Poly (2-(dimethylamino)ethyl methacrylate)/plasmid nanoplexes: towards optimal size for transfection of cells

Liliana Patricia Ramírez Ríos

Supervisors: Prof. Dr. W. E. Hennink

Dr. E. Mastrobattista

International Master in Drug Innovation

Department of Pharmaceutics

Utrecht Institute for Pharmaceutical Sciences

Utrecht, 07 February 2008

1 Introduction

Gene delivery using non-viral approaches has become increasingly a relevant field of research and development in view of its further application on gene therapy. In order to achieve an efficient gene transfer to the nucleus of the cell and to promote efficient gene expression, the non-viral carrier has to overcome different barriers such as to condense the DNA into polyplexes, to bind to the cell surface, to mediate cell entry, and to avoid endo-lysosomal degradation.

Thus, nanocomplexes formed by self-assembly of both naked DNA and Poly (2-(dimethylamino)ethyl methacrylate) (pDMAEMA) (see Fig. 1) in solution have to fulfill several criteria to be suitable for the use as gene delivery carriers. The particle size of the nanocomplexes is very important in order to facilitate the intracellular transport into the nucleus.[1] The nucleus is surrounded by a double membrane, which contains pores for transport. The pores of the nucleus are around 40 nm of diameter when opened and serve as size exclusion barriers. The smaller the particle size, the better the transfection efficiency.

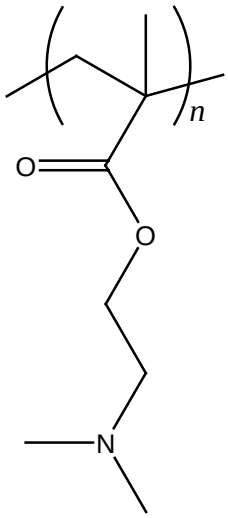


Fig. 1: Chemical structure of the cationic polymer used in this study: Poly (2-(dimethylamino)ethyl methacrylate) (pDMAEMA)

pDMAEMA is used as synthetic carrier to deliver the DNA into the cell. This polymer has advantageous properties: it is easy to prepare, feasible to work at different ph conditions, e.g. physiological conditions, and environmentally friendly (water soluble), along with other properties that make this polymer very suitable for its application in gene therapy.

The size of the DNA/polymer complexes can be influenced predominantly by changing different parameters[2], such as the way of mixing and the mixing time, the ratio of nitrogen residues to DNA phosphate-groups (N/P)[3], ionic strength of the solution, DNA concentration, pH, temperature, molecular weight of the polymer[4] and DNA, etc. Other parameters that have not been explored before in the formation of the polyplexes are the restriction of the polyplexes to a confinement in emulsions or miniemulsions and the use of solvents to induce condensation of DNA and further condensation of the polyplexes.

Gene delivery by chemical methods use the condensation of DNA by cationic lipids, peptides and polymers. Often the cationic lipids are formulated with a non-charged phospholipid or cholesterol to form liposomes. Thus, lipoplexes are formed when cationic

liposomes are mixed with DNA. The structure and size of the final lipoplexes are influenced by several factors during their preparation and, hence, the resulting polyplexes are generally heterogeneous in size and morphology.

By lipid condensation of DNA, very small particles can be obtained. Single DNA molecules were condensed into particles by a cationic cysteine-based detergent[5]. After aerobic dimerization, cystine-lipid/DNA particles were obtained. The particle size of the final lipid/DNA complexes was between 20 and 30 nm.

Besides pDMAEMA several cationic polymers such as polyethylenimine (PEI) have been used to condensate the DNA into particles. Ogris et al[2] found that the size of the DNA complexes with PEI or transferrin-PEI can be a relevant factor for transfection efficiency. They discovered that PEI condensates the DNA into small particles at low ionic strength and aggregates into bigger particles (up to 1000nm) at physiological salt concentration. They prepared in their studies DNA/PEI complexes with a particle size of between 30 and 60 nm.

pDMAEMA/DNA polyplexes can be synthesized in our lab by polyelectrolyte condensation of DNA. The particle size varies from approx. 100 nm to 1 μm depending on the reaction conditions, such as N/P ratio, DNA and polymer concentration and the type and ionic strength of the buffer used.

There are several ways to prepare polyplexes. One way is to first add the polymer solution to the plasmid DNA and then to stir the resulting mix. Another way is to add the polymer solution to the DNA solution which is being stirred at one given rpm. The latter approach could reduce the particle size of the final dispersion even more, since the continuously agitation could destroy possible aggregates in the formation of polyplexes. In this report,

this way of mixing the polyplexes as well as the time of mixing needed to produce smaller polyplexes are discussed in section 3.1.

Compacted state of DNA is preferred at higher temperatures.[6] Since DNA is responsive to temperature, the particle size of the final pDMAEMA/DNA polyplexes may depend on the temperature. The formation of the polyplexes at different temperatures is discussed in section 3.2. The temperature is varied between 10 and 60 ºC.

The pH value of the polymer solution has a very strong influence on the final particle size of the polyplexes due to the change in the proportion of the charged amine groups.[3] On the other hand, changing the pH of DNA destabilizes the helix and lowers the thermal melting point of the DNA.[7] Both factors could contribute strongly to changing the particle size of the polyplexes. Two approaches of dropping the pH of the polymer/DNA mixture at high pH with the aim of reducing the size of the polyplexes are considered in the section 3.3.

Another method that could potentially produce smaller polyplexes is the use of confinement effects to restrict the environment of the polyplexes. Such confinement of the polyplexes can be generated by emulsions. An emulsion is a heterogeneous system consisting of two immiscible liquids, in which small droplets of one fluid (disperse phase) are dispersed in the other fluid (continuous phase) by means of shaking, mechanical agitation or ultrasound. Depending on which compound is forming the continuous phase, the emulsions can be classified as *direct*, oil in water (O/W), and *inverse*, water in oil (W/O).[8]

Emulsions are divided in macroemulsions, miniemulsions or microemulsions, depending on the droplet size and the stabilization mechanism. The droplet size usually varies from

about 100 nm to several μm for macroemulsions, between 50 and 500 nm for miniemulsions and between 1 and 100 nm for microemulsions, approximately.

An emulsion tends to break over time because the system tends to be in the state of minimum energy, to go back to its original state, that is lower surface and interface tension and larger volume. The principal instabilization mechanisms are the coalescence and the Ostwald ripening. Coalescence is the process of aggregation of two droplets to form one larger droplet through collision, while Ostwald ripening is the process whereby large droplets grow at the expense of smaller ones due to the transport of dispersed phase molecules from the smaller to the larger droplets through the continuous phase.

The classification by size is more or less ambiguous and thus it is that emulsions are also classified depending on the stability mechanisms, amount of surfactant, etc. The predominant difference between emulsions lies in their respective stability. Macroemulsions are kinetically stable, microemulsions are thermodynamic stable and miniemulsions are stable against molecular diffusion (Ostwald ripening) and against coalescence.

Section 3.4 focuses on the use of a direct emulsion as a medium for the confinement of the pDMAEMA and plasmid into droplets at high pH -without charge interaction- to favor the homogeneous condensation within the droplet by decreasing the pH. It is assumed that the particle size of the droplet is reduced once the polyplexes are formed. The idea is to calculate the amount of DNA necessary to have one molecule of DNA per particle in the aqueous phase and so to ensure that the complex formed in the organic phase has the smallest particle size possible. Once the polyplexes are formed in the emulsions droplets, the next and more challenging step is to break the emulsion in a view to remove surfactant

and organic solvent without any further aggregation. Besides the particle size of the polyplexes, the zeta potential of the final polyplexes will also be discussed.

Miniemulsions were also explored as a confinement way to generate smaller polyplexes.

Miniemulsions[9] are emulsions wherein the droplets are stabilized against molecular diffusion degradation (Ostwald ripening, a unimolecular process or τ_1 mechanism) and against coalescence by collisions (a bimolecular process or τ_2 mechanism).

Stabilization against coalescence can be obtained in colloidal chemistry by means of the addition of suitable surfactants which can act as steric, electrostatic, or electrosteric stabilizer agents.

When an emulsion is prepared, a specific distribution of the droplet size is obtained. Even when the surfactant provides the droplets with sufficient colloidal stability, the outcome of this size distribution is determined by their droplet or Laplace pressures, which increase with decreasing droplet size, resulting in a net mass flux by diffusion between the droplets. If the droplets are not stabilized against diffusional degradation, small ones will disappear, thereby increasing the average droplet size (Ostwald ripening).

The addition of a small amount of a third component that is almost completely insoluble in the continuous phase and is trapped within the droplets can stop the Ostwald ripening in the system. The ripening inhibitors are called “ultrahydrophobe” or “hydrophobe” in O/W miniemulsions and “lipophobe” and in W/O miniemulsions.

