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Optimizing the Novel Formulation of Liposome-Polycation-DNA Complexes (LPD) by Central Composite Design

Xun Sun and Zhirong Zhang

West China School of Pharmcy, Sichuan University, No. 17, Section 3, Renmin South Road, Chengdu 610041, China

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LPD vectors are non-viral vehicles for gene delivery comprised of polycation-condensed plasmid DNA and liposomes. Here, we described a novel anionic LPD formulation containing protamine-DNA complexes and pH sensitive liposomes composed of DOPE and cholesteryl hemisuccinate (Chems). Central composite design (CCD) was employed to optimize stable LPD formulation with small particle size. A three factor, five-level CCD design was used for the optimization procedure, with the weight ratio of protamine/DNA (X_1), the weight ratio of Chems/DNA (X_2) and the molar ratio of Chems/DOPE in the anionic liposomes (X_3) as the independent variables. LPD size (Y_1) and LPD protection efficiency against nuclease (Y_2) were response variables. Zeta potential determination was utilized to define the experimental design region. Based on experimental design, responses for the 15 formulations were obtained. Mathematical equations and response surface plots were used to relate the dependent and independent variables. The mathematical model predicted optimized X_1-X_3 levels that achieve the desired particle size and the protection efficiency against nuclease. According to these levels, an optimized LPD formulation was prepared, resulting in a particle size of 185.3 nm and protection efficiency of 80.22%.

Key words: Liposome-Polycation-DNA complexes (LPD), Central composite design (CCD), Formulation optimization

INTRODUCTION

Non-viral delivery systems for gene therapy have been increasingly proposed as safer alternatives to viral vectors. They have the potential to be administered repeatedly with minimal host immune response, stable in storage, easy to produce on a large scale. These advantages have provided the impetus to continue their development. So far, several non-viral delivery systems have been developed, such as liposomes (Stuart and Allen, 2000), nanoparticles (Corsi *et al.*, 2003), hydrogel (Vinogradov *et al.*, 2002), emulsion (Kim *et al.*, 2003) and peptide nucleic acid (Koppelhus *et al.*, 2003). Complexes formed between cationic liposomes and plasmid DNA have been the predominant non-viral vectors employed for the transfection of eukaryotic cells in research laboratories (Audouy *et al.*, 2002). However, their utility in gene therapy is hampered by toxicity of the cationic lipids, limited *in vivo* transfection efficiency and low tissue specificity. These shortcomings may due to inadequate DNA condensation, poor colloidal stability and incompatibility with the abundance of negatively charged macromolecules present in the physiological environment.

In an effort to seek an *iv*-injectable, targetable vector for gene transfer, Lee and Huang developed a new anionic lipidic vector LPD (liposome-polycation-DNA complexes) in 1996 (Lee and Huang, 1996). It contained a highly condensed core composed of poly-L-lysine (PLL) and DNA and an anionic lipid shell. LPD was prepared by the following procedure: DNA was first condensed with PLL with positive charge in moderate excess; the resulting complex was then mixed with anionic liposomes carrying a targeting ligand. Structurally, this formulation was virus-like particles, each containing a condensed genome as the core and a negative lipidic shell as the envelope.

Protamine sulfate, as a naturally occurring substance found in sperm, has been suggested that it gave higher transfection levels than synthetic polymer PLL (Kabanov, 1995). This may due to the presence of nuclear localization signal in protamines amino acid sequence, which can potentate gene expression by increasing the nuclear

Correspondence to: Zhirong Zhang, West China School of Pharmcy, Sichuan University, No.17, Section 3,Renmin South Road, Chengdu 610041, China
Tel: 86-28-8550-1566; Fax: 86-28-8545-6898
E-mail: zrzzl@vip.sina.com

translocation of DNA. Furthermore, this polycation has a favorable antigencity profile based on its extensive history of clinical applications, both in a slow-release insulin formulation and as an antidote to heparin-induced anti-coagulation (Jeannette *et al.*, 2002). As a condensing agent, protamine sulfate has widely been used in the formulation of cationic LPD (Ueno, *et al.*, 2002; Arangoa, *et al.*, 2003), however its application in anionic LPD need to be further investigated and optimized.

For effective optimization of LPD formulation, a systemic approach is required. Optimization strategies can in general be classified as follows: univariate approach, sequential techniques and simultaneous techniques. The univariate approach, which is the simplest one, is typically used in such cases when the effect of a limited number of factors without mutual interactions is examined. It has widely been used for the optimization of LPD formulation (Guo *et al.*, 2002). However, the ratios of the three components in the LPD are interdependent to form the small size, effective vectors. Therefore, we employed central composite design (CCD) to optimize the LPD formulation.

