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Research paper

The effect of formulation parameters on the size of poly-((2-dimethylamino)ethyl methacrylate)-plasmid complexes

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Abstract

The aim of this study was to gain insight into the formulation parameters affecting the size of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes (polyplexes). Experimental designs were applied to screen and optimize several variables, which may influence the complex size. In a screening design, it was demonstrated that at a fixed concentration of plasmid (40 μg/ml) after incubation with polymer, the size of the resulting polyplexes was highly dependent on the polymer/plasmid ratio as well as on the pH, viscosity (i.e. sucrose concentration) and ionic strength of the aqueous solution. However, the temperature, PEG 600 (up to 5% (v/v)) and Tween 80 (up to 0.2%) had a marginal effect on the size of the polyplexes. In an optimization design, the effect of the pH, polymer/plasmid ratio and Tween on the size of the polymer/plasmid complexes prepared at relatively high concentration of plasmid (50–200 μg/ml) was evaluated. Based on the results of the optimization design, a mathematical model was derived, which describes the relationship between the size of the polyplexes and the different formulation parameters. This model shows that even at high plasmid concentration (200 μg/ml), small sized polyplexes were formed at low pH and ionic strength, especially when the solution contains 20% (w/v) sucrose. This concentrated polyplex dispersion (polymer/plasmid ratio $>$3/1 (w/w), 200 μg plasmid/ml) can be diluted down to 5 μg/ml plasmid without significant changes in particle size and transfection potential. At lower ratios, a growth in particle size was observed upon dilution of the complexes, which might also explain the low transfection efficiency of these polyplexes in vitro.

Keywords: Experimental design; Formulation parameter; Polymer/plasmid complexes; Polyplexes; Poly((2-dimethylamino)ethyl methacrylate; Transfection

1. Introduction

Cationic polymers are widely under investigation as non-viral transfectants to introduce DNA into a target cell [1,2]. These polymers bind via electrostatic interactions to the negatively charged DNA which results in the formation of polymer/plasmid complexes (for these complexes the name ‘polyplexes’ was suggested [3]). The size of the polyplexes is an important factor for the transfection efficiency as has been recently demonstrated by us for poly((2-dimethylamino)ethyl methacrylate)/plasmid complexes [4–7]. Small, positively-charged and stable polymer/plasmid complexes could be formed in an aqueous buffer solution (20 mM Hepes, pH 7.4) at a plasmid concentration not exceeding 40 μg/ml. At higher plasmid concentration severe aggregation was observed and these aggregates possessed a low transfection potential [4,5]. However, for in vivo studies, relatively high concentrations of plasmid DNA have to be administered in order to obtain a detectable transfection level [8–12]. The physico-chemical and biopharmaceutical properties of polyplexes and lipoplexes has been studied in literature [13–15]. However, no systematic study of the

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influence of formulation parameters on the characteristics of polyplexes has been published.

The aim of this study is to gain insight into the formulation parameters (pH, ionic strength, temperature, viscosity of the aqueous solutions, polymer/plasmid ratio and the presence of stabilizers for colloidal systems) which affect the size of polymer/plasmid complexes. This information can be utilized to increase the polyplex concentration by rational method instead of trial and error. Since the formation of polyplexes is a multivariate problem, we decided to use experimental designs. Experimental design approaches are a powerful method to investigate a multivariate problem with a minimum of effort [16,17]. Moreover, with an experimental design, not only the influence of each variable on the determinant (response) can be evaluated, but also the interaction between independent variables can be assessed [18,19].

2. Materials and Methods

2.1. Materials

pCMV-lacZ plasmid contains a bacterial lacZ gene preceded by a nuclear location signal under control of the CMV promoter [20]. 2-(Dimethylamino)ethyl methacrylate (DMAEMA) was obtained from Fluka. RPMI-1640 medium and DMEM (Dulbecco's modified Eagles medium) were obtained form Gibco, Breda, The Netherlands. Fetal Calf Serum (FCS) was purchased from Integron, Zaandam, The Netherlands. Cells were cultured in complete DMEM medium, which was prepared by supplementing plain DMEM with FCS (final concentration 5%), Hepes (final concentration 25 mM, pH 7.4), penicillin (final concentration 100 IU/ml), streptomycin (final concentration 100 μg/ml) and amphotericin B (final concentration 0.25 μg/ml). X-Gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) was from Gibco, Breda, The Netherlands. XTT reagent (proliferation Kit II) was a product of Boehringer, Mannheim, Germany. All other chemicals and reagents used were of analytical grade.

2.2. Synthesis and characterization of PDMAEMA

PDMAEMA was prepared by a radical polymerization of 2-(dimethylamino)ethyl methacrylate essentially as described previously [4]. The number average molecular weight (M_n) and weight average molecular weight (M_w) relative to dextran as determined by gel permeation chromatography were 47×10^3 and 280×10^3 g/mol, respectively.

2.3. Experimental design

Two experimental designs were used, namely a screening and an optimization design. CARD software (S-matrix, Eureka, CA) was used to define the sets of experiments and analyze the data. Experimental designs based on D-optimality were utilized since the experimental domain (space of factors) in which the range of factors is restricted, is an irregular polyhedron area [21–23]. Moreover, D-optimal designs are frequently used because these are suitable for factors evaluated at more than two levels [24].