Once the pDMAEMA/DNA polyplexes are formed, the DNA is protected by the cationic polymer, and it is possible to use ultrasound in the miniemulsion process without any fragmentation of the DNA. Kuo et al[10] showed that only cationic polymers provide

protection of plasmid DNA against ultrasonic degradation. Generating a miniemulsion of standard polyplexes within the aqueous phase, mineral oil as organic phase, and hepes as a lypophobe, small polyplexes either disrupted or separated from the original polyplexes can be obtained. In the section 3.5, miniemulsion droplets used as confinement to produce single polyplexes are discussed. The zeta potential and the particle size of the final polyplexes after removal of organic phase are also discussed.

Several kinds of buffers or solvents such as hepes, hepes buffer saline, glucose are often used to prepare nanoplexes and their effect on the particle size has already been studied.[3] Nevertheless, the use of a solvent to induce the condensation of DNA before electrolyte condensation is still unexplored. The solvent-induced condensation is known to generate more compact DNA globules.[11] Once the DNA is condensed in water-miscible organic solvent, a further condensation by an electrolyte such a pDMAEMA may produce even smaller polyplexes. In the last section, section 3.6, a novel approach to reduce the particle size of the polyplexes substantially by the combination of two different ways of condensation of DNA to form polyplexes is presented. A great deal of solvents and water-solvent-concentrations are screened to study their effect on the particle size of the polyplexes. Thus, a new approach to produce polyplexes with a particle size of around 80 nm in a biocompatible solvent and at low concentrations is described.

The N/P ratio and the concentration of DNA are also parameters known to have a strong influence on the final particle size of the polyplexes and in their future transfection activity. For this reason, these parameters were also varied during this study.

The objective of the project described here is to optimize the nanocomplexes consisting of polymer and DNA that can be used as artificial viruses[12] in gene therapy to introduce a

specific gene into a diseased cell. The optimization implies reduction of the particle size of the final polyplexes in order to enhance the further transport into the nucleus.

2 Materials and methods

2.1 Materials

The following compounds are used as received:

Hepes (99%, Acros Organics, USA), Tween 80 (Sigma, USA), Span 80 (Sigma, USA), mineral oil (Sigma, USA), diethyl ether (air stabilized, Biosolve, The Netherlands), polyaspartic acid (Sigma, USA), methanol GR for analysis (Merck, Germany), ethanol GR for analysis (absolute, Merck, Germany), n-propanol (99+%, Across organics), tert-butanol (purum, Fluka), n-butanol (99% extra pure, Acros Organics, USA), dymethyl sulfoxide for synthesis (Merck, Germany), 1,2-dimethoxyethane (Janssen Chimica, Belgium), and tetrahydrofuran (air stabilized, Biosolve, The Netherlands)

Synthesis and characterization of p(DMAEMA)

PDMAEMA was synthesized by radical polymerization of the corresponding monomer as previously described.[13] The first batch of polymer used for studying the effect of the mixing time and manner of preparation of polyplexes on the particle size was pDMAEMA 990527 GWB J. The molecular weight (Mw) and polidispersity index (Mn/Mw) was 2600 kDa and 17, respectively. The second batch of pDMAEMA used for the rest of the experiments was labeled with the name 990527 GWB K with a molecular weight of 3500 and a polidispersity index of 16.

Plasmid

The plasmid pCMV-LacZ containing a bacterial lacZ gene preceded by a nuclear localization signal under control of a CMV promoter was used. The particle size of this plasmid was approx. 7kb. The concentration is 3 mg/ml in TE buffer (Plasmid Factory).

2.2 Dynamic Light Scattering (DLS)

The z-average particle size and polydispersity index (PDI) of the polyplexes was determined by dynamic light scattering (DLS) at 25 ºC using a Malvern CGS-3 multi-angle goniometer (Malvern Ltd., Malvern), consisting of a HeNe laser source(λ=632.8 nm, 22 mW output power), temperature controller (Julabo water bath), and a digital correlator ALV-5000/EPP). Time correlation functions were analyzed using the ALV-60X0 software V.3.X. provided by Malvern, to obtain the hydrodynamic diameter of the particles (Zave) and the polydispersity index, PDI).

DLS measurements were made at a scattering angle of 90° and at 25 °C, 30 minutes after the preparation of polyplexes without any further dilution (500 µl of sample). The measurements were repeated three times and all experiments were performed in triplicate. The refractive indices of the solvents used for the data analysis were determining using a Bench-type, Abbe-Refractometer-Carl Zeiss, model 51807 and the viscosities were measured using a rheometer (AR1000-N, TA Instruments, Etten-Leur, The Netherlands) equipped with a standard steel cone geometry (40 mm and 1° cone). A solvent trap was used to prevent evaporation of the solvent. The shear rate was continuously measured from 0 to 100 1/s during 5 min at 25 °C.

2.3 Zeta potential

The zeta potential of the complexes was determined at 25 ºC using a Zetasizer 2000 (Malvern Instruments, UK). The instrument was checked with a standard polystyrene dispersion of known zeta potential. The measurements were made 30-45 min after complex formation and without any further dilution (1 ml of sample). The measurements were made three times and experiments were performed in duplicate.

2.4 Gel electrophoresis

Treatment of polyplexes with polyaspartic acid and/or NaOH were used to dissociate the DNA from the complex. Polyplex samples, whether or not treated with polyaspartic acid or NaOH solution, were analyzed by electrophoresis in 0.7% agarose gel containing 0.5 μg/ml ethidium bromide, in TAE buffer (pH 7.4). The gels were exposed to UV light for visualization of the DNA present in the gels. A DNA marker of 1 kb (Fermentas) was used as a control.

2.5 Mixing time and manner

N/P ratio denotes the molar ratio of pDMAEMA Nitrogen to DNA Phosphate.

PDMAEMA and plasmid DNA stock solutions were diluted to the required concentration with 20mM Hepes pH 7.4 at N/P ratio of 5 and at final 10 μg/ml of DNA concentration. Three nmol of P per μg of DNA and 5 nmol of N per μg of polymer were used for calculations of the concentration of stock polymer solution.

A thermomixer comfort (Eppendorf AG, 1.5 ml) was used to mix the polymer and the DNA solutions.

Four hundred microliters of polymer solution were added to 100 μl of DNA at 25 ºC and 1400 rpm (the thermomixer is set at these conditions before mixing both solutions), and for predefined mixing times. The so-formed polyplexes were kept for 30 min at room temperature to allow stabilization of the complex.

2.6 Temperature-induced condensation of DNA and polyplexes

Polyplexes were formed using the a.m. method with a mixing time of 20 s and at temperatures of 10, 20, 25, 30, 35, 40, 45, 50 and 60 ºC. Two different N/P ratio of 5 and 1.3 and final DNA concentration of 10 μg/ml were used to calculate the corresponding

polymer concentration. In short, after setting the thermomixer at the given temperature and at 1400 rpm, 400 µl of polymer solution were added to 100 µl of DNA solution at 1400 rpm. The particle size was measured after 30 min after complex formation.

2.7 Reduction of pH

2.7.1 By continuously adding of hydrochloric acid solution to the mixture of pDMAEMA/DNA at pH 12

pDMAEMA stock solution and plasmid DNA were diluted to the corresponding concentration with 0.1M NaOH. Eight hundred microliters of polymer were added to 200 µl of DNA at 25 ºC and 1400 rpm, and 20 s of mixing. 1.2 M HCl was used to drop the pH of the mixture. The hydrochloric acid was added during the stirring at 1400 rpm and 20 s of mixing time. The DLS measurements were made after 30 min.

2.7.2 By adding a given volume of chlorhydric acid to the mixture of pDMAEMA and Plasmid DNA at pH 12

pDMAEMA stock solution and plasmid DNA were diluted to the corresponding concentration with 0.1M NaOH at N/P of 5 and 10 µg/ml DNA concentration. Eight hundred microliters of polymer solution was added to 200 µl of DNA at 25 ºC, 1400 rpm and 20 s of mixing. Each sample was formed by adding a given volume of 1.2 M HCL to the mixture of pDMAEMA and plasmid DNA at 1400 rpm and 20s of stirring. DLS measurements were made after 30 min after adding the amount of HCl.

2.8 Confinement in emulsions

2.8.1 Preparation of the emulsion of polyplexes

Preparation of the organic phase (oil-surfactant mixture):

The following concentration of mixture of surfactants were prepared:

- *High amount of surfactants* - 4.5% of Span 80 and 0.5% of Tween 80:

  Fifty milliliters of stock organic phase was prepared by thoroughly mixing 2.25ml of Span 80 and 250µl of Tween 80 and the resting amount of mineral oil.

- *Low amount of surfactants* - 0.3% (wt./wt.) of Span 80 and 0.03% of Tween 80:

  Fifty milliliters of stock organic phase was prepared by thoroughly mixing 0.126 g of Span 80 and 0.0126 g of Tween 80 and the resting amount of mineral oil.

2.8.2 Preparation of the aqueous phase:

The aqueous phase consisting of a mixture of polymer and plasmid DNA in NaOH solution (at pH 12) at N/P of 5 and a final DNA concentration of 80 µg/ml was prepared by mixing 400 µl of pDMAEMA solution in 0.1M NaOH and 100 µl of DNA solution in water at 1400 rpm en 25 °C for 20 s. At these conditions, at pH of 12, pDMAEMA is not charged and therefore no interaction with the DNA is taking place.

