International Journal of Pharmaceutics 392 (2010) 261–267

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Rational development of a stable liquid formulation for nanomedicine products

Enikő R. Tőke[a], Orsolya Lőrincz[a], Eszter Somogyi[a], Julianna Lisziewicz[a,b,*]

[a] Genetic Immunity Kft, H-1045 Budapest, Berlini u. 47-49, Hungary
[b] Genetic Immunity LLC, 8300 Greensboro Drive, Suite 800 Mclean, VA 22102, United States

ARTICLE INFO

Article history:
Received 2 February 2010
Received in revised form 17 March 2010
Accepted 19 March 2010
Available online 25 March 2010

Keywords:
DermaVir
Polyethylenimine
Plasmid DNA
pDNA/PEIm nanoparticle
Liquid formulation

ABSTRACT

DermaVir vaccine is a novel "pathogen-like" nanomedicine containing a plasmid DNA complexed with a polyethylenimine that is mannobiosylated to target antigen-presenting cells and to induce immune responses (pDNA/PEIm). To develop a commercially viable vaccine product we have systematically investigated the variability of raw materials and their relationship with the product's biological activity. We demonstrated that the cGMP quality requirements are not sufficient to reproducible formulate the nanomedicine with optimal biological activity. Unexpectedly, we found that the high cationic concentration of the pDNA favored the biological activity, but did not support the stability of the nanomedicine. Similarly, the presence of EDTA in the pDNA increased the size of the nanoparticle to microparticles causing the drop of its biological activity. A new parameter, the Cl/N ratio of the PEIm, also influenced the biological activity together with the chemical properties of the solvent. Based on these findings we have developed a pDNA/PEIm formulation capable to maintain the physical stability and the biological activity of the nanomedicine. This work illustrates some of the key steps that must be taken for the implementation of "Quality by Design" (QbD) approach for a biotech product.

1. Introduction

DermaVir is a novel immunotherapeutic nanomedicine product—presently under phase II clinical development for the treatment of HIV/AIDS (Electronic Source, 2010). Its active biologic ingredient is a single plasmid DNA (pDNA) encoding nine HIV-1 antigens. DermaVir has been developed to stimulate HIV-specific immune responses therefore its biological activity depends on the expression of the pDNA-encoded antigens in the cells. To obtain potent antigen expression the pDNA is formulated to a pathogen-like nanoparticle with a mannobiosylated linear polyethylenimine (PEIm) in a glucose/dextrose solution (Lisziewicz et al., 2001, 2005a,b; Lori et al., 2005). The immunization procedure is performed topically with DermaPrep device (Lisziewicz et al., 2005a). DermaPrep is the first lymph node-targeting transdermal device, a unique alternative for syringes, especially appropriate for topical administration of vaccines and nanomedicines.

As DermaVir and other pDNA- and polymer-based nanomedicinal products approach a pharmaceutical reality, a number of issues need to be comprehensively addressed beyond their clinical efficiency and safety including the development of stable formulations that make them suitable for the global market (Ohana et al., 2004). Up to date there were no studies on the formulation of pDNA/PEIm nanomedicines. However, there are challenges using polymeric carriers, since the nanoparticles often suffer from poor physical stability leading to decomposition and consequently to the loss of transfection efficiency. Preparation of nanoparticles is generally performed shortly before their use because their tendency to aggregate (Neu et al., 2005). Previous studies established lyophilized formulations for pDNA/PEI nanoparticles formed with branched PEI (Molina et al., 2001; Anchordoquy et al., 2005; Hobel et al., 2008), but linear PEI based complexes could not be lyophilized (Hobel et al., 2008). Data obtained with branched PEI is not useful for formulation development with linear PEIm because branched PEI is more toxic and has different physico-chemical properties (von Harpe et al., 2000; Choosakoonkriang et al., 2003).

DermaVir nanomedicine clinical investigational product is presently prepared from three components that are stored separately at three different temperatures: pDNA at −80 °C, PEIm at −20 °C and the glucose solution at room temperature (25 °C). For phases I and II clinical trials the nanomedicine is formulated from these components at the clinical pharmacy according to our standard operating procedures. DermaVir is needed to be administered within 3 h because of the instability of the final nanomedicine product in glucose solution. The presence of a clinical pharmacist to formulate the product is not commercially favorable and permits human errors. In addition, since the pDNA is stored at −80 °C, immunization could be carried out only at clinical sites having low

* Corresponding author at: Genetic Immunity, H-1045 Budapest, Berlini u. 47-49, Hungary. Tel.: +36 1 272 0364; fax: +36 1 272 0364.
E-mail address: lisziewj@geneticimmunity.com (J. Lisziewicz).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.03.048

temperature freezers, not a common equipment even in leading clinical sites.

Our aim was to develop a liquid, commercially viable pDNA/PEIm formulation that is ready to use by nurses at the doctor's office. Here we present a rational, target product profile-oriented analysis of the pDNA/PEIm nanomedicines as a platform technology. We introduce first the detailed physico-chemical analysis of the nanomedicine components and their effect on the product quality. We show that the new liquid nanomedicine formulation maintains its stability and biological activity. Since DermaVir is our lead immunotherapeutic product candidate, a "typical" pDNA/PEIm nanomedicine, the general features and conclusions of the new nanoformulation are applicable to other pDNA/PEIm nanomedicine product candidates presently under development for cancer, allergy and infectious disease indications.

2. Materials and methods

2.1. Materials

pDNA: pLWXu1 encoding HIV-1 specific antigens (12.5 kbp). Different pDNA lots were contract manufactured by Althea Technologies Inc., San Diego (pDNA-A) or Aldevron LLC Fargo (pDNA-B) and delivered with Certificate of Analysis as 1 mg/ml solutions.

PEIm, polyethylenimine containing 3% grafted mannobiose: 22 kDa linear polyethylenimine lots (13.6 mM solution calculated as N concentration) were either contract manufactured by PolyPlus Transfection Illkirch, France (PEIm-A) or manufactured inhouse (PEIm-B). PEIm samples with different degree of cationic character (Cl/N ratio) were prepared starting from PEIm-A 13.6 mM solution (calculated as N concentration) and gradually deprotonated with 0.1–0.6 molar equivalents of 0.1 M NaOH solution, respectively.

