Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 261 (2003) 115–127
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The influence of formulation variables on in vitro transfection efficiency and physicochemical properties of chitosan-based polyplexes

Kristine Romøren [a,*], Solveig Pedersen [a], Gro Smistad [a], Øystein Evensen [b], Beate J. Thu [b]

[a] *Department of Pharmaceutics, School of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo, Norway*
[b] *Department of Morphology, Genetics and Aquatic Biology, The Norwegian School of Veterinary Science, Oslo, Norway*

Received 9 October 2002; received in revised form 8 May 2003; accepted 13 May 2003

Abstract

The aim of this study was to investigate how a selection of formulation variables affects the in vitro transfection efficiency and physicochemical properties (particle size, zetapotential and chitosan–plasmid association) of chitosan-based polyplexes. Experimental designs in combination with multivariate data analysis were applied to reveal the effects of the formulation variables on the responses. The following formulation variables were studied: molecular weight and degree of acetylation of chitosan, pH and ionic strength of the buffer in which chitosan was dissolved, charge ratio of polyplexes, plasmid concentration and inclusion of a coacervation agent in the plasmid solution. The in vitro transfection efficiency in *Epithelioma papulosum cyprini* (EPC) cells was affected by the polyplex charge ratio, the DNA concentration in the complexes as well as the molecular weight and degree of acetylation of the chitosans. Two favourable formulations were identified in a more thorough investigation. These formulations were made of SC113 (theoretical charge ratio 10) and SC214 (theoretical charge ratio 3). The size of the complexes was affected by the degree of acetylation, concentration of DNA, pH, inclusion of a coacervation agent and the charge ratio. The charge ratio, pH and ionic strength determined the zetapotential of the particles, while the charge ratio was important for the association between the plasmid and chitosan.

Keywords: DNA vaccine; Chitosan; In vitro transfection efficiency; Physicochemical properties; Multivariate data analysis

1. Introduction

Genetic immunisation techniques have gained increasing interest following the documentation that DNA could elicit immune response a decade ago (Tang et al., 1992). There are several reasons why plasmid vectors are attractive for the development of vaccines and, among others, the high stability of plasmid DNA, the relatively easy manufacturing process, the ability of the vaccine to elicit both humoral and cellular immune responses, the lack of infection risk (which is associated with attenuated viral vaccines) and low manufacturing costs are often mentioned (Restifo et al., 2000; Webster and Robinson, 1997). The DNA vaccine technology has also been applied in teleost fish and protection against various diseases

* Corresponding author. Tel.: +47-22-85-65-98; fax: +47-22-85-44-02.
E-mail address: kristine.romoren@farmasi.uio.no (K. Romøren).

0378-5173/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0378-5173(03)00301-6

has been well documented (Lorenzen et al., 1998; Anderson et al., 1996; Gomez-Chiarri et al., 1996; Heppell and Davis, 2000).

Chitosan is a biodegradable polysaccharide composed of the subunits D-glucosamine and *N*-acetyl-D-glucosamine, linked together by β(1,4)-glycosidic bonds. Chitosan was first described as a delivery system for plasmids by Mumper et al. (1995). It is an attractive vector for gene delivery due to its low toxicity and, in addition, the polymer can be obtained in, or modified to, different molecular weights and different degrees of acetylation. From a pharmaceutical point of view, a characterisation of the properties of the chitosan–DNA polyplexes is important. Many formulation parameters, like salt concentration, pH, polymer charge density, polymer molecular weight and the charge ratio, may influence the polymer–DNA complexation and the characteristics of the complexes (Richardson et al., 1999). Although the effects of such variables on the in vitro transfection efficiency, size, zetapotential and chitosan–plasmid association are reported (Koping-Hoggard et al., 2001; Mao et al., 2001; MacLaughlin et al., 1998; Sato et al., 2001; Lee et al., 2001; Ishii et al., 2001), a systematic and simultaneous investigation of the respective formulation variables' effect on the properties has not been performed.

Evaluating the effect of a high number of formulation variables usually require many experiments, which are often costly and time consuming. It is therefore interesting to minimise the total number of performed experiments. In statistical experimental designs, many factors are varied simultaneously in a systematic way using the concept of factorial designs. This approach has several advantages compared to traditional "one variable at a time" strategy, e.g. it is more efficient as more information can be gained from a smaller number of experiments. Using a multivariate data analysis in combination with the experimental design adds further benefit, since an analysis of all the formulation variables can be performed at the same time. Experimental designs and multivariate data analysis are described by Esbensen et al. (2000).

The aim of this study was to investigate how a selection of formulation variables affects in vitro transfection efficiency in *Epithelioma papulosum cyprini* (EPC) cells. In addition, the effect of the formulation variables on physicochemical properties (size, zetapotential and chitosan–plasmid association) of the polyplexes was investigated. The formulation variables investigated were molecular weight and degree of acetylation of chitosan, pH and ionic strength of the buffer in which chitosan was dissolved, charge ratio in the polyplexes, plasmid concentration and inclusion of a coacervation agent in the plasmid solutions. To reduce the high number of possible formulation combinations, a fractional factorial design was created. The data were evaluated with Partial Least Squares Regression (PLSR). In addition to these screening studies, a more detailed characterisation was performed on the in vitro transfection efficiency of the polyplexes, aimed at finding the most efficient formulation.

2. Materials and methods

2.1. Chemicals

Chitosans (SeaCure 113 CL (SC113), SeaCure 211 CL (SC211), SeaCure 214 CL (SC214) and SeaCure 312 CL (SC312)) with different molecular weights and degree of acetylation (F_A) (Table 1) were kindly provided by Pronova Biopolymers (Drammen, Norway). As described previously, the chitosan samples were dialysed against distilled, de-ionised water to remove excess chloride ions (Romøren et al., 2002). L-α-Dioleoylphosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

The plasmid construct pcDNA3-luc, a gene encoding firefly luciferase inserted into the polylinker of pcDNA3 (Invitrogen, Groningen, The Netherlands),

Table 1
The different types of chitosan used in the factorial design

Types of chitosan	Average number molecular weight (M_n) (kDa)	Degree of acetylation (F_A)
SC113	6.6	0.15
SC211	90	0.32
SC214	160	0.15
SC312	160	0.25

The average number molecular weight (M_n) and the degree of acetylation (F_A) are determined after dialysis and freeze-drying according to the methods of Anthonsen et al. (1993) and Muzzarelli and Rocchetti (1986), respectively.

was obtained from GeneCare (Lyngby, Denmark). The purity and integrity of the plasmid DNA were assessed by UV spectroscopy (A_{260}/A_{280} ratio) and by agarose gel electrophoresis. The DNA homogeneity was calculated as described by Varley et al. (1999) and the plasmid DNA was also tested for endotoxins (72 EU/mg). The concentration of the plasmid was determined by UV spectroscopy (1 OD = 50 μg/ml at 260 nm) (Felgner et al., 1997).