2.8.3 Preparation of pDMAEMA/DNA mixture containing emulsion and the polyplexes containing emulsion thereof

To prepare the emulsion of pDMAEMA/DNA mixture,[14] 5 aliquots of 100 µl of this mixture were added to 950 µl of stock oil surfactant solution over a period of 2 min in a round bottom cryo tube vial. After the addition was finished, the emulsion was stirred for 3 min in an ultra-turrax (Ika Labortechnik T-8, Staufen, Germany) at the maximal speed in

an ice-cooled bath. By adding 40 µl of 1M HCL to the continuous oil phase during stirring, the pH within the water droplets were lowered to neutral or acid conditions allowing formation of the polyplexes within the droplets of the emulsion.

2.8.4 Breaking up of the emulsion

High amount of surfactants

Removal of oil phase and surfactants:

- The emulsion droplets were sedimented at 5000 rpm for 10 min at 25 °C. The upper oil/surfactant phase was disposed.
- 10 ml of mineral oil was added to the emulsion, mixed and centrifuged at 5000 rpm for 10 min at 25 °C. The upper phase was disposed. This extraction was performed at least for 5 times.

Low amount of surfactants

Removal of oil phase and surfactants:

- The emulsion droplets were set a 5000 rpm for 10 min at 25 °C. The upper phase was disposed.
- 10 ml of mineral oil was added to the emulsion, mixed and centrifuged at 5000 rpm for 10 min at 25 °C. The upper phase was disposed. This extraction was performed 3 times.

Control emulsion:

A sample using the same emulsion procedure but without surfactant was prepared and used as control.

2.8.5 Dissociation of the polyplexes by treatment with polyaspartic acid and monitored by agarose gel electrophoresis

Plasmids (if they are complexed with pDMAEMA) were dissociated from the polymer by incubating 20 µl of broken miniemulsion with 10 µl of polyaspartic acid (20 mg/ml) in

10 mM phosphate buffer (pH 7.4) with 145mM NaCl for 1, 2 or 3 days at 25 °C prior to electrophoresis.

2.8.6 Dissociation of the polyplexes by treatment with 0.1 M NaOH and monitored by agarose gel electrophoresis

Plasmids (if they are complexed with pDMAEMA) were dissociated from the polymer by incubating 30 µl of broken miniemulsion with 30 µl of 0.1 M NaOH for 1, 2 or 3 days at 25 °C prior to electrophoresis.

2.9 Confinement in miniemulsions

2.9.1 Preparation of the miniemulsion

Preparation of the organic phase (oil-surfactant mixture):

Two different concentrations of mixture of surfactants are prepared:

- 4.5% of Span 80 and 0.5% of Tween 80:

  Fifty milliliters of stock organic phase was prepared by thoroughly mixing 2.25 ml of Span 80 and 250 µl of Tween 80 and the resting amount of mineral oil.

- 0.3%(wt/wt) of Span 80 and 0.003%(wt/wt) of Tween 80:

  Fifty milliliters of stock organic phase was prepared by thoroughly mixing 0.126 g of Span 80 and 0.0126 g of Tween 80 and the resting amount of mineral oil.

2.9.2 Preparation of the aqueous phase:

The aqueous phase consisting of the polyplexes in 20mm Hepes solution at N/P of 5 and a final DNA concentration of 10 µg/ml was prepared by mixing 400 µl of pDMAEMA solution and 100 µl of DNA solution at 1400 rpm and 25 °C for 20 s.

2.9.3 Preparation of miniemulsion of polyplexes

To prepare the emulsion of polyplexes,[14] 5 aliquots of 100 µl of polyplexes were added to 9.5 ml of stock oil surfactant solution over a period of 2 min in a round bottom cryo tube vial. After the addition was finished, the emulsion was stirred for 1h at 1400rpm. After this pre-emulsification, the miniemulsion was obtained by sonication for 30 s at 50% amplitude in an ice-cooled bath.

2.9.4 Breaking up of the miniemulsion

Removal of oil phase and surfactants:

Ten milliliters of water-saturated diethyl ether was added to the miniemulsion, mixed and centrifuged at 13,000g for 5 min at 25 °C. The upper phase was disposed. This extraction was performed three times.

To avoid denaturation or precipitation of the aqueous phase during the removal of the oil phase, the diethyl ether must be water saturated: One hundred milliliters of water and 100 ml of diethyl ether were vigorously mixed in a separating funnel. The upper phase is water saturated diethyl ether.

Removal of residual solvent

Diethyl ether was removed from the dispersion of polyplexes with air reflux.

2.10 Solvent-induced condensation of DNA and the polyplexes

pDMAEMA/DNA polyplexes with an N/P ratio of 5 and a final DNA concentration of 10 µg/ml were prepared at different concentrations of solvent ranging from 0.2 to 80% (v/v). Three thousand microgram per milliliter plasmid DNA stock solution was diluted to 50 µg/ml with a given %(v/v) solvent-water mixture which had been filtered using a cellulose acetate (CA-S) Whatman filters of 0.2 µm (in the case of THF and DME, the filtration is

avoided). 5000 μg/ml of pDMAEMA stock solution in water was diluted to the corresponding concentration of polymer solution in the same solvent used for dilution of DNA stock solution. For the calculations, there are 3 nmol of Phosphate /μg of DNA and 5 nmol of Nitrogen/μg of Polymer. The percentages (v/v) of solvent are 0.2, 0.5, 1, 10, 20, 40, 50, 60, and 80. DNA and polymer alcohol solutions are prepared around 1 hr before preparation of the polyplexes.

Eight hundred microliters of polymer solution were added to 200 μl of DNA solution at 25 ºC and 1400 rpm (Thermomix), and 20 s of mixing. Thus, the final dispersion consisted of 30 μg/ml of pDMAEMA, 10 μg/ml of plasmid DNA in a given %(v/v) solvent-water mixture.

Where the final polyplexes were in solvent/hepes mixture, a similar procedure as described above was used. In this case the polymer and plasmid solution were prepared in 20 mM Hepes.

Dissociation of the polyplexes by treatment with polyaspartic acid and monitored by agarose gel electrophoresis

Plasmids (if they are complexed with pDMAEMA) were dissociated from the polymer by incubating 100 μl of polyplexes with 50 μl of polyaspartic acid (20mg/ml) in 10 mM phosphate buffer (pH 7.4) with 145mM NaCl for 18 h at 25 °C prior to electrophoresis.

3 Results and discussion

3.1 Mixing time and manner

In order to study the effect of the mixing time on the particle size of the polyplexes, the polymer solution was added to the DNA solution at N/P of 5 and 10 µg/ml DNA concentration during the stirring at 1400 rpm and for *t* mixing time in a thermomixer.

Table 1: Effects of mixing time on the particle size of the polyplexes at N/P=5 and DNA concentration: 10 µg/ml

Sample	*mixing time t / s*	*diameter / nm*	*PDI*
070109lr01	5	158	0.40
070109lr04	10	159	0.356
070109lr07	20	158	0.35
070109lr10	30	166	0.40
070109lr13	45	175	0.35
070109lr161	60	177	0.40

Table 1 shows clearly that the particle size of the final polyplexes is constant when the mixing time of the polymer and plasmid solutions is between 5 and 20 s. A 20 s time period is chosen as being the most appropriate mixing time for the preparation of the polyplexes. It should be stressed that a thermomixer is used to mix both solutions at a given temperature during the whole research in order to have a more homogeneous agitation and mixing of both components. The polydispersity is relatively high (a polydispersity of < 0.2 is generally considered as monodisperse), perhaps due to the decomposition or contamination of the polymer used.

3.2 Temperature-induced condensation of DNA and polyplexes

After changing the batch of the polymer used to form the polyplexes, smaller particle size and polydispersity are obtained.

To monitor the effect of the temperature on the formation of the polyplexes and their respective particle size, temperature ranges from 10 to 60 °C was used to prepare the polyplexes. Both pDMAEMA and DNA solution were kept at a given temperature for at least 15 min in the thermomixer before mixing.

As shown in Table 2 and Table 3, when the N/P of the polyplexes is 5, the polyplexes were relatively stable up to 40 °C; but for high temperatures, the particle size increases strongly.

Provided N/P is 1.3, the situation changes notably. The particle size is relatively stable up to 40 °C. At 40 °C, the particle size reduces almost 10% relative to the standard polyplexes. Above 40 °C, the particle size begins to increase slightly. Presumably, a change in the DNA conformation at 40 °C could be the cause of the reduction of the particle size of the polyplexes. Heating the DNA solution causes a thermal denaturation of the DNA due to the dehydration or release of water from the surroundings of the DNA molecule. Thus, a globular structure can be formed within the DNA molecules. Lows N/P ratios could favor this process.

