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International Journal of Pharmaceutics 211 (2000) 79–88

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Sterilization of poly(dimethylamino) ethyl methacrylate-based gene transfer complexes

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Received 18 May 2000; received in revised form 30 August 2000; accepted 21 September 2000

Abstract

Parental administration of polyplex formulations for gene therapy or genetic vaccination requires sterile preparations. The possibilities and limitations of autoclaving, filtration and a combination of both methods for sterilization of poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA) based gene transfer complexes were assessed. Agarose gel electrophoresis and circular dichroism spectroscopy showed that sterile filtration of polyplexes did not change the topology and integrity of the DNA. The transfection potential was fully retained in COS-7 and OVCAR-3 cells, although the concentration of DNA was slightly decreased by the filtration process. Pre-coating of the filter with polyplexes reduced the material loss. In contrast, autoclaving dramatically affected physical characteristics of polyplexes, resulting in complete loss of transfection potential. Sterile filtration or autoclaving of polymer alone did not result in material loss, or in decreased transfection potential after complexation with plasmid DNA. 'Naked' DNA could easily be sterilized by filtration as well. In conclusion, sterilization of complexes between pDMAEMA-based cationic polymeric gene transfer agents and DNA plasmid is feasible by filtration. Depending on the filter type used, the filtered volume should be high enough, to prevent substantial material loss. Separate sterilization of the polymer by autoclaving or filtration and DNA by filtration offers a good alternative to filtration of formed polyplexes.

Keywords: DNA; Transfection; Sterilization; Poly(2-(dimethylamino) ethyl methacrylate); Cationic polymeric carriers; Polyplexes

1. Introduction

The aim of gene therapy is to treat inherited or acquired genetic deficiencies (e.g. cystic fibrosis) or viral diseases (e.g. Hepatitis B, HIV) by introduction of DNA encoding a therapeutic protein or a specific virus antigen into the target cells,

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0378-5173/00/$ - see front matter
PII: S0378-5173(00)00593-7

respectively. As naked DNA will barely pass cellular membranes, a carrier system is required for transfection (Behr, 1993; Ledley, 1997; Godbey et al., 1999; De Smedt et al., 2000). A promising class of non-viral transfection agents is constituted of cationic polymers, which condense DNA by ionic interactions. Well known examples of these polymers are DEAE dextran, poly(L-lysine), and poly(ethyleneimine) (Kawai and Nishizawa, 1984; Curiel et al., 1992; Boussif et al., 1995; Godbey et al., 1999).

Recently, it has been shown that poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA) as well as copolymers based on this polymer are able to introduce DNA into cells (Cherng et al., 1996; Van de Wetering et al., 1997, 1998a, 1999; Hinrichs et al., 1999; Van de Wetering et al., 2000). Since then, several pharmaceutical aspects of this transfection system have been addressed. Freeze-drying was shown to be an excellent method to preserve the size and transfection potential of pDMAEMA/plasmid complexes (polyplexes), even after aging at 40°C (Cherng et al., 1997, 1999b). In another study, it was demonstrated that the DNA topology affected pDMAEMA-mediated transfection: the circular forms of DNA (supercoiled and open circular) had a higher transfection activity than the linear forms (Cherng et al., 1999a).