Earle's Minimum Essential Medium (EMEM) containing 25 mM HEPES, gentamicin and L-glutamine were purchased from BioWhittaker (Verviers, Belgium). Foetal bovine serum (FBS) was acquired from Sigma-Aldrich Chemicals (Steinheim, Germany). The luciferase assay was obtained from Promega (Madison, WI, USA), the PicoGreen® assay from Molecular Probes (Leiden, The Netherlands) and the Bio-Rad Protein Assay from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were of analytical grade.

2.2. Experimental design

The effects of the seven formulation variables (molecular weight and degree of acetylation of chitosan, pH and ionic strength of the HAc/NaOAc buffer in which chitosan was dissolved, the charge ratio in the polyplexes, plasmid concentration and inclusion of a coacervation agent in the plasmid solutions) on four responses (in vitro transfection efficiency, size, zetapotential and association between the plasmid and chitosan) were studied in a screening design (Table 2).

Table 2
Experimental levels of the formulation variables in the screening design

Formulation variables	Experimental levels	
Molecular weight (M_n)[a] (kDa)	6.6/90	160
Degree of acetylation (F_A)[a]	0.15	0.25/0.32
pH (HAc/NaOAc buffer)	5.5	7
Ionic strength (HAc/NaOAc buffer) (mM)	15	35
Plasmid concentration[b] (μg/ml)	50 (4[c])	100 (20[c])
Charge ratio of polyplex	0.5 2	5
Coacervation agent (Na_2SO_4)[b] (mM)	0	50

[a] The molecular weight and degree of acetylation of the chitosan types are as described in Table 1.
[b] Concentrations prior to mixing with chitosan.
[c] DNA concentrations used for the transfection studies.

The design (48 different samples) had a resolution of IV, meaning that main effects are not confounded with two-factor interactions, while two-factor interactions are confounded with other two-factor interactions.

A multilevel design was created to find the most favourable formulation for in vitro transfection efficiency. The formulation variables used were the charge ratio (0.5, 1, 2, 3, 4, 5 and 10) and DNA concentration (0.5, 1.5 and 2.5 μg per well). All the four types of chitosan with different molecular weights and degrees of acetylation were investigated. The other formulation variables used in the screening design were kept constant (pH 5.5, ionic strength 25 mM and no coacervation agent present in the plasmid solution). Each formulation was analysed in triplicate.

The creation and evaluation of the statistical experiments were performed using the computer program Unscrambler® (The Unscrambler 7.5, Camo ASA, Trondheim, Norway). PLSR was performed on each response to assess which formulation variables that are of significant importance for the different responses in the screening design. The responses were weighed by auto-scaling (1/S.D.) before any statistical operations were performed and all models were calculated employing cross validation. The approximated uncertainty variance of the PLSR coefficients was estimated by the Jack-knife uncertainty test ($P = 0.05$) as described by Martens and Martens (2000).

2.3. Preparation of chitosan solutions and polyplex

The chitosans were dissolved overnight in HAc/NaOAc buffers of different pH and ionic strength. The particles were made by mixing equal volumes of the plasmid and chitosan solutions. The compound in "charge shortage" was added to the one in "charge excess" under intense stirring on a vortex mixer. The chitosan–DNA mixtures were further vortexed for 20 s and were allowed to rest for at least 30 min before use. According to the nomenclature (Felgner et al., 1997), the charge ratio is defined as the ratio between the maximum number of protonable primary amines in chitosan and the number of negative phosphates on DNA. Polyplexes of various charge ratios were obtained by diluting chitosan with the corresponding buffer to appropriate concentrations. In half of the samples in the screening design, a coacervation

agent was included in the plasmid solution (50 mM Na_2SO_4).

2.4. Characterisation of the in vitro transfection efficiency of the polyplexes

EPC cells, a cell line from carp (*Cyprinus carpio*) (Fijan et al., 1983), were grown in EMEM/HEPES supplemented with 10% FBS, 3.5 mM L-glutamine and 50 μg/ml gentamicin at 25 °C. In the transfection experiments, cells were seeded in a concentration of 5×10^5 cells per well in a 24-well plate. After 24 h, the FBS-containing medium was removed from the cells and replaced with a FBS-free medium and polyplexes (750 μl FBS-free medium and 250 μl formulation per well). After 24 h of incubation, the FBS-free medium and the formulations were removed and replaced with the complete medium. The cells were analysed for luciferase after further 2 days. The cells were then lysed in a lysating buffer (25 mM Tris–phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton® X-100) and assayed for luciferase activity according to the manufacturer's description in a Lumat LB 9507 Luminometer (EG&G Berthold, Germany). The relative light units (RLU) were normalised to the protein concentration as determined by the Bio-Rad assay. Lipoplex 2 made of DOPE:DOTAP 1:1 (w/w) complexed with pcDNA3-luc in a 2:1 charge ratio was used as a positive control.

2.5. Characterisation of the physicochemical properties of the polyplexes

2.5.1. Particle size

The size of the particles was determined by photon correlation spectroscopy at 25 °C using a Coulter N4 MD (Hialeah, FL, USA) at a 90° angle. The refractive index and viscosity of pure water were used as calculation parameters and each sample was measured in triplicate using the unimodal model for size distribution.

2.5.2. Zetapotential

The zetapotential was measured by micro-electrophoresis at 25 °C (Zetasizer 3000 HS, Malvern Instruments, UK). The viscosity and dielectric constant of pure water were used as calculation parameters. All samples were diluted in the corresponding buffer to a chitosan concentration of 1 μg/ml.

2.5.3. Chitosan–plasmid association

The association between the chitosan and plasmid was determined quantitatively with the PicoGreen® assay according to the manufacturer's instructions. Briefly, 100 μl aliquots of polyplexes (diluted to a DNA concentration of 0.5 μg/ml) was added to 100 μl PicoGreen® reagent (diluted 1:200 in 20 mM NaOAc, pH 6) and vortexed. After 2–5 min of incubation, the fluorescence of the mixture was measured in a Hellma ultra-micro cell (Ex. 480 nm, Em. 520 nm, Luminiscence Spectrofotometer LS50B, Perkin-Elmer, Bodenseewerk, Germany).

