## Table of Contents

### Chapter 1: Oligonucleotide delivery – Aims of this thesis

1. Introduction 1
2. Biological barriers for the delivery of antisense oligonucleotides (ONs) 2
3. Cationic polymers and cationic liposomes as pharmaceutical carriers for ONs
   3.1. Physicochemical properties of polyplexes
   3.2. Physicochemical properties of lipoplexes
   3.3. Physicochemical properties of polymeric matrices containing ONs
4. Physicochemical properties of ON containing nanoparticles 6
5. Cellular behavior of ON-nanoparticles
   5.1. Cellular behavior of polyplexes
   5.2. Cellular behavior of lipoplexes
   5.3. Cellular behavior of polymeric matrices containing ONs
6. Aims and outline of this thesis 13
7. References 15

### Chapter 2: A brief introduction to fluorescence correlation spectroscopy

1. Introduction 19
2. The FCS set-up 20
3. Measuring the mobility of fluorescent molecules by FCS 22
4. Conclusions 25
5. References 25

### Chapter 3: On the complex formation between cationic polymethacrylates and oligonucleotides

1. Introduction 26
2. Materials and methods
   2.1. Oligonucleotides
   2.2. Cationic polymers
   2.3. Preparation of polyplexes
   2.4. Particle size measurements
   2.5. Zeta potential measurements
   2.6. Fluorescence measurements
   2.7. Gel electrophoresis experiments
   2.8. Fluorescence correlation spectroscopy (FCS)
3. Results and Discussion 32
   3.1. Size and zeta potential of pDMAEMA/ON complexes
   3.2. Fluorescence properties of pDMAEMA/Rh-ON complexes
   3.3. Fluorescence correlation spectroscopy on polyplexes
4. Summary and Conclusions 45
5. References 46
Chapter 4: Photon counting histogram analysis to analyze the fluorescence fluctuations of oligonucleotide/cationic polymer complexes

1. Introduction 49
2. Photon Counting Histogram analysis 50
3. Materials and methods 53
4. Results & Discussion 54
5. Conclusion 60
6. References 62

Chapter 5: Interactions between oligonucleotides and cationic polymers investigated by fluorescence correlation spectroscopy

1. Introduction 63
2. Materials and methods 64
   2.1. Cationic polymers
   2.2. Preparation of polyplexes
   2.3. Agarose gel electrophoresis on the polyplexes
   2.4. Exchange of Rh-ON in polyplexes with dextran sulfate as monitored by gel electrophoresis
   2.5. Fluorescence correlation spectroscopy
3. Results & Discussion 67
4. Summary and conclusions 79
5. References 80

Chapter 6: Cellular behavior of oligonucleotide/carrier complexes

1. Introduction 82
2. Materials and methods 83
   2.1. Cell and Reagents
   2.2. Oligonucleotides
   2.3. Cationic carriers
   2.4. Inhibition of ICAM-1 expression in A549 cells
   2.5. Laser scanning confocal microscopy analysis
   2.6. Cellular uptake of ONs measured by flow cytometry
   2.7. Agarose gel electrophoresis experiments
3. Results & Discussion 88
   3.1. Biological activity of ICAM-1 antisense ON in the presence or absence of cationic carriers
   3.2. Cellular uptake of free and complexed ONs
   3.3. Intracellular localization of ONs
4. Summary and conclusions 96
5. References 100
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>102</td>
</tr>
<tr>
<td>Samenvatting</td>
<td>109</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td>116</td>
</tr>
</tbody>
</table>
Chapter 1

Oligonucleotide delivery - Aims of this thesis

1. Introduction

Over the past 10 years, the field of antisense oligonucleotides (ONs) has made remarkable advances. In August 1998, the first antisense molecule (Fomiveren®) to treat cytomegalovirus infection was approved by the FDA.

The fidelity of ONs arises from their ability to form Watson-Crick hydrogen bonds with the target mRNA. As shown in Figure 1, different mechanisms can account for the biological effect of ONs. Translational arrest may occur by blockade of ribosomal read-through (Figure 1B) or by activation of ribonuclease H (RNase H) (Figure 1C). RNase H is an endonuclease that recognizes RNA-DNA duplexes and selectively cleaves the mRNA strand. The mechanism is catalytic: once a mRNA molecule is cleaved, the antisense dissociates from the mRNA chain and is able to bind to a second mRNA (1).

Figure 1. Schematic representation of the biochemical working mechanism of ONs. The translation of mRNA (green) into proteins is accomplished by ribosomes, which travel along the mRNA transcripts (A). Binding of an antisense oligonucleotide (orange) can inhibit the translation (B): it can prevent the ribosomes from beginning or completing their journey. Also RNase H can be activated. RNase H cuts the mRNA at the site where the ON has bound (C).
2. Biological barriers for the delivery of antisense oligonucleotides (ONs)

The biological efficacy of administered ONs is restrained as they must overcome several obstacles before they reach the intracellular target. The degradation of ONs by extra- and intracellular nucleases can essentially be solved by chemical modifications of the natural phosphodiester backbone. For example, to overcome the metabolic instability of phosphodiester oligonucleotides (PO-ONs), phosphorothioate oligonucleotides (PS-ONs) were under investigation (2). As reviewed by Engels and Uhlmann, many other chemical modifications were proposed (3). However, backbone modifications are not able to solve the problem of low cell membrane permeability, another barrier for ONs delivery. Since ONs are relatively large hydrophilic molecules (5-10 kDa) they do not passively diffuse across cell membranes (step B in Figure 2). Naked ONs have to enter the cell predominantly by adsorptive endocytosis and fluid phase endocytosis (2). After endocytosis it is of key importance for antisense ONs to escape from endosomal/lysosomal compartments into the cytoplasm (step C in Figure 2), as it has been demonstrated that ONs may either be exocytosed or may be partially digested in the endosomes (4).

Figure 2. Biological barriers for ON delivery: (A) The ON-carrier complex must be stable in the extracellular matrix. (B) The ON-carrier complex has to overcome the cellular membrane and (C) must escape from the endosomes. Once the ONs are released from their carrier in the cytoplasm (D), the ONs must be resistant to nuclease activity. In case the nucleus is the biological target for the ONs, nuclear pore transport is obligatory (E).
Therefore, the efficiency of antisense ONs can be enhanced when delivered into cells using transfecting agents, which promote the escape of the ONs from the endosomes (5). Once they are localized in the cytoplasm the ONs rapidly diffuse to the nucleus (step E in Figure 2), which appears to also be a location of antisense activity (6).

It has been shown that fluorescently labeled ONs microinjected in the cytoplasm quickly diffuse in the nucleus, and it is assumed that cytoplasmic diffusion and uptake in the nucleus are not major biological barriers for ONs (6). Nuclear pores allow antisense ONs to diffuse freely between the nucleus and the cytoplasm. To the contrary, DNA plasmids slowly diffuse through the cytoplasm and have a difficult time passing from the cytosol to the nucleus. This difference between ONs and DNA plasmids may in part be due to the size discrepancy. Lukacs et al. recently showed that molecular crowding and collisional interactions are primarily responsible for the slowed diffusion of DNA fragments in the cytosol (8).

As explained in the following section, drug delivery research is evaluating the potential and benefits of different types of pharmaceutical carriers for ONs delivery. Most experiments have been done with cationic lipids and cationic polymers, which spontaneously form interpolyelectrolyte complexes with the negatively charged nucleic acids (called lipoplexes and polyplexes, respectively) (9). As illustrated in Figure 2, an absolute requirement is that the ONs remain associated to their carriers as long as they are located extracellularly (step A in Figure 2). However, once the complexes arrive in the cytosol of the cell, the ONs have to be released from their carriers (step D in Figure 2).

3. Cationic polymers and cationic liposomes as pharmaceutical carriers for ONs

Table 1 represents the chemical structures and some molecular features of the most frequently studied cationic polymers in the context of ON delivery. Some of the polymers have a linear structure (like poly-L-lysine (pLL)) while others like poly-ethylene-imine (pEI) and dendrimers are highly branched. The branched polymers can be further distinguished by their symmetry of branching: the dendrimers are radially branched, while pEI has no defined center of symmetry. The positive charge can be situated either on the backbone (like in pEI) or on the side groups (like in pLL). However, the major differences
are the type and the number of protonable amines. Some polymers like pLL are fully protonated at physiological pH in the case of pEI and dendrimers only part of the amine groups are protonated.

To improve the aqueous solubility and stability of the resulting polyplexes and to reduce aspecific interactions with e.g. serum proteins, chemists synthesized copolymers by conjugating the polymer with hydrophilic segments like polyethylene glycol (pEG) (10-12, Table 1). The simplest form is where the hydrophilic and the polycationic segments are joined at their ends to give an A-B type diblock structure (Figure 3A) (e.g. poly(ethylene glycol)-poly-L-lysine (pEG-b-pLL)) (Table 1). If several monomers of the polycationic polymer are linked to hydrophilic polymers like pEG, then the resulting copolymer has a graft architecture (pEG-g-pLL) (Figure 3B) (Table 1).

![Figure 3](image_url)

**Figure 3.** (A) A block copolymer is created when the polycationic polymer (red) is attached at one end to the hydrophilic polymer like pEG (blue). (B) A graft copolymer is created when several units of the polycationic polymer are linked to the hydrophilic polymer.

Table 2 represents the chemical structure of some (cationic) lipids widely investigated for ON delivery. Cationic lipids are amphiphilic molecules consisting of a hydrophobic portion and a hydrophilic head group carrying a single or multiple positive charges. From the cationic lipids, cationic liposomes are made which can complex with the ONs. In general, most cationic liposomes are composed of a neutral lipid (“helper lipid”) and a cationic lipid. Often used as helper lipid is dioleoylphosphatidylethanolamine (DOPE). The role of the helper lipid is e.g. to facilitate the endosomal escape of the lipoplexes through the fusion to and/or the disruption of the endosomal membrane (13). Often used cationic lipids are DOTMA (1, 2-dioleoylpropyl-3-N,N,N-trimethylammonium chloride) and DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane).
<table>
<thead>
<tr>
<th><strong>Table 1. Cationic polymers studied as ON carriers</strong></th>
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<tbody>
<tr>
<td><strong>CATIONIC HOMOPOLYMERS</strong></td>
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<tr>
<td><strong>linear structure</strong></td>
</tr>
<tr>
<td>1. <strong>polylysine (pLL)</strong>: pKa between 9 and 10</td>
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<tr>
<td><img src="image1" alt="Polylysine structure" /></td>
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<tr>
<td><strong>branched structure</strong></td>
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<tr>
<td>2. <strong>branched pEI</strong>: also linear pEI exists. pKa of the primary amines is around 5.6</td>
</tr>
<tr>
<td><img src="image2" alt="Branched pEI structure" /></td>
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<tr>
<td>3. <strong>DAB64-dendrimer</strong>: it consists of a 1,4- diaminobutane core (DAB) and 64 terminal amine groups. It has a highly branched structure; the pKa of the primary amine group is 7 whereas it is about 4 for the interior amine groups.</td>
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<td><img src="image3" alt="DAB64-dendrimer structure" /></td>
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<td><strong>CATIONIC COPOLYMERS</strong></td>
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<td><strong>block-copolymers</strong></td>
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<td>4. <strong>pEG-b-pLL</strong></td>
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<td><img src="image4" alt="pEG-b-pLL structure" /></td>
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<td><strong>graft-copolymers</strong></td>
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<tr>
<td>5. <strong>pEG-g-pLL</strong> (R = pEG)</td>
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<td><img src="image5" alt="pEG-g-pLL structure" /></td>
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### Table 2: Lipids used in the preparation of liposomes for ON delivery

<table>
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</tr>
<tr>
<td>2</td>
<td>DOTAP</td>
<td><img src="image2" alt="DOTAP Structure" /></td>
</tr>
<tr>
<td>3</td>
<td>DOTMA</td>
<td><img src="image3" alt="DOTMA Structure" /></td>
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### 4. Physicochemical properties of ON containing nanoparticles

#### 4.1 Physicochemical properties of polyplexes

Generally speaking, little information is presently available about the self-assembly and the structural features of cationic polymer/ON complexes. As it takes at least between 6 and 10 salt bonds to form a cooperative system and, as compared with DNA, the length of an ON is rather short, the number of available groups for cooperative binding with the polycation is limited (10, 14). Due to the presence of a relatively low number of salt bonds cationic polymer/ON complexes may dissociate during dilution. Therefore one should be cautious when comparing results of experiments performed at high polyplex concentrations with data obtained at much greater dilutions (15).
In general, as schematically represented in Figure 4, the size, surface charge and aqueous solubility of polyplexes based on homopolymers (like pLL, pEI, ..) depend on their charge ratio ($\varphi$), being the ratio of the positive charge equivalents on the polymer to the negative charge equivalents on the ON (16). At low values of $\varphi$ ($< 1$), water soluble negatively charged polyplexes form in which the amount of protonated amines is lower than the amount of phosphate anions. As Figure 4 schematically shows, upon increasing the concentration of cationic polymer, the polyplexes become larger due to aggregation, which is a result of the lowered negative charge. The largest aggregates exist at a value of $\varphi$ close to 1, explained by the near neutral zeta potential ($\zeta$) of the complexes in this $\varphi$ region. A further increase in the polycation concentration reduces the size of the polyplexes. This is due to electric repulsion, arising from their positive zeta potential (17).

![Figure 4. Schematic representation of the size (•) and zeta potential (■) of cationic polymer/ON complexes as a function of their charge ratio ($\varphi$). Typically, the size of polyplexes is in the order of hundreds of nanometers.](image)

When block copolymers (like pEG-pEI, pEG-pLL) are used to complex ONs, “core-shell” particles are formed (Figure 5). As an example, Kataoka et al. showed that mixing pEG-pLL with ONs results in small (60 nm) core-shell particles with a relatively narrow size distribution (11). It is explained that the charge neutralization of the cationic polymer segments upon binding to ONs leads to the formation of local hydrophobic sites resulting in a micel like structure; particles arise consisting of a hydrophobic core surrounded by pEG segments (10). Figure 6 shows schematically the size and $\zeta$ of such core-shell particles. At low $\varphi$ values, $\zeta$ of the particles sharply increases when the block copolymers
are added to the ONs solution. However, at higher $\phi$ ratios, $\zeta$ levels off and remains neutral. This neutral value of $\zeta$ is consistent with the formation of nanoparticles having a core-shell structure as the neutral segments of the block copolymer are at the surface of the particle (Figure 5).

Figure 5. Complexation of ONs to polycationic block copolymers results in polyplexes with a core-shell architecture. Core-shell polyplexes are also called “polymeric micelles”.

![Figure 5](image)

To prevent dissociation of core-shell polyplexes in the extracellular matrix, several authors have suggested cross-linking the core or the shell by bonds that can be cleaved intracellularly but not extracellularly (18). The cross-links fix their structure and permanently suppress the dissociation. As an example, Kataoka et al. prepared pEG-pLL/ON micelles with cores cross-linked by disulfide bonds: thiol groups were introduced in the side chains of the lysine units of pEG-pLL and were oxidized (19). The reduction of the disulfide bond is expected to occur only in the cells since the intracellular fluid is a

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-8-
strong reducing environment (glutathione concentration 3 mM) compared to the extracellular fluid (glutathione concentration 10 µM).

Most of the cationic polymers have the disadvantage that they are polydisperse with regard to their molecular weight. This fact probably also contributes to the heterogeneous properties (with regard to their size) of the resulting polyplexes. Therefore some groups considered cationic dendrimers as ON carriers (20). As shown in Table 1, dendrimers have a well-defined highly ordered, spherical structure. Kabanov et al. showed that Astramol dendrimers are fully penetrable by linear polyanions like polyacrylic acid and polystyrene sulfonate (21). Consequently, upon complexation with dendrimers, ONs probably do not only wind around the dendrimer species but also enter their interior.

4.2 Physicochemical properties of lipoplexes

As conventional non-charged liposomes resulted in a poor encapsulation of DNA and low levels of transfection, investigators were encouraged to develop other types of liposomes. Felgner et al. proposed the use of cationic liposomes because they were able to spontaneously form stable complexes with the polyanionic nucleic acids based on electrostatic interaction. Although cationic liposome/ON complexes are often used in transfection experiments, relatively little information is available on their physicochemical characteristics and morphology (22-24). Direct comparison between cationic liposome/ON and cationic liposome/plasmid DNA complexes should be avoided, because ONs are flexible, single-stranded molecules while plasmid DNA is a rather stiff, double-stranded, supercoiled molecule. Moreover, while plasmid DNA collapses upon interaction with cationic carriers, ONs do not.

In analogy with the core-shell polyplexes (Figure 5), Meyer et al. studied in detail ON/cationic liposome complexes in which the lipids were modified with pEG (22). They observed that binding of ONs to these cationic liposomes was very efficient although they contained a relatively high amount of pEG-modified lipids (like poly(ethylene glycol)-phosphatidylethanolamine, pEG-PE). It seemed that the hydrophilic pEG coating did not shield the positive charge on the liposome from interaction with ONs. Compared with cationic liposomes lacking pEG-PE, which showed progressive aggregation and/or precipitation, pegylated lipoplexes are more stable. Probably the pEG coat stabilizes the
complexes both by preventing cross-linking of the vesicles by ON molecules and by avoiding bilayer contact between the liposomes, which may induce their aggregation.

4.3 Physicochemical properties of polymeric matrices containing ONs

Besides cationic polymers and cationic liposomes, also nanoscopic “polymeric matrices” have been suggested as ON carriers (25-29). One can entrap the ONs in the polymeric matrix or adsorb them on the surface of the nanoparticle. Release of the ONs from these nanoparticles can occur by diffusion of the ON through the matrix, by bulk erosion of the polymeric matrix or by desorption of the ON from the surface of the polymeric matrix. In comparison to lipoplexes and polyplexes such nanoscopic polymer matrices are more stable in biological fluids and in storage. Another advantage is the ability to control the rate of ON release by e.g. changing the structure of the polymeric matrix.

Chavany et al. preferred to lead ONs into polyalkylcyanoacrylate (PACA) nanoparticles by adsorption of the ONs to the surface (25). This association could only occur in the presence of a hydrophobic counter-ion e.g. quaternary ammonium salts such as cetyltrimethylammonium bromide (CTAB) or by using a hydrophobic conjugate of the ON (25-27). The poor yield of association in the absence of a hydrophobic moiety was explained by the repulsion between the polyanionic ONs and the negatively charged particles. In the case of the PACA nanoparticles, ONs are adsorbed at the surface, which makes them susceptible to degradation by nucleases. Another disadvantage of the PACA nanoparticles is the low loading efficiency (less than 1 %) (28). To solve this problem, Berton et al. used poly-(D,L-lactic acid) (PLA) nanoparticles (prepared by emulsification) which permitted loading significantly larger amounts of ONs (entrapment efficiency of 27%). Loading of the ONs was possible by complexing them with CTAB. The addition of the complexed ON in the organic phase of the emulsion led to their entrapment in the core of the polymeric matrix.

Recently an original process for ON loading into alginate nanosponges was introduced (29). Loading of the ONs could occur in two different ways. First, through the single addition of pLL/ON complexes to a calcium alginate “pregel”. The amount of ON binding to these nanoparticles was higher than the amount of ONs associated with the pLL. This suggests that free ON was also able to associate with the alginate gel, possibly by
calcium ions that from a bridge between carboxylic acids on the alginate polymer and phosphate groups on the ONs. Because the surface charge of the ON loaded and non-loaded particles was the same, it was suggested that the ONs were incorporated inside the nanoparticle matrix rather than being adsorbed on their surface. The second method for preparing these particles was through addition of ONs to the already formed alginate particles (containing pLL). In this case the ONs were attracted by ionic driving forces into the alginate particles. Compared with PACA and PLA nanoparticles, a big advantage of the nanosponges is that they are prepared in an aqueous medium without using any organic solvent and that the ONs are localized in the core of the particles.

5. Cellular behavior of ON nanoparticles

5.1 Cellular behavior of polyplexes

It is generally accepted that polycation/ON complexes enter the cell by spontaneous endocytosis (30) (step B in Figure 2). It is also believed that certain polycations not only carry the ONs but also help the ONs escape from the endosomes, a necessity to avoid lysosomal degradation. Particularly, it has been suggested that pEI and dendrimers promote the endosomal escape based on their “proton sponge effect” (31). After endocytosis of pEI/ON or dendrimer/ON complexes, both polymers are able to buffer the endosomal acidification. As a consequence, protons further accumulate in the endosomes accompanied by a passive influx of chloride anions. These events cause osmotic swelling of the endosomes and a subsequent disruption of the endosomal membrane, which allows the ONs to enter the cytosol.

There are only a few examples of the delivery of ONs using pEI as carrier. Boussif et al. showed that pEI was able to target ON to the nucleus of neurons (32). Their experiments suggested that some pEI/ON complexes crossed the nuclear membrane while other pEI/ON complexes disassembled in the cytoplasm (33). However, opinions on the cellular behavior of pEI/ON complexes differ greatly (30, 34).

Following the proton sponge hypothesis, cationic polymers were equipped with proton sponge like molecules. Pichon et al. coupled histidine, having imidazole groups, to oligolysine, which could also buffer the acid lumen of the endosomes (35). To facilitate the endosomal escape of ONs, the group of Pichon also developed peptides that were able to
fuse with the endosomal membrane (36). The peptide has a random structure at neutral pH while it adopts a helical structure upon acidification of the endosomes, which precedes its interaction with the endosomal membrane and results in disruption of the membrane.

5.2 Cellular behavior of lipoplexes

It is now believed that lipoplexes do not simply fuse with the plasma membrane like suggested earlier (37, 38). This would lead to a direct delivery of ONs into the cell cytoplasm, thus avoiding endosomal entrapment. Many authors suggest that ON-lipid complexes are taken into the cell via endocytosis, as it is the case for polyplexes.