Since polyplex formulations are intended for parenteral administration, sterilization is an essential part of the production process. So far, no studies have been published in which sterilization methods for polyplex formulations were evaluated. As gamma and UV irradiation are known to destroy the structure of DNA (e.g. Francis and Regan, 1986; Mustonen et al., 1999), these methods are not suitable for sterilization of polyplexes. Ethylene oxide commonly used for sterilizing medical disposable devices, kills microorganisms by replacing labile hydrogen atoms with hydroxyethyl groups. Ethylene oxide treatment of DNA results in *N*-7-(2-hydroxyethyl) guanine residues (e.g. Segerback, 1994), and is therefore not suitable for polyplex sterilization. Heat sterilization damages the structure of DNA by breakage of intramolecular hydrogen bonds (e.g. Brannen, 1970). However, the polymer present in the polyplexes may stabilize the DNA structure and, therewith, prevent high temperature induced degradation. This stabilization may result from ionic interactions between the positively charged polymer and the negatively charged DNA that may limit the movement of the chains, thus slowing down the breakdown process. Alternatively the polymer might help the DNA to assume the right conformation upon cooling of the autoclaved polyplexes. Besides chemical degradation, autoclaving might also induce aggregation of the colloidal polyplex particles. The addition of sucrose to the solution may prevent aggregation during autoclaving, as it also prevents aggregation at high polymer and DNA concentrations (Cherng et al., 1999c). Finally, microorganisms are removed from parenteral solutions by using filters with a cut-off of 0.2 μm. Since the size of polyplexes is generally between 80 and 180 nm (Cherng et al., 1996; Van de Wetering et al., 1997), filtration is potentially suitable as sterilization method for polyplex formulations. However, aggregates present in the polyplex formulation and adsorption to the filter might result in blockage of the filter. Also, the shear forces applied to the polyplexes might induce degradation of the plasmid molecules, e.g. conversion from supercoiled to circular and linear forms.

In this paper the possibilities and limitations of autoclaving, sterile filtration and also a combination of both methods for sterilization of pDMAEMA-based gene transfer complexes are assessed.

2. Materials and methods

2.1. Materials

Ammonium peroxodisulfate (APS), α,α′-Azoisobutyronitril (AIBN), dextran standards, 2-(dimethylamino) ethyl methacrylate (DMAEMA), β-galactosidase, 2-mercaptoethanol, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis-(4-methoxy-6-nitro)benzene sulfonic acid (XTT), *N*-methyl dibenzopyrazine methylsulfate (NPS), ortho - nitrophenyl - β - D - galactopyranoside (ONPG) and poly(L-aspartic acid) (M_w 15 000–

50 000 g/mol) (Sigma-Aldrich, Bornem, Belgium), monomethoxy-polyethylene glycol (PEG)-5000 and disuccinimidyl carbonate (Fluka, Bornum, Belgium), dimethyl aminopyridine (DMAP, Acros, Bornum, Belgium), RPMI 1640 (Gibco BRL, Breda, The Netherlands), diethylether, ethylacetate, and sucrose (Merck, Haar, Germany), *N*,*N*-dimethyl formamide (DMF, Biosolve, Valkenswaard, The Netherlands), agarose (Pronase D-1, Hispanagar, Burgos, Spain) and SYBR Green I (Molecular Probes, Eugene, OR, USA) were used as received.

Plasmid pCMV*lacZ*, which contains a bacterial *lacZ* gene preceded by a nuclear localization signal under control of a CMV promoter (Bout et al., 1993), was isolated from *Escherichia coli* as described before (Cherng et al., 1996).

HEPES buffered saline (HBS) was composed of 150 mM NaCl, 20 mM 4-(2-hydroxy ethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4.

Cell culture flasks and microtiter plates were obtained from Falcon (Micronic, Lelystad, The Netherlands). OVCAR-3 cells originate from the American Type Culture Collection (ATCC), no. HTB 161 (Maryland, USA) (Hamilton et al., 1983). COS-7 cells, derived from African Green monkey kidneys, originated from the ATCC (no. CRL 1651). Cells were cultured as described previously (Arigita et al., 1999).