In the screening design, seven variables which might influence the complex size were rationally selected (Table 1); 11 trials were required for the complete screening of the experimental domain. The *z*-average particle size as determined by dynamic light scattering (DLS) was used as a response. In samples where aggregation occurred (visual inspection), the size of the complexes was arbitrarily taken as 1000 nm. The results of the first design were used to define a second (optimization) design in which the effect of three factors on the size of the complexes was evaluated in more detail. Therefore, 27 trials including four replicates (see optimization design: sample 1 and 27, sample 3 and 6, sample 4 and 21, sample 7 and 15; Table 2) were performed and the average particle size of the formed complexes was used as a response. For both designs, a second-order polynomial model was applied to fit the obtained data [25].

2.4. Preparation of PDMAEMA-plasmid particles

First, a stock solution of PDMAEMA in HBS (Hepes buffered saline; 20 mM Hepes, 0.9% (w/v) NaCl, pH 7.2) was prepared (final concentration 5 mg/ml). Aliquots of this solution were diluted with 20 mM acetate (pH 5.0 or 5.7) or 20 mM Hepes buffers (pH 6.5, 7.4 or 8.0) to the required polymer concentration. The plasmid stock solution (3.0 mg DNA/ml in 10 mM Tris/1 mM EDTA, pH 7.4) was diluted with the same buffers. Solutions with varying concentrations of polymer and plasmid were made to reach the polymer/plasmid ratios defined by the experimental design. Next, 100 μl plasmid solution was mixed with 500 μl aqueous solution containing different concentrations of sucrose, Tween 80, PEG 600 and NaCl. If necessary, the solutions were brought at the required temperature (0 or 40°C). Subsequently, 400 μl polymer solution was added to the plasmid solution and gently mixed for 5 s (Vortex Genie 2) and then incubated for 30 min at ambient temperature.

2.5. The effect of pH and NaCl on size and charge of polyplexes

To study the influence of pH on the characteristics of the polyplexes, samples were prepared at a fixed plasmid concentration (200 μg/ml) and polymer concentration (1mg/ml) in the presence of 20% sucrose at varying pH (ranging from 6.5 to 8.0, 20 mM Hepes). The size and charge (zeta potential) of the polyplexes was determined after 30 min incubation at room temperature.

Table 1

Levels of the independent variables evaluated in the screening design and the effect of independent variables on the size of polyplexes (a standard deviation in the size around 0.01 μm was observed ($n = 3$))

Sample	(X_1)	(X_2)	(X_3)	(X_4)	(X_5)	(X_6)	(X_7)	$(X_3)^2$	$X_1{*}X_2$	Observed size (μm)	P.d.	Predicted size (μm)
1	1	1	−1	1	1	1	1	1	1	0.109	0.34	0.109
2	1	−1	1	1	1	1	1	1	−1	0.213	0.14	0.225
3	1	−1	0	1	1	−1	0	0	−1	0.135	0.14	0.133
4	1	0	1	1	−1	−1	1	1	0	0.124	0.25	0.123
5	1	1	1	−1	−1	−1	−1	1	1	0.178	0.21	0.178
6	1	−1	−1	−1	1	1	1	1	−1	0.234	0.65	0.226
7	−1	1	1	1	1	1	1	1	−1	0.158	0.20	0.155
8	−1	−1	1	1	1	1	1	1	1	1*	n.d.	0.883
9	−1	1	−1	1	1	−1	1	1	−1	0.121	0.35	0.122
10	−1	−1	0	−1	1	1	1	0	1	0.314	0.52	0.323
11	−1	−1	−1	−1	−1	−1	−1	1	1	0.423	0.43	0.429

Independent variables	Units	Ranges/levels (Normalized values)		
		1	0	− 1
Polymer/plasmid ratio (X_1)	(w/w)	(At a ratio of 5/1)		(At a ratio of 1/1)
Ionic strength (X_2)	NaCl (M)	0	0.25	0.5
Sucrose (X_3)	%, (w/v)	0	20	40
pH (X_4)		5		8
Tween 80 (X_5)	%, (v/v)	0		0.2
PEG 600 (X_6)	%, (w/v)	0		5
Mixing temperature (X_7)	°C	0	20	40

*Size of aggregates is arbitrarily taken as 1 μm.

To study the effect of the ionic strength on the polyplex formation, samples were prepared at a fixed plasmid concentration (200 μg/ml) and a polymer concentration (1mg/ml) in the presence of 20% sucrose at pH 6.5, 20 mM Hepes and a varying NaCl concentrations (0, 0.25 and 0.5 M).

2.6. Particle size and zetapotential measurements

The *z*-average size and polydispersity index (p.d.) of the PDMAEMA-plasmid particles were determined by dynamic light scattering (DLS) at 25°C with a Malvern 4700 system using a 25 mW He-Ne laser (NEC, Tokyo, Japan) and an automeasure version 3.2 software (Malvern, Malvern, UK). As a measure of the homogeneity of the colloid dispersion, the system reports a p.d. This index ranges from 0.0 for an entire homogeneous up to 1.0 for a completely inhomogeneous dispersion. Each sample was measured in triplicate; for the non-aggregated samples the accuracy was around 10 nm. The refractive index of the aqueous solutions was determined using a refractometer. The viscosity of the solutions was determined by DLS measurements, using a standard latex with known diameter (100 nm) essentially according to the method described by De Smidt and Crommelin [26].