The mechanism of release of lipoplexes from the endosomes is not fully understood. However, Zelphati and Szoka proposed a model in which the cationic lipids of the complex interact with the anionic lipids in the cytoplasmic face of the endosomal membrane and “flip-flop” into the outer leaflet of the endosomal membrane (39, 40). It is suggested that during this flip-flop event the ONs escape from the endosome to the cytoplasm while the cationic lipid remains in the outer leaflet. At present, it is unclear why endocytosis seems to be required for cellular entering of the lipoplexes, as fusion of the complex with the plasma membrane would also permit the same anionic lipids to flip to the surface. One possibility is that internalization of the complexes into a compartment with a high radius of curvature promotes close contact between the two surfaces that are suitable for fusion (41). Consequently, like polymers, liposomes not only enhance the extent of ON uptake into cells, but also markedly change the intracellular distribution of the ONs.

Lappalainen et al. showed that the nuclear envelope could form a barrier against the penetration of ON-lipoplexes into the nucleus (42). They suggested that part of the ONs stay associated with the liposomes in the cytosol and, due to size limitation, never reach the nucleus. In agreement with the observation by Lappalainen, Marcusson et al. demonstrated by confocal microscopy that the lipoplexes must dissociate before the ON can gain access to the nucleus (43).

The use of cationic liposomes is still greatly limited, most critically by their toxicity and the decreased activity in the presence of serum. Two explanations come to mind: (1) the charged surface of the complex can be coated with serum proteins and (2) the serum proteins may dissociate the lipoplexes. Newer cationic lipid formulations like GS 2888...
(Cytofectin®) have been developed which efficiently transfect ONs into many cell types in the presence of serum (44). To overcome the “serum barrier” pEG-modified cationic liposomes are also under investigation for ON delivery (22).

5.3 Cellular behavior of polymeric matrices containing ONs

The mechanism of delivery of ONs from the polymeric matrices described in 4.3 is not well understood. By confocal microscopy it was shown that these nanoparticles are mainly taken up in the cell by an endocytic/phagocytotic pathway, as they were present in lysosomes and phasomes (25). The presence of intact ONs in the cell nucleus suggests that part of the internalized ONs is able to escape from the endosomal/lysosomal compartment. It was shown that this was the case for ON delivered by PACA nanoparticles containing CTAB, however, for nanoparticles containing diethylaminoethyl (DEAE) almost no escape was observed. Possibly CTAB may destabilize the lysosomal membrane.

6. Aims and outline of this thesis

Research on the use of oligonucleotide drugs started more than ten years ago. As antisense oligonucleotides interact with their target by Watson-Crick base pairing, it was expected that this class of therapeutic molecules would act very specific. Treatment of viral diseases, cancer, … would become possible without the occurrence of severe side effects.

However, oligonucleotides are still far from becoming therapeutic agents. A few of the hurdles that antisense therapy must overcome are the failure of the oligonucleotide to cross cell membranes, the inefficient targeting, and the rapid degradation by nucleases…

To overcome these barriers, many groups are currently screening a high number of pharmaceutical carriers for ON delivery. However, while much effort is oriented towards the synthesis of new carriers, the optimization of the physicochemical and pharmaceutical features of the complexes is frequently neglected. We believe that rational (as opposed to empirical chemical) design of improved vectors requires a better knowledge of the multistage process by which pharmaceutical vectors deliver ONs into cells. In this optimization process we should pay attention to all the critical steps involved in the delivery of ONs by pharmaceutical carriers: (1) the preparation of the ON/carrier complexes, (2) the stability of the complexes in biological fluids like serum, (3) the
transport of the complexes from the extracellular space to the intracellular environment and (4) the cellular behavior of the complexes. An important step, which has to occur once the complexes arrive in the cells, is the release of the ONs from their carrier.

Although the association and dissociation of ON/pharmaceutical carrier complexes have been studied extensively in vitro (i.e. in buffer), information on the complexation in biological media, and especially in the cellular environment is limited. Therefore, very challenging is the development of innovative methods to study the behavior of ON/pharmaceutical carrier complexes in the cytoplasm and nucleus of cells. With this scope in mind, this thesis deals with fluorescence correlation spectroscopy. The main aim of this thesis is to evaluate the potential this method has to study the complexation between ONs and their carriers.

In this chapter (Chapter 1) a detailed literature overview on the physicochemical characteristics of different types of ON complexes (lipo- and polyplexes, polymeric matrices) was summarized. Also differences in the cellular uptake of the different complexes were highlighted.

As more advanced physicochemical methods must be developed to do intracellular experiments, we were interested to evaluate the possibility to use FCS to study the dissociation/association of ONs from/to their carrier. First, in Chapter 2, we explain briefly the main principle of FCS to better understand the chapters dealing with this method. In Chapter 3 we elucidate the complexation behavior between oligonucleotides and polymethacrylates by FCS, in combination with other well known physicochemical techniques like dynamic light scattering (DLS), zeta potential measurements, fluorimetric and gel electrophoresis experiments.

We quickly concluded that fluorescence fluctuation spectroscopy (FFS), instead of FCS could provided information about the ON-cationic polymer complexes.

In Chapter 4 we try to assess which type of FFA extracts the most information on polyplexes. The method of analysis introduced in Chapter 3, based upon large time bins, was compared to an alternative statistical technique presented in literature, based upon sub-ms time bins.
In Chapter 5 we evaluate to what extent FFS is generally applicable in the characterization of complexation of ONs with other types of cationic carriers, as the search to new pharmaceutical carriers never seems to stop.

In Chapter 6 of this thesis we wondered to which extent methods like flow cytometry and confocal scanning laser microscopy (CSLM) were able to provide us with information on the cellular delivery of the ONs from their carriers.

7. References


Chapter 2

A brief introduction to Fluorescence Correlation Spectroscopy

1. Introduction

When measuring the fluorescence of a sample by a conventional fluorimeter, the emission from a large number of fluorescent molecules is collected. A Fluorescence Correlation Spectroscopy (FCS) instrument measures the fluorescence of only one or a few fluorescent molecules present in a very small observation volume (Figure 1).

![Figure 1](image)

**Figure 1.** Schematic representation of the small observation volume (typically \( \sim 1 \ \mu m^3 (10^{-15} \ l) \)) of an FCS instrument. Only the molecules in the observation volume are excited.

The parameters of primary interest in FCS are the fluctuations in the fluorescence intensity in the small observation volume (Figure 2A) caused by the diffusion of fluorescent molecules in and out the observation volume (Figure 1). However, fluorescence fluctuations may arise not only from Brownian diffusion but also from flow and chemical reactions. In the fluorescence fluctuation experiments described in this thesis, the fluorescence fluctuations are only due to the diffusion of fluorescently labeled oligonucleotides (ONs) through the observation volume.
Figure 2. The passage of fluorescent molecules in and out the observation volume gives rise to fluctuations in measured fluorescence (A). An autocorrelation function (B) can be derived from the fluctuations and allows to calculate the average residence time in the observation volume.

2. The FCS set-up

The FCS set-up usually consists of a laser beam which is projected into a microscope water immersion objective (via a dichroic mirror) and focused on the sample (Figure 3). The instrument measures the fluorescence fluctuations in the excitation volume (the observation volume) of the microscope. The emitted light is collected by the same objective, passes through the dichroic mirror and the emission filter to reach the detector. The pinhole in the image plane blocks any fluorescence light not originating from the focal region. The dichroic mirror basically serves as a wavelength-dependent beam-splitter: it deflects excitation light and transmits the fluorescence. The fluorescence fluctuations (Figure 2A) are processed by a hardware correlator and the resulting autocorrelation curve (Figure 2B) is analyzed with specific software, yielding information on the diffusion coefficient and the concentration of the fluorescent molecules in the solution.
Figure 3. Schematic representation of the FCS set-up.
3. Measuring the mobility of fluorescent molecules by FCS

One way to calculate the diffusion coefficient of the fluorescent molecules from their fluorescence fluctuations is the use of autocorrelation analysis (Figure 2B). Autocorrelation analysis measures how long a fluctuation persists in time by measuring the self-similarity of a time series signal. In autocorrelation analysis, the fluorescence intensity at time $t$ $F(t)$ ($\bullet$) is compared to (multiplied with) the fluorescence intensity at time $t + \tau$, where $\tau$ is a variable interval (Figure 4). This product is averaged over all possible pairs of intensities that can be found in the measurement. The resulting function with variable $\tau$ is the autocorrelation function $G(\tau)$. When the deviation from the average fluorescence at a time $t$ is positive, there is a large chance (in the case $\tau$ is small ($\bullet$)) that the fluorescence deviation a time interval $\tau$ later is also positive. The two values are correlated and most products are positive. When $\tau$ is progressively longer ($\bullet$), the correlation is lost and more products will occur between a positive and negative intensity deviation. The product average will decrease with increasing time interval. The autocorrelation function decays from a maximum value at $t = 0$ to zero for large delay time $\tau$ (Figure 2B). The faster the component moves, the faster the correlation is lost (it shows a faster decay as a function of time).

The autocorrelation function of the fluctuations allows us to compute the translational diffusion time ($\tau_d$) and the average total number of fluorescent molecules in the observation volume ($N$). The translational diffusion time is the average time a fluorescent molecule spends in the observation volume. Typically, fluorescence fluctuations are registered during 20 to 30 seconds which is long compared to $\tau_d$. Consequently, a large number of single diffusion events occur during an FCS experiment. When the fluorescence fluctuations are only due to diffusion through the confocal volume, which is approximated as a cylinder with radius $\omega_1$ and height $2\omega_2$ (Figure 1), $G(\tau)$ takes the following form (1):

$$G(\tau) = 1 + \frac{1}{N} f(\tau/\tau_d)$$

with

$$f(\tau/\tau_d) = \left[ \frac{1}{1 + \tau/\tau_d} \right] \left[ \frac{1}{1 + (\omega_1/\omega_2)^2 \tau/\tau_d} \right]^{1/2}$$

The ratio $\omega_2/\omega_1$ is called the structural parameter of the confocal volume element.
For a mixture of two types of fluorescent molecules exhibiting a fast ($\tau_{\text{fast}}$) and a slow ($\tau_{\text{slow}}$) translational diffusion time, $G(\tau)$ reads (1):

$$G(\tau) = 1 + \frac{I}{N} \left[ (1 - y) \cdot f\left(\frac{\tau}{\tau_{\text{fast}}}\right) + y \cdot f\left(\frac{\tau}{\tau_{\text{slow}}}\right) \right]$$  \hspace{1cm} (3)

where $(1-y)$ and $y$ are the molar fractions of the fast and the slow component, respectively.

The translational diffusion coefficient (D) is calculated from $\tau_d$ by the following equation:

$$D = \frac{\omega_i^2}{4\tau_d}$$  \hspace{1cm} (4)

To resolve two species (a slow and a fast one) from an FCS experiment, their difference in diffusion coefficient must be large enough. It is often assumed that a difference in diffusion coefficient of a factor two is sufficient to resolve the two species by FCS. Approximating a molecule by a homogeneous sphere, the hydrodynamic radius will scale with the third root of the molecular weight. Therefore, the molecular weight of the slow molecule need to be at least 8 times the molecular weight of the slow compound. When the difference in molecular weight is smaller than 8, the difference in diffusion time will be too narrow to resolve by means of autocorrelation (Figure 5).

As the amplitude of the autocorrelation function is inversely proportional to the occupation number N (see Figure 2B), it allows the calculation of the concentrations of fluorescent molecules by the following equation:

$$C\left(\text{mol} \, / \ell\right) = \frac{N}{6.02 \cdot 10^{23} \cdot 2 \cdot \pi \cdot \omega_i^2 \cdot \omega_s^2}$$  \hspace{1cm} (5)

However, calculating the concentration of the fluorescent molecules is not always so straightforward. Since FCS experiments are applied on nM concentrations, a variety of complications has to be dealt with. First, many fluorescent labeled molecules adhere to the surface of the cuvet. In the nM and sub-nM range, a significant amount of the molecules contained in the sample may simply disappear that way. Second, photophysical damage may reduce the number of detected molecules even further, so that an overall accuracy for N of more than 20-30% is hard to achieve.
Figure 4. The fluorescence fluctuations are distributed around the average fluorescence $\langle F(t) \rangle$. Autocorrelation analysis measures how long a fluctuation persists in time. The fluorescence intensity at time $t$ (●) is compared to the fluorescence intensity at time $t + \tau$, where $\tau$ is a variable interval. This product is averaged over all possible pairs of intensities that can be found in the measurement. The resulting function with variable $\tau$ is the autocorrelation function $G(\tau)$. When the deviation from the average fluorescence at a time $t$ is positive, there is a large chance that for a small interval $\tau$ (●) the fluorescence deviation a time interval $\tau$ later is also positive: the two values are correlated. When $\tau$ is longer (●), the correlation is lost.

Figure 5. Measurement principle for FCS. The smallest partner is fluorescently labeled (red). The amplitude of the autocorrelation function is constant during reaction. The change in molecular weight after binding to the large ligand shows up in the autocorrelation function.
4. Conclusions

FCS can be considered as a versatile technique, useful in vitro as well as in vivo. The large number of parameters that can be measured by FCS makes the method applicable in a large domain.

A major advance of FCS is that it is a non-invasive technique, consequently very useful for the investigation of the precious biological environment. No external perturbations are necessary as this method is concerned with minute spontaneous fluctuations around the thermodynamic equilibrium. Also the possibility to measure the diffusion of different species without the need for separation makes the method very interesting.

A disadvantage of the method is certainly that the interpretation of the autocorrelation is rather difficult. Frequently additional methods are needed to confirm the FCS results.

5. References

Chapter 3

On the complex formation between cationic polymethacrylates and oligonucleotides

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1. Introduction

The use of oligonucleotides (ONs) to down regulate the production of specific gene products requires the entry of ONs into the cytoplasm and/or nucleus of the cells, to be able to hybridize with their targets. Entry into the nucleus is required when considering triple helix therapy, where the ONs form triple-stranded helices with DNA and consequently inhibit transcription. When the antisense approach is considered, which aims to bind the ONs to natural sense mRNA in order to inhibit translation, the hybridization can take place in the cytoplasm as well as in the nucleus. Generally, the cellular uptake of ONs is very poor due to their large size, hydrophilicity and negatively charged backbone. Furthermore, especially unmodified phosphodiester ONs, are very susceptible to nuclease activity (1). To bypass these problems, cationic lipids and cationic polymers, which spontaneously form soluble interpolyelectrolyte complexes with the negatively charged nucleic acids (called lipoplexes and polyplexes, respectively), are under investigation as pharmaceutical carriers for ONs (2-6).

Like already mentioned in Chapter 1, the physicochemical and biological features that govern the activity of ON delivery systems are not very well understood. Despite major efforts to understand the physicochemical behavior of interpolyelectrolyte complexes, the mechanism of complex formation between polycations and nucleic acids is still unknown due to the high complexity of the phenomena. Nevertheless, the association to and dissociation of ONs from such carriers are critically important both in vitro as well as in vivo. If the affinity between the ONs and the cationic carriers is too low, the complex will dissociate prematurely,
e.g. in the blood stream, while a strong affinity might prevent the release of the ONs intracellularly, a necessity to allow interaction with their target.

Many methods exist to study the association and dissociation of ONs and plasmids to/from their carrier in non-biological environments. Often applied for this purpose are gel electrophoresis and density gradient analysis (7). Szoka et al. investigated the release of ONs from a cationic liposome complex by fluorescence resonance energy transfer (FRET) (8). However, FRET needs a proper orientation of the donor and acceptor molecules, which is hard to obtain in case of ON/cationic polymer complexes. When labeling ON and polymer with donor and acceptor molecules, respectively, quenching of the donor was not accompanied by an increased emission of the acceptor, consequently on could not talk about energy transfer (data not shown). Very recently, electron paramagnetic resonance and surface plasmon resonance spectrometry were introduced to investigate the interactions between nucleic acids and polycations (9-10).

However, to obtain real breakthroughs in the design and the understanding of the dissociation of polyplexes in biological environments like cells, there is an urgent need for advanced physicochemical methods, which allow us to characterize these critical steps not only in vitro but also in biological media.

In the current chapter, we aimed to understand the complex formation between rhodamine labeled ONs (Rh-ONs) and poly(2-dimethylamino)ethyl methacrylate (pDMAEMA; Figure 1), a cationic polymer, which is currently under investigation as a pharmaceutical carrier for nucleic acids (11-15).

![Figure 1.](Figure 1. 2-(dimethylamino)ethyl methacrylate, being the monomer of pDMAEMA. The pKa of the amine equals 7.5.)
Moreover, we have introduced fluorescence correlation spectroscopy (FCS), a technique that shows potential to be applied on a cellular scale, as a novel tool for studying the interactions between cationic polymers and ONs. Basically, as illustrated in Figure 2 and as explained in Chapter 2, FCS monitors the fluorescence fluctuations caused by the diffusion of fluorescently labeled ONs through the confocal volume of a microscope. Upon binding to large cationic polymers, which are themselves not excitable by the laser light, one can expect that the diffusion of the Rh-ONs slows down, which may consequently influence the fluorescence fluctuations (16).

**Figure 2.** The passage of fluorescently labeled molecules (•) through the confocal volume element (A) gives rise to fluctuations in measured fluorescence (B). An autocorrelation function (C) can be derived from the fluctuations and allows calculating the average residence time in the confocal volume.

2. Materials and methods

2.1. Oligonucleotides.

The 25-mer phosphodiester ONs (5’-TCT-GGG-TCA-TCT-TTT-CAC-GGT-TGG-C-3’) and 25-mer rhodamine (Rh) labeled phosphodiester ONs were purchased from Eurogentec (Seraign, Belgium). The fluorescent labeling occurred at the 5’ end of the ONs: each Rh-ON molecule contained one Rh label. The concentration of the ON and Rh-ON stock solutions (in Tris-buffer at pH 8) was determined by measuring the absorption at 260 nm (1 OD\textsubscript{260} = 33 µg ON/mL) and equaled 64 and 46 µg/mL, for the ON and Rh-ON stock solution, respectively. The molecular mass of the ONs and Rh-ONs was 7 646 g/mol and 8 253 g/mol, respectively.
2.2. Cationic polymers.

Poly(2-dimethylamino)ethyl methacrylate, a kind gift of the University of Utrecht, was synthesized and characterized as described elsewhere (14). The molecular mass of the DMAEMA monomer equaled 157 g/mol. The number average molecular mass \( M_n \) of pDMAEMA was estimated to be around 60 000 g/mol as determined by gel permeation chromatography (GPC). As no pDMAEMA molecular weight standards are available, dextran molecular weight standards were used to estimate \( M_n \) from GPC. The pKa of the amine group in pDMAEMA equaled 7.5 and was determined by titration as described elsewhere (17).

2.3. Preparation of polyplexes.

pDMAEMA/Rh-ON complexes differing in charge ratio \( (\varphi) \), but having the same Rh-ON concentration (10 µg/mL, 1.2 µM) were prepared. \( \varphi \) is the ratio \((+/-)\) of the positive charge equivalents on pDMAEMA to the negative charge equivalents on Rh-ON. \( \varphi \) was calculated assuming that 1 µg of 25-mer Rh-ON contains 2.91 nmol negative charges and 1 µg of pDMAEMA contains 3.54 nmol positive charges as calculated from the molecular mass of the DMAEMA monomer and the pKa of the pDMAEMA. Two methods of preparing the pDMAEMA/Rh-ON complexes were considered in this study. In the “fast addition method”, the pDMAEMA/Rh-ON complexes (varying in \( \varphi \)) were prepared by adding different volumes of the pDMAEMA stock solution (347 µg/mL in 20 mM Hepes buffer at pH 7.4) to a fixed volume of the Rh-ON stock solution in one step. After addition of the polymer solution, the dispersion was vortexed for 10 seconds. To obtain the final Rh-ON concentration of 10 µg/mL, the dispersions were further diluted with Hepes buffer at pH 7.4. The polyplexes were allowed to equilibrate for 30 min at room temperature before use. In the “slow addition method”, the complexes were prepared by adding drop by drop the pDMAEMA/Hepes mixture (with the same volume but with varying concentration of pDMAEMA to obtain polyplexes with a different charge ratio) to a fixed volume of the Rh-ON stock solution. After the addition of each drop of polymer solution, the dispersion was vortexed. The polyplexes were allowed to equilibrate for 30 min at room temperature before use. Particle size, zeta potential, fluorescence measurements and gel electrophoresis were all done on the same pDMAEMA/Rh-ON dispersions, having a final Rh-ONs concentration of 10 µg/mL (1.2 µM).
For FCS measurements, the pDMAEMA/Rh-ONs dispersions were prepared by the fast addition method as described above. However, the final Rh-ONs concentrations equaled 0.25 µg/mL (30 nM).

2.4. Particle size measurements.

Dynamic light scattering measurements (DLS) on pDMAEMA/Rh-ON complexes were carried out on a Malvern 4700 instrument (Malvern, Worcestershire, U.K.) at 25 °C and at an angle of 90 degrees. The incident beam was a HeNe laser beam (633 nm). The pDMAEMA/Rh-ON complexes were prepared as described above. To avoid dust particles, the pDMAEMA solutions were filtered before being added to the nucleotide solutions. Average pore size of the filter was 0.45 µm (Schleicher & Schuell, Dassel, Germany). The particle size was measured 30 minutes after the preparation of the pDMAEMA/Rh-ON complexes. For calculating the z-average hydrodynamic diameter from the DLS data, the viscosity and refractive index of water at 25 °C (0.89 mPa.s and 1.333, respectively) were used. Polystyrene nanospheres (220 ± 6 nm; Duke Scientific Corp, Palo Alto, CA) were used to verify the performance of the instrument. The particle size of each dispersion (as characterized by φ) was measured three times.

2.5. Zeta potential measurements.

Zeta potential (ζ) measurements on the pDMAEMA/ON complexes were performed at 25 °C on a Malvern Zetasizer 2000 (Malvern, Worcestershire, UK), which is based on electrophoretic light scattering. ζ was measured within 1 hour after the preparation of the pDMAEMA/ON complexes. Polystyrene nanospheres (- 50 mV; Duke Scientific Corp, Palo Alto, CA) were used to verify the performance of the instrument. ζ of each dispersion was measured three times.