2.2. Synthesis of pDMAEMA and pDMAEMA-PEG

2-(Dimethylamino) ethyl methacrylate (DMAEMA) was purified by distillation under reduced pressure shortly before use. pDMAEMA (Fig. 1, $M_n = 2.1 \times 10^5$ and $M_w = 2.4 \times 10^6$ g/mol) was prepared by a radical polymerization of DMAEMA, as described previously (Van de Wetering et al., 1997). pDMAEMA-PEG block-copolymer was synthesized via a two-step synthesis. pDMAEMA of intermediate molecular weight with a terminal hydroxyl group was synthesized using a chain transfer agent (CTA, 2-mercaptoethanol). DMAEMA and 2-mercaptoethanol (monomer/CTA 93/1, mol/mol) were dissolved in toluene and AIBN in toluene was added (monomer/initiator 100/1, mol/mol). After 22 h at 60°C the polymer was isolated by precipitation in petroleum ether and dried in vacuum. Meanwhile, the *N*-hydroxysuccinimide (NHS)-ester of PEG-5000 was synthesized from monomethoxy-PEG-5000 (1 mmol) and disuccinimidylcarbonate (5 mmol), using dimethyl aminopyridine (DMAP, 5 mmol) as catalyst in 15 ml DMF (Elbert and Hubbell, 1998). After 24 h the reaction mixture was precipitated in 150 ml diethyl ether, filtered on a P4 filter, and washed with 25 ml diethyl ether. The product was dissolved in ethyl acetate, cooled to 4°C, filtered on a P4 filter and dried to air. The product was washed with 25 ml ethyl acetate and dried again, resulting in a white powder. Nuclear magnetic resonance (NMR)-spectroscopy showed that 80% of the monomethoxy-PEG was activated.

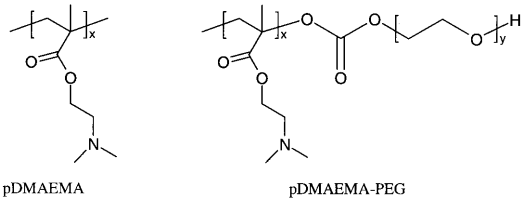

Fig. 1. Structure of poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA) and PEGylated pDMAEMA (pDMAEMA-PEG).

Next, the NHS-ester of PEG-5000 (292 mg) was coupled to pDMAEMA-OH (503 mg) in a triethylamine (100 μl)/dimethyl formamide (DMF, 30 ml) mixture at ambient temperature. After 4 days 10 ml water was added and the pH was lowered to 7 using 1 M HCl. After dialysis, the product was freeze-dried using a Christ 'alpha 1–2' freeze dryer at a pressure below 1 mbar (pDMAEMA-PEG, Fig. 1, $M_n = 1.0 \times 10^4$ and $M_w = 2.3 \times 10^4$ g/mol). The molecular weights of the polymers relative to dextran were determined by gel permeation chromatography, as described previously (Van de Wetering et al., 1997), using only the HMW column (Shodex OHpak KB-806M) instead of a series of columns (Shodex OHpak KB-802 and KB-806M), resulting in sharper peaks and an improved resolution. The PEG-content of the co-polymer was 25 % (w/w), as determined from NMR-spectroscopy after cation exchange chromatography using Biorad Macroprep High S and 2.5 M NaCl in PBS.

2.3. Preparation of pDMAEMA/plasmid particles (polyplexes)

Polyplexes were prepared as described before (e.g. Van de Wetering et al., 1997; Cherng et al., 1999a). In short, pDMAEMA or pDMAEMA-PEG was dissolved in HBS or 20 mM HEPES buffer at pH 7.4 supplemented with 10% (w/v) sucrose (HB/sucrose). The polymer solutions were subsequently diluted to a final concentration of 56 μg/ml in the same buffer. The plasmid stock solution was diluted in the same buffer to a concentration of 75 μg/ml. Next, 4 ml polymer solution was added to 1 ml plasmid solution, the solution was gently mixed and left for 30 min at ambient temperature. The final polymer and DNA concentration in the formulation is 45 and 15 μg/ml, respectively. Assuming that around 50% of the side groups in pDMAEMA are protonated at pH 7.4, this corresponds to a polymer/DNA ratio of 3/1 (w/w) and a charge ratio of 2.6 (P/N) for pDMAEMA and 1.9 (P/N) for pDMAEMA-PEG.

2.4. Autoclaving of polyplexes, polymer, and plasmid

Polyplexes (5 ml) were autoclaved in sealed 10-ml glass ampoules using a SANOclav, type M-ECZ (Wolf, Geislingen, FRG). The temperature was raised to 100°C (ca. 15 min). The samples were kept at this temperature for 2 min. Then the autoclave was closed, and the temperature and pressure increased. The ampoules were kept at 121 ± 1°C for 17 min, including 2 min for heating the content of the ampoules, before being cooled down to ambient temperature. Besides polyplexes, polymer (5 ml, 56 μg/ml) and DNA (2.5 ml, 75 μg/ml) solutions were autoclaved separately before preparation of polyplexes.