Table 2: Effects of temperature on the particle size of nanoplexes during their formation. * denotes the polyplexes prepared at standard conditions in 20 mM Hepes

Sample/ N/P	T(°C)									
	10		25*		30		35		40	
	d/nm	PDI	d/nm	PDI	d/nm	PDI	d/nm	PDI	d/nm	PDI
070117lr/ 5	129(± 4)	0.222	125 (± 4)*	0.231*	131 (± 5)	0.216	131 (± 2)	0.237	129 (± 2)	0.231
070123lr 1.3	124(± 2)	0.181	125 (± 4)	0.194	122 (± 7)	0.191	124 (± 4)	0.205	113(± 10)	0.223

Table 3: Effects of temperature on the particle size of nanoplexes during their formation. Continuation of Table 2.

Sample/ N/P	T(°C)					
	45		50		60	
	d/nm	PDI	d/nm	PDI	d/nm	PDI
070117lr/ 5	139 (±5)	0.235	149 (± 5)	0.293	163 (± 2)	0.252
070123lr 1.3	124 (± 8)	0.216	126 (± 6)	0.255	140 (± 9)	0.387

3.3 Dropping the pH

Polymer and DNA were mixed at N/P of 5 at pH 12 to prevent interaction of polymer with DNA and subsequent formation of polyplexes. Lowering the pH was induced by adding the indicated amounts of HCl. Two different approaches were used to drop the pH of the polymer/DNA mixture. The first approach was to reduce the pH of the polymer DNA mixture by continuous addition of acid. In the second approach, the dropping of pH was realised step-wise. This means that a new sample was prepared for every predefined amount of HCL.

Table 4: Influence of continuously reduction of pH on the particle size of the polyplexes at N/P=5 and 10 µg/ml of DNA

µl of 1.2M HCl added to sample 070202lr04	*pH*	*diameter / nm*	*PDI*	**Mean count rate**
0	12.5	-	-	-
30	12.0	176	0.325	127
60	10.5	203	0.294	185
65	9.5	217	0.280	193
70	7.5	167	0.286	715
75	3.0	182	0.319	635

As can be observed in the Table 4 and Fig. 2, when the acid was added continuously, there was formation of polyplexes at pH 7.5, as shown by the high amount of counts. Light increase of the particle size with the addition of HCl can be observed, but at pH 7.5 the particle size reduces again. These results run counter to expectations.

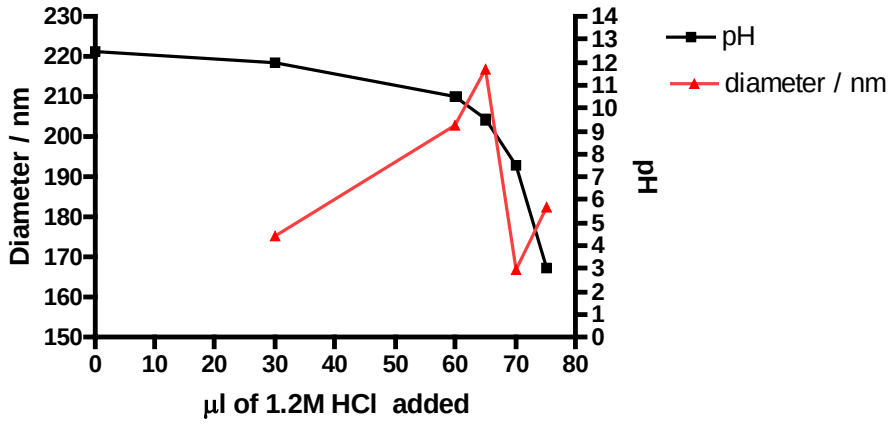


Fig. 2: Influence of continuous reduction of pH on the particle size of the polyplexes at N/P=5 and 10 µg/ml of DNA

The aim of this project was to reduce the particle size of the polyplexes by slowly dropping the pH of a very alkaline solution that does not allow polyplex formation in order to better control the polyplex formation, but it appears that other factors, such as DNA concentration, can also affect the formation of the particle during this process. For this reason, the final DNA concentration was reduced to 1 μg/ml. The particle size of the polyplexes can be seen in the Table 5. Again, formation of polyplexes can be seen by increasing the number of particles with the decreasing of the pH. A light decrease of the account rate can be observed at pH 3.5 possibly due to degradation of dissociation of DNA from the polyplexes at this low pH.

Table 5: Influence of continuously reduction of pH on the particle size of the final polyplexes at N/P=5 and 1μg/ml of DNA

μl of 1.2M HCl added to sample 070205lr	***pH***	***diameter / nm***	***PDI***	**mean count rate**
0	12.5	-	-	87
5	12.5	298	0.467	341
10	12.0	324	0.420	395
15	12.0	317	0.295	537
20	12.0	343	0.463	717
25	-.	311	0.389	1022
30	-.	307	0.416	1061
35	7.5	336	0.435	1147
45	3.5	334	0.307	781

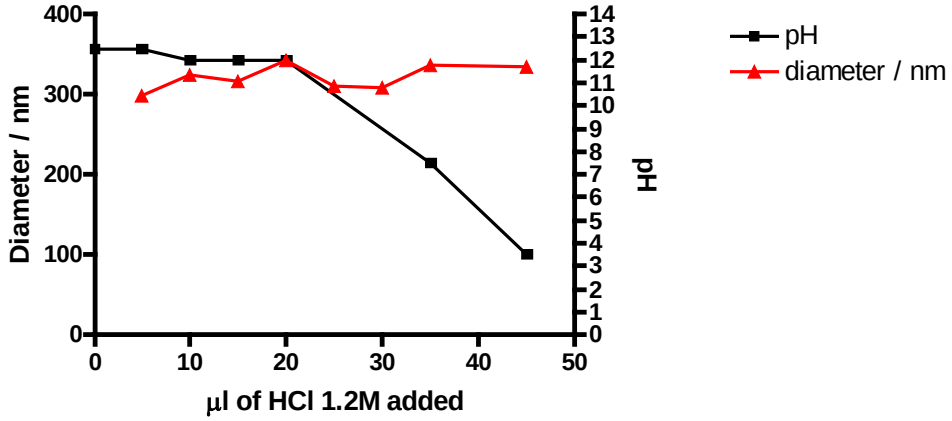


Fig. 3: Influence of continuous reduction of pH on the particle size of the polyplexes at N/P=5 and 10 µg/ml of DNA

The polyplexes are bigger than in the previous case where the DNA concentration was higher. Decreasing the DNA concentration increases the particle size of the formed polyplexes. This result again runs counter to expectations. Furthermore, there is an increase in the particle size with the decrease of pH, which contradicts previous work.[3] However, the polydispersity is very high and, thus, no affirmation can be made. Without doubt, there are even more factors that may influence the nucleation of the polyplexes with the decreasing of the pH. Another factor might be the degradation of polymer at high pH conditions.

To promote the homogenous nucleation of the polyplexes by dropping the pH, the reduction of the pH is performed quickly and with a given amount of acid per each sample. Table 6 reveals that the particle size of the polyplexes reduces with dropping of the pH, as expected. However, the particle size of the polyplexes obtained by this approach are around the same value of the standard polyplexes at the same conditions in 20mM Hepes (Table 2).

Table 6: Influence of step-wise reduction of pH on the particle size of the polyplexes at N/P=5 and 10 µg/ml of DNA

Sample	*µl of 1.2M HCl added*	*pH*	*diameter / nm*	*PDI*	*mean count rate*
070323lr15	60	12	-	-	-
070323lr17	70	12	-	-	-
070323lr19	75	11	288	0.459	-
070323lr23	77	7	197	0.326	178
070323lr21	78	3	121	0.279	311
070323lr25	80	2	121	0.258	332

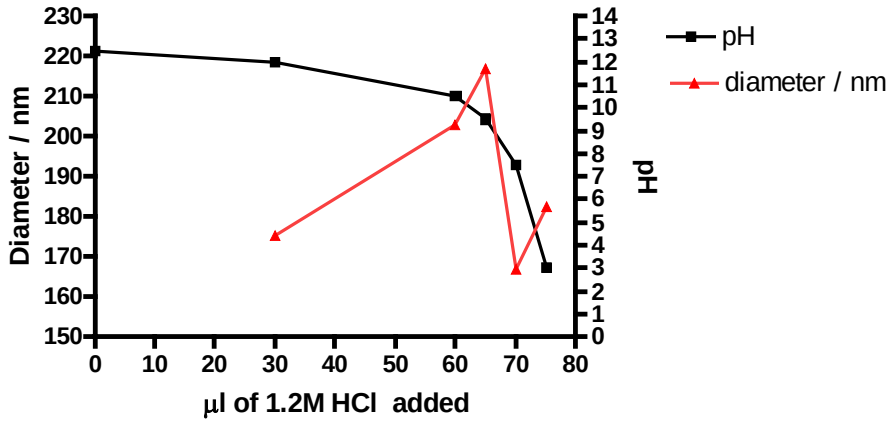


Fig. 4 Influence of step-wise reduction of pH on the particle size of the polyplexes at N/P=5 and 10 µg/ml of DNA

3.4 Confinement in emulsions

The polymer/DNA mixture at alkaline conditions –at pH 12, there is no interaction between the polymer and DNA molecules due to the fact that they are negatively charged- was added step-wise to the oil/surfactant mixture and after stirring by ultra-turrax an emulsion was obtained. After addition of the pH to drop the pH, a polyplexes-containing emulsion was formed.

