_3 groups in PrMA and MAA). Anti-transferrin receptor antibody was digested to F(ab')$_2$ following the mouse IgG1 Fab and F(ab')$_2$ preparation kit procedure (Pierce, Rockford, IL). Maleimide-PEG$_{169}$-*b*-P(PrMA$_{31}$-*co*-MAA$_{62}$) **5** was coupled to the freshly reduced Fab'-SH (1.5 eq.) overnight in degassed PBS pH 7.2. The excess Fab' was removed through an anion exchange column (HiTrap Q FF, GE Healthcare, Glattbrugg, Switzerland), and the product concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce). Unreacted maleimide groups were quenched with 5 eq. cysteine. The final Fab'-PEG$_{169}$-*b*-P(PrMA$_{31}$-*co*-MAA$_{62}$) **6** purity was assessed by sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) (Fig. 3.2).

Table 3.3. Characteristics of PEG-*b*-P(PrMA-*co*-MAA)s.

Diblock copolymer	M_n[a]	M_n[b]	M_w/M_n[b]
PEG_{115}-*b*-P($PrMA_{28}$-*co*-MAA_{53})	13,300	13,500	1.19
PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45})	11,700	15,600	1.06
H_2N-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62})	16,900	14,800	1.10

[a] Determined by ^{1}H NMR spectroscopy.
[b] Determined by gel permeation chromatography (GPC).

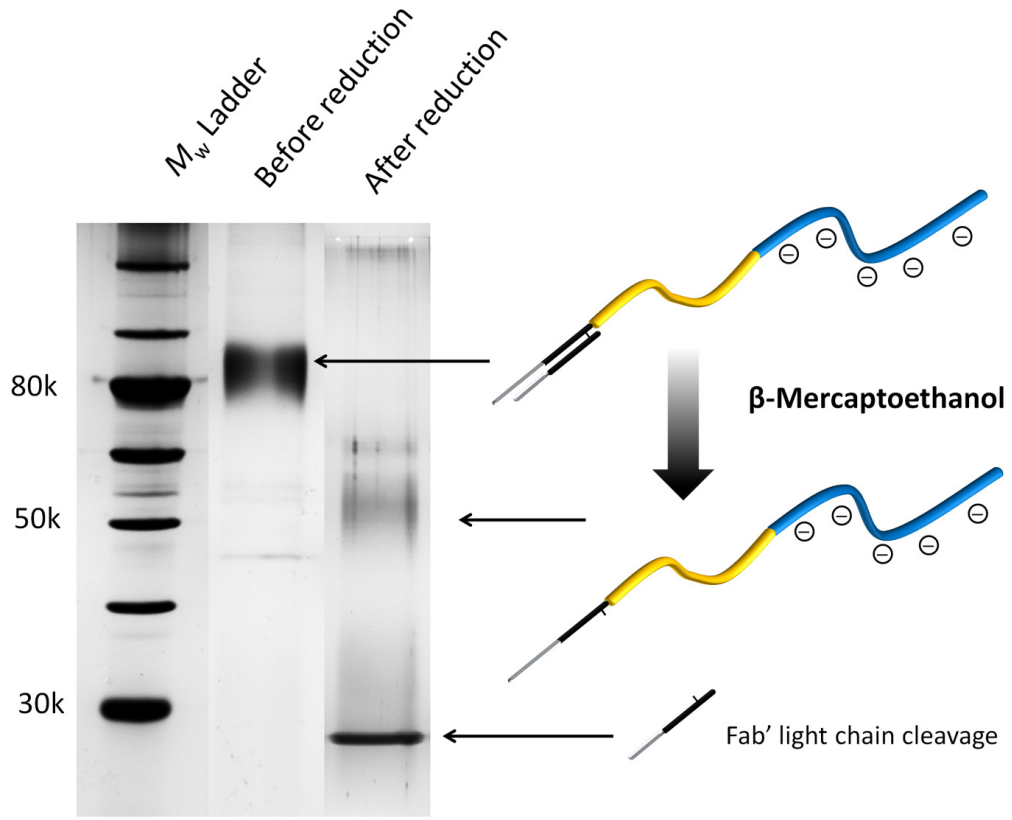


Figure 3.2. SDS-PAGE of Fab'-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}) under non-reducing and reducing conditions. Under reducing conditions, the Fab' light chain was cleaved, resulting in a decrease in the conjugated copolymer molecular weight from 80 to 50 kDa. The molecular weight of intact Fab' is 48 kDa.

3.2.3. NMR spectroscopy analysis and molecular weight determination

^{1}H NMR spectra were recorded on a Bruker Av400 spectrometer (Bruker BioSpin, Fällanden, Switzerland). GPC measurements were performed in 10 mM Tris-HCl buffer (pH 8.0), using a Viscotek TDAmax system (Viscotek, Houston, TX) equipped with a differential refractive index and light scattering detectors. Adequate molecular weight separation was achieved using two ViscoGEL columns (GMPWXL) in series at a flow rate of 0.7 mL/min and a temperature of 35 °C.

3.2.4. Modified oligonucleotide synthesis

Standard phosphoramidite solid-phase synthesis conditions were used for the synthesis of all modified oligonucleotides [34] on an Applied Biosystems (Carlsbad, MA) 3400 DNA Synthesizer at a 1 μmol scale with Unylink CPG support (ChemGenes, Wilmington, MA). All phosphoramidites were prepared as 0.15 M solutions in ACN. 5-ethylthiotetrazole (0.25 M in ACN) was used to activate phosphoramidites for coupling. Detritylations were accomplished with 3% trichloroacetic acid in CH_2Cl_2 for 110 s. Capping of failure sequences was achieved with acetic anhydride in tetrahydrofuran (THF) and 16% *N*-methylimidazole in THF. Oxidation was done using 0.1 M I_2 in 1:2:10 pyridine:water:THF. Coupling times were 600 s for 2'F-ANA and 2'F-RNA phosphoramidites, with the exception of the guanosine phosphoramidites which were allowed to couple for 900 s. 5'-phosphorylation of modified antisense strands was achieved using bis(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite at 0.15 M (600 s coupling time). Deprotection and cleavage from the solid support was accomplished with 3:1 NH_4OH:EtOH for 48 h at room temperature (RT). Purification of crude oligonucleotides was done by preparative denaturing PAGE using 24% acrylamide gels. Gel bands were extracted overnight in diethyl pyrocarbonate-treated autoclaved Millipore water, and lyophilized to dryness. Purified oligonucleotides were desalted with Nap-25 Sephadex columns from GE Healthcare. siRNAs were prepared by annealing equimolar quantities of complementary oligonucleotides in siRNA buffer (100 mM KOAc, 30 mM HEPES-KOH, 2 mM $Mg(OAc)_2$, pH 7.4) by slowly cooling from 96 °C to RT.

3.2.5. PICM preparation

PICMs were prepared in a 100-μL volume of Tris buffer (10 mM, pH 7.4) and then diluted such that the final concentration of MAA copolymer was 0.1 mg/mL. Briefly, negatively-charged micelle components (PEG_{115}-*b*-P(PrMA-*co*-MAA), Fab'-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}) and siRNA) were combined in Tris buffer and mixed with PAMAM dendrimer at increasing N/(P + COOH) molar ratios. N corresponds to the number of primary amine groups of the PAMAM while P and COOH account for the phosphate and carboxylate groups of the nucleic acid and MAA copolymer, respectively. The samples were stirred for 20 min at RT to allow micelle formation. Typical P/COOH ratio was 0.013 for PICM tested at 50 nM siRNA. Micelles formed at different N/(P + COOH) ratios were characterized by dynamic light scattering (DLS) and laser Doppler anemometry using a Beckman Coulter Delsa Nano C particle analyzer (Krefeld, Germany). The parameters recorded were the hydrodynamic diameter, polydispersity index (PI), scattering intensity and ζ-potential. Unless otherwise

indicated, the selected optimal ratio was 2.0 because the micelles formed at this ratio exhibited a low hydrodynamic diameter, the lowest PI, the highest scattering intensity, and a near-neutral ζ-potential. PEG copolymers bearing antibody fragments were only used for *in vitro* cell experiments, to mediate cellular uptake of the PICMs. Fab'(CD71)-PICMs, Fab'(MOPC)-PICMs, and PICMs stand for nanocarriers bearing anti-CD71 Fab', MOPC Fab' or no antibody fragment, respectively.

3.2.6. Transmission electron microscopy (TEM) analysis of particles

Samples (4 μL) of the PICM suspension, prepared at a concentration of 0.5 mg/mL MAA copolymer, were adsorbed to glow discharged carbon-coated copper grids for 2 min. They were then negatively stained with 2% (w/v) uranyl acetate solution for 30 s and air-dried after removal of excess liquid using filter paper. The specimens were examined in a Philips CM12 (tungsten cathode) transmission electron microscope (FEI, Hillsboro, OR) at 100 kV, and images were recorded with a Gatan CCD 794 camera (Gatan Inc., Pleasanton, CA).

3.2.7. Stability of siRNA in serum

The decrease of fluorescence resonance energy transfer (FRET) from fluorescein-labeled antisense strand to the DY547-labeled-sense strand (Table 3.2) was exploited to follow the degradation of the siRNA in serum-containing media (Equation 3.1) [28]:

$$\%\ \text{of siRNA degradation} = \left[\frac{\left(I_{(Fl/Dy)\,t=t} - I_{(Fl/Dy)\,t=0}\right)}{\left(I_{(Fl/Dy)\,t=\infty} - I_{(Fl/Dy)\,t=0}\right)}\right] \times 100 \qquad (3.1)$$

where $I_{(Fl/Dy)}$ is the fluorescence intensity of fluorescein (λ_{em} = 525 nm)/DY547 (λ_{em} = 565 nm) when they are both excited at 488 nm, measured using a Tecan Safire plate reader (Tecan, Durham, NC). $I_{(Fl/Dy)t=0}$, $I_{(Fl/Dy)t=t}$ and $I_{(Fl/Dy)t=\infty}$ correspond to the FRET before siRNA degradation, at different time intervals and at the end of the experiment, respectively. At the end of the experiment, heparin was added to destabilize the complexes. The FRET corresponding to 100% degradation was determined after 24-h incubation in the heparin solution. To study the effect of serum on the stability of entrapped siRNA, PEG_{115}-*b*-P($PrMA_{28}$-*co*-MAA_{53})/PAMAM PICMs were incubated with increasing FBS concentrations. The effects of albumin (40 mg/mL), α- and β-predominant globulins (15 mg/mL), γ-globulins (10 mg/mL) and heparin (6×10^{-4} mg/mL) on the degradation profile of the siRNA entrapped in the micelles were also examined in 20% serum using the same method. These protein concentrations are representative of the corresponding plasma levels.

3.2.8. Cell culture

Prostate adenocarcinoma cells (PC-3) were obtained from the American Type Culture Collection (Rockville, MD). They were grown in complete RPMI medium (RPMI containing 10% FBS, supplemented with 1% non essential amino acids, 1% sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin). Stock cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Only cells in the exponential phase of growth were used.

3.2.9. Characterization of cellular uptake via flow cytometry

PC-3 cells were seeded one day prior to the experiment in a 12-well plate at a density of 200,000 cells/well in complete RPMI containing 10% FBS. Following overnight culture, the cells were treated with complexes containing 100 nM of fluorescently labeled Bcl-2 siRNA (DY547-siRNA, Table 3.2) in Opti-MEM I medium supplemented with or without 10% FBS for 3 h. The cells were then washed twice with PBS, trypsinized (0.5 mg/mL trypsin in 0.4 mM EDTA tetrasodium salt) for 3 min at 37 °C, and washed twice by centrifugation (10 min, 300 x *g*) in ice-cold PBS supplemented with 2% FBS. The resulting cell pellet was resuspended in ice-cold PBS supplemented with 2% FBS. The fluorescence analysis of a minimum of 10,000 events per sample was performed with a FACScanto flow cytometer (BD Biosciences, San Jose, CA).

3.2.10. Cell viability assay

PAMAM dendrimers were dissolved in Tris buffer and sterilized through 0.2-μm filter. The *in vitro* cytotoxicity was assessed by CellTiter 96 AQueous One Cell Proliferation Assay (Promega, Madison, WI). PC-3 cells were seeded the day before the experiment in a 96-well plate at a density of 6000 cells/well in 100 μL of complete RPMI medium containing 10% FBS. The medium was then replaced by 100 μL/well fresh Opti-MEM I medium containing different concentration of PAMAMs ranging from 10 to 500 μg/mL. After a 5-h incubation period, cells were rinsed once with PBS and fed with 100 μL of fresh complete RPMI medium. After a total incubation period time of 72 h, the cells were rinsed once with PBS, and fed with 100 μL per well of fresh medium plus 20 μL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) solution (Promega, Madison, WI) containing the tetrazolium compound. The absorbance was measured at 490 nm after a 3.5-h incubation period using a Tecan Infinite M200 plate reader

(Tecan, Männedorf, Switzerland). Cell viability was calculated according to the following equation (Equation 3.2):

$$\text{Cell viability (\%)} = (OD_{490}\ \text{sample}/OD_{490}\ \text{control}) \times 100 \quad (3.2)$$

where OD_{490} sample represents the optical density of the wells treated with polymers and OD_{490} control is the wells treated with complete medium only.

3.2.11. Assessment of mRNA levels by real-time PCR

PC-3 cells were seeded one day prior to the experiment in a 6-well plate at a density of 200,000 cells/well in complete RPMI containing 10% FBS. PICMs containing Bcl-2 siRNA were incubated with the 60–70% confluent cells for 5 h in Opti-MEM I containing 10% or 50% FBS. Subsequently the transfection medium was changed to complete RPMI containing 10% FBS. After a 48-h incubation time, the cells were harvested and total RNA was isolated using RNeasy Mini kit (QIAGEN, Valencia, CA). Purified RNA ($OD_{260}/OD_{230} \geq 1.8$) was used as a template to assess the gene expression level of Bcl-2 *via* a two-step quantitative reverse-transcription PCR (qRT-PCR). Briefly, reverse transcription reaction was carried out using high capacity cDNA reverse transcriptase kit (Applied Biosystems). Following the cDNA synthesis, the qPCR was run using Power SYBR Green PCR Master Mix (Applied Biosystems) with a 7900HT Fast Real-Time PCR system (Applied Biosystems). The data were normalized to the internal control; β-actin. The primers for Bcl-2 and β-actin were purchased from QIAGEN (Primer set ID QT00025011 and QT01680476, respectively). All procedures followed the manufacturer's protocol. Relative gene expression levels were calculated using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method.

3.2.12. Analysis of Bcl-2 protein knockdown by Western blot

PC-3 cells were seeded one day prior to the experiment in a 6-well plate at a density of 150,000 cells/well in complete RPMI containing 10% FBS. PICMs containing Bcl-2 siRNA were incubated with the 50–60% confluent cells for 5 h in Opti-MEM I containing 10% FBS. Subsequently, the transfection medium was changed to complete RPMI containing 10% FBS. Following a 72-h incubation period, the cells were washed with PBS and lysed by incubation in lysis buffer at 4°C for 1 h. Cell debris were removed by centrifugation at 8000 x *g* for 15 min at 4°C, and protein concentration was determined using the BCA assay (Pierce). 15–25 µg of protein aliquots were resolved by SDS-PAGE and transferred to a poly(vinylidene

difluoride) membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% bovine serum albumin in PBS containing 0.1% polysorbate 20 for 1 h at RT, and subsequently probed with anti-Bcl-2 monoclonal antibody (Dako, Glostrup, Denmark) (diluted 1:200 in blocking buffer) overnight at 4 °C. Horseradish peroxidase-labeled polyclonal goat anti-mouse secondary antibody (Millipore, Billerica, MA) was incubated at 1:1200 dilution with the membrane in PBS containing 0.1% polysorbate 20 and 5% milk for 1.5 h at RT. Protein bands were revealed with ECL Plus Western blotting detection reagents (GE healthcare) and recorded on a ChemiDoc XRS (Bio-Rad). The intensities of the bands were analyzed using Quantity One software (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as internal control and was detected using an anti-GAPDH monoclonal antibody at a dilution of 1:2500 (Advanced Immunochemical Inc., Long Beach, CA).

3.2.13. Statistics

All statistical analyses were performed using the computer program *OriginPro* (Northampton, MA). Experiments were analyzed using a one-way analysis of variance followed by Tukey's test to determine the significance of all paired combinations. The level of statistical significance was fixed at $p < 0.05$.

3.3. Results and discussion

3.3.1. Synthesis of polymer and formation of PICMs

PEG_{115}-*b*-P(PrMA-*co*-MAA)s containing *ca.* 25 and 50 PrMA and MAA units were synthesized by ATRP (Table 3.3) as described previously [32]. MAA provides the copolymer with pH-sensitivity, while PrMA is used to minimize polymer aggregation due to hydrogen-bond formation between repeating MAA units and the PEG block [27, 32, 35]. Because of their well-defined structure and tunable functional groups, polycationic dendrimers are attractive condensing agents for nucleic acid delivery [36, 37]. Among them, PAMAMs have been widely studied for their relatively high transfection efficiency *in vitro*, especially in mammalian cells [38]. In a previous study, we reported that PEG_{115}-*b*-P(PrMA-*co*-MAA) could, in the presence of conventional PAMAM G5 and short stranded nucleic acids, self-assemble to produce PICMs with nanoscale size and low polydispersity [28]. An interesting property of this approach resides in the possibility to produce PEGylated nanocarriers with different cationic cores without the need to derivatize the block copolymer component. As summarized in Table 3.1, PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45}) was mixed with several PAMAM structures (Fig. 3.3). Four different PAMAMs, *i.e.*, two generation 4 (PAMAM G4 and PAMAM $G4_{C12}$) and two generation 5 dendrimers (PAMAM G5 and PAMAM $G5_{S\text{-}S}$), were tested. The criterion for PAMAM selection were based on i) their potential to form narrowly distributed PICMs, ii) their intrinsic cytotoxicity, and iii) their transfection efficiency when complexed in the carrier. PAMAM G4 and G5 only differ in size (*i.e.*, the number of terminal primary amines). PAMAM $G4_{C12}$ bears 25% *N*-(2-hydroxydodecyl) chains on its surface whereas PAMAM $G5_{S\text{-}S}$ possesses a cystamine core (Table 3.1, Fig. 3.3). We hypothesized that using a PAMAM $G4_{C12}$ could enhance transfection, as already reported for hydrophobized PAMAMs [39] and polyethylenimine (PEI) [40, 41]. PAMAM $G5_{S\text{-}S}$ was selected for the potential lability of its disulfide core [42], which could, in principle, reduce toxicity and favor nucleic acid release. In all cases, micelles of less than 100 nm with near-neutral ζ-potential were obtained at the optimal N/(P+COOH) ratio (Table 3.1). Larger PICMs (~100 nm) were generated with PAMAM $G4_{C12}$, which may be the consequence of a greater aggregation number in the presence of alkyl chains [43]. As illustrated in Figure 3.4A (PAMAM G5 dendrimer), the stoichiometric charge annealation of the polyion mixture was typically around a N/(P+COOH) ratio of 2. At this ratio, micelle size was minimal and scattering intensity was maximal, indicating higher concentration of PICMs. TEM analysis

(See methods 3.2.6) revealed that the micelles were spherical in shape (Fig. 3.4B), with a size similar to those obtained by DLS.

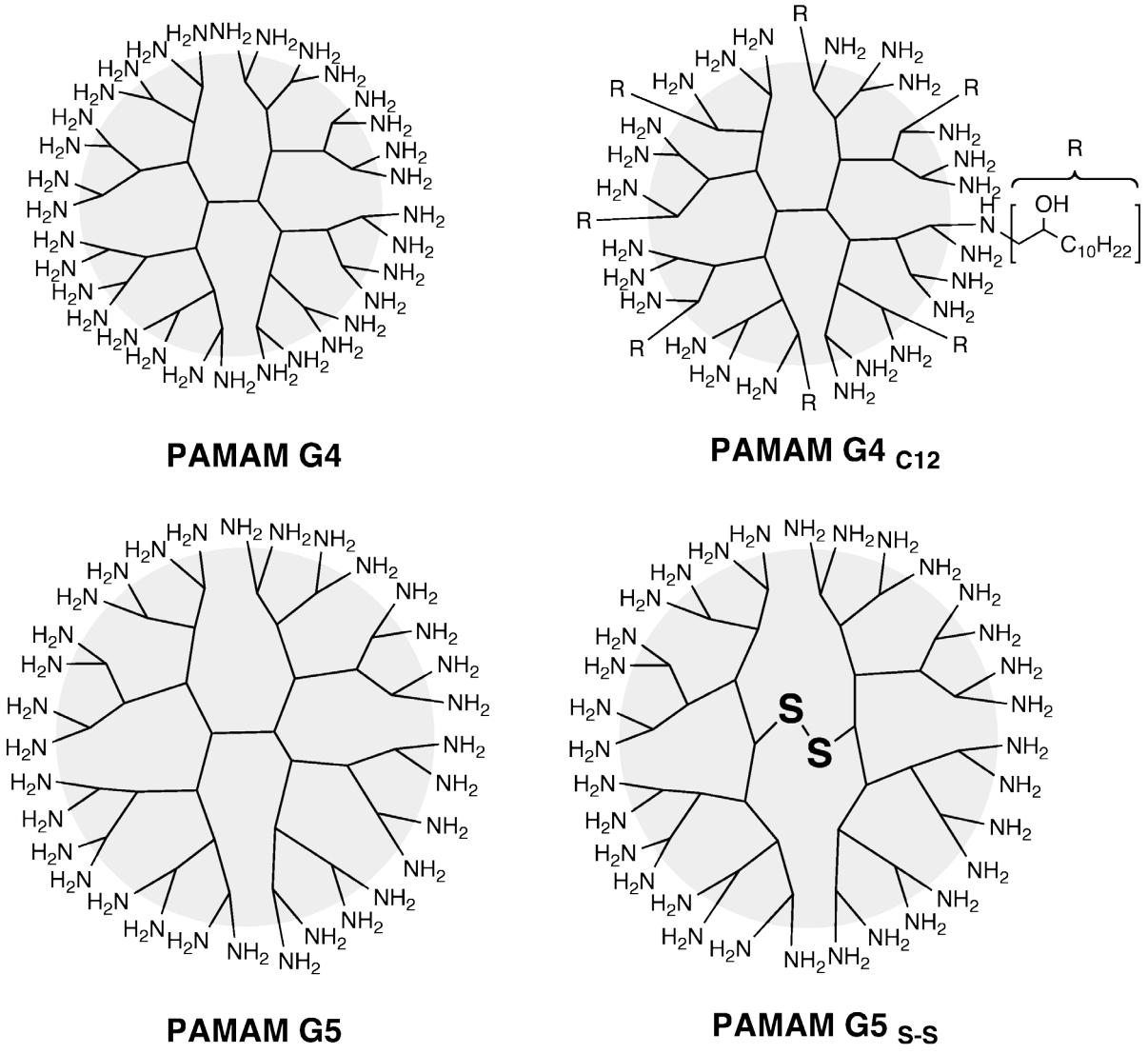


Figure 3.3. Schematic representation of the different PAMAM dendrimers employed. Number of surface amine groups is 64 and 128 for PAMAM G4 and G5, respectively. PAMAM $G4_{C12}$ has been functionalized with 25% N-(2-hydroxydodecyl) (C_{12}, R chain). PAMAM $G5_{S\text{-}S}$ has a cystamine core. For clarity reasons, the inner chemical structure of dendrimers was omitted. It consists of amido amine branches with an ethylenediamine core.

In order to produce PICMs with enhanced and specific uptake, a PEG-*b*-P(PrMA-*co*-MAA) derivatized with an anti-transferrin receptor (anti-CD71) antibody fragment was prepared. As previously reported [28], a key feature of PEG_{115}-*b*-P(PrMA-*co*-MAA) is its ability to dissociate from the micelle PAMAM/nucleic acid core upon protonation of MAA units at endosomal pH. This could be potentially useful if a significant fraction of the receptor is recycled back such as in the case of the transferrin receptor [44, 45]. However, this issue will have to be studied in more detail since the cross-linking of the transferrin receptor has been previously shown to alter its cellular trafficking [46]. The freshly reduced Fab' fragment was coupled to H_2N-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}) (Scheme 3.1, **4**) through a maleimide/activated ester bifunctional linker. The excess Fab' was removed by elution over an ion exchange column. To maximize the accessibility of the targeting ligand to the receptor, the Fab' was attached to a PEG chain longer than that used to prepare the PICMs (7.5 *vs.* 5 kDa). The conjugate was characterized by SDS-PAGE under reducing and non-reducing conditions. A smeared band was observed at a molecular weight of ~80 kDa for the native Fab'-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}). The addition of β-mercaptoethanol caused the cleavage of the antibody light chain, giving rise to two bands at 50 and 30 kDa (Fig. 3.2), which correspond to the block copolymer coupled with the Fab' heavy chain and the light chain, respectively. When 1.5 mol eq. of Fab' over reactive maleimides were used, the conjugation reaction typically yielded 60–80% functionalization of the maleimide groups. This conjugation approach for PEG-*b*-P(PrMA-*co*-MAA) should be more stable *in vivo* than the one previously described in our group relying on the disulfide linkage. Indeed, Trail *et al.* [47] observed that when coupling the anti-cancer drug doxorubicin to a monoclonal antibody *via* a stable thioether bond, the circulation half-life was almost doubled compared to the cleavable disulfide conjugate.