A qualitative analysis of the association of plasmid DNA to chitosan was performed by gel electrophoresis. Twenty microlitres of polyplex mixed with 2 μl of 0.4% bromophenol blue was applied in the slots of a 0.8% agarose gel containing 1× TAE-buffer. After electrophoresis, the gel was coloured with 0.5 μg/ml ethidium bromide for 30 min and visualised by UV. Photographs were made by Bio-Rad GelDoc 2000 digital gel documentation system.

3. Results

3.1. The influence of the formulation variables on the in vitro transfection efficiency of the polyplexes

In the PLSR model, 71% of the variation in the in vitro transfection efficiency is explained by 20% of the variance in the formulation variables and two PLSR components were necessary for this variance explanation. The plot of the weighted regression coefficients from the PLSR analysis on the transfection efficiency (Fig. 1) revealed that the charge ratio is strongly positively correlated to the transfection efficiency. Polyplexes with a negative charge (polyplex 0.5) hardly give any transfection of the EPC cells, while the use of polyplex 2 and polyplex 5 most often results in transfection (Table 3). Furthermore, the molecular weight (M_n) and DNA concentration (DNA conc) are also positively correlated to the transfection efficiency, an increase in these values therefore increases the transfection efficiency of the formulations. In contrast, the

Table 3
The average values ($n = 3$–4) of the responses in the fractional factorial design

Samples	Experimental levels							Responses				
	A	B	C	D	E	F	G	Size	Zetapotential	Migration	% Association	Transfection efficiency
1	6.6	0.15	1	5.5	15	50	5	275	23.03	0	97.0	5.51
2	160	0.15	1	5.5	15	0	0.5	159	−38.23	1	81.2	0.00
3	90	0.32	1	5.5	15	0	0.5	188	−30.97	1	92.1	0.00
4	160	0.25	1	5.5	15	50	5	213	26.83	0	96.8	4.29
5	6.6	0.15	2	5.5	15	0	5	141	23.46	0	97.1	5.24
6	160	0.15	2	5.5	15	50	0.5	212	−39.50	1	82.7	1.59
7	90	0.32	2	5.5	15	50	0.5	271	−33.60	1	87.6	0.00
8	160	0.25	2	5.5	15	0	5	239	25.57	0	97.2	4.07
9	6.6	0.15	1	7	15	0	0.5	222	−44.20	1	41.7	0.00
10	160	0.15	1	7	15	50	5	194	8.33	0	95.4	2.88
11	90	0.32	1	7	15	50	5	230	3.17	0	96.0	0.76
12	160	0.25	1	7	15	0	0.5	177	−44.40	1	80.9	0.00
13	6.6	0.15	2	7	15	50	0.5	204	−48.30	1	72.6	0.00
14	160	0.15	2	7	15	0	5	214	9.23	0	95.1	2.25
15	90	0.32	2	7	15	0	5	257	1.20	0	95.5	4.51
16	160	0.25	2	7	15	50	0.5	257	−43.53	1	76.7	3.30
17	6.6	0.15	1	5.5	35	50	0.5	214	−33.30	1	54.3	0.00
18	160	0.15	1	5.5	35	0	5	180	17.10	0	96.7	2.84
19	90	0.32	1	5.5	35	0	5	195	20.60	0	96.4	0.00
20	160	0.25	1	5.5	35	50	0.5	184	−35.10	1	72.4	0.00
21	6.6	0.15	2	5.5	35	0	0.5	168	−36.33	1	66.7	0.00
22	160	0.15	2	5.5	35	50	5	222	26.50	0	97.3	2.22
23	90	0.32	2	5.5	35	50	5	260	21.80	0	93.9	4.00
24	160	0.25	2	5.5	35	0	0.5	161	−34.67	1	74.3	0.00
25	6.6	0.15	1	7	35	0	5	175	0.30	0	95.9	5.40
26	160	0.15	1	7	35	50	0.5	263	−42.93	1	48.7	0.00
27	90	0.32	1	7	35	50	0.5	252	−41.37	1	60.3	0.00
28	160	0.25	1	7	35	0	5	154	4.57	0	95.1	4.29
29	6.6	0.15	2	7	35	50	5	261	−0.60	0	94.6	4.82
30	160	0.15	2	7	35	0	0.5	258	−42.63	1	79.8	3.95
31	90	0.32	2	7	35	0	0.5	284	n.d.	1	70.8	2.29
32	160	0.25	2	7	35	50	5	266	6.13	0	96.0	4.85
33	6.6	0.15	1	5.5	15	0	2	104	18.40	0	91.3	4.34
34	160	0.15	1	5.5	35	0	2	190	19.17	0	95.3	3.26
35	90	0.32	1	5.5	35	50	2	271	16.36	0	93.1	0.00
36	160	0.25	1	5.5	15	50	2	195	24.70	0	95.1	5.32
37	6.6	0.15	2	5.5	35	50	2	Ag.	16.70	0	95.6	4.48
38	160	0.15	2	5.5	15	50	2	335	25.77	0	95.5	6.09
39	90	0.32	2	5.5	15	0	2	243	18.00	0	93.9	0.00
40	160	0.25	2	5.5	35	0	2	233	19.37	0	95.1	5.30
41	6.6	0.15	1	7	15	50	2	Ag.	−21.30	0	89.7	0.00
42	160	0.15	1	7	35	50	2	Ag.	−1.13	0	95.0	5.67
43	90	0.32	1	7	35	0	2	275	−3.17	0	94.6	0.00
44	160	0.25	1	7	15	0	2	259	−0.87	0	91.8	5.39
45	6.6	0.15	2	7	35	0	2	Ag.	−3.50	0	96.0	4.94
46	160	0.15	2	7	15	0	2	Ag.	−3.77	0	88.1	6.15
47	90	0.32	2	7	15	50	2	376	−0.83	0	87.5	2.74
48	160	0.25	2	7	35	50	2	299	−4.43	0	95.5	4.74

The experimental values are M_n (kDa) (A), F_A (B), low (1)/high (2) values of the DNA concentration (C), pH of the buffer (D), ionic strength of the buffer (mM) (E), concentration of coacervation agent (mM) (F) and the theoretical charge ratio (G). The responses are the size (nm), zetapotential (mV), migration of plasmid in an agarose gel (1 = migration, 0 = no migration), % association of plasmid to chitosan and transfection efficiency (log(RLU/mg total protein)). Ag.: aggregation.