To determine the size of the pDMAEMA/DNA mixture-containing emulsion droplets at high pH, where no interaction is possible, the w/o emulsion was diluted ten-fold. Taking into account the viscosity and the refractive index of the mineral oil, the particle size was determined as shown in Table 7. The particle size of water droplets at high pH was 123 nm. With this diameter, the amount of DNA necessary to have at least one DNA molecule per droplet (the number of droplets can be calculated from Eq.1) is around 8.5 mg/ml of DNA.

$$N_{droplets} = 6 * 10^{18} \frac{V_{water\ phase}}{\pi d^3} \qquad \text{Eq. 1}$$

Where d is the droplet diameter in nm and $V_{water\ phase}$ is the volume of water phase in µl.

Assuming an optimal N/P ratio of 5, this would require 25.5 mg of pDMAEMA to obtain monomolecular polyplexes (i.e. polyplexes containing a single pDNA molecule).

This concentration is very high to work with and, therefore, only 5% of this DNA concentration is taken as initial concentration to prepare the polyplexes.

Table 7: Particle size of the pDMAEMA/DNA mixture-containing emulsion

Sample	***%Surfactant (related to the oil phase)***	***diameter / nm***	***PDI***
070710lr03	4.5 % Span 80 0.5% Tween 80	123	0.363

After addition of HCL, the polyplexes containing emulsion droplets increase in size to 160 nm as seen in Table 8, contrary to expectations.

It is possible that the rapid addition of concentrated HCl to reduce the particle size creates even stronger electrostatic interactions producing bigger particles or aggregates.

At these conditions, the polyplexes are stable against coalescence by electrostatic interactions and against Ostwald ripening by hepes sodium salt which is present in excess.

Table 8: Particle size of the polyplexes-containing emulsion droplets after addition of HCL

Sample	***%Surfactant (related to the oil phase)***	***diameter / nm***	***PDI***
070710lr03	4.5 % Span 80 0.5% Tween 80	160	0.327
070816lr01	0.3 % Span 80 0.03% Tween 80	220.7	0.2135

After breaking the emulsion, the particle size of the polyplexes increases, indicating that the polyplexes aggregate again as shown in Table 9 for sample 070710lr03. Assuming an equal distribution of DNA and polymer over the emulsion droplets, 1 in every 20 droplets will contain a DNA molecule; consequently, 19 out of 20 will only contain polymer. Starting with a N/P of 5 in the bulk, those droplets containing DNA will end up with an N/P ratio of ~ 0.25. This N/P ratio is too low to form polyplexes, so probably polyplexes are being formed during breakage of the emulsion droplets, thereby releasing the free polymer contained in 19 out of 20 emulsion droplets. This uncontrolled situation might create aggregation of the polyplexes and, thus, an increase in the particle size of the final dispersion.

Surprisingly, the zeta potential is found to be negative, contrary to expected. Presumably, the use of mixture of surfactants to prepare the emulsion has a very strong effect on the final zeta potential. To verify this assumption, a control emulsion consisting of a sample under the same conditions for the emulsion of the polyplexes but without any surfactant to

prepare it was used. Table 9 reveals that the zeta potential for the control emulsion is indeed positive.

For the formulation of the emulsion, two kinds of surfactants with different hydrophile-lipophile balance (HLB) were used. A low HLB(<9), in this case HLB of 4 for Span 80 (Fig. 6) refers to oil soluble surfactant (lipophilic surfactant) and high HLB(>11) refers to water soluble surfactant (hydrophilic surfactant) in this case for Tween 80 (Fig. 5) with a HLB of 15. This mixture of surfactants is used to produce a more effective stabilization in the system. Some mixture of surfactants could form a complex at the interface producing a strong interfacial film.[15, 16] It is still not clear how Spans and Tweens interact at the oil-water interface, but some studies have been made to elucidate this question in air-water interface. Tween 80 has more polar headgroups due to the polyethylenglycol groups.

Lu and Rodhes[16] found that a film of Tween 80 and Span 80 presented some discontinuities suggesting changes in the composition or structure within the film. A subphase could be formed in the film similar to the Fig. 7.

Such a ethylene glycol-containing subphase may create hydrogen bond with the water molecules and so contribute to the negative zeta potential of the dispersion. Hence the polyplexes are possibly coated with a layer of polyethylenglycol from the surfactant.

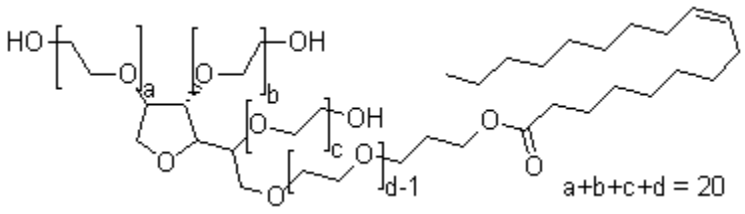


Fig. 5.: Structure of the surfactant Tween 80 or Polyethylene oxide (20) sorbitan monooleate

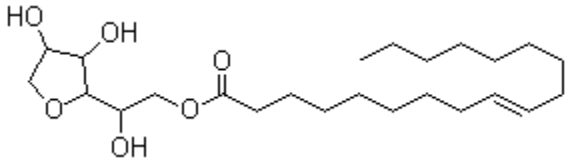


Fig. 6: Structure of the surfactant Span 80 or Sorbitan monooleate

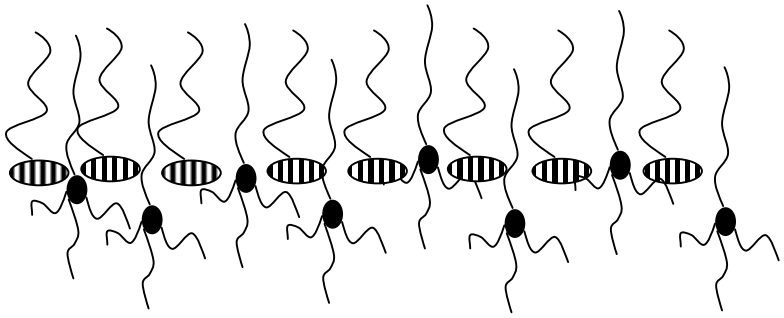


Fig. 7: Tween 80 molecule (solid) are forced into the subphase

Table 9: Characteristics of the polyplexes after breaking the emulsions

Sample	*%Surfactant (related to the oil phase)*	*diameter / nm*	*PDI*	*ZP/mV*	*ZP deviation/mV*
Control emulsion	0	-	-	38	16
070710lr03(after 3 washings)	4.5 % Span 80 0.5% Tween 80	185	0.406	-19	5
070710lr03 after filtration	4.5 % Span 80 0.5% Tween 80	126	0.242	-9	5
070725lr01 (after 6 washings)	4.5 % Span 80 0.5% Tween 80	168	0.410	4	13
070816lr01 (after tree washings)	0.3 % Span 80 0.03% Tween 80	273	0.244	33	12

On the other hand, the effect of the pEGylation (coating the polyplexes with polyethylene glycol) on the surface charge of the polyplexes was previously studied in our group. Steenis[17] found that PEGylation of polyplexes indeed reduces the zeta potential of the polyplexes to near neutral surface charge and sometimes drops to negative values depending on the N/P ratio. The method proposed here, i.e. to produce polyplexes by confinement of the polymer/DNA mixture in emulsion droplets and further reduction of the pH to generate polyplexes in the droplets, could be a rapid alternative to the PEGylation approaches used in the past. It is to note that any copolymerization or activation of DMAEMA with other comonomers, such as 2-aminoethyl methacrylate (AEMA) or N-3 – aminopropyl methacrylamide (NAPMAm), is necessary.

Precisely because of the negative zeta potential of the final polyplexes, a filter of regenerated cellulose of 0.2 μm (13 mm HPLC Syr flt) can be used without any loss of polyplexes due to electrostatic interactions with the cellulose. After filtration, the particle size of the polyplexes is around 120 nm. This diameter is the same as that of the polymer/DNA mixture containing emulsions. Thus, there is some fraction of the polyplexes that have not undergone any change in the particle size after addition of acid. Furthermore, as shown in Fig. 8, in the lane Caf, the polyplexes are still present and condensed after filtration.

After more washing steps, the zeta potential is turning slightly positive. At this stage, the stability of the dispersion could decrease notably. Finally, if the mixture surfactant concentration is decreased by 15%, it is easier to get rid of surfactant reaching the high positive value of zeta potential after only tree washing steps (see Table 9).

To study the DNA complexation, polyplexes were incubated in an excess of polyaspartic acid during three days. Aliquots were taken every day and immediately frozen. Polyaspartic acid is used as a competitor with DNA in the DNA/pDMAEMA interaction.

The sample 070710lr03(after 3 washings) was subjected to treatment with polyaspartic acid during three days but any dissociation can be observed as shown in lane Apaa1 to Apaa3 of the Fig. 8. Veron and coworkers[18] found that the negative charged polyplexes are more difficult to destabilize by polyaspartic acid than positive charged polyplexes. In our case, polyaspartic acid did dissociate the DNA from the positive charged standard polyplexes (data not shown) without any trouble.