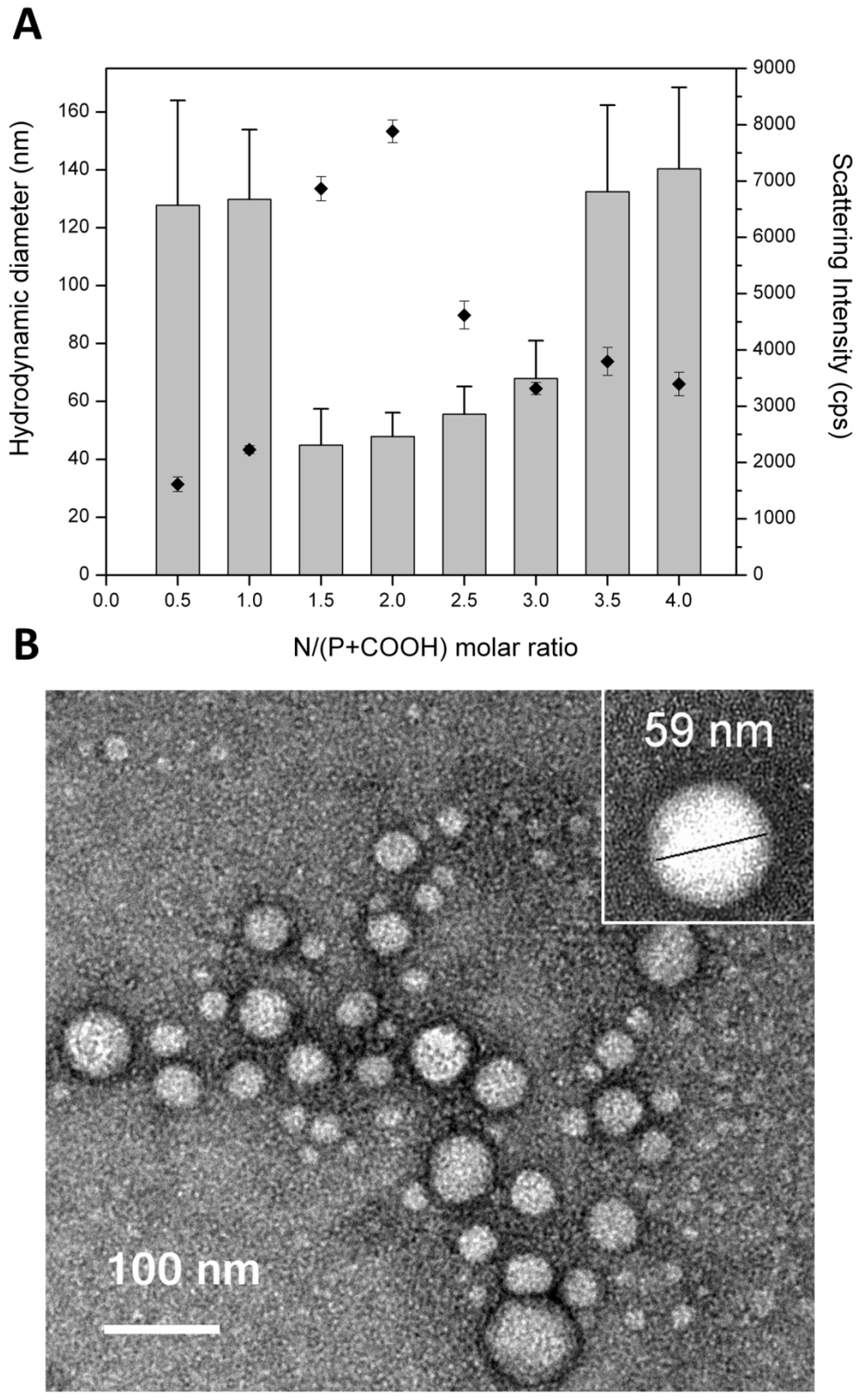


Figure 3.4. Evaluation of PICMs prepared with PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45}), PAMAM G5 and Bcl-2 siRNA. (A) PICM characteristics as a function of the N/(P + COOH) molar ratio. The average scattering intensity is represented by a diamond dot (◆). The average PI recorded at a 2.0 N/(P+COOH) ratio was the lowest and equal to 0.24. (B) TEM image of PICMs at the optimal N/(P+COOH) ratio of 2.0. Inset represents a highly magnified PICM.

3.3.2. Cell viability assay

The toxicity of the different tested PAMAMs was assessed in the absence of PEG_{115}-*b*-P(PrMA-*co*-MAA) (Fig. 3.5). In our previous study, it was shown that the complexation of PAMAMs to PEG_{115}-*b*-P($PrMA_{28}$-*co*-MAA_{53}) greatly reduced the overall cytotoxicity [28] and therefore, this could mask potential differences between the PAMAMs. Lower generation PAMAM G4 was associated with a lower cytotoxicity than PAMAM G5. Reduction of PAMAM cytotoxicity is desirable but it has been shown to be often linked to reduced transfection efficiency [48, 49]. The greatest toxicity was observed for PAMAM $G4_{C12}$, and may be related to the greater membrane destabilizing properties of such an amphiphilic polycation [50].

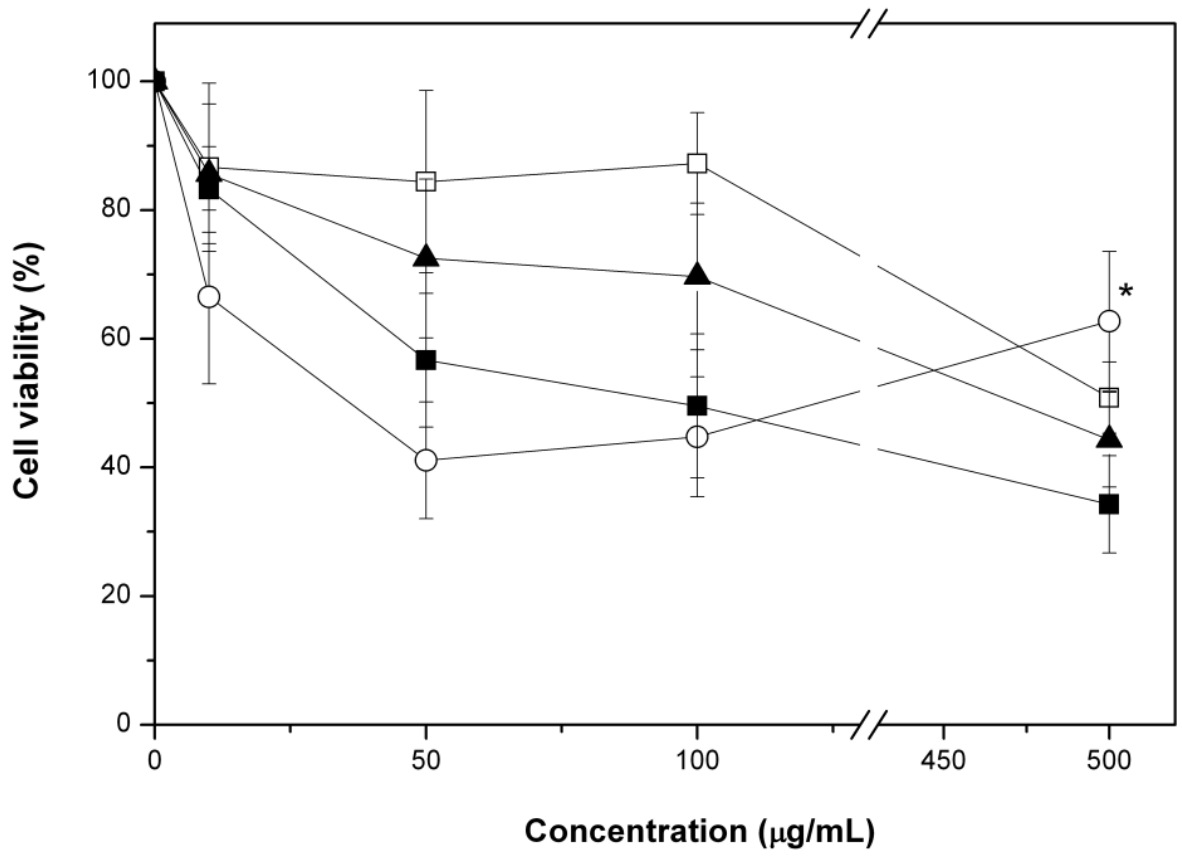


Figure 3.5. Cell viability of PC-3 cells exposed for 5 h to increasing concentrations (0–500 μg/mL) of PAMAM G4 (□), PAMAM $G4_{C12}$ (○), PAMAM G5 (▲) or PAMAM $G5_{S\text{-}S}$ (■). (★) Aggregates of PAMAM $G4_{C12}$ (~80 nm) were detected at this concentration. Results are expressed as mean ± S.D. (n = 4).

Surprisingly, the cytotoxicity of the PAMAM $G4_{C12}$ decreased at the highest tested concentration. This could be attributed to the formation of larger aggregates at high concentrations (~80 nm), which may reduce the internalization of the dendrimer. Unexpectedly, the PAMAM $G5_{S\text{-}S}$ derivative seemed more toxic than the G5. A possible explanation could be that, upon dendrimer reduction, the increase of surface area may lead to a net increase of positive charge density following protonation of originally buried amines. Even though this has to be confirmed, the resulting PAMAM $G5_{S\text{-}S}$ halves could have higher membranolytic properties, hence higher cytotoxicity, than the intact PAMAM G5.

3.3.3. Serum stability of the siRNA

To study the effect of serum and different plasma proteins on the stability of entrapped siRNA, ternary PICMs prepared with PAMAM G5 were incubated with increasing concentrations of serum. The degradation of siRNA was monitored by FRET as described elsewhere [28]. The rate of siRNA degradation depended on the serum concentration (Fig. 3.6A). After an incubation time of 4 h, siRNA degradation went from 10 to 40% upon increasing the serum content from 20 to 100%, respectively. To evaluate the contribution of the different serum components towards the destabilization of the PICMs, the complexes were incubated with albumin, α- and β-predominant globulins, γ-globulins, and heparin at levels typically found in plasma, and the siRNA degradation was monitored in 20% serum (Fig. 3.6B). All plasma proteins and heparin were found to contribute equally to micelle destabilization. A slightly stronger effect was observed for α- and β-globulins. Chen *et al.* [51] have recently shown that α- and β-globulins are major factors for the destabilization of PEG-*b*-poly(D,L-lactide) micelles while other plasma proteins play minor roles. The formation of PICMs involves electrostatic interactions and, accordingly, such micelles are vulnerable to destabilization by charged plasma components. However, our data clearly show that the micelles display some stability in serum, with more than 50% intact siRNA after 4 h, while free siRNA was completely degraded within 50 min. These results also indicate that siRNA can be gradually released from the PICMs, and potentially become bioavailable to the cells.

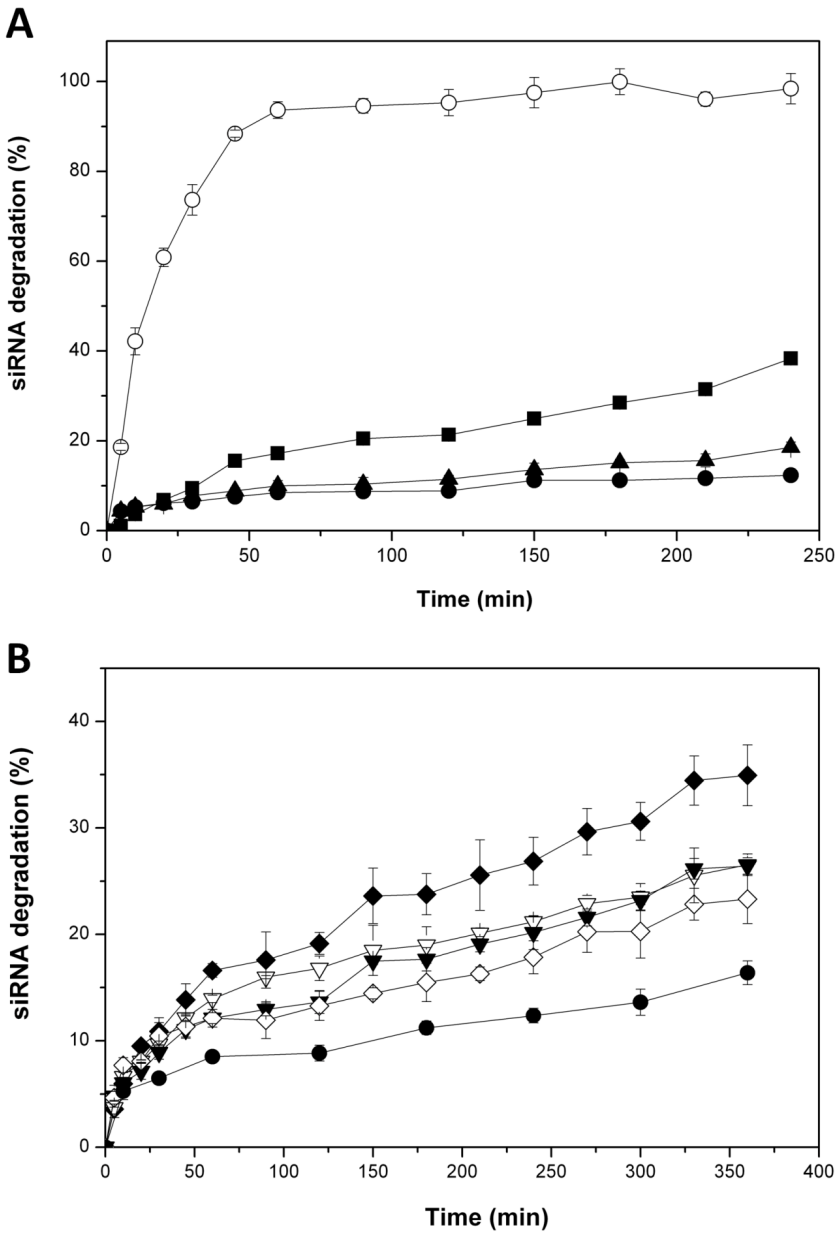


Figure 3.6. (A) Effect of serum concentration on the stability of siRNA complexed in PEG_{115}-*b*-P($PrMA_{28}$-*co*-MAA_{53})/PAMAM G5 PICMs (10 µg/mL siRNA, 37°C, pH 7.4). The formulations were incubated in 20% (●), 50% (▲) or 100% (■) FBS. Naked siRNA control in 20% FBS is also shown by an empty circle (○). (B) Effect of albumin (40 mg/mL, ▽), γ-globulins (10 mg/mL, ▼), α- and β-predominant globulins (15 mg/mL, ◆), and heparin (6 x 10^{-4} mg/mL, ◇) on the stability of siRNA formulated in PEG_{115}-*b*-P($PrMA_{28}$-*co*-MAA_{53})/PAMAM G5 PICM (10 µg/mL siRNA, 37°C, pH 7.4) when incubated with 20% FBS. Formulations in 20% FBS without any protein addition are represented by solid circles (●). Results are expressed as mean ± S.D. (n = 3). For some data points error bars are smaller than symbols.

3.3.4. Cellular uptake of PICMs

In order to determine the optimal targeting ligand concentration, PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45})/PAMAM G5 micelles loaded with fluorescently labeled siRNA (Table 3.2) were doped with increasing amounts of Fab'-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}), and the uptake by PC-3 cells was monitored by flow cytometry. The uptake increased with increasing proportions of ligand-functionalized polymer to reach a plateau at 1.25 mol% Fab' per anionic polymer chain (Fig. 3.7). The high negative charge density of heparin is known to interact and disassemble PICMs *via* competition of charges [23]. To confirm that Fab'(CD71)-PICMs were internalized and not just bound to the CD71 receptors on the cell surface, cells were washed with a heparin solution before flow cytometry analysis. No difference in fluorescence intensity was observed compared to the PBS washing step (data not shown), suggesting that the micelles were internalized after 3 h incubation.

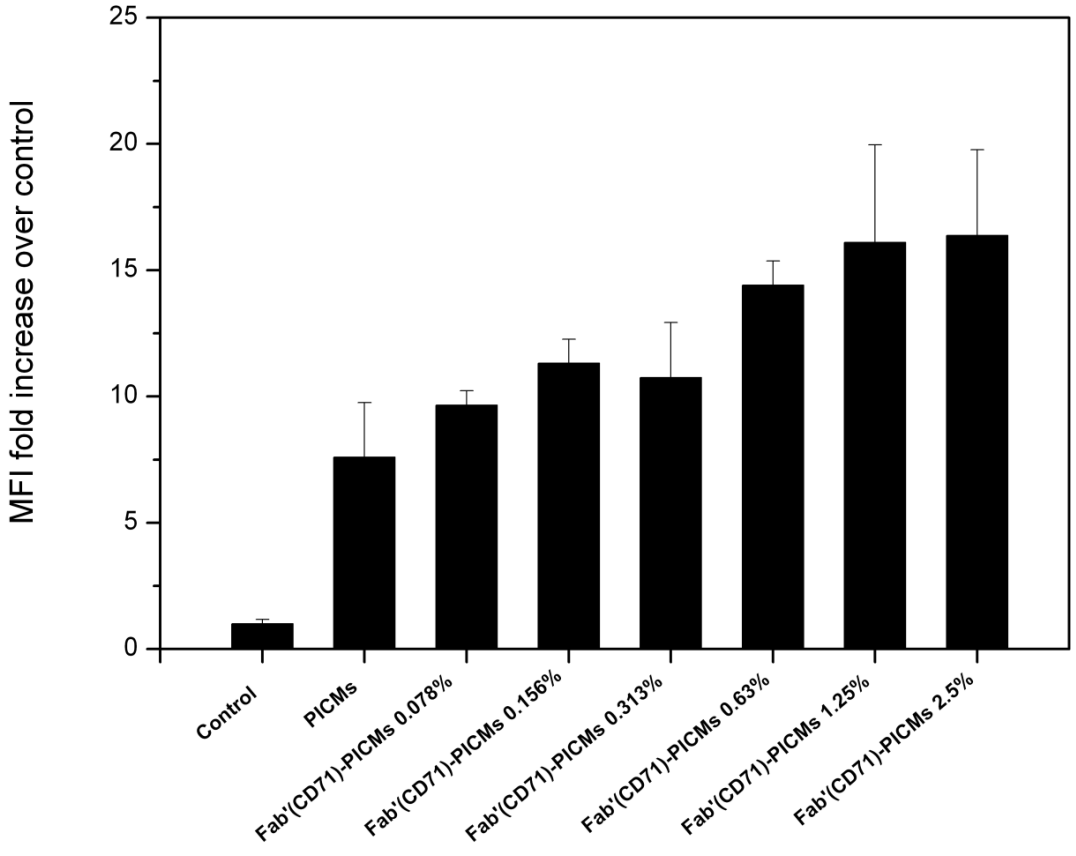


Figure 3.7. Flow cytometry results for the uptake of Fab'(CD71)-PICMs containing fluorescently-labeled siRNA by PC-3 cells. Effect of increasing Fab'-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62})/PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45}) molar ratio on the cellular uptake of the micelles. All experiments were performed in 10% FBS. Results are expressed as mean ± S.D. (n = 3).

As illustrated in Figure 3.8, the targeted micelles were taken up to a greater extent than both isotype-matched control Fab'-coated PICMs and unmodified micelles. The uptake was not influenced by the addition of 10% serum, which is consistent with the serum stability data reported previously (Fig. 3.6A).

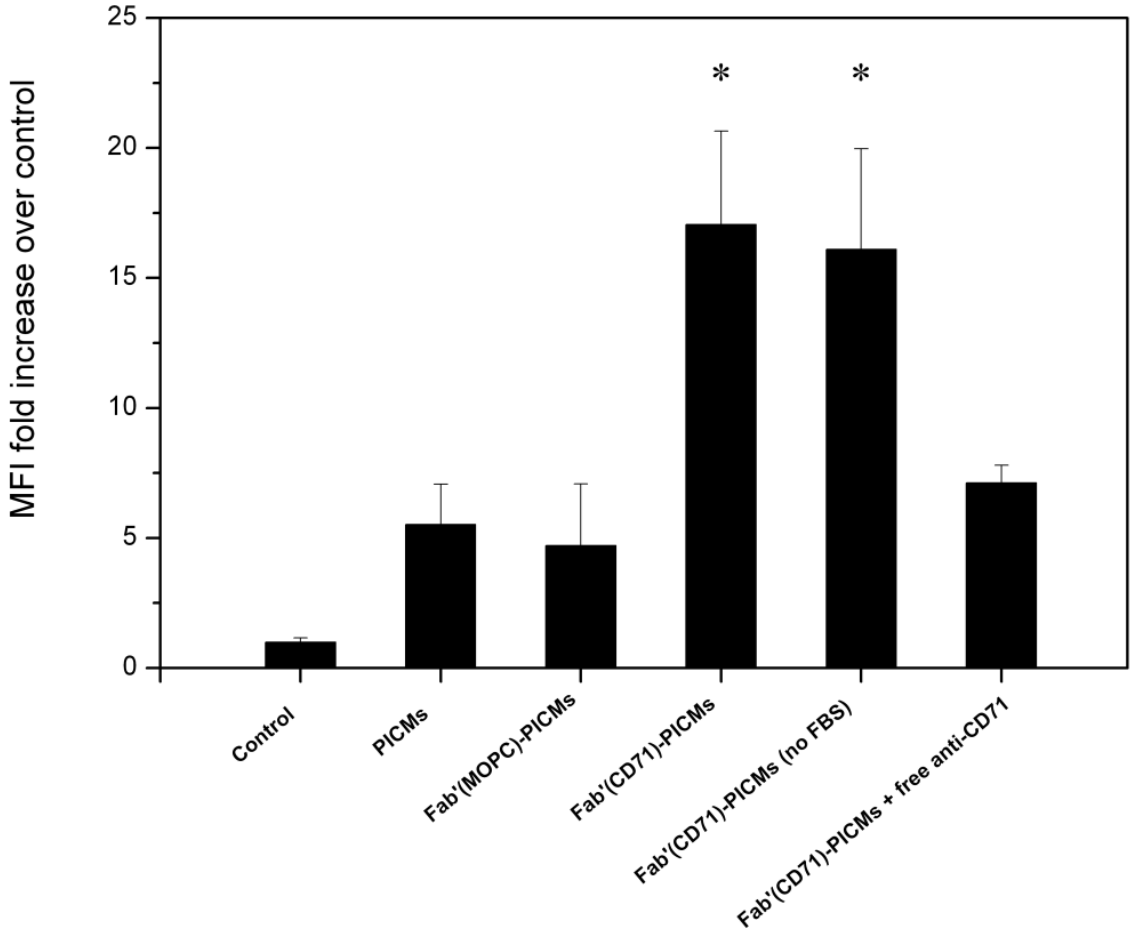


Figure 3.8. Uptake of different PICM formulations by PC-3 cells. All experiments were performed in 10% FBS unless otherwise indicated. PICMs presented in this figure were all prepared with PAMAM G5 dendrimer and loaded with DY547-Bcl-2 siRNA. MOPC immunoglobulin has unknown specificity and serves as isotype control. The last histogram represents competitive binding assays of Fab'(CD71)-PICMs performed in the presence of a 20-fold excess of free anti-CD71 antibody. Results are expressed as mean ± S.D. (n = 3); (*) $p < 0.05$ vs. undecorated or MOPC decorated PICMs.

Moreover, preincubating the cells for 30 min with a 20-fold excess of free anti-CD71 antibody led to a substantial decrease of the uptake of Fab'(CD71)-PICMs, confirming the specificity of the interaction (Fig. 3.8, last column). These data strongly suggest that Fab'(CD71)-PICMs are mainly taken up *via* the transferrin receptor. Preparation of polyplexes exploiting the binding of the anti-CD71 antibody to the transferrin receptor for targeted delivery is known to internalize in clathrin-coated pits through receptor-mediated endocytosis [52]. Once the siRNA complex is endocytosed, the predominant fate is enzymatic degradation in the lysosome or recycling for extracellular clearance [18]. We have previously studied the intracellular trafficking of nucleic acid cargos by confocal microscopy and observed that escape from lysosomal pathway was favored by using this type of ternary pH-sensitive PICMs compared to a control siRNA/polycation complex [28]. Part of the effect may be attributed to the membrane destabilizing properties of the polyanion [53], although this hypothesis remains to be verified.

3.3.5. Gene expression inhibition of siRNA loaded PICMs

Ternary PICMs prepared with different PAMAMs (Table 3.1) and 2 siRNAs (unmodified and 2'-modified with 2'F-RNA and 2'F-ANA) (Table 3.2) were assessed for their silencing activity towards the Bcl-2 oncogene. Figure 3.9 reports the anti-Bcl-2 activity of PICMs containing PAMAM G5. The targeted PICMs (Fab'(CD71)-PICMs) prepared with a 50 nM concentration of the unmodified siRNAs decreased the Bcl-2 mRNA levels to 40% of its normal expression (Fig. 3.9A). On the opposite, the non-targeted PICMs or targeted PICMs containing mismatched siRNA sequence had only a marginal effect on the RNA expression. The small decrease in the mRNA level when cells were exposed to non-targeted PICMs can be explained by residual unspecific cellular uptake (Fig. 3.8). Furthermore, to evaluate possible immunostimulation of the carrier [54] which could lead to modulation of the mRNA expression, targeted PICMs devoid of siRNA (Fig. 3.9A, last column) were also tested. These showed no effect on Bcl-2 mRNA levels. The inhibition observed with the Fab'(CD71)-PICM system was only slightly less than for lipofectamine, a widely used cationic vector known for its high *in vitro* efficiency but important toxicity [55].