Central composite design (CCD), also called Response Surface Methodology (RSM), is a rapid technique used to empirically derive a functional relationship between an experimental response and a set of input variables. Furthermore, it may determine the optimum level of experimental factors required for a given response. A factor is defined as an input variable whose value can be set during an experiment. The response variable is a measured quantity whose value is affected by levels chosen for the factors. CCD reduces the number of experimental runs that are necessary to establish a mathematical trend in the experimental design region.

In the present study, anionic LPD was prepared using protamine sulfate as the condensing agent. CCD was employed to optimize LPD formulation with small particle size and colloidal stability.

MATERIALS AND METHODS

Materials

The plasmid pORF lacZ (3.54 kb) was purchased from Invivogen (USA). β-Galactosidase reporter gene staining kit, protamine sulfate (derived from salmon), cholesteryl hemisuccinate (Chems) and Dioleoyl phosphatidylethanolamine (DOPE) was purchased from Sigma. Qiagen Giga Endo-free plasmid purification kit was purchased from Qiagen (CA, USA). DNA clean-up kit was obtained from V-gene (Hangzhou, China). All the other chemicals and reagents used were of the analytical grade obtained commercially.

Plasmid DNA preparation

The plasmid pORF lacZ (3.54 kb), is a eukaryotic expression vector which contains the EF-1/HTLV hybrid promoter within an intron. The lacZ gene codes for the enzyme β-galactosidase, whose activity allows for the quick determination of cells expressing the lacZ gene. pORF-lacZ plasmid DNA was isolated and purified from DH5-α *E. coli* using the Qiagen Giga Endo-free plasmid purification kit. DNA concentration and purity were quantified by UV absorbance at 260 nm and 280 nm on a GBC UV cintra 10e Spectrophotometer. The structural integrity and topology of purified DNA was analyzed by agarose gel electrophoresis.

LPD preparation

Anionic liposomes composed of Chems/DOPE were prepared with the molar ratio according to the central composite design. The lipid mixture was dissolved in appropriate chloroform and a thin lipid film was formed in a round-bottomed flask by drying the solvent under a stream of nitrogen gas. Residual chloroform was further moved by placing the flask in a desiccator vacuum for 30 min. The film was hydrated with the addition of 10 mM Hepes buffer (pH 7.4). Resuspended lipids then underwent ten passes through using an extruder with 200, 100 nm polycarbonate membranes respectively.

LPD were formed by first mixing equal volumes of DNA and protamine together. DNA and protamine were both diluted from the stock with 10 mM Hepes buffer to get a final DNA concentration of 30 μg/mL. After mixing the solution was briefly vortexed, and the resulted polyplexes were incubated for 10 min at room temperature. Preformed anionic liposomes were subsequently added to the DNA/Protamine mixture under mild vortexing to achieve the desired final component concentrations and ratios.

Size and zeta potential

Diameter and surface charge of LPD were measured by photon correlation spectroscopy (PCS) (Malvern zetasizer 3000 HS, Malvern instruments Ltd., UK) with a 50 mV laser. 20 μL of LPD was diluted by 3 mL of 10 mM Hepes buffer and added into the sample cell. The measurement time was set to 2 min (rapid measurement) and each run consisted of 10 subruns. The measurements were done at 25°C at an angel of 90°. The size distribution follows a lognormal distribution.

The potential of the lipid carriers at the surface of spheres, called the zeta potential (ζ), which was derived from mobility of particles in electric field by applying the smoluchowsky relationship, was measured at least three times and averaged at appropriate concentrations of samples.

Resistance to DNase I action

Resistance of LPD complexes to DNase I was deter-

mined by spectrofluorimetry with Hoechst 33258 as the fluorophores. LPD complexes were submitted to DNase I action (5 units DNase I/g of DNA) in a buffer solution (50 mM pH 7.4 Tris-HCl; 10 mM $MnCl_2$). After 60 min at 37°C, the enzyme was inactivated upon incubation with 0.5M EDTA (1 μL/unit of DNase I). Afterwards, 1%(v/v) Triton and 0.9% (w/v) heparin (Moret, *et al.*, 2001) were employed to destroy the liposomal bilayer and dissociate plasmid from protamine.

Fluorescence spectra and quantum yields are generally more dependent on the environment than absorption spectra and extinction coefficients. In the case of fluorophores Hoechst 33258, pH, ion and other compounds would affect fluorescence detection. Therefore, DNA clean-up kit was employed to remove protein, salts and nuclear acids smaller than 100 bp. Meanwhile, Hoechst 33258 stock solution was prepared in Tris NaCl-EDTA buffer (TNE; 10 mM Tris base, 0.2 M NaCl, 1 mM EDTA, pH 7.4) to keep relatively stable detection environment.