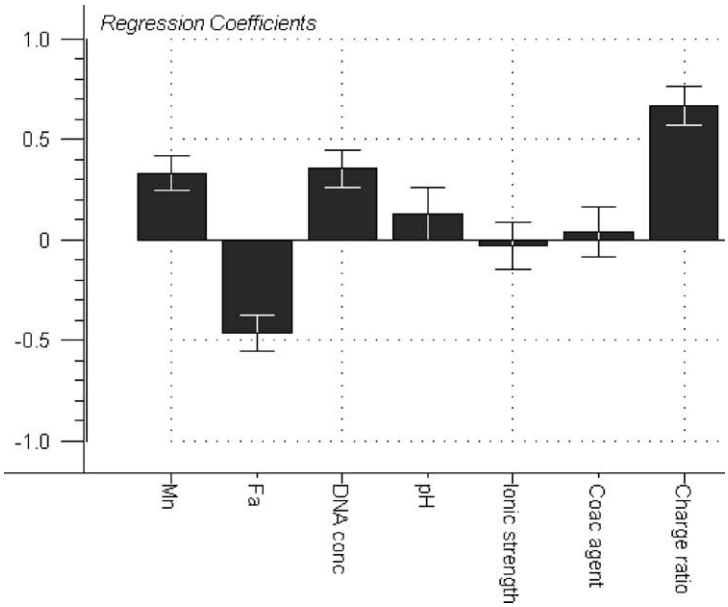

Fig. 1. The regression coefficients obtained by a PLSR analysis of the influence of formulation parameters on the in vitro transfection efficiency. Only the main effects are shown. The approximated uncertainty variance of the PLSR coefficients was estimated by the Jack-knife uncertainty test and corresponds to $P = 0.05$.

degree of acetylation (F_A) is negatively correlated. The other three main variables (pH, ionic strength and coacervation agent) were not significantly correlated to the transfection efficiency of the formulations.

An interaction between the molecular weight and charge ratio was discovered when the in vitro transfection efficiency of the polyplexes was investigated more thoroughly in a multilevel design (Fig. 2A). A high molecular weight of chitosan (in this experiment SC214, 160 kDa) gives a high transfection at charge ratios $\leq$4, while a low molecular weight of chitosan (SC113, 6.6 kDa) is beneficial at higher charge ratios. An interaction between the charge ratio and the degree of acetylation on the chitosan was also revealed (Fig. 2B). SC214 ($F_A = 0.15$) gave higher or similar transfection levels as SC312 ($F_A = 0.25$) at the charge ratios 0.5, 1, 2 and 3, while SC312 gave highest levels at the charge ratios of 5 and 10. Decreasing the DNA concentrations from 2.5 to 0.5 μg per well decreased the amount of luciferase expressed in the cells but did not alter the charge ratio optimum for the different types of chitosan. The highest luciferase levels in the EPC cells were found after transfection with SC113 (polyplex 10) and SC214 (polyplex 3).

3.2. The influence of the formulation variables on the physicochemical properties of the polyplexes

3.2.1. Particle size

A substantial aggregation of the polyplexes with a charge ratio of 2 occurred when some of the formulation parameters were combined (Table 3). Measuring the size of these aggregates was not possible as they were polydispersed and too large for an accurate determination at the 90° angle using photon correlation spectroscopy. These samples were therefore omitted in the PLSR analysis of the formulation variables' influence on the particle size.

Fig. 3 shows the weighted regression coefficients for the model obtained by a PLSR analysis of the formulation variables' influence on the particle size. The model employed two PLSR components, which were able to explain 77% of the variation in the particle size based on 32% of the variance in the formulation variables. Four of the formulation variables (F_A, DNA concentration, pH and content of coacervation agent) were positively correlated with the particle size, meaning that the particle size will increase when the values of these variables increase. The charge ratio does not

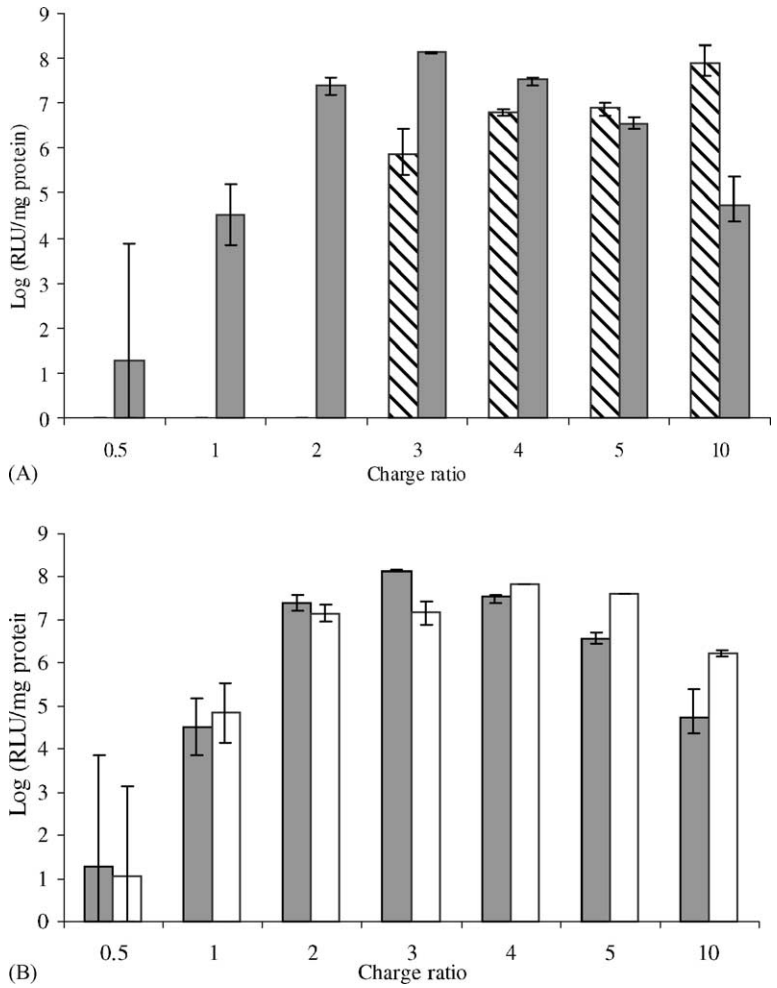

Fig. 2. Transfection of EPC cells with polyplexes made of (A) chitosans with a high molecular weight (SC214, filled bars) and low molecular weight (SC113, striped bars) but similar F_A (0.15) and (B) chitosans with a low F_A (SC214, filled bars) and high F_A (SC312, open bars) but with similar molecular weight (160 kDa). The concentration of DNA was 2.5 μg per well. Each bar represents the mean of three parallels ±max./min. values.

come out as a significant variable, the squared effect of the theoretical charge ratio (Char**2), however, is negatively correlated to the particle size. This means that the further the charge ratio is from charge ratio 1 (charge neutrality), the smaller the particles. The last two formulation variables, the molecular weight and the ionic strength, were not significant.