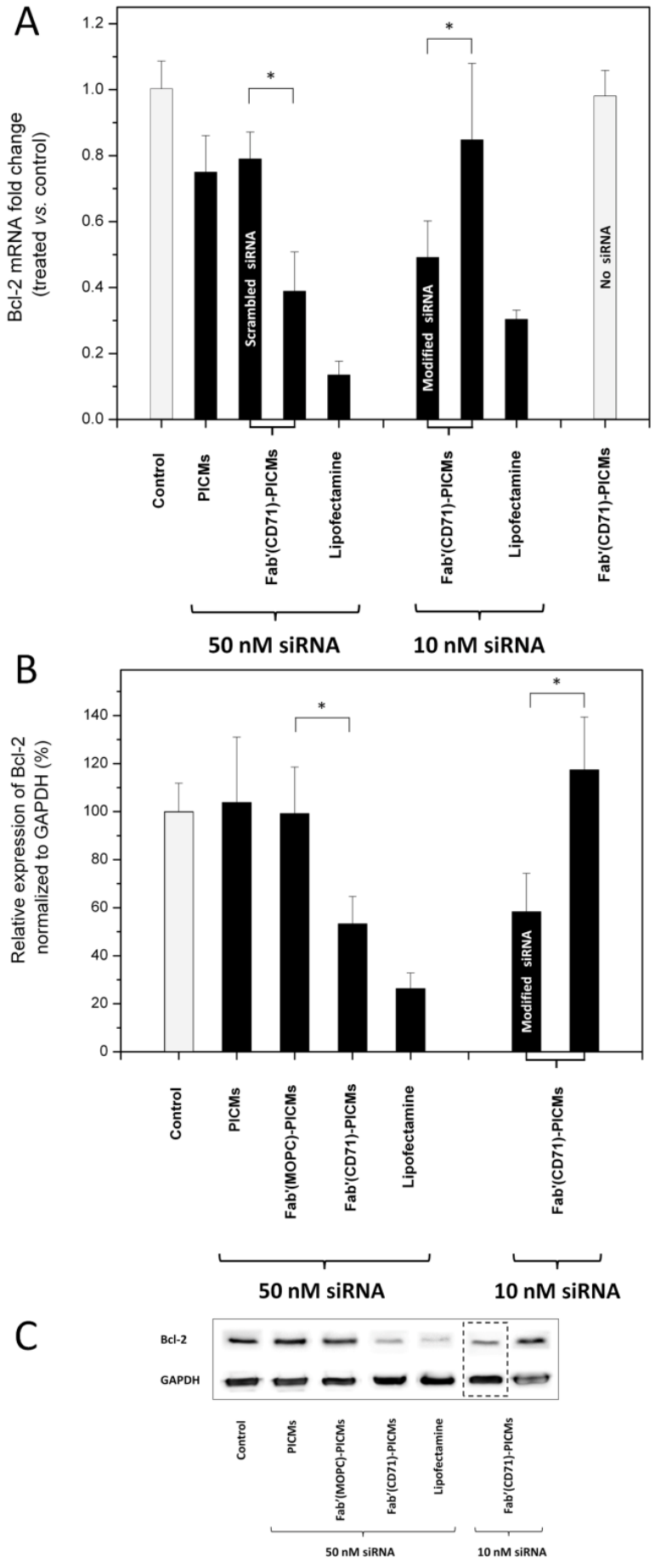

A
Bcl-2 mRNA fold change
(treated vs. control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Scrambled siRNA
Modified siRNA
No siRNA
Control
PICMs
Fab'(CD71)-PICMs
Lipofectamine
Fab'(CD71)-PICMs
Lipofectamine
Fab'(CD71)-PICMs
50 nM siRNA
10 nM siRNA
B
Relative expression of Bcl-2
normalized to GAPDH (%)
140
120
100
80
60
40
20
0
*
*
Modified siRNA
Control
PICMs
Fab'(MOPC)-PICMs
Fab'(CD71)-PICMs
Lipofectamine
Fab'(CD71)-PICMs
50 nM siRNA
10 nM siRNA
C
Bcl-2
GAPDH
Control
PICMs
Fab'(MOPC)-PICMs
Fab'(CD71)-PICMs
Lipofectamine
Fab'(CD71)-PICMs
50 nM siRNA
10 nM siRNA

Figure 3.9. Analysis of Bcl-2 silencing in PC-3 cells incubated for 5 h at 37 °C with different complexes containing Bcl-2 siRNA or scrambled sequence (Table 3.2). Unlabeled black bars represent formulations prepared with unmodified Bcl-2 siRNA. Final siRNA concentration in cell culture was 50 or 10 nM. PICMs presented in this figure were all prepared with PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45}) and PAMAM G5. Controls were treated with medium alone. All experiments were performed in 10% FBS. (A) Bcl-2 mRNA expression was evaluated by real time PCR 48 h after transfection. Data was processed using the delta delta CT ($2^{-\Delta\Delta CT}$) method. The β-actin gene was used as endogenous control. (B) Bcl-2 protein knockdown was evaluated by Western blots 72 h after treatment. GAPDH served as endogenous control. (C) Immunoblot bands of (B) conditions after Bcl-2 and GAPDH protein revelation. Dashed rectangle represents the bands with the modified Bcl-2 siRNA. Results are expressed as mean ± S.D. (n = 3); (*) $p < 0.05$ for the comparison indicated.

Incubation of PICMs in 50% FBS during transfection resulted in slightly higher mRNA levels compared to formulations incubated in 10% FBS (Fig. 3.10). The effects of PICM observed on the Bcl-2 mRNA expression were also translated to the protein expression, with strong inhibitory activity achieved with the Fab'(CD71)-PICMs at 50 nM siRNA (Fig. 3.9B). This system was then tested with a 2'-modified siRNA (Table 3.2). The nucleic acid structure was chosen from a previous study which showed that this modification pattern produced potent gene silencing for several siRNA sequences and targets [17]. Fab'(CD71)-PICMs containing the 2'-modified siRNA produced the same silencing effect, at both the mRNA and protein levels, as those loaded with unmodified siRNA, but at a 5-fold lower concentration (10 *vs.* 50 nM) (Fig. 3.9). Fab'(CD71)-PICMs loaded with this reduced concentration of unmodified siRNA exhibited significantly lower transfection efficiency (Fig. 3.9). In addition Fab'(CD71)-PICMs loaded with a chemically modified but mismatched sequence did not suppress mRNA nor the protein Bcl-2 levels (data not shown). These results demonstrate the positive contribution of using a chemically modified siRNA with an advanced nucleic acid delivery vehicle.

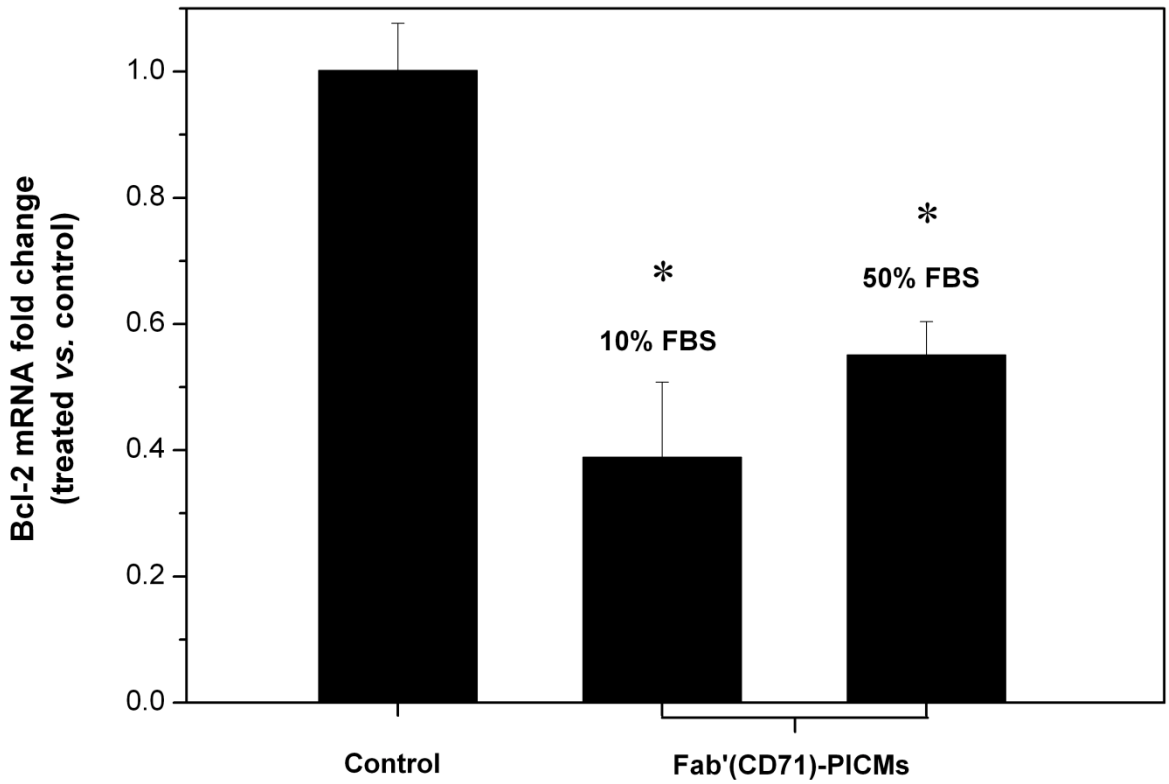


Figure 3.10. Analysis of Bcl-2 mRNA knockdown in PC-3 cells incubated for 5 h at 37 °C with complexes containing unmodified Bcl-2 siRNA. Experiments were performed in 10% or 50% FBS as indicated on the graph. Final siRNA concentration in cell culture was 50 nM. PICMs presented in this figure were all prepared with PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45}) and PAMAM G5. Controls were treated with medium alone. Bcl-2 mRNA knockdown was evaluated by real time PCR 48 h after treatment. β-actin served as endogenous control. Results are expressed as mean ± S.D. (n = 4); (*) $p < 0.05$ vs. control.

Lastly, PICMs prepared with different PAMAM cores (Table 3.1) and with a 50 nM Bcl-2 siRNA concentration were tested for their potential knockdown of the Bcl-2 mRNA levels. Formulations prepared with PAMAM G4 and PAMAM $G5_{S\text{-}S}$ were also able to significantly knockdown the Bcl-2 mRNA down to 50% of its initial expression (Fig. 3.11). However, PICMs containing a PAMAM $G4_{C12}$ core were highly cytotoxic hence preventing isolation of sufficient RNA for downstream processing (data not shown). PAMAM G4-based micelles, while less toxic (Fig. 3.5), were apparently as efficient as PAMAM G5-based micelles (Fig. 3.11). These combined results clearly show the versatility of the PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45})-based complexes, which is a key point necessary for the preparation of an optimal formulation presenting features such as i) low cytotoxicity and ii) high transfection efficiency.

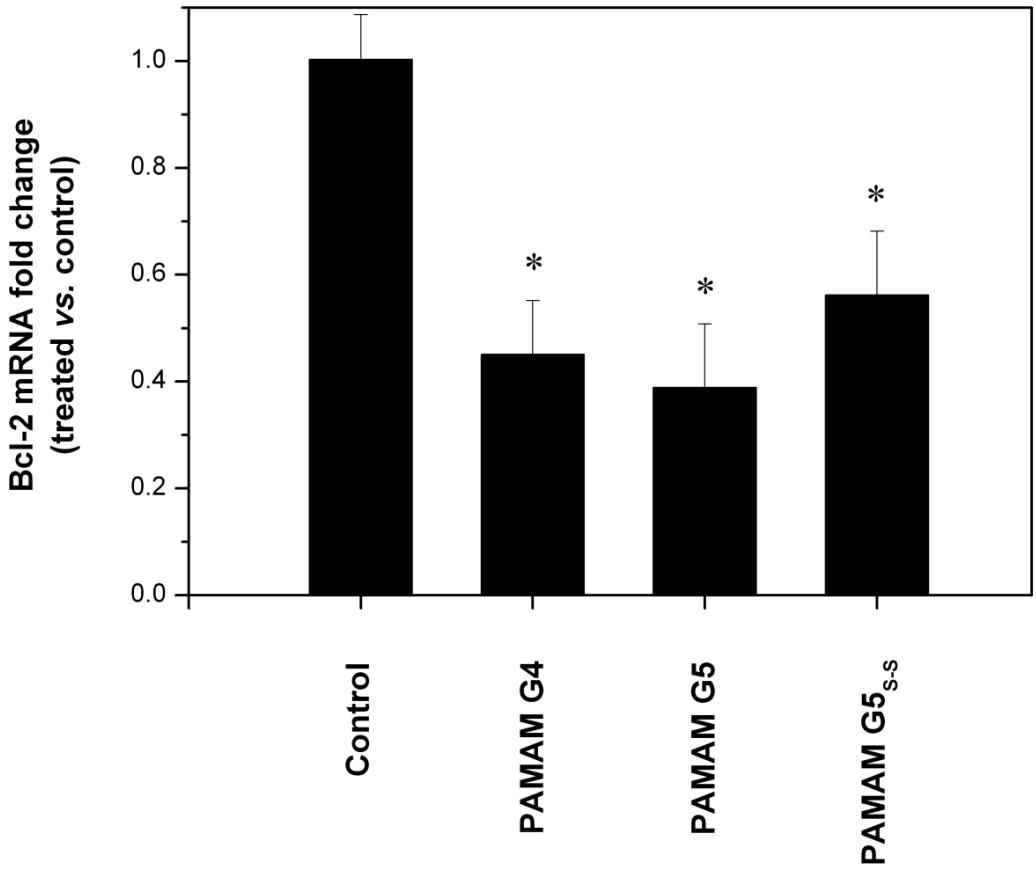


Figure 3.11. Analysis of Bcl-2 silencing in PC-3 cells incubated for 5 h at 37 °C with Fab'(CD71)-PICMs prepared with PEG_{115}-*b*-P($PrMA_{21}$-*co*-MAA_{45}) and different PAMAM dendrimers at their optimal N/(P+COOH) ratios (Table 3.1). Final siRNA concentration in cell culture was 50 nM and unmodified siRNA was used. Controls were treated with medium alone. All experiments were performed in 10% FBS. Bcl-2 mRNA knockdown was evaluated by real time PCR 48 h after treatment. β-actin served as endogenous control. Results are expressed as mean ± S.D. (n = 4); (*) $p < 0.05$ vs. control.

3.4. Conclusions

pH-sensitive PICMs based on methacrylic acid copolymers and PAMAMs were prepared and shown to have good stability under serum conditions, efficiently protecting the siRNA cargo against enzymatic degradation. These nanocarriers, when stably functionalized with a selected antibody fragment, demonstrated significantly higher cellular uptake than native untargeted PICMs. Furthermore, the targeted PICMs downregulated expression of the Bcl-2 mRNA and oncoprotein. Optimal transfection efficacy was achieved using chemically 2'-modified siRNA and a PAMAM G5 polycationic core. Further work will aim at evaluating these versatile pH-responsive PICMs under *in vivo* conditions.

3.5. Acknowledgements

This work was financially supported by an ETH Research Grant to J.-C. Leroux (ID ETH-0209-3), and by a CIHR Operating Grant to M.J. Damha. Dr. Marlen Knobloch and Sinem Karaman are gratefully acknowledged for their help with real-time PCR. The authors thank Dr. Marc A. Gauthier for his help with the schematic illustrations of the complexes and acknowledge the support by the Electron Microscopy Center of ETH Zurich (EMEZ).

3.6. References

[1] Aagaard L, Rossi JJ. RNAi therapeutics: Principles, prospects and challenges. Adv Drug Deliv Rev. 2007;59:75-86.

[2] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391:806-11.

[3] Miyagishi M, Taira K. U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nat Biotechnol. 2002;20:497-500.

[4] Kawasaki H, Taira K. Short hairpin type of dsRNAs that are controlled by $tRNA^{Val}$ promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res. 2003;31:700-7.

[5] Novina CD, Sharp PA. The RNAi revolution. Nature. 2004;430:161-4.

[6] Kircheis R, Kichler A, Wallner G, Kursa M, Ogris M, Felzmann T, et al. Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. Gene Ther. 1997;4:409-18.

[7] Wang X-L, Xu R, Wu X, Gillespie D, Jensen R, Lu Z-R. Targeted systemic delivery of a therapeutic siRNA with a multifunctional carrier controls tumor proliferation in mice. Mol Pharm. 2009;6:738-46.

[8] Bartlett DW, Su H, Hildebrandt IJ, Weber WA, Davis ME. Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality *in vivo* imaging. Proc Natl Acad Sci U S A. 2007;104:15549–54.

[9] Jones RA, Cheung CY, Black FE, Zia JK, Stayton PS, Hoffman AS, et al. Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by pH-sensitive disruption of endosomal vesicles. Biochem J. 2003;372:65-75.

[10] Li SD, Huang L. Gene therapy progress and prospects: Non-viral gene therapy by systemic delivery. Gene Ther. 2006;13:1313-9.

[11] Braasch DA, Jensen S, Liu Y, Kaur K, Arar K, White MA, et al. RNA interference in mammalian cells by chemically-modified RNA. Biochemistry. 2003;42:7967-75.

[12] Meyer M, Dohmen C, Philipp A, Kiener D, Maiwald G, Scheu C, et al. Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA-polymer conjugate. Mol Pharm. 2009;6:752-62.

[13] Leblond J, Mignet N, Largeau C, Spanedda M-V, Seguin J, Scherman D, et al. Lipopolythioureas: A new non-cationic system for gene transfer. Bioconjugate Chem. 2007;18:484-93.

[14] Corey DR. Chemical modification: the key to clinical application of RNA interference? J Clin Invest. 2007;117:3615-22.

[15] Bumcrot D, Manoharan M, Koteliansky V, Sah DWY. RNAi therapeutics: A potential new class of pharmaceutical drugs. Nat Chem Biol. 2006;2:711-9.

[16] Watts JK, Deleavey GF, Damha MJ. Chemically modified siRNA: Tools and applications. Drug Discov Today. 2008;13:842-55.

[17] Deleavey GF, Watts JK, Alain T, Robert F, Kalota A, Aishwarya V, et al. Synergistic effects between analogs of DNA and RNA improve the potency of siRNA-mediated gene silencing. Nucleic Acids Res. 2010;38:4547-57.

[18] Medina-Kauwe LK, Xie J, Hamm-Alvarez S. Intracellular trafficking of nonviral vectors. Gene Ther. 2005;12:1734-51.

[19] Wattiaux R, Laurent N, Wattiaux-De Coninck S, Jadot M. Endosomes, lysosomes: Their implication in gene transfer. Adv Drug Deliv Rev. 2000;41:201-8.

[20] Hawker CJ, Wooley KL. The convergence of synthetic organic and polymer chemistries. Science. 2005;309:1200-5.

[21] Kataoka K, Togawa H, Harada A, Yasugi K, Matsumoto T, Katayose S. Spontaneous formation of polyion complex micelles with narrow distribution from antisense oligonucleotide and cationic block copolymer in physiological saline. Macromolecules. 1996;29:8556-7.

[22] Mastrobattista E, van der Aa MA, Hennink WE, Crommelin DJA. Artificial viruses: A nanotechnological approach to gene delivery. Nat Rev Drug Discov. 2006;5:115-21.

[23] Yessine M-A, Dufresne M-H, Meier C, Petereit H-U, Leroux J-C. Proton-actuated membrane-destabilizing polyion complex micelles. Bioconjugate Chem. 2007;18:1010-4.
[24] Harada A, Togawa H, Kataoka K. Physicochemical properties and nuclease resistance of antisense-oligodeoxynucleotides entrapped in the core of polyion complex micelles composed of poly(ethylene glycol)-poly(L-lysine) block copolymers. Eur J Pharm Sci. 2001;13:35-42.
[25] Dufresne M-H, Elsabahy M, Leroux J-C. Characterization of polyion complex micelles designed to address the challenges of oligonucleotide delivery. Pharm Res. 2008;25:2083-93.
[26] Satturwar P, Eddine MN, Ravenelle F, Leroux J-C. pH-responsive polymeric micelles of poly(ethylene glycol)-*b*-poly(alkyl(meth)acrylate-*co*-methacrylic acid): Influence of the copolymer composition on self-assembling properties and release of candesartan cilexetil. Eur J Pharm Biopharm. 2007;65:379-87.
[27] Sant VP, Smith D, Leroux J-C. Enhancement of oral bioavailability of poorly water-soluble drugs by poly(ethylene glycol)-*block*-poly(alkyl acrylate-*co*-methacrylic acid) self-assemblies. J Control Release. 2005;104:289-300.
[28] Elsabahy M, Wazen N, Bayó-Puxan N, Deleavey G, Servant M, Damha MJ, et al. Delivery of nucleic acids through the controlled disassembly of multifunctional nanocomplexes. Adv Funct Mater. 2009;19:3862-7.
[29] Yessine M-A, Lafleur M, Meier C, Petereit H-U, Leroux J-C. Characterization of the membrane-destabilizing properties of different pH-sensitive methacrylic acid copolymers. Biochim Biophys Acta. 2003;1613:28-38.
[30] Dufès C, Uchegbu IF, Schätzlein AG. Dendrimers in gene delivery. Adv Drug Deliv Rev. 2005;57:2177-202.
[31] Hong S, Leroueil PR, Janus EK, Peters JL, Kober M-M, Islam MT, et al. Interaction of polycationic polymers with supported lipid bilayers and cells: Nanoscale hole formation and enhanced membrane permeability. Bioconjugate Chem. 2006;17:728-34.
[32] Sant VP, Smith D, Leroux J-C. Novel pH-sensitive supramolecular assemblies for oral delivery of poorly water soluble drugs: preparation and characterization. J Control Release. 2004;97:301-12.
[33] Ranger M, Jones M-C, Yessine M-A, Leroux J-C. From well-defined diblock copolymers prepared by a versatile atom transfer radical polymerization method to supramolecular assemblies. J Polym Sci, Part A: Polym Chem. 2001;39:3861-74.
[34] Damha MJ, Ogilvie KK. Oligoribonucleotide synthesis: The silyl-phosphoramidite method. In: Agrawal S, editor. Protocols for Oligonucleotides and Analogs. Totowa: The Humana Press Inc.; 1993. p. 81-114.
[35] Holappa S, Kantonen L, Winnik FM, Tenhu H. Self-complexation of poly(ethylene oxide)-*block*-poly(methacrylic acid) studied by fluorescence spectroscopy. Macromolecules. 2004;37:7008-18.
[36] Stiriba SE, Frey H, Haag R. Dendritic polymers in biomedical applications: From potential to clinical use in diagnostics and therapy. Angew Chem-Int Edit. 2002;41:1329-34.
[37] Thomas M, Klibanov AM. Non-viral gene therapy: Polycation-mediated DNA delivery. Appl Microbiol Biotechnol. 2003;62:27-34.
[38] Esfand R, Tomalia DA. Poly(amidoamine) (PAMAM) dendrimers: From biomimicry to drug delivery and biomedical applications. Drug Discov Today. 2001;6:427-36.
[39] Kono K, Akiyama H, Takahashi T, Takagishi T, Harada A. Transfection activity of polyamidoamine dendrimers having hydrophobic amino acid residues in the periphery. Bioconjugate Chem. 2005;16:208-14.
[40] Oskuee RK, Dehshahri A, Shier WT, Ramezani M. Alkylcarboxylate grafting to polyethylenimine: A simple approach to producing a DNA nanocarrier with low toxicity. J Gene Med. 2009;11:921-32.
[41] Gabrielson NP, Pack DW. Acetylation of polyethylenimine enhances gene delivery *via* weakened polymer/DNA interactions. Biomacromolecules. 2006;7:2427-35.
[42] Tomalia DA, Huang B, Swanson DR, Brothers HM, Klimash JW. Structure control within poly(amidoamine) dendrimers: Size, shape and regio-chemical mimicry of globular proteins. Tetrahedron. 2003;59:3799-813.

[43] Kim HJ, Ishii A, Miyata K, Lee Y, Wu S, Oba M, et al. Introduction of stearoyl moieties into a biocompatible cationic polyaspartamide derivative, PAsp(DET), with endosomal escaping function for enhanced siRNA-mediated gene knockdown. J Control Release. 2010;145:141-8.
[44] Lim C-J, Shen W-C. Comparison of monomeric and oligomeric transferrin as potential carrier in oral delivery of protein drugs. J Control Release. 2005;106:273-86.
[45] Daniels TR, Delgado T, Rodriguez JA, Helguera G, Penichet ML. The transferrin receptor part I: Biology and targeting with cytotoxic antibodies for the treatment of cancer. Clin Immunol. 2006;121:144-58.
[46] Marsh EW, Leopold PL, Jones NL, Maxfield FR. Oligomerized transferrin receptors are selectively retained by a lumenal sorting signal in a long-lived endocytic recycling compartment. J Cell Biol. 1995;129:1509-22.
[47] Trail PA, Willner D, Knipe J, Henderson AJ, Lasch SJ, Zoeckler ME, et al. Effect of linker variation on the stability, potency, and efficacy of carcinoma-reactive BR64-doxorubicin immunoconjugates. Cancer Res. 1997;57:100-5.
[48] Merkel OM, Mintzer MA, Librizzi D, Samsonova O, Dicke T, Sproat B, et al. Triazine dendrimers as nonviral vectors for *in vitro* and *in vivo* RNAi: The effects of peripheral groups and core structure on biological activity. Mol Pharm. 2010;7:969–83.
[49] Liu X-x, Rocchi P, Qu F-q, Zheng S-q, Liang Z-c, Gleave M, et al. PAMAM dendrimers mediate siRNA delivery to target Hsp27 and produce potent antiproliferative effects on prostate cancer cells. ChemMedChem. 2009;4:1302-10.
[50] Wakefield DH, Klein JJ, Wolff JA, Rozema DB. Membrane activity and transfection ability of amphipathic polycations as a function of alkyl group size. Bioconjugate Chem. 2005;16:1204-8.
[51] Chen H, Kim S, He W, Wang H, Low PS, Park K, et al. Fast release of lipophilic agents from circulating PEG-PDLLA micelles revealed by *in vivo* Förster resonance energy transfer imaging. Langmuir. 2008;24:5213-7.
[52] Daniels TR, Delgado T, Helguera G, Penichet ML. The transferrin receptor part II: Targeted delivery of therapeutic agents into cancer cells. Clin Immunol. 2006;121:159-76.
[53] Convertine AJ, Benoit DSW, Duvall CL, Hoffman AS, Stayton PS. Development of a novel endosomolytic diblock copolymer for siRNA delivery. J Control Release. 2009;133:221-9.
[54] Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol. 2005;23:457-62.
[55] Wu Y, Wang W, Chen Y, Huang K, Shuai X, Chen Q, et al. The investigation of polymer-siRNA nanoparticle for gene therapy of gastric cancer *in vitro*. Int J Nanomed. 2010;5:129-36.

Chapter 4

The Interactions of Amphiphilic Antisense Oligonucleotides with Serum Proteins and their Effects on *in vitro* Silencing Activity

Arnaud E. Felber[1], Núria Bayó-Puxan[1,2], Glen F. Deleavey[2], Bastien Castagner[1], Masad J. Damha[2], Jean-Christophe Leroux[1]

[1] Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland

[2] Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC H3A OB8, Canada

This chapter is published:
Biomaterials, **2012**; 33:5955-65.