2.6. Fluorescence measurements.

The fluorescence of pDMAEMA/Rh-ON complexes was measured 30 minutes after their preparation on an SLM-Aminco Bowman fluorimeter (SLM-Aminco Bowman, Rochester, NY; λ_{ex} = 525 nm, λ_{em} = 584 nm). Fluorescence of each dispersion was measured three times.
CHAPTER 3

2.7. Gel electrophoresis experiments.

Gel electrophoresis was performed on the same polyplex dispersions as used for particle size, \( \zeta \) and fluorescence measurements. After preparation, the dispersions were allowed to stand at room temperature for 30 min. They were diluted with loading buffer (90% dispersion + 10% loading buffer which consisted of 50% sucrose in distilled water containing bromophenol blue). 20 \( \mu \text{L} \) of this mixture was placed in the wells of a 2.5% agarose gel. A TBE buffer was used containing 10.8 g/L Tris base, 5.5 g/L boric acid and 0.58 g/L EDTA. The total amount of oligonucleotides in the wells was 0.18 \( \mu \text{g} \). A potential of 100 V was applied for 50 minutes. The oligonucleotides were detected by staining the gel with a SYBR Green I solution (Molecular Probes, Leiden, The Netherlands; 100 \( \mu \text{L} \) SYBR Green I in 1L TBE buffer adjusted to a pH between 7.5 and 8).

2.8. Fluorescence correlation spectroscopy (FCS).

The translational mobility of Rh-ONs and polyplexes in buffer was measured using FCS, which is based on the analysis of intensity fluctuations of excited fluorescent molecules (as discussed in Chapter 2) caused by diffusion through the confocal volume of a microscope (Figure 2A) (18). After excitation of the fluorescent molecules by a stationary laser beam, the emitted photons are detected by a highly sensitive avalanche photodiode. This results in an autocorrelation curve as shown in Figure 2C which allows us to compute the translational diffusion time (\( \tau_d \)) which is the average time a fluorescent molecule spends in the excitation volume. The translational diffusion coefficient (D) is calculated from \( \tau_d \) by the following equation:

\[
D = \frac{\omega_i^2}{4\tau_d}
\]

A commercial FCS instrument (Confocor, Zeiss-Evotec, Jena-Hamburg, Germany) was used. Briefly, the light of a HeNe laser operating at 543 nm was projected into the microscope water immersion objective (C-apochromat 40x magnification) via a dichroic mirror. The laser beam was focused at about 180 \( \mu \text{m} \) above the bottom of the cuvettes (Nalge Nunc International, Naperville, IL), which contained the Rh-ON solutions or polyplex dispersions. Laser power was adapted by inserting a neutral density filter. The emitted light was collected by the same objective, passed through the dichroic mirror and the 45 \( \mu \text{m} \)
CHAPTER 3

pinhole to finally reach the avalanche photodiode. Measurements on the Rh-ON solution were performed at room temperature on samples of 200 µL. The measuring time on the Rh-ON solution was 50 seconds and the sample was measured 10 times. For FCS measurements on the pDMAEMA/Rh-ON complexes, 200 µL of the dispersions were prepared by the fast addition method in an Eppendorf tube (Eppendorf, Hamburg, Germany), vortexed and immediately transferred into the FCS Nunc cuvettes to begin the FCS measurement. Each dispersion (as characterized by ϕ) was measured 60 times. For each measurement, the fluorescence fluctuations were recorded during 50 seconds.

As the size of the excited sample region (Figure 2A) is affected by small variations in optical alignment, before starting the measurements, the volume element must be calibrated. ω₁ and ω₂ were determined using a rhodamine 6G solution (5 nM in water) with known diffusion coefficient (2.8 × 10⁻⁶ cm²/s). FCS measurements on the rhodamine 6G solution were performed at room temperature, using a 0.5 neutral density filter, while the fluorescence signal was autocorrelated over 25 seconds. τ₁ of the rhodamine 6G molecules, as calculated from G(τ) equaled 72 ± 2 µs. ω₁, as calculated from Eq. 4, equaled 0.28 µm. The structural parameter of the confocal volume element (ω₂/ω₁) equaled 8.17 as determined from G(τ) of the rhodamine 6G solution and allowed us to calculate ω₂, which equaled 2.29 µm.

3. Results & Discussion

3.1. Size and zeta potential of pDMAEMA/ON complexes.

None of the pDMAEMA/Rh-ON dispersions used for size and zeta potential measurements were apparently turbid. However, in spite of their transparent appearance, light scattering increased upon adding pDMAEMA to the Rh-ON solutions, suggesting the formation of nanosized particles through the complexation of the Rh-ON with pDMAEMA. Figure 3 shows the hydrodynamic size and ζ of pDMAEMA/Rh-ON polyplexes as a function of ϕ.

Both a “slow addition method” and a “fast addition method” were used to prepare the polyplexes, as it was reported previously that the way of preparation might have a substantial influence on the properties of the resulting polyplexes (19). As each point originates from
DLS and zeta potential measurements on three polyplex dispersions, which were independently prepared, the way we prepared the polyplexes resulted in complexes, which were fairly reproducible with regard to their size and surface properties. Only at low values of \( \varphi \), the way of adding the pDMAEMA solution to the Rh-ON solution seemed to have an influence on the size of the resulting polyplexes.

**Figure 3.** The z-average hydrodynamic diameter (A) and zeta potential (B) of pDMAEMA/Rh-ON complexes, as a function of the charge ratio. The polyplexes were prepared by fast and slow addition of the pDMAEMA solution to the Rh-ON solution, respectively. The Rh-ON concentration in the dispersions was 10 µg/mL. Each measuring point is the mean value of measurements on 3 dispersions, which were independently prepared. Each dispersion was measured 3 times.
The dependence of the size and surface properties of the pDMAEMA/Rh-ON complexes on $\varphi$ shows a typical profile as often observed for complexes formed between oppositely charged polyions (20). At low values of $\varphi$, non-stoichiometric polyplexes form, in which the amount of protonated amines is lower than the amount of phosphate anions and which are negatively charged. The interpretation of this is shown in Figure 4A.

**Figure 4.** Representation of interactions between Rh-ONs and pDMAEMA under different conditions. (A) at low values of $\varphi$ ($\varphi < 2$), where there is an excess negative charge (B) at intermediates values of $\varphi$ ($2 < \varphi < 3$) with equal amount of negative and positive charge (C) at high values of $\varphi$ ($\varphi > 3$) when there is an excess of positive charge.

A fraction of the short and flexible Rh-ON chains binds only partially to the longer pDMAEMA chains which implies that only a few of the phosphate anions on the Rh-ON chains are involved in complex formation. The non-neutralized anions provide for the water solubility of the polyplexes and for their negative net charge. Another fraction of the Rh-ONs chains remains free in solution. Electrophoresis indeed confirmed that, at values of $\varphi < 1$, free Rh-ONs are present (data not shown).

We wondered whether, at values of $\varphi < 1$ (Figure 3), the particle size and $\zeta$ correspond to individual polyplexes or aggregated polyplexes. As the molecular mass of an individual polyplex can be estimated some hundreds of thousands g/mol (e.g. in case several Rh-ONs bind to 1 pDMAEMA chain), the hydrodynamic diameter must be lower than the values measured in Figure 3, suggesting that aggregated polyplexes are present. As Figure 4A illustrates, aggregation may occur by Rh-ONs, which bridge individual polyplexes. A striking observation in Figures 3A and 3B is that, at values of $\varphi < 1$, the size of the particles gradually
increases, while their $\zeta$ gradually decreases, upon adding more pDMAEMA chains. We suggest therefore that increasing pDMAEMA concentration in this region lead to more polyplex formation, and that aggregation of these polyplexes results in larger structures with lower net negative charge. DLS reveals that fully aggregated polyplexes are formed for values of $\varphi$ between 1 and 3. For these polyplexes, almost no repulsion occurs between the particles, as $\zeta$ approximates zero, which leads to a clustering of individual polyplexes, as presented in Figure 4B (19). Upon further increasing the concentration of pDMAEMA, $\zeta$ reaches a positive plateau indicating a shortage of Rh-ON to compensate the charge. Electrophoresis data prove that at a high value of $\varphi$ all the Rh-ONs are bound (data not shown). Probably, as illustrated in Figure 4C, as a higher number of pDMAEMA chains are present, the average number of Rh-ONs bound to one chain is strongly decreased and the non-neutralized cations explain the positive net charge of the polyplexes. The electric repulsion between the positively charged pDMAEMA chains in the polyplexes decreases the size of the pDMAEMA/Rh-ON aggregates at higher $\varphi$-values (Figure 3A). However, intermolecular bridging of individual polyplexes, as described above, may still occur and explains why the hydrodynamic diameter is much larger than what is expected for individual polyplexes.

An unanswered question is why the interaction between pDMAEMA and Rh-ONs shows a non-stoichiometric behavior, i.e. why $\zeta$ crosses-over from a negative to a positive value at $\varphi > 1$. Previous studies showed that for many types of cationic polymers, $\zeta$ crosses-over when the number of positive amine charges on the polymers equals the number of negative phosphate groups on DNA (5). Interestingly, upon complexation of salmon sperm DNA instead of ONs to pDMAEMA, $\zeta$ crossed over from a negative to a positive value of $\varphi = 1$ (data not shown).

### 3.2. Fluorescence properties of pDMAEMA/Rh-ON complexes.

Figure 5 shows that upon complexation of Rh-ON to pDMAEMA, its fluorescence decreases. At values of $\varphi$ lower than 1, the fluorescence decreases monotonously when the concentration of pDMAEMA increases. The pDMAEMA/Rh-ON complexes having a charge ratio between 2 and 3 show the lowest fluorescence, while at higher $\varphi$-values their fluorescence again increases. A similar decrease of the fluorescence as seen in Figure 5 was observed in e.g. the complexation of phosphorothioate ONs to polyamine-poly(ethylene

-35-
glycol) copolymers and the complexation of fluorescently labeled DNA to poly-L-lysine (21-22). We hypothesize that the gradual decrease of the fluorescence of the Rh-ONs solutions at low $\varphi$-values is due to a partial extinction of the fluorescence upon binding of the Rh-ONs to the pDMAEMA chains.

![Figure 5](image)

**Figure 5.** Fluorescence of pDMAEMA/Rh-ON dispersions as a function of the charge ratio. The polyplexes were prepared by fast and slow addition of the pDMAEMA solution to the Rh-ON solution, respectively. The Rh-ON concentration in the dispersions was 10 $\mu$g/mL. The fluorescence of the Rh-ON solution in the absence of pDMAEMA (i.e. at $\varphi = 0$) was set to 100 %. Each measuring point is the mean value of measurements on 3 dispersions, which were independently prepared. Each dispersion was measured 3 times.

Following the work of Trubetskoy et al. who recently reported on the self-quenching of fluorescently labeled DNA upon condensation with polycations, we further investigated whether self-quenching occurs in the pDMAEMA/Rh-ONs polyplexes (22). Figure 6 shows the fluorescence of pDMAEMA/Rh-ON polyplexes of different charge ratio, prepared by adding the pDMAEMA solution to a mixture of Rh-labeled and non-labeled ON (“ON + Rh-ON mixture”). While the total ON concentration (10 $\mu$g/mL) was fixed, only the percentage of Rh-labeled to unlabeled ON was varied. Three observations can be made from Figure 6.
First, at all compositions of the “ON + Rh-ON mixture”, adding pDMAEMA decreases the fluorescence of the “ON + Rh-ON mixture”.

Second, at all $\varphi$-values, the higher the amount of labeled ONs in the polyplexes, the stronger the fluorescence of the complexes deviates from the fluorescence of the corresponding “ON + Rh-ON mixture” without pDMAEMA. This can only be attributed to self-quenching, which occurs between the Rh-ONs and which becomes more pronounced when more Rh-ONs are present in the complex.

Figure 6. Fluorescence of pDMAEMA/ON + Rh-ON polyplexes prepared by adding (fast addition method) pDMAEMA to “ON + Rh-ON mixtures”. The x-axis indicates the percentage of Rh-ON in the “ON + Rh-ON mixtures”. $\varphi = 0$ (●) shows the fluorescence of the “ON + Rh-ON mixture” when there was no pDMAEMA present. The values of $\varphi$ are: $\varphi = 0.5$ (■), $\varphi = 1$ (▲), $\varphi = 3$ (▽) and $\varphi = 6$ (▲). The ON concentration in the dispersions was 10 µg/mL.

Third, the extent of self-quenching as a function of $\varphi$, indicated by the slope of the line, increases at low values of $\varphi$, is maximal when $\varphi$ equals 3 and decreases again at high $\varphi$-values. As illustrated in Figure 4, this has to be explained by a change in the average number of Rh-ONs per complex. At low $\varphi$-values, by adding more polymer strands to the oligonucleotide solution, self-quenching can occur due to the formation of multimolecular polyplexes. The Rh molecules come into close proximity of each other and can quench their neighbors. At high $\varphi$-values, self-quenching decreases as the average amount of Rh-ON per complex probably decreases. For monomolecular complexes, self-quenching would probably not occur as all the Rh-ONs are on different pDMAEMA strands.
3.3. Fluorescence correlation spectroscopy on polyplexes.

As explained in Materials and Methods, the fluorescence fluctuations, which are monitored in FCS, are caused by the diffusion of the fluorescently labeled molecules through the laser beam. Upon binding of fluorescent labeled molecules (Rh-ONs) to non-fluorescent ones (pDMAEMA), slow fluctuations would have to appear that could be quantified by autocorrelation analysis. Recently, Meseth et al. studied the resolution limit of FCS for two different particles in solution (23). It was shown that, under optimal conditions, the diffusion times should differ by at least a factor of 1.6 which means that the particles must have a molecular weight difference of a factor of 4 assuming the molecular weight to be proportional to \(D^{-3}\). As the molecular mass of the Rh-ONs was 8253 g/mol, while \(M_n\) of pDMAEMA was estimated to be 60 000 g/mol, FCS was evaluated to study the complexation between Rh-ON and pDMAEMA, moreover as FCS shows potential to be applicable in cellular studies (24-27).

Rhodamine was preferred as the fluorescent label because; compared to fluorescein, its photophysical characteristics are more suitable for FCS measurements. Fluorescein needs a higher laser light intensity to obtain an acceptable value for the counts per molecule; it is more sensitive to photobleaching and gives rise to significant triplet state. However, a main disadvantage of Rh is its ability to stick to all types of materials. Especially, as FCS is applied in the nanomolar range of the fluorescent molecules it is important to take into account this behavior when binding studies are considered. FCS revealed that the storage of Rh-ON solutions in Eppendorf tubes for 10 minutes decreased the number of molecules in the confocal volume element from \(3.9 \pm 0.1\) to \(3.0 \pm 0.1\), which coincides, with a decrease in concentration from 7.1 nM to 5.6 nM. The cuvettes used in the FCS measurements did not adsorb Rh-ONs.

Figure 7 shows a compressed representation of the fluorescence fluctuations in the confocal volume (Figure 7A) and the autocorrelation curve (Figure 7B) of Rh-ONs in buffer. From the autocorrelation function and the mean diffusion time of the molecules \((193 \pm 4 \mu s)\) a diffusion coefficient of \(1.05 \times 10^{-6}\) cm²/s was calculated, which agrees with values in literature. Politz and co-workers measured with FCS a diffusion coefficient of \(0.5 \times 10^{-6}\) cm²/s for a 43-mer ON (27).
Figure 7. FCS measurements on Rh-ONs in buffer: fluorescence fluctuation profile (A), autocorrelation curve (B) and fluorescence intensity distribution (C). The Rh-ON concentration was 0.25 µg/mL.
Figure 8 compares the fluorescence fluctuations of a 30 nM (0.25 µg/mL) Rh-ON solution before and after complexation with pDMAEMA. The association of the Rh-ONs with pDMAEMA clearly influences the fluorescence fluctuation profile.

![Figure 8](image)

**Figure 8.** Fluorescence fluctuation profile for respectively a Rh-ON solution (A) and pDMAEMA/Rh-ON dispersions (B: \( \phi = 8 \) and C: \( \phi = 17 \)). Adding dextran sulfate to the pDMAEMA/Rh-ON dispersions (\( \phi = 17 \)) partially recovered the fluorescence of the baseline, while the highly intense fluorescence peaks almost disappeared (D). The Rh-ON concentration equaled 0.25 µg/mL.

First, the fluorescence intensity significantly decreases. Especially, a gradual decrease can be observed when \( \phi \) of the pDMAEMA/Rh-ON complexes is increased. This agrees with the fluorimetric observations as explained in Figure 5 and 6.

Second, highly intense fluorescence peaks became visible upon complexation of Rh-ON to pDMAEMA. Considering the time frame of one measuring point to be 100 ms and the (estimated) diffusion time of polyplexes to be between 1 and 10 ms (see below), one highly intense fluorescence peak does not necessarily originate from the diffusion of one single, very bright polyplex into the confocal volume. It could also be attributed to the diffusion of a number of less bright polyplexes in the confocal volume during the 100 ms time frame.

As multimolecular pDMAEMA polyplexes were revealed from Figure 6, it is highly likely that the highly intense fluorescence peaks originate from the presence of pDMAEMA chains, which bear several Rh-ON. The heterogeneous distribution of the height of the highly intense fluorescence peaks probably indicates that polyplexes with a polydisperse composition, with regard to the number of Rh-ONs per polyplex, are formed. However, as the
discussion above pointed out, the fluorescence of pDMAEMA/Rh-ON complexes may be influenced by many factors, one can not state that an increase of the height of the highly intense fluorescence peaks by e.g. factor of two is attributed to a doubling of the number of Rh-ONs per complex.

In the measurements of Figure 5, we evaluated to which extent dextran sulfate, an anionic polymer, could destabilize the pDMAEMA/Rh-ON complexes. Upon adding 100 µL of a dextran sulfate solution (1 mg/mL in Hepes buffer) to 900 µL pDMAEMA/Rh-ON dispersion (ϕ = 1), the fluorescence of the pDMAEMA/Rh-ON dispersion partially restored (data not shown), which indicates that dextran sulfate competes with the Rh-ONs for binding to pDMAEMA (28). This could also be observed in the FCS data. Figure 8 shows that upon addition of dextran sulfate the fluorescence is partially restored, while the highly intense fluorescence peaks almost disappear.

Although the highly intense fluorescence peaks indicated the presence of the pDMAEMA/Rh-ON complexes (e.g. in Figure 9A), they greatly disturbed the determination of the autocorrelation function from the fluorescence fluctuations (Figure 9B). Especially, using equation 3 of Chapter 2, it was difficult to calculate the diffusion times and the amount of free and complexed Rh-ON from $G(\tau)$ of the pDMAEMA/Rh-ONs dispersions.

This forced us to analyze the fluorescence fluctuation profile in an alternative way. Recently, a new method was proposed to analyze fluorescence fluctuation profiles of a mixture of different types of fluorescent molecules where one type of the molecules shows much brighter fluorescence with respect to the other, applicable when integration over long time bins has been done (29). Van Craenenbroeck et al. performed FCS on mixtures of fluorescein and highly fluorescent 100 nm polystyrene beads. The fluorescence fluctuation profiles, with the appearance of highly intense fluorescence peaks arising from the passage of the fluorescent beads through the confocal volume, resembled the fluorescence fluctuation profiles of pDMAEMA/Rh-ON dispersions.
Figure 9. FCS measurements on pDMAEMA/Rh-ON dispersion ($\phi = 8$): fluorescence fluctuation profile (A), autocorrelation curve (B) and fluorescence intensity distribution (C). The Rh-ON concentration equaled 0.25 µg/mL.
They showed that a linear relation exists between the total fluorescence observed in highly intense fluorescence peaks in the fluorescence fluctuation profile and the concentration of the highly fluorescent beads in the mixture. We applied this type of analysis to the fluorescence fluctuation profile of the pDMAEMA/Rh-ON dispersions. When only free Rh-ONs were present, the fluorescence intensities in the fluorescence fluctuation profile (Figure 7A) showed a Gaussian distribution (Figure 7C) with a mean value influenced by the mean number of molecules in the confocal volume and their fluorescence quantum yield (29). The fluorescence fluctuation profile of the pDMAEMA/Rh-ON dispersion showed highly intense fluorescence peaks (Figure 9A) whose intensity did no longer belong to the Gaussian distribution (indicated as “highly intense fluorescence peaks”; Figure 9C). We used the Kolmogorov-Smirnov based statistical method, as described by Van Craenenbroeck et al., to determine above which value of the fluorescence intensities the measured intensities no longer contributed to the Gaussian distribution (“highly intense fluorescence peaks”) and were therefore regarded as originating from pDMAEMA/Rh-ON complexes (29). Figure 10 shows the number of the highly intense fluorescence peaks in the fluorescence fluctuation profile of pDMAEMA/Rh-ON complexes. For pDMAEMA/Rh-ON complexes having $\phi$-values lower than 15, increasing $\phi$ gave rise to an increase in the number of highly intense fluorescence peaks. As the Rh-ON concentration was the same in all the pDMAEMA/Rh-ON dispersions, a higher number of pDMAEMA chains may decrease the average number of Rh-ON bound per pDMAEMA chain as discussed above. This may increase the number of polyplexes, which may explain the increase in the number of highly intense fluorescence peaks. However, upon further increasing the number of pDMAEMA chains one could expect that, from a certain value of $\phi$, polyplexes would arise which consist of only one or a few Rh-ONs bound per pDMAEMA chain and whose fluorescence intensities belong to the Gaussian distribution. Such polyplexes will not result in highly intense fluorescence peaks in the fluorescence fluctuation profile and consequently it is expected that the number of highly intense fluorescence peaks would decrease.