The liberated DNA was purified by DNA clean-up kit Parallel experiments were performed by incubating samples under the same experimental conditions, except that DNase I was previously inactivated. The extent of DNA stability was determined according to the following equation:

$$\text{DNA stability (\%)} = (F_a - F_0)/(F_i - F_0) \times 100$$

Where F_a is the fluorescence value emitted by Hoechst 33258 in the presence of the complexes treated with the active enzyme, F_i is the fluorescence value emitted by Hoechst 33258 in the presence of the complexes treated with the inactive enzyme and F_0 is the residual fluorescence of Hoechst 33258.

Definition of the experimental design region

The experimental range of a factor must be chosen in order to adequately measure its effects on the response variable. The range must not be too large, leading to non-realistic experiments and not too small, far from an optimal region. Zeta potential measurements can be a useful tool for characterizing colloidal drug delivery systems. They can give information about the surface properties of the carrier and therefore help to determine how the constituent molecules are organized. The formation of polycation-DNA complexes resulted in a moderate net positive charge of the colloidal solution. Hence zeta potential determination can be utilized to marker the formation of polycation-DNA complexes and anionic LPD. In this study, the fields of protamine/DNA and Chems/DNA were set according to the size and zeta potential measurements of polycation-DNA complexes and LPD respectively.

Central composite design

The size of the condensed DNA is thought to be critical for in vivo delivery because particle size influences not only the biodistribution but also the efficiency of cellular uptake through endocytosis. At an appropriately condition, polycation can precondense plasmid DNA more effectively than cationic liposomes (Liu *et al.*, 2001). Nuclease resistance ability was also a very important index to evaluate the non-viral delivery system. Therefore LPD size and LPD protection efficiency against nuclease were chosen as responses of CCD.

In general, CCD is constructed in such a way that $2^f + 2f + 1$ experiments are required where f represents the number of factors to be studied, which affect experimental results. Accordantly, a three-factor CCD requires 15 experimental points, each of which being a result of different formulations. In order to estimate the pure experimental uncertainty of CCD, it is important to measure repeatedly the response function to the conditions determined by the central points. In this case, five repeated experiments were performed. The non-linear quadratic model generated by the design is of the form: $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_1^2 + b_5X_2^2 + b_6X_3^2 + b_7X_1X_2 + b_8X_2X_3 + b_9X_1X_3$, where Y is the measured response associated with each factor level combination; b_0 is an intercept; b_1-b_9 are the regression coefficients; X_1, X_2, and X_3 are the factors studied. The constant and the regression coefficients were calculated using the statistical package Statistica (vision 5.0, Tulsa, OK). In order to check the validity of the model, analysis of variance was used. F-ratios and correlation coefficients were the criteria for validation.

RESULTS AND DISCUSSION

The experimental design region

The first step in preparing LPD was the condensation of DNA with protamine sulphate. In this study, we made use of zeta potential measurements to define the fields of protamine/DNA, in which DNA-polycation complexes were moderately positive. From Fig. 1, the experimental range of protamine/DNA was set between 2 to 3.5. The size of the resulted particles averaged between 90-150 nm with a polydispersity of 0.20-0.32. Afterwards, we measured the zeta potential of LPD by first fixing protamine/DNA at the ratio of 2 to define the experimental range of Chems/DNA. Fig. 2 showed that when the ratio of Chems/DNA was higher than 7, the zeta potential of LPD could be lower than -25mv. Therefore, the ratio of Chems/DNA was defined from 7 to 15. After the experimental regions were determined, experimental values for individual points (axial star, factor and center) were determined as shown in Table I.

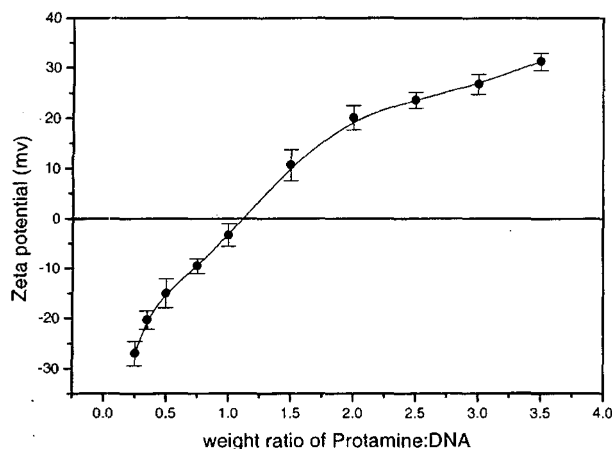

Fig. 1. Zeta potential measurements of protamine-DNA complexes at different weight ratios. All samples were prepared in 10 mM Hepes buffer at a final DNA concentration of 30 µg/mL by mixing the solution with brief vortexing and incubating for 10 min at room temperature.