4.1. Introduction

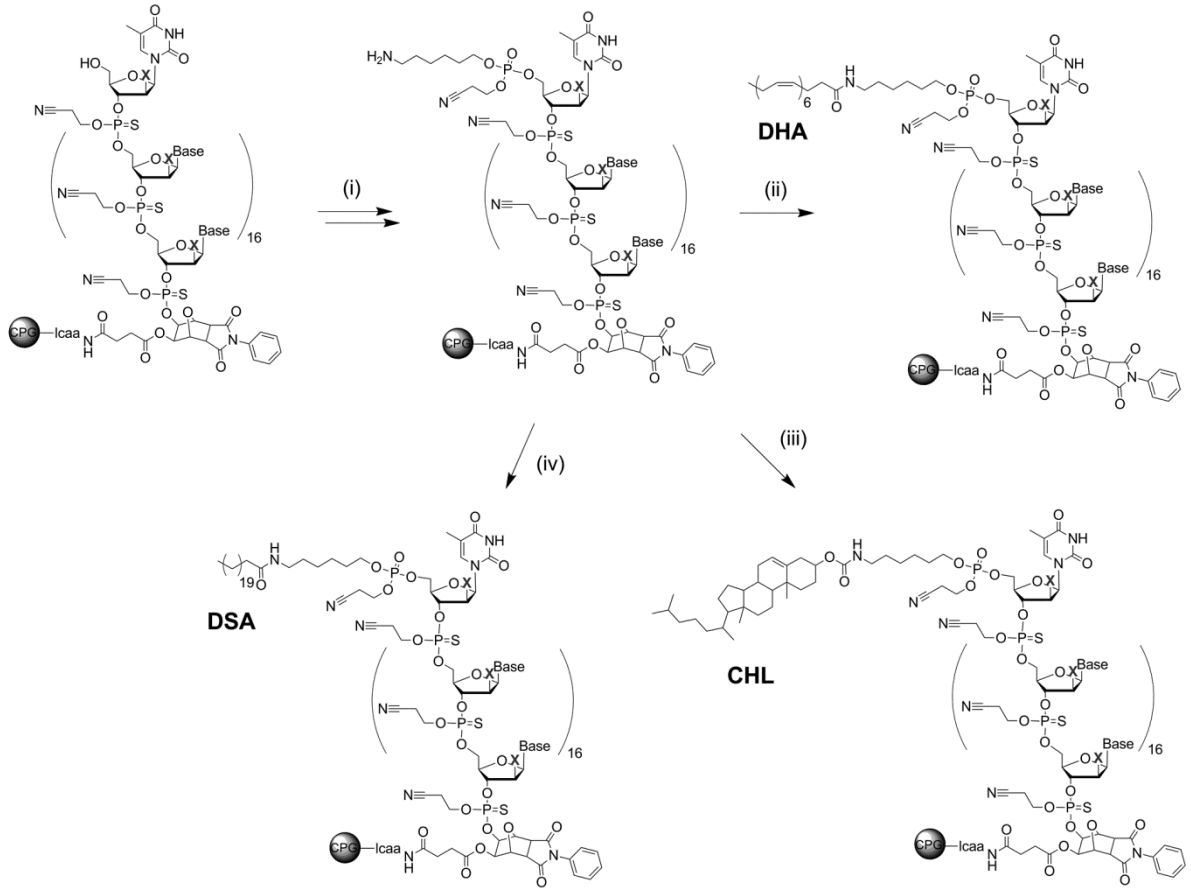
Since the late 1970's starting with the pioneer work of Zamecnik and Stephenson, antisense oligonucleotides (AONs) have been investigated as a means to achieve targeted and specific knockdown of gene expression [1, 2]. The success of AON therapeutics depends not only on the intracellular recognition event between the mRNA of the gene to be inhibited and the synthetic oligonucleotide drug, but also on many upstream pharmacokinetic processes. The anionic nature of AONs renders them almost impermeable to the negatively-charged cell membranes [3, 4]. Additionally, unmodified AONs are rapidly degraded by nucleases [5]. In order to achieve the desired pharmacological effect, a state of the art AON drug should exhibit i) a favorable pharmacokinetic profile, ii) resistance to nucleases, iii) intracellular bioavailability, iv) high affinity for target mRNA, and v) potent activation of the cellular machinery (*i.e.*, ribonuclease H (RNase H)), to yield reliable and effective knockdown of the targeted gene. Attempts to imbue AONs with these desirable properties have produced an impressive number of backbone chemical modifications compatible with AON-mediated gene silencing (for reviews on these modifications, see ref. [6, 7]). The first generation of backbone chemical alterations utilized 2′-deoxyribonucleotide phosphorothioate (PS) modifications. It entailed AONs with increased stability in biological systems and significantly increased their biological half-life [8]. The second generation of AON constructs typically employed chemically modified sugars, paired with a PS backbone, in order to further enhance stability towards nucleases, and in some cases potency. Common examples of chemically-modified sugars for these applications include 2′-fluoro, 2′-O-methyl and 2′-O-methoxyethyl analogues of RNA. The 2′-fluoro-arabinonucleic acid (2′F-ANA) modification is another example of a sugar chemical modification that can be readily applied to AON constructs [9, 10]. 2′F-ANA improves the nuclease resistance of oligonucleotides [11], increases binding stability to target mRNA [12], and structurally mimics DNA when bound with an RNA target, preserving RNase H activation [10, 12].

Today, most clinical trials with AONs and other nucleic acid drug candidates are performed in the absence of a delivery system [13]. Unfortunately, chemical modifications of the backbone structure do not generally solve permeability problems [14, 15] and only modestly improve the pharmacokinetic and/or biodistribution profiles [16, 17]. Strategies aimed at solving these problems can be divided into two categories: (i) the use of carrier-based systems and (ii) the chemical derivatization of the nucleic acid with functional conjugates. The first approach consists of protecting and transporting the antisense drugs with

a colloidal delivery system, such as viral capsids [18], lipoplexes (cationic lipids) [19], polyplexes (cationic polymers) [20], and different types of nanoparticles [21-24]. However, issues such as the toxicity of the formulating agents [25], and insufficient transfection efficacy *in vivo*, are hampering the clinical applicability of this approach to some extent [26]. On the other hand, strategies based on the chemical derivatization of nucleic acids involve the direct administration of an uncomplexed AON which is linked to a targeting ligand [27], cell penetrating peptide [28], or hydrophobic tail [29]. For example, it has been reported that covalent attachment of steroids and various other hydrophobic moieties to the 5′-end of AONs could extend their systemic circulation time and enhance cellular uptake [6, 30, 31]. These types of hydrophobized macromolecular structures are often referred to as amphiphilic AON conjugates. Even though promising results have been generated *in vitro* with such derivatives in the absence of transfecting agent, acceptable silencing levels are usually achieved with high nucleic acid doses (in the 2–10 μM range). Furthermore, the interactions between these amphiphilic conjugates and serum proteins, such as human serum albumin (HSA), have often not been well characterized [32].

In the present study, a library of amphiphilic AON derivatives was synthesized and characterized. The model AONs were all derived from the model Oblimersen (OB) sequence. OB is an 18-mer PS oligodeoxynucleotide complementary to the initial coding region of Bcl-2 mRNA, which was designed to inhibit the expression of the Bcl-2 oncoprotein [14]. The lipophilic moieties, docosahexaenoic acid (DHA), cholesterol (CHL), and docosanoic acid (DSA) were attached *via* an aliphatic amino-hexanol-linker to the 5′-end of PS-DNA AONs and PS-DNA analogs containing a fluorine substituent at the 2′-position of the arabinose sugar (*i.e.*, 2′F-ANA) (Scheme 4.1, Appendix 4.7.1). CHL was selected as control due its extensively reported conjugation to nucleic acid [29, 32, 33]. DHA, an ω-3 polyunsaturated C_{22} natural fatty acid, was chosen due to the reported reduced side-effects and enhanced anti-tumor efficacy observed when conjugated to paclitaxel [34]. DSA was selected as polysaturated counterpart of DHA. The *in vitro* downregulation of Bcl-2 by the amphiphilic AONs was assessed using carrier-free and carrier-mediated transfection conditions. The selected nucleic acid carrier was pH-sensitive polyion complex micelle (PICM) targeting the transferrin receptor (*i.e.*, CD71). This nanosized system was previously shown to improve the intracellular bioavailability and efficacy of AONs and siRNAs [22, 35]. The impact of serum proteins addition, and more specifically, HSA addition, on the transfection activity of the

unformulated and formulated AONs was also examined, with the aim of identifying new strategies for improving the *in vivo* delivery of amphiphilic AONs.



Scheme 4.1. Synthetic pathway for the preparation of the various amphiphilic AON derivatives. (i) MMT-6-amino-hexanol phosphoramidite, 5-ethylthiotetrazole; I_2 solution; no capping; 3% TCA in DCM; 5% DIPEA in DCM. (ii) DHA, PyBOP, DIPEA, DMF, 4h, RT. (iii) Cholesteryl chloroformate, DIPEA, DCM/DMF (5:1), 4h, RT. (iv) DSA, PyBOP, DIPEA, THF/DMF (9:1), 4 h, 40 ºC. The conjugates were then deprotected under standard conditions. X = H (DNA) or F (2′F-ANA).

4.2. Materials and methods

4.2.1. Materials

RPMI medium, Opti-MEM I medium, fetal bovine serum (FBS), trypsin, Lipofectamine™ 2000 (used according to the supplier's instructions), SYBR® Gold nucleic acid gel stain, and phosphate-buffered saline (PBS; 1 mM KH_2PO_4, 150 mM NaCl, 3 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) were obtained from Invitrogen (Carlsbad, CA). Unmodified siRNA sequence (5′-GCA UGC GGC CUC UGU UUG AUU-3′, sense strand) was designed and synthesized by Dharmacon (Chicago, IL). Low binding microcentrifugation tubes (DNA Lobind®) were purchased from Eppendorf-Vaudaux (Schönenbuch, Switzerland). DHA, DSA, cholesteryl chloroformate, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), *N*,*N*-Diisopropylethylamine (DIPEA), HSA, generation 5.0 poly(amido amine) (PAMAM), and decanoic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Acrylamide/bis-acrylamide solution (30%) was from Bio-Rad Laboratories (Hercules, CA). Boric acid was purchased from Hänseler AG (Herisau, Switzerland). Ethylenediaminetetraacetic acid (EDTA) was obtained from AppliChem (Darmstadt, Germany). Poly(ethylene glycol)-*b*-poly(propyl methacrylate-*co*-methacrylic acid) (PEG_{115}-*b*-$P(PrMA_{22}$-*co*-$MAA_{58})$ (M_n 13,000; M_w/M_n = 1.06) and fragment antigen binding (Fab′)-modified PEG copolymer (Fab′-PEG_{169}-*b*-$P(PrMA_{31}$-*co*-$MAA_{62})$) were synthesized as previously reported [35, 36]. All other products, unless otherwise specified, were purchased from Fisher Scientific AG (Wohlen, Switzerland).

4.2.2. Synthesis of oligonucleotide

Standard phosphoramidite solid-phase synthesis conditions were used for the synthesis of the oligonucleotides [37]. Syntheses were performed on an Applied Biosystems (Carlsbad, MA) 3400 DNA Synthesizer on a 1-μmol scale using Unylink CPG as the solid support (ChemGenes, Wilmington, MA). 2′F-ANA phosphoramidites were prepared as 0.15 M solutions in dry acetonitrile (ACN), and DNA phosphoramidites were prepared as 0.1 M in dry ACN. 5-ethylthiotetrazole (0.25 M in ACN, ChemGenes) was used to activate the phosphoramidites for coupling. Detritylations were accomplished with 3% trichloroacetic acid (TCA) in dichloromethane (DCM) for 110 s. Capping of failure sequences was achieved with acetic anhydride in tetrahydrofuran (THF) and 16% *N*-methylimidazole in THF. Oxidation was done using 0.1 M I_2 in 1:2:10 pyridine:water:THF. Sulfurizations of PS backbone oligonucleotides were accomplished using a 0.1 M solution of xanthane hydride (TCI

America, Portland, OR) in 1:1 (*v/v*) pyridine:ACN. The sulfurization step was allowed to proceed for 2.5 min, with new sulfurization reagent added to the column after 1.25 min. Phosphoramidite coupling times were 600 s for 2′F-ANA, with the exception of guanosine phosphoramidite, which was allowed to couple for 900 s. DNA coupling times were 110 s, and 270 s for guanosine. For amphiphilic AON conjugates preparation, mono-methoxytrityl (MMT)-protected 6-amino-hexanol phosphoramidite (ChemGenes) was attached to oligonucleotide 5′-ends using a coupling cycle (without capping) with a coupling time of 600 s, followed by oxidation and detritylation (3% TCA in DCM, 240 s). Prior to the coupling with the corresponding aliphatic moiety (next section), oligomers were successively washed with DCM (1 x 5 min), 5% DIPEA in DCM (5 x 1 min), DCM (5 x 1 min) and ACN (5 x 1 min). Washes were removed by ultracentrifugation and final residual solvent was evaporated under reduced pressure.

4.2.3. Synthesis of amphiphilic AONs conjugates

The solid support-bound amino modified oligonucleotide (1.0 μmol) was placed in a low-binding microcentrifugation tube. *DHA coupling*: 150 μL of a DHA (0.25 M), PyBOP (0.25 M) and DIPEA (0.75 M) solution in anhydrous dimethylformamide (DMF) was added to the AON and the reaction was stirred for 4 h at room temperature. *DSA coupling:* 150 μL of a DSA (0.25 M), PyBOP (0.25 M) and DIPEA (0.75 M) solution in anhydrous THF:DMF (9:1) was added to the AON, and the reaction was stirred for 4 h at 40 °C. *CHL coupling:* 150 μL of a cholesteryl chloroformate (0.25 M) and DIPEA (2.25 M) solution in anhydrous DCM:DMF (5:1) was added to the AON and the reaction was stirred for 4 h at room temperature. *bisDHA coupling*: Following a standard 1 μmol solid phase oligonucleotide synthesis, a symmetrical branching linker (ChemGenes) (Appendix 4.7.2) was attached to the 5′-end of the growing oligonucleotide using a 900 s coupling time. The MMT-protected 6-amino-hexanol phosphoramidite (ChemGenes) was then added (coupling performed at 0.2 M concentration for 600 s, with no subsequent capping step, and a 240 s detritylation step following oxidation). The amino modified oligonucleotide attached to the CPG was placed in a low binding microcentrifugation tube. Coupling of the DHA moiety was performed with 24 mg of DHA, PyBOP (38 mg) and DIPEA (40 μL) in 150 μL anhydrous DMF and the reaction was shaken for 4 h at room temperature. Excess of reagents was removed by washing the solid support as follows: DMF (5 x 1 min), ACN (5 x 1 min). Washes were removed by ultracentrifugation and final remaining solvent was evaporated under reduced pressure (rotavap).

For all conjugates, excess of reagents was removed by washing the solid support as follows: DMF (5 x 1 min), ACN (5 x 1 min). Washes were removed by ultracentrifugation, and final residual solvent was evaporated under reduced pressure. In all cases, lipophilic moiety coupling conversions were more than 75%, as determined by high-performance liquid chromatography (HPLC) (Appendices 4.7.1 and 4.7.2).

4.2.3.1. Cleavage and purification

Deprotection and cleavage from the solid support was accomplished with 1 mL of 3:1 NH_4OH:EtOH for 48 h at room temperature [38]. Cleavage solution was removed under reduced pressure. The product was extracted with 2 x 250 µL H_2O and 2 x 250 µL EtOH. Solvent was removed, and the pellet was precipitated from 50 µL NaOAc (3 M, pH = 5.5) and 1 mL of cold *n*-BuOH for 3 h in a dry ice bath. The pellet was dissolved in 1 mL H_2O:ACN (1:1) and subjected to ultraviolet quantification. Purification of crude oligonucleotides was done either by preparative denaturing polyacrylamide gel electrophoresis (PAGE) using 24% acrylamide gels or by reverse phase HPLC. For the PAGE purification, gel bands were extracted overnight in diethylpyrocarbonate-treated autoclaved ultrapure deionized water, and lyophilized to dryness. Reverse phase HPLC purification was carried out on a Waters 1525 HPLC (Milford, MA) using a Varian Pursuit 5 (Agilent Technologies, Mississauga, ON, Canada) reverse phase C18 column (250 x 10 mm) with solvent A as 100 mM triethylammonium acetate in water supplemented with 5% ACN (pH 7.0), and solvent B as ACN. HPLC flow was set at 4 mL/min and a gradient was run for 30 min at 50 °C from 80:20 to 60:40 (solvent A:solvent B) for DHA conjugates, and from 70:30 to 20:80 (solvent A:solvent B) for DSA, CHL, and bisDHA conjugates. All purified oligonucleotides were desalted with Nap-25 Sephadex columns from GE Healthcare (Glattbrugg, Switzerland) according to standard protocol. Characterization of the amphiphilic conjugates can be found in the Appendices 4.7.1 and 4.7.2.

4.2.4. Cell culture

Prostate adenocarcinoma cells (PC-3) were obtained from the American Type Culture Collection (Rockville, MD). They were grown in complete RPMI medium (RPMI containing 10% FBS, supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 100 units/mL penicillin, and 100 µg/mL streptomycin). Stock cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed on mycoplasma-free cell lines and only cells in the exponential phase of growth were used.

4.2.5. Assessment of mRNA levels by real-time PCR

Following adequate post-transfection incubation time (*i.e.,* 72 or 48 h for carrier-free or PICM-loaded AONs, respectively), cells were harvested and RNA was isolated using RNeasy Mini kit (QIAGEN, Valencia, CA). Purified RNA ($OD_{260}/OD_{230} \geq 1.8$) was used as a template to assess the gene expression level of Bcl-2 *via* a two-step quantitative reverse-transcription PCR (qRT-PCR). Briefly, reverse transcription reaction was carried out using high capacity cDNA reverse transcriptase kit (Applied Biosystems). Following the cDNA synthesis, the qPCR was run using Power SYBR Green PCR Master Mix (Applied Biosystems) with a 7900HT Fast Real-Time PCR system (Applied Biosystems). The data were normalized to the internal control; β-actin. The primers for Bcl-2 and β-actin were purchased from QIAGEN. All procedures followed the manufacturer's protocol. Relative gene expression levels were calculated using the delta delta Ct ($2^{-\Delta\Delta Ct}$) method. Results are expressed as the Bcl-2 mRNA level fold change between treated and non-treated (*i.e.,* control) samples.

4.2.6. Carrier-free transfection

PC-3 cells were seeded one day prior to the experiment in a 12-well plate at a density of 100,000 cells/well in complete RPMI containing 10% FBS. Solutions (500 µL) containing 800 nM (0.4 nmol) oligonucleotides were prepared in Opti-MEM I containing i) 0% or ii) 20% (*v/v*) FBS, or iii) the desired HSA concentration. The samples were then stirred for 20 min at room temperature and directly incubated with the 60–70% confluent cells overnight. Subsequently, the transfection medium was changed to complete RPMI containing 10% FBS. After a 72-h total incubation time, RNA was isolated and the gene expression levels of Bcl-2 were assessed as described above (see section 4.2.5.).

In another series of experiments, the influence of preincubating HSA with decanoic acid on the transfection efficacy was investigated. PC-3 cells were seeded one day prior to the experiment in a 12-well plate at a density of 100,000 cells/well in complete RPMI containing 10% FBS. HSA (1 or 4 molar excess over AON) and decanoic acid (35 molar excess over HSA) were pre-incubated in 0.5 mL Opti-MEM I for 1 h at 37 °C under constant shaking. FANA2-DSA (0.4 nmol, 800 nM) was then directly added to the solution and allowed to mix for another 20 min prior to overnight incubation with the 60–70% confluent cells. Subsequently, the transfection medium was changed to complete RPMI containing 10% FBS. After a 72-h total incubation time, RNA was isolated and gene expression levels of Bcl-2 were assessed as described above (see section 4.2.5.).

4.2.7. Carrier-mediated transfection

PC-3 cells were seeded one day prior to the experiment in a 12-well plate at a density of 150,000 cells/well in complete RPMI containing 10% FBS. PICMs loaded with nucleic acid were prepared in a 100-µL volume of Tris buffer (10 mM, pH 7.4) as previously described [35, 36]. Briefly, negatively-charged micelle components (PEG_{115}-*b*-P($PrMA_{22}$-*co*-MAA_{58}), 3.2 nmol; Fab′-PEG_{169}-*b*-P($PrMA_{31}$-*co*-MAA_{62}), 0.04 nmol; nucleic acid, 0.2 nmol or 0.025 nmol for AON and siRNA, respectively) were combined in Tris buffer and mixed with PAMAM dendrimer at a N/(P+COOH) molar ratios equal to 1.5. N corresponds to the number of primary amine groups of the PAMAM while P and COOH account for the phosphate and carboxylate groups of the nucleic acid and MAA copolymer, respectively. The samples were stirred for 20 min at room temperature to allow micelle formation. Samples were then incubated with the 60–70% confluent cells for 5 h in a total of 500 µL Opti-MEM I containing no HSA or 8 mg/mL HSA. Subsequently, the transfection medium was changed to complete RPMI containing 10% FBS. After a 48-h total incubation time, RNA was isolated and gene expression levels of Bcl-2 were assessed as described above (see section 4.2.5.).

4.2.8. HSA stability of AON-loaded PICMs

PICMs were prepared to contain 0.2 nmol of either OB or FANA2-DSA; or 0.06 nmol of siRNA in a final volume of 100 µL, using similar conditions as previously reported [35, 36]. When appropriate, HSA was added to the sample to have a final protein concentration of 8 mg/mL (*i.e.*, equivalent to 20% serum). The solution was allowed to incubate for 3 h at 37 °C under constant shaking. Directly before gel electrophoresis, 5x loading buffer (Tris buffer 50 mM, pH 8.0, 25% (*v/v*) glycerol, 5 mM EDTA) was added to 12 µL of samples, resulting in final nucleic acid amounts per well of *ca*. 150 ng for OB and FANA2-DSA, or *ca*. 100 ng for siRNA. Formulations were then loaded onto a 5% (*w/v*) stacking gel and 20% (*w/v*) acrylamide running gel prepared from a Tris–borate–EDTA (TBE) buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) adjusted to pH 7.4. Gel was then immersed in TBE buffer (same composition as above) and electrophoresed at constant voltage of i) 80 V for about 20 min, followed by ii) 150 V for about 50 min. Finally, nucleic acid was revealed following manufacture's protocol for SYBR® Gold nucleic acid gel stain and fluorescence was recorded on a ChemiDoc XRS (Bio-Rad).

4.2.9. Characterization of AON-HSA interactions

Samples were prepared to contain 0.1 nmol of FANA2-DSA in a final volume of 50 µL (*i.e.,* [FANA2-DSA] = 2000 nM). Firstly, increasing amount of HSA (*n* x 0.1 nmol) and decanoic acid (*n* x 3.5 nmol) were pre-incubated in Tris buffer (10 mM, pH 7.4) for 1 h at 37 °C under constant shaking (*n* = 0, 1, 2, 4, and 10). FANA2-DSA was then added and the solution was further incubated for 5 h at 37 °C under mild agitation. For samples not containing decanoic acid, Tris buffer (10 mM, pH 7.4) was added to replace the missing volume. Directly before gel electrophoresis, 5x loading buffer (Tris buffer [50 mM, pH 8.0], 25% glycerol, 5 mM EDTA) was added to 12 µL of samples (*i.e.*, 150 ng FANA2-DSA per well). Formulations were loaded onto a 5% (*w/v*) stacking gel and 20% (*w/v*) acrylamide running gel prepared from a TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) adjusted to pH 7.4. Electrophoresis conditions and nucleic acid detection were performed under the same condition as previously mentioned (see section 4.2.8).

Gel was then immersed in TBE buffer (same composition as above) and electrophoresed at constant voltage of i) 80 V for about 20 min, followed by ii) 150 V for about 50 min. Finally, nucleic acid was revealed following manufacture's protocol for SYBR® Gold nucleic acid gel stain and fluorescence was recorded on a ChemiDoc XRS (Bio-Rad).

4.2.10. Statistical analysis

All statistical analyses were performed using the computer program *OriginPro* (OriginLab, Northampton, MA). Experiments were analyzed using a one-way analysis of variance followed by Tukey's test to determine the significance of all paired combinations. The level of statistical significance was fixed at $p \leq 0.05$.

4.3. Results

Table 4.1. Sequences of synthesized oligonucleotides targeting the Bcl-2 mRNA.

Designation	Sequence (5′–3′) [a]
OB	TCT CCC AGC GTG CGC CAT
FANA1	**tct** CCC AGC GTG CGC **cat**
FANA2	TCT **ccc** AGC **gtg** CGC **cat**
FANA3	**tct ccc** AGC GTG **cgc cat**

[a] Where ATGC and **atgc** are PS-DNA and PS-2′F-ANA, respectively.

The PS-DNA sequence (OB, Table 4.1), targeting the initiation codon region of the human Bcl-2 mRNA, was chosen as reference AON due to its well established ability to inhibit the Bcl-2 expression at both mRNA and protein levels [14]. This AON was used in this study only as an *in vitro* model, since OB has been associated with off-target effects [39, 40]. Three additional OB-based AON sequences containing PS-2′F-ANA regions were also synthesized.

4.3.1. Synthesis of amphiphilic AONs

The synthetic approach to obtain amphiphilic oligonucleotide conjugates (Scheme 4.1) was designed to be versatile in order to easily access a small library of compounds. This strategy allowed an exploration of the impact of chemical modifications and choice of lipophilic moiety on transfection efficacy. The key point in the procedure was the use of an MMT-protected amino-hexanol phosphoramidite as a linker between the oligonucleotide and the lipophilic moiety. The oligonucleotide sequences with appropriate chemical modification were assembled in parallel by automated solid support synthesis (1.0 µmol scale). The amino-hexanol phosphoramidite linker was then incorporated and subsequently deprotected within the automated program using a modified coupling cycle. No capping step was performed after coupling to avoid acetylation of the amine, and longer (*i.e.*, 240 s) MMT-removal time was implemented to ensure complete amine deprotection. Finally, beads were removed from the columns to be subjected to coupling reactions using PyBOP and/or DIPEA with the corresponding lipophilic moiety in a microcentrifugation tubes. Conditions were optimized for each lipophilic moiety to take into consideration their differing solubilities. Cleavage from

the solid support and deprotection was performed using standard protocols [38, 41]. The amphiphilic AONs were purified by reverse phase HPLC. For all coupling reactions, good conversions were obtained (70–80%), as observed in HPLC chromatograms (Appendix 4.7.1).

Additionally, a bis-aliphatic conjugate (bisDHA) was obtained by incorporating a symmetrical branching phosphoramidite linker (Appendix 4.7.2) prior to the amino-hexanol phosphoramidite linker addition. DHA was then coupled to the bis-amino-OB AON (Appendix 4.7.2B). OB-bisDHA was purified by reverse phase HPLC (Appendix 4.7.2C) and characterized by MS (Appendix 4.7.2D). Conversions of 50% were obtained. Attempts to produce the bisCHL and bisDSA conjugates failed, probably due to steric hindrance in the second amide coupling reaction.

Table 4.2. Library of synthesized compounds.