Triethanolamine buffer (TEA) and solutions of different pH, were prepared at 10 mM concentration from triethanolamine (Fluka, *Ultra*) and triethanolamine–hydrochloride (Fluka, *Puriss*). Triethanolamine buffer containing 10% mannitol pH 7.5 (TEAM) was prepared with mannitol (M, Aldrich, *Ultra*).

All reagents were of Ph. Eur or USP grade, for solutions Milli-Q water (W, Millipore) was used.

2.2. Methods

2.2.1. Preparation and storage of DermaVir (pDNA/PEIm) nanomedicine

pDNA (1 mg/ml) was diluted with 6 volume equivalent solvent and then 13.6 mM PEIm was added at N/P ratio of 3 (except otherwise indicated). Nanoparticle formation was allowed to proceed for 20 min at room temperature (RT). The actual types of the solvent used are described separately at every formulation. In one case the solvent contained EDTA (Gibco) in 1 mM final concentration. Nanoparticles were then either used directly ("fresh" or "control" nanoparticles) or stored at 4 °C, room temperature (RT) or 37 °C for periods of times as indicated.

2.2.2. Biological activity test

293T cells were cultured in DMEM culture medium (Invitrogen). 0.5 μg pDNA in pDNA/PEIm formulation were added to 50,000 cells per each well. After 21 h HIV-1 p24 antigen expression was quantified from the supernatants by ELISA (Zeptometrix, USA). This potency assay was validated according to ICH guidelines. Experiments were performed with 5 parallels, at least two times.

2.2.3. Inductively coupled plasma mass spectrometry (ICP-MS)

Measurements were performed from 0.5 ml samples in triplicates according to EPA 6020 standardized procedure.

2.2.4. Total organic carbon (TOC) assay

Measurements were performed from 0.5 ml samples in triplicates according to MSZ EN 1484:1998 standardized procedure.

2.2.5. Adsorbable bonded organic halogens (AOX) assay

Measurements were performed from 0.5 ml samples in triplicates according to MSZ EN ISO 9562:2005 standardized procedure, modified accordingly.

2.2.6. UV/vis spectrophotometry

Measurements were performed from 0.5 ml samples in triplicates, using quartz cuvette in Jasco V-630 instrument. Full spectra were recorded from 190 to 1100 nm with 5 nm step width.

2.2.7. Particle size measurements

Measurements were performed from samples containing 10 μg/ml pDNA in DermaVir (in triplicates), using Brookhaven ZetaPALS^{TM} instrument equipped with BI-MAS OPTION software Ver. 3.88 and quartz cuvette. Parameters: automatic mode; angle: 90.00; wavelength: 659.0 nm; 3 runs; temperature: 25 °C. All samples were diluted with Milli-Q water before measurement.

2.2.8. Agarose gel electrophoresis (AGE)

0.8% agarose gel (containing ethidium bromide) and 1× TAE buffer pH 8.3 (Invitrogen) were used for all experiments. pDNA amount loaded were 250 ng/well in triplicates. The *oc*/*ccc* ratio of the pDNA inside the nanoparticles was determined by AGE after decomplexation with 2% SDS (sodium dodecyl sulphate) solution (100 V, 120 min running) (Okamoto et al., 2005). Evaluation of the gel was performed using ImageJ software from NIH.

2.2.9. Statistical analysis

To assess the significance of the results, Student's *t*-test was performed on selected data and all *P*-values were calculated at a significance level of 0.05.

3. Results

We were surprised to find different biological activities of DermaVir nanomedicine formulated from two lots of pDNA that were identical according to the presently accepted cGMP quality requirements. Both pDNAs were manufactured from the same master cell bank and were solved in sterile deionized water. The two pDNA lots were equivalent based on release testing criteria of the manufacturers described in the certificate of analysis (CoA): proper identity, low or undetectable quantities of impurities (protein: <1%, w/w; RNA: <2%), endotoxin levels (<5 EU/mg) and supercoiled form content (*ccc* = 75 ± 10%). It is generally accepted that the potency of pDNA-based products is related to the supercoiled form content (ccc %) of the pDNA (Evans et al., 2000; Cupillard et al., 2005; Tumanova et al., 2005). Repeated testing revealed that despite of the quality equivalency of these pDNAs the biological activity of pDNA/PEIm nanomedicine was significantly different ($p < 0.0001$) (Fig. 1a).

To find the reason we have performed a detailed elemental analysis on the pDNA solutions and found that there are significant differences in the ionic strength (Table 1). Specially, pDNA-A contained over 5 molar equivalents of sodium, mostly as sodium-chloride, calculated to the pDNA concentration. In contrast, the pDNA-B contained only 0.3 molar equivalents of sodium-chloride.

To investigate whether the different NaCl content of pDNA solutions was responsible for the different biological activities we have supplemented the pDNA-B solution with 3 and 6 molar equivalents of NaCl before formulating pDNA/PEIm nanomedicine (initial NaCl concentration: 0.3 molar equivalents). NaCl supplementation increased the biological activity of the nanomedicine compared