While it was unclear whether the size, $\zeta$ and fluorimetric data in Figures 3 and 5 concern individual or aggregated polyplexes, due to the use of highly diluted dispersions in FCS, it is highly probable that the diffusion behavior of individual polyplexes is studied in FCS. This can also be observed from the diffusion time, estimated from the time at which $G$ equals the half of $G(0)$, in Figure 9B: the estimated diffusion time is around 1-10 milliseconds. In case clustered polyplexes are present, the diffusion time would probably be
much higher. The decrease of the number of Rh-ONs per pDMAEMA chains starts from values of $\phi$ around 15 (Figure 10), while this is observed at $\phi$-values of 3 in Figure 3A, this might be a consequence of the much lower concentration of the complexes.

![Figure 10](image_url)

**Figure 10.** Number of highly intense fluorescence peaks observed in the fluorescence fluctuation profile of pDMAEMA/Rh-ON dispersions as a function of the charge ratio. The Rh-ON concentration equaled 0.25 µg/mL.

Generally, the FCS data could be interpreted based on the properties of the pDMAEMA/Rh-ON complexes as revealed from other methods. Although, a more quantitative interpretation of the FCS data remains complicated. Especially, the polydispersity of the complexes (both in fluorescence as in molecular size) and the fluorescence quenching upon binding the Rh-ONs to the pDMAEMA chains largely hamper the interpretation of the fluorescence fluctuation profiles.
4. Summary and conclusions

DLS, $\zeta$, fluorimetric and gel electrophoresis experiments, which could be performed when a micromolar Rh-ON concentration (10 µg/mL) was used, pointed out that complexation occurs between Rh-ONs and pDMAEMA. In the dependence of size, $\zeta$ and fluorescence of the pDMAEMA/Rh-ON polyplexes on their charge ratio three regions could be clearly distinguished. (1) At values of $\varphi < 1$, only a part of the Rh-ON chains binds to the pDMAEMA chains (as revealed from gel electrophoresis) by a few of their phosphate anions. The non-neutralized anions of the bound ONs provide for the net negative charge of the polyplexes. As the molar mass of an individual polyplex has to be in the order of some hundreds of thousands g/mol and as the hydrodynamic diameters measured on the dispersions ranged from about 80 nm to 250 nm, it was suggested that aggregation of individual polyplexes occurs by e.g. Rh-ONs which bridge individual polyplexes. At $\varphi < 1$, the gradual decrease of the fluorescence of the polyplex dispersions upon increasing the amount of pDMAEMA was explained by self-quenching of the fluorescence, as, upon binding, the ONs significantly concentrate. Consequently, the quenching as observed in the fluorescence measurements confirmed the existence of “multimolecular complexes”. (2) At values of $\varphi$ between 1 and 3, DLS revealed that fully aggregated polyplexes were formed. As in this region $\zeta$ crosses-over from a negative to a positive value and approximates zero, the clustering of the individual polyplexes was attributed to the absence of any repulsion between the particles. (3) At values of $\varphi > 3$, the polyplexes were positively charged indicating that the polycations in excess incorporated into the complexes. Electrophoresis data indicated that all the Rh-ONs were bound. As in this region the fluorescence of the dispersions again increased it was suggested that, when a higher number of pDMAEMA chains are present, the average number of Rh-ONs bound to one chain begins to decrease which results in less quenching of the fluorescence. It was suggested that the free cations explain the positive net charge of the polyplexes and that the electric repulsion between the positively charged pDMAEMA chains on the polyplexes probably decreased the size of the pDMAEMA/Rh-ON complexes.

FCS experiments, which were performed on polyplexes prepared from nanomolar solution of Rh-ONs, also revealed that interactions occur between the Rh-ONs and pDMAEMA. In agreement with the fluorimetric measurements on the micromolar pDMAEMA/Rh-ON dispersions, the fluorescence intensity in the fluorescence fluctuation profiles showed a similar $\varphi$ dependence. Specifically, highly intense fluorescence peaks
appeared in the fluorescence fluctuation profile when complexation between Rh-ON and pDMAEMA occurred. As multimolecular pDMAEMA polyplexes were revealed from fluorimetric measurements on the micromolar pDMAEMA/Rh-ON dispersions, it is very likely that the highly intense fluorescence peaks originated from the presence of pDMAEMA chains, which bear several Rh-ONs. For $\varphi < 15$, the number of highly intense fluorescence peaks increased upon adding more pDMAEMA chains to the Rh-ON solution. This was expected as, the more pDMAEMA chains, the more polyplexes and the lower the number of Rh-ONs per polyplex as suggested from the fluorimetric measurements. As expected, from a certain value of $\varphi$ (around 15) the number of highly intense fluorescence peaks dropped as pDMAEMA/Rh-ON polyplexes arose which probably consist of only one or a few Rh-ONs bound per pDMAEMA.

5. References


Chapter 4

Photon Counting Histogram Analysis to analyze the Fluorescence Fluctuation of oligonucleotide/cationic polymer complexes

1. Introduction

In Chapter 3 we have demonstrated that correlation analysis was not a straightforward method to analyze fluorescence fluctuations of the pDMAEMA/ON polyplexes as, due to quenching, the quantum yield of the fluorescent oligonucleotides changed substantially upon binding to the polymer. Moreover, the very bright species, due to the fact that several fluorescent labeled ONs are present in one complex dominated the fluorescence fluctuation profile. Therefore we were forced to study the distribution of the amplitudes of the fluorescence fluctuations instead of their time course. As described in Chapter 3 we used the method as established by Van Craenenbroeck et al. to study the distribution of the amplitude of the fluorescence fluctuations of pDMAEMA/ON complexes (1, 2). The fluorescence fluctuations of the pDMAEMA complexes in Figures 7, 8 and 9 of Chapter 3 were displayed with a time resolution of 100 ms.

By the time we investigated how to extract information from the amplitude of the signals in the fluorescence fluctuation profile, the group of Prof. Gratton (Illinois, USA) developed the “photon counting histogram analysis” (PCH) (3). Essential in this method is that the fluorescence fluctuations are recorded in sub-ms resolution.
In this chapter we tried to find answers to the following questions:

- Do we lose essential information by using 100 ms time bins instead of sub-ms time intervals? As explained above, the fluorescence intensities in Figures 7, 8 and 9 of Chapter 3 were recorded with a time resolution in the µs-range but were displayed with a time resolution of 100 ms. Therefore, the fluorescence intensities measured at µs resolution were summated to obtain 100 ms time intervals. On the contrary, the home-built fast data acquisition card, which was used in the experiments described below, allows access to the complete time resolved sequence of the photon events.

- Is it possible by PCH analysis of the fluorescence fluctuations to extract more (quantitative) information on the polyplexes?

2. Photon Counting Histogram analysis

Essential for PCH analysis is that a complete access to the photon events is required; in principle every single photon must be detected separately (Figure 1A). The current data acquisition cards are almost able to fulfill this requirement as can be seen in Figure 1A: the time intervals are now about 25 ns and hence no more than about 10 photons are counted per time interval (4). This explains why a typical PCH histogram shows a super-Poissonian profile (Figure 1B) instead of a Gaussian distribution (Figure 2B) like for the data displayed by Confocor I (Figure 2A). The larger the time bins in which the data are recorded, the higher the number of photons detected in each bin and the more a Poissonian distribution approaches a Gaussian one.
Figure 1. Time sequence (sub-µs time interval) of the number of photons as detected by the fast data acquisition card (A). Histogram of the number of photons showing a Poissonian profile (red) (B).

Figure 2. Confocor I records photons with a time resolution in the sub-µs range. However the photons are summated and displayed in time intervals of 100 ms (A). The histogram shows a Gaussian profile (red) (B).
Three sources of fluctuations account for the shape of the photon counting histogram. When photons reach the photodetector and are converted into photon counts, the detector generates inevitable shot noise. Even when the light source has an absolute constant intensity, the photon count is not constant and the resulting photon count distribution is given by a Poissonian distribution. The additional broadening of the Poissonian distribution is caused by (1) fluorescence intensity fluctuations, originating from the diffusion of molecules in the spatially inhomogeneous excitation profile and (2) by the particle number fluctuations within the observation volume. The fact that the count distribution follows super-Poissonian instead of Poissonian statistics is crucial for extracting information from the histogram.

PCH analysis uses exactly the same experimental fluorescence fluctuation data as used for autocorrelation analysis, but focuses on a different property of the signal. In PCH analysis, species are separated based on molecular brightness rather than on their molecular weight. The distribution of photon counts is uniquely described by two parameters for each fluorescent species: the molecular brightness of the particle ($\varepsilon$) and the average number of particles within the observation volume ($N$). The concept is quite straightforward. A particle with a given brightness produces a characteristic intensity fluctuation as it travels through the observation volume. If another particle with a higher molecular brightness enters the observation volume, a strong intensity fluctuation occurs in the fluorescence signal. The distribution of the molecular brightness values and their recurrence frequency is hidden in the statistics of the amplitude distribution. Therefore molecular brightness values of the particles and their respective concentrations can be extracted from the amplitude statistics. The deviation of the PCH from a Poisson function is most pronounced in the tail and therefore a logarithmic data representation is very useful to make the super-Poissonian character of the PCH more visible.
3. Materials and methods

Fluorescence fluctuations measurements.

For fluorescence fluctuation experiments “single-photon” and “two-photon” excitation set-ups were used in this study. As the single-photon excitation system the Confocor I (Zeiss-Evotec, Jena-Hamburg, Germany) as described in Chapter 3 was used. The integration time in the fluorescence fluctuation profiles was about 100 ms.

The FCS measurements which were analyzed by PCH analysis were done on a two-photon excitation system, a mode-locked Ti:Sapphire laser (Mira 900; Coherent Inc., Santa Clara, CA) pumped by a diode-pumped intracavity doubled Nd:YVO4 Vandyke laser (Verdi, Coherent Inc., Santa Clara, CA), was used as the two-photon excitation source. Two-photon excitation is the simultaneous absorption of two photons (usually of the same energy) by a molecule, which is normally excited by a single photon with twice the energy (6). For example, a fluorophore can reach the excited state either by absorption of a single 400 nm photon or absorption of two 800 nm photons. The advantage of this way of excitation is that the wavelength of the fluorescence and the wavelength of the excitation light are widely separated. However, the main advantage is its inherent optical sectioning effect. The high photon flux required for two-photon excitation only occurs at the microscope focus. Especially important for cellular experiments is that no photodamage will occur outside the excitation volume.

For all measurements, an excitation wavelength of 780 nm was used. Photon counts were detected with an avalanche photodiode (APD) (SPCM-AQ-161; EG&G). The output of the APD unit, which produces TTL pulses, was directly connected to the data acquisition card. The recorded and stored photon counts were analyzed with PV-WAVE version 6.10 (Visual Numerics). A Zeiss Axiovert 135 TV microscope (Thornwood, NY) with a 63x Plan Apochromat oil immersion objective (NA = 1.4) was used. Measurements on the Rh-ON solution and polyplex dispersions were performed at room temperature on samples of 1000 µL. Each sample (differing in ϕ) was prepared 3 times (n = 3) and the fluorescence fluctuations were recorded during 500 or 800 s for the Rh-ON solutions and polyplex dispersions, respectively. The measurements were performed in the time-mode; the photon counts were sampled at 20 kHz resulting in a sampling time of 50 µs. The PCH analysis of the fluorescence fluctuations was performed as described in detail by Chen et al (3).
4. Results & Discussion

As described in this chapter, the PCH is experimentally determined from the fluorescence fluctuation profile and shows the probability to detect a certain number of photons ($k$) per sampling time. To try PCH analysis we measured the fluorescence fluctuation profiles of the polyplexes on the PCH-instrument using a sampling time of 50 µs (compared to 100 ms for Confocor I measurements).

Figure 3A shows a time averaged trace of the photon counts of an 8 nM Rh-ON solution (in the absence of pDMAEMA) as measured by the two-photon excitation setup. The symbols connected by the solid line in the semilogarithmic plot of Figure 3B show the corresponding photon count distribution. Based on the computer algorithm as developed by Chen et al., which fits the experimental PCH, we were able to extract the average number of Rh-ONs in the observation volume ($N$) and the molecular brightness of the Rh-ONs from the fitting (3). The fit recovered $N$ to be 0.66 while $\varepsilon$ equaled 5200 cpsm (counts per second per molecule). As Figure 3C shows, the residuals between the data and the fit of the PCH (displayed in units of standard deviations) vary randomly and yield a reduced $X^2$ close to 1, indicating a good description of the data by a single species model.
Figure 3. Fluorescence fluctuation profile (as measured by PCH set-up) for 8 nM Rh-ON solution (A). (B) shows the corresponding photon counting histogram expressed in a semilogarithmic scale. (C) shows the residuals between the data and the fit of the PCH (displayed in units of standard deviations).

Figure 4A shows an integrated time trace of the photon counts upon complexation of Rh-ONs to pDMAEMA ($\varphi = 1$) as measured by the PCH setup. As observed in the single-photon measurements on Confocor I (Chapter 3), the fluorescence intensity decreases while highly intense fluorescence peaks arise. The highly intense fluorescence peaks affect the PCH at higher counts (Figure 4B). By fitting these data to a 2-species model, we resolved 2 species with totally different molecular brightness values: a rather dim species with $\varepsilon_1 = 5200$ cpsm ($N_1 = 0.69$), which corresponds to the free oligonucleotide and a much brighter species with $\varepsilon_2 = 70000$ cpsm ($N_2 = 0.001$), the multimolecular complex.
Figure 4. Fluorescence fluctuation profile (as measured by PCH set-up) of a pDMAEMA/Rh-ON dispersion having a value of $\varphi = 1$ (A) and the corresponding photon counting histogram with experimental data (■) and PCH fit (●) (B). The Rh-ON concentration equaled 8 nM. The rectangle shows the fluorescence data, between two highly intense fluorescence peaks, used for PCH analysis. (C) shows the residuals between the data and the fit of the PCH (displayed in units of standard deviations).

However, a strictly quantitative interpretation of the PCH parameters for the bright species remains problematic. The difficulty lies in the ‘rarity’ of the events associated with the bright species and the heterogeneity of the formed pDMAEMA/Rh-ON complexes. To illustrate this point, consider the fluctuation trace in Figure 4A. If we split the data around 400 seconds into two sets (set 1 and set 2) and analyze each sequence individually, we arrive at the same result for the dim species. However, for the bright species the number of molecules and the brightness differ in both cases (data not shown). This fact is readily explained by visual inspection of Figure 4A: no strong intensity fluctuations are observed for the first 400 seconds, while a few events are visible in the later part of the fluctuation trace. The molecular brightness as obtained by PCH analysis of the fluorescence fluctuations between highly intense fluorescence peaks (the fluctuations indicated by the rectangle in Figure 4A) allowed
us to conclude that these fluctuations originated from the diffusion of the free Rh-ONs as $\varepsilon$ (5000 cpsm) agreed quite well with $\varepsilon$ of free Rh-ONs as obtained from Figure 3.

Figure 5 presents an integrated time trace of photon counts of a pDMAEMA/Rh-ON dispersion having $\varphi = 4$. The fluorescence intensity further decreases while the amount of highly fluorescence peaks significantly increases which might indicate that the number of polyplexes increases. Despite the larger number of highly intense fluorescence peaks, the interpretation of the PCH analysis remains difficult. Probably, the sample is very heterogeneous and even if many events are observed, the number of events per particular polyplex is still rare. The insert in Figure 5 shows the complete time resolved sequence of the photon events underlying the fluorescence peak indicated by the red arrow in the integrated time trace of photon counts. The peak in the integrated time trace seems to originate from the passage of one bright pDMAEMA/Rh-ON complex. Remarkable is the long residence time of the complex in the confocal volume, almost 500 ms. Also interesting is the fine structure in the pattern; upon passing through the confocal volume, at a certain moment the fluorescence significantly drops. Three hypotheses may explain this pattern: (1) although highly unlikely, immediately after the passage of a polyplex through the confocal volume a second polyplex may come in; (2) considering the dynamic features of the polyplexes, quenching and dequenching phenomena in the polyplex may cause blinking of the complex while it stays in the confocal volume; (3) the polyplex may pass through the focus, consequently it may move toward the periphery of the confocal volume element and may move back into the focus.

To rule out optical laser trapping, we repeated the same experiments at lower laser powers. The use of high laser power often results in an entrapment of the particles in the laser beam, resulting in a longer residence time of the particles in the observation volume. We observed qualitatively, the same features and residence times as at the higher laser power; a strong argument against a mechanism based on laser trapping.
Figure 5. Fluorescence fluctuation profile (as measured by PCH set-up) of a pDMAEMA/Rh-ON dispersion having a value of $\varphi = 4$ (A) and the corresponding photon counting histogram with experimental data (■) and PCH fit (■) (B). The Rh-ON concentration equaled 8 nM. The insert shows the complete time resolved sequence of the photon events of the fluorescence peak that appeared at 183 s in the integrated fluctuation profile. (C) shows the residuals between the data and the fit of the PCH (displayed in units of standard deviations).

As explained in Chapter 3, at a high excess of pDMAEMA (i.e. at extremely high values of $\varphi$) one expects the presence of monomolecular (i.e. one Rh-ON per pDMAEMA chain) instead of multimolecular complexes. This was indeed observed in the fluorescence fluctuation profile of pDMAEMA/Rh-ON dispersions having $\varphi = 1000$ (Figure 6) which did not show highly intense fluorescence peaks. This allowed PCH analysis with a single species model, revealing values for $N$ and $\varepsilon$ of respectively 0.52 and 8700 cpsm. Remarkably, $\varepsilon$ of such monomolecular pDMAEMA/Rh-ON complexes was higher compared with $\varepsilon$ of the free Rh-ON (5200 cpsm).
Figure 6. Fluorescence fluctuation profile (as measured by PCH set-up) of the pDMAEMA/Rh-ONs dispersion having a value for $\phi = 1000$. The Rh-ON concentration equaled 8 nM.

The increase in $\varepsilon$ agrees with the increase in the fluorescence intensity of the fluctuation profiles (from 3500 Hz in Figure 3 to 4600 Hz in Figure 6) and was also reflected in the fluorescence of the polyplexes as measured using a conventional fluorimeter. As shown in Figure 7 the fluorescence of the pDMAEMA/Rh-ON complexes from a charge ratio of 20 onwards is higher than the fluorescence of the free Rh-ON solution. Although the reason is unclear, the presence of the pDMAEMA chains results in another surrounding of the Rh-ONs, which may alter their fluorescence properties. Several groups indeed reported that the fluorescence of dyes such as 5-carboxytetramethylrhodamine could be partially quenched upon binding to an oligonucleotide, the degree of quenching being dependent on the fluorophore’s proximity to purines and its position in the oligonucleotide (7). It is hypothesized that the pDMAEMA chains might lead to a “dequenching” of the Rh molecules bound to the ONs.
5. Conclusion

In general, complexation between Rh-ONs and pDMAEMA can be observed in the fluorescence fluctuation profiles. Although, we must admit it is hard to obtain quantitative information. Contrary to the analysis method as developed by Van Craenenbroeck et al., PCH allows the calculation of the number of molecules (N) in the confocal volume element as well as the molecular brightness (ε) of the particles. When highly intense fluorescence peaks do not appear in the fluorescence fluctuations (at high charge ratios), indicating the existence of monomolecular complexes, the method of Van Craenenbroeck et al. is not applicable, whereas PCH analysis can be applied resulting in the molecular brightness and the number of these polyplexes in the confocal volume. However a necessary condition for the application of this method is that sufficient data can be obtained for all the particles in solution.

When intensity bursts appear in the fluctuation profile, due to the presence of multimolecular complexes, the interpretation of the PCH analysis remains difficult. Also the heterogeneous distribution of the height of the highly intense fluorescence peaks probably indicates that polyplexes with a polydisperse composition, with regard to the number of Rh-ONs per polyplex, are formed. However, we must stress that one cannot state that the height of the highly intense fluorescence peaks is directly proportional to the number of Rh-ON per complex, because the fluorescence of pDMAEMA/Rh-ON complexes may be influenced by many factors. Also, considering the inhomogeneous excitation profile in the confocal volume, the fluorescence of a polyplex as detected by the photon detector depends on the way it moves through the confocal volume. An event in which a relatively bright complex enters the
periphery of the laser beam may not be distinguished from an event in which a less fluorescent complex passes through the focus (8).

Therefore, if one is interested in the analysis of rare events (like detection of β-amyloid proteins in cerebrospinal fluid of Alzheimer’s patients), preferably the method of Van Craenenbroeck et al. is used in which the number of fluorescence bursts can be considered as a relative amount of the number of multimolecular complexes in solution (9). In this case Poisson fit procedures can not be longer applied as rare events do not allow us to sample long enough to sample the proper statistics. In case the solution contains a few rare aggregates, several hundred thousand events must be collected before one can be sure that the fit is correct.

The only condition for working with Confocor I is, that when data must be compared, they must be collected with the same total measurements time, as each data set always contains 509 intensity values, independent on the measurement time used. We must also emphasize that the size of the time bin width is not unlimited. When using time bins much larger than the diffusion time of the bright particles, this will lead to a decrease in the average count rate in the time bin due to averaging out of the intensities, consequently a reduced discrimination of spikes is possible. In this case it is possible that essential information is lost. However, small differences in fluorescence signal between species can not be resolved by the latter method. On the contrary, Müller et al. demonstrated that PCH has the possibility to separate a mixture of biomolecules with a brightness ratio difference of two, which is of considerable biological interest (10).

When comparing advantages and disadvantages of both methods, we agree with Van Craenenbroeck et al. that both methods are able to provide complementary information, which should prove useful in tackling biological problems (11). One can state that the data acquisition time window can be chosen appropriate to the system studied. For analyzing rare events, time bin widths of $10^{-3}$ s are advised; otherwise inadequate statistics will influence data analysis. A sub-ms time window ($10^{-5} – 10^{-4}$ s) is preferable when confronted with small difference in molecular brightness per molecule (analysis of mixtures of binary dyes, single and double labeled proteins). Furthermore, the observations seen with the two photon set-up match the results obtained with the Confocor I (one photon set-up).
6. References


Chapter 5

Interactions between oligonucleotides and cationic polymers investigated by Fluorescence Correlation Spectroscopy.