No.	Antisense sequence	Lipophilic moiety	Mass found	Mass calculated
1	OB	-	-	-
2	OB	DHA	6173.1	6174.0
3	OB	CHL	6275.3	6276.9
4	OB	DSA	6185.2	6186.4
5	OB	bisDHA	6833.1	6831.4
6	FANA 1	DHA	6281.1	6282.0
7	FANA 1	CHL	6383.6	6384.9
8	FANA 1	DSA	6293.4	6294.3
9	FANA 2	DHA	6335.7	6335.0
10	FANA 2	CHL	6439.0	6438.8
11	FANA 2	DSA	6347.2	6348.3
12	FANA 3	DHA	6389.5	6389.9
13	FANA 3	CHL	6491.2	6492.8
14	FANA 3	DSA	6401.1	6402.3

4.3.2. Silencing activity of amphiphilic AONs

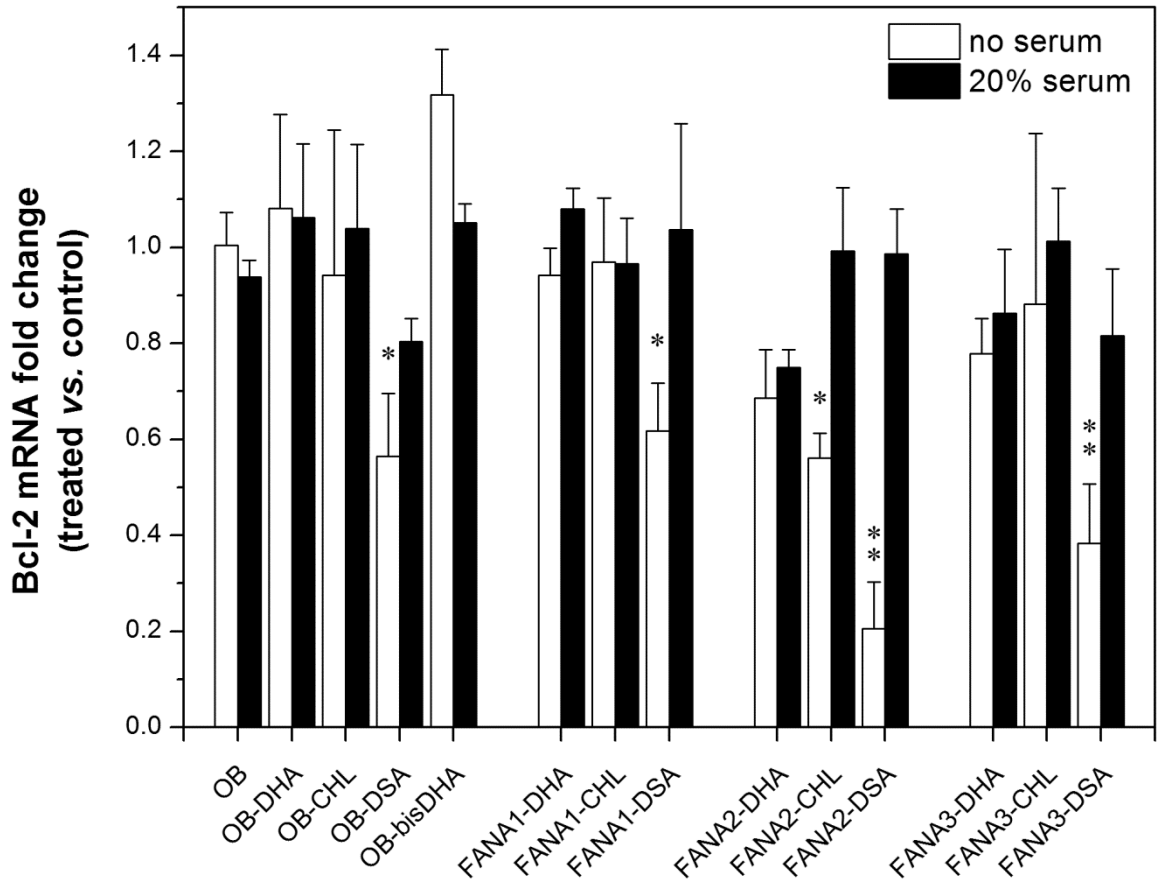


Figure. 4.1. Analysis of Bcl-2 mRNA silencing in PC-3 cells incubated overnight at 37 °C, with different amphiphilic AON derivatives (all targeting Bcl-2 mRNA). AON concentration in cell culture was 800 nM. Controls were treated with medium alone. Experiments were performed in 0% (empty bars) or 20% (filled bars) FBS supplemented transfection medium. Bcl-2 mRNA expression was evaluated by real time PCR 72 h after transfection. Results are expressed as mean ± S.D. (n = 3). (*) $p \leq 0.01$ or (**) $p \leq 0.001$ *vs*. control.

The silencing activity of the amphiphilic AON derivatives and unmodified controls (Table 4.2) was investigated *in vitro* on PC-3 cells. For carrier-free conditions, the AONs were incubated overnight with cells at a concentration of 800 nM in the presence or absence of serum (Fig. 4.1). The level of the Bcl-2 gene product was determined *via* qRT-PCR 72 h post-transfection. A preliminary kinetic study revealed that maximal RNA downregulation was obtained after 72 h (data not shown). The level of β-actin in the same sample was used as an internal standard. As illustrated in Figure 4.1, conjugates featuring the DSA moiety exhibited the highest knockdown efficiencies under serum-free conditions (empty bars) compared to all other lipophilic-modified compounds. In particular, AONs containing PS-2′F-

ANA in an altimer (*vide infra*) modification design [42] (FANA2, Table 4.1) produced the highest antisense activity, with 80% mRNA suppression for FANA2-DSA (Fig. 4.1). Under similar conditions, amphiphilic derivatives containing either CHL or DHA induced less silencing activity, with maximum knockdown values of 31 and 44% for FANA2-DHA and FANA2-CHL, respectively. It is noteworthy that, in the absence of a transfection agent, the PS-DNA (OB) did not have any effect on the targeted mRNA (Fig. 4.1). However, its conjugation with DSA resulted in a 44% inhibition of Bcl-2 mRNA. The highly lipophilic AON bearing two DHA chains, OB-bisDHA, did not show any downregulating effect.

The addition of serum to the transfection medium (*i.e.*, 20% FBS) drastically reduced the *in vitro* knockdown efficiency of the amphiphilic conjugates (Fig. 4.1, filled bars). This result suggests that the AONs might interact with one (or multiple) serum protein(s) (*e.g.*, HSA) in the extracellular milieu.

4.3.3. Effect of HSA on the activity of amphiphilic AONs

The effect of HSA alone, the most abundant serum protein, on the knockdown activity of selected amphiphilic AON conjugates was also examined (Fig. 4.2). FANA2-DSA was selected for this experiment because it produced the highest silencing activity under serum-free conditions (Fig. 4.1). Two additional PS-DNA AONs, one modified with a DSA moiety and one unmodified, were also tested for comparison. In this experiment, cells were transfected with 800 nM of nucleic acid in a carrier-free fashion in the presence of 8 mg/mL HSA, which corresponds to the HSA concentration in 20% serum. As shown in Figure 4.2A, the PS-DNA lacking the DSA moiety, remained inactive upon HSA addition. Interestingly, HSA alone was able to abolish the silencing activity of both DSA-modified AONs (OB-DSA and FANA2-DSA). The HSA effect was comparable to that of 20% serum (Fig. 4.1). Under these experimental conditions (*i.e.*, 800 nM AON and 8 mg/mL HSA), the molar excess of HSA over FANA2-DSA was *ca*. 150. To further investigate the impact of HSA complexation on the silencing activity, the conjugate:HSA molar ratio was varied (Fig. 4.2B). It was found that reducing the lipophilic AON to protein molar ratios in the transfection medium to 1:1 and 1:2 partially restored the downregulation levels (56% and 31%, respectively, Fig. 4.2B). The activity of the DSA-modified antisense drug was fully inhibited by HSA when the molar excess of HSA over the conjugate was ≥ 4 (Fig. 4.2B).

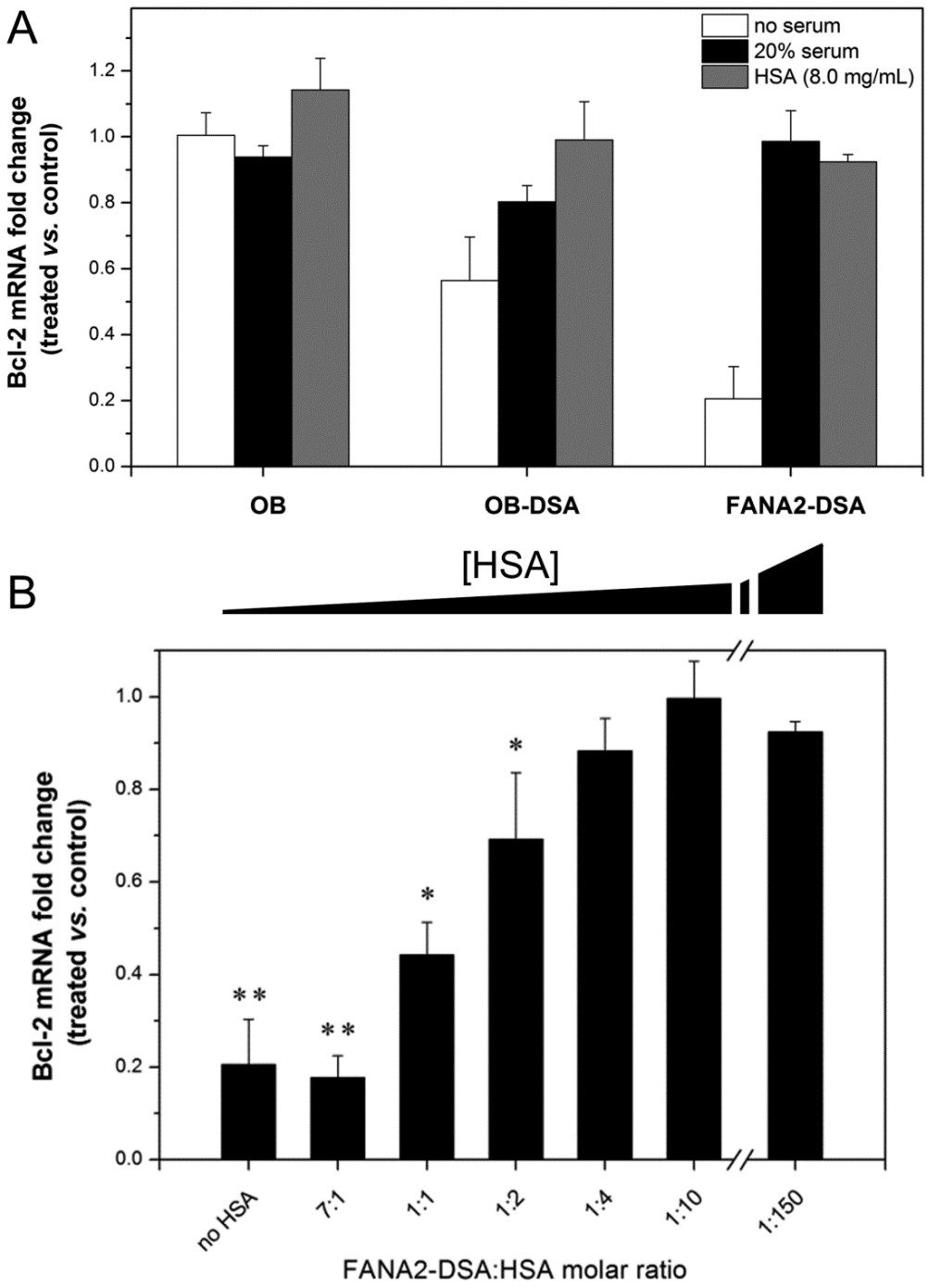


Figure 4.2. Effect of HSA on Bcl-2 mRNA silencing. (A) Analysis of Bcl-2 mRNA silencing in PC-3 cells incubated overnight at 37 °C with OB, OB-DSA, and FANA2-DSA conjugates. AON concentration in cell culture was 800 nM. Experiments were performed in Opti-MEM I supplemented with i) 0%, ii) 20% (v/v) FBS, or iii) 8 mg/mL HSA (ca. 150 molar excess over AON). (B) Analysis of Bcl-2 mRNA silencing following overnight incubation with various FANA2-DSA:HSA molar ratios. Final AON concentration in cell culture was 800 nM. Bcl-2 mRNA fold change is expressed relative to an untreated control (medium alone). Bcl-2 mRNA expression was evaluated by real time PCR 72 h after transfection. Results are expressed as mean ± S.D. (n = 3). (*) $p \leq 0.01$ or (**) $p \leq 0.001$ *vs*. control.

4.3.4. Silencing activity of amphiphilic AON loaded in PICMs

The silencing activity of selected nucleic acids was further evaluated following their complexation into a polymeric nanocarrier. The conjugates were complexed with anionic PEG-*b*-P(PrMA-*co*-MAA) and cationic PAMAM dendrimers to yield pH-sensitive PICMs (Fig. 4.3A). Such short nucleic acid-loaded ternary micelles typically have hydrodynamic diameters of 50–60 nm, narrow size distribution, and near-neutral zeta potential (data not shown). Furthermore, our group previously reported that coupling a Fab′ targeting ligand directed against the CD71 (*i.e.*, transferrin) receptor on the surface of such PICMs favored their specific cellular uptake through receptor mediated endocytosis [22, 35]. In this experiment, PC-3 cells were transfected with nucleic acid-loaded PICMs (400 nM or 50 nM for AON and siRNA, respectively) for 5 h in absence or presence of 8 mg/mL HSA (Fig. 4.3B). The level of the Bcl-2 gene product was determined *via* qRT-PCR 48 h post-transfection. Figure 4.3B shows that compared to OB and OB-DSA, PICM-loaded with the FANA2-DSA AON exhibited the highest knockdown efficiency (60% mRNA suppression) in the absence of HSA. Under similar conditions, PICMs loaded with the other two AON sequences induced less silencing activity, with maximum knockdown values of 30% and 34% for OB and OB-DSA, respectively (Fig. 4.3B). The silencing efficiency of PICM-loaded FANA2-DSA significantly decreased to 38% upon incubation with 8 mg/mL HSA (Fig. 4.3B). A similar trend was also observed for OB and OB-DSA sequences (Fig. 4.3B). However, these reductions in silencing activity were not statistically significant. Then, the silencing activities of the PICM-delivered AONs +/- HSA in the culture medium were compared to that of a 21-mer anti-Bcl-2 phosphodiester siRNA incorporated into the PICMs. In the absence of HSA, the encapsulated siRNA (50 nM) reduced of 43% the expression of Bcl-2 mRNA. However, in the case of the double stranded nucleic acid, HSA had no effect on the transfection efficiency (Fig. 4.3B). As discussed below this could be related to differences in the stability of the PICMs loaded with siRNA or AON.

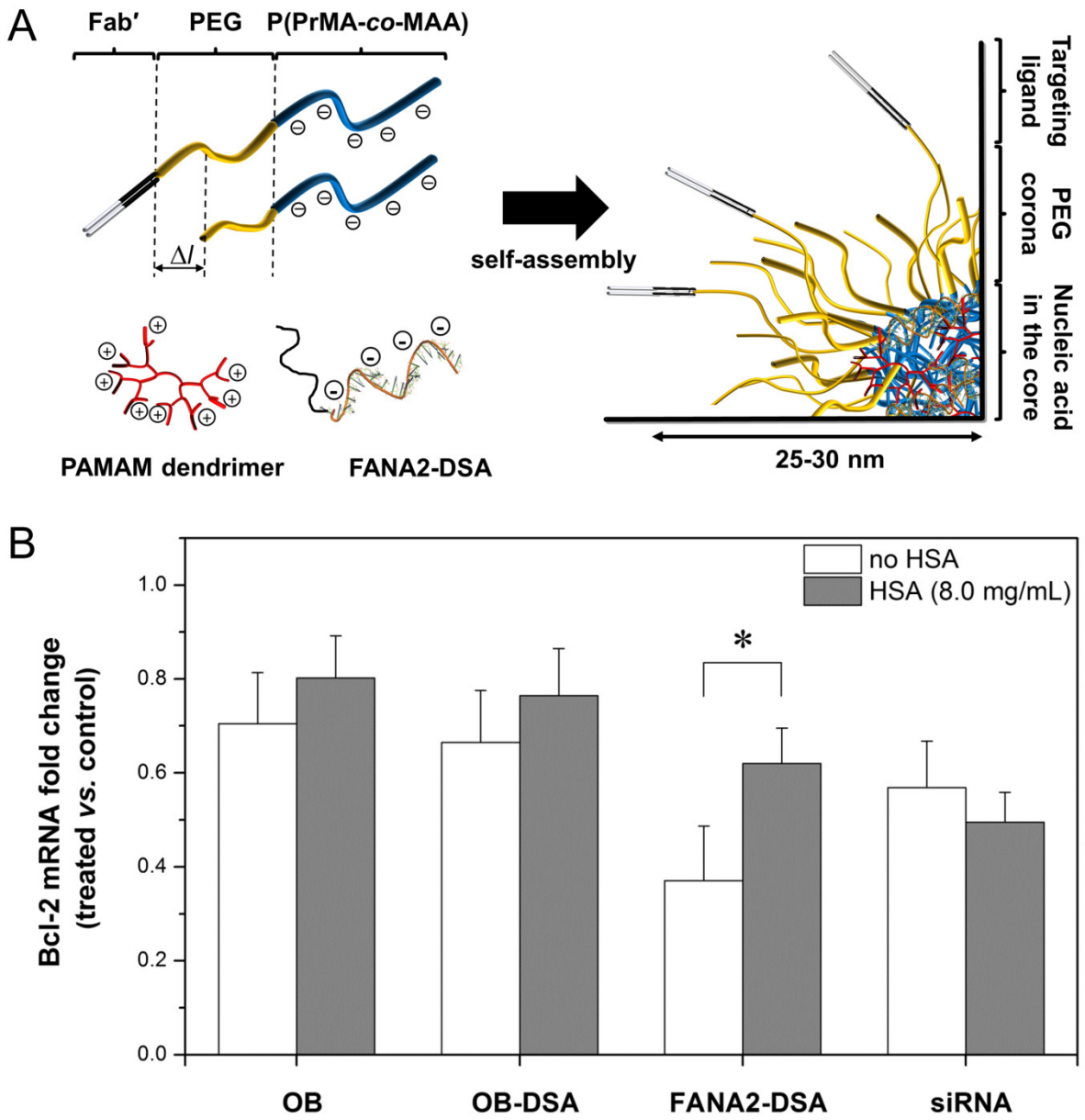


Figure 4.3. Bcl-2 mRNA silencing using AON and siRNA-loaded PICMs. (A) Schematic illustration of PICM formation. Complexation of FANA2-DSA is represented here. Figure is partially reproduced from Felber *et al.*, with permission from Elsevier [35]. (B) Analysis of Bcl-2 mRNA silencing in PC-3 cells incubated for 5 h at 37 °C with PICMs loaded with the nucleic acids. Final AON concentration in cell culture was 400 or 50 nM for AON and siRNA, respectively. Controls were treated with medium alone. Experiments were performed in HSA-free (empty bars) or HSA (8 mg/mL) supplemented (filled bars) transfection medium. Bcl-2 mRNA expression was evaluated by real time PCR 48 h after transfection. Results are expressed as mean ± S.D. (n = 3–6). (*) $p \leq 0.05$ *vs*. indicated bar.

4.3.5. Interaction of HSA with the PICMs

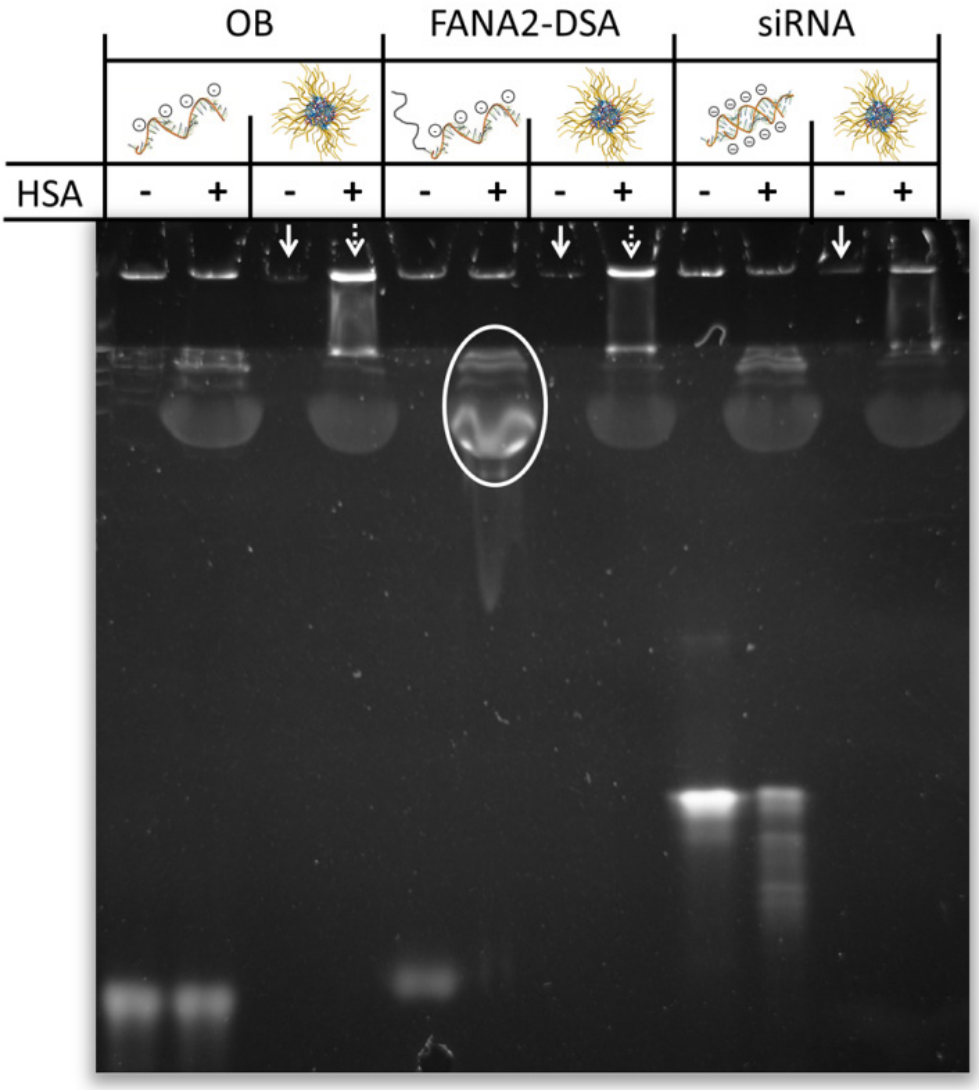


Figure 4.4. Nucleic acid gel retardation assay of carrier-free and PICM-loaded OB, FANA2-DSA, and siRNA formulations. Delivery method is illustrated above the wells. Samples were incubated without (-) or with (+) 8 mg/mL HSA. Each well contained 150 ng of OB and FANA2-DSA, or 100 ng of siRNA. Solid and dashed arrows indicate intact and degraded PICM structure, respectively. Encircled region points out FANA2-DSA conjugates complexed with HSA.

To better understand the role of HSA in the reduction of transfection activity, the stability of the PICMs loaded with OB, FANA2-DSA or the siRNA was evaluated by gel migration assays in absence and presence of 8 mg/mL HSA (Fig. 4.4). The HSA had no influence on the migration of the free OB lacking the DSA, while the FANA2-DSA seemed to

interact strongly with the protein (Fig. 4.4, encircled band). The chemically unmodified siRNA sequence was degraded slightly upon albumin addition as indicated by the smear band observed along its migration lane. In the absence of HSA (-), the incorporation of all 3 nucleic acid sequences into PICMs led to the loss of the fluorescence signal, reflecting the inaccessibility of entrapped nucleic acid/siRNA to the staining dye (Fig. 4.4, solid arrows). The addition of HSA (+) led to the destabilization of the PICMs prepared with single stranded nucleic acid (*i.e.*, OB, FANA2-DSA) as shown by the bright fluorescent band at the bottom of the loading well (exposition of the unshielded AON/PAMAM core to the nucleic acid staining dye) (Fig. 4.4, dashed arrows). Interestingly, PICMs containing siRNA appeared more resistant to the destabilizing effect of HSA (Fig. 4.4, last well).

4.3.6. Fatty acid saturation assay

In a last set of experiments, it was determined if blocking the sites for the binding of amphiphilic AONs to HSA could allow the recovery of the gene silencing of the non-formulated FANA2-DSA. The interactions between HSA and fatty acids have been extensively studied [43, 44]. It has been reported that up to 10 decanoic acid molecules could bind to one HSA molecule [44]. The relatively good aqueous solubility of this short (*i.e.*, C_{10}) fatty acid made it an interesting candidate to compete with DSA for binding HSA (Fig. 4.5A). In order to visualize the gradual sequestration of the DSA-modified AON with increasing HSA concentrations, mixtures were prepared at different conjugate:HSA molar ratios and loaded onto a polyacrylamide gel. Retardation in the migration of the amphiphilic AON derivative as a result of HSA binding was then evaluated using a nucleic acid staining dye (Fig. 4.5B). In the absence of fatty acid (-), FANA2-DSA migration was retarded upon increasing HSA molar excess (arrows), with loss of the free-nucleic acid band signal at a conjugate:HSA molar ratio of 1:10 (Fig. 4.5B). Pre-incubating HSA with a 35 molar excess of decanoic acid prevented substantially the FANA2-DSA sequestration by HSA (Fig. 4.5B). Figure 4.5C shows the impact of decanoic acid addition on the silencing efficacy of FANA2-DSA. While in the absence of the fatty acid, the Bcl-2 downregulation decreased progressively with increasing HSA concentrations, the incubation of HSA with a 35 molar excess of decanoic acid completely restored the transfection activity of the amphiphilic AON.