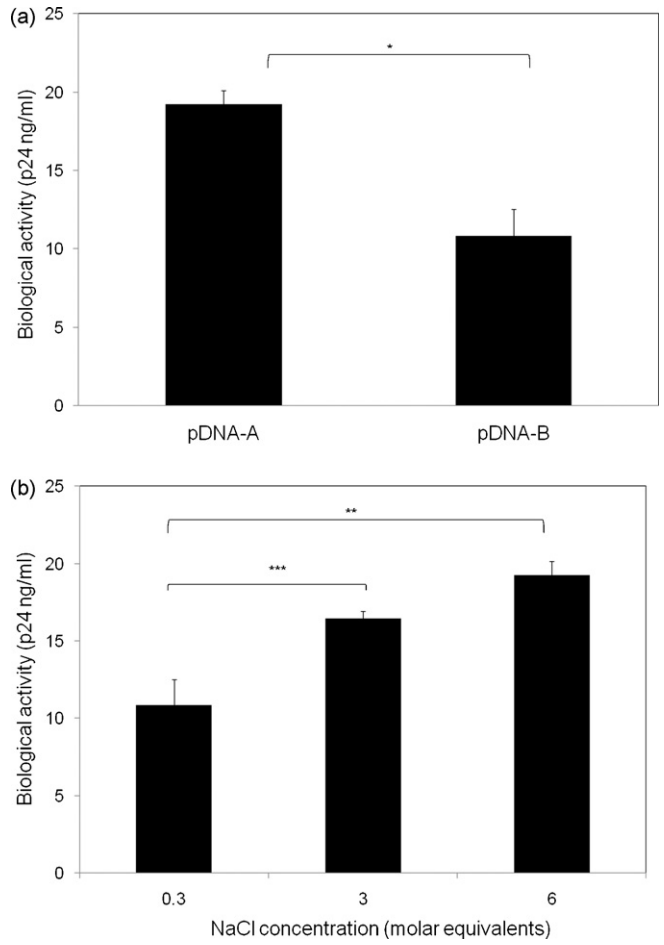

Fig. 1. Effect of the NaCl content of pDNA solution on the biological activity of the DermaVir nanomedicine. (a) Biological activity of DermaVir nanomedicine prepared from the two lots of pDNA (A and B) identical based on presently accepted release testing criteria and same PEIm. (b) NaCl content of the pDNA solution determines the biological activity of DermaVir nanomedicine. $^{*}p < 0.0001$, $^{**}p < 0.0001$, $^{***}p < 0.0003$, others are not significant ($p = 0.55$).

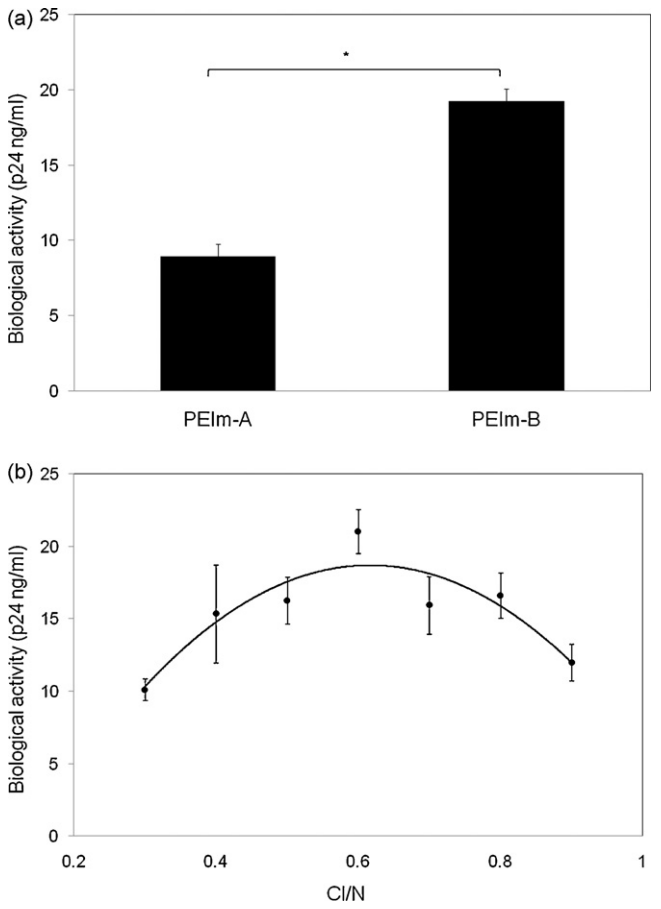

Fig. 2. Effect of the Cl/N ratio of the PEIm solution on the biological activity of the DermaVir nanomedicine. (a) Biological activity of DermaVir prepared from the two lots of PEIm (A and B) identical based on presently accepted release testing criteria and same pDNA, formulated in TEAM pH 7.5. (b) Effect of Cl/N ratio of the PEIm on the biological activity of DermaVir formulated in TEAM pH 7.5. $^{*}p < 0.0001$, $y = -83.6x^2 + 103.1x - 13.1$, $R^2 = 0.83$.

to the pDNA-B having 0.3 molar equivalent salt concentrations (Fig. 1b). When the pDNA-B was complemented with the same amount of NaCl found in pDNA-A (6 molar equivalent) the biological activity reached the one prepared from pDNA-A. These results suggest that the ionic strength of the pDNA solution, especially the NaCl content can influence the gene expression and consequently the biological activity of pDNA-based nanomedicinal products.

Next we formulated pDNA/PEIm nanomedicine with two lots of PEIm of the same quality and found significant differences in the biological activity of the nanomedicine ($p < 0.0001$) (Fig. 2a). Characterization of the two lots of PEIm demonstrated comparable quality; the linear structures were confirmed by NMR, the molecular mass and polydispersities were similar, the grafted mannobiose content and lack of unbounded sugars were the same. Based on the data obtained with pDNA we suspected that the NaCl might cause these discrepancies. However, after conducting a detailed elemental analysis we did not find considerable amount of cations in the PEIm solutions, instead we found significant differences in the Cl anion (Table 1). Since the chlorine content of the PEIm solution represents the protonated N atoms in salt form ($-NH_2{}^+Cl^-$) we calculated the Cl/N ratio. Our analysis revealed Cl/N molar ratio of 0.9 for PEIm-A and 0.6 for PEIm-B.

Table 1
Elemental analysis of two lots pDNA and PEIm solutions.

Lot	C[a]	N[b]	P	Na	Mg	Cl[c]
	mg/l (±10%)					
pDNA-A	405	NR	100	403	32	521
pDNA-B	390	NR	93	25	1	3
PEIm-A	387	191	22	10	3	436
PEIm-B	389	192	41	7	2	193

NR: not relevant.
[a] Measured by TOC.
[b] Calculated using the C/N molar ratio of PEIm structure as 2.36 (other organic impurities are excluded by NMR).
[c] Measured by AOX.

To understand whether the different Cl/N ratio of the PEIm solutions was responsible for the different biological activity of pDNA/PEIm nanomedicine, we made identical PEIm preparations with different Cl/N ratio. To obtain these samples, PEIm-A having Cl/N ratio of 0.9 was "neutralized" with NaOH solution. pDNA/PEIm nanomedicine was formulated from PEIm samples (0.3–0.9 Cl/N ratio) and their biological activities were tested (Fig. 2b). We found that Cl/N ratio has an optimum at 0.6, neither higher nor lower Cl/N ratio results in optimal biological activity of DermaVir nanomedicine. We found that when decreasing the Cl/N ratio of PEIm-A from 0.9 to 0.6, which was the Cl/N ratio of PEIm-B, the biological activity became the same suggesting that the differences between the two PEIm preparations were due to the different Cl/N ratio. The relation between the biological activity of the nanomedicines and the Cl/N ratio of PEIm can be math-

Table 2
Effect of the N/P ratio on the biological activity of the DermaVir nanomedicine prepared freshly and after 1-week storage at 23 ± 2 °C (RT).