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1. Introduction

At the moment the knowledge on the self-assembly of cationic polymers and oligonucleotides (ONs) is limited (1). However, to develop efficient DNA vectors, it is essential to be able to synthesize well-defined DNA complexes and characterize their physicochemical properties. Especially important is the understanding of their biological behavior. To obtain real breakthroughs in the design and the understanding of the dissociation of DNA complexes in cellular environment, there is a need for physicochemical methods, which allow us to characterize these critical steps not only in vitro but also in the cytoplasm of the cell where the dissociation has to occur.

With this purpose in mind we have introduced in Chapter 3, fluorescence correlation spectroscopy (FCS) for studying the complexation of ONs to poly(2-dimethylamino)ethyl methacrylate (pDMAEMA) (2, 3). FCS works on a microscopic scale and, consequently, shows potential to be applied in cells (4). As frequently new types of cationic polymers are developed and as many types of cationic polymers are currently under investigation for DNA therapy, the present chapter aims to evaluate to which extent FCS is generally applicable in the characterization of the complexation of DNA to cationic polymers. Polyplexes formed by three different types of cationic polymers were investigated. We have compared complexes based upon pDMAEMA, a linear polymer with positively charged side groups (discussed in...
Chapter 3) with core-shell polyplexes based on poly(ethylene glycol)-poly-ethylene-imine (pEG-pEI), a block copolymer with a hydrophilic pEG segment (Figure 1) (1, 5, 6). Several groups reported on the increased activity of ONs incorporated in such systems (7).

![Figure 1. Structure of the block copolymer pEG-pEI.](image)

Also we investigated the complexation between ONs and dendrimers which are uniform in size, show a highly branched spherical structure and have a surface covered with charged primary amino groups (for the structure see Table 1, Chapter 1) (8).

2. Materials and Methods

2.1 Cationic polymers.

Poly(ethylene glycol)-poly-ethylene-imine (pEG-pEI) was synthesized and characterized as described by Vinogradov et al. (1). The total content of nitrogen was 3.75 µmol/mg polymer. The weight average molecular weight $M_w$ was determined to be 12400 g/mol by static light scattering.

DAB-dendrimer-(NH$_2$)$_{64}$ (DAB$_{64}$) was synthesized by published methods (9, 10). This type of dendrimers has 1,4-diaminobutane (DAB) as central core molecule and bears 64 surface charges (amine groups). The molar mass is 7168 g/mol.

2.2. Preparation of polyplexes.

Complexes with different w/w (weight of the cationic polymer/weight of the Rh-ON) ratios were prepared: a w/w value of 1 indicates that an equal mass of cationic polymer and Rh-ON was present in the polyplex dispersion. The Rh-ON concentration in the polyplex dispersion was always 10 µg/mL (1.2 µM). The complexes (varying in w/w ratio) were
prepared by adding in one step different volumes of the cationic polymer stock solution (in Hepes buffer at pH 7.4) to a fixed volume of the Rh-ON stock solution. After addition of the polymer solution, the dispersion was vortexed for 10 seconds. To obtain the final Rh-ON concentration of 10 µg/mL, the dispersions were further diluted with 20 mM Hepes buffer at pH 7.4. The polyplexes were allowed to equilibrate for 30 min at room temperature before use. Particle size, zeta potential and fluorescence measurements were all done on the same cationic polymer/Rh-ON dispersions.

For FCS measurements, the cationic polymer/Rh-ONs dispersions were prepared as described above. However, the final Rh-ON concentration equaled 0.2 µg/mL (24 nM).

2.3. Agarose gel electrophoresis on the polyplexes.

The polyplex dispersions used in the gel electrophoresis experiments were prepared as described above but contained 26.7 µg/mL Rh-ON (3.2 µM). After their preparation the polyplexes were allowed to equilibrate for 30 min at room temperature. 30 µL of these dispersions was mixed with 5 µL of a 50% sucrose solution in distilled water and placed in the wells of a 1.1% agarose gel. A TBE buffer was used containing 10.8 g/L Tris base, 5.5 g/L boric acid and 0.58 g/L EDTA. A potential of 100 V was applied for 60 min. The oligonucleotides were detected based upon their fluorescence.

2.4. Exchange of Rh-ON in polyplexes with dextran sulfate as monitored by gel electrophoresis.

pDMAEMA/Rh-ON, pEG-pEI/Rh-ON and DAB64/Rh-ON polyplex dispersions were prepared, all having a w/w ratio of 4 and containing 26.7 µg/mL Rh-ON. Thirty min after their preparation, dextran sulfate, at various concentrations (10, 50, 100, 150, 200, 250, 300, 400 and 500 µg/mL) was added to the cationic polymer/Rh-ON complexes. Another 30 min later, 30 µL of these mixtures were diluted with 5 µL of a 50% sucrose solution in distilled water and placed in the wells of a 1.1% agarose gel to start the electrophoresis experiments as described above.

2.5. Fluorescence correlation spectroscopy
A commercial FCS instrument (Confocor, Zeiss-Evotec, Jena-Hamburg, Germany) was used. The beam of the HeNe laser (543 nm) was focused at about 180 µm above the bottom of the cuvettes (Nalge Nunc International, Naperville, IL), which contained the Rh-ON solutions or polyplex dispersions. Laser power was adapted by inserting a neutral density filter. For FCS measurements on the cationic polymer/Rh-ON complexes, 200 µL of the dispersions was prepared as previously described in an Eppendorf tube (Eppendorf, Hamburg, Germany), vortexed and immediately transferred into the FCS Nunc cuvettes to begin the FCS measurement. In each FCS experiment the fluorescence fluctuations were measured for 50 s.
3. Results & Discussion

3.1 Complex formation as revealed from dynamic light scattering and zeta potential

Figure 2 shows the results of size and $\zeta$ measurements on the different types of polyplexes as a function of their w/w ratio.

As already discussed in Chapter 3, for pDMAEMA/Rh-ON dispersions, at low values of w/w, the size of the polyplexes gradually increases upon increasing the amount of
pDMAEMA. At w/w of 2, aggregation of the pDMAEMA/Rh-ON polyplexes occurs which is attributed to the almost neutral ζ of the polyplexes. Further increasing the amount of pDMAEMA results in a positive ζ, which probably indicates that a charged polycation ‘corona’ surrounds the complexes. The positive charge of the pDMAEMA/Rh-ON polyplexes seems to prevent aggregation (Figure 2A). Electrophoresis data proved that at high values of w/w all the Rh-ONs were bound (Figure 6).

As Figure 2A shows, in case of pEG-pEI, the size of the complexes hardly changes upon varying the w/w ratio. While at low w/w values ζ sharply increases (Figure 2B), at higher w/w ratios ζ levels off and remains neutral. It indicates that the excess of pEG-pEI does not incorporate into the electroneutral particles, in contrast to the pDMAEMA/Rh-ON polyplexes at high w/w value. This (nearly) neutral value of ζ is consistent with the formation of a core-shell structure in which a pEG corona surrounds the ONs complexed to the pEI segments (Figure 5, Chapter 1) (11, 12). The pEG corona prevents aggregation as clearly observed from the DLS data (Figure 2A).

The addition of DAB₆₄ to the Rh-ON solutions results in large aggregates, comparable with the complexation of pDMAEMA to Rh-ONs. A main difference with pDMAEMA is that only at extremely high amounts of DAB₆₄ (w/w > 15) a decrease in the particle size can be observed, although positively charged DAB₆₄/Rh-ON polyplexes exist already at w/w around 5 (Figure 2B). Large aggregates by complexation of dendrimers with plasmid DNA were also reported by Kukowska-Latallo (13).

3.2. Complex formation as revealed from fluorescence measurements

Figure 3 shows that at lower w/w values the fluorescence of the Rh-ON solution decreases upon complexation with all types of cationic polymers studied. While the maximal degree of quenching seems to be independent on the type of cationic polymer, there was a clear difference in the degree of quenching at higher w/w values.
Figure 3. Fluorescence (as measured by a fluorimeter) of pDMAEMA/Rh-ON (■), pEG-pEI/Rh-ON (▲), and DAB64/Rh-ON (●) dispersions as a function of the w/w ratio. The Rh-ON concentration in all the dispersions was 10 µg/mL; consequently, the number of Rh-ON chains was the same in all the dispersions. The fluorescence of the Rh-ON solution in the absence of cationic polymer (i.e. at w/w = 0) was set to 100% (n = 3). The insert on the right is a close up of the fluorescence of the dispersions having a w/w ratio between 0 and 5.

In Chapter 3 we showed experimental evidence that the decrease of the fluorescence of Rh-ONs upon complexation with pDMAEMA is attributed to partial quenching of the fluorescence of the Rh-label due to presence of several Rh-ON chains in one complex (i.e. “multimolecular complexes”) (4). Thereby, the labels are in close proximity and quench each other. The increased fluorescence of the pDMAEMA/Rh-ON polyplexes at higher w/w was explained by a decrease in the number of Rh-ON per polyplex: at high w/w values, a higher number of polyplexes but with a lower number of Rh-ONs per polyplex were present resulting in less quenching between the Rh labels. Also when pDMAEMA/Rh-ON polyplexes with high w/w were prepared by titrating a pDMAEMA/Rh-ON dispersion having low w/w with a pDMAEMA solution (instead of adding the pDMAEMA solution in one step to the Rh-ON stock solution as in Figure 3), the fluorescence increased at high w/w values (Figure 4). This indicated that a “redistribution” of the Rh-ONs took place: Rh-ONs originally present in a specific complex were released from this complex to become attached to other cationic polymers. As discussed in Chapter 3, the reason why at high w/w values the fluorescence exceeds largely the fluorescence of the free Rh-ON solution is unclear but must be attributed
to the presence of the pDMAEMA chains which probably results in another surrounding of the Rh-ONs which may alter their fluorescence properties.

![Graph showing fluorescence of pDMAEMA/Rh-ON dispersions at different w/w ratios.](image)

**Figure 4.** Fluorescence (as measured by a fluorimeter) of pDMAEMA/Rh-ON (▲) dispersions at different w/w ratio’s obtained by titrating 10 µg/mL Rh-ON with pDMAEMA stock solution. The fluorescence of the Rh-ON solution in the absence of cationic polymer (i.e. at w/w = 0) was set to 100 % (n = 3).

In case of pEG-pEI, at high w/w ratios the fluorescence remained constant indicating that the structure of the pEG-pEI/Rh-ON complexes at high w/w ratios seems similar to the one at lower w/w ratios. This is supported by Figure 2: size and ζ of the complexes hardly changed at higher w/w ratios. Also when the Rh-ON solutions were titrated with pEG-pEI solutions, the fluorescence remained constant indicating that a “redistribution” of the Rh-ON chains did not occur (Figure 5). It seems that the Rh-ON chains are entrapped in the core of the complexes and are not able to leave the complex to become attached to other pEG-pEI strands.
CHAPTER 5

Figure 5. Fluorescence (as measured by a fluorimeter) of pEG-pEI/Rh-ON (%) dispersions at different w/w ratio’s obtained by titrating 10 µg/mL Rh-ON with pEG-pEI stock solution. The fluorescence of the Rh-ON solution in the absence of cationic polymer (i.e. at w/w = 0) was set to 100 % (n = 3).

Like for the pDMAEMA/Rh-ON polyplexes, the fluorescence of the DAB₆₄/Rh-ON polyplexes increased at higher w/w values, however only from w/w value > 15 onwards.

3.3 Complex formation as revealed from agarose gel electrophoresis

Results of agarose gel electrophoresis experiments on pDMAEMA/Rh-ON polyplexes showed that when the amount of pDMAEMA was increased, the amount of free Rh-ON was diminished, while the amount of complexed Rh-ON (which remains in the slots) increased (Figure 6). From a w/w value of 2 onwards, all the Rh-ON chains were associated to the pDMAEMA chains. This is exactly the w/w ratio at which $\zeta$ approximates zero (Figure 2B) while the fluorescence of the pDMAEMA/Rh-ON polyplexes was minimal at this w/w value (Figure 3 and 4).

Similarly, in case of pEG-pEI, the amount of free Rh-ON was also decreasing upon adding more pEG-pEI, while no free Rh-ON could be detected anymore from a w/w value of 2 onwards (Figure 7). In case of DAB₆₄ all the Rh-ONs were already associated to the cationic polymer at a w/w value of 0.5. The insert in Figure 3 shows that the maximal degree of fluorescence quenching, which can be expected to occur when all the Rh-ONs are bound, was also obtained at a w/w value around 0.5. The reason why complete binding of Rh-ONs to
DAB\textsubscript{64} occurs at a lower w/w value is probably attributed to the higher charge density of DAB\textsubscript{64} compared to pDMAEMA and pEG-pEI.

**Figure 6.** Gel electrophoresis on pDMAEMA/Rh-ON complexes with different w/w values. The Rh-ON concentration in all the dispersions is 26.7 µg/mL Rh-ON. Lane 1 and 10 contain Rh-ON alone. Lane 2-9 contain pDMAEMA/Rh-ON dispersions having a w/w value of respectively 0.2; 0.5; 0.7; 1; 1.5; 2; 3 and 5.

**Figure 7.** Gel electrophoresis on pEG-pEI/Rh-ON complexes with different w/w values. The Rh-ON concentration in all the dispersions is 26.7 µg/mL Rh-ON. Lane 1 and 11 contain Rh-ON alone. Lane 2-10 contain pEG-pEI/Rh-ON dispersions having a w/w value of respectively 0.2; 0.5; 0.7; 1; 1.5; 2; 3; 5 and 10.

### 3.4 Complex formation as revealed by FCS

As many types of cationic polymers are currently under investigation for antisense therapy, we were interested to evaluate to which extent FCS is generally applicable in the
characterization of the complexation of ONs to cationic polymers like pEG-pEI or dendrimers.

The “A-data” in Figure 8 and 9 are the fluorescence fluctuations in the excitation volume of free Rh-ONs (24 nM in Hepes buffer pH 7.4). The association of the Rh-ONs with the cationic polymers (pDMAEMA and DAB$_{64}$, respectively) clearly influenced the fluctuation profiles (B series in Figure 8 and 9). Similar observations were made in case of pEG-pEI (data not shown).

![Figure 8](image-url)

**Figure 8.** Left panel: fluorescence fluctuations in the excitation volume of the FCS instrument for a Rh-ON solution (A) and pDMAEMA/Rh-ON dispersions (B: w/w =10). The Rh-ON concentration equaled 0.2 µg/mL (24 nM). Right panel: adding 3 µg/mL dextran sulfate (C) to the pDMAEMA/Rh-ON dispersions partially recovered the fluorescence of the baseline. The addition of 30 µg/mL (D) dextran sulfate resulted in a partial recovery of the fluorescence and a disappearance of the highly intense fluorescence peaks.
Figure 9. Left panel: fluorescence fluctuation profile for respectively a Rh-ON solution (A) and DAB$_{64}$/Rh-ON dispersions (B: w/w = 10). The Rh-ON concentration equaled 0.2 µg/mL (24 nM). Right panel: adding 3 µg/mL dextran sulfate (C) to the DAB$_{64}$/Rh-ON dispersions did not influence the fluorescence fluctuation profile of the polyplex. The addition of 12 µg/mL (D) and 30 µg/mL dextran sulfate (E) results respectively in a partial and total recovery of the fluorescence and an almost complete disappearance of the highly intense fluorescence peaks.

For all the polymers studied, the fluorescence intensity in the excitation volume significantly decreased which agreed with the fluorimetric measurements (Figure 3). Moreover, highly intense fluorescence peaks appeared in the fluorescence fluctuations which were assumed to derive from the diffusion of ‘multimolecular’ complexes (i.e. complexes bearing numerous ON molecules, each ON molecule being labeled with a fluorescent tag) into the excitation volume. The diffusion coefficient as obtained by autocorrelation analysis of the fluorescence fluctuations between highly fluorescence peaks (further indicated as “baseline”) allowed us to conclude that these fluctuations originate from the diffusion of the free Rh-ONs (data not shown). Apparently, this seems contradictory to the gel electrophoresis results which showed that at higher w/w values, all the Rh-ONs are bound. However, as FCS can detect single fluorescent molecules it is much more sensitive than the fluorescence detection used in gel electrophoresis. Moreover, while the concentration used in FCS is in the nanomolar range the concentration used in the electrophoresis experiments is in the micromolar range. Diluting the dispersions may dissociate the polyplexes which increases the concentration of free Rh-ONs.
As illustrated in Figure 10, fluorescence measurements on pDMAEMA/Rh-ON dispersions which were diluted a 100 times clearly show the shift of the equilibrium \( \text{RhON} + \text{polymer} \rightleftharpoons [\text{RhONpolymer}] \) to the left. For the 0.1 µg/mL solution the maximal quenching of the fluorescence is obtained at w/w = 20 while it is at a w/w value of 2.5 for the 10 µg/mL solution.

![Fluorescence (as measured by a fluorimeter) of pDMAEMA/Rh-ON dispersions. The Rh-ON concentration in all the dispersions was 10 µg/mL (square) and 0.1 µg/mL (circle), respectively. The fluorescence of the Rh-ON solution in the absence of cationic polymer (i.e. at w/w = 0) was set to 100 % (n = 3).](image)

**Figure 10.** Fluorescence (as measured by a fluorimeter) of pDMAEMA/Rh-ON dispersions. The Rh-ON concentration in all the dispersions was 10 µg/mL (■) and 0.1 µg/mL (●), respectively. The fluorescence of the Rh-ON solution in the absence of cationic polymer (i.e. at w/w = 0) was set to 100 % (n = 3).

Unfortunately, like mentioned in Chapter 3, the highly fluorescence peaks drastically disturbed the analysis of the fluctuation profiles by autocorrelation and, consequently, binding studies by means of autocorrelation analysis were no longer feasible. To obtain information on the state of complexation of the Rh-ONs one has to rely on the presence or absence of highly fluorescence peaks in the fluctuation profile. To fully confirm that the highly fluorescence peaks are caused by complexed Rh-ONs, we further investigated (1) the average fluorescence of the (highly fluorescence) peaks of complexes which were prepared from cationic polymers and mixtures of non labeled and rhodamine labeled ON, (2) the fluorescence fluctuation profile at high w/w values and (3) the change in the fluctuation profiles upon adding dextran sulfate to the polyplex dispersions, which (partially or fully) dissociate the polyplexes.
Figure 11 shows the average fluorescence per peak as a function of the composition of the “ON + Rh-ON mixture” used in the preparation of pDMAEMA based polyplexes; while the total ON concentration (0.2 µg/mL) was fixed, only the ratio of Rh-labeled to unlabeled ON was varied. Three types of pDMAEMA polyplexes were prepared differing in w/w ratio. To identify whether a certain fluorescence value could be considered a highly fluorescence peak (i.e. to find out whether it differed significantly from the intensity of the baseline) and to calculate the average fluorescence of the peaks (correcting for the intensity of the baseline attributed to free Rh-ON) we used the method described recently by Van Craenenbroeck et al. which was previously discussed in Chapter 3 (14).

![Figure 11](image)

Figure 11. The average fluorescence/peak of pDMAEMA/Rh-ON dispersions at different w/w ratios (w/w = 1, ■, w/w = 5 ○ and w/w = 20, x) as measured by FCS. The total ON concentration was fixed (0.2 µg/mL), the ratio of Rh-labeled to unlabeled ON was varied. The x-axis indicates the percentage of Rh-ON in the “ON + Rh-ON mixtures” or fraction of fluorescent oligonucleotide.

Three observations were made from Figure 11. First, large standard deviations were present which is due to the heterogeneous composition of the complexes (e.g. with regard to the number of Rh-ONs per complex) that results in a broad distribution of the height of the highly intense fluorescence peaks.

Second, in all w/w values, the higher the amount of labeled ONs in the “ON + Rh-ON mixture”, the higher the average fluorescence of the peaks. It confirms that the highly intense fluorescence peaks derive from multimolecular complexes that are composed of a number of Rh-ONs. However, pDMAEMA based polyplexes prepared from a “ON + Rh-ON mixture”
containing a low percentage of Rh-ON (e.g. 15 %) were not always significantly less fluorescent than polyplexes prepared from only Rh-ONs (i.e. 100 % Rh-ON). This can be attributed to the quenching of the fluorescence, which becomes especially pronounced when many Rh-ONs are present in the complex.

Third, in all compositions of the “ON + Rh-ON mixture”, the higher the w/w ratio, the lower the average fluorescence per peak, indicating that the individual polyplexes become less fluorescent. This agrees with the fluorescence (as measured by a fluorimeter) of the 0.1 µg/mL (i.e. 12 nM) polyplex dispersions as shown in Figure 10.

At high w/w ratios highly intense fluorescence peaks in the fluctuations were not observed anymore in the case of Rh-ON/pDMAEMA and Rh-ON/DAB₆₄ (data not shown). As discussed above, the increase in the fluorescence at higher w/w values as observed in Figure 3 was explained by less quenching between the Rh labels due to a lower number of Rh-ONs in the complexes. Probably, the existence of ‘monomolecular’ complexes, consisting of only one or a few Rh-ONs explains why highly fluorescence peaks are not observed at high w/w values. Contrary to pDMAEMA and DAB₆₄, highly intense fluorescence peaks remained present in the fluorescence fluctuations of Rh-ON/pEG-pEI dispersions at high w/w ratios (Figure 12).

![Figure 12](image_url)

**Figure 12.** Number of highly intense fluorescence peaks observed in the fluorescence fluctuation profile of pEG-pEI/Rh-ON dispersions as a function of the w/w ratio. The Rh-ON concentration equaled 0.25 µg/mL.
Again, this agrees with the observations in Figure 3 indicating that multimolecular Rh-ON/pEG-pEI complexes continue to exist at higher w/w ratios.