However, when the polyplexes were incubated in an excess of NaOH, a clear DNA dissociation could be observed after 24 h. Alkaline degradation or denaturation of the DNA due to high excess of NaOH could be a possible reason of the vanished bands in the lane.

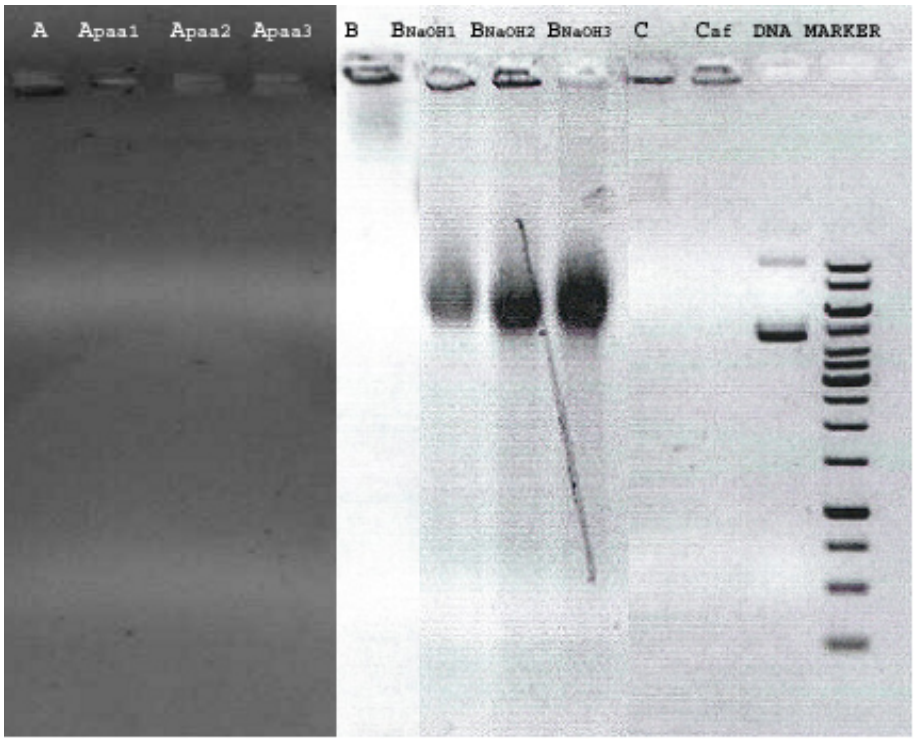


Fig. 8: Agarose gel electrophoresis of the pDMAEMA/DNA polyplexes after tree washing steps and of the DNA dissociated by polyaspartic acid (paa) and NaOH from pDMAEMA/DNA polyplexes. Dissociation by paa: lane A indicates polyplexes after breaking the emulsion by three washing steps (zero day), and lane Apaa1 to Apaa3 indicates DNA after treatment of the polyplexes A with an excess of paa from day one to day three. Dissociation by NaOH. Lane B indicates polyplexes after breaking the emulsion by three washing steps and lane BnaOH1to BnaOH3 indicates DNA after treatment of the polyplexes B with an excess of NaOH solution. Lane C and Caf denote the polyplexes after breaking the emulsion with and without filtration through generated cellulose filter

The negative value of the zeta potential is presumably due to the emulsion process and the use of surfactants.

When a high amount of surfactants is used, the zeta potential is decreased to 3.68 by increasing the washing steps, but it is still very low in comparison with the value of the standard polyplexes at the same conditions(+30).

When a low amount of surfactants is used, the zeta potential of the polyplexes reaches the standard value after 3 washings. Thus, it is easier to get rid of surfactant.

3.5 Confinement in miniemulsions

The polymer/DNA polyplexes in hepes were transferred step-wise to the surfactant-oil mixture. After stirring for one hour, a polyplexes-containing macroemulsion was generated. By sonication of this macroemulsion, a miniemulsion was produced. The hepes buffer acts as a osmotic agent to prevent the Oswald ripening.

Provided the pDMAEMA/DNA polyplexes prepared at an N/P of 5 and a final DNA concentration of 10 μg/ml comprise aggregates of polyplexes, the miniemulsion process could be an option to disrupt them. As shown in Table 10 at high concentrations of surfactant a stable miniemulsion with a droplet size of 60 nm was achieved. Due to hydrophobic effect, the polyplexes are expected to localize within the droplets. If the amount of mixture of surfactants is decreased, the obtained droplet size is bigger than expected.

Table 10: Droplet size of the miniemulsion of polyplexes. The amounts of the miniemulsion recipe were 10-fold scaled-down

Sample	*%Surfactant (related to the oil phase)*	*diameter / nm*	*PDI*
070328lr01	4.5 % Span 80 0.5% Tween 80	60 nm	0.25
070418lr01	0.3% Span 80 0.003% Tween 80	200 nm	0.366

Since the aim of this project is to decrease the size of the polyplexes, the miniemulsion of polyplexes with the highest amount of surfactants was used in further experiments because of the small size of the polyplexes. It is important to point out that we are interested in water-based polyplexes. For this reason the miniemulsion must be broken.

The particle size of the final broken dispersion is 166 nm; its polydispersity is 0.254, as can be observed in Table 11. Thus, the particle size of the polyplexes is slightly bigger than the particle size of the standard polyplexes at the same conditions in hepes solution. Hence, the confinement of the polyplexes on miniemulsions does decrease the particle size of the polyplexes in the organic phase, in this case, mineral oil. However, the polyplexes aggregate again forming bigger complexes after removal of the organic phase. After filtration through a regenerated cellulose filter of 0.2 μm, a particle size of 68.8 nm with a high polydispersity of 0.558 is obtained. Due to the high polydispersity, it is difficult to make affirmations about the particle size. Nevertheless, the zeta potentials of both the broken dispersion and the final broken dispersion after filtration are negative, suggesting—as in the case of emulsion—a coating of polyethylene oxide in the surface of the polyplexes.

Table 11: Characteristics of the polyplexes after breaking the miniemulsions

Sample	*%Surfactant (related to the oil phase)*	*diameter / nm*	*PDI*	*ZP/mV*	*ZP deviation/mV*
070521lr06 Final broken dispersion	4.5 % Span 80 0.5% Tween 80	166	0.254	-5	14
070521lr06 Final broken dispersion after filtration	4.5 % Span 80 0.5% Tween 80	68	0.5	-13	7

3.6 Solvent-induced condensation of DNA and polyplexes

Table 12 shows some characteristics of the polyplexes prepared in different alcohol-water mixture ranging from 0.2 to 80%(v/v) of ethanol. Both pDMAEMA and DNA solutions were very stable and no precipitation was observed. This observation coincides with those of Herskovits et al[11] who found that the DNA can still be stable in 100% ethanol in absence of salt. Precipitation of DNA after exposure to ethanol can be the consequence of either impurities like proteins within the DNA or DNA molecular weight.[19] In contrast to the polyplexes prepared in hepes where the polymer and DNA solution were prepared immediately before the preparation of the polyplexes, in ethanolic solutions, the polymer and DNA solutions were kept for at least 30 min in contact with the ethanol solution before preparation of the polyplexes. This ensures the maximal dehydration or compaction of the DNA before the second condensation occurs.

Up to a 10% ethanol, the particle size of the polyplexes decreases approx. 30% to 81 nm compared with the standard polyplexes at the same condition but in 20mM Hepes, as shown in Table 12. Thus, the condensation of DNA in the polyplexes is achieved by combination of two different ways. First, the solvent-induced condensation and, second, the

polycation condensation. In the solvent-induced condensation the DNA is denatured and dehydrated.[11] The conformation of DNA molecules in such water-ethanol mixtures is less expanded as in pure water. DNA denaturation may take place by loosing water molecules from the surroundings. The result is compaction of DNA into an idealized globular form. Once the DNA is solvent-precondensated, a second kind of condensation takes place, the polyelectrolyte condensation. Due to electrostatic interactions, the negative charged condensate DNA becomes even more compacted after contact with positive charged polycation, in this case pDMAEMA.

Table 12: Characteristic of the polyplexes prepared in different concentrations of ethanol-water solutions

Sample	***Ethanol concentration (v/v)***	***diameter / nm***	***PDI***	***mean count rate***	***pH***	***Zeta Potential /mV***	***Zeta potential Deviation /mV***
standard polyplexes in water 070911lr13	0	107 (±4)	0.219	224	7	24.8	10.0
standard polyplexes in 20mM Hepes 070117lr07/	0	120 (± 6)	0.215	445	7	26	6.13
071107lr01	0.2	83 (±2)	0.269	243	4.5	61.1	18.6
071107lr04	0.5	83 (±3)	0.238	314	5	-	-
071107lr07	1	81 (±2)	0.267	232	5	-	-
070903lr01	10	82 (±2)	0.226	232	5	-	-
070903lr07	40	97 (± 4)	0.298	259	5	-	-
070903lr10	50	92 (± 8)	0.304	264	5	-	-
070903lr13	60	93 (±3)	0.300	216	5	-	-
070903lr16	80	92 (± 3)	0.295	213	5	-	-

As shown in Table 12, at ethanol concentrations higher than 40%, up to 80%, the particle size of the polyplexes increases with a sharp transition at 40% ethanol. At these higher ethanol concentrations, the polydispersity index is bigger, implying a broader distribution of the particle size. These results are in line with the observation of Roy et. al.[20] of a conformational transition of DNA at 40% ethanol. Without doubt, the removal of water by ethanol has a great influence on the structure and conformation of DNA. Thus, increasing ethanol concentrations has an even greater impact on the conformation of DNA and, subsequently, on the particle size of the pDMAEMA/DNA polyplexes. The type and change of DNA conformation with the type and the concentration of the solvent is beyond the scope of this research.