2.5. Sterile filtration of polyplexes, polymer, and plasmid

To sterilize polyplexes, at least 3 ml of sample were filtered by hand through 0.22 μm syringe filters (30 mm diameter, cellulose acetate, Schleicher & Schuell), which in some cases were primed with 3 ml sample. Besides polyplexes, polymer (56 μg/ml) and DNA (15 μg/ml) solutions were filtered separately before preparation of polyplexes. The optical density at 225 nm was used as a measure for the polymer concentration before and after sterile filtration.

2.6. Particle size measurements

The *z*-average particle size of the polyplexes was determined by dynamic light scattering at 25°C with a Malvern 4700 system. This system comprises a Model 2013 air-cooled Argon ion laser (75 mW, 488 nm), equipped with a model 2500 remote interface controller (Uniphase) and the PCS version 1.35 software (Malvern, Malvern, UK). The particle size distribution of the colloid dispersion is reported as a polydispersity index (PD), ranging from 0 for an entire monodisperse up to 1 for a completely heterodisperse system.

2.7. Agarose gel electrophoresis

Naked plasmid was analyzed by electrophoresis in a 0.7% agarose gel containing ethidium bromide, as described previously (Cherng et al., 1999b). To analyze plasmid complexed with polymer, the polyplexes were dissociated by incubation with a 100-fold excess of poly(aspartic acid) to DNA (w/w) for at least 24 h (Katayose and Kataoka, 1997; Arigita et al., 1999). DNA present in the gel was visualized by exposure to UV light.

2.8. Circular dichroism measurements

Circular dichroism (CD) spectra were recorded from 330–230 nm at 25°C in quartz cells (path length 1 cm) with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines/mm, blaze wavelength 230 nm), and 1.24-mm slits. Each measurement was the average of at least six repeated scans (step resolution 2 nm, 1 s each step)

Table 1
Recovery and size of sterile polyplexes[a]

Polyplex preparation	Solvent	Recovery (%)[b]	Size (μm)	PD[c]
pDMAEMA				
Non-sterilized	HBS	100 ± 1	0.17 ± 0.01	0.17
Filtered	HBS	66 ± 1[e]	0.16 ± 0.01	0.14
filtered, primed filter	HBS	89 ± 1[e]	0.16 ± 0.01	0.16
Autoclaved	HBS	Aggregation[e]	>2 μm[e]	0.37
Autoclaved polymer[d]	HBS	103 ± 2	0.18 ± 0.01	0.20
Non-sterilized	HB/sucrose	102 ± 1	0.10 ± 0.01[f]	0.12
Autoclaved	HB/sucrose	Aggregation[e]	0.63 ± 0.03[e]	0.12
pDMAEMA-PEG				
Non-sterilized	HBS	100 ± 1	0.16 ± 0.01	0.15
Filtered	HBS	88 ± 1[e]	0.16 ± 0.01	0.07
Filtered, primed Filter	HBS	87 ± 1[e]	0.17 ± 0.01	0.08
Autoclaved	HBS	Aggregation[e]	>1 μm[e]	0.26
Autoclaved polymer[d]	HBS	103 ± 1	0.17 ± 0.01	0.06
Non-sterilized	HB/sucrose	101 ± 1	0.13 ± 0.01[f]	0.12
Autoclaved	HB/sucrose	Aggregation[e]	0.53 ± 0.04[e]	0.62

[a] A polymer/plasmid ratio of 3/1 (w/w) was used. DNA concentration was 15 μg/ml. The data were analyzed by one-way ANOVA corrected for multiple comparison by the Bonferroni method.
[b] Recovery as determined by OD260; 100% represents the OD260 of the untreated samples in HBS.
[c] PD, polydispersity index.
[d] Polymer autoclaved and added to non-treated DNA at a polymer/plasmid ratio of 3/1 (w/w).
[e] Significantly different from non-sterilized polyplexes in the same buffer ($P<0.01$).
[f] Significantly different from non-sterilized polyplexes in HBS ($P<0.01$).

from which the background (polymer in buffer) spectrum was subtracted (Arigita et al., 1999; Cherng et al., 1999a).