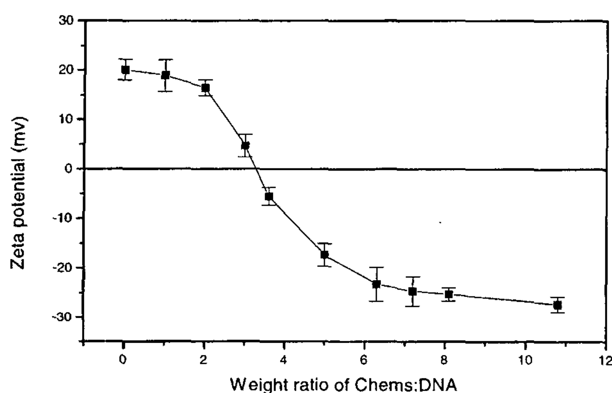

Fig. 2. Zeta potential measurements of LPD at different weight ratios of Chems and DNA. All samples were prepared by adding preformed anionic liposomes composed of Chems/DOPE to the DNA/Protamine complexes under mild vortexing to achieve the desired ratios.

Table I. Determination of experimental values for the CCD with a specified axial spacing

	Axial star point (lower)	Factor point (lower)	Central point	Factor point (upper)	Axial star point (upper)
Protamine/DNA (w:w)	2.00	2.32	2.75	3.18	3.50
Chems/DNA (w:w)	7.00	8.70	11.00	13.91	15.00
Chems/DOPE (mol:mol)	0.60	0.73	0.90	1.07	1.20

Observed responses for the CCD design

The experimental runs and the observed responses for the 15 formulations were given in Table II, in which X_1 and X_2 were the ratio of protamine/DNA (w/w) and Chems/DNA (w/w) respectively; X_3 was the molar ratio of Chems/DOPE in the anionic liposomes; Y_1 and Y_2 were the LPD size and LPD protection efficiency against nuclease respectively. The constant, the regression coefficients and the statistical parameters for each response variable, were listed in Table III. The two equations represented the quantitative effect of process variables (X_1, X_2, and X_3) and their interactions on their responses Y_1 and Y_2 respectively. As seen from Table IV and V, the measured and predicted values for Y_1 and Y_2 were in good agreement, which supported the applicability of CCD.

Table II. Observed responses for the CCD design

Run	X_1	X_2	X_3	Y_1	Y_2
1	2.320	8.700	0.730	191.900	63.120
2	3.180	8.700	0.730	461.000	78.170
3	2.320	13.910	0.730	156.600	16.160
4	3.180	13.910	0.730	177.100	30.970
5	2.320	8.700	1.070	213.600	76.720
6	3.180	8.700	1.070	382.400	95.900
7	2.320	13.910	1.070	200.500	44.030
8	3.180	13.910	1.070	180.600	64.410
9	2.000	11.000	0.900	175.000	42.440
10	3.500	11.000	0.900	371.700	76.010
11	2.750	7.000	0.900	392.200	110.910
12	2.750	15.000	0.900	197.400	47.020
13	2.750	11.000	0.600	181.000	26.710
14	2.750	11.000	1.200	260.000	70.990
15	2.750	11.000	0.900	182.900	61.030
16	2.750	11.000	0.900	195.200	62.340
17	2.750	11.000	0.900	189.400	61.890
18	2.750	11.000	0.900	198.500	63.070
19	2.750	11.000	0.900	202.700	62.670
20	2.750	11.000	0.900	208.400	64.360

X_1: the weight ratio of protamine/DNA
X_2: the weight ratio of Chems/DNA
X_3: the molar ratio of Chems/DOPE
Y_1: LPD size (nm)
Y_2: LPD protection efficiency against nuclease (%)

Table III. Regression equations for the responses

$Y_1 = -53.1275 + 231.3364 X_1 - 33.7485 X_2 + 127.6429 X_3 + 119.4688X_1^2 + 5.2766X_2^2 + 161.7047 X_3^2 - 48.5827 X_1 X_2 + 26.5528 X_2 X_3 - 240.595 X_1 X_3$ (R^2=0.9697; $F_{12,7}$=93.16; P=0.000002)