Another indication that the highly intense fluorescence peaks are related with complexation of the ONs was obtained from dissociation of the polyplexes by the poly-anion dextran sulfate. Especially, characterization of the dissociation of DNA complexes in cells is a question of principal importance with regard to the use of cationic polymers as DNA carriers. When dextran sulfate was mixed with the polyplex dispersions, two features in the fluorescence fluctuation profiles indicated the release of Rh-ONs from the multimolecular complexes (compare profiles C, D and E versus B in Figure 8 and 9). First, the intensity of the baseline increased which indicates a higher amount of free Rh-ONs. Second, the highly intense fluorescence peaks (partially or fully) disappeared. In the case of pDMAEMA (Figure 8), the addition of dextran sulfate (3 or 30 µg/mL) could only partially release the Rh-ONs from the complexes. This agreed with the results from electrophoresis measurements that also indicated even a very high concentration (500 µg/mL) of dextran sulfate was not able to release all the oligonucleotides from the pDMAEMA/Rh-ON complexes (Figure 13).

![Complexed Rh-ON and Free Rh-ON](image)

**Figure 13.** Gel electrophoresis on pDMAEMA/Rh-ON complexes (w/w = 4). The Rh-ON concentration in all the dispersions is 26.7 µg/mL Rh-ON. Lane 2-12 contain 0, 10, 50, 100, 150, 200, 250, 300, 400 and 500 µg/mL dextran sulfate. Lane 1 contains Rh-ON alone (reference).

In the case of DAB₆₄ (Figure 9) the addition of 3 µg/mL of dextran sulfate did not influence the fluorescence fluctuations; 30 µg/mL was necessary to have a complete
disappearance of the multimolecular complexes. In the case of pEG-pEI, 3 µg/mL of dextran sulfate resulted in the dissociation of all the multimolecular complexes (data not shown).

4. Summary and conclusions

Our results on the complexation between Rh-ONs and respectively pDMAEMA and pEG-pEI can be summarized as schematically represented in Figure 14 en 15.

At low polymer/Rh-ON ratios, a decrease in the fluorescence of the Rh-ONs, as measured by conventional fluorimetry, was observed upon binding of the Rh-ONs to both types of polymers. This was explained by the creation of ‘multimolecular complexes’ in which the Rh-labels are close in contact and can quench each other (Figure 14A-15A). The multimolecular complexes, which are highly fluorescent as they carry a number of Rh-ONs, resulted in highly intense fluorescence peaks in the fluorescence fluctuation profile as measured by FCS.

In case of pDMAEMA, at higher polymer/Rh-ON ratios the fluorescence of the polyplexes increased, caused by the formation of ‘monomolecular complexes’ which consist of only one Rh-ON per polymer (Figure 14C). In the case of pEG-pEI, as the fluorescence stayed constant upon further increasing the polymer/Rh-ON ratio, multimolecular polyplexes remained to exist under the form of a core-shell structure (Figure 15B).

Figure 14. Representation of interactions between Rh-ONs and pDMAEMA under different conditions. A) at low values of w/w, B) at intermediate values of w/w and C) at high values of w/w.
FCS confirmed these results as the high fluorescence peaks disappeared in case of pDMAEMA/Rh-ON dispersions, but remained present for pEG-pEI/Rh-ON dispersions. For DAB$_{64}$, at high w/w values the highly intense fluorescence peaks disappeared in the fluctuations profiles. This suggests that the complexation between Rh-ONs and DAB64 resembles the complexation between Rh-ONs and pDMAEMA.

Finally, FCS seems applicable to study interactions between fluorescently labeled ONs and cationic polymers, independent of the type of cationic polymer. As FCS measurements can be done in cells, it may become a tool to investigate the complexation between DNA and cationic carriers in cells which is a crucial step in the optimization of pharmaceutical carriers for DNA.

5. References


Chapter 6

Cellular behavior of oligonucleotide/carrier complexes

Parts of this chapter are submitted to Pharmaceutical Research

1. Introduction

In the previous chapters we focused on the physicochemical characteristics of different polymer/oligonucleotide complexes in buffer. Currently, a major challenge in antisense therapy is the efficient delivery of oligonucleotides (ONs) to their biological site of action. Therefore, we were interested in knowing whether the polymers investigated in previous chapters are good candidates for pharmaceutical ON carriers, which means having efficient binding and cellular delivery of the ONs.

Today, most studies on the cellular behavior of DNA carriers deal with the delivery of plasmid DNA. The cellular delivery of ONs from their carriers has been investigated less systematically (4). This may be partially explained by the fact that the cellular delivery of plasmids can be evaluated rather easily from measuring the cellular expression of the corresponding protein. However, the biological evaluation of antisense ON effects is more difficult because of the “high background”: antisense ONs only decrease the signal from a preexisting high level of target RNA or translated protein.

While a biological assay can give an answer on whether a certain carrier can successfully deliver ONs or not, it can not give an answer on the important question why one carrier is successful while another one fails. Therefore, in this chapter we do not only investigate the biological antisense effect of ONs complexed to the different carriers. We were especially interested in knowing how cellular methods like flow cytometry and confocal scanning laser microscopy (CSLM) could provide us with additional information on the cellular delivery of the ONs from their carriers.
The cell line we preferred for the determination of the biological efficiency of the ONs is the human lung epithelial cell line, A549, which expresses the intracellular adhesion molecule-1 (ICAM-1), a 90-110 kDa membrane glycoprotein. ICAM-1 is normally expressed at low levels on the surface of these cells. In response to tumor necrosis factor-α (TNF-α), an increased expression (“upregulation”) of ICAM-1 has been demonstrated (5). When targeted to human ICAM-1 mRNA, antisense ONs can inhibit the upregulated expression in A549 cells by different mechanisms. The oligonucleotide “ISIS 1939”, which targets specific sequences in the 3’-untranslated region of the mRNA, is biological active by degradation of the targeted mRNA by RNase H. The oligonucleotide “ISIS 1570” targets the AUG translation initiation codon. In the latter case, the protein level is reduced but not the ICAM-1-mRNA level (6).

2. Materials and methods

2.1. Cells and Reagents.

Human lung carcinoma cells (A549) (DSMZ, Braunschweig, Germany ACC107) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; without phenol red) (Gibco, Merelbeke, Belgium) containing 2 mM glutamine, 10% heat inactivated fetal bovine serum (FBS), 1% penicilline-streptomycine. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. Cells were prophylactically treated against mycoplasma with Plasmocin (Invivogen, Cayla, France). Hoechst 33258 (bisbenzimidazole) (ICN Biochemicals, Inc. Aurora, Ohio) was used to prove that cells were free of mycoplasma.

2.2. Oligonucleotides.

The 20-mer (5’-CCC CCA CCA CTT CCC CTC TC-3’) ICAM-1 antisense ONs employed in the present study target the 3’ non-coding region of ICAM-1 mRNA. They were supplied by Eurogentec (Seraign, Belgium) as phosphorothioate ONs (PS-ONs) and phosphodiester ONs (PO-ON). Also the 5’ rhodamine green (Rh) labeled ONs were supplied by Eurogentec. This 20-mer antisense ON corresponds to ISIS 1939.
2.3. Cationic carriers.

Lipofectin (DOTMA:DOPE (1:1)) (dioleylphosphatidylethanolamine: 1, 2-dioleoylpropyl-3-N,N,N-trimethylammonium chloride (1:1)) was purchased from Life Technologies (Gaithersburg, MD) (for the structures see Table 2, Chapter 1).

Poly(ethylene glycol)-poly-ethylene-imine (pEG-pEI)) was synthesized by Prof. dr. S. Vinogradov (University of Nebraska; for the structure see Figure 1, Chapter 5). The weight average molecular weight ($M_w$), as determined from static light scattering, was 12400 g/mol. PEI (25 kDa) was obtained by Sigma-Aldrich (Bornem, Belgium; for the structure see Table 1, Chapter 1).

Poly(2-dimethylamino)ethyl methacrylate-co-aminoethylmethacrylate (pDMAEMA-co-AEMA; abbreviated as $graft$ pDMAEMA) was a kind gift from the University of Utrecht. Part of AEMA (max 25 %) is bound to pEG 5000 (MW 1700 kg/mol) (Figure 1).

![Figure 1. Structure of $graft$ pDMAEMA](image-url)
2.4. Inhibition of ICAM-1 expression in A549 cells.

A549 cells were plated onto 96-well microtiter plates (10^4 cells/well). On day 3 (at 90% confluency) the cells were washed three times with PBS buffer. In case of Lipofectin, 100 µL DMEM containing 20 µg/mL Lipofectin was added to each well of the plate, followed by 50 µL of the ONs solution in DMEM. Lipofectin is a combination of a cationic lipid (1, 2-dioleoylpropyl-3-N,N,N-trimethylammonium chloride) (DOTMA) and a neutral lipid dioleylphosphatidylethanolamine (DOPE).

Where graft pDMAEMA/ON polyplexes (w/w = 4) were used, 150 µL of the polyplex dispersion was added to the cells, 30 min after their preparation. Graft pDMAEMA is a copolymer with graft architecture. We must emphasize that in this case, contrary to the complexation with Lipofectin, the complexation takes place before the polyplexes were put on the cells.

The expression of ICAM-1 was measured as follows. Four hours after adding the free or complexed ONs to the cells, 100 µL culture medium supplemented with 10 ng/mL TNF-α was added to induce overexpression of ICAM-1. After 18 hours, the cells were washed, fixed for 15 min at room temperature in PBS containing 20 mg/mL paraformaldehyde and blocked with 2% normal goat serum (NGS) in a 1% BSA/PBS solution. Consequently, as shown in Figure 1B, cells were washed and incubated for 90 min at 37°C with mouse anti-human ICAM-1 antibody (0.5 µg/mL) (ImmunoSource, Zoersel, Belgium). Cells were washed and incubated for 1 h at 37°C with sheep anti-mouse antibody-horseradish peroxidase conjugate (1:1000) (Figure 2C). After 3 washes, the peroxidase activity was assessed using 100 µL of o-phenylenediamine dihydrochloride (OPD; Sigma, Bornem, Belgium) (Figure 2D). After 15 min incubation at 37°C, the reaction was stopped by adding 100 µL 2 M H_2SO_4 and the absorbance was read on an LISA plate reader at 490 nm. A_0 is the absorption of the cells without cytokine induction (“basal expression”). A_{TNF} is the absorption of the cells upon induction with cytokine but in the absence of ONs. A_{ON,TNF} is the absorption of the cells upon induction with cytokines and after treating the cells with (free or complexed) ONs.
Figure 2. (A) A549 cells expressing ICAM-1 protein. (B) Cells are incubated with mouse anti-human ICAM-1 antibody (▲). (C) Antibodies bound to the cells were detected by incubation with sheep anti-mouse antibody-horseradish peroxidase (HRP) (●) conjugate. (D) Peroxidase activity was determined by the addition of o-phenylenediamine dihydrochloride (OPD) which can be reduced to a colored agent, which can be detected at 490 nm. The higher the amount of ICAM-1 protein, the more OPD can be reduced by HRP, the higher the absorption at 490 nm.
2.5. Laser scanning confocal microscopy analysis.

The cells were seeded onto sterile 8-well Nunc chambers (Nalge Nunc Int., Naperville, IL) and allowed to adhere for 1 day. 150 µL of free Rh-ONs (in DMEM), 150 µL of Rh-ONs in the presence of Lipofectin (13 µg/mL) or 150 µL of the \textit{graft} pDMAEMA/Rh-ONs (w/w = 4) dispersion were added to the cells and incubated for 3 hours at 37°C. The cells were washed three times with PBS before investigating them by confocal laser scanning microscopy (Bio-Rad MRC 1024; Hemel Hempstead, UK). We used a 60x water immersion objective and a krypton/argon laser (488 nm) for the excitation of the Rh.

2.6. Cellular uptake of ONs measured by flow cytometry.

A549 cells were harvested by trypsin and 3 x 10^5 cells were incubated at 37 °C during 4 hours in DMEM containing Rh-ONs either in the absence or presence of a cationic carrier. After incubation, the cells were centrifuged 3 times (200 g for 5 min); after each centrifuging step the pellet was washed with PBS. Consequently, the cells were resuspended in DMEM. The fluorescence intensity of the cells was recorded at 520 nm using a flow cytometer (Becton Dickinson, Erebodegem, Belgium) at an excitation wavelength of 488 nm. 5000 cells were analyzed.

2.7. Agarose gel electrophoresis experiments

The polyplex dispersions (pEI/ON; w/w 1.6) and \textit{graft} pDMAEMA/ON; w/w = 4) used in the “gel electrophoresis experiments” contained 26.7 µg/mL Rh labeled PO-ON or Rh labeled PS-ON. Thirty minutes after the preparation of the polyplex dispersions, unlabeled PS-ONs (or PO-ONs) were added to the dispersions. Another 30 minutes later, 30 µL of these dispersions was mixed with 5 µL of a 50% sucrose solution in distilled water and placed in the wells of a 1.1 % agarose gel. A TBE buffer was used containing 10.8 g/L Tris base, 5.5 g/L boric acid and 0.58 g/L EDTA. A potential of 100 V was applied for 60 min. The oligonucleotides in the gel were detected based upon their fluorescence.
3. Results and discussion

3.1. Biological activity of ICAM-1 antisense ON in the presence or absence of cationic carriers

To evaluate the biological activity of ONs bound to different carriers we used the ICAM-1 assay. In this assay we determined the inhibition of the ICAM-1 protein expression on the surface of A549 cells induced by TNF-α. ISIS 1939 PS-ON and its phosphodiester analog (PO-ON) were chosen as ONs as they are more active than ISIS 1570 (6).

It was important to assure that ICAM-1 experiments were done on mycoplasma-free cells. Indeed, we observed that in mycoplasma infected cells the basal ICAM-1 expression ($A_0$) was increased to the same level as the induced ICAM-1 expression ($A_{TNF}$) (data not shown). We observed that the treatment of the cells with Plasmocin successfully eliminated the mycoplasma: extranuclear Hoechst 33258 fluorescence was absent while the ICAM-1 expression was low. Our observations agreed with the study of Fabisiak et al. who reported that cytokine levels (as measured by ELISA) in mycoplasma infected cells were 50-fold higher than in the non-infected lines. They also observed that treatment of these cells with mycoplasma removal agents eliminated Hoechst fluorescence and significantly reduced the cytokine levels (9).

To verify whether the cationic carriers themselves influence the ICAM-1 expression, we determined the ICAM-1 expression of the A549 cells in the presence and absence of the carriers. While pDMAEMA (for structure see Figure 1 in Chapter 3) influenced both the basal and the induced ICAM-1 expression, graft pDMAEMA (structure see Figure 1 of this chapter), Lipofectin (for structures see Table 2 of Chapter 1) and pEG-pEI (for structure see Figure 1 of Chapter 5) did not influence the ICAM-1 expression. Figure 3 on the following page shows the effect of Lipofectin on the ICAM-1 expression. Consequently, the biological effect of antisense ONs complexed with pDMAEMA could not be studied by this assay.
Figure 3. Basal and TNF induced ICAM-1 expression of A549 cells in both the presence and absence of Lipofectin.

In the absence of a carrier, the PS-ONs and PO-ONs failed to inhibit TNF-α induced ICAM-1 expression even at concentrations of up to 1 µM (data not shown). Probably, this can be explained by the inability of naked ONs to diffuse passively through the cellular membrane as they are large (5-10 kDa), negatively charged hydrophilic molecules.

Figure 4A shows that after treatment with Lipofectin/PO-ON no significant change in the level of induced ICAM-1 protein expression was observed for different concentrations of PO-ON (0.25-1.2 µM). Other groups already showed that cationic lipids were not able to confer antisense activity on PO-ONs (6, 10). Although, as shown in Figure 4B, Lipofectin/PS-ON complexes were able to significantly decrease the ICAM-1 expression.
Figure 4. Inhibition of induced ICAM-1 expression in A549 cells by PO-ONs (A) and PS-ONs (B) in the presence of Lipofectin. A549 cells were treated with serum-free medium containing Lipofectin (13.3 mg/mL) and the indicated concentration of ONs (3: 100 nM, 4: 250 nM, 5: 500 nM, 6: 750 nM, 7: 1 µM, 8: 1,2 µM). Bar 1 and 2 show respectively the basal and the induced ICAM-1 expression. The ICAM-1 expression of cytokine-treated cells was set to 100 %. All the data are expressed as mean values of 4 measurements (n = 4).

Figure 5 compares the inhibition of ICAM-1 expression by PO-ONs and PS-ONs upon complexation to respectively Lipofectin and graft pDMAEMA. The “% inhibition” in the y-axis was calculated as follows:

\[ \left(\frac{A_{ON}^{TNF} - A_0}{A_{TNF} - A_0}\right) \times 100 \]
Figure 5. % Inhibition of TNF induced ICAM-1 expression in A549 cells by graft pDMAEMA/PO-ON (▲), graft pDMAEMA/PS-ON (■), Lipofectin/PO-ON (▲) and Lipofectin/PS-ON (■).

Figure 5 shows that, in contrast to Lipofectin, graft pDMAEMA efficiently delivers both PO-ONs as well as PS-ONs to ICAM-1 mRNA. Especially, a very efficient inhibition of the ICAM-1 expression is obtained when graft pDMAEMA/PS-ON complexes are used, even at very low PS-ON concentrations.

One of the hypotheses to explain the discrepancy between Lipofectin and graft pDMAEMA in the delivery of PO-ONs, is that the PO-ONs dissociate from the cationic lipid already during the endosomal escape. Marcusson et al. suggested from CLSM measurements that the lipid indeed stays in the endosomal compartment while the ONs travel through the cell (11). Consequently, the PO-ONs are in a free form in the cytoplasm and may become degraded by the intracellular nucleases (12). Graft pDMAEMA may stay associated with the ONs in the cytoplasm for a longer time and, consequently, may protect the ONs from nuclease degradation (13). Godbey et al. recently reported that in case of pEI/pDNA complexes, the polymer as well as the plasmid appeared in the cell nucleus (14). This suggests that the plasmid DNA is protected against enzymatic degradation and that non-dissociated pEI/pDNA complexes are delivered in the nucleus.
We further observed that the ICAM-1 expression, whenever the PS-ONs and the PO-ONs were complexed with pEG-pEI (which have a core-shell structures, see Figure 5 in Chapter 1), was not inhibited (data not shown).

3.2. Cellular uptake of free and complexed ONs

The cellular uptake of the ONs was evaluated by flow cytometry. The ONs (PO-ONs and PS-ONs) were labeled with rhodamine green. A major advantage of flow cytometry is that in a short time a huge population of cells can be evaluated for the absence or presence of fluorescent ONs. A disadvantage of the method is that we can not obtain information on the intracellular localization of the ONs. Although, some groups did distinguish between endosomal or cytoplasmic localization of ONs from flow cytometric studies. Therefore, they labeled the ONs with the pH sensitive dye fluorescein isothiocyanate (FITC) (15).

Cells were exposed to free Rh-labeled PO-ON and PS-ON. In the case of Rh-labeled PS-ONs, all cells showed a higher fluorescence as compared to the blanco cells, also after a washing step with acid glycine buffer (pH 2.0) (data not shown). However, we suggest that the increase in fluorescence of the cells was attributed to surface bound PS-ONs and not to internalized PS-ONs as the efficacy of the washing method for PS-ONs is open to question. As Figure 6A shows, in the case of Rh labeled PO-ON no enhancement of cell-associated fluorescence compared to the blanco could be observed, as all data were located in the upper left side of the graph. It indicated that the cells did not take up free Rh labeled ONs. This probably explains why free ONs did not show any biological effect in the ICAM-1 assay (see 3.1).

Figure 6B shows that complexation of Rh-PO-ON with Lipofectin resulted in an increased fluorescence in 90% of the cells. Figure 4A and Figure 5 showed that the Lipofectin/Rh-PO-ON complexes did not suppress the ICAM-1 expression. Combining the results from the flow cytometric measurements and the ICAM-1 assay indicates that the absence of antisense activity of Lipofectin/Rh-PO-ON complexes is not attributed to an inefficient entrance of the complexes in the cell. Rather the inability of the PO-ONs to detach intracellularly from their carrier and/or the enzymatic degradation of the PO-ONs causes the absence of antisense activity.
Figure 6. Cellular uptake of ONs as studied by flow cytometry. A549 cells were treated for 3 hours with 0.5 µM free PO-ON (A), 0.5 µM PO-ON (B) and 0.5 µM PS-ON in combination with Lipofectin (13 µg/mL) (C). The threshold of the upper left part is calculated after measuring the fluorescence of blanco cells. The x-axis is a measure of the fluorescence intensity of the cells. The y-axis shows the forward scattering (FSC) and is a measure of cell size.
Figure 6C shows instances of complexation of Rh-PS-ON with Lipofectin almost all the cells showed increased fluorescence compared to the blanco cells. Even very bright cells could be detected.

Complexation of Rh-PO-ONs (0.5 µM) with graft pDMAEMA (w/w = 6) resulted in an enhanced cell-associated fluorescence for 73% of the cells, while about 10% of the cells were dead as detected by propidium iodide (data not shown). Also, the graft pDMAEMA/PS-ON were efficiently taken up by the cells (data not shown).

When labeled PO-ONs (0.5 µM and 1 µM) were complexed with pEG-pEI (w/w = 6) no enhancement of cell-associated fluorescence could be observed (data not shown). Probably the absence of antisense activity in the ICAM-1 assay can be explained by the inability of these complexes to enter the cells.

3.3. Intracellular localization of the ONs

In contrast to flow cytometry, CLSM can be used to localize the ON, and even the carrier, inside the cell. Also “colocalization” experiments using differently labeled ONs and carriers can be useful to find out whether the labeled ON and the labeled carrier are close together.

The intracellular localization experiments were done on unfixed cells as non-viable cells have the property of internalizing significantly more ONs than viable ones. CLSM experiments showed that A549 cells did not take up free Rh-PO-ONs and Rh-PS-ONs. In case of Rh-PS-ONs a faint staining of the cell surface could be detected (data not shown), whereas in Rh-PO-ONs no fluorescence at all was observed. These results agree with those of the flow cytometric experiments and the ICAM-1 assay.