2.9. Transfection

Cells were transfected as described previously (Cherng et al., 1996; Van de Wetering et al., 1997). Briefly, 24 h before transfection, cells were seeded at a concentration of 3.0×10^4 cells/cm^2 in 96-well plates. Cells were supplied with fresh medium before transfection and subsequently overlaid with the polyplex dispersion for 1 h at 37°C in a humidified 5% CO_2-containing atmosphere (1 μg plasmid/well). After further culture for about 48 h, one series was tested for β-galactosidase reporter gene expression by using the substrate ONPG (LaPage et al., 1973; Arigita et al., 1999), whereas the second series was used to determine the cytotoxic effect of the polyplexes using an XTT colorimetric assay (Scudiero et al., 1988; Arigita et al., 1999).

2.10. Statistical analysis

The data were analyzed by one-way ANOVA corrected for multiple comparison by the Bonferroni method.

3. Results and discussion

3.1. Autoclaving of polyplexes

Autoclaving is known to destruct naked plasmid (e.g. Brannen, 1970). However, one could hypothesize that the polymer present in the polyplexes might stabilize the DNA structure and thereby prevent high temperature induced degradation. Furthermore, it has been demonstrated that the ester bonds in pDMAEMA are not susceptible towards hydrolysis even under extreme conditions (Van de Wetering et al., 1998b). The physical characteristics of polyplexes dramatically changed during the autoclaving process (Table 1).

Both in HBS and in HB/sucrose severe aggregation was observed visually as well as spectrophotometrically (high optical density at 400 nm). These results were supported by DLS measurements, which showed that large particles were present after autoclaving. Previously it was shown that particles larger than 0.30 μm are hardly or not at all able to transfect cells (Van de Wetering et al., 1997). Also, after autoclaving the DNA could not be dissociated from the polymer anymore. In this respect, autoclaved polyplexes resembled polyplexes aged for several months at 40°C (Cherng et al., 1999b). In that study, it was shown that upon prolonged storage of polyplexes in aqueous solution at elevated temperature, the aggregation of polyplexes was accompanied by a gradual breakdown of the DNA from supercoiled, via open circular and linear DNA to fragmented DNA. Probably in autoclaving a similar process takes place, but much faster.

Remarkably, although aggregation occurred, the size distribution (PD) of pDMAEMA-polyplexes autoclaved in HB/sucrose remained narrow. The CD spectrum of polyplexes autoclaved in HB/sucrose resembled that of non-autoclaved material, although the intensity of the signal was decreased (Fig. 2).

Autoclaving pDMAEMA alone did not change the physical characteristics of polyplexes prepared from untreated DNA with the autoclaved polymer (Table 1). This observation is in agreement with the stability of pDMAEMA towards hydrolysis under extreme conditions reported previously (Van de Wetering et al., 1998b).

The topology and integrity of DNA from autoclaved and non-sterilized polyplexes were studied by agarose gel electrophoresis (Fig. 3). After autoclaving the plasmid could not be dissociated from pDMAEMA (Fig. 3A), even by incubation for 24 h with a 100-fold excess of poly(aspartic acid). The polyplex autoclaved in HBS showed only a light stain near the slot in which it was applied, whereas a very bright stain was observed close to the slot for the polyplex autoclaved in HB/sucrose, indicating the presence of substantial amounts of DNA. For polyplexes prepared with pDMAEMA-PEG in HBS similar results were obtained (Fig. 3B), except that now a bright stain was observed for both HBS and HB/sucrose. Apparently, both sucrose and PEG protect the DNA present in pDMAEMA/DNA complexes to some extent from degradation during autoclaving.

During autoclaving of plasmid alone, severe degradation occurred, as was detected by agarose gel electrophoresis after staining the gel with SYBR Green I. This staining method is more sensitive that ethidium bromide (not illustrated).