Zeta potential measurements were conducted by determining the electrophoretic mobility at a temperature of 25°C with Zetasizer 2000 (Malvern, Malvern, UK). Each sample was determined in triplicate.

2.7. Cell culture and transfection

Gene transfer studies without chloroquine were performed essentially as described previously, using COS-7 cells (cells of SV-40-transformed African green monkey kidney) [4]. Before transfection, the polymer/plasmid complexes were further diluted with RPMI to a plasmid concentration of 5 μg/ml and carried out by adding 1 μg plasmid with varying amount of PDMAEMA upon 1.1×10^4 cells in 96-well plates. Expression of the pCMV-lacZ gene was established of fixed cells (0.25% glutaraldehyde; 5 min at 4°C) with X-gal (0.8 mg/ml) in phosphate buffer, pH 7.4) for 24 h.

Transfection values expressed as relative transfection efficiency were normalized to the number of transfected cells, found after incubation of the cells with freshly prepared polymer/plasmid complexes in Hepes (polymer and plasmid in these samples were 120 and 40 μg/ml, respectively; final concentrations during transfection were 15 μg polymer/ml and 5 μg plasmid/ml). The cell viability was evaluated using an XTT-assay as described previously [4].

Table 2

Levels of the independent variables evaluated in the optimization design and the effect of independent variables on the size of polyplexes in the optimization design (a standard deviation in the size around 0.01 μm was observed (n = 3))

Sample	(X_1)	(X_2)	(X_3)	(X_4)	$(X_1)^2$	$(X^2)_2$	$(X_3)^2$	$(X_4)^2$	X_1*X_2	X_1*X_3	X_1*X_4	X_2*X_3	X_2*X_4	X_3*X_4	Observed size (μm)	P.d.	Predicted size (μm)
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.181	0.31	0.207
2	0	0	0	−1	0	0	0	1	0	0	0	0	0	0	0.199	0.30	0.195
3	0	0	−1	0	0	0	1	0	0	0	0	0	0	0	1*	n.d.	0.73
4	1	−1	−1	−1	1	1	1	1	−1	−1	−1	1	1	1	0.239	0.42	0.229
5	1	1	−1	1	1	1	1	1	1	−1	1	−1	1	−1	0.290	0.42	0.282
6	0	0	−1	0	0	0	1	0	0	0	0	0	0	0	1*	n.d.	0.73
7	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0.121	0.32	0.118
8	0.25	0.25	0.25	0.25	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.182	0.28	0.166
9	1	1	−1	−1	1	1	1	1	1	−1	−1	−1	−1	1	0.226	0.36	0.235
10	1	−1	−1	1	1	1	1	1	−1	−1	1	1	−1	−1	0.294	0.42	0.308
11	−1	−1	−1	−1	1	1	1	1	1	1	1	1	1	1	1*	n.d.	0.735
12	−1	1	−1	1	1	1	1	1	−1	1	−1	−1	1	−1	1*	n.d.	1.068
13	−1	1	1	−1	1	1	1	1	−1	−1	1	1	−1	−1	1*	n.d.	1.113
14	1	−1	1	1	1	1	1	1	−1	1	1	−1	−1	1	0.103	0.47	0.099
15	0	1	1	1	0	1	1	1	0	0	0	1	1	1	0.115	0.30	0.118
16	1	1	1	−1	1	1	1	1	1	1	−1	1	−1	−1	0.153	0.25	0.14
17	−1	1	−1	−1	1	1	1	1	−1	1	1	−1	−1	1	1*	n.d.	0.891
18	−1	0	0	0	1	0	0	0	0	0	0	0	0	0	1*	n.d.	0.559
19	1	−1	1	−1	1	1	1	1	−1	1	−1	−1	1	−1	0.137	0.35	0.141
20	−1	−1	1	−1	1	1	1	1	1	−1	1	−1	1	−1	1*	n.d.	1.024
21	1	−1	−1	−1	1	1	1	1	−1	−1	−1	1	1	1	0.224	0.43	0.229
22	−1	−1	1	1	1	1	1	1	1	−1	−1	−1	−1	1	0.220	0.16	0.225
23	−1	−1	−1	1	1	1	1	1	1	1	−1	1	−1	−1	1*	n.d.	1.288
24	0	−1	0	0	0	1	0	0	0	0	0	0	0	0	0.176	0.39	0.175
25	1	1	1	0	1	1	1	0	1	1	0	1	0	0	0.118	0.31	0.127
26	−1	0	−1	−1	1	0	1	1	0	1	1	0	0	1	1*	n.d.	3.445
27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.181	0.31	0.207