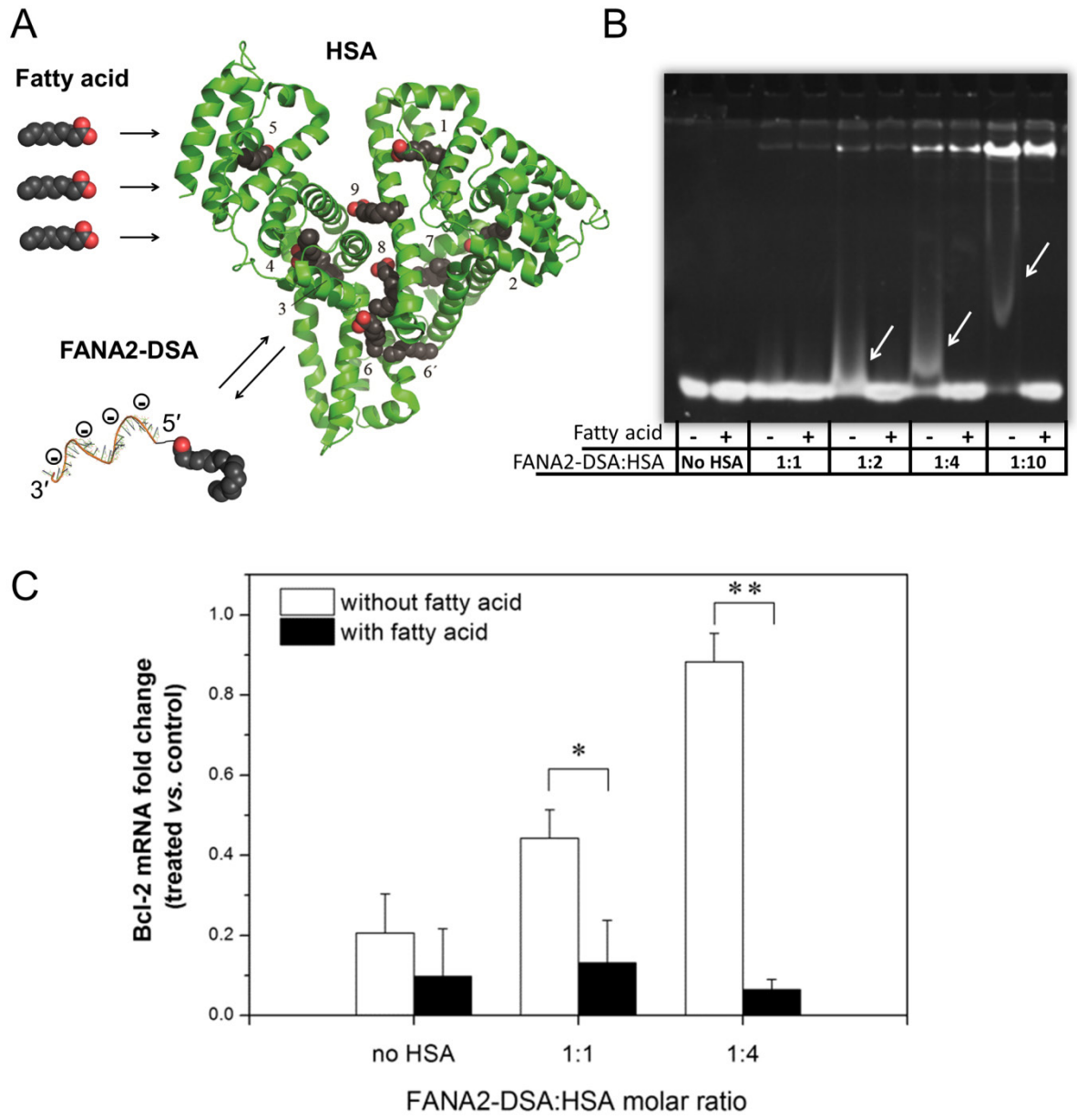


Figure 4.5. Fatty acid displacement assay. (A) Schematic representation of decanoic acid, FANA2-DSA, and HSA (not on scale). HSA structure containing 10 decanoic acid moieties is represented (PDB ID code 1E7E). Figure was prepared using PYMOL. (B) Nucleic acid gel retardation assay at different FANA2-DSA:HSA molar ratios. The protein was pre-incubated with (+) or without (-) 35 molar excess of fatty acid prior to FANA2-DSA addition. Each well contained 150 ng of FANA2-DSA. Solid arrows indicate FANA2-DSA migration retardation. (C) Analysis of Bcl-2 mRNA silencing in PC-3 cells incubated overnight with different FANA2-DSA:HSA molar ratios. For competition assay (filled bars), HSA was pre-incubated for 30 min at 37 °C with a 35 molar excess of fatty acid prior to amphiphilic AON addition. Final AON concentration in cell culture was 800 nM for all experiments. Bcl-2 mRNA fold change is expressed relative to an untreated control (medium alone). Results are expressed as mean ± S.D. (n = 3–5). (*) $p \leq 0.01$ or (**) $p \leq 0.001$ *vs*. indicated bar.

4.4. Discussion

Conjugation of lipophilic molecules, such as CHL or fatty acids, to the nucleic acid backbone has been hailed as a promising approach to overcoming some of the hurdles associated to the carrier-free delivery of genetic material *in vivo* [29, 33, 45]. These modifications significantly improved the biological half-life and the cellular uptake of nucleic acids [29, 33]. Petrova *et al*. recently reported that the uptake depended on the nature of lipophilic molecule and, more interestingly, on the length of the linker [32]. For a siRNA-CHL conjugate, a linker bearing 6 to 10 carbon atoms displayed the highest uptake and gene silencing activity following carrier-free delivery [32]. In the present study, a library of modified oligonucleotides was prepared using standard phosphoramidite solid-phase synthesis conditions (Table 4.2) [30, 46]. In addition to the PS-DNA sequence (OB), three 2′F-ANA AONs were selected to enhance nuclease resistance with minimal modification (FANA1, modified at the termini to resist exonuclease degradation), or with significant modification using previously described potent construct designs (FANA2 (altimer design), and FANA3 (gapmer design)) (Table 4.1). These chemically-modified AON constructs have been studied previously in the context of gene silencing potency comparisons [10]. The gapmer design for chemically modified AONs has the benefits of imparting exonuclease resistance, while maintaining a DNA core that is able to elicit the activity of the RNase H enzyme [13]. The altimer construct, however, is not always compatible with RNase H when some of the common AON modifications (2′-O-methyl, locked nucleic acid) are used, but is known to function well with the 2′F-ANA modification since these AONs retains the ability to recruit RNase H [10, 12]. The AONs were conjugated to different lipophilic moieties *via* an aliphatic (6 carbons) linker located at the 5′-ends of the oligonucleotide backbones (Scheme 4.1, Appendices 4.7.1 and 4.7.2). The 5′-hydroxyl-position of the oligodeoxyribonucleotides was used for the introduction of the aliphatic compounds because it is more straightforward to modify on solid-support. This synthetic pathway returned high coupling conversions (70–80%) (Table 4.2, Appendices 4.7.1 and 4.7.2).

This small library allowed us to evaluate the influence of three lipophilic moieties on mRNA inhibition following carrier-free incubation of different AONs. The activity of the amphiphilic AON was dependent upon the pattern of PS-2′F-ANA chemical modifications and the type of lipophilic residues used. Among the screened amphiphilic conjugates, the highest mRNA knockdowns were observed with AON sequences conjugated to the DSA moiety, which is a fully saturated C_{22} fatty acid (Fig. 4.1). In particular, AONs containing PS-

2′F-ANA in an altimer chemical modifications design (FANA2, Table 4.1) produced the highest antisense activity in absence of serum (Fig. 4.1). In general, DSA-conjugates showed higher activity than either DHA or CHL modified ones. This suggests that DSA has stronger interaction with the cellular membrane, resulting in enhanced uptake. The partition coefficients (log*P*) of the lipophilic moieties DHA, CHL, and DSA have been reported to be 5.20 [47], 7.17 [48], and 10.24 [47], respectively. Therefore, DSA-conjugates are expected to be slightly more hydrophobic than the others amphiphilic AONs, which could result in superior affinity with the cell membrane. As observed previously [32], there seems to be an optimal degree of lipophilicity for an efficient silencing effect. The highly lipophilic AON bearing two DHA chains did not show any effect (Fig. 4.1). Although additional mechanistic studies are needed, nucleic acids which are too lipophilic may remain trapped in the endosomes [32].

The low activity of the mono DHA conjugate was somewhat disappointing (Fig. 4.1). In the early 2000's, DHA had been hailed as a promising tumor targeting candidate [34]. Preclinical animal studies have suggested that DHA-paclitaxel may have an improved therapeutic index when compared to paclitaxel alone [34]. The encouraging results obtained in the animal models led to the development of various other DHA-flanked anti-cancer drug conjugates, such as DHA-doxorubicin [49] and DHA-docetaxel [50]. In clinical phase II, the activity of DHA-paclitaxel (Taxoprexin®) in patients with gastric or esophageal adenocarcinoma [51] and non-small cell lung cancer (NSCLC) [52] was, however, found to be insufficient to pursue the clinical development of this conjugate. Recently, the orphan G protein-coupled receptor 120 (GPR120) was shown to be a functional DHA receptor/sensor and to mediate the potent insulin sensitizing and antidiabetic effects of DHA *in vivo* by repressing macrophage-induced tissue inflammation [53]. These interesting new findings suggest that perhaps cells expressing GPR120 could still represent interesting targets for DHA-modified AONs.

The efficacy of the free amphiphilic AON was drastically reduced in the presence of 20% serum (Fig. 4.1). These results contrast with the findings of other groups who showed that the association of nucleic acids with serum proteins was participating to the silencing activity [30, 33, 45, 54]. Under *in vivo* conditions, binding to serum proteins enhances the circulation time of nucleic acids. This increases the exposure time of the oligonucleotide to the target cells, eventually leading to an increase in activity. In some cases, the association

with serum proteins may improve the uptake of the nucleic acids by specific entry pathways. For instance, it was suggested that the internalization of AON-CHL by mouse spleen cells could be mediated in part through its association with low-density lipoprotein (LDL) and subsequent binding to the LDL receptor [30]. More recently, Wolfrum *et al.* studied the uptake of siRNA-CHL by HepG2 cells. They hypothesized that the internalization of siRNA-CHL occurred *via* a mammalian homolog of the *Caenorhabditis elegans* transmembrane protein Sid-1 and that lipoproteins were required to mediate the delivery of the nucleic acid to these cells [33]. These different observations suggest that the entry route of amphiphilic oligonucleotides is complex and remains to be further characterized. However, it is well-established that the cellular bioavailability of such hydrophobized AONs depends on i) the lipophilic moiety, ii) the type of nucleic acid, and iii) the targeted tissue.

Our experiments clearly demonstrate that a strong *in vitro* association to serum proteins, such as HSA, may prevent the nucleic acid from entering into tumoral PC-3 cells. The silencing potential of the AON-DSA was dependent upon the molar ratio of AON to albumin in the transfection medium (Fig. 4.2B). In mammalians, fatty acids are presented to cells as fatty acid/albumin complexes. Their dissociation from albumin represents the first step of the cellular uptake process and involves the participation of membrane proteins with high affinity for fatty acid [55]. Protein-facilitated cellular uptake is believed to be the dominant means for fatty acids transport into the cells [56]. The prevalent view is that the membrane-associated protein "fatty acid translocase" (CD36) accepts fatty acids at the cell surface to increase their local concentration, thus increasing the number of fatty acid diffusion (often referred to as "flip-flop") events [56]. Albumin (and other serum proteins) may indeed prevent the local membrane accumulation of the amphiphilic oligonucleotides by acting as an extracellular sink. Interestingly, it was recently reported (although only succinctly) that the cellular uptake of amphiphilic siRNA by several cell lines decreased in the presence of serum [32].

In order to verify whether shielding the amphiphilic AON from the albumin and modifying the uptake pathway would improve the silencing activity, the nucleic acid was incorporated into pH-sensitive PICMs targeting the transferrin receptor (*i.e.*, CD71). This type of polymeric carrier was shown to complex and efficiently deliver unmodified AONs [22] and siRNAs [22, 35]. Previous reports demonstrated that the core-shell structure of the delivery vehicle did not only protect the anionic antisense drugs against serum proteins, but also

enhanced their intracellular bioavailability due to: i) facilitated cellular uptake *via* the transferrin receptor, ii) micelle destabilization in the endosomes *via* the decomplexation of the polymeric shell, and iii) improved endosomal escape of the transported cargo *via* the use of a PAMAM dendrimer. Compared to the carrier-free incubation conditions, incorporation of the FANA2-DSA into the PICMs allowed reducing substantially the incubation time from 16 to 5 h, to achieve good transfection efficacy. This probably corresponds to a faster intracellular availability when the nucleic acid is complexed to a targeted nanoparticle having endosomolytic properties. Similar observations were reported by Petrova *et al.* using siRNA-CHL and Lipofectamine 2000 [32]. They hypothesized that when formulated with Lipofectamine 2000, siRNA-CHL could escape from the endosomes more efficiently and with faster kinetics than when delivered in a carrier-free fashion [32]. In the presence of HSA, the PICMs loaded with FANA2-DSA also lost some activity due to partial destabilization of the micellar structure (Fig. 4.4). Such a destabilizing effect was, however, not observed with siRNA (and the activity was not altered), possibly because of the different loading level or the siRNA double-stranded structure, which increased the cooperative electrostatic complexation with the cationic PAMAM of the PICMs.

Finally, in the case of non-formulated AON, we showed that by blocking the fatty acid binding sites in HSA, it was possible to preserve the silencing activity of the FANA2-DSA (Fig. 4.5). This observation may open new research directions in the field of nucleic acid delivery. Displacement of small drug molecules from albumin by endogenous fatty acids has been extensively reported [57-59]. However, while albumin binding sites can be easily blocked *in vitro* by adding an excess of free fatty acids, this strategy could not be realistically applied *in vivo* after intravenous injections of low doses of AONs. Between 0.1 and 2 moles of fatty acids are typically bound to one mole of albumin under normal physiological conditions, but the molar ratio of fatty acids to HSA can rise up to 6:1 or greater in the peripheral vasculature during fasting or extreme exercise [60], or under pathological conditions such as diabetes, liver, and cardiovascular disease [61]. This implies that circulating HSA is not easily saturated by the administration of exogenous fatty acid. However, it could be envisaged in the future to encapsulate amphiphilic AONs such as FANA2-DSA, into polymeric/lipophilic implants or nanostructures capable of releasing the nucleic acid in the interstitium (where protein concentrations is lower) at the vicinity of the site of action. Such delivery systems could be prepared with neutral biocompatible lipids [62] or polymers [63] thus avoiding the use of potentially toxic cationic systems. They could also

contain high amounts of free fatty acids to locally saturate the surrounding proteins, thus allowing the amphiphilic nucleic acid to interact with the cell membrane.

4.5. Conclusion

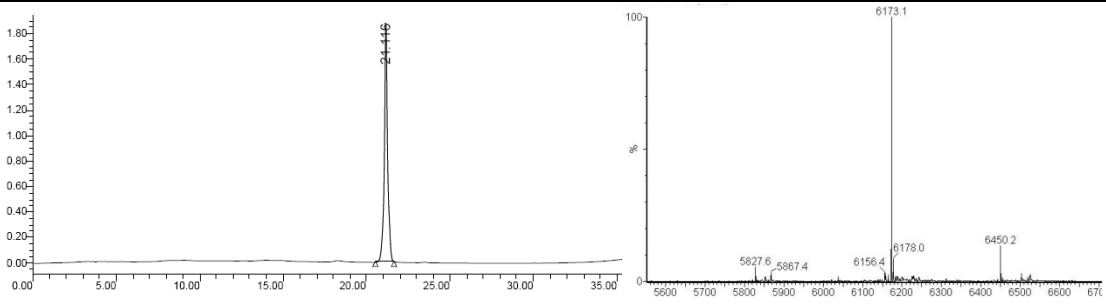
In this study, different amphiphilic AON derivatives were synthesized and tested for the downregulation of Bcl-2 mRNA. In protein free buffer, it was found that the most potent structure was the FANA2-DSA. However, introduction of serum or HSA in the transfection media decreased the antisense activity whether the AON was free or encapsulated into PICMs. In the case of PICMs, HSA destabilized to some extent the micellar structure, while for the free amphiphilic AONs, the loss of activity could be explained by a reduced intracellular bioavailability due to the association with extracellular HSA. Previous *in vivo* experiments have shown that the association of free nucleic acid to circulating blood proteins can be beneficial to improve the pharmacokinetic properties of the antisense drugs. However, when highly hydrophobic moieties such as DSA are linked to AONs, strong binding to albumin or any other plasma proteins could have a detrimental effect on the cellular bioavailability. This would happen in cases where the plasma proteins are not involved in the uptake of the AONs. While it has been shown that lipoproteins could promote the internalization of amphiphilic AONs, such studies remain isolated and deserve further validation. We believe that a strong association to plasma proteins would in most cases impair the AON activity and therefore strategies aiming at locally preventing the association of hydrophobized AONs to serum proteins could be tested in the future.

4.6. Acknowledgments

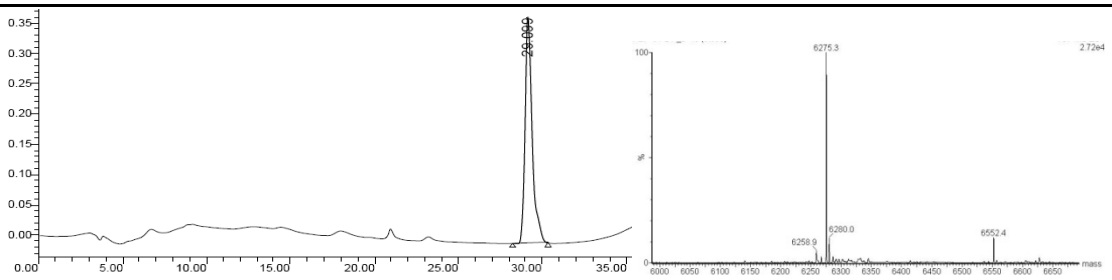
This work was financially supported by an ETH Research Grant to AEF and JCL (ID ETH-0209-3), NSERC Vanier CGS to GFD, and a CIHR Operating Grant to MJD. NBP is a Beatriu de Pinós fellow (Generalitat de Catalunya, Spain, ID 2007 BP-A 00028).

4.7. Appendices

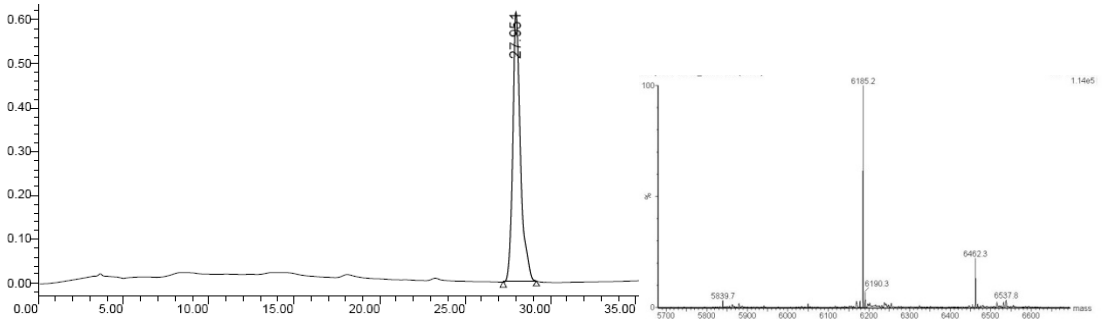
Appendix 4.7.1. (**A**) HPLC chromatograms and mass spectra of conjugates. HPLC conditions: C_{18}, gradient from 5% to 100% B in 30 min at 50 °C.