Table 2 shows the relative in vitro transfection efficiency and relative cell viability of pDMAEMA-based polyplexes. In line with what could be expected from the electrophoresis results,

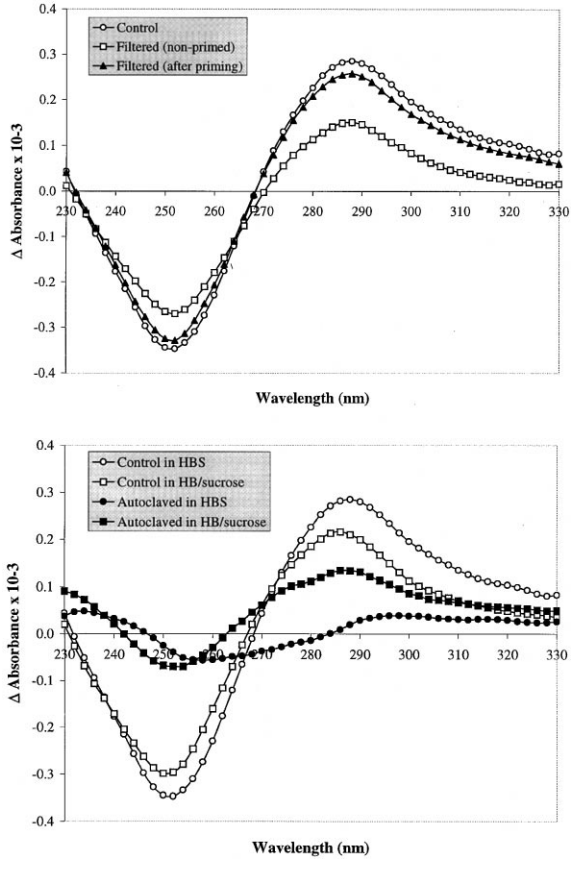

Fig. 2. Effect of autoclaving on circular dichroism (CD) spectra of polyplexes. Typical spectra of poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA)-plasmid polyplexes (3/1 w/w ratio) in HEPES buffered saline (HBS) or HB/sucrose before and after autoclaving. DNA concentration was 15 μg/ml before sterile filtration. The spectra are non corrected for the DNA-concentration.

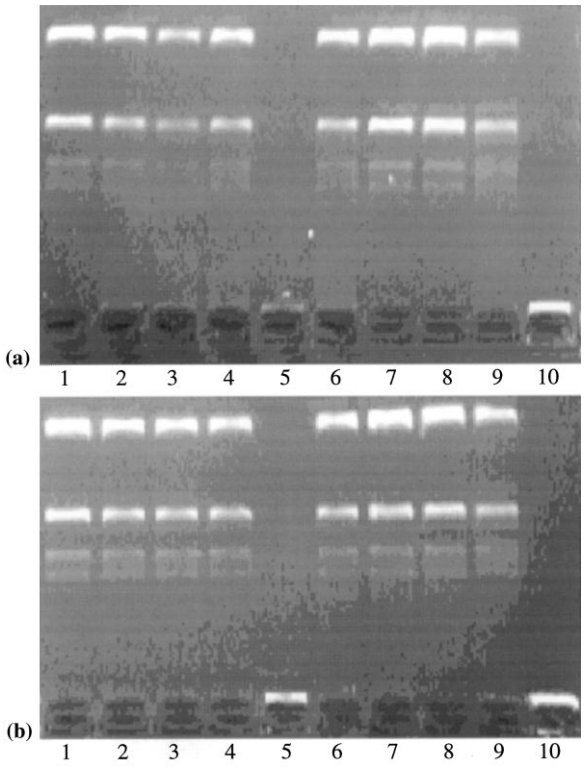

Fig. 3. Agarose gel electrophoresis of sterile and non-sterilized polyplexes. Polyplexes were incubated for 24 h at ambient temperature with a 100-fold excess of poly(aspartic acid) (w/w) prior to electrophoresis. (a) poly(2-(dimethylamino) ethyl methacrylate) (pDMAEMA); (b): pDMAEMA-PEG. Lanes 1 and 7, free plasmid in HEPES buffered saline (HBS); lane 8, free plasmid in HB/sucrose; lanes 2 and 9, non-sterilized polyplexes in HBS and HB/sucrose respectively; lane 3, filtered polyplexes; lane 4, polyplexes filtered with polyplex-primed filter; lanes 5 and 10, polyplexes autoclaved in HBS and HB/sucrose respectively; lane 6, polyplexes prepared with autoclaved polymer.