Independent variables	Units	Ranges/ levels	(Normalized values)		
		1	0.25	0	− 1
pH (X_1)		5	5.7	6.5	8
Tween 80 (X_2)	%, (v/v)	0.1	0.2	0.3	0.5
Plasmid concentration (X_3)	μg/ml	50	87.5	125	200
Polymer/ plasmid ratio (X_4)	(w/w)	(At a ratio of 5/1)	(At a ratio of 3/1)	(At a ratio of 2/1)	(At a ratio of 1/1)

*Size of aggregates is arbitrarily taken as 1 μm

3. Results

3.1. Selection of the formulation parameters

PDMAEMA and plasmid DNA bind to each other via electrostatic interactions. It might therefore be expected that the charge density on the polymer is an important parameter affecting the size of the polyplexes. Since the charge density of the polymer depends on the pH of the aqueous solution, we selected pH values ranging from pH 5.0 where the tertiary amine side-groups of the polymer are all protonated, to pH 8.0 where around 25% of the side groups are protonated (the pK_a of the polymer is around 7.5). The ionic strength of the solution (NaCl concentration) is an important factor as well, because it decreases the double layer, thereby affecting the stability of the colloidal polymer/plasmid particles. Moreover, a high ionic strength might dissociate the polymer-DNA complexes. In a previous study, it was found that the formation of polymer/plasmid aggregates is dependent on the polymer/plasmid ratio [4]; this ratio was therefore taken as a variable too. The polymer/plasmid ratios evaluated were 1/1, 3/1 and 5/1, which correspond with a N/P (amine/phosphate) ratio around 2/1, 6/1 and 10/1, respectively. Besides, it might be expected that the temperature and the viscosity of the solution are important factors for the stability of colloidal systems. Moreover, the effect of PEG and Tween 80 on particle size of the polymer/plasmid complexes was evaluated as well, since these compounds are known stabilizers for colloidal systems.

3.2. Screening design

In the screening design, we evaluated the effect of the variables discussed above on the size of the polymer/plasmid complexes (Table 1). The complexes were prepared at a relatively low concentration of plasmid (40 μg/ml). The data were fitted with a linear and a polynomial model. However, these models had a low predictive value (low R^2, data not shown). We therefore used data transformation in a second order polynomial model, as suggested by Box and Draper [27]. The analysis resulted in the following Eq. (1):

$$Y = 8.452 + 0.915 * X_1 + 1.911 * X_2 - 1.188 * X_3 + 1.195 * X_4 - 1.567 * X_5 + 0.316 * X_6 + 0.645 * X_7 - 3.160 * (X_3)^2 - 0.734 * (X_1X_2) \quad (1)$$

$R_2 = 0.99$

in which Y = 1/size (μm) and X_{1-7} represent the normalized values for the different independent variables (Table 1). Only one non-linear term $(X_3)^2$ and one interaction term are included (X_1X_2) in Eq. (1), since the variation inflation factors for the other non-linear and interaction terms were >10 [28]. Table 1 shows that, by the model, the predicted sizes of the particles are in very good agreement with the observed values. From Eq. (1), the following trends can be seen. A low ionic strength favors the formation of small particles: at $I = 0.5$ (normalized value -1, Table 1), Y is on the average 2×1.91 smaller than at $I = 0$ (normalized value 1); therefore the particle size ($1/Y$) increases with ionic strength. Since the coefficient values for PEG and temperature are relatively small, the effect of these variables on particle size is marginal. The effect of sucrose on the size of the particles is complicated. The linear coefficient value suggests that smaller particles are formed at high viscosity. However, the non-linear coefficient suggests that larger particles are formed at both low and high sucrose concentration (normalized values of X_3 are 1 and -1, respectively; X_3^2 is 1) and that the smallest particles are formed at 20% sucrose (normalized values is 0); this is shown in Fig. 1.

3.3. Optimization design

Based on the results of the screening design, the temperature (20°C), the concentration of sucrose (20%) and the ionic strength (20 mM buffer without NaCl) were fixed, whereas PEG was excluded. In the optimization design the effect of pH, polymer/plasmid ratio, Tween 80 and plasmid concentration on particle size was studied. Factors with continuous ranges were chosen to allow evaluation of small changes of variables in response across a compact of design space. Table 2 gives the results of determined and predicted size. It appears that the preparation of the polyplexes was very reproducible (see the replicates: sample 1 and 27, sample 3 and 6, sample 4 and 21, sample 7 and 15). Besides, it can also be seen in Table 2 that most polyplexes, especially the ones prepared at high plasmid concentration (normalized value -1), have a polydispersity index around 0.3. This means that the particles are not fully homodisperse. However, a polydispersity of 0.3 is not uncommon for polymer/plamid particles [6]. As for the screening design, a second order polynomial model with data transformation was utilized to fit the obtained data (shown as Eq. (2)):

$$Y = 4.815 + 2.176 * X_1 + 0.055 * X_2 + 1.637 * X_3 + 0.688 * X_4 - 0.850 * (X_1)^2 + 0.950 * (X_2)^2 - 1.808 * (X_3)^2 + 0.978 * (X_4)^2 + 0.034 * (X_1X_2) + 0.777 * (X_1X_3) - 0.130 * (X_1X_4) + 0.040 * (X_2X_3) + 0.099 * (X_2X_4) + 1.011 * (X_3X_4) \quad (1)$$

$R_2 = 0.98$

Again, the predicted polyplex sizes are in very good agreement with the observed values (Table 2). Since the model contains both non-linear as well as interaction terms, a direct analysis of the effect of the various independent variables on particle size is not possible.