N/P	2		3		4	
	Fresh	1 week, RT	Fresh	1 week, RT	Fresh	1 week, RT
Biol. activity (p24 ng/ml)	10.7 ± 0.1	18.8 ± 1.9	19.0 ± 1.8	17.0 ± 0.4	15.1 ± 1.9	13.6 ± .0.8
D_{Eff} (nm)	339 ± 133	280 ± 7	227 ± 14	214 ± 5	205 ± 9	213 ± 9

ematically described with a polynomial second-degree equation of $y = -83.6x^2 + 103.1x - 13.1$ having a maximum at Cl/N = 0.61. Therefore, the Cl/N value is an important parameter for the biological activity of pDNA-based nanomedicines prepared from cationic polymers and the optimum Cl/N value is 0.6 representing 60% protonation of the PEIm molecule.

We have observed that the biological activity of pDNA/PEIm nanomedicines sometime improves during the storage. To explain this phenomenon we have investigated the effect of the N/P ratio (N content of PEIm and P content of pDNA) on the biological activity (Table 2). We have demonstrated that the biological activity of the nanomedicine prepared freshly at N/P = 2 had significantly improved after 1 week of storage. The same improvement during storage did not occur when the nanomedicine was formulated with higher N/P ratio. We hypothesized that at N/P = 2 the freshly formed nanoparticles have not enough stable structures and this structure might stabilize during storage, because the low amount of PEIm perhaps needs more time to reach the equilibrium of the pDNA/PEIm complex. We measured the size of the particles and found that the freshly prepared DermaVir at N/P = 2 has an effective diameter of 400 nm with very high SD ($D_{Eff} = 339 \pm 133$), while after storage the nanoparticles became more compact of 280 nm with very low SD ($D_{Eff} = 280 \pm 7$) suggesting that compact nanoparticles are required for optimal biological activity of DermaVir. In contrast, nanomedicines formulated at N/P ≥ 3 were either stable or their stability decreased with storage. The compact size and small SD were characteristic for the other fresh or stored pDNA/PEIm nanomedicine formulations with N/P ratios of 3 and 4 (average particle size is 214 ± 10 nm). The biological activity of the nanoparticles prepared freshly at N/P ratio of 3 and 4 was comparable ($p = 0.059$). However, after 1 week of storage the biological activity of both the DermaVir formulated at N/P ratio of 3 and 4 slightly decreased compared to the freshly prepared samples ($p = 0.111$ and $p = 0.262$, respectively). Interestingly, the biological activity of the stored DermaVir nanomedicine prepared at N/P = 4 was significantly lower ($p < 0.003$) than the biological activity of the one prepared at N/P = 3 (Table 2). We concluded that the biological activity and stability of the DermaVir nanomedicine is affected by the N/P ratio of the PEIm and pDNA components and the optimal N/P ratio is 3.

We have investigated the effect of the pH to the structure and biological activity of the pDNA/PEIm nanomedicine. Increasing the pH up to 9 increases the pDNA stability because double stranded pDNA in aqueous solution degrades through a depurination/β-elimination process and the first step in this process is acid catalyzed (Evans et al., 2000). Therefore, we prepared nanomedicine in formulation solvents from pH 3.6 to pH 9.3 and tested the effects on the biological activity. We have performed two sets of experiments: DermaVir formulated freshly and DermaVir stored in refrigerator for 1 week (Fig. 3a). The pH of the solvent influenced the biological activity of the pDNA/PEIm nanomedicine. The relationship between the biological activity of the freshly prepared nanomedicines and the pH of the solvent can be mathematically described with a polynomial second-degree equation: $y = -3.5x^2 + 43.7x - 41.1$ with a maximum at pH 6.2 of the solvent. We found that after storage the biological activity decreased at pH 6.2 (Fig. 3a). The pH of the formulation solution between 7 and 7.5 supported the highest biological activity of both the fresh and the stored DermaVir. We concluded that the optimum value of the solvent pH is at the physiological range (pH 7–7.5) and the pH of the solvent is an important parameter for the biological activity and stability of the pDNA-based nanomedicines.

Since EDTA is known to increase the stability of pDNA aqueous solutions, we investigated the effect of this excipient on the stability and biological activity of nanomedicines (Evans et al., 2000). We found that 1 mM EDTA in DermaVir nanomedicine solution significantly decreased the biological activity compared to the control formulation without EDTA (Fig. 3b). The remarkable drop of biological activity is certainly the result of the modified structure of the pDNA/PEIm nanoparticles, which is indicated by the large particle size with very high SD ($D_{Eff} = 1208 \pm 403$ nm) (Fig. 3b). We hypoth-