autoclaving of the polyplexes resulted in total loss of transfection capacity, both in COS-7 and OVCAR-3 cells. After autoclaving, polyplexes prepared from pDMAEMA-PEG were unable to transfect cells as well. Autoclaving of the polyplexes did not significantly alter the viability of cells incubated with these polyplexes.

Transfection and viability results obtained with polyplexes, prepared from autoclaved polymer and non-autoclaved plasmid, were comparable to those found for non-sterilized polyplexes (Table 2).

3.2. Sterile filtration of polyplexes

Sterile filtration of pDMAEMA polyplexes did not significantly decrease their size, as shown by dynamic light scattering measurements (Table 1), but it affected the concentration of pDMAEMA polyplexes. For pDMAEMA/DNA the optical density at 260 nm decreased by 35% by the sterile filtration procedure. The reduction in DNA concentration might be explained by retention of particles with a size larger than the cut-off of the filter combined with non-specific binding of polyplexes to the surface of the filter material. A similar drop in the intensity of the CD spectrum as well as in the number of photon counts in the DLS measurement supports the 35% reduction in DNA concentration. By priming the filter with 3 ml polyplex prior to use, the polyplex recovery was increased to 90%. Thus, the best way to avoid product loss is to filter larger batches of polyplexes and discard the first fraction of filtrate. Recovery using cellulose acetate filters from Sartorius (Minisart), Acrodisc PF 0.8/0.2 Supor or Acrodisc 0.2 LPB (Gelman Sciences) did not significantly differ from the regularly used filters (73 ± 3, 69 ± 2 and 66 ± 3, respectively, for pDMAEMA-polyplexes).

Sterile filtration did not significantly influence the size of pDMAEMA-PEG polyplexes. Also, the recovery of the material during sterile filtration (no priming) was substantially higher than for pDMAEMA polyplexes (90%), which probably results from a decrease in non-specific binding of the polymer to the cellulose acetate surface of the filter, due to the shielding effect of PEG. For pDMAEMA-PEG/DNA priming of the filter with polyplex did not significantly influence the DNA concentration in the filtrate.

Pre-coating of the filter with free polymer will also prevent material loss. However, in this case the filter should be washed after priming in order to remove free polymer from the filter, which may result in increased toxicity of the filtered polyplex preparation (Van de Wetering et al., 1997).

During sterile filtration of polymers only, virtually no material was lost, as determined by UV, using OD 225 as a measure for the polymer concentration (Table 3). No significant difference

Table 2
Relative transfection efficiency[a] and relative cell viability[b] for sterile polyplexes[c]

Polyplex preparation	Solvent	COS 7		OVCAR 3	
		Transfection	Viability	Transfection	Viability
pDMAEMA					
Non-sterilized	HBS	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
Filtered	HBS	0.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
Filtered, primed filter	HBS	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
Autoclaved	HBS	0.0 ± 0.0[e]	0.9 ± 0.1	0.0 ± 0.0[e]	0.8 ± 0.2
Autoclaved polymer[d]	HBS	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
Non-sterilized	HB/sucrose	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.6 ± 0.1[f]
Autoclaved	HB/sucrose	0.0 ± 0.0[e]	0.9 ± 0.1	0.0 ± 0.0[e]	0.7 ± 0.2