The models obtained by PLSR modelling of the formulation variables' influence on the size of polyplexes of different charge ratios separately explained 86, 91 and 93% of the variance in the size of polyplex 0.5, polyplex 2 and polyplex 5, respectively. Table 4 shows the magnitude of the weighted regression coefficients and thus the influence of the different formulation variables on the particle size of the polyplexes of different charge ratios. The weighted regression coefficient and thus the influence of F_A increases from a non-significant value of 0.216 (polyplex 0.5) to significant values of 0.248 and 0.344 (polyplex 2 and polyplex 5, respectively). The weighted regression coefficient of the pH is largest and thus most important for the size of polyplex 0.5 (0.548), less so for polyplex 2 (0.318) and not significant for polyplex 5.

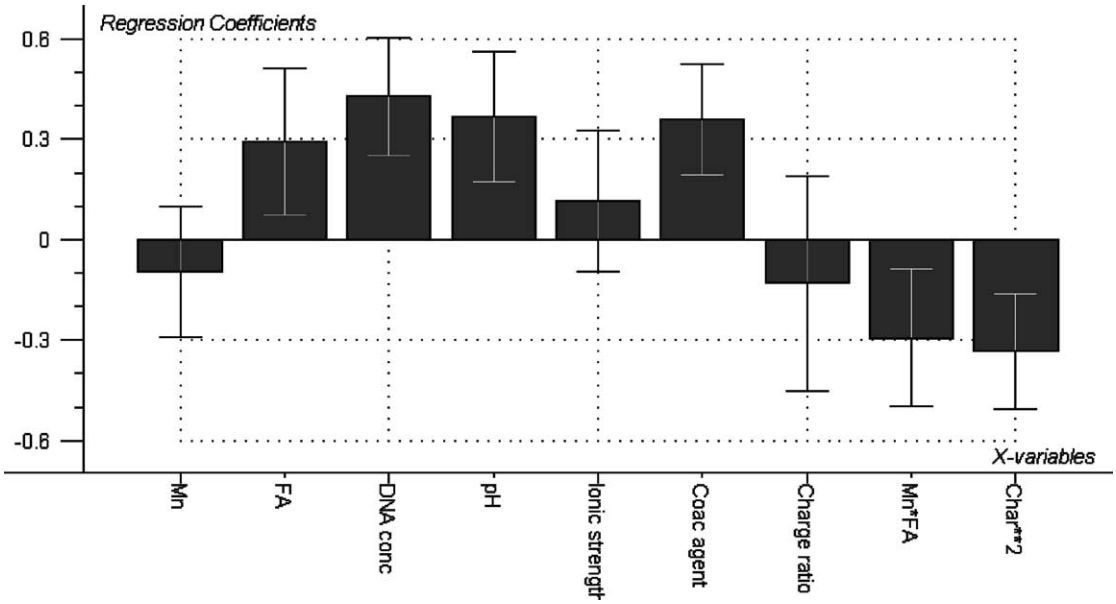

Fig. 3. The weighed regression coefficients obtained by a PLSR analysis of influence of formulation parameters on the particle size. The approximated uncertainty variance of the PLSR coefficients was estimated by the Jack-knife uncertainty test and corresponds to $P = 0.05$.

3.2.2. Zetapotential

The PLSR model obtained for the formulation variables' influence on the zetapotential used 24% of the variation in the formulation variables to explain 68% of the variation in *Y*, two PLSR components were necessary. Only two of the formulation variables had a significant influence on the model, the charge ratio and the pH. The charge ratio is positively correlated whereas the pH is negatively correlated to the zetapotential of the polyplexes. The effect of these formulation parameters can be seen in the score plot in Fig. 4. The score plot illustrates how the samples are placed relatively to the score vectors and therefore also how they group compared to each other. The zetapotentials are highest for polyplexes with a charge ratio of 5 and a pH of 5.5 (Fig. 4, grouped and marked with C) while the lowest zetapotentials are found for complexes with a charge ratio of 0.5 and pH 7 (grouped and marked with A). Some of the different polyplexes are also grouped together (polyplex 2 (pH 7) and polyplex 0.5 (pH 5.5)), as the zetapotentials of the particles are comparable (grouped and marked with B).

Upon separate PLSR modelling of polyplexes with different charge ratios, one more formulation variable showed its significance. The ionic strength of the buffer was positively correlated when there was a net negative charge of the polyplexes (polyplex 0.5), but negatively correlated for polyplexes with a net positive charge (polyplex 5) (data not shown). There was no significance of this formulation variable for the zetapotential of polyplex 2.

Table 4
The trends of the weighed regression coefficients of the main formulation parameters, obtained by PLSR modelling, on the size of the polyplexes

Formulation variables	Polyplex 0.5	Polyplex 2	Polyplex 5
Molecular weight (M_n)	−0.165*	0.058	−0.138
Degree of acetylation (F_A)	0.216	0.248*	0.344*
DNA concentration	0.163*	0.306*	0.146*
pH	0.548*	0.318*	0.089
Ionic strength	0.144	−0.025	0.020
Coacervation agent	0.138*	0.290*	0.249*

The polyplexes of the different theoretical charge ratios are here modelled separately. The asterisk (*) indicates a significant correlated effect. The significance of the regression coefficients has been determined with the Jack-knife uncertainty test and corresponds to $P = 0.05$.

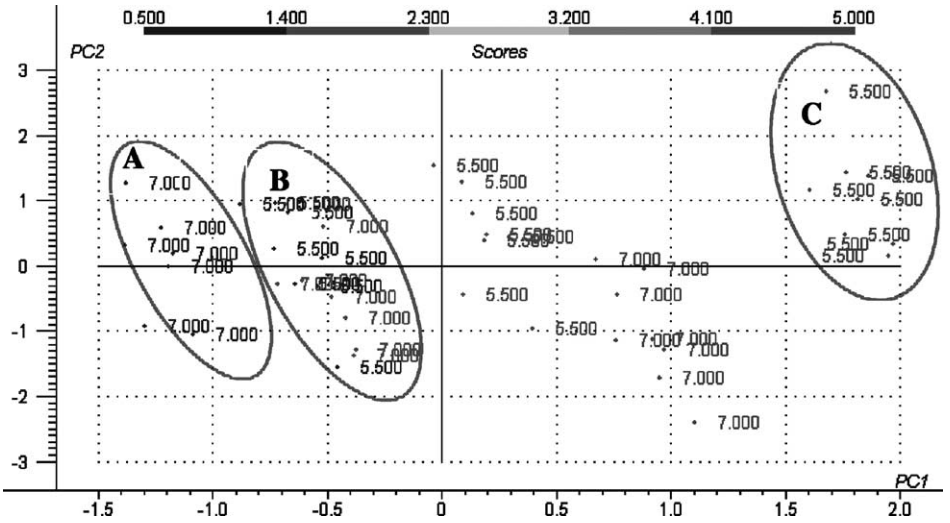

Fig. 4. PLSR score plot for the influence of the formulation variables pH and theoretical charge ratio on the zetapotential. The figure shows how the samples are placed relatively in the connection to the score vectors as well as how they group in regard to pH (shown with numbers) and charge ratios (A, charge ratio 0.5; B, charge ratio 0.5 and 2; and C, charge ratio 5).