$Y_2 = 60.6118 + 53.9503 X_1 - 35.4222 X_2 + 238.2008 X_3 - 8.8115 X_1^2 + 0.8936 X_2^2 - 169.967 X_3^2 + 0.064 X_1 X_2 + 8.3006 X_2 X_3 + 16.5869 X_1 X_3$ (R^2=0.9954; $F_{12,7}$=3.68; P=0.0465)

X_1: the weight ratio of protamine/DNA
X_2: the weight ratio of Chems/DNA
X_3: the molar ratio of Chems/DOPE
Y_1: LPD size (nm)
Y_2: LPD protection efficiency against nuclease (%)

Table IV. Observed and predicted values of the response Y_1

Run	Observed	Predicted	Residuals
1	191.9	192.30	-0.40
2	461.0	441.80	19.20
3	156.6	151.81	4.79
4	177.1	183.62	-6.52
5	213.6	223.42	-9.82
6	382.4	402.57	-20.17
7	200.5	229.97	-29.47
8	180.6	191.43	-10.83
9	175.0	161.50	13.50
10	371.7	367.70	4.00
11	392.2	391.30	0.90
12	197.4	172.36	25.04
13	181.0	197.21	-16.21
14	260.0	226.70	33.30
15	182.9	197.40	-14.50
16	195.2	197.40	-2.20
17	189.4	197.40	-8.00
18	198.5	197.40	1.10
19	202.7	197.40	5.30
20	208.4	197.40	11.00

Y_1: LPD size (nm)

Table V. Observed and predicted values of the response Y_2

Run	Observed	Predicted	Residuals
1	63.12	63.22	-0.10
2	78.17	78.83	-0.66
3	16.16	16.28	-0.12
4	30.97	32.18	-1.21
5	76.72	77.83	-1.11
6	95.90	98.29	-2.39
7	44.03	45.59	-1.56
8	64.41	66.34	-1.93
9	42.44	41.89	0.55
10	76.01	73.57	2.44
11	110.91	109.45	1.46
12	47.02	44.52	2.50
13	26.71	26.64	0.07
14	70.99	68.15	2.84
15	61.03	62.69	-1.66
16	62.34	62.69	-0.35
17	61.89	62.69	-0.80
18	63.07	62.69	0.38
19	62.67	62.69	-0.02
20	64.36	62.69	1.67

Y_2 LPD protection efficiency against nuclease (%)

Effect of formulation ingredients on particle size and protection efficiency

Various investigators have reported the importance of size of plasmid DNA and DNA carriers for gene transfection (Kreiss *et al.*, 1999; Cherng *et al.*, 1999). Plasmid DNA of smaller size was shown to have higher transfection efficiency. It was suggested that the larger particles are too voluminous to be taken up by the cell via endocytosis resulting in lower transfection. The responses surfaces for Y_1 (LPD size) were shown in Fig. 3-4. The response

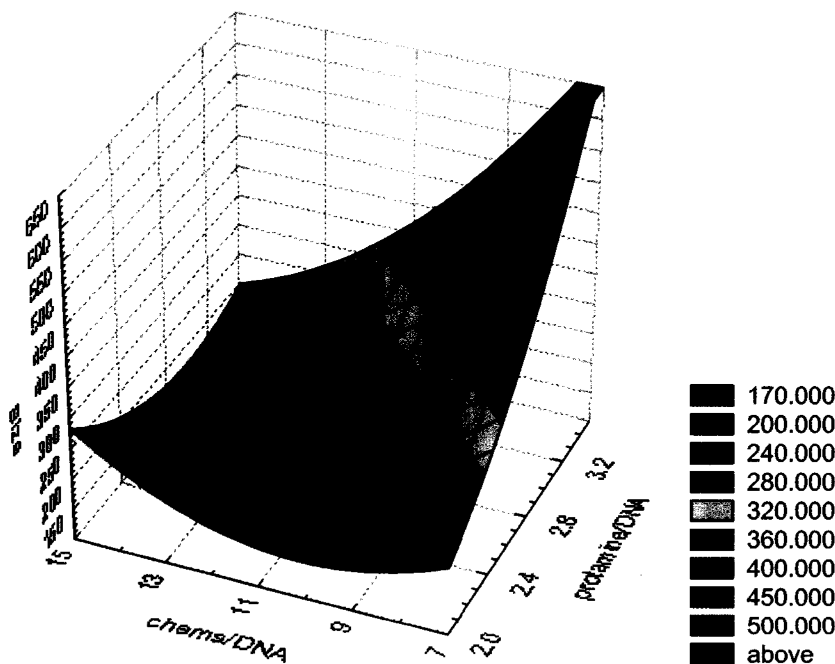

Fig. 3. Response surface plot showing the effect of Chems/DNA and Protamine/DNA weight ratios on the response of LPD size (nm) while keeping the molar ratio of Chems/DOPE at 0.90. LPD were prepared by adding preformed anionic liposomes composed of Chems/DOPE to the DNA/ Protamine complexes under mild vortexing in 10 mM Hepes buffer.