[a] Transfection values normalized (per cell type) to the maximum number of transfected cells after incubation with non-filtered pDMAEMA/DNA 3/1 (w/w) polyplexes prepared in HBS.
[b] Cell viability was normalized to the number of viable cells not treated with polyplexes.
[c] A polymer/plasmid ratio of 3/1 (w/w) was used. DNA concentration was 15 μg/ml. $n = 3–6 \pm$ S.D. The data were analyzed by one-way ANOVA corrected for multiple comparison by the Bonferroni method.
[d] Polymer autoclaved and added to non-treated DNA at a polymer/plasmid ratio of 3/1 (w/w).
[e] Significantly different from non-sterilized polyplexes in the same buffer ($P < 0.01$).
[f] Significantly different from non-sterilized polyplexes in HBS ($P < 0.01$).

was observed between the various filters mentioned above (data not shown). Also for the DNA, the loss during sterile filtration (measured by OD 260 nm) is small ($91 \pm 4\%$ recovery).

The topology and integrity of DNA in filtered and non-sterilized polyplexes were studied by agarose gel electrophoresis after dissociation of the polyplexes with a 100-fold excess of poly(aspartic acid). Results for polyplexes prepared with pDMAEMA are shown in Fig. 3A. After 24 h incubation of polyplexes with poly(aspartic acid), the dissociation was almost complete. Plasmids dissociated from non-sterilized polyplexes as well as from filtered polyplexes showed a DNA pattern similar to that of naked plasmid, although the intensity of the DNA bands from the filtered polyplexes was lower, as was expected from the decreased concentration in the filtrate. For polyplexes prepared with pDMAEMA-PEG similar results were found with the exception that no apparent decrease of band intensity due to sterile filtration was observed (Fig. 3B).

Sterile filtration of DNA alone did not affect the topology of the DNA (Fig. 4). This means that shear forces applied on the plasmid are not causing conversion into other topological forms.

The influence of sterile filtration on the transfection efficiency and the cell viability was evaluated in a tissue culture system in the presence of serum. Table 2 shows the relative in vitro transfection efficiency and relative cell viability of the polyplexes. Both in COS-7 and OVCAR-3 cells, sterile filtration of pDMAEMA polyplexes resulted in a substantial decrease in transfection efficiency of 20–30%, which can be explained by the observed decrease in DNA concentration. As expected, priming the filter with polyplex almost fully prevented this reduction in transfection efficiency. The same trends were observed for pDMAEMA-PEG.

Polyplexes prepared from separately filtered polymer and DNA showed a relative transfection efficiency (1.00 ± 0.05) and a relative viability

Table 3
Effect of sterile filtration on concentration of polymer and DNA[a]

pDMAEMA	$100 \pm 1\%$
pDMAEMA-PEG	$97 \pm 1\%$
plasmid	$91 \pm 4\%$

[a] Polymer was filtered at 5 mg/ml, plasmid at 10 μg/ml, using a 0.22 μm filter.

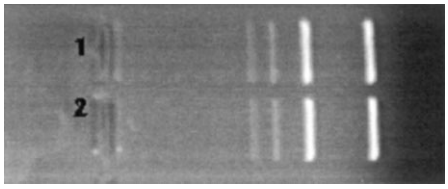

Fig. 4. Agarose gel electrophoresis showing naked DNA before (1) and after (2) sterile filtration.

(0.64 ± 0.06) comparable to non-sterilized polyplexes (0.98 ± 0.10 respectively 0.65 ± 0.05).

4. Conclusions

Sterilization of complexes between pDMAEMA-based cationic polymeric gene transfer agents and DNA plasmid is feasible by sterile filtration. Depending on the filter type used, the filtered volume should be high enough, to prevent substantial material loss. A pre-filter with a larger pore-size may prevent occlusion of the filter in cases where relatively large quantities are filtered. Autoclaving of polyplexes results in severe aggregation, extensive degradation and complete loss of transfectivity.

Separate sterilization of the polymer by autoclaving or filtration and DNA by filtration offers a good alternative to filtration of the formed polyplexes.

Acknowledgements

This research has been supported by the European Union (PL 97 0002). Furthermore, we thank Martha M.G. van Herwaarden for autoclaving the samples. We thank Dr W.N.E. van Dijk-Wolthuis for synthesizing the pDMAEMA-PEG.

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