3.2.3. Chitosan–plasmid association

The amount of plasmid associated with chitosan was determined quantitatively by the PicoGreen® assay. Upon PLSR modelling of the formulation variables' influence on the association efficiency, the charge ratio was the only formulation variable with a significant effect on the association efficiency. This variable was positively correlated, an increase in the theoretical charge ratio therefore caused increased association between the plasmid and chitosan (data not shown).

Electrophoresis in agarose gels was used to study the association between the plasmid and chitosan qualitatively. Differences between the polyplex formulations of different charge ratios could be observed (Table 3). Migration of the plasmid in the gel was observed for all the polyplex 0.5 formulations, while no migration was observed for the polyplex 2 and polyplex 5 formulations. No effects of other formulation parameters could be observed.

4. Discussion

4.1. The influence of the formulation variables on the in vitro transfection efficiency of the polyplexes

The formulation variables molecular weight and degree of acetylation of chitosan, DNA concentration and the charge ratio were of importance for the in vitro transfection efficiency of the polyplexes. The highest values of luciferase expression in the EPC cells were found when SC113 (polyplex 10) and SC214 (polyplex 3) were used in the polyplexes. Dependent on the application, other factors like stability and solubility of the chitosans, amounts of chitosan needed as well as the costs, may be considered when deciding which type to use. Also, properties like the size of the complexes should to be taken into account.

The highest weighted regression coefficient, hence the largest influence on the in vitro transfection efficiency, was due to the formulation variable charge ratio. The charge ratio has also earlier been shown to be of importance for transfection efficiency in vitro (Ishii et al., 2001; Koping-Hoggard et al., 2001). While polyplex 0.5 hardly managed to transfect the EPC cells, polyplex 2 and polyplex 5 most often resulted in expression of luciferase. An increased positive charge on the complexes most likely promotes the interaction with the negatively charged cell surface and such an association of the complex with the cell surface is probably a requirement for cell uptake. Furthermore, enzymatic degradation of chitosan is a likely mechanism for the endosomal escape of the polyplexes (Koping-Hoggard et al., 2001). The degradation products, oligo- and monosaccharides,

increase the osmolarity in the endosomes. This increase is followed by water influx, swelling and in the end, rupture of the membranes and release of the plasmid. Increasing the charge ratio in the polyplex implies an increase in the chitosan concentration in the complex. Higher amounts of chitosan in the polyplexes may lead to a higher osmotic pressure in the endosomes and as a consequence, the efficiency of plasmid release may increase.

The molecular weight of chitosan in the polyplex was another important formulation variable and in the optimisation experiment, an interaction between the molecular weight of the chitosan and the charge ratio was also found to be of importance. As previously mentioned, a low molecular weight chitosan is beneficial at high charge ratios of the polyplexes, but at lower charge ratios the opposite is the case. Conflicting data are found in the literature regarding the effect of the molecular weight on the transfection efficiency. MacLaughlin et al. (1998) performed a transfection on Cos-1 cells with polyplex 2 made of chitosans with different molecular weight (7–540 kDa). The results indicated that the chitosan molecular weight had a limited influence on plasmid expression in vitro. Similar observations were made by Koping-Hoggard et al. (2001). Transfections with polyplexes made of chitosans with different molecular weight performed by Ishii et al. (2001) are in contradiction to these results. They used, however, a different cell line (SOJ cells) and a N/P ratio of 5 (the N/P ratio is defined as the ratio between the polymer nitrogen (N) per DNA phosphate (P) (Borchard, 2001)). High levels of transfection were observed with the chitosan–plasmid complexes containing 40 and 84 kDa chitosan, while no expression was observed for 1 and 110 kDa chitosans. As shown by Sato et al. (2001), there may be a different molecular weight optimum of chitosan in different cell lines. Different origin of cell lines may therefore be the most important reasons for the conflicting results reported in literature. In addition, the importance of the charge ratios must also be taken into consideration. As shown in our studies with the EPC cell line, there are small differences in transfection efficiency between the low/high molecular weight chitosans for some charge ratios, while for other charge ratios the difference is large (Fig. 2). This interaction would not have been easily detected without the use of a factorial design.

Another important formulation variable was the degree of acetylation (F_A), and the optimisation study also revealed an interaction between this factor and the charge ratio. In the EPC cell line, low F_A values were beneficial at charge ratios ≤ 3. At higher charge ratios, however, a higher F_A value was advantageous. Low F_A values imply that the density of NH_3^+ groups able to react with the negative phosphate groups of the plasmid is high. As a consequence, the interaction between the polymer and plasmid may be stronger. The high degree of association may therefore be of hindrance to the release of DNA after uptake into the cells. Our studies illustrated that the importance of the F_A differed at high and low charge ratios. Thus, the low F_A may be of hindrance to the release of DNA at high charge ratios.

The concentration of DNA was the fourth factor of importance for the magnitude of expressed luciferase in the EPC cells. Increasing the concentration from 0.5 to 2.5 μg per well lead to an increase in luciferase expression. This is a normal and expected dose–response phenomenon. By a further increase in the DNA concentration to 5 μg per well, saturation in the expression levels was observed (data not shown).

The other three formulation variables in the screening study, inclusion of a coacervation agent and the pH and ionic strength of the buffer, were not important for the transfection efficiency of the polyplexes. The pH has previously been shown to be of importance for the transfection of cells (Sato et al., 2001; Ishii et al., 2001). The effect of both pH and the ionic strength, however, may be hidden in our studies because the polyplexes are added to the cells together with the cell medium. The ionic strength and pH of the cell medium may therefore over-run the effects from the formulation itself. The content of coacervation agent (50 mM in the plasmid solution) did not influence the transfection efficiency. In a more elaborated study, a further increase in the concentration of the coacervation content in the plasmid solution (up to 100 mM) also did not show enhanced transfection (data not shown).