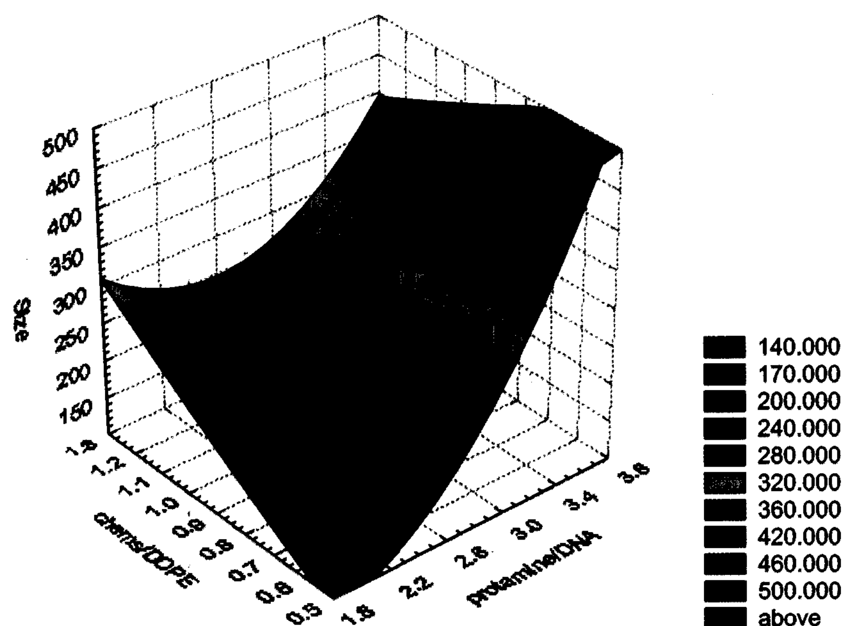

Fig. 4. Response surface plot showing the effect of molar ratios of Chems/DOPE and weight ratios of Protamine/DNA on the response of LPD size (nm) while keeping the weight ratio of Chems/DNA at 11. LPD were prepared by adding preformed anionic liposomes composed of Chems/DOPE to the DNA/Protamine complexes under mild vortexing in 10 mM Hepes buffer.

model was mapped against two experimental factors while the third was held constant at its middle level. Fig. 3 showed that at Chems/DOPE molar ratio of 0.9, with the increasing of protamine/DNA, the ratios of Chems to DNA should increase accordingly to obtain the LPD particle sizes smaller than 170 nm. This was because higher amount of anionic liposomes was needed to interact with protamine-DNA complexes and form stable anionic LPD complexes with a large negative zeta potential. It was obvious that if LPD had a large negative zeta potential, they would repel each other and there would be dispersion stability. On the other hand, if LPD had low zeta potential values, then there would be no force to prevent the particles coming together and they would aggregate gradually resulting large particles.

When the ratio of Chems to DNA was 11, the lower protamine/DNA ratios within the experimental field would lead to smaller particle sizes of LPD (Fig. 4). This suggested the higher positive zeta potential of polycation-DNA complexes would electronically attract more anionic liposomes around each polyplex, which might cause the increase of LPD sizes. Meanwhile the increase of Chems/DOPE would result in larger sizes of LPD. This might be because the increased charge density of anionic liposomes enhanced the interaction with polyplexes, which also caused more anionic liposomes associate around each polyplex.

The contour plots for Y_2 were shown in Fig. 5-6. It could be seen from Fig. 5, at protamine/DNA ratio of 2.75, the molar ratios of Chems/DOPE in the range of 0.9-1.2 would result in relatively high protection efficiencies. The pH sensitive component Chems, could stabilize fusogenic lipid DOPE, a lipid which preferentially adopted the inverted hexagonal phase above 10 °C, into the lamellar phase at pH 7.4. The reduced amount of Chems in the formulation, would lead to decreased stability of LPD during 60 min incubation at 37 °C. However, the ratio of Chems/DNA was more dominating factor than the ratio of Chems/DOPE in term of obtaining high protection efficiencies. Lower Chems/DNA ratios would obtain higher protection efficiencies, which were also found in Fig. 6.