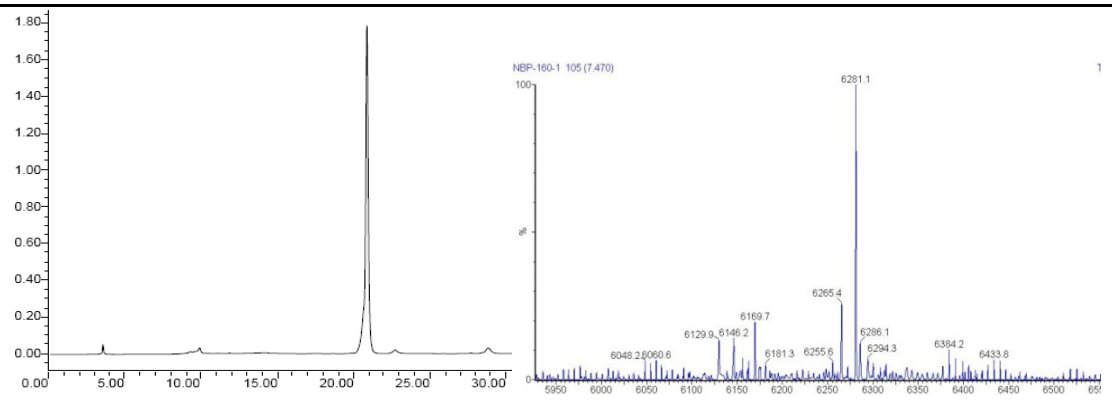
2 OB-DHA



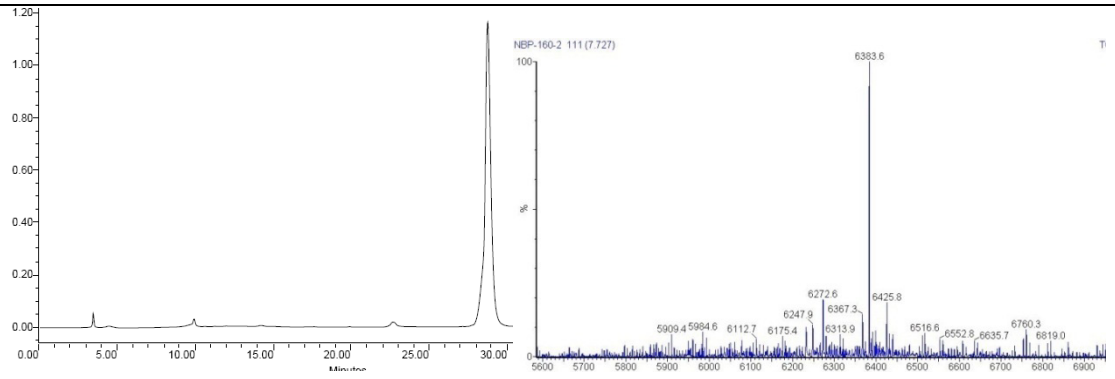
3 OB-CHL



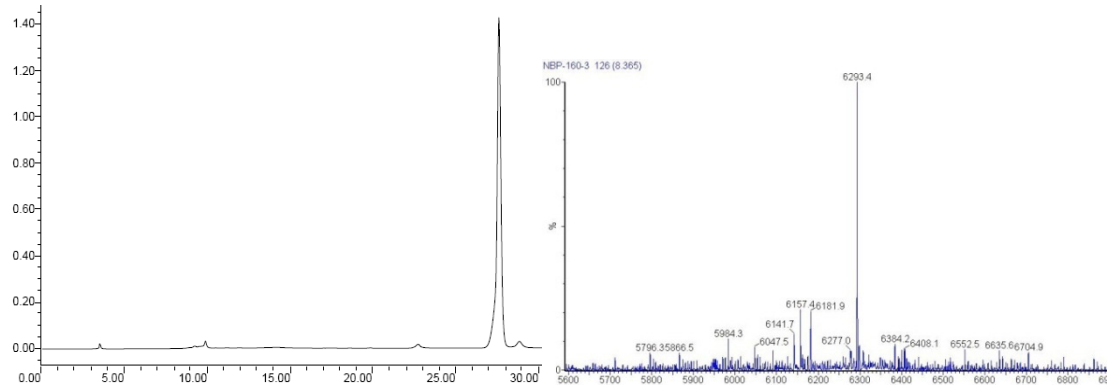
4 OB-DSA



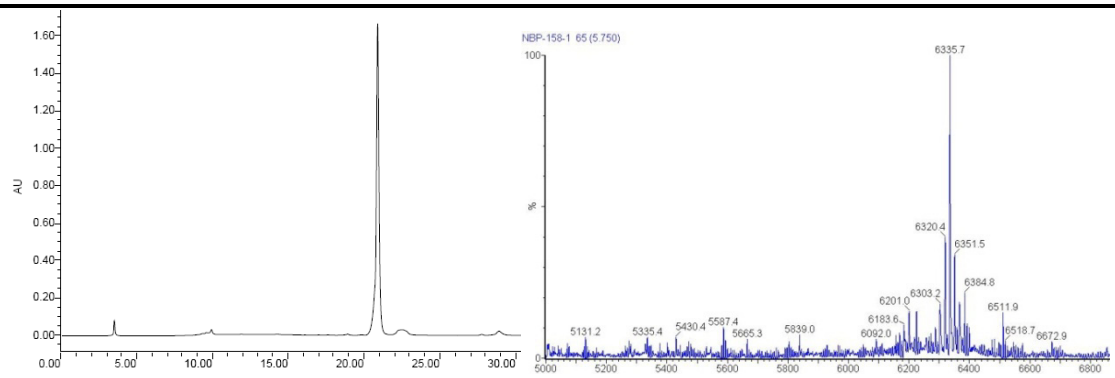
6 FANA1-DHA



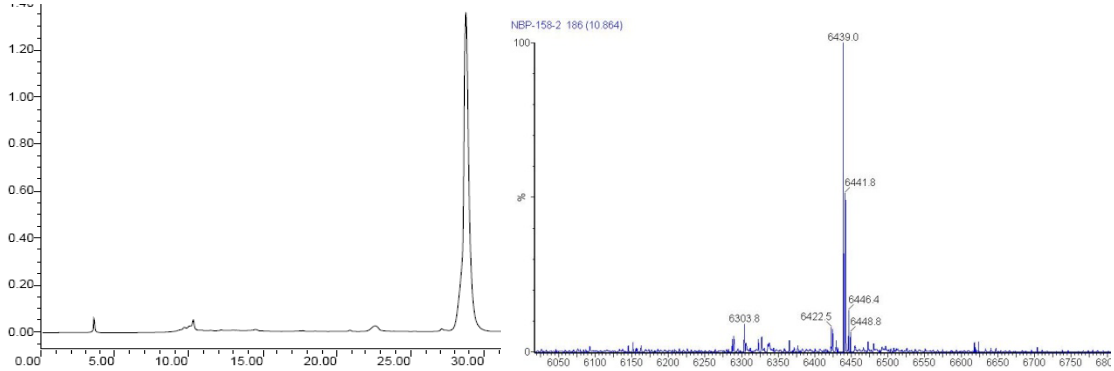
7 FANA1-CHL



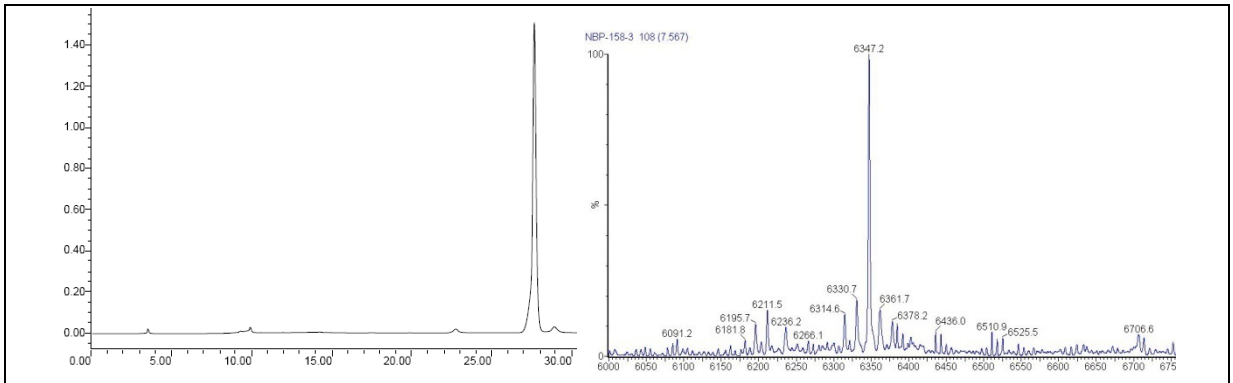
8 FANA1-DSA



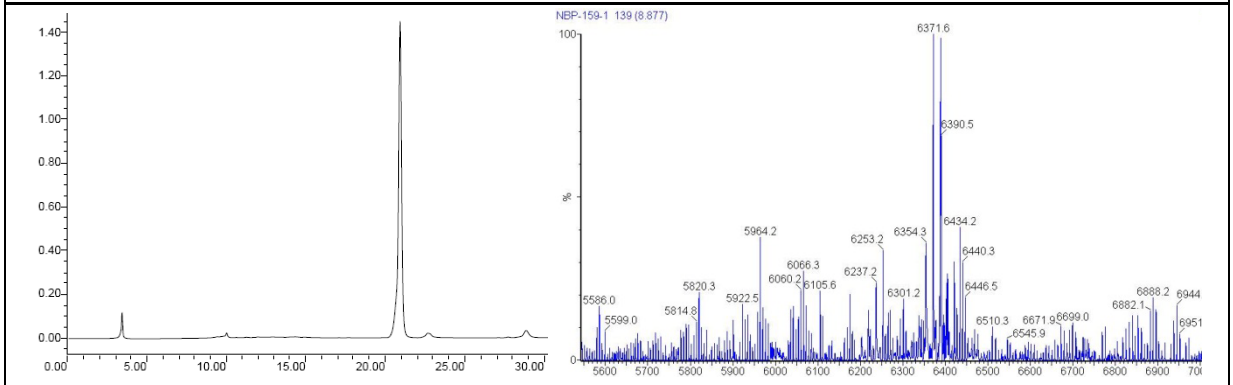
9 FANA2-DHA



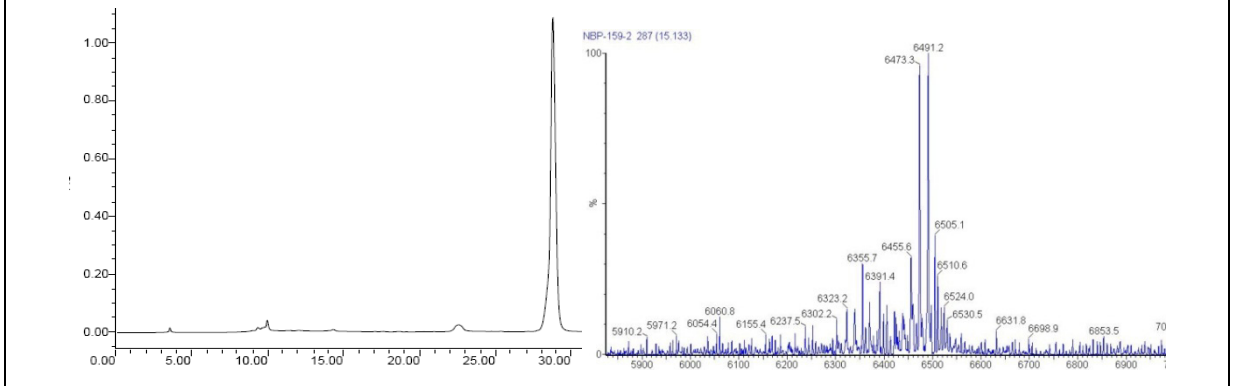
10 FANA2-CHL



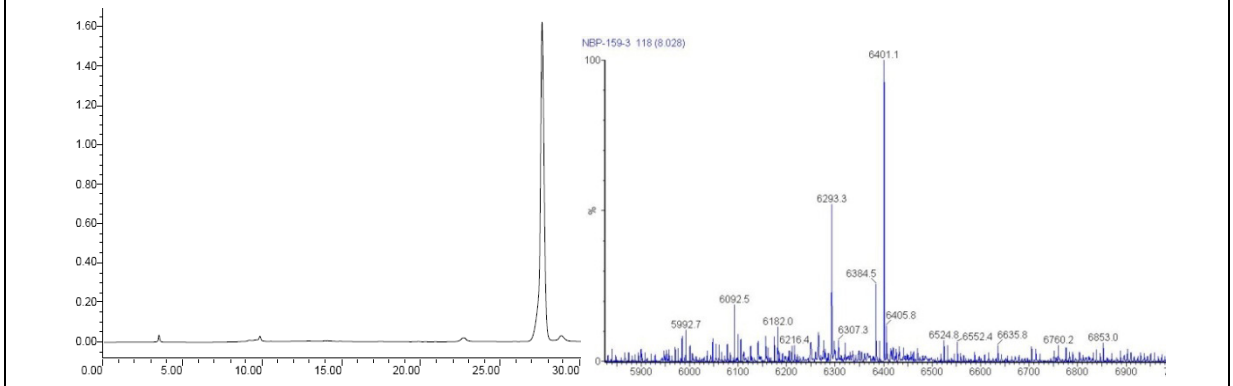
11 FANA2-DSA



12 FANA3-DHA



13 FANA3-CHL



14 FANA3-DSA

Appendix 4.7.1. (**B**) Characterization of amphiphilic AONs

General remarks: Solvent A: H_2O with 100 mM triethylammonium acetate and 5% ACN; solvent B: ACN.

2. OB-DHA:

21.1 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6173.1 $[M+H]^+$ (Calculated 6174.0).

3. OB-CHL:

29.1 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6275.3 $[M+H]^+$ (Calculated 6276.9).

4. OB-DSA:

28.0 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6185.2 $[M+H]^+$ (Calculated 6186.4).

5. OB-bisDHA:

Refer to Appendix 4.7.2.

6. FANA1-DHA:

21.5 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6281.1 $[M+H]^+$ (Calculated 6282.0).

7. FANA1-CHL:

29.6 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6383.6 $[M+H]^+$ (Calculated 6384.9).

8. FANA1-DSA:

28.6 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6293.4 $[M+H]^+$ (Calculated 6294.3).

9. FANA2-DHA:

21.8 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6335.7 $[M+H]^+$ (Calculated 6335.0).

10. FANA2-CHL:

28.8 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6439.0 $[M+H]^+$ (Calculated 6438.8).

11. FANA2-DSA:

28.8 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6347.2 $[M+H]^+$ (Calculated 6348.3).

12. FANA3-DHA:

21.9 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6389.5 $[M+H]^+$ (Calculated 6389.9), 6371.6 [M-18].

13. FANA3-CHL:

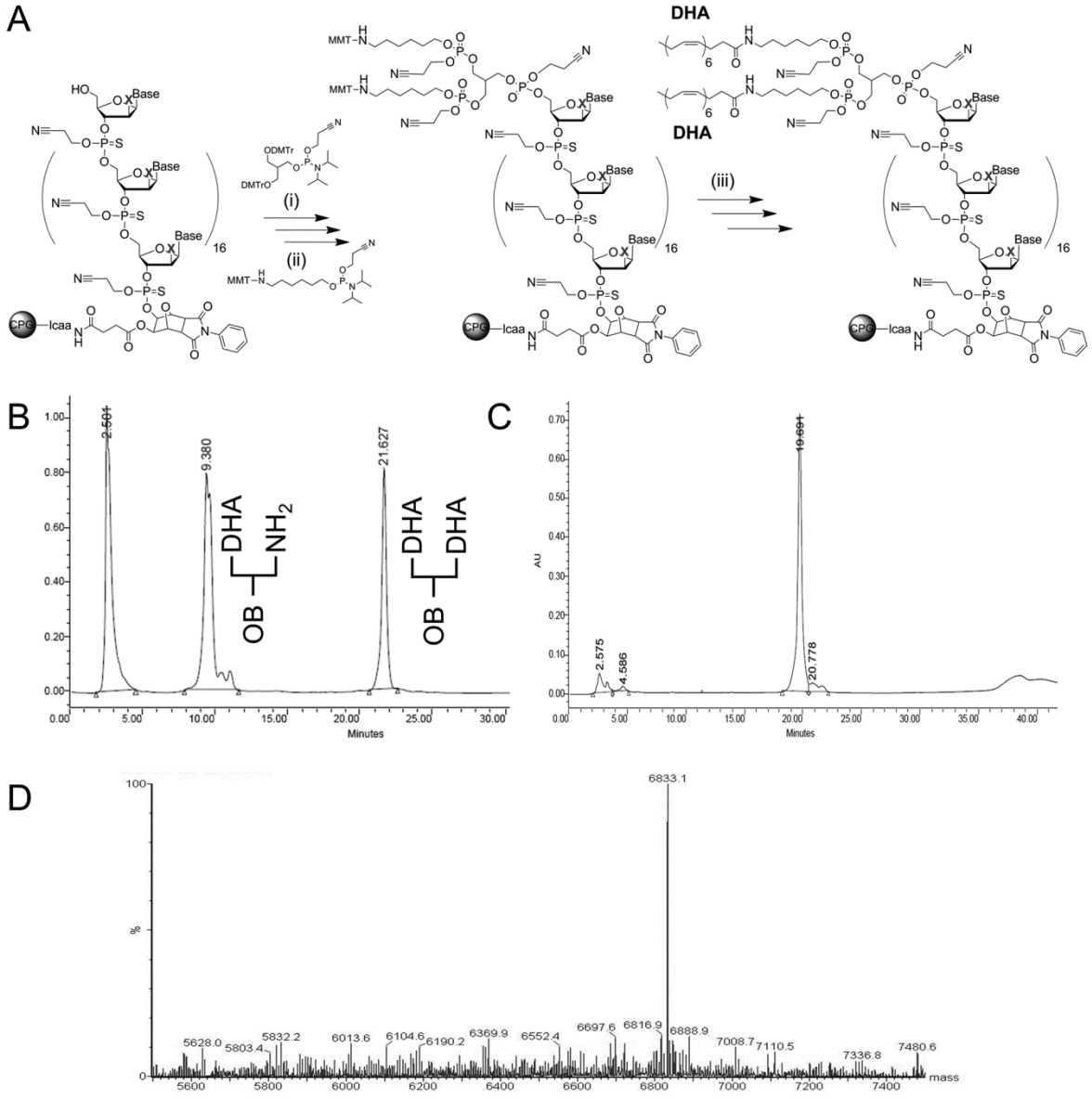
28.9 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6491.2 $[M+H]^+$ (Calculated 6492.8), 6473.3 [M-18].

14. FANA3-DSA:

28.9 min (C_{18}, gradient from 5% B to 100% B in 30 min at 50 °C), 6401.1 $[M+H]^+$ (Calculated 6402.3).

Appendix 4.7.2. bisDHA synthesis and characterization.

A) Synthetic scheme to obtain bisDHA conjugates by using a symmetrical branched phosphoramidite linker. (i) symmetrical branching phosphoramidite (Chemgenes), 5-ethylthiotetrazole; I_2 solution; 3% TCA in DCM; (ii) MMT-6-amino-hexanol phosphoramidite, 5-ethylthiotetrazole, I_2 solution; 3% TCA in DCM; (iii) DHA, PyBOP, DIPEA in DCM. The conjugates were then deprotected under standard conditions. X = H (DNA) or F (2′F-ANA). B) HPLC chromatogram following DHA coupling to the bis-amino PS-DNA sequence (HPLC conditions: gradient from 5 to 100 % of solvent B in 30 min at 50 ºC). C) HPLC chromatogram of OB-bisDHA (HPLC conditions: gradient from 50 to 90 % of solvent B in 30 min at 50 °C). D) MS spectrum of OB-bisDHA.



4.8. References

[1] Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A. 1978;75:280-4.

[2] Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc Natl Acad Sci U S A. 1978;75:285-8.

[3] Akhtar S, Basu S, Wickstrom E, Juliano RL. Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes). Nucleic Acids Res. 1991;19:5551-9.

[4] Lebedeva I, Stein CA. Antisense oligonucleotides: Promise and reality. Annu Rev Pharmacol Toxicol. 2001;41:403-19.

[5] Harada A, Togawa H, Kataoka K. Physicochemical properties and nuclease resistance of antisense-oligodeoxynucleotides entrapped in the core of polyion complex micelles composed of poly(ethylene glycol)-poly(L-lysine) block copolymers. Eur J Pharm Sci. 2001;13:35-42.

[6] Manoharan M. Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. Antisense Nucleic Acid Drug Dev. 2002;12:103-28.

[7] Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta. 1999;1489:69-84.

[8] Chan JHP, Lim S, Wong WSF. Antisense oligonucleotides: From design to therapeutic application. Clin Exp Pharmacol Physiol. 2006;33:533-40.

[9] Damha MJ, Wilds CJ, Noronha A, Brukner I, Borkow G, Arion D, et al. Hybrids of RNA and arabinonucleic acids (ANA and 2'F-ANA) are substrates of ribonuclease H. J Am Chem Soc. 1998;120:12976-7.

[10] Kalota A, Karabon L, Swider CR, Viazovkina E, Elzagheid M, Damha MJ, et al. 2'-Deoxy-2'-fluoro-β-d-arabinonucleic acid (2'F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. Nucleic Acids Res. 2006;34:451-61.

[11] Watts JK, Katolik A, Viladoms J, Damha MJ. Studies on the hydrolytic stability of 2'-fluoroarabinonucleic acid (2'F-ANA). Org Biomol Chem. 2009;7:1904-10.

[12] Lok C-N, Viazovkina E, Min K-L, Nagy E, Wilds CJ, Damha MJ, et al. Potent gene-specific inhibitory properties of mixed-backbone antisense oligonucleotides comprised of 2'-Deoxy-2'-fluoro-d-arabinose and 2'-deoxyribose nucleotides. Biochemistry. 2002;41:3457-67.

[13] Bennett CF, Swayze EE. RNA targeting therapeutics: Molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu Rev Pharmacol Toxicol. 2010;50:259-93.

[14] Dias N, Stein CA. Potential roles of antisense oligonucleotides in cancer therapy. The example of Bcl-2 antisense oligonucleotides. Eur J Pharm Biopharm. 2002;54:263-9.

[15] Stein CA. Phosphorothioate antisense oligodeoxynucleotides: Questions of specificity. Trends Biotechnol. 1996;14:147-9.

[16] Agrawal S, Temsamani J, Tang JY. Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. Proc Natl Acad Sci U S A. 1991;88:7595-9.

[17] Sands H, Gorey-Feret LJ, Cocuzza AJ, Hobbs FW, Chidester D, Trainor GL. Biodistribution and metabolism of internally 3H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. Mol Pharmacol. 1994;45:932-43.

[18] Couto LB, High KA. Viral vector-mediated RNA interference. Curr Opin Pharmacol. 2010;10:534-42.

[19] Zelphati O, Szoka FC. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. Pharm Res. 1996;13:1367-72.

[20] Lungwitz U, Breunig M, Blunk T, Göpferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm. 2005;60:247-66.

[21] Dufresne M-H, Elsabahy M, Leroux J-C. Characterization of polyion complex micelles designed to address the challenges of oligonucleotide delivery. Pharm Res. 2008;25:2083-93.

[22] Elsabahy M, Wazen N, Bayó-Puxan N, Deleavey G, Servant M, Damha MJ, et al. Delivery of nucleic acids through the controlled disassembly of multifunctional nanocomplexes. Adv Funct Mater. 2009;19:3862-7.

[23] Kakizawa Y, Kataoka K. Block copolymer micelles for delivery of gene and related compounds. Adv Drug Deliv Rev. 2002;54:203-22.

[24] Chavany C, Doan TL, Couvreur P, Puisieux F, Hélèna C. Polyalkylcyanoacrylate nanoparticles as polymeric carriers for antisense oligonucleotides. Pharm Res. 1992;9:441-9.
[25] Lv H, Zhang S, Wang B, Cui S, Yan J. Toxicity of cationic lipids and cationic polymers in gene delivery. J Control Release. 2006;114:100-9.
[26] Xu L, Anchordoquy T. Drug delivery trends in clinical trials and translational medicine: Challenges and opportunities in the delivery of nucleic acid-based therapeutics. J Pharm Sci. 2011;100:38-52.
[27] Ming X, Alam MR, Fisher M, Yan Y, Chen X, Juliano RL. Intracellular delivery of an antisense oligonucleotide via endocytosis of a G protein-coupled receptor. Nucleic Acids Res. 2010;38:6567-76.
[28] Astriab-Fisher A, Sergueev DS, Fisher M, Ramsay Shaw B, Juliano RL. Antisense inhibition of P-glycoprotein expression using peptide–oligonucleotide conjugates. Biochem Pharmacol. 2000;60:83-90.
[29] Crooke ST, Graham MJ, Zuckerman JE, Brooks D, Conklin BS, Cummins LL, et al. Pharmacokinetic properties of several novel oligonucleotide analogs in mice. J Pharmacol Exp Ther. 1996;277:923-37.
[30] Krieg AM, Tonkinson J, Matson S, Zhao Q, Saxon M, Zhang LM, et al. Modification of antisense phosphodiester oligodeoxynucleotides by a 5' cholesteryl moiety increases cellular association and improves efficacy. Proc Natl Acad Sci U S A. 1993;90:1048-52.
[31] Raouane M, Desmaële D, Urbinati G, Massaad-Massade L, Couvreur P. Lipid conjugated oligonucleotides: A useful strategy for delivery. Bioconjugate Chem. 2012;23:1091-104.
[32] Petrova NS, Chernikov IV, Meschaninova MI, Dovydenko IS, Venyaminova AG, Zenkova MA, et al. Carrier-free cellular uptake and the gene-silencing activity of the lipophilic siRNAs is strongly affected by the length of the linker between siRNA and lipophilic group. Nucleic Acids Res. 2012;40:2330-44.
[33] Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, et al. Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. Nat Biotechnol. 2007;25:1149-57.
[34] Bradley MO, Swindell CS, Anthony FH, Witman PA, Devanesan P, Webb NL, et al. Tumor targeting by conjugation of DHA to paclitaxel. J Control Release. 2001;74:233-6.
[35] Felber AE, Castagner B, Elsabahy M, Deleavey GF, Damha MJ, Leroux J-C. siRNA nanocarriers based on methacrylic acid copolymers. J Control Release. 2011;152:159-67.
[36] Bayó-Puxan N, Dufresne M-H, Felber AE, Castagner B, Leroux J-C. Preparation of polyion complex micelles from poly(ethylene glycol)-*block*-polyions. J Control Release. 2011;156:118-27.
[37] Wilds CJ, Damha MJ. 2'-Deoxy-2'-fluoro-β-d-arabinonucleosides and oligonucleotides (2'F-ANA): Synthesis and physicochemical studies. Nucleic Acids Res. 2000;28:3625-35.
[38] Bellon L. Oligoribonucleotides with 2'-O-(tert-butyldimethylsilyl) groups. In: Beaucage SL, Bergstrom DE, Glick GD, Jones RA, editors. Curr Protoc Nucleic Acid Chem. New York: John Wiley & Sons, Inc.; 2000. p. 3.6.1-3.6.13.
[39] Winkler J, Stessl M, Amartey J, Noe CR. Off-target effects related to the phosphorothioate modification of nucleic acids. ChemMedChem. 2010;5:1344-52.
[40] Anderson EM, Miller P, Ilsley D, Marshall W, Khvorova A, Stein CA, et al. Gene profiling study of G3139- and Bcl-2-targeting siRNAs identifies a unique G3139 molecular signature. Cancer Gene Ther. 2006;13:406-14.
[41] Damha MJ, Ogilvie KK. Oligoribonucleotide synthesis: The silyl-phosphoramidite method. In: Agrawal S, editor. Protocols for Oligonucleotides and Analogs. Totowa: The Humana Press Inc.; 1993. p. 81-114.
[42] Min K-L, Viazovkina E, Galarneau A, Parniak MA, Damha MJ. Oligonucleotides comprised of alternating 2'-Deoxy-2'-fluoro-β-d-arabinonucleosides and d-2'-deoxyribonucleosides (2'F-ANA/DNA 'Altimers') induce efficient RNA cleavage mediated by RNase H. Bioorg Med Chem Lett. 2002;12:2651-4.
[43] Simard JR, Zunszain PA, Ha C-E, Yang JS, Bhagavan NV, Petitpas I, et al. Locating high-affinity fatty acid-binding sites on albumin by x-ray crystallography and NMR spectroscopy. Proc Natl Acad Sci U S A. 2005;102:17958-63.

[44] Bhattacharya AA, Grüne T, Curry S. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. J Mol Biol. 2000;303:721-32.
[45] Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature. 2004;432:173-8.
[46] MacKellar C, Graham D, Will DW, Burgess S, Brown T. Synthesis and physical properties of anti-HIV antisense oligonucleotides bearing terminal lipophilic groups. Nucleic Acids Res. 1992;20:3411-7.
[47] Bavafa S, Mahboub M, Ardakan RB, Mashhadi FF. Quantitative structure-property relationship (QSPR) study of n-octanol-water partition coefficients ($logP_{o/w}$) of fatty acids using multiple linear regression (MLR). OUAC. 2011;22:79-86.
[48] Chiosa V, Mandravel C, Teodoreanu C-L, Stanculescu I. Comparison of QSPR properties for cholesterol and cholestanol oligomeric association forms. Rev Roum Chim. 2006;51:135-40.
[49] Wang Y, Li L, Jiang W, Yang Z, Zhang Z. Synthesis and preliminary antitumor activity evaluation of a DHA and doxorubicin conjugate. Bioorg Med Chem Lett. 2006;16:2974-7.
[50] Shaikh IAA, Brown I, Schofield AC, Wahle KWJ, Heys SD. Docosahexaenoic acid enhances the efficacy of docetaxel in prostate cancer cells by modulation of apoptosis: The role of genes associated with the NF-κB pathway. Prostate. 2008;68:1635-46.
[51] Jones R, Hawkins R, Eatock M, Ferry D, Eskens F, Wilke H, et al. A phase II open-label study of DHA-paclitaxel (Taxoprexin) by 2-h intravenous infusion in previously untreated patients with locally advanced or metastatic gastric or oesophageal adenocarcinoma. Cancer Chemother Pharmacol. 2007;61:435-41.
[52] Payne M, Ellis P, Dunlop D, Ranson M, Danson S, Schacter L, et al. DHA-paclitaxel (Taxoprexin) as first-line treatment in patients with stage IIIB or IV non-small cell lung cancer: Report of a phase II open-label multicenter trial. J Thorac Oncol. 2006;1:984-90.
[53] Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 2010;142:687-98.
[54] Lau S, Graham B, Cao N, Boyd BJ, Pouton CW, White PJ. Enhanced extravasation, stability and *in vivo* cardiac gene silencing via *in situ* siRNA-albumin conjugation. Mol Pharm. 2012;9:71-80.
[55] Stremmel W, Pohl J, Ring A, Herrmann T. A new concept of cellular uptake and intracellular trafficking of long-chain fatty acids. Lipids. 2001;36:981-9.
[56] Schwenk RW, Holloway GP, Luiken JJFP, Bonen A, Glatz JFC. Fatty acid transport across the cell membrane: Regulation by fatty acid transporters. Prostaglandins Leukot Essent Fatty Acids. 2010;82:149-54.
[57] Tesseromatis C, Alevizou A. The role of the protein-binding on the mode of drug action as well the interactions with other drugs. Eur J Drug Metab Pharmacokinet. 2008;33:225-30.
[58] Sansom LN, Evans AM. What is the true clinical significance of plasma protein binding displacement interactions? Drug Saf. 1995;12:227-33.
[59] Vorum H, Honoré B. Influence of fatty acids on the binding of warfarin and phenprocoumon to human serum albumin with relation to anticoagulant therapy. J Pharm Pharmacol. 1996;48:870-5.
[60] Brodersen R, Andersen S, Vorum H, Nielsen SU, Pedersen AO. Multiple fatty acid binding to albumin in human blood plasma. Eur J Biochem. 1990;189:343-9.
[61] Cistola DP, Small DM. Fatty acid distribution in systems modeling the normal and diabetic human circulation. A 13C nuclear magnetic resonance study. J Clin Invest. 1991;87:1431-41.
[62] Ng KK, Lovell JF, Zheng G. Lipoprotein-inspired nanoparticles for cancer theranostics. Acc Chem Res. 2011;44:1105-13.
[63] Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov. 2005;4:581-93.

Chapter 5

General Conclusion and Outlook

The limited clinical success of synthetic nucleic acid therapeutics has challenged the scientific community to develop a large number of strategies to impart these atypical drugs with more desirable properties. While most systems offer interesting features, they often remain poorly characterized and are associated, in most cases, with suboptimal pre-clinical and/or clinical outcomes. In this context, the principal objective of this Ph.D. thesis was to develop and thoroughly characterize two non-viral strategies for nucleic acid *delivery*. The major achievements of this work are summarized hereunder:

- An optimized approach to prepare PICMs based on methacrylic acid copolymers was developed. Furthermore, different features of PICMs were characterized, including: size, zeta potential, stability, pH-sensitivity, cytotoxicity, uptake, and *in vitro* transfection efficiency (**chapter 3**)

- The *in vitro* silencing activity of a selection of hydrophobized AON conjugates was studied. In addition, a strategy to restore their efficiency upon interaction with HSA was proposed and validated *in vitro* (**chapter 4**)

It was observed that PEG-*b*-P(PrMA-*co*-MAA) interacted with PAMAM dendrimers to form pH-sensitive core-shell type PICMs (**chapter 3**). Moreover, a selection of nucleic acids such as siRNA, AON, amphiphilic AON conjugates, and plasmid DNA (pDNA) could be incorporated in the nanocomplexes (see Fig. 5.1). The resulting constructs had diameters in the 50–100 nm range and low polydispersity indices. Interestingly, and despite the relatively low contribution of the nucleic acid molecules in the preparation of the PICMs (*i.e.*, typically 1–5 mol% of total negative charges), the diameter of the nanocarrier was found to depend on the type of nucleic acid payload. For instance, PICMs prepared with siRNA were smaller than

those loaded with pDNA (Fig. 5.1). A tangible explanation could come from the size of the nucleic acid used (*ca*. 13 kDa for siRNA *vs*. > 3000 kDa for pDNA). It is noteworthy that the proposed delivery platform displayed an optimal size for an enhanced circulation time upon systemic administration. Indeed, a delivery system should be larger than *ca.* 10 nm to escape glomerular filtration in the kidney, but preferably smaller than *ca*. 100 nm (particularly for lipid-based nanoparticles) to limit unspecific uptake by the mononuclear phagocytic system [1].