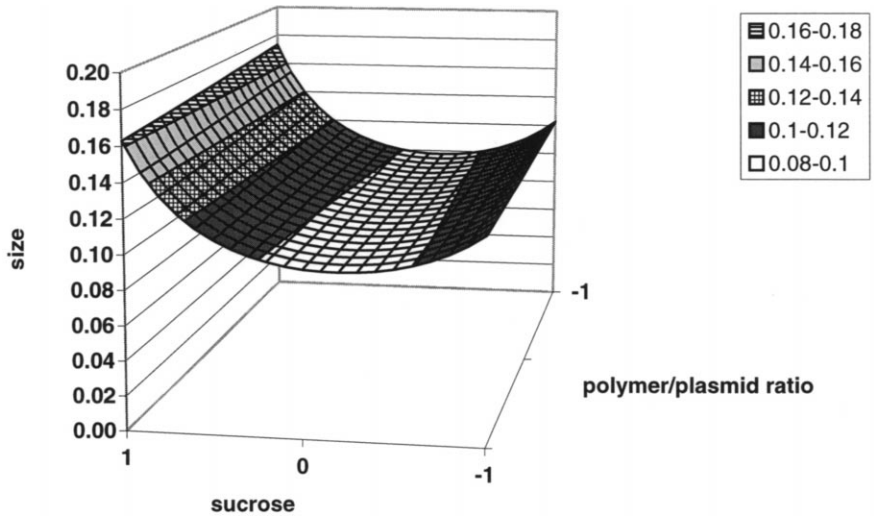

Fig. 1. Three dimensional surface plot illustrating the effect of sucrose and the polymer/plasmid ratio on the size of the polyplexes prepared at pH 5.0 and 20°C without Tween 80, PEG and NaCl. Normalized values of sucrose: 1 = 0%, 0 = 20% and −1 = 40%. For polymer/plasmid ratio: 1 = 5/1 and −1 = 1/1.

The influence of pH and plasmid concentration on particle size of polyplexes is shown in Fig. 2a. For the pH values ranging from 5 to approximately 6.8, small particles with a size between 0.2–0.3 μm are formed when 200 μg/ml plasmid is used for the preparation of the particles. However, the size tremendously increased when the pH is above 7. As can be seen, the preparation of non-aggregated complexes at pH 8.0 and a plasmid concentration of 200 μg/ml is not possible.

Fig. 2b shows the particle size as a function of polymer/plasmid ratio and plasmid concentration. Only a slight effect of these variables on the size is observed. Also Tween 80 has a marginal effect on the particle size (Fig. 2c). All figures demonstrate that the particle size increases with increasing plasmid concentration.

The model derived from the optimization design was used to predict under which conditions the smallest polyplexes can be formed at a plasmid concentration of 200 μg/ml. The outcome of this analysis indicates that a buffer of pH 5.7, 0.1% Tween 80 and a polymer/plasmid ratio of 1/1 (w/w) have to be used to prepare polyplexes with a predicted size of 0.28 μm. Particles which were prepared under these conditions possessed a size of 0.23 μm (Table 3), demonstrating the robustness of the model.

We also prepared particles at a polymer/plasmid ratio of 3/1 (w/w) in the absence and presence of Tween, and at varying plasmid concentration. Table 3 shows that in agreement with previous experience, the size of the polyplexes increases with increasing plasmid concentration. In addition, a good agreement between the predicted and observed sizes is seen, again demonstrating the good predictive power of the model. No significant differences between the size of particles prepared in absence or presence of Tween is observed (Table 3).

3.4. Effect of the pH on particle size and zetapotential of polyplexes

Fig. 3 shows the characteristics (size and charge) of polyplexes prepared at different pH values (polymer/plasmid ratio, 5/1). This figure also shows the size of the particles as predicted with our model. It can be seen that the polyplexes prepared between pH 6.5 and 7.5 have a small size. However, severe aggregation occurred at pH 7.7 and 8.0 (particle size arbitrarily taken as 1 μm, see Section 2). Although the predicted and found sizes do not fully agree, especially at pH 7.5. and 7.7, the model perfectly predicted the found trend: an increase in particle size with pH. Fig. 3

Table 3

The size of polyplexes prepared under different conditions*

Formulation	Polymer/plasmid ratio (w/w)	Plasmid concentration (μg/ml)	Tween 80 (0.1%)		Without Tween 80
			Predicted value in size (μm)	Observed value in size (μm)	Observed value in size (μm)
I	(At a ratio of 3/1)	50	0.12	0.13	0.13
II	(At a ratio of 3/1)	100	0.12	0.18	0.18
III	(At a ratio of 3/1)	150	0.16	0.23	0.26
IV	(At a ratio of 3/1)	200	0.34	0.28	0.27
V	(At a ratio of 1/1)	200	0.28	0.23	0.24

*A standard deviation = 0.01μm was observed in all size measurements (n = 3).

also shows that zetapotential of polyplexes decreases with increasing pH.