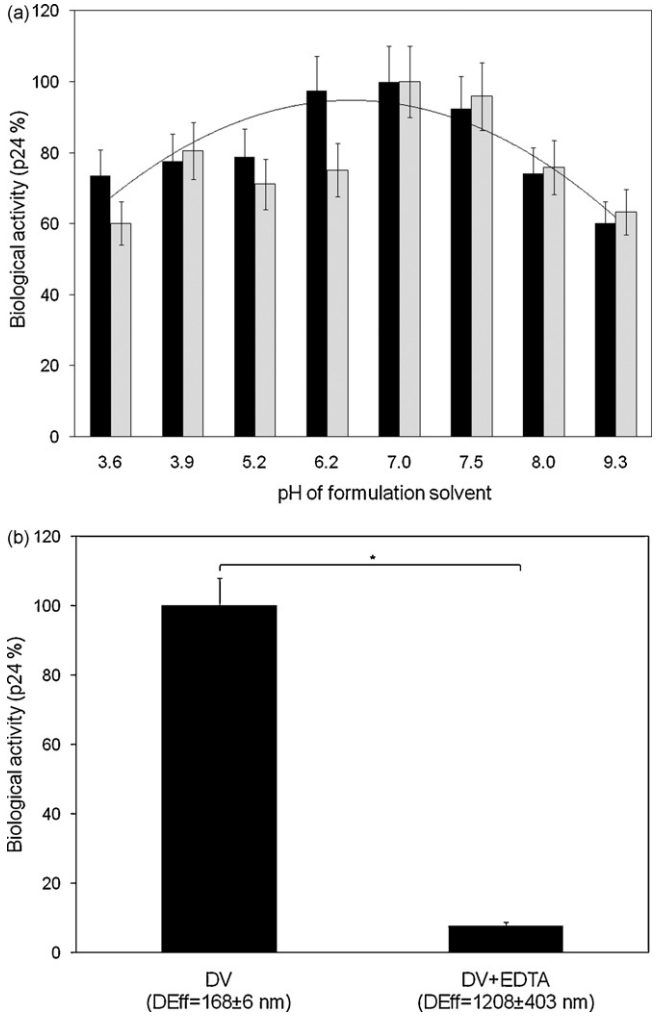

Fig. 3. Effect of the formulation solvent and excipient on the biological activity of DermaVir nanomedicine. (a) Effect of the formulation solvent pH on the biological activity of the DermaVir prepared from pDNA-B and PEIm-B in TEAM at different pH, (■) fresh, (□) 1-week 4 °C, (▬) polynomial curve (fresh), $y = -3.5344x^2 + 43.71x - 41.146$, $R^2 = 0.7533$. (b) Effect of the EDTA on the biological activity of DermaVir nanomedicine, $^*p < 0.0001$.

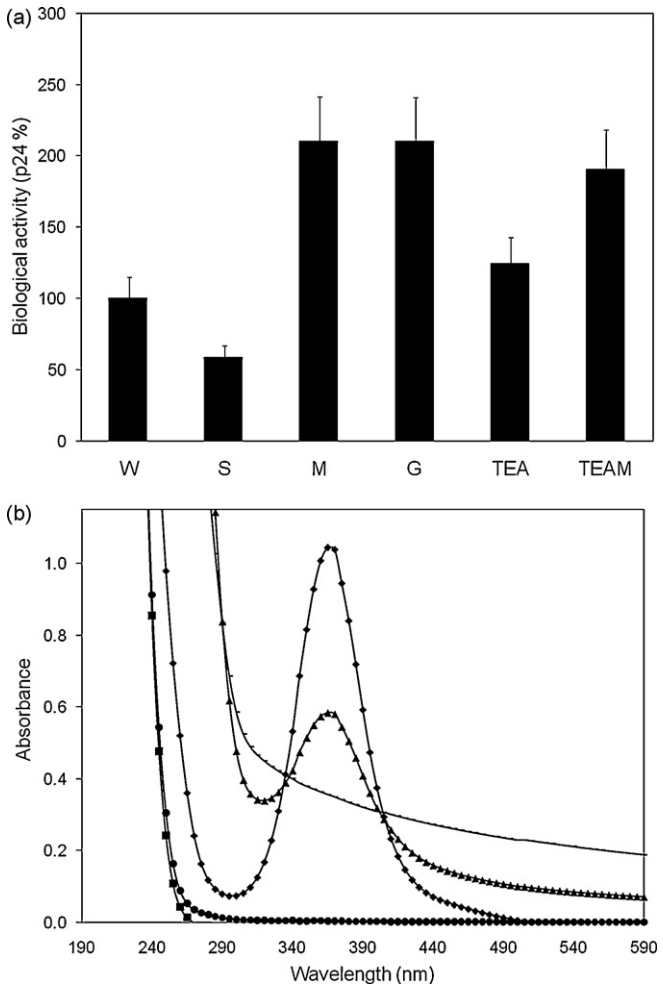

Fig. 4. Effect of formulation solvent on the biological activity and stability of DermaVir nanomedicine. (a) Effect of the formulation solvent on the biological activity. Solvents: W (sterile water), S (150 mM NaCl solution), M (10% mannitol solution), G (10% glucose solution), TEA (triethanolamine buffer pH 7.5), TEAM (triethanolamine buffer pH containing 10% mannitol). (b) Effect of the formulation solvent on the chemical stability. PEIm solution (●) mixture of PEIm with glucose (ϑ) and mannitol (■), respectively, DermaVir formulations with glucose (▲) and mannitol (▬). The solutions were stored at 23 ± 2 °C for 2 months and the UV spectra were recorded without dilution.

esized that the EDTA competes with the pDNA to interact with the cationic polymer (PEIm), consequently nano-sized particles can be only formed in the absence of EDTA (D_{Eff} = 168 ± 6 nm) (Fig. 3b). These results show that EDTA, used as stabilizer in the pDNA solutions, disrupts the structure of pDNA/PEIm nanoparticles.

We further investigated the effect of the formulation solvents on the biological activity of the nanomedicine. We found that complementing the formulation solvent with sugar-based compounds (e.g. glucose and mannitol) improved the biological activity of the nanomedicine compared to water, physiological saline or pH buffer (Fig. 4a). Glucose is a commonly used solvent in medicinal products and we used it in the DermaVir nanomedicine formulation during the phases I and II trials. However, we observed that the color of the clinical DermaVir formulations turns to yellow from a clear solution during storage. We have investigated the reason of the yellow color and found that during storage the glucose interacts with the PEIm forming a yellow adduct which has an absorbance maximum at 365 nm (Fig. 4b). The yellow adduct is probably the so called "Amadori" product (Tjan and Ouweland, 1974; Bhattacharyya, 1995), however its isolation and identification was not performed. This yellow adduct did not appear if the nanomedicine was formulated with mannitol. The difference between glucose and mannitol is that glucose contains a carbonylic group that could react with the amino group of the PEIm. The same side reactions could not take place with mannitol because it is not a reductive sugar. Therefore, to optimize the biological activity of DermaVir nanomedicine we decided to complement the formulation solution with mannitol instead of glucose.