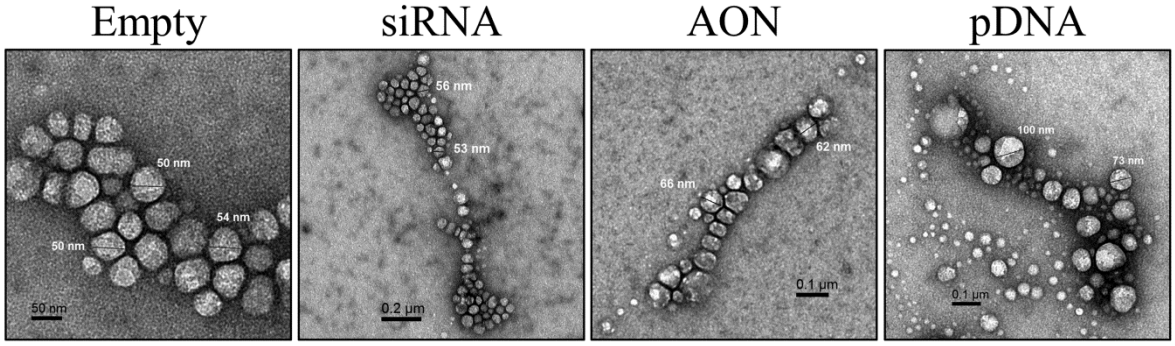


Figure 5.1. PICMs can support a large variety of nucleic acid molecules. TEM images of the nanocarriers prepared without cargo (empty) or with siRNA, AON, and pDNA payload. Samples were negatively stained, as previously described (**chapter 3**, section 3.2.6). The average sizes obtained by DLS were 48, 57, 68, and 71 nm for empty, siRNA, AON, and pDNA containing nanoparticles, respectively (unpublished data).

PICMs prepared with siRNA were further characterized and were shown to have good stability in the presence of serum and to efficiently protect the nucleic acid cargo against enzymatic degradation. It was observed that more than 50% intact siRNA remained in PICMs after a 4-h incubation in 100% serum, while free siRNA was completely degraded within 50 min. However, when translated to an *in vivo* model, dilution effects (following intravenous injection) and interactions with charged plasma molecules (*e.g.*, α- and β-globulins and heparin) could impair the stability of the PICMs. Should this problem occur, it could be envisaged in the future to strengthen the core of PICMs by, for example, crosslinking them. The enhanced stability in serum of a delivery vehicle is pivotal and generally allows it to exhibit improved circulation time in the blood, which is crucial to exploit the EPR effect. Numerous nanoparticles, including PICMs, have been evaluated for possible systemic cancer treatment due to their potential ability to exploit the EPR effect and passively accumulate at the vicinity of certain types of solid tumors [2]. However, the usefulness of the EPR effect in humans is often questioned, for example because its heterogeneity reduces its broad applicability [3]. Therefore, in order to produce PICMs with enhanced and specific uptake,

the nanocarriers were stably functionalized with an anti-CD71 Fab′. The resulting targeted PICMs demonstrated significantly higher cellular uptake in PC-3 cells than isotype-matched control Fab′-coated PICMs and native unmodified PICMs. Furthermore, the flow cytometry data obtained suggested that the targeted micelles were specifically internalized after 3 h incubation. Overall, our findings strongly indicate that the targeted PICMs are mainly taken up *via* receptor-mediated endocytosis. Interestingly, the strategy developed to decorate the PICMs with an antibody fragment is, in principle, applicable to a large variety of other targeting ligands. However, it was observed that special considerations had to be taken when selecting the monoclonal IgG. In fact, the number of cysteine residues presents in the hinge region of an IgG (which differ among species and subclasses) affected the efficiency of the coupling procedure, thus requiring significant optimization (unpublished observations).

It is generally agreed that an optimal nucleic acid delivery vehicle must display endosomal escape properties. In a preliminary study, it was observed that substituting the PAMAM cationic condensing agent of the pH-sensitive PICMs by PLL had little influence on the particle size, but drastically reduced their *in vitro* transfection efficiency (unpublished observations). This can be partially explained by the lower buffering capacity of PLL compared to PAMAM at endosomal pH [4]. Another crucial issue to consider when using delivery vehicles is toxicity. Non-viral systems are generally viewed as safer platform compared to virus-based ones. However, the presence of large (non-biodegradable) positively charged polymers has often been associated with severe cellular toxicity [5]. Our results suggested that the cytotoxicity of the PAMAM-based formulations on PC-3 cells was relatively low. In particular, PAMAM dendrimers of generation four exhibited a combination of both low cytotoxicity and promising transfection efficiency (when used for PICM preparation). The right balance between transfection efficiency and low cytotoxicity is a key requirement for a clinically viable delivery system. Nevertheless, should the PICMs display non-negligible toxicity *in vivo*, biodegradable PAMAM dendrimers with high buffering capacity (for endosomal escape) and high charge density (for stability) could be prepared.

A major challenge to nucleic acid delivery is to achieve high transfection efficiency. Our results showed that targeted PICMs (loaded with as low as 10 nM of chemically-modified siRNA) significantly downregulated the expression of the Bcl-2 mRNA and protein levels in PC-3 cells. These results compare advantageously with similar non-viral delivery platforms [6]. Interestingly, and despite a significantly lower cellular uptake (*ca*. 5–6 fold, unpublished

observations), the proposed system produced gene silencing in the same range as the commercially available Lipofectamine™ transfecting agent. This may be partially explained by the better endosomal escape of siRNA when formulated into the pH-sensitive PICMs. However, this hypothesis remains to be verified. Finally, it was found that the optimal transfection efficiency (in PC-3 cells) was achieved when the PICMs were prepared using chemically 2′-modified siRNA and a PAMAM generation five polycationic core. Interestingly, these results suggest that combining both optimized nucleic acid chemistry with a suitable delivery vehicle can improve the biological effect of the drug.

Unfortunately, data on the *in vivo* efficacy, long-term toxicity, pharmacokinetics, and biodistribution of multi-functional polymeric micelles are still relatively scarce. Despite the use of PEG or other hydrophilic polymers to confer steric stability and stealthiness to the particles, part of the injected dose will irremediably accumulate in the liver [7]. A substantial fraction may then be taken up by Kupffer cells, where unwanted toxic effects can potentially occur. Furthermore, due to their size, polymeric micelles (and nanoparticles in general) can only reach tissues where the endothelium presents gaps large enough for them to be "leaky", such as liver, spleen, and some types of solid tumors. Therefore, several potential disease targets will not be addressable by particle-based delivery systems.

In contrast, free nucleic acid conjugates (*i.e.*, not embedded in a carrier) are usually far smaller than the pores in normal vascular endothelium and thus able to access virtually all tissues (with exception to the central nervous system). Covalent modification of short nucleic acid therapeutics with hydrophobic molecules is now often performed to improve their biological efficiency [8]. In the present work, different amphiphilic AON derivatives were tested for the downregulation of Bcl-2 mRNA (**chapter 4**). In protein-free buffer, conjugates bearing a DSA moiety exhibited the highest knockdown efficiencies in PC-3 cells. In particular, AON containing PS-2′F-ANA in an altimer modification design (*i.e.*, FANA2) was found to be the most potent structure, with 80% mRNA suppression with 800 nM of FANA2-DSA. Due to its relatively higher hydrophobicity, it was suggested that DSA could have stronger interaction with the cellular membrane. Furthermore, the altimer construct is known to function well with the 2′F-ANA modification since these AONs retains the ability to recruit RNase H [9].

An interesting observation was that the introduction of serum in the transfection media drastically decreased the antisense effect of the lipophilic conjugates. Our results showed that this loss of silencing activity could be attributed to the strong association of the conjugates with extracellular HSA and other serum proteins, which was suggested to limit their interactions with PC-3 cells. These cell culture observations have to be contrasted with previous *in vivo* experiments reporting that the association of hydrophobized nucleic acids to circulating blood proteins was beneficial to improve their pharmacokinetic properties [10, 11]. The results obtained in this study from both gel retardation and mRNA knockdown assays further suggest that the *in vitro* silencing potential of the AON-DSA conjugate was dependent upon the molar ratio of AON to albumin in the transfection medium. It was found that a molar excess of HSA over the conjugate of 4 or above in the transfection medium completely inhibited their activity. However, decreasing the HSA molar excess to 2 partially restored the silencing potential of the DSA-modified antisense drug. This observation led to the proposal that pre-incubating HSA with an excess of a free fatty acid could partially prevent the FANA2-DSA association to HSA. Our data validated the proposition by showing that supplementing the transfection medium with an excess of decanoic acid prior to adding the conjugate completely restored its transfection efficiency. The fate of hydrophobized nucleic acids following systemic administration and, in particular, their interactions with certain plasma proteins, is still vaguely understood. Based on our observations, it is believed that a strong association to plasma proteins will impair the AON activity. In particular, this would happen *in vivo* in cases where the plasma proteins are not involved in the uptake of the AON conjugates. Should this problem occurs, strategies aiming at locally preventing the association of hydrophobized AONs to serum proteins could be developed in the future. Furthermore, the entry route and intracellular trafficking of these entities is complex and remains to be better characterized. Despite displaying improved cellular interactions, hydrophobized conjugates still lack features such as specific targeting moiety and endosomolytic properties, which would make them more broadly applicable. Therefore, and even though their small sizes potentially allow them to widely distribute, it is believed that the clinical potential of hydrophobized nucleic acids is principally limited to local delivery and/or to easily accessible tissues such as liver or kidney.

In this doctoral thesis, the model AON and siRNA sequences were chosen from the literature for their ability to inhibit the expression of the Bcl-2 oncogene, at both mRNA and protein levels. In principle, the two delivery approaches described above can be transposed to

any standard nucleic acid drug with an antisense mechanism (independently of its sequence). While the selected sequences are suitable *in vitro*, their clinical value remains to be established. For instance, the Oblimersen sequence (G3139 or Genasense®) used in **chapter 4** has been associated with suboptimal activity in patients, and its development in clinical phase III trials has been largely impaired [12]. Even though other therapeutic sequences may now be more relevant for *in vivo* applications, it should be considered that they might also reveal unexpected outcome upon further clinical characterization. While our research was focused on the *delivery* of nucleic acids, it is obvious that sequence selection and validation is a crucial initial step for successful clinical results [13].

In conclusion, this Ph.D. thesis presented two options for improving the delivery of nucleic acid therapeutics. In particular, the PICMs were stable at high serum concentrations, had relatively low cytotoxicity, and efficiently transfected PC-3 cells with a therapeutic siRNA. Considering this solid *in vitro* proof-of-concept, a collaboration aiming at evaluating the *in vivo* applicability of the PICMs was initiated with the group of prof. Calon (Université Laval, Canada). Within this collaboration, the ability of PICMs to target and transfect the epithelium of the blood brain barrier in a mouse model will be tested [14]. In a preliminary experiment, PICMs decorated with a rat anti(mouse)-CD71 Fab′ showed significant *in vitro* cellular uptake on mouse brain endothelial (b.End5) cells (unpublished observations). As for the hydrophobized nucleic acids, the knowledge gained on the role of pendant hydrophobic moieties on their interactions with blood proteins may create new research opportunities to deliver this type of drug. These contributions could be instrumental for the development of novel non-viral delivery strategies for nucleic acids. This thesis provided some evidence that PICM-based and hydrophobized conjugates delivery systems could both be useful platforms to improve the biological activity of nucleic acid therapeutics. In all likelihood, the type of tissue targeted will favor one approach over the other. The next step would be to determine if these systems are viable and safe in a pre-clinical context.

5.1. References

[1] Daka A, Peer D. RNAi-based nanomedicines for targeted personalized therapy. Adv Drug Deliv Rev. 2012;64:1508-21.

[2] Wang AZ, Langer R, Farokhzad OC. Nanoparticle delivery of cancer drugs. Annu Rev Med. 2012;63:185-98.

[3] Denison TA, Bae YH. Tumor heterogeneity and its implication for drug delivery. J Control Release. 2012;164:187-91.

[4] Liang W, Lam JKW. Endosomal escape pathways for non-viral nucleic acid delivery systems. In: Ceresa B, editor. Molecular Regulation of Endocytosis: InTech; 2012. p. 421-67.

[5] Ballarín-González B, Howard KA. Polycation-based nanoparticle delivery of RNAi therapeutics: Adverse effects and solutions. Adv Drug Deliv Rev. 2012;64:1717-29.

[6] Aliabadi HM, Landry B, Sun C, Tang T, Uludağ H. Supramolecular assemblies in functional siRNA delivery: Where do we stand? Biomaterials. 2012;33:2546-69.

[7] Bertrand N, Leroux J-C. The journey of a drug-carrier in the body: An anatomo-physiological perspective. J Control Release. 2012;161:152-63.

[8] Juliano RL, Ming X, Nakagawa O. The chemistry and biology of oligonucleotide conjugates. Acc Chem Res. 2012;45:1067-76.

[9] Kalota A, Karabon L, Swider CR, Viazovkina E, Elzagheid M, Damha MJ, et al. 2'-Deoxy-2'-fluoro-β-d-arabinonucleic acid (2'F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. Nucleic Acids Res. 2006;34:451-61.

[10] Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, et al. Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. Nat Biotechnol. 2007;25:1149-57.

[11] Petrova NS, Chernikov IV, Meschaninova MI, Dovydenko IS, Venyaminova AG, Zenkova MA, et al. Carrier-free cellular uptake and the gene-silencing activity of the lipophilic siRNAs is strongly affected by the length of the linker between siRNA and lipophilic group. Nucleic Acids Res. 2012;40:2330-44.

[12] Stein C, Hoehn B, Rossi J. Oligonucleotide therapeutics. In: Garrett-Mayer E, editor. Principles of Anticancer Drug Development: Springer New York; 2011. p. 569-87.

[13] Lightfoot HL, Hall J. Target mRNA inhibition by oligonucleotide drugs in man. Nucleic Acids Res. 2012;40:10585-95.

[14] Paris-Robidas S, Emond V, Tremblay C, Soulet D, Calon F. *In vivo* labeling of brain capillary endothelial cells after intravenous injection of monoclonal antibodies targeting the transferrin receptor. Mol Pharmacol. 2011;80:32-9.

List of Abbreviations

2′F-ANA	2′-fluoro-arabinonucleic acid
2′F-RNA	2′-fluoro RNA analog
2′-O-MOE	2′-O-methoxyethyl
AA	acrylic acid
ACN	acetonitrile
AlClPc	aluminum chloride phthalocyanine
Al(M)A	alkyl(meth)acrylate
AMD	age-related macular degeneration
AON	antisense oligonucleotide
apoB	apolipoprotein B
ATRP	atom transfer radical polymerization
AUC	area under the blood (plasma) concentration *vs.* time curve
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BMA	butyl methacrylate
CHL	cholesterol
CMC	critical micelle concentration
CPP	cell penetrating peptide
DCM	dichloromethane
DHA	docosahexaenoic acid
DIPEA	*N*,*N*-Diisopropylethylamine
DLS	dynamic light scattering
DMAA	dimethylacrylamide
DMD	Duchenne muscular dystrophy
DME	diabetic macular edema
DMF	dimethylformamide
DOPE	dioleoylphosphatidylethanolamine
DSA	docosanoic acid
dsRNA	double-stranded RNA
EA	ethyl acrylate
EDTA	ethylenediaminetetraacetic acid
EMA	ethyl methacrylate

Fab′	fragment antigen binding
FBS	fetal bovine serum
FDA	United States Food and Drug Administration
FRET	fluorescence resonance energy transfer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GI	gastrointestinal
GPC	gel permeation chromatography
HEMA	2-hydroxyethyl methacrylate
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HSA	human serum albumin
*i*BA	*iso*-butyl acrylate
LCST	lower critical solution temperature
LNA	locked nucleic acid
MAA	methacrylic acid
MMA	methyl methacrylate
MMT	mono-methoxytrityl
mRNA	messenger RNA
N/(P+COOH)	primary amines over phosphates and carboxylate groups charge ratio
*n*BA	*n*-butyl acrylate
NIPAM	*N*-isopropylacrylamide
OB	Oblimersen (also known as G3139 or Genasense®)
ODA	octadecyl acrylate
PAA	poly(acrylic acid)
PAGE	poly(acrylamide) gel electrophoresis
PAMAM	poly(amido amine)
PAsp	poly(L-aspartic acid)
PBS	phosphate-buffered saline
PC-3	prostate adenocarcinoma cell
PCL	poly(ε-caprolactone)
PCR	polymerase chain reaction
PDMAEMA	poly(*N*,*N*-dimethylaminoethyl methacrylate)
pDNA	plasmid DNA
PEG	poly(ethylene glycol)

PEGMA	PEG methacrylate
PEI	poly(ethylene imine)
PG	poly(glycidol)
PGA	poly(L-glutamic acid)
PI	polydispersity index
PICM	polyion complex micelle
PLA	poly(D,L-lactide)
PM	polymeric micelle
PMAA	poly(methacrylic acid)
PMO	phosphorodiamidate morpholino-oligomer
PNA	peptide nucleic acid
PrMA	propyl methacrylate
PS	phosphorothioate
PSMA	prostate specific membrane antigen
PUA	poly(10-undecenoic acid)
PyBOP	(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
qRT-PCR	quantitative reverse-transcription PCR
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase H	ribonuclease H
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-PAGE
siRNA	small interfering RNA
TBE	Tris-borate-EDTA (buffer)
*t*BMA	*tert*-butyl methacrylate
TCA	trichloroacetic acid
TEM	transmission electron microscopy
THF	tetrahydrofuran
UA	10-undecenoic acid
VBODENA	4-(2-vinylbenzyloxy)-*N,N*-(diethylnicotinamide)
VP	*N*-vinyl-2-pyrrolidone

Curriculum Vitae

Arnaud Éleuthère Felber

born in Le Locle, Switzerland

on March 14th, 1985

✉ arnaud.felber@gmail.com

EDUCATION

02/2009 – 03/2013 **ETH Zürich, Switzerland**
Ph.D. in Drug Formulation & Delivery, Institute of Pharmaceutical Sciences, Department of Chemistry (D-CHAB)
PhD thesis: "*Non-viral strategies for nucleic acid delivery*"
Supervisors: Prof. Dr. J.-C. Leroux and Dr. Bastien Castagner
Auxiliary courses completed: Entrepreneurship in Technology Ventures, Accounting for Managers, and Strategic Management

07/2007 – 08/2008 **University of California Los Angeles (UCLA), USA**
Master's thesis abroad (1 year research project)
Department of Chemical and Biomolecular Engineering
Master's thesis: "*Gene delivery from responsive hyaluronic acid hydrogels for directed angiogenesis*"
Advisors: Prof. Dr. Tatiana Segura and Prof. Dr. Jeffrey Alan Hubbell

07/2006 – 08/2008 **EPF Lausanne, Switzerland**
Master in Bioengineering with a Minor in Biomedical Engineering
Semester project in the Microsystems for space technologies laboratory, CSEM, Neuchâtel, Switzerland
Project: "*Fabrication and characterization of PDMS conductive fibers*"

09/2003 – 07/2006 **EPF Lausanne, Switzerland**
Bachelor in Life Sciences and Technology with a specialization in Neurosciences
Bachelor's project : "*Development of immunomarkers to identify cell degeneration in a genetic model of Parkinson's disease* "

07/2000 – 07/2003 **Lycée Denis-de-Rougemont, Neuchâtel, Switzerland**
High School diploma in physic and mathematics

PROFESSIONAL EXPERIENCE

02/2009 – present	**Research and Teaching Assistant, ETH Zürich, Switzerland** D-CHAB, Institute of Pharmaceutical Sciences, Prof. J.-C. Leroux Student supervision, Publication writing and review
08/2008 – 02/2009	**Industrial trainee, Genzyme (Sanofi), Cambridge, MA, USA** R&D department Development of therapeutic polymers-based anti-cancer drugs
09/2007 – 12/2007	**Intramural Sports Official, UCLA, Los Angeles, CA, USA** Contract with the John Wooden Center, UCLA Recreation Referring soccer and volleyball games
07/2006 – 09/2006	**Intern, UCLA, Los Angeles, CA, USA** Department of Chemical and Biomolecular Engineering, Prof. Segura Programing a database for the group's chemical inventory
03/2006 – 06/2006	**Research assistant, EPF Lausanne, Switzerland** Brain and Mind Institute, Neurodegenerative Disease Laboratory Prof. Patrick Aebischer and Dr. Jean-Charles Bensadoun Data analysis
08/2005 – 03/2006	**Research Assistant, EPF Lausanne, Switzerland** Regenerative Medicine and Pharmacobiology Laboratory Prof. Jeffrey A. Hubbell and Dr. Tatiana Segura Characterization of siRNA-based drug delivery system

ORGANISATIONS

02/2010 – 03/2013	**Board member** of the Pharmaceutical Scientist Association, ETHZ
02/2012 – 03/2013	**Member** of the American Chemical Society (ACS) and the Controlled Release Society (CRS)
08/2005 – 08/2006	**Vice-President** of the Life Sciences Student Association, EPFL
08/2006 – 08/2007	**Logistic officer** for the Harvard WorldMUN, hosted by EPFL

LANGUAGES

French:	Native Language
English:	Fluent
German:	Written and Spoken

Scientific Contributions

Publications (for citations on Google scholar: Author = AE Felber)

Felber AE, Bayó-Puxan N, Deleavey GF, Castagner B, Damha MJ, Leroux JC. The interactions of amphiphilic antisense oligonucleotides with serum proteins and their effects on *in vitro* silencing activity. Biomaterials. 2012;33:5955-65. **(IF_{2013} = 7.404)**

Felber AE, Dufresne M-H, Leroux JC. pH-sensitive vesicles, polymeric micelles, and nanospheres prepared with polycarboxylates. Adv Drug Deliv Rev. 2012;64:979-92.
(IF_{2013} = 11.502)
Remark: AEF and MHD contributed equally to this work.

Bayó-Puxan N, Dufresne M-H, **Felber AE**, Castagner B, Leroux JC. Preparation of polyion complex micelles from poly(ethylene glycol)-*block*-polyions. J Control Release. 2011;156:118-27. **(IF_{2013} = 6.499)**

Felber AE, Castagner B, Elsabahy M, Deleavey GF, Damha MJ, Leroux JC. siRNA nanocarriers based on methacrylic acid copolymers. J Control Release. 2011;152:159-67. **(IF_{2013} = 6.499)**

Rosset S, Niklaus M, Stojanov V, **Felber AE**, Dubois P, Shea HR. Ion-implanted compliant and patternable electrodes for miniaturized dielectric elastomer actuators. In: Bar-Cohen Y, editor. Electroactive Polymer Actuators and Devices. San Diego, CA: Proc. of SPIE Vol. 6927; 2008.

Oral Presentations

Felber AE, Castagner B, Leroux JC. Carrier-free and carrier-loaded delivery of nucleic acid. Zurich-Basel-Geneva Joint Seminar Series in Drug Formulation & Delivery, ETH Zurich, Switzerland. January 26 – 27, 2012.

Felber AE, Castagner B, Leroux JC. Smart pH-responsive nanocarriers for nucleic acid drug delivery. "Doktorandentag", Institute of Pharmaceutical Sciences, ETH Zurich, Switzerland. September 13, 2011.

Felber AE, Deleavey GF, Castagner B, Damha MJ, Leroux JC. Polymeric micelles with detachable shell prepared by ATRP for siRNA delivery. 242nd American Chemical Society (ACS) National Meeting, Denver, CO. August 28 – September 1, 2011.

Felber AE, Castagner B, Leroux JC. Introduction to nucleic acid drug delivery. International Year of Chemistry 2011 – "Tag der Chemie", ETH Zurich, Switzerland. June 18, 2011.

Felber AE, Castagner B, Elsabahy M, Leroux JC. Smart pH-responsive nanocarriers for nucleic acid delivery. 3rd International NanoBio Conference, ETH Zurich, Switzerland. August 24 – 27, 2010.

Felber AE, Castagner B, Leroux JC. Smart pH-responsive nanocarriers for the delivery of nucleic acid drugs. Zurich-Basel-Geneva Joint Seminar Series in Drug Formulation & Delivery, University of Geneva, Switzerland. June 21 – 22, 2010.

Poster Presentations

Felber AE, Bayó-Puxan N, Deleavey GF, Castagner B, Damha MJ, Leroux JC. *In vitro* silencing activity of amphiphilic antisense oligonucleotides. Swiss Pharma Science Day, University of Bern, Switzerland. August 29, 2012

Felber AE, Bayó-Puxan N, Deleavey GF, Castagner B, Damha MJ, Leroux JC. *In vitro* silencing activity of amphiphilic antisense oligonucleotides. 39th Controlled Release Society (CRS) Annual Meeting & Exposition, Québec City, Canada. July 15 – 18, 2012.

Felber AE, Castagner B, Deleavey GF, Damha MJ, Leroux JC. Smart pH-responsive nanocarriers for nucleic acid drug delivery. 6th Graduate Symposium of the Materials Research Center (MRC), ETH Zurich, Switzerland. June 8, 2011.

Felber AE, Castagner B, Leroux JC. Smart pH-responsive nanocarriers for nucleic acid delivery. 3rd Symposium Swiss Society of Chemical Industries (SSCI), ETH Zurich, Switzerland. November 5, 2010.

Felber AE, Castagner B, Leroux JC. Smart pH-responsive nanocarriers for nucleic acid delivery. Industry Day 2010, ETH Zurich, Switzerland. September 7, 2010.

Acknowledgments

To begin with, I would like to express my sincerest thanks to Prof. Dr. Jean-Christophe Leroux for giving me the opportunity to be part of his dynamic research team. I am deeply grateful for his extensive scientific input, as well as his honesty and availability.

I further thank Dr. Bastien Castagner, my co-supervisor and co-examiner. His expert advice regarding the chemistry aspect of this work has been invaluable. More importantly, the friendship we developed allowed me to approach him at any given time, especially during difficult and stressful phases of the project.

I am thankful to Prof. Dr. Jonathan Hall for accepting to be a co-examiner of this Ph.D. thesis.

My sincerest thanks go to our different collaborators. Thank you to Prof. Dr. Masad J. Damha and his Ph.D. student Glen F. Deleavey from McGill University (Montreal, Canada) for providing us with the chemically modified siRNA sequences. Thank you to Prof. Dr. Frédéric Calon and his Ph.D. student Sarah Paris-Robidas from ULaval (Québec, Canada) for testing our nanoparticles *in vivo*.

Many thanks also go to Prof. Dr. Michael Detmar and Prof. Dr. Dario Neri and their respective groups for the use of several of their instruments.

I gratefully acknowledge all present and former members of the *Drug Formulation & Delivery* group for the time spent together inside and outside of the lab. In particular, thanks to Dr. Marie-Hélène Dufresne for co-writing a review with me and Dr. Núria Bayó-Puxan for the synthesis and characterization of the amphiphilic AON conjugates library. I would like to specially thank Vincent Forster, Mattias Ivarsson, Virginie Schmid, Dr. Marc A. Gauthier, Jessica Schulz, Lorine Brülisauer, Prof. Bruno Gander, Athanasia Dasargyri, Dr. Paola Luciani, Monica Langfritz, Elena Moroz, Anna Polomska, Maurizio Roveri, Katrin & Gregor Fuhrmann, Ander Estella, Mi Liu, Dr. Soo Hyeon Lee, and Dr. Davide Brambilla for their priceless support and friendship during the past years.

Finally, I would like to thank my family and friends for their constant support and advice.