A little bit of excess anionic liposomes might be necessary for them to attach to and fuse around the cationic core, and further condense the complexes to obtain smaller LPD. However, in the nuclease resistance assay, LPD were exposed to DNase I in the reaction buffer (50 mM pH 7.4 Tris-HCl; 10 mM $MnCl_2$). This saline solution might help anionic liposomes to release DNA from LPD during incubation at 37 °C. In the literature of polyplexes, DNA can be released from the complexes by anionic polycation such as heparin (Moret, *et al.*, 2001), polymethacrylic acid (Trubetskoy, *et al.*, 1999) and polyaspartic acid (Mastrobattista, *et al.*, 2001). The higher the salt concentration the easier polyanion can release DNA from the complex (Trubetskoy, *et al.*, 1999). Guo and Lee reported the use of excess amounts of anionic liposomes resulted in the release of DNA from the PEI-DNA complexes (Guo and Lee, 2000). Similarly, we also found that by electrophoretic mobility analysis of LPD, anionic

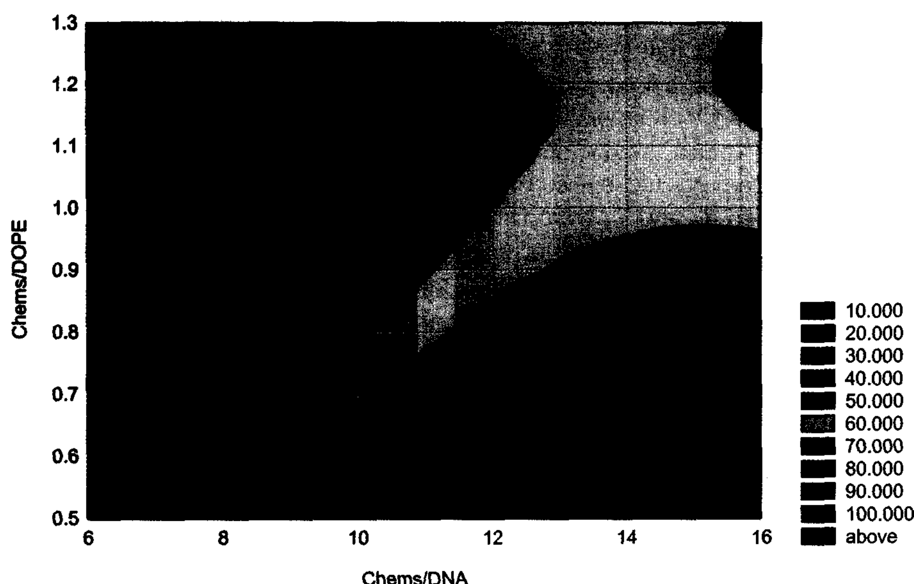

Fig. 5. Contour plot showing the effect of weight ratios of Chems/DNA and molar ratios of Chems/DOPE ratios on the response of LPD protection efficiency against nuclease (%) while keeping the weight ratio of protamine/DNA at 2.75. LPD were prepared by adding preformed anionic liposomes composed of Chems/DOPE to the DNA/Protamine complexes under mild vortexing in 10 mM Hepes buffer.