After establishing the liquid nanomedicine formulation optimal for the biological activity we have performed stability testing at 37 °C and 4 °C. Four lots of DermaVir were manufactured using two lots of pDNA (see Fig. 1 and Table 1) and two lots of PEIm (see Fig. 2 and Table 1) (Fig. 5a). The biological activity of the DermaVir product (DV-AA) prepared from the pDNA solution of high ionic strength and the highly protonated PEIm was significantly lower than the freshly prepared control sample (Fig. 5b). We formulated DermaVir (DV-AB) with PEIm of low degree of cationic character. DV-AB had more potent biological activity than DV-AA and was not influenced by storage temperatures of 4 and 37 °C. However, DV-AB biological activity after storage was significantly lower that the fresh DermaVir formulation (Fig. 5b).

We investigated the supercoiled pDNA form content (*ccc%*) of the DermaVir nanomedicine after SDS decomplexation by AGE (Table 3) (Okamoto et al., 2005). We found partial degradation of pDNA inside some formulations. Specifically, 14.2 and 48.4% supercoiled pDNA form remained after 37 °C storage in DV-AA and DV-AB, respectively, from the original 78%. This pDNA degradation inside the nanomedicine could be responsible for the reduced biological activity. In both DV-AA and DV-BA formulations we observed aggregations (D_{Eff} = 355 ± 28 nm) that reduced significantly the biological activity of these preparations during storage (Fig. 5b and c). Both contained the PEIm-A, which had high Cl content and 90% protonation (0.9 Cl/N ratio). However, the biological activity after storage of the DV-BB prepared with the low ionic strength of pDNA-B and 60% protonated PEIm-B was comparable with the fresh one (Fig. 5c). Further testing of the DV-BB at 4 °C revealed the consistent stability of the formulation for 8 weeks without any loss of biological activity and without any aggregation (D_{Eff} = 194 ± 12 nm) (Fig. 5d).

4. Discussion

Explicit characterization of the starting materials is one of the key parameters of the formulation development for nanomedicines as they have crucial effect on their biological activity and stability. We demonstrated that the current cGMP quality requirements for pDNA and PEIm are not sufficient for the characterization of these materials. Chemical parameters of these biological products have significant effect on the quality of the nanomedicine products.

We showed that the ionic strength of the pDNA solution, especially the sodium chlorine residues, increased the biological activity of the nanomedicine. However, such positive effect diminished during the storage of DermaVir, because the presence of high NaCl concentration decreased its biological activity compared to the one prepared freshly. The sodium ions influence the binding affinity of the pDNA to the PEIm cations. If more sodium ions are present in the pDNA solution more of the phosphate groups of pDNA would form sodium salt, therefore the secondary amines of PEIm could not competitively displace these sodium ions having much lower basic properties. Consequently, the inherent structures of the pDNA/PEIm nanoparticles are less tight (loose) than the tight particles formed between higher number of anionic phosphates (less sodium salt) and the polycationic PEIm. The loose structure of the nanoparticles is favorable only for the biological activity, i.e. fast release of the pDNA from the complex core, but for long-term storage a more compact structure is required which protects the pDNA from degradation (Lechardeur et al., 1999; Schaffer et al., 2000).

(a)

DermaVir	pDNA-A	pDNA-B	PEIm-A	PEIm-B
DV-AA	+	-	+	-
DV-AB	+	-	-	+
DV-BA	-	+	+	-
DV-BB	-	+	-	+