Furthermore, we studied the effect of the NaCl concentration on the size of the polyplexes (polymer/plasmid ratio 5/1, plasmid concentration 200 μg/ml, 20% sucrose, 20 mM Hepes, pH 6.5). In the absence of NaCl small polyplexes with a size around 0.3 μm were obtained. In agreement with the results of our screening design, polymer/plasmid aggregates were formed at 0.25 and 0.5 M NaCl.

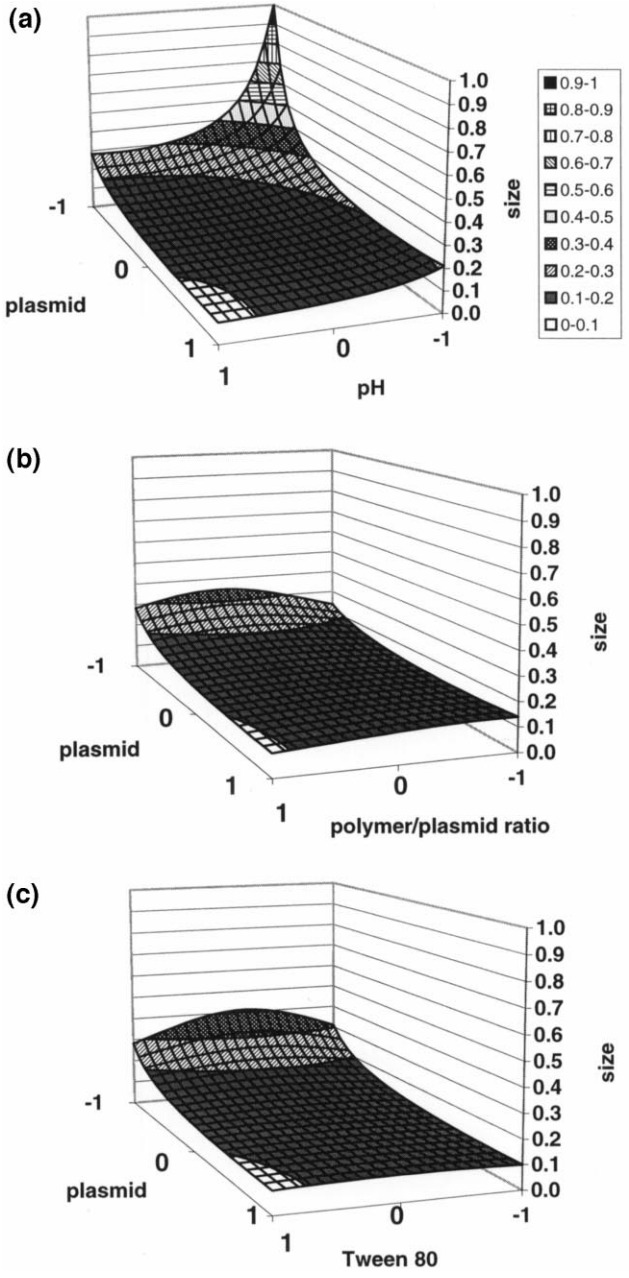

Fig. 2. (a) Three dimensional surface plot illustrating the effect of pH and plasmid concentration on the size of polyplexes prepared in the presence of 0.1% Tween 80 and at polymer/plasmid ratio of 5/1 (w/w). Normalized values of plasmid concentration: 1 = 50 μg/ml, 0 = 125 μg/ml and −1 = 200 μg/ml. For pH: 1 = 5, 0 = 6.5 and −1 = 8. (b) Three dimensional surface plot illustrating the effect of polymer/plasmid ratio and plasmid concentration on the size of polyplexes in the presence of 0.1% Tween 80 and at pH = 5.0. Normalized values of polymer/plasmid ratio: 1 = 5/1, 0 = 2/1 and −1 = 1/1. (c) Three dimensional surface plot illustrating the effect of Tween 80 and plasmid concentration on the size of the polyplexes prepared at a polymer/plasmid ratio 5/1 and pH = 5.0. Normalized values of Tween 80, 1 = 0.1%, 0 = 0.3% and −1 = 0.5%.

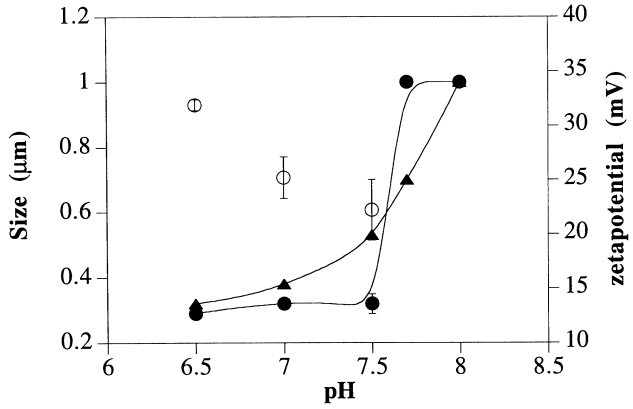

Fig. 3. Effect of pH on the size and zetapotential (○) of polyplexes. Polyplexes were prepared at a polymer/plasmid ratio of 5:1 and at a fixed plasmid concentration of 200 μg/ml in 20 mM Hepes containing 20% (w/v) sucrose. The observed particle sizes (●) and predicted values (▲) were compared. The results are expressed as mean values ± S.D. of three experiments. Zetapotential was not determined for those samples results in aggregates at pH 7.7 and 8.0.