4.2. The influence of the formulation variables on the physicochemical properties of the polyplexes

4.2.1. Particle size

The formulation variables F_A, concentration of DNA, pH, inclusion of a coacervation agent in the

plasmid solution as well as the squared effect of the charge ratio was positively correlated to the particle size.

The degree of acetylation (F_A) was positively correlated to the size of the polyplexes, as the size of polyplexes increased when the degree of acetylation in chitosan increased. Similar tendencies are described by Koping-Hoggard et al. (2001). The number of amino groups available for protonation and hence the charge ratio of the polymer increases as the degree of acetylation decreases, and a strong interaction with DNA is possible. In the opposite situation with a high F_A, the interaction between the polymer and plasmid may be weaker and larger particles are formed. An increase in the content of acetylated groups in chitosan also results in a stiffer and more extended polymer chain (Anthonsen et al., 1993) that may contribute to the increased particle size. Upon an increase in F_A, the concentration of chitosan must be increased to obtain the desired charge ratio. At a constant DNA concentration, the chitosan concentration in polyplex 5 is higher than in polyplex 0.5. Consequently, there is a larger increase in the chitosan concentration in polyplex 5 compared to polyplex 0.5 upon increasing the F_A value. In our studies, where separate PLSR models for the three charge ratios were made (Table 4), the weighted regression coefficient for the degree of acetylation increased in magnitude as the charge ratio increased from 0.5 to 5.

The average pK_a of the amino groups in a chitosan oligomer is about 6.5 (Anthonsen and Smidsrød, 1995), hence, the charge density of the polymer is also reduced when the pH of the chitosan solution increases from 5.5 to 7. Due to the reduced charge density of chitosan at pH 7, a weaker interaction between the DNA and chitosan is expected. As a result, the size of the polyplexes increases as the pH increases.

The plasmid concentration was also positively correlated to the particle size, an increase in this formulation variable therefore results in increased size of the polyplexes. Increasing the concentration of DNA is followed by an increase in the concentration of chitosan to obtain the desired charge ratio, and this may lead to an increased particle size. This is in accordance with the studies of MacLaughlin et al. (1998). They found an increased particle size upon increasing the DNA concentration in the chitosan–DNA complexes for both polyplex 2 and polyplex 6.

Complex coacervation is a process of spontaneous phase separation that occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution. The electrostatic interaction between the two macromolecules results in the separation of a coacervate (Leong et al., 1998). In order to increase the phase separation, a coacervation agent (here, Na_2SO_4) may be used in the preparation of polyplexes. In our study, particles made by this method were generally larger compared to complexes without a coacervation agent. Similar studies have been performed by Mao et al. (2001). However, they did not find a significant influence when they increased the content of Na_2SO_4 in the plasmid phase from 2.5 to 50 mM. It is important to notify that their complexes were made under different conditions and that polyplexes made without a coacervation agent were not included in this study.

The charge ratio is a formulation variable often varied when preparing polyplexes. Polyplex 0.5 and polyplex 2 theoretically have a 1:2 and 2:1 ratio of positively charged amino groups (chitosan) to negatively charged phosphate groups (plasmid), respectively. This implies that charge of the complexes in theory will be in the same distance from charge ratio 1, i.e. charge neutrality. To give polyplex 0.5 and polyplex 2 equal influence, the charge ratio 0.5 was changed to -2 during modelling. Then, the quadratic effect of the charge ratios could be included and came out as a significant factor. Smaller particles were observed at higher values of (charge ratio)^2, i.e. the further away from charge neutrality. This is in accordance with earlier studies performed on polyplexes (Mumper et al., 1995; Erbacher et al., 1998; Mao et al., 2001).

The molecular weight was not found to be significant for the particle size in our studies. This is in contrast to studies of MacLaughlin et al. (1998). They found the particle size to depend on the molecular weight of chitosan and an increase in molecular weight lead to an increase in the particle size. However, the complexes were made under different conditions and the degree of acetylation, varying from 0.11 to 0.2, was not taken into consideration in this study.

4.2.2. Zetapotential

The zetapotential is, due to its measure of the particle charge, in theory only dependent on the positive or negative charge of the polyplex. For this reason, it is expected that only variables with an influence

on the charge are significant for the response. In the PLSR modelling of all the samples together, the charge ratio and pH were the only significant formulation variables.

The charge ratio is directly connected to the zetapotential and a positive and negative charge ratio consequently lead to a positive and negative zetapotential. This is also shown by other groups (Mao et al., 2001; Erbacher et al., 1998; Pouton et al., 1998). The charge of the complex, however, is not only dependent on the concentration of DNA and chitosan in the polyplex. The pH of the buffer, in which the polymer is dissolved, also plays an important role. The charge density of chitosan is dependent on the pH. At pH 5.5–5.7, about 90% of the amino groups are protonated (Mao et al., 2001). At a neutral pH the degree of protonation is reduced, meaning that the actual charge of the polyplex is different from the charge of a polyplex of the same charge ration made at pH 5.5. As a consequence, the zetapotential of the polyplexes is reduced with an increased pH. Mao et al. (2001) measured the zetapotential of chitosan-based polyplexes and described a similar pH tendency.

Possibly, the effects of other formulation variables are hidden due to the huge impact of the charge ratio and pH on the response. An additional PLSR modelling was performed on polyplex 0.5, polyplex 2 and polyplex 5 separately and the ionic strength of the buffer now became significant. The ionic strength influences the charge of the particles due to its effects on the Debÿe–Huckel length parameter ($1/\kappa$). When the ionic strength increases, κ increases. Thereby, the electrical double layer is compressed and the zetapotential decreases. As observed in our studies, the particles become less negatively charged for polyplex 0.5 and less positively charged for polyplex 5. The variable is not significant for polyplex 2, probably due to an actual charge of the complexes close to zero.

4.2.3. Chitosan–plasmid association

Only the charge ratio could be distinguished as an important formulation variable for the association efficiency as measured by the PicoGreen® assay and the gel electrophoresis. The methods used are probably not specific enough for formulation studies, as adding different buffers and solutions to the polyplexes during sample preparation complicates the analyses and may hide effects. Nevertheless, studies like this are important as information concerning the state of the plasmid quickly can be gained.