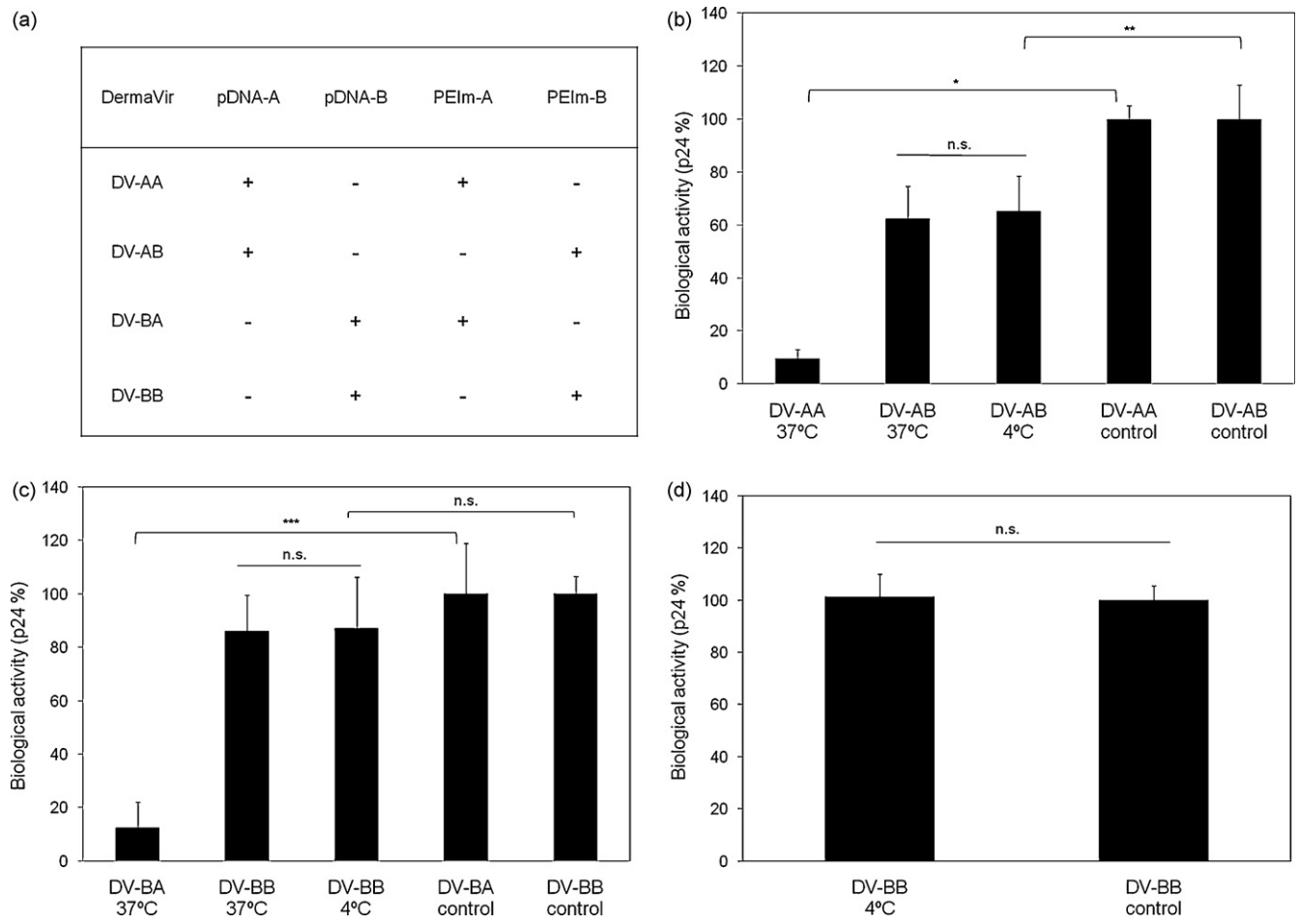

Fig. 5. Stability testing at 4 and 37 °C of the DermaVir nanomedicine liquid formulations. (a) DermaVir formulations prepared at N/P ratio of 3 in TEAM. (b) Biological activity of DermaVir formulations after 3 weeks storage. (c) Biological activity of DermaVir formulations after 3 weeks storage. (d) Full conservation of the biological activity of DermaVir nanomedicine after storage at 4 °C for 8 weeks. $^{*}p < 0.0001$, $^{**}p < 0.001$, $^{***}p < 0.0003$, n.s. not significant.

Characterization of the PEIm components of the nanomedicine we identified the Cl/N molar ratio as one of the key parameters for the biological activity of the nanomedicine. The chlorine ion is the negative counter ion of the cationic N of PEIm. During synthesis of the PEIm the conversion of poly-(2-ethyl-2-oxazoline) is performed by acidic hydrolysis using HCl, consequently the chlorine content is relevant in the structure of the PEIm (Jeong et al., 2001). The chlorine content, more precisely the Cl/N ratio quantifies the cationic degree of the PEIm. The PEIm has double role in the biological activity of the pDNA/PEIm nanomedicines: its cationic (protonated) amino groups interact with the polyanionic pDNA and by its "neutral" (protonable) amino groups act as a "proton-sponge" against the low pH of the endosome protecting the nanomedicine from the degradation (Boussif et al., 1995). These two processes require a PEIm solution with an optimum degree of cationic character that according to our investigations is 60% protonation of its total N atoms.

The formulation development for pDNA/PEIm nanomedicines had to take into consideration the stability requirements of medicinal products. We determined the optimal mixing ratio of the pDNA and PEIm components (N/P ratio) for the formation of the nanomedicine. N/P ratio of 3 was selected to be optimal for the biological activity and the stability of the nanomedicine. Higher N/P ratio not only alters the stability of the nanomedicine but might increase its toxicities (Bragonzi et al., 1999; Chollet et al., 2002; Fahrmeir et al., 2007).

Investigating the formulation solvent for pDNA/PEIm nanomedicine, we found that the optimal pH value of the solvent is at the physiological pH range (pH 7–7.5). This pH required for both the optimal biological activity and the stability of the nanomedicine. Taking into consideration the pH values required for pDNA (pH ~ 9) and PEIm stability (pH ~ 8 (Choosakoonkriang et al., 2003)), we have selected pH of 7.5 for the formulation of the DermaVir nanomedicine product. For buffering the nanomedicine formulation we selected triethanolamine (TEA), because it is a pharmaceutically acceptable pH buffer with maximum buffer capacity around pH 7.5. This buffer contains non-reactive tertiary amines and it is a weaker base than the secondary amine containing linear PEIm, therefore suitable to exclude potential side reactions with the acidic pDNA. For the liquid formulation, sterile water and 10% glucose or dextrose solutions are the commonly used and pharmaceutically acceptable solvents, but none of them were

Table 3
Analysis of the pDNA forms in the DermaVir formulations by AGE after decomplexation with SDS.

Sample	pDNA	pDNA-SDS	DV-AA-37 °C	DV-AB-37 °C	DV-AB-control
ccc% (±2%)	78.6	80.5	14.2	48.4	75.8

acceptable for formulation of pDNA/PEIm nanomedicines (Behr et al., 2000). We have shown that reductive sugars like glucose or mannose do not support the stability of DermaVir because they react with PEIm. We found that sugar-alcohols like mannitol improve biological activity of the nanomedicine without side reactions with PEIm. Therefore, in the pDNA/PEIm nanomedicine formulation we have combined the positive effects of the TEA pH buffer and the mannitol and selected a solvent consisting of TEA buffer at pH 7.5 containing 10% mannitol (TEAM).

We presented here a rational product profile-oriented analysis of the PEIm/pDNA nanomedicines that includes the detailed characterization of the starting materials and takes into consideration the biological activity and the stability of the medicinal product. This approach of developing nanomedicine formulation is in accordance with FDA's (US Food and Drug Administration) current "Quality by Design" program claiming that the product and process knowledge base must include an understanding of the variability in raw materials and the relationship between a process and product's critical quality attributes (CQA) (Rathore and Winkle, 2009). The optimal pDNA/PEIm liquid formulation requires the pDNA in a low ionic strength solution and the PEIm with 60% degree of cationic character mixed at N/P ratio of 3 and formulated in triethanolamine buffered (pH 7.5) mannitol solution (TEAM). This new liquid formulation was stable, no aggregation, decomplexation and loss of biological activity was detected during storage. Our liquid formulation did not require any additional covalently attached protecting groups to the PEI or surfactants to prevent the aggregation as described previously (Ikonen et al., 2008).

Acknowledgments

This work was supported by the National Office for Research and Technology (NKTH), Hungary Grants: HIKC05 and DVCLIN01. We thank József Pandur, Ildikó Pulinka, Éva Szikora, Mónika Lakatos, Erika Horváth for their contributions and Gabriela Barbieri for editorial assistance.

References

Anchordoquy, T.J., Armstrong, T.K., Molina, M.C., 2005. Low molecular weight dextrans stabilize nonviral vectors during lyophilization at low osmolalities: concentrating suspensions by rehydration to reduced volumes. J. Pharm. Sci. 94, 1226–1236.

Behr, J.P., Demeneix, B., Lezoualch, F., Mergny, M., Scherman, D., Boussif, O., 2000. Nucleic acid containing composition, preparation and uses of same, US Patent 6,013,240, 11 January.