The particle size is reduced around 50% from the published value in our group by using only ethanol-water solutions.

At concentrations as low as 0.2% ethanol, a small change in the aqueous environment of the polyplexes generates a strong reduction in their particle size. Because of the future applications in gene delivery, it is very convenient to create polyplexes in aqueous or hepes-buffered media, more reminiscent of the physiological conditions. As shown in Table 13, polyplexes prepared in 1 and 10% v/v ethanol in 20mM Hepes do not reveal any significant change in the particle size if they are compared with polyplexes prepared in mixed ethanol water solution.

Table 13: Characteristics of the polyplexes prepared in different concentrations of ethanol/hepes mixtures. Hepes concentration: 20 mM

Sample	*Ethanol concentration/ %(v/v)*	*diameter / nm*	*PDI*	*mean count rate*	*pH*	*Zeta Potential /mV*	*Zeta Deviation /mV*
070911lr04-06	1	84 (± 2)	0.187	412	7	36	14
070911lr10-12	10	87 (± 2)	0.228	259	7	30	14

To observe the effect of the chain length of the alcohol in the particle size, polyplexes were prepared in methanol, n-propanol and n-butanol at different concentrations. As can be seen in Table 14, the sample 070920lr01 shows a reduction of the particle size of around 4% with respect to the polyplexes prepared at the same conditions but with 1% ethanol as a medium. Thus, methanol with only one carbon has the tendency to reduce the particle size of the polyplexes. This reduction in the particle size with respect to the polyplexes prepared in ethanol-water mixtures could be neglected. Furthermore, the use of methanol in biomedical application is completely ruled out.

Table 14: Characteristic of the polyplexes prepared in different concentrations of methanol-water solutions

Sample	*Methanol concentration % (v/v)*	*diameter / nm*	*PDI*	*mean count rate*	*pH*	*Zeta Potential /mV*	*Zeta Deviation /mV*
	0.2	-	-	-	-	51	16
	0.5	-	-	-	-	55	19
070920lr01	1	78 (± 2)	0.220	389	5	57	18
070920lr04	10	84 (± 4)	0.214	398	5	59	21
070920lr07	20	83 (± 2)	0.227	315	5	46	21
070920lr10	50	84 (± 1)	0.255	232	5	-	-
070920lr13	60	90 (± 4)	0.274	226	5	-	-
070920lr16	80	95 (± 4)	0.279	259	5	-	-

As shown in Table 15 and Table 16, the use of n-propanol and n-butanol water mixtures has a tendency to increase the particle size of the polyplexes. In the case of polyplexes prepared in n-butanol-water mixtures, the particle size increase around 14% relative to the particle size of the polyplexes in 1% ethanol-water system.

Thus, it appears that when the chain length of the alcohol used to prepared the polyplexes is increased, the particle size of the complexes increases. However, it is necessary to consider that the solubility of n-butanol in water is only 9.1 ml/100ml of water at 25 ºC in contrast to methanol, ethanol and n-propanol which are fully miscible in water.

Table 15: Characteristics of the polyplexes prepared in different concentrations of n-propanol-water mixtures

Sample	***n-Propanol concentration % (v/v)***	***diameter / nm***	***PDI***	***mean count rate***
071029lr10	0.2	88 (± 2)	0.239	367
071029lr13	0.5	88 (± 3)	0.211	401
071029lr01	1	86 (±4)	0.215	348
071029lr04	10	93 (± 2)	0.226	343
071029lr07	20	98 (± 8)	0.245	312

Table 16: Characteristics of the polyplexes prepared in different concentrations of n-butanol-water mixtures

Sample	***n-Butanol concentration % (v/v)***	***diameter / nm***	***PDI***	***mean count rate***
071029lr16	0.2	89 (±3)	0.235	408
071029lr19	0.5	86 (±6)	0.261	338
071031lr05	1	94 (± 3)	0.183	630
071031lr07	4	96 (± 2)	0.218	422

For this reason, tert-butanol water solution is also tested as of solvent to induce condensation of DNA and subsequently condensation of the polyplexes thereof.

Table 17: Characteristics of the polyplexes prepared in different concentrations of tert-butanol-water mixtures

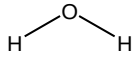
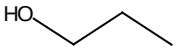
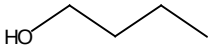
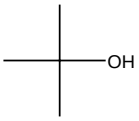
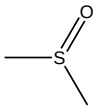
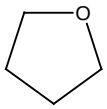
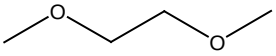
Sample	*tert-Butanol concentration % (v/v)*	*diameter / nm*	*PDI*	*mean count rate*
071010lr10	0.2	82 (± 2)	0.238	289
071010lr13	0.5	80 (± 2	0.252	268
071011lr01	1	88 (± 2)	0.235	324
071011lr04	10	90 (± 3)	0.245	258
071011lr07	20	87 (± 5)	0.336	152

Table 17 shows the particle size of polyplexes prepared in different concentrations of tert-butanol water mixtures. The particle size of the polyplexes does not increase sharply with increasing the concentration of tert-butanol as in the case of n-butanol. Thus, presumably the solubility of the solvent in water has a strong influence on the final particle size of the polyplexes.

The addition of an organic solvent to an aqueous system generates a decrease in the dielectric constant of the medium. As a consequence, the solubility of DNA or pDMAEMA/DNA in the solvent should also decrease. At a critical point, if the solubility of DNA or pDMAEMA/DNA in the solvent is too low, aggregates or precipitation could occur. Hence, the preparation of polyplexes in methanol water mixtures produces the smallest polyplex due to the fact that the decrease in dielectric constant and simultaneously solubility is smaller than in the case of n-butanol (see Table 18). However, in the case of

tert-butanol no affirmation can be made because of the balance between reduction of the dielectric constant and increase the solubility of the tert-butanol in water.

Table 18 Dielectric constants of the solvents used

Solvent	*Structure*	*Dielectric constant ξ*
Water		78.4 at 77ºF
Methanol		32.6 at 77ºF
Ethanol		24.3 at 77ºF
n-Propanol		20.1 at 77ºF
n-Butanol		17.8 at 68ºF
tert-Butanol		12.4 at 77ºF
Dimethyl sulfoxide (DMSO)		48.0 at 68ºF
Tetrahydrofuran (THF)		7.6 at 77ºF
1,2-Dimethoxyethane (DME)		3.5 at 77ºF

According with the above explanation, since the dielectric constant of DMSO is higher than that of methanol, a strong reduction of the particle size would be expected, but the results shown in Table 19 reveal stable polyplexes whose particle size is in the range of the polyplexes prepared with 1% ethanol without further reduction of the particle size.

Table 19: Characteristics of the polyplexes prepared in different concentrations of DMSO-water mixtures

Sample	*DMSO concentration % (v/v)*	*diameter / nm*	*PDI*	*mean count rate*
071011lr10	0.2	83 (± 3)	0.231	283
071011lr13	0.5	86 (± 3)	0.247	304
071011lr16	1	84 (± 1)	0.255	179
071011lr16(after 3h)	1	83 (± 1)	0.240	254

In conclusion, changing the water in the DNA molecule by a water-miscible solvent generates a great change in the physicochemical properties of the solution, such as dielectric constant, viscosity, density, refractive index among others, that causes very strong effects in the behavior of the DNA molecule. One of these effects can be measured by changing in the particle size of the DNA molecule.

By adding a water-organic solvent binary mixture to the plasmid DNA and further formation of the polyplexes by cationic condensation with pDMAEMA in the same binary mixture, polyplexes with even more smaller particle size with respect to standard polyplexes in 20 mM Hepes are obtained. For the solvents tested, the best results could be obtained if the solvent was an alcohol of less than two carbon atoms and the alcohol concentration was very low. The low concentration of the alcohol used to induce condensation of the DNA and the polyplexes was very advantageous because in this way it is not necessary to get rid of the solvent (e.g. by dialysis) after formation of the polyplexes. Similar results were achieved for DMSO as solvent. It is important to note that for the future biomedical application, the best choice in solvent would be ethanol at the lowest

concentration. A concentration as low as 0.2% ethanol allows further applications without any further removal of ethanol.