5. Conclusion

The in vitro transfection efficiency was affected by the polyplex charge ratio, by the DNA concentration in the complexes as well as the molecular weight and degree of acetylation of the chitosans. The highest levels of luciferase expression in the EPC cells were obtained with polyplexes made of SC113 (theoretical charge ratio 10) and SC214 (theoretical charge ratio 3). All the formulation variables were of importance for physicochemical properties of the chitosan-based polyplexes. The fractional factorial design combined with multivariate data analysis was thus a suitable tool for effective formulation studies of polyplexes.

Acknowledgements

The authors wish to thank Dr. K. Dyrstad, Department of Pharmaceutical Research, Amersham Health AS, for valuable input regarding the multivariate data analysis. The chitosans were kindly provided by Pronova Biopolymers. We are also grateful to Department of Analytical Characterisation, Amersham Health AS, for the use of the Zetasizer. This work was supported by EC FAIR CT-98-4087 and Alpharma AS.

References

Anderson, E.D., Mourich, D.V., Fahrenkrug, S.C., LaPatra, S., Shepherd, J., Leong, J.A., 1996. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol. Mar. Biol. Biotechnol. 5, 114–122.

Anthonsen, M.W., Vårum, K.M., Smidsrød, O., 1993. Solution properties of chitosans: conformation and chain stiffness of chitosans with different degrees of *N*-acetylation. Carbohydr. Polym. 22, 193–201.

Anthonsen, M.W., Smidsrød, O., 1995. Hydrogen ion titration of chitosans with varying degrees of *N*-acetylation by monitoring induced ^{1}H-NMR chemical shifts. Carbohydr. Polym. 26, 303–305.

Borchard, G., 2001. Chitosans for gene delivery. Adv. Drug Del. Rev. 52, 145–150.

Erbacher, P., Zou, S., Bettinger, T., Steffan, A.-M., Remy, J.-S., 1998. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. Pharm. Res. 15, 1332–1339.

Esbensen, K.H., Gyout, D., Westad, F., 2000. Multivariate Data Analysis—In Practice. Camo ASA, Trondheim, Norway.

Felgner, P.L., Barenholz, Y., Behr, J.P., Cheng, S.H., Cullis, P., Huang, L., Jessee, J.A., Seymour, L., Szoka, F., Thierry, A.R., Wagner, E., Wu, G., 1997. Nomenclature for synthetic gene delivery systems. Hum. Gene Ther. 8, 511–512.

Fijan, N., Sulimanovic, D., Bearzotti, M., de Kinkelin, P., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F., 1983. Some properties of the *epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio*. Ann. Virol. (Inst. Pasteur) 134E, 207–220.

Gomez-Chiarri, M., Brown, L.L., Levine, R.P., 1996. Protection against *Renibacterium salmoninarum* infection by DNA-based immunization. Aquaculture Biotechnol. Symp. Proc. 155.

Heppell, J., Davis, H.L., 2000. Application of DNA vaccine technology to aquaculture. Adv. Drug Del. Rev. 43, 29–43.

Ishii, T., Okahata, Y., Sato, T., 2001. Mechanism of cell transfection with plasmid/chitosan complexes. Biochim. Biophys. Acta 1514, 51–64.

Koping-Hoggard, M., Tubulekas, I., Guan, H., Edwards, K., Nilsson, M., Vårum, K.M., Artursson, P., 2001. Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. Gene Ther. 8, 1108–1121.

Lee, M., Nah, J.-W., Kwon, Y., Koh, J.J., Ko, K.S., Kim, S.W., 2001. Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. Pharm. Res. 18, 427–431.

Leong, K.W., Mao, H.-Q., Truong-Le, V.L., Roy, K., Walsh, S.M., August, J.T., 1998. DNA-polycation nanospheres as non-viral gene delivery vehicles. J. Control. Release 53, 183–193.

Lorenzen, N., Lorenzen, E., EinerJensen, K., Heppell, J., Wu, T., Davis, H., 1998. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss*, Walbaum) following DNA vaccination. Fish Shellfish Immunol. 8, 261–270.

MacLaughlin, F.C., Mumper, R.J., Wang, J.J., Tagliaferri, J.M., Gill, I., Hinchcliffe, M., Rolland, A.P., 1998. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J. Control. Release 56, 259–272.

Mao, H.-Q., Roy, K., Troung-Le, V.L., Janes, K.A., Lin, K.Y., Wang, Y., August, J.T., Leong, K.W., 2001. Chitosan–DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. J. Control. Release 70, 399–421.

Martens, H., Martens, M., 2000. Modified Jack-knife estimation of parameter uncertainty in bilinear modelling by partial least squares regression (PLSR). Food Qual. Pref. 11, 5–16.

Mumper, R.J., Wang, J., Claspell, J.M., Rolland, A.P., 1995. Novel polymeric condensing carriers for gene delivery. Proc. Int. Symp. Control. Release Bioact. Mater. 22, 178–179.

Muzzarelli, R.A.A., Rocchetti R., 1986. The determination of the degree of acetylation of chitosans by spectrophotometry. In: Muzzarelli, R.A.A., Jeuniaux, C., Gooday, G.W. (Eds.), Chitin in Nature and Technology. In: Proceedings of the International Conference of Chitin and Chitosans. Plenum Press, New York, pp. 385–388.

Pouton, C.W., Lucas, P., Thomas, B.J., Uduehi, A.N., Milroy, D.A., Moss, S.H., 1998. Polycation-DNA complexes for gene delivery: a comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. J. Control. Release 53, 289–299.

Restifo, N.P., Ying, H., Hwang, L., Leitner, W.W., 2000. The promise of nucleic acid vaccines. Gene Ther. 7, 89–92.

Richardson, S.C.W., Kolbe, H.V.J., Duncan, R., 1999. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. Int. J. Pharm. 178, 231–243.

Romøren, K., Thu, B.J., Evensen, Ø., 2002. Immersion delivery of plasmid DNA II. A study of the potentials of a chitosan based delivery system in rainbow trout (*Oncorhynchus mykiss*) fry. J. Control. Release 85, 215–225.

Sato, T., Ishii, T., Okahata, Y., 2001. In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 22, 2075–2080.

Tang, D., DeVit, M., Johnston, S.A., 1992. Genetic immunization is a simple method for eliciting an immune response. Nature 356, 152–154.

Varley, D.L., Hitchcock, A.G., Weiss, A.M.E., Horler, W.A., Cowell, R., Peddie, L., Sharpe, G.S., Thatcher, D.R., Hanak, J.A.J., 1999. Production of plasmid DNA for human gene therapy using modified alkaline cell lysis and expanded bed anion exchange chromatography. Bioseparation 8, 209–217.

Webster, R.G., Robinson, H.L., 1997. DNA vaccines: a review of developments. Biodrugs 8, 273–292.