Figure 7 shows that, in the case of complexation with Lipofectin, a part of the Rh-PO-ONs was in the nuclei, as they stained green, while another part of the Rh-PO-ONs was in the endosomes as revealed from the punctate regions in the cytosol. Similar CLSM results were observed for Lipofectin/Rh-PS-ON. The CLSM experiments show that Lipofectin is able to
CHAPTER 6

deliver both types of ONs in the cellular environment, which agrees with the flow cytometric observations. However, only the Lipofectin/PS-ON complexes showed antisense activity.

Dheur et al. obtained a similar intracellular distribution for PS-ONs and PO-ONs upon delivery by Lipofectin (13). Studying Lipofectin/PO-ONs they attributed the enhancement of the nuclear fluorescence to the nuclear accumulation of the fluorophore after degradation of the ON by nucleases. It is clear that confocal microscopy and flow cytometry experiments can not be interpreted properly if the Rh-ONs are degraded within cells: in this situation the fluorescence relates to free dye.

Where graft pDMAEMA is concerned, for both types of ONs, almost the same pictures as those with Lipofectin could be obtained.

Complexation of ONs with pEG-pEI did not result in a significant staining of the cytoplasm and nuclei of the A549 cells. Consequently, the absence of the antisense effect, as observed in the ICAM-1 assay, can be explained by a low cellular uptake or by the dissociation of the complex in the culture medium. As explained in Chapter 5, pEG-pEI/ON complexes have a core-shell structure while their zeta potential is close to zero (Figure 5 in Chapter 1). This probably explains the low cellular uptake. However, it is also possible that the cells take up the pEG-pEI/ON complexes but that the complexes do not dissociate intracellularly. As observed in Figure 5 of Chapter 5, upon complexation of Rh labeled ONs to pEG-pEI a significant quenching of the fluorescence occurs which may make the complexes invisible to CLSM.
4. Summary and Conclusion

In this chapter we have evaluated the biological activity of PS-ONs and PO-ONs both in the absence and presence of a pharmaceutical carrier.

Free PO-ONs and PS-ONs failed to decrease the ICAM-1 protein level, probably due to the inability of naked ONs to diffuse passively through the cellular membrane, as they are large, negatively charged hydrophilic molecules. The hypothesis that free ONs are not able to enter the cellular environment was confirmed by CLSM as well as by flow cytometric measurements.

Although flow cytometric and CLSM experiments clearly showed cellular uptake for PS-ONs and PO-ONs complexed with Lipofectin, Lipofectin/PO-ON did not show a decrease in the ICAM-1 protein level while Lipofectin/PS-ON showed a biological effect (Figure 4 and Figure 5). Therefore, we suggested that the absence of antisense activity of Lipofectin/Rh-PO-ON complexes is not attributed to an inefficient entrance of the complexes in the cells but rather to enzymatic degradation of the PO-ONs. Like mentioned in Chapter 1, PS-ONs are more stable in cells than the PO-ONs (16).

Contrary to Lipofectin, graft pDMAEMA efficiently increased the antisense activity of both types of ONs (Figure 5). Noticeably, graft pDMAEMA/PS-ONs showed a very efficient inhibition of the ICAM-1 expression. As expected, flow cytometric and CLSM experiments
showed cellular uptake of both \( \text{graft} \) pDMAEMA/PS-ONs and \( \text{graft} \) pDMAEMA/PO-ONs complexes.

We wondered whether the chemical modification of PO-ONs into PS-ONs influence the interaction of the oligonucleotides with their carriers and consequently their biological effect. Therefore, Rh-PO-ON/carrier and Rh-PS-ON/carrier complexes were exposed to increasing concentrations of respectively (unlabeled) PS-ONs and PO-ONs followed by gel electrophoresis analysis. Figure 8A shows the results of \( \text{graft} \) pDMAEMA/Rh-PO-ON complexes exposed to unlabeled PS-ONs; Figure 8B shows the results of \( \text{graft} \) pDMAEMA/Rh-PS-ON complexes exposed to unlabeled PO-ONs.

The “competition experiment” in Figure 8 shows that \( \text{graft} \) pDMAEMA forms more stable complexes with Rh-PS-ON (Figure 8B) than with Rh-PO-ON (Figure 8A): while PS-ON are able to displace Rh-PO-ONs from \( \text{graft} \) pDMAEMA, PO-ONs cannot displace the Rh-PS-ONs from their carrier.

Similar results were obtained when using the polymer pEI (Figure 9): PS-ONs were able to displace Rh-PO-ONs from pEI while PO-ONs could not displace Rh-PS-ONs from pEI. Dheur et al. used this result to explain why in their experiment the pEI/PO-ON complexes exerted antisense activity while the pEI/PS-ON complexes did not (13). Following this hypothesis and considering the results of Figure 8, one would expect no antisense activity for the \( \text{graft} \) pDMAEMA/PS-ON complexes as Figure 8B shows that these complexes may have difficulties in releasing the ONs.

Gel electrophoresis experiments like those in Figure 8 are reported in literature very often as a means to obtain information on the ease of dissociation of DNA complexes. However, our experiments illustrate that it is hard to correlate the outcome of such measurements with the cellular behavior of DNA complexes. Also, although methods like CLSM and flow cytometry are useful in trying to understand the cellular behavior of DNA complexes, they do not allow to explain our biological results in Figure 5. This chapter clearly shows the need for more advanced biophysical methods to be able to optimize pharmaceutical carriers for DNA. As Fluorescence Correlation Spectroscopy (FCS) allows studying the complexation between DNA and its carriers (as shown in this thesis) and as FCS shows potential to measure within cells (17, 18), we believe that FCS could contribute to the understanding of the cellular fate of DNA complexes.
Figure 8. (A) Gel electrophoresis on graft pDMAEMA/Rh-PO-ON complexes (w/w = 4) exposed to increasing concentration of unlabeled PS-ONs. The Rh-PO-ON concentration in all the dispersions is 26.7 μg/mL. Lane 1 contains Rh-PO-ON alone. Lane 2 contains graft pDMAEMA/Rh-PO-ON complexes. In lanes 3-9 unlabeled PS-ONs were added to the graft pDMAEMA/Rh-PO-ON complexes in 0.5; 1; 1.5; 3; 4; 5 and 6-fold to the Rh-PO-ON concentration. (B) Gel electrophoresis on graft pDMAEMA/Rh-PS-ON complexes (w/w = 4) exposed to increasing concentrations of unlabeled PO-ONs. The Rh-PS-ON concentration in all the dispersions is 26.7 μg/mL. Lane 1 contains Rh-PS-ON alone. Lane 2 contains graft pDMAEMA/Rh-PS-ON complex. In lanes 3-9 unlabeled PO-ONs were added to the pDMAEMA/Rh-PS-ON in 0.5; 1; 1.5; 3; 4; 5 and 10-fold to the Rh-PS-ON concentration.
Figure 9. (A) Gel electrophoresis on pEI/Rh-PO-ON complexes (w/w = 1.6). The Rh-PO-ON concentration in all the dispersions is 26.7 µg/mL. Lane 1 contains Rh-PO-ON alone. Lane 2 contains pEI/Rh-PO-ON complexes. For lanes 3-9 unlabeled PS-ONs were added to the pEI/Rh-PO-ON complexes in 0.5; 1; 1.5; 3; 4; 5 and 6-fold to the Rh-PO-ON concentration. (B) Gel electrophoresis on pEI/Rh-PS-ON complexes (w/w = 1.6). The Rh-PS-ON concentration in all the dispersions is 26.7 µg/mL. Lane 1 contains Rh-PS-ON alone. Lane 2 contains pEI/Rh-PS-ON complexes. For lanes 3-9 unlabeled PO-ONs were added to the pEI/Rh-PS-ON complexes in 0.5; 1; 1.5; 3; 4; 5 and 6-fold to the Rh-PS-ON concentration.
5. References


Since the early 1980’s the idea has emerged that by using oligonucleotides (ONs) one could inhibit the synthesis of proteins based on the ability of complementary (antisense) oligonucleotides to bind intracellular RNA. In those days it was expected that this should lead to drugs with a high specificity for their target. Consequently, many groups screened all types of ONs to target genes which are known to be responsible for dysfunction of cells or tissues. However, despite their exciting potential, until now only one antisense oligonucleotide was approved by the FDA. This can be partially explained by the fact that ONs suffer from numerous limitations like rapid degradation and difficult transport through cell membranes.

Pharmaceutical scientists were challenged to develop a ‘package’ for ONs to overcome their limitations. They investigated to which extent liposomes and polymers are suitable to carry the ONs to their target.

However, nowadays, carriers able to successfully delivery ONs to their target after parenteral or mucosal administration are still missing. This is partially due to a lack of knowledge on the intracellular mechanisms which govern the delivery of the ONs by their carrier. Indeed, the release of an ON from its carrier is a crucial step to exert its activity in the cytoplasm or nucleus. This dissociation from its carrier must occur at the right time and the right place: in case the ONs dissociate from their carrier outside the cell, the ONs will be degraded by extracellular nucleases.

A major idea, which was at the origin of this thesis, was that in order to optimize pharmaceutical carriers for ONs one should be able to study the association/dissociation of ONs to/from their carriers intracellularly. In that way we became interested in Fluorescence Correlation Spectroscopy (FCS).
FCS measures the fluorescence fluctuations in a volume element of about \(1 \mu m^3\) (Figure 1 in Chapter 2). The fluctuations are due to the diffusion of fluorescent molecules (Brownian motion) in and out this volume element. As the concentration of the fluorescent molecules in FCS experiments is in the nanomolar range, only one or a few fluorescent molecules are present in the volume element. As shown in Figure 2 of Chapter 2, from the fluorescence fluctuations an autocorrelation curve can be derived. The faster the fluorescent molecules move, the faster the autocorrelation is lost (i.e. the correlation function will show a faster decay as a function of time). From the autocorrelation curve the diffusion coefficient of the fluorescent molecules can be calculated. One can expect that the diffusion of a relatively small fluorescent molecule (like a fluorescently labeled oligonucleotide) will slow down upon binding to a larger molecule or particle (like a polymer or a liposome). Consequently, this will influence the fluorescence fluctuations in the volume element of the FCS instrument. As FCS allows to measure in cells (the volume element of \(1 \mu m^3\) is much smaller than the average volume of a cell) and as we expected that the release of a fluorescently labeled ON from its carrier could be observed from a change in the fluorescence fluctuations, we started the FCS work.

First we focused on the complexation between the 25-mer rhodamine labeled oligonucleotides (Rh-ONs) and the cationic polymer poly(2-dimethylamino)ethyl methacrylate (pDMAEMA; Figure 1 in Chapter 3) which is currently investigated as a pharmaceutical carrier for (high molecular weight) plasmid DNA by the group of Prof. dr. W. Hennink (Department of Pharmaceutics, University of Utrecht). It was the first time that the complexation of pDMAEMA with (low molecular weight) ONs was considered. Therefore, before studying the pDMAEMA/Rh-ON complexes by FCS we tried to obtain information on the properties of these complexes (“polyplexes”) from alternative methods like dynamic light scattering (DLS), zeta potential measurements, fluorimetric and gel electrophoresis experiments. It is generally known that the properties of complexes between poly-electrolytes depend on the ‘+/- ratio’ (\(\phi\)) being the ratio of the positive charge equivalents on the polycation to the negative charge equivalents on the polyanion. Therefore, we prepared pDMAEMA/Rh-ON dispersions at different +/- ratio (by varying the pDMAEMA concentration and keeping the Rh-ON concentration constant) and measured the properties of the pDMAEMA/Rh-ON complexes.
The characteristics of the pDMAEMA/Rh-ON complexes could be summarized as follows (as illustrated in Figure 4 in Chapter 3):

- At low +/- ratio (φ < 1) only a part of the Rh-ONs is bound to the pDMAEMA; in other words, there were too few pDMAEMA chains to bind all the Rh-ONs. Indeed, gel electrophoresis revealed free Rh-ONs. The hydrodynamic diameter of the complexes varied between 80 nm and 250 nm (Figure 3A, Chapter 3). The pDMAEMA/Rh-ON complexes showed a negative zeta potential (Figure 3B, Chapter 3) which indicates that not all the phosphate anions on the ONs were neutralized by positive charges on the polymer; in other words, the ONs were bound to the pDMAEMA chains by only a few of their phosphate anions. Upon increasing the charge ratio (from 0 to 1) the pDMAEMA/Rh-ON dispersions became gradually less fluorescent (as measured by a conventional fluorimeter) (Figure 5, Chapter 3). This was explained by self-quenching of the fluorescence, as, upon binding of the Rh-ONs to the pDMAEMA the Rh-ONs significantly concentrate. Consequently, the quenching as observed in the fluorescence measurements indicated the existence of “multimolecular complexes” which exist of many Rh-ONs bound to one or more pDMAEMA chains.

- At φ-values between 1 and 3, DLS revealed that fully aggregated polymer/ON complexes were formed. As in this region ζ crosses-over from a negative to a positive value and approximates zero (Figure 3B, Chapter 3), the clustering of the individual polyplexes was attributed to the absence of any repulsion between the particles.

- At φ-values higher than 3, the polyplexes were positively charged (Figure 3B, Chapter 3) indicating that the polycations in excess incorporated into the complexes. As in this region the fluorescence of the dispersions again increased (Figure 5, Chapter 3) it was suggested that, when a higher number of pDMAEMA chains are present, the average number of Rh-ONs bound to one chain begins to decrease which results in less quenching of the fluorescence. At higher φ-values probably “monomolecular complexes” exist which consist of only one Rh-ON per pDMAEMA chain. It was suggested that, at high φ-values, the free cations explain the positive charge of the polyplexes and that the electric repulsion between the
positively charged pDMAEMA chains on the polyplexes probably decreased the size of the pDMAEMA/Rh-ON complexes.

Knowing the size, zeta potential and fluorescence properties of the pDMAEMA/Rh-ON complexes we investigated these complexes by FCS. As Figure 8 in Chapter 3 shows, the addition of pDMAEMA to the Rh-ONs clearly influenced the fluorescence fluctuations of the Rh-ONs (indicating interactions). Especially, highly intense fluorescence peaks appeared in the fluorescence fluctuation profiles when complexation between Rh-ONs and pDMAEMA occurred. As multimolecular complexes were revealed from the fluorimetric measurements on the dispersions (Figure 6, Chapter 3) we attributed the highly intense fluorescence peaks to the passage through the volume element of very bright complexes which are pDMAEMA chains bearing many Rh-ONs.

The highly intense fluorescence peaks clearly indicated the existence of Rh-ONs complexed to pDMAEMA. However, due to the highly intense fluorescence peaks it became impossible to calculate the autocorrelation function from the fluorescence fluctuations. This forced us to analyze the fluorescence fluctuations in an alternative way. In stead of using information related to the time decay of the fluctuations (like in autocorrelation analysis) we analyzed the amplitude distribution of the fluorescence fluctuations.

As we assumed that a highly intense fluorescence peak corresponded to a multimolecular pDMAEMA/Rh-ON complex we were interested to calculate the number of highly intense fluorescence peaks in the fluorescence fluctuation profile of an FCS experiment. This number should be a relative measure of the amount of multimolecular pDMAEMA/Rh-ON complexes in the dispersion. To calculate this number we first applied the method which was developed by Dr. E. Van Craenenbroek of the group of Prof. dr. Y. Engelborghs (Catholic University of Leuven). This method is only applicable when the data points in the fluorescence fluctuation profiles are collected over relatively long ‘time bins’ (minimal 1 ms): i.e. one data point in the fluorescence fluctuation profile is the average fluorescence measured in a time period of 1 ms (Figure 10, Chapter 3). Using this type of analysis we observed that the number of highly intense fluorescence peaks increased upon increasing the $\varphi$-value of the complexes. This was expected as the more pDMAEMA chains the more complexes and the lower the number of Rh-ON per complex as suggested from the fluorimetric measurements. As expected, from a certain value of $\varphi$ (around 15) the number of...
highly intense fluorescence peaks dropped (Figure 10, Chapter 3) as pDMAEMA/Rh-ON complexes arose which probably consist of only one Rh-ON bound per pDMAEMA (monomolecular complexes).

Although the determination of the number of highly intense fluorescence peaks is a straightforward method to qualitatively register complexed Rh-ONs, we wondered whether it was possible to obtain more quantitative information on the complexation between poly-electrolytes from FCS measurements. By the time we performed the FCS study on the complexation between pDMAEMA and Rh-ONs, the group of Prof. dr. E. Gratton (Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign) published their ‘Photon Counting Histogram’ (PCH) theory as another alternative to analyze fluorescence fluctuation profiles. This method is only applicable when the data in the fluorescence fluctuation profiles are collected over short time bins (0.01 ms – 0.1 ms); PCH analysis calculates the brightness and the concentration of the fluorescent species from the fluorescence fluctuation profile. To study the complexation between pDMAEMA and Rh-ONs by PCH analysis we retake the fluorescence fluctuations measurements of the pDMAEMA/Rh-ONs dispersions at much smaller time bins (Figure 3-6, Chapter 4). Generally speaking, analyzing the fluorescence fluctuations of the pDMAEMA/Rh-ONs complexes by PCH analysis was not superior compared with the analysis method of Van Craenenbroeck et al.; this was especially due to the rarity of the highly intense fluorescence peaks which do not provide sufficient data statistics. However, when monomolecular complexes were formed, i.e. at high values of the charge ratio, highly intense fluorescence peaks were not present which allowed a more straightforward interpretation of the fluorescence fluctuations by PCH analysis (Figure 6, Chapter 4). By PCH analysis we calculated that the monomolecular complexes were more fluorescent (brighter) than free Rh-ONs. This agreed with the fluorescence measurements on the pDMAEMA/Rh-ONs dispersions (using a conventional fluorimeter; Figure 7, Chapter 4) which also showed that, at high values of $\varphi$, the fluorescence of the pDMAEMA/Rh-ONs dispersions was higher than the fluorescence of the free Rh-ON solution.

As currently numerous cationic polymers are screened for the delivery of ONs while the synthesis of new types of cationic polymers by organic chemistry laboratories goes on, it was useful to evaluate to which extent FCS is generally applicable in the characterization of the complexation of ONs to cationic polymers. With this purpose in mind we investigated cationic polymer/ON complexes formed by three different types of cationic polymers. We
compared polyplexes based upon pDMAEMA (as investigated in Chapter 3 and 4) with polyplexes based upon poly(ethylene glycol)-poly-ethylene-imine (pEG-pEI; for the structure see Figure 1 of Chapter 5), which show a 'core-shell' structure (Figure 5 of Chapter 1), and complexes prepared with the polycationic dendrimer DAB$_{64}$ (for the structure see Table 1 of Chapter 1). Generally speaking, highly intense fluorescence peaks appeared in the fluorescence fluctuation profiles of all the polyplexes (no matter which type of cationic polymer was used; Figure 8 and 9, Chapter 5) which allowed to conclude that FCS is indeed applicable to study the complexation between fluorescently labeled ONs and cationic polymers, independent of the type of cationic polymer. Furthermore, FCS revealed a difference in the complexation of Rh-ONs to respectively pDMAEMA and pEG-pEI. As Figure 3 of Chapter 5 shows, at low polymer/Rh-ON ratios, a decrease in the fluorescence of the Rh-ONs (as measured by conventional fluorimetry) was observed upon binding of the Rh-ONs to both pDMAEMA and pEG-pEI. As explained above, this was attributed to the creation of ‘multimolecular complexes’ in which the Rh-labels are close in contact and can quench each other. In case of pDMAEMA, at higher charge ratio’s, the fluorescence of the polyplexes again increased (due to the formation of ‘monomolecular complexes’) while the highly intense fluorescence peaks disappeared in the fluorescence fluctuation profiles. However, in case of pEG-pEI, at higher charge ratio’s, the fluorescence stayed constant (Figure 3, Chapter 5) indicating that the multimolecular complexes remain to exist. Indeed, as expected, highly intense fluorescence peaks also remained in the fluorescence fluctuation profiles of these dispersions.

After studying the physicochemical properties and especially the complexation behavior of polycationic polymer/ON complexes in buffer, we were interested to know which complexes deliver the ONs successfully in cells. The biological activity of the ONs in human epithelial cells (A549) was evaluated by measuring their ability to inhibit the expression of the intracellular adhesion molecule (ICAM-1) (Figure 2 in Chapter 6). In Chapter 6 of this thesis we wondered to which extent methods like flow cytometry and confocal scanning laser microscopy (CSLM) were able to provide us with information on the cellular delivery of the ONs from their carriers.

In absence of a pharmaceutical carrier, phosphodiester ONs (PO-ONs) as well as phosphorothioate ONs (PS-ONs) failed to decrease the ICAM-1 protein level. CSLM and flow cytometry revealed that free PS-ONs and PO-ONs (Figure 6A in Chapter 6) do not enter
the cellular environment which explains why they failed to decrease the ICAM-1 protein level.

When complexing the ONs with cationic liposomes based upon Lipofectin (for structure see Table 2 of Chapter 1), only the complexes based on PS-ONs showed antisense activity (Figures 4 and 5 of Chapter 6). The Lipofectin/PO-ONs complexes showed no activity, although CLSM and flow cytometry suggested cellular uptake (Figures 6B and 7B of Chapter 6). Probably the absence of antisense activity can be explained by enzymatic degradation of the PO-ONs after they are released from the Lipofectin liposomes.