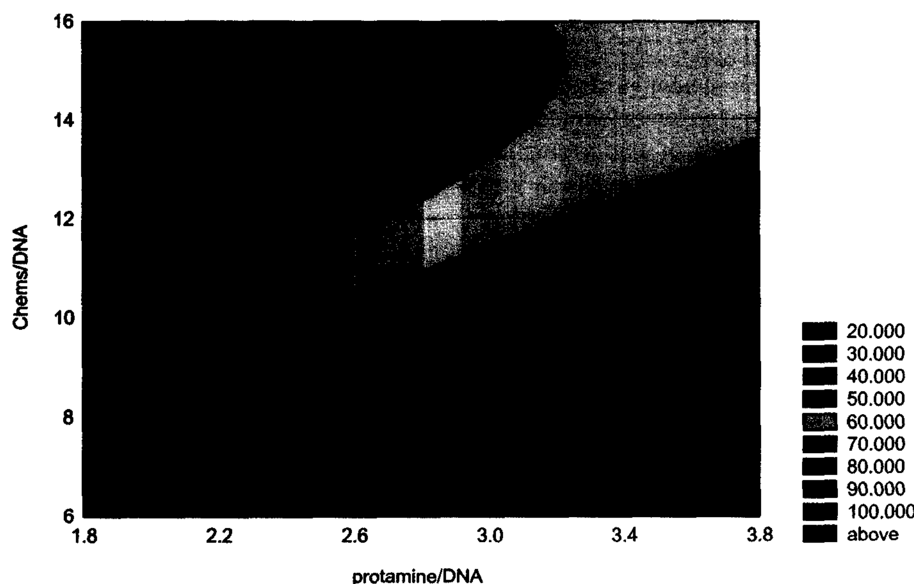

Fig. 6. Contour plot showing the effect of Chems/DNA and Protamine/DNA weight ratios on the response of LPD protection efficiency against nuclease (%) while keeping the molar ratio of Chems/DOPE at 0.90. LPD were prepared by adding preformed anionic liposomes composed of Chems/DOPE to the DNA/Protamine complexes under mild vortexing in 10 mM Hepes buffer.

liposomes had the ability to strip off protamine from plasmid DNA (data not shown). Therefore, the increased Chems/DNA ratios might cause the decrease of DNase resistance.

Comparing particle size and protection efficiency of LPD with protamine/DNA ratio of 3.4 and 2.0, we might assumed that at lower protamine/DNA ratio, smaller LPD could be formed to get the same protection efficiency. The underlying reason might be that polyplexes with higher positive surface charge would attract more negative liposomes around each polyplex and form thicker lipid coat, which resulted in larger particle sizes.

Optimization of the formulation ingredients

After we generated the polynomial equations relating the dependent and independent variables, the process was optimized for the response Y_1 and Y_2. Formulation compositions were optimized to obtain the LPD sizes

smaller than 200 nm and the protection efficiency high than 80%. The optimized X_1-X_3 levels that achieved the desired particle size and the protection efficiency against nuclease and their predicted Y_1 and Y_2 responses were given in Table VI. To verify these values, a new formulation was prepared according to the predicted levels of X_1-X_3. As shown in Table VI, obtained Y_1 and Y_2 were in a close agreement with the predicted value. The given results demonstrated the reliability of the optimization procedure in predicting the particle size of LPD and the protection efficiency against nuclease.

The interaction between DNA and polycation manifested itself in a number of biological processes that could account for their improved transfection characteristics *via* the endocytic pathway due to the small size of the condensed structures. Following endocytosis, polycations might act to protect DNA from nucleuses, and enhance the subsequent routing of DNA to the nucleus. However, it was found in this study that after protamine contents reach the ratio to effectively condense plasmid DNA, continual increase of protamine/DNA ratios in the formulations would lead to increased particle sizes. Therefore, optimized protamine content is critical to improving the efficiency of LPD-mediated transfection.

Both cationic and anionic liposomal vectors have been evaluated for DNA delivery. Cationic liposomal formulations have shown high transfection activities *in vitro* relying on an excess of cationic charge to interact with negatively cell surface. However, they are only marginally active when administered intravenously, since they interact nonspecifically and highly promiscuously with negatively charged vascular walls, as well as serum components. Anionic liposomes are an alternative to cationic liposomes as gene transfer vectors. They are more compatible with the physiological environment of the systemic circulation. Yet, their effective uptake by the cells relies on the linked targeting ligand through the receptor mediated endocytic pathway. In this study, anionic lipid coating of polyplexes fulfilled the following functions. First, the coating shielded the positive surface charge of polyplexes that might cause aggregation of polyplexes upon exposure to the biological environment. Second, the shield of positive surface charges prevented the non-specific binding to cells and the lipidic coatings provided a site for ligand or antibody attachment to obtain specific cellular uptake. Moreover, the pH sensitive liposomes consisting of Chems and DOPE could provide endosomal disruption activity. Our further work is focused on the conjugation of hepatoma cell specific ligand to the lipid to obtain specific celluar uptake.

Table VI. Optimized values obtained by the constraints applied on Y_1 and Y_2

Variable	Nominal values	Response	Expected values	Observed values
X_1	2.0	Y_1	190.94	185.30
X_2	7.4	Y_2	81.10	80.22
X_3	0.85			

X_1: the weight ratio of protamine/DNA
X_2: the weight ratio of Chems/DNA
X_3: the molar ratio of Chems/DOPE
Y_1: LPD size (nm)
Y_2: LPD protection efficiency against nuclease (%)

CONCLUSIONS

The present investigation concerned itself with the physical stability of plasmid DNA vectors and focused on the influence of different ratios of formulation components on particle sizes and nuclease resistance ability of LPD (liposome-polycation-DNA complexes). It was concluded that central composite design (CCD) could be successfully used to optimize the formulation of LPD. The optimized formulation could be obtained with a relatively limit number of experimental runs using the appropriate statistical design and optimizing technique.

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