It is known that the condensation process of the DNA can also be affected by small changes in several variables, such as concentration of DNA. Table 20 shows the characteristics of the polyplexes prepared at different concentrations of DNA in 0.2% ethanol and a N/P ratio of 5. The particle size of the polyplexes decreases with decrease of the DNA concentration, as expected. The smallest particle size is found for polyplexes 071107lr08 formed at 1 μg/ml final concentration of DNA. Note that the small count rate of these polyplexes makes it almost impossible to measure the particle size accurately.

Table 20: Influence of the concentration of DNA on particle size of polyplexes prepared in 0.2% ethanol water at N/P of 5

Sample	*DNA concentration / μg/ml*	*diameter / nm*	*PDI*	*mean count rate*
071107lr08	1	70 (± 5)	0.367	22
071107lr04	2	74 (± 6)	0.324	42
071106lr10	10	83 (± 2)	0.269	243
071107lr01	20	93 (± 2)	0.275	663

Another parameter that could have a strong impact on the particle size of the polyplexes, as in the case of the standard polyplexes prepared in hepes, is the nitrogen/phosphate ratio of the polyplexes. For this reason, the N/P ratio of the polyplexes were varied from 1.3 to 5 as shown in Table 21. Surprisingly, no change in the particle size with the decrease of the N/P ratio from 5 to 2 was observed, but the polydispersity of the dispersion of the polyplexes decreases substantially by decreasing the N/P ratio or the polymer/DNA ratio. Increasing

the amount of phosphate charges in 0.2% ethanol makes the condensed DNA particles even more homogeneous and, therefore, the final polyplexes after further condensation by the polymer. Zeta potential of polyplexes prepared at low N/P ratios was positive and decreased slightly with the decrease of the N/P ratio.

At an N/P ratio of 1.3, an increase in the particle size can be seen. Interestingly, the zeta potential did not turn negative, in contrast to the results obtained by Verbaan[21]. One possible explanation is that once the DNA molecules are homogeneously condensed by using an ethanolic solution, the cationic polymer envelops the already condensed negatively charged DNA molecule and, then, the positive charge density overweighs the negative charge density of the polyplexes.

Table 21: Influence of the N/P ratio on the particle size of the polyplexes prepared in 0.2% of ethanol and at 10 μg/ml of DNA

Sample	*N/P ratio*	*diameter / nm*	*PDI*	*mean count rate*	*pH*	*Zeta Potential /mV*	*Zeta potential deviation*
071106lr01 (A)	5	83 (± 2)	0.269	243	4.5	61	19
071106lr04 (C)	4.3	83 (± 1)	0.231	303	4.5	63	16
071106lr07 (E)	3.4	83 (± 1)	0.218	328	4.5	56	17
071106lr10 (G)	2	83 (± 1)	0.165	416	4.5	49	17
071106lr13 (I)	1.3	92 (± 3)	0.134	532	4.5	46	17

In order to determine if the pDMAEMA was able to condense the DNA in 0.2% ethanol solution, the association and the dissociation of DNA by polyaspartic acid were studied by gel electrophoresis. As shown in Fig. 9, the samples A, C, E, G, and I prepared at different N/P ratios are completely compacted. No free DNA(open circular and supercoiled) can be detected. These polyplexes showed naked DNA (open circular and supercoiled) at all ratios studied. These results are in agreement with those of Arigita[22] from previous work in our

group on association/dissociation of polyplexes in hepes buffer and reaffirm that by using ethanol/water mixtures a good compaction or condensation of the DNA could be achieved.

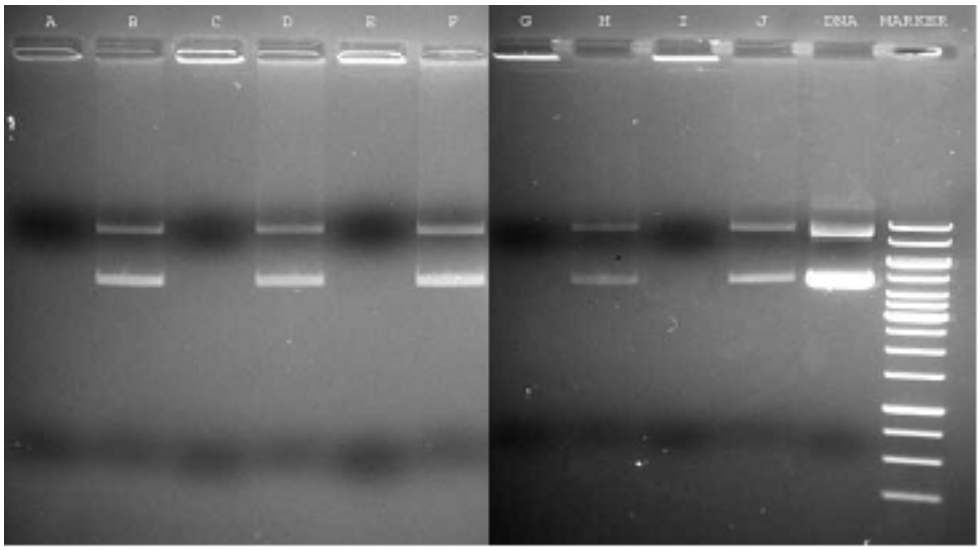


Fig. 9: Agarose gel electrophoresis of pDMAEMA/DNA polyplexes in 0.2% ethanol at N/P ratio of: 5 (A), 4.3 (C), 3.4 (E), 2 (G), and 1.3 (I) and agarose gel electrophoresis of the DNA dissociated by p(ASP) from pDMAEMA/DNA polyplexes in 0.2% ethanol at N/P ratio of: 5 (B), 4.3 (D), 3.4 (F), 2 (H) and 1.3 (J). Free DNA and the marker GeneRuler of 1kb were used as references.

Table 22: Characteristics of the polyplexes prepared in 1,2-dimethoxyethane-water mixtures

Sample	*DME concentration % (v/v)*	*diameter / nm*	*PDI*	*mean count rate*
011101lr01	0.2	81 (± 1)	0.229	306
011101lr04	0.5	85 (± 1)	0.244	282
011101lr07	1	89 (± 3)	0.212	370

Table 22 shows the particle size of the polyplexes prepared in DME but any further decrease of the particle size can be observed in comparison to the polyplexes prepared in ethanol-water solutions. THF was also tested as a solvent, but no reduction of the particle

size was achieved. In contrast, an increase of the particle size with the THF concentration was observed (data not shown).

4 Conclusions and Outlook

In order to achieve the main goal of this scientific research regarding the optimization of the polyplexes consisting of pDMAEMA and DNA, several approaches were used in which different parameters were varied. Parameters such as mixing time and manner of mixing, temperature-induced condensation, pH dropping from alkaline conditions, confinement by macro and miniemulsions, and solvent-induced condensation were studied during this research.

In the first approach presented in this report concerning the mixing time and the manner of mixing, it was shown that the most appropriate mixing time for the formation of smaller polyplexes were 20 s. Polymer solution was always added to DNA solution during the stirring by a thermomixer to favor the reduction of the polyplex size.

Secondly, the approach to reduce the size of the polyplexes by increasing the temperature of formation of polyplexes was explored. At an N/P ratio of 1.3 and at 40 ºC, 10% reduction of the particle size relative to the standard polyplexes at the same conditions was achieved. For future experiments, it would be necessary to vary even more the N/P ratios of the polyplexes and to study their effect on the particle by increasing the temperature.

In the third approach, the effect of the pH dropping from a DNA/polymer mixture at alkaline conditions on the final size of the polyplexes was studied. Unfortunately, no reduction of the particle size of the standard polyplexes could be observed.

In the forth and fifth approaches, the effect of space restriction on the reduction of the size of the polyplexes was studied. There was no reduction of the size by confinement in emulsions. Instead, after breaking the emulsions to get the water-based polyplexes, a slight

increase of the particle size was observed. Furthermore, a very interesting negative potential of the polyplexes was observed. This negative potential could be the consequence of the formation of an ethylene glycol-containing subphase on the surface of the polyplexes. The use of Tween 80 and Span 80 as surfactant mixture for the formation of the mini(emulsion) favored the formation of this ethylene glycol coating and, thus, a rapid alternative to PEGylation could be generated.

Finally, it was presented that the use of both solvent-water mixtures to induce the condensation of DNA and polyelectrolyte condensation decreased at least 30% the particle size of the standard polyplexes. **80 nm** polyplexes were formed by using a concentration as low as 0.2 % ethanol-water mixture.

A future approach could be the combination of three kinds of DNA condensations in order to reduce the size of the polyplexes: temperature-, solvent- and polyelectrolyte-induced condensation of DNA and polyplexes.

Eighty nm polyplexes in 0.2% ethanol could be used for transfection of cells as a main application of these polyplexes.

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Título: Reduction of the particle size by confinement of the polyplexes in miniemulsions
Asunto:
Autor: 0508225
Palabras clave:
Comentarios:
Fecha de creación: 08/02/2008 01:47
Cambio número: 3
Guardado el: 08/02/2008 01:49
Guardado por: Liliana
Tiempo de edición: 3 minutos
Impreso el: 08/02/2008 01:49
Última impresión completa
Número de páginas: 53
Número de palabras: 11.114 (aprox.)
Número de caracteres: 55.572 (aprox.)