Upon complexation to graft pDMAEMA complexes (for the structure see Figure 1 of Chapter 6), both types of ONs showed an efficient inhibition of ICAM-1 expression (Figure 5 of Chapter 6). Flow cytometry and CLSM indeed showed that the complexes were taken up by the cells. Compared with graft pDMAEMA/PO-ONs, the graft pDMAEMA/PS-ONs showed a better antisense activity (Figure 5 of Chapter 6). However, gel electrophoresis experiments showed that graft pDMAEMA forms more stable complexes with Rh-PS-ONs (Figure 8B of Chapter 6) than with Rh-PO-ONs (Figure 8A of Chapter 6). Only based upon the outcome of gel electrophoresis experiments, one could expect no antisense activity for PS-ONs as the intracellular dissociation of the ONs from graft pDMAEMA may not occur. Such experiments illustrate that it is hard to correlate the outcome of such type of measurements with the cellular and biological behavior of DNA complexes. Although methods like CLSM, flow cytometry and gel electrophoresis are useful in trying to understand the cellular behavior of DNA complexes, they do not allow to explain all the biological results which were obtained. These measurements also clearly showed that more advanced biophysical methods which can be applied in cells (like FCS) may help to better understand the cellular behavior of polyplexes and may contribute to the optimization of pharmaceutical carriers for DNA.
Samenvatting

Het idee om met behulp van oligonucleotiden (ONs) de synthese van eiwitten te inhiberen, door binding van (antisense) oligonucleotiden op het intracellulair RNA, dateert reeds van de jaren tachtig. Men hoopte dat ONs zouden leiden tot geneesmiddelen met hoge specificiteit voor hun target. Echter tot op heden wordt slechts één antisense oligonucleotide als geneesmiddel gebruikt. Dit kan gedeeltelijk verklaard worden doordat ONs snel afgebroken worden in het lichaam en zeer moeilijk doorheen celmembranen kunnen diffunderen.

De farmaceutische wereld stond voor de uitdaging een gepaste verpakking te ontwikkelen voor oligonucleotiden. Zo werd intensief onderzocht in welke mate liposomen en polymeren in staat zijn om ONs te beschermen tegen afbraak en hun opname door cellen te bevorderen.

Echter, er zijn nog steeds geen dragers die oligonucleotiden na parenterale of mucosale toediening succesvol naar hun target leiden. Dit is gedeeltelijk te wijten aan een gebrekkige kennis over de intracellulaire mechanismen die de vrijgave van ONs uit hun drager bewerkstelligen. Deze vrijgave is immers een cruciale stap opdat het ON biologisch actief zou zijn in het cytoplasm a of de nucleus. De vrijstelling van de ONs moet gebeuren op de juiste plaats en het juiste tijdstip: zo kunnen ONs afgebroken worden door extracellulaire nucleasen indien ze te vroeg in de extracellulaire omgeving vrijgesteld worden.

Teneinde farmaceutische dragers voor ONs te optimaliseren moet men de vrijgave uit hun drager intracellulair kunnen bestuderen. Dit was het uitgangspunt van deze thesis. Met dit perspectief voor ogen verdiepten we ons in Fluorescentie Correlatie Spectroscopie (FCS).

FCS meet de fluorescentie-fluctuaties in een volume-element van ongeveer 1 µm$^3$ (Figuur 1 van Hoofdstuk 2). De fluorescentie-fluctuaties worden veroorzaakt door de fluorescente moleculen die in en uit het volume-element diffunderen (Brownse beweging). Door de lage concentratie van de fluorescente moleculen in FCS experimenten (nanomolair), is er slechts één (of hoogstens enkele moleculen) in het volume-element aanwezig. Zoals
geïllustreerd in Figuur 2 van Hoofdstuk 2 kan uit de fluorescentie-fluctuaties een autocorrelatie-curve afgeleid worden. Hoe sneller een fluorescente molecule beweegt, hoe sneller de autocorrelatie verloren gaat (de correlatie-functie zal een sneller verval vertonen in functie van de tijd). Uit de autocorrelatie-curve kan men vervolgens de diffusiecoëfficiënt van de fluorescente moleculen berekenen. Men kan verwachten dat de diffusie van een relatief kleine fluorescente molecule (zoals een fluorescent gemerkt oligonucleotide) zal vertragen bij binding aan een grotere molecule of partikel (zoals een polymer of een liposoom). Deze binding zal bijgevolg de fluorescentie-fluctuaties in het volume-element van het FCS instrument beïnvloeden. Omdat FCS toelaat in cellen te meten (het volume-element van 1 µm³ is veel kleiner dan het gemiddelde volume van een cel) en omdat we verwachtten dat de vrijgave van een fluorescent gemerkt ON uit zijn drager de fluorescentie-fluctuaties verandert, besloten we om te exploreren in welke mate FCS kan ingezet worden om de intracellulaire vrijgave van ONs te bestuderen.

Eerst focussen we op de complexatie van een 25-mee rhodamine gemerkt oligonucleotide (Rh-ONs) aan het kationisch polymer poly(2-dimethylamino)ethyl methacrylaat (pDMAEMA; Figuur 1 van Hoofdstuk 3). pDMAEMA wordt momenteel onderzocht als farmaceutische drager voor (hoog moleculair gewicht) plasmide DNA door de groep van Prof. dr. W. Hennink (Universiteit Utrecht). Het was de eerste maal dat de complexatie van pDMAEMA aan (laag moleculair gewicht) ONs werd bekeken. Alvorens de pDMAEMA/Rh-ON complexen te bestuderen met FCS probeerden we informatie over deze complexen te bekomen via dynamische lichtverstrooing (DLS), zeta-potentiaal metingen, fluorimetrie en gel electroforeseexperimenten. Het is algemeen gekend dat de eigenschappen van complexen tussen poly-electrolieten afhankelijk zijn van de ‘+/- verhouding’ (φ) zijnde de verhouding van het aantal positieve ladingen op het polykation tegenover het aantal negatieve ladingen op het polyanion. Daarom werden pDMAEMA/Rh-ON complexen aangemaakt die van elkaar verschillen in φ-waarde (door de ON concentratie constant te houden en de pDMAEMA concentratie te laten variëren).
De karakteristieken van de pDMAEMA/Rh-ON complexen kunnen als volgt samengevat worden (geïllustreerd in Figuur 4 van Hoofdstuk 3):

- Indien de +/- verhouding laag is ($\varphi < 1$), zijn slechts een deel van de Rh-ON moleculen gebonden aan pDMAEMA; met andere woorden, er zijn te weinig pDMAEMA ketens om alle Rh-ONS te binden. Inderdaad, gel electroforese toonde vrije Rh-ONS. De hydrodynamische diameter van deze complexen varieerde tussen 80 nm en 250 nm (Figuur 3A, Hoofdstuk 3). De pDMAEMA/Rh-ON complexen vertoonden een negatieve zeta-potentiaal (Figuur 3B, Hoofdstuk 3). Dit gaf aan dat niet alle fosfaat-anionen van de gecomplexeerde ONs geneutraliseerd waren door de positieve ladingen van het polymer; met andere woorden, de ONs waren gebonden aan pDMAEMA ketens door slechts enkele van hun fosfaat-anionen. Bij verhoging van $\varphi$ (van 0 tot 1) werden de pDMAEMA/Rh-ON dispersies geleidelijk minder fluorescent (zoals gemeten met een conventionele fluorimeter) (Figuur 5 van Hoofdstuk 3). Dit verklaarden we door “self-quenching”: in de complexen komen de Rh-ONS dicht bij elkaar te zitten waardoor hun fluorescentie uitdooft. De self-quenching in deze fluorescentie-metingen toonde aan dat deze pDMAEMA/Rh-ON complexen “multimoleculair” zijn: ze bestaan uit verschillende Rh-ONS gebonden aan één of meerdere pDMAEMA ketens.

- Voor $\varphi$-waarden tussen 1 and 3 toonde DLS het bestaan aan van grote (geaggregeerde) polymer/ON complexen (Figuur 3A in Hoofdstuk 3). Figuur 3B in Hoofdstuk 3 toont aan dat in dit $\varphi$-gebied de zeta-potentiaal ($\zeta$) van de complexen overgaat van een negatieve naar een positieve waarde. Dit duidt erop dat de grote aggregaten ontstonden door klustering van neutrale polyplexen die elkaar te weinig afstoten.

- Voor $\varphi$-waarden > 3 waren de polyplexen positief geladen (Figuur 3B van Hoofdstuk 3). Dit toonde aan dat het pDMAEMA in overmaat aanwezig was in de complexen. In dit $\varphi$-gebied nam de fluorescentie van de dispersies terug toe (Figuur 5 van Hoofdstuk 3). Dit verklaarden we doordat het gemiddeld aantal Rh-ONs per complex afnam waardoor er minder self-quenching bestond. Bij hoge $\varphi$-waarden zijn de pDMAEMA/Rh-ON complexen vermoedelijk “monomoleculair” en bestaan ze uit één of enkele Rh-ONS gebonden aan één pDMAEMA keten. Bij hogere $\varphi$-waarden hebben de complexen een positieve zeta-potentiaal (Figuur 3B)
van Hoofdstuk 3). De elektrische afstoting tussen de polyplexen verklaart waarom de pDMAEMA/Rh-ON complexen in dit gebied niet aggregeren.

Nadat we de grootte, zeta-potentiële en fluorescentie-eigenschappen van de pDMAEMA/Rh-ON complexen in beeld gebracht hadden, onderzochten we deze complexen met behulp van FCS. De toevoeging van pDMAEMA aan de Rh-ONS beïnvloedde duidelijk de fluorescentie-fluctuaties van de Rh-ONS (Figuur 8, Hoofdstuk 3). In het bijzonder werden sterk intense pieken waargenomen in het fluorescentie-fluctuatie-profiel. Omdat we op basis van Figuur 6 in Hoofdstuk 3 aantoonden dat pDMAEMA/Rh-ON complexen multimoleculair kunnen zijn, stelden wij dat de sterk intense pieken te wijten zijn aan dergelijke multimoleculaire complexen (die meerdere Rh-ONS bevatten en daardoor sterk fluorescent zijn) die in en uit het volume-element van het FCS toestel diffunderen.

De sterk intense fluorescentie-pieken toonden duidelijk aan dat de Rh-ONS gecomplexeerd worden aan pDMAEMA. Echter, deze sterk intense fluorescentie-pieken verhinderden om een autocorrerelatie-functie te berekenen uit de fluorescentie-fluctuaties. Dit zette ons aan om de fluorescentie-fluctuaties op een alternatieve manier te bestuderen. In plaats van het tijdsverval van de fluctuaties te bestuderen (zoals bij autocorrerelatie analyse) analyseerden we de amplitude-distributie van de fluorescentie-fluctuaties.

We berekenden het aantal sterk intense fluorescentie-pieken in het fluorescentie-fluctuatie-profiel van een FCS experiment. Aangezien we veronderstelden dat een sterk intense fluorescentie-piek afkomstig was van één multimoleculair pDMAEMA/Rh-ON complex zou dit een maat zijn voor het aantal multimoleculaire pDMAEMA/Rh-ON complexen in de dispersie. Om het aantal sterk intense fluorescentie-pieken te berekenen, gebruikten we eerst de methode ontwikkeld door Dr. E. Van Craenenbroek van de groep van Prof. dr. Y. Engelborghs (Katholieke Universiteit Leuven). Deze methode is enkel toepasbaar wanneer de data in het fluorescentie-fluctuatie-profiel gecollecteerd worden over relatief lange ‘time bins’ (minimaal 1 ms): in dit geval komt één datapunt in het fluorescentie-fluctuatie-profiel overeen met de gemiddelde fluorescentie gemeten in een tijdsperiode tussen 1 ms and 100 ms. We stelden vast dat het aantal sterk intense fluorescentie-pieken toenam bij hogere ϕ-waarde (Figuur 10, Hoofdstuk 3). Dit duidt aan dat bij een hogere pDMAEMA concentratie (i.e. hogere ϕ-waarde) meer pDMAEMA/Rh-ON complexen gevormd worden. Aangezien de Rh-ON concentratie van de dispersie constant is, betekent dit dat bij een hogere
SAMENVATTING

$\varphi$-waarde het aantal Rh-ON per complex daalt. Inderdaad, voor $\varphi > 15$ nam het aantal sterk intense fluorescentie-pieken zelfs af.

Alhoewel de bepaling van het aantal sterk intense fluorescentie-pieken een voor de hand liggende methode was om kwalitatief de complexatie van Rh-ONs te registreren, vroegen we ons af of het mogelijk was om meer kwantitatieve informatie omtrent de complexatie tussen polyelectrolieten te bekomen met behulp van FCS. Op het moment dat wij FCS studies uitvoerden op pDMAEMA/Rh-ONs complexen, publiceerde de groep van Prof.dr. E. Gratton (Universiteit Illinois) hun ‘Photon Counting Histogram’ (PCH) theorie als een alternatieve methode om fluorescentie-fluctuaties te analyseren. Deze methode is enkel toepasbaar wanneer de data in het fluorescentie-fluctuatie-profiel gecollecteerd worden in een korte ‘time bins’ (0,01 ms – 0,1 ms); PCH analyse berekent de fluorescentie van de fluorescente species (alsmede hun concentratie) uit het fluorescentie-fluctuatie-profiel. Om de complexatie tussen pDMAEMA en Rh-ONs met behulp van PCH analyse te bestuderen, hernamen wij de fluorescentie-fluctuatie metingen op de pDMAEMA/Rh-ONs dispersies met kleine ‘time bins’ (Figuur 3-6, Hoofdstuk 4). Echter, de analyse van de fluorescentie-fluctuaties van de pDMAEMA/Rh-ONs complexen via de PCH methode bleek, vergeleken met de analyse methode van Van Craenenbroeck et al., niet superieur te zijn: de sterk intense fluorescentie-pieken in het fluorescentie-fluctuatie-profiel kwamen te zeldzaam voor. Echter, de fluorescentie-fluctuaties van monomoleculaire complexen (bekomen bij hoge $\varphi$-waarde) vertoonden geen sterk intense fluorescentie-pieken waardoor PCH analyse wel mogelijk was (Figuur 6, Hoofdstuk 4). Via PCH analyse berekenden we o.m. dat de monomoleculaire complexen meer fluorescent zijn dan de vrije Rh-ONs. Dit was in overeenstemming met de fluorescentie metingen op pDMAEMA/Rh-ONs dispersies (d.m.v. een conventionele fluorimeter; Figuur 7, Hoofdstuk 4) die aantoonden dat, bij hoge $\varphi$-waarden, de fluorescentie van de pDMAEMA/Rh-ONs dispersies hoger was dan de fluorescentie van de vrij Rh-ON oplossing.

Omdat frequent nieuwe soorten kationische polymeren als mogelijke dragers voor ONs gesynthetiseerd worden, was het zinvol om na te gaan in welke mate FCS kan ingezet worden om de polymer/ON complexen te bestuderen. Met dit doel voor ogen bestudeerden we kationische polymeer/ON complexen aangemaakt met 3 verschillende soorten kationische polymeren. We hebben complexen op basis van pDMAEMA (zoals bestudeerd in Hoofdstuk 3 en 4) vergeleken met polyplexen op basis van poly(ethylene glycol)-poly-ethylene-imine.
(pEG-pEI; structuur zie Hoofdstuk 5, Figuur 1), welke een ‘core-shell’ structuur vertonen (Figuur 5, Hoofdstuk 1). Bovendien hebben we complexen bereid op basis van het polykationisch dendrimeer DAB_{64} (structuur zie Hoofdstuk 1, Tabel 1). Er verschenen sterk intense fluorescentie-pieken in het fluorescentie-fluctuatie-profiel van alle polyplexen (Figuren 8 en 9, Hoofdstuk 5). De fluorescentie-metingen (met de conventionele fluorimeter) in Figuur 3 van Hoofdstuk 5 toonden aan dat, bij lage pDMAEMA/Rh-ON verhoudingen, de fluorescentie van de Rh-ONs afneemt bij complexatie met pDMAEMA zowel als met pEG-pEI. Dit werd verklaard door de vorming van multimoleculaire complexen waarin de Rh-moleculen dicht bij elkaar zitten en elkaar uitdoven. Hieruit concludeerden we dat FCS toelaat om de complexatie tussen fluorescent gemerkte ONs en kationische polymeren, onafhankelijk van het type kationisch polymeer, te bestuderen. Meer zelfs, FCS bevestigde het verschil in complexatiedrag van Rh-ONs met respectievelijk pDMAEMA en pEG-pEI. In het geval van pDMAEMA, bij hoge ladingsverhouding, stijgt de fluorescentie van de polyplexen (door de vorming van monomoleculaire complexen; Figuur 3 van Hoofdstuk 5) terwijl de sterk intense fluorescentie-pieken verdwijnen in de fluorescentie-fluctuatie-profielen. Echter de fluorescentie van pEG-pEI/Rh-ON complexen blijft constant (bij verhoging van φ), (Figuur 3, Hoofdstuk 5) wat aanduidt dat multimoleculaire complexen blijven bestaan. Inderdaad, sterk intense fluorescentie-pieken bleven aanwezig in de fluorescentie-fluctuatie-profielen van deze dispersies.

Na het bestuderen van de fysicochemische eigenschappen en het complexatiedrag van polykationische polymeer/ON complexen in buffer vroegen we ons af welke complexen ONs succesvol in cellen kunnen brengen. De biologische activiteit van de ONs in humane epitheelcellen (A549) werd geëvalueerd door te bepalen in welke mate ze de expressie van de intracellulaire adhesie molecule-1 (ICAM-1) kunnen inhiberen (Figuur 2 in Hoofdstuk 6). Verder evalueerden we in welke mate methoden zoals ‘flow cytometry’ en ‘confocale laser scanning microscopie’ (CLSM) ons informatie kunnen verschaffen over de intracellulaire vrijgave van ONs uit hun drager.

In afwezigheid van een farmaceutische drager slaagden fosfodiëster ONs (PO-ONs) en fosforothioaat ONs (PS-ONs) er niet in de expressie van het ICAM-1 proteïne te onderdrukken. CLSM en ‘flow cytometry’ toonden dat vrije PS-ONs en PO-ONs (Figuur 6A in Hoofdstuk 6) niet in de cellen geraken, hetgeen verklaart waarom ze er niet in slagen het ICAM-1 proteïne niveau te doen dalen.
SAMENVATTING

Bij complexatie van de ONs met kationische liposomen op basis van Lipofectine (structuur zie Hoofdstuk 1, Tabel 2) vertoonden alleen de complexen op basis van PS-ONs antisense activiteit (Figuren 4 en 5, Hoofdstuk 6). De Lipofectine/PO-ONs complexen vertoonden geen activiteit, alhoewel CLSM en ‘flow cytometry’ cellulaire opname suggereerden (Figuren 6B en 7B van Hoofdstuk 6). De afwezigheid van antisense activiteit kan waarschijnlijk verklaard worden door enzymatische degradatie van PO-ONs na de vrijstelling uit de Lipofectine liposomen.

Bij complexatie met behulp van graft pDMAEMA (structuur zie Figuur 1 van Hoofdstuk 6) vertoonden beide types van ONs een efficiënte inhibitie van ICAM-1 expressie (Figuur 5 van Hoofdstuk 6). ‘Flow cytometry’ en CLSM toonden inderdaad dat de complexen door de cellen opgenomen werden. Echter de graft pDMAEMA/PS-ONs gaven een betere antisense werking dan de graft pDMAEMA/PO-ONs (Figuur 5 van Hoofdstuk 6). Nochtans toonden gel electroforese experimenten dat graft pDMAEMA meer stabiele complexen vormt met Rh-PS-ONs (Figuur 8B van Hoofdstuk 6) dan met Rh-PO-ONs (Figuur 8A van Hoofdstuk 6). Louter gebaseerd op het resultaat van de gel electroforese experimenten zou men geen antisense activiteit verwachten voor PS-ONs omdat de intracellulaire dissociatie van de ONs uit graft pDMAEMA mogelijk niet plaatsvindt. Deze experimenten illustreren dat het moeilijk is om het resultaat van dergelijke metingen te corréleren met het cellulaire en biologisch gedrag van DNA complexen. Alhoewel methoden zoals CLSM, ‘flow cytometry’ en gel electroforese nuttig zijn om te proberen het cellulair gedrag van DNA complexen te begrijpen, laten ze toch niet toe om alle verkregen biologische resultaten te verklaren. Deze waarnemingen tonen aan dat er duidelijk een nood is aan meer geavanceerde biofysische methoden die kunnen toegepast worden op cellen (zoals FCS). Dit zou leiden tot het beter begrijpen van het cellulair gedrag van polyplexen en tot de optimalisatie van farmaceutische dragers voor DNA.
### List of abbreviations and symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CSLM</td>
<td>confocal scanning laser microscopy</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DAB</td>
<td>1, 4-diaminobutane</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>DOPE</td>
<td>dioleylphosphatidylethanolamine</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-dioleoyl-3-trimethylammoniumpropane</td>
</tr>
<tr>
<td>DOTMA</td>
<td>1, 2-dioleoylpropyl-3-N,N,N-trimethylammonium chloride</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno-Sorbent Assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>HeNe laser</td>
<td>helium neon laser</td>
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<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>$M_n$</td>
<td>number average molecular mass</td>
</tr>
<tr>
<td>$M_w$</td>
<td>weight average molecular mass</td>
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<tr>
<td>ONs</td>
<td>oligonucleotides</td>
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<tr>
<td>OPD</td>
<td>o-phenylenediamine dihydrochloride</td>
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<td>NGS</td>
<td>normal goat serum</td>
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<td>PACA</td>
<td>polyalkylcyanoacrylate</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCH</td>
<td>photon count histogram</td>
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<td>pDMAEMA</td>
<td>poly(2-dimethylamino)ethyl methacrylate</td>
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<td>pEG</td>
<td>polyethylene glycol</td>
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<td>pEG-b-pEI</td>
<td>poly(ethylene glycol)-poly-ethylene-imine</td>
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<tr>
<td>pEG-g-pLL</td>
<td>poly(ethylene glycol)-poly-L-lysine</td>
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<tr>
<td>pEG-PE</td>
<td>poly(ethylene glycol)-phosphatidylethanolamine,</td>
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<tr>
<td>pEI</td>
<td>poly-ethylene-imine</td>
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<td>PLA</td>
<td>poly-(D,L-lactic acid)</td>
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<td>PO-ONs</td>
<td>phosphodiester ONs</td>
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<td>ribonuclease H</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>TBE</td>
<td>buffer based on Tris, Boric Acid and EDTA</td>
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<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)methylamine</td>
</tr>
<tr>
<td>w/w ratio</td>
<td>weight/weight ratio</td>
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<tr>
<td>$\zeta$</td>
<td>zeta potential</td>
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