Bhattacharyya, S., 1995. Reductive alkylation of dimethylamine using titanium(IV) isopropoxide and sodium borohydride: an efficient, safe, and convenient method for the synthesis of N,N-dimethylated tertiary amines. J. Org. Chem. 60, 4928–4929.

Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P., 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7301.

Bragonzi, A., Boletta, A., Biffi, A., Muggia, A., Sersale, G., Cheng, S.H., Bordignon, C., Assael, B.M., Conese, M., 1999. Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. Gene Ther. 6, 1995–2004.

Chollet, P., Favrot, M.C., Hurbin, A., Coll, J.L., 2002. Side-effects of a systemic injection of linear polyethylenimine–DNA complexes. J. Gene Med. 4, 84–91.

Choosakoonkriang, S., Lobo, B.A., Koe, G.S., Koe, J.G., Middaugh, C.R., 2003. Biophysical characterization of PEI/DNA complexes. J. Pharm Sci. 92, 1710–1722.

Cupillard, L., Juillard, V., Latour, S., Colombet, G., Cachet, N., Richard, S., Blanchard, S., Fischer, L., 2005. Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats. Vaccine 23, 1910–1916.

Electronic Source (14.01.2010): http://www.clinicaltrial.gov/ct2/results?term=dermavir.

Evans, R.K., Xu, Z., Bohannon, K.E., Wang, B., Bruner, M.W., Volkin, D.B., 2000. Evaluation of degradation pathways for plasmid DNA in pharmaceutical formulations via accelerated stability studies. J. Pharm. Sci. 89, 76–87.

Fahrmeir, J., Gunther, M., Tietze, N., Wagner, E., Ogris, M., 2007. Electrophoretic purification of tumor-targeted polyethylenimine-based polyplexes reduces toxic side effects *in vivo*. J. Control. Release 122, 236–245.

Hobel, S., Prinz, R., Malek, A., Urban-Klein, B., Sitterberg, J., Bakowsky, U., Czubayko, F., Aigner, A., 2008. Polyethylenimine PEI F25-LMW allows the long-term storage of frozen complexes as fully active reagents in siRNA-mediated gene targeting and DNA delivery. Eur. J. Pharm. Biopharm. 70, 29–41.

Ikonen, M., Murtomaki, L., Kontturi, K., 2008. Controlled complexation of plasmid DNA with cationic polymers: effect of surfactant on the complexation and stability of the complexes. Colloids Surf. B. Biointerfaces 66, 77–83.

Jeong, J.H., Song, S.H., Lim, D.W., Lee, H., Park, T.G., 2001. DNA transfection using linear poly(ethylenimine) prepared by controlled acid hydrolysis of poly(2-ethyl-2-oxazoline). J. Control. Release 73, 391–399.

Lechardeur, D., Sohn, K.J., Haardt, M., Joshi, P.B., Monck, M., Graham, R.W., Beatty, B., Squire, J., O'Brodovich, H., Lukacs, G.L., 1999. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. Gene Ther. 6, 482–497.

Lisziewicz, J., Gabrilovich, D.I., Varga, G., Xu, J., Greenberg, P.D., Arya, S.K., Bosch, M., Behr, J.P., Lori, F., 2001. Induction of potent human immunodeficiency virus type 1-specific T-cell-restricted immunity by genetically modified dendritic cells. J. Virol. 75, 7621–7628.

Lisziewicz, J., Trocio, J., Whitman, L., Varga, G., Xu, J., Bakare, N., Erbacher, P., Fox, C., Woodward, R., Markham, P., Arya, S., Behr, J.P., Lori, F., 2005a. DermaVir: a novel topical vaccine for HIV/AIDS. J. Invest. Dermatol. 124, 160–169.

Lisziewicz, J., Trocio, J., Xu, J., Whitman, L., Ryder, A., Bakare, N., Lewis, M.G., Wagner, W., Pistorio, A., Arya, S., Lori, F., 2005b. Control of viral rebound through therapeutic immunization with DermaVir. AIDS 19, 35–43.

Lori, F., Trocio, J., Bakare, N., Kelly, L.M., Lisziewicz, J., 2005. DermaVir, a novel HIV immunisation technology. Vaccine 23, 2030–2034.

Molina, M.C., Allison, S.D., Anchordoquy, T.J., 2001. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. J. Pharm. Sci. 90, 1445–1455.

Neu, M., Fischer, D., Kissel, T., 2005. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J. Gene Med. 7, 992–1009.

Ohana, P., Gofrit, O., Ayesh, S., Al-Sharef, W., Mizrahi, A., Birman, T., Schneider, T., Matouk, I., de Groot, N., Tavdy, E., Sidi, A., Hochberg, A., 2004. Regulatory sequences of the H19 gene in DNA based therapy of bladder cancer. Gene Ther. Mol. Biol. 8, 181–192.

Okamoto, H., Sakakura, Y., Shiraki, K., Oka, K., Nishida, S., Todo, H., Iida, K., Danjo, K., 2005. Stability of chitosan–pDNA complex powder prepared by supercritical carbon dioxide process. Int. J. Pharm. 290, 73–81.

Rathore, A.S., Winkle, H., 2009. Quality by design for biopharmaceuticals. Nat. Biotechnol. 27, 26–34.

Schaffer, D.V., Fidelman, N.A., Dan, N., Lauffenburger, D.A., 2000. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. Biotechnol. Bioeng. 67, 598–606.

Tjan, S.B., Ouweland, G.A.M.V.D., 1974. PMR investigation into the structure of some n-substituted 1-amino-1-deoxy-D-fructoses (amadori rearrangement products): evidence for a preferential conformation in solution. Tetrahedron 30, 2891–2897.

Tumanova, I., Boyer, J., Ausar, S.F., Burzynski, J., Rosencrance, D., White, J., Scheidel, J., Parkinson, R., Maguire, H., Middaugh, C.R., Weiner, D., Green, A.P., 2005. Analytical and biological characterization of supercoiled plasmids purified by various chromatographic techniques. DNA Cell Biol. 24, 819–831.

von Harpe, A., Petersen, H., Li, Y., Kissel, T., 2000. Characterization of commercially available and synthesized polyethylenimines for gene delivery. J. Control. Release 69, 309–322.