3.5. Transfection and cytotoxicity study

Polymer/plasmid particles prepared at a relatively high plasmid concentration (formulation IV and V, Table 3) were evaluated for their transfection potential after dilution with RPMI to a final plasmid concentration ranging from 5–50 μg /ml. A formulation prepared at a plasmid concentration of 200 μg/ml and a polymer/plasmid ratio of 5/1 (w/w) was evaluated too.

The relative transfection efficiency of these complexes as a function of plasmid concentration is shown in Fig. 4a. It can be seen that after dilution to a plasmid concentration of 5 μg/ml, the complexes prepared at a ratio of 3/1 and 5/1 possessed the same transfection efficiency as particles prepared at a 3/1 ratio and a plasmid concentration of 5 μg/ml (relative transfection efficiency around 1). For the particles prepared at 3/1 and 5/1 ratios, the transfection efficiency increases with plasmid concentration up to 25 μg/ml and 15 μg/ml, respectively; thereafter the transfection efficiency decreases. In a previous study, we reported that in presence of chloroquine, a maximum in transfection efficiency was observed at a plasmid concentration of 10 μg/ml [4]. The difference with the results found in that paper might be explained by the toxicity of chloroquine [6]. At a plasmid concentration of 5 μg/ml, particles prepared at a 1/1 ratio were hardly able to transfect cells. A slight increase in transfection efficiency with plasmid concentration is observed for these polyplexes. Table 4 gives the sizes of the polyplexes used for the transfection experiments shown in Fig. 4a. It appears that the polyplexes prepared at a 3/1 and 5/1 polymer/plasmid ratio are stable after dilu-

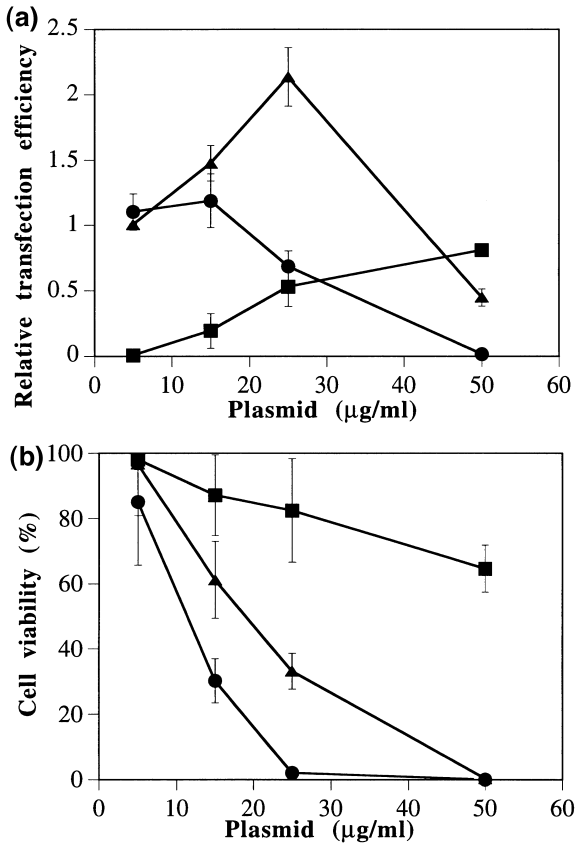

Fig. 4. (a) Relative transfection efficiency of polyplexes after dilution with RPMI to a varying plasmid concentration. The results are expressed as mean values ± S.D. of two to three experiments. Polyplexes were prepared at a polymer/plasmid ratio of 5:1 (●), 3:1 (▲) and 1:1 (■) at a fixed plasmid concentration of 200 μg/ml in 20 mM acetate buffer (pH 5.7) containing 20% (w/v) sucrose. (b) Effect of polyplexes on cell viability. The results are expressed as mean values ± S.D. of two to three experiments. Polyplexes were prepared at a polymer/plasmid ratio of 5:1 (●, 3:1 (▲) and 1:1 (■) at a fixed plasmid concentration of 200 μg/ml in 20 mM acetate buffer (pH 5.7) containing 20% (w/v) sucrose. Subsequently, polyplexes were diluted in RPMI to the desired plasmid concentration.

tions with RPMI. However, the polyplexes prepared at a 1/1 ratio showed aggregation after dilution. It is shown in Fig. 4b that the cell viability drops with increasing polymer/plasmid concentration. This reduction was more pronounced for the polyplexes prepared at a high polymer/plasmid ratio.

4. Discussion

We have previously described that small polyplexes (size around 0.2 μm) were formed by simply mixing equal volumes of aqueous solutions of Hepes buffer (20 mM, pH 7.4) containing plasmid (final concentration 40 μg/ml) and polymer (final concentration 120 μg/ml). At higher plasmid concentration severe aggregation was observed. By application of experimental designs, we now succeeded to prepare polyplexes at a relatively high concentration of plasmid (final concentration 200 μg/ml). The designs also showed that a low pH favored the formation of small polyplexes. A likely explanation is that at pH 5, the side groups of the polymers are all protonated, resulting in a polymer with a high charge density. Therefore strong plasmid-polymer interactions occur which results in small polyplexes. It was shown that low ionic strength conditions also favor the formation of small polyplexes. Again, the size of the particles decreases with increasing polymer-plasmid interaction. We observed a tremendous change in the size of the polyplexes from pH 7–8 (Fig. 3). Since the degree of protonation changes substantially in this pH range (pKa of the polymer is around 7.5), the strength of the polymer-DNA interactions reduces accordingly. Moreover, a lower degree of protonation also resulted in particles with a lower zeta-potential (Fig. 3), which might be the reason for the formation of aggregates at pH values around 8.

We showed that PEG, Tween 80 and temperature had a marginal effect on the size of the polyplexes. Sucrose had a rather complex effect on the size of the polyplexes. Going from 0–20% sucrose, the particle size decreases. The higher viscosity of the 20% sucrose solution (compared with the viscosity of Hepes buffer) may prevent the aggregation of formed polyplexes. Also DNA-sucrose interactions might play a role (cf. discussion on PEG). However, going from 20 to 40% sucrose, the size of the formed polyplexes increases. A possible explanation for the tendency to form aggregates at a concentration >20% sucrose may be that the interaction between the positively charged polymer and negatively charged plasmid DNA is hindered by a viscous 'barrier'. Although the viscosity of a 5% PEG solution is about the same as a 20% sucrose solution (1.66 and 2.07 mPa, respectively, as determined by DLS analysis [25]), PEG does not favor the formation of small particles. This

Table 4
The size of polyplexes upon dilution with RPMI*

	Initial plasmid concentration	Plasmid concentration after dilution with RPMI			
	200 μg/ml	50 μg/ml	25 μg/ml	15 μg/ml	5 μg/ml
Polymer/plasmid ratio 1/1	0.22 ± 0.01 μm	Aggregation	Aggregation	Aggregation	Aggregation
Polymer/plasmid ratio 3/1	0.25 ± 0.01 μm	0.21 ± 0.0 μm	0.21 ± 0.01 μm	0.22 ± 0.01 μm	0.22 ± 0.01 μm
Polymer/plasmid ratio 5/1	0.28 ± 0.02 μm	0.21 ± 0.01 μm	0.21 ± 0.01 μm	0.20 ± 0.01 μm	0.20 ± 0.01 μm

*These polyplexes were prepared at a fixed plasmid concentration (200 μg/ml) at polymer/plasmid ratio of 5/1, 3/1 and 1/1 in 20 mM acetate buffer (pH 5.7) containing 20% (w/v) sucrose.

might indicate that specific sucrose-DNA interactions [29] play a role in the formation of small polyplexes.

In the transfection study, the results showed that polyplexes prepared at a relatively high concentration of plasmid (200 μg/ml) and at a polymer/plasmid ratio of 3/1 or 5/1 retained there full transfection potential after dilution to a plasmid concentration of 5 μg/ml (relative transfection efficiency around 1). At higher plasmid concentration ($>$15 μg/ml) the transfection efficiency decreases (for the particle prepared at a 5/1 ratio) or first increases and then decreases ($>$25 μg/ml plasmid for the particles prepared at a 3/1 ratio). This can be explained by the cytotoxicity of the polymer either in its free form or complexed to plasmid DNA resulting in the decrease of transfection efficiency (Fig. 4b). It should be mentioned that although PDMAEMA is toxic for cells, poly(lysine), a frequently used transfectant [2], is substantially more toxic [6]. In addition, the toxicity of PDMAEMA is masked once complexed with DNA [4,7]. Interestingly, polyplexes prepared at a 1/1 ratio were hardly able to transfect cells, in spite of the fact that the size of the polyplexes prepared at this ratio before dilution was about the same as for the particles prepared at a 3/1 and 5/1 ratio (Table 4). However, after dilution severe aggregation was observed in solutions containing polyplexes prepared at a 1/1 ratio, whereas the particle size (around 0.2 μm) and zeta potential (around +24 mV) of the polyplexes prepared at the other ratios did not change after dilution (Table 4). The instability of polyplexes prepared at ratio 1/1 is in agreement with the finding of Pouton et. al. [30] for lipid/plasmid complexes. The reasons for the particle aggregation is not yet clear. However, the observed aggregation of the polyplexes after dilution could explain their low transfection. Although the findings reported in this paper are, strictly speaking, only applicable for PDMAEMA/plasmid complexes, the obtained insights can be used for the formulation of other polymer-based polyplexes.

5. Conclusion

This study shows that the presence of sucrose, low pH and low ionic strength favor formation of relatively small polyplexes. This information allows us to prepare polyplexes at a plasmid concentration of 200 μg/ml with a small size (approximately 0.2 μm). This concentrated complex dispersion can be diluted without significant changes in particle size and in vitro transfection efficiency if a polymer/plasmid ratio above three was used. For lower ratios, particle size growth was observed upon dilution of the complexes with RPMI, which might also explain the low transfection efficiency of these complexes in vitro.

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