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Evaluation of the stability of polymer-based plasmid DNA delivery systems after ultrasound exposure

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Abstract

Under ultrasound exposure, the stability of plasmid DNA protected by polymer-based gene delivery system is an important factor for achieving optimal transfection into cells. We have evaluated the effectiveness of various polymer-based plasmid DNA delivery systems, which are interactive polymers and cationic polymers, to avoid shear degradation induced by ultrasound exposure. Alternatively, it is shown that sonication of plasmid DNA for exposure time as low as 10 s resulted in total DNA fragmentation and the loss of transfection potency in NIH/3T3 cells. Among these polymer-based plasmid DNA delivery systems, only cationic polymers had the ability to provide the protection of plasmid DNA from ultrasonic degradation as indicated by the reservation in supercoiled circular (SC) and open circular (OC) forms of plasmid DNA on the agarose gel electrophoresis. The DNA stability protected by cationic polymers decreased after ultrasound exposure in 1 M sodium chloride solution. Also, higher molecular weight of cationic polymers and sufficient cationic polymer/DNA weight ratios are essential to prevent DNA from degradation under ultrasound exposure in aqueous or salt solution. These results suggest that the protective mechanism by cationic polymers is due to the attractive bonding between cationic polymer and negative plasmid DNA. Whereas, DNA condensation alone provoked by the addition of polyethylene glycols was not sufficient to resist the DNA fragmentation induced by ultrasound exposure.

Keywords: DNA; Stability; Ultrasound; Polymer-based plasmid DNA delivery systems; Gel electrophoresis

1. Introduction

Gene therapy, based on non-viral vectors, has recently been acknowledged as an attractive alternative for overcoming some of the disadvantages associated with viral vectors (Ledley, 1995; Felgner et al., 1997). Among non-viral vectors used so far, polymers are one of the more promising delivery vehicles for non-viral gene therapy applications (Tang and Szoka, 1997; Gebhart and Kabanov, 2001). Two approaches of polymers for gene delivery are commonly employed. One strategy is to form condensed complexes by mixing negatively charged DNA with cationic polymers in aqueous medium (De Smedt et al., 2000). Plasmid DNA in complexed form is protected from enzymatic degradation, thus increasing the efficiency of gene delivery within a cell (Bielinska et al., 1997). Another approach is to apply interactive polymers,

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which do not offer DNA condensation, for sustained gene expression to muscle (Mumper et al., 1996).

In order to obtain optimal gene expression, plasmid DNA has to retain its supercoiled circular (SC) and open circular (OC) forms (Kimoto and Taketo, 1996). Therefore, polymer-based delivery systems must provide the protection of plasmid DNA from physical, chemical, and enzymatic degradation. The degradative mechanisms of plasmid DNA occur in any aqueous solution near neutral pH by the two-step process of depurination and β-elimination, leading to cleavage of the phosphodiester backbone (Suzuki et al., 1994). When a delivery gene system is fabricated, the sensitivity of plasmid DNA especial to shear degradation must be taken into serious consideration. The introduction of structural changes by shear stress will eventually convert SC plasmid DNA to the OC, linear, or even fragmented DNA (Levy et al., 1999).

Ultrasound has been used for dispersing colloidal gene delivery systems such as cationic liposomes (Eastman et al., 1997). Early studies also demonstrated that ultrasound can be used as an effective mean for delivering plasmid DNA into cells (Lawrie et al., 2000; Wyber et al., 1997; Greenleaf et al., 1998). However, shear stress generated by ultrasound apparently cause major structural damage to plasmid DNA (Elsner and Lindblad, 1989). The stability profile of plasmid DNA in peptide and cationic lipid-based gene delivery systems after ultrasound exposure has previously been reported (Wasan et al., 1996; Adami et al., 1998; Mckenzie et al., 2000). However, due to tight bonding between plasmid DNA and peptide or cationic lipid, the direct analysis of plasmid DNA stability is difficult. For cationic lipid gene delivery systems, the stability of plasmid DNA was only analyzed by low-resolution gel permeation chromatography (Wasan et al., 1996). As for peptide-based gene delivery systems, the direct analysis of plasmid DNA condensates after ultrasonication was partially interfered or resolved even by trypsin digestion of peptides on the gel electrophoresis (Adami et al., 1998).

Thus, it is of interest to develop and evaluate the stability profile of polymer-based plasmid DNA delivery systems after ultrasound exposure. In this text, we have studied the effectiveness of various polymer-based gene delivery systems in order to obtain stable plasmid DNA to avoid shear degradation by ultrasound exposure. For cationic polymer gene delivery systems, direct analysis of plasmid DNA stability by gel electrophoresis has been accomplished via polyaspartic acid incubation. Excess oppositely charged polyelectrolytes such as polyaspartic acid have tended to disassemble cationic polymer–DNA complexes by displacing DNA (Trubetskoy et al., 1999). These stability properties help to achieve optimal gene expression from polymer-based gene delivery systems after ultrasound exposure.

2. Materials and methods

2.1. Plasmid DNA

The plasmid (pSG5*lacZ*), which encodes the *lacZ* gene for the β-galactosidase, was driven by a SV40 promoter to assess gene expression and provided by Dr. H.-S. Liu (Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC). Plasmid DNA was amplified in *E. Coli* and purified by column chromatography (QIAGEN-Mega kit, The Netherlands). The purity of plasmid DNA was established by UV spectroscopy (E260/E280 nm ratio ranging from 1.87 to 1.89 were used). Agarose (0.7%) gel electrophoresis analysis using restriction enzymes showed that plasmid DNA was mainly in the supercoiled form and one band corresponding to a size of 8 kb was visible.

2.2. Chemicals

Polyaspartic acid, polyvinylpyrrolidone (PVP, Mw = 10 kDa), poly-L-lysine (PLL) hydrobromide (Mw = 500–2000 Da, PLLL), PLL hydrobromide (Mw = 70–150 kDa, PLLM), PLL hydrobromide (Mw > 300 kDa, PLLH), and poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (Pluronic F-68, F127) were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Polyehylene glycol (PEG) 200, 1000, 6000, and 20,000 were purchased from Fluka Chemie GmbH (Switzerland). Polyethyleneimine (PEI, Mw = 800 kDa) was obtained from Sigma as 50% (w/v) solution. The PEI solutions were adjusted to desired aqueous concentrations and neutralized (final pH = 7.0) with HCl.

2.3. Preparation of polymer-based DNA delivery systems and ultrasound exposure

For cationic polymer-based delivery systems (PEI and PLL), the plasmid DNA (20 μg) and the appropriate amount of cationic polymers were each diluted into 150 μl of sterile ddH_2O or 1 M NaCl aqueous solution. Cationic polymers (75 μl) was added to plasmid DNA solution and allowed to incubate at room temperature. After 10 min, the resulting solution was vortexed for 30 s and then spun down. The resulting solution was sat for 30 min and then the rest of 75 μl of cationic polymers was added to the resulting solution following the above described preparations. For interactive polymers and polyethylene glycols, 20 μg of plasmid DNA was added into the desired weight concentration of polymer solution and the final volume of the resulting solution was 300 μl. Ultrasonication was applied using a 600 W probe-type homogenizer (VCX 600, Sonics and Materials Inc., Connecticut, USA) with a frequency of 20 kHz. The 1.5 ml microfuge tube containing 300 μl of sample was placed in an ice bath vessel to avoid thermal effects of ultrasound exposure. An ultrasound probe was inserted directly into the samples and operated at vibrational amplitude of 20% in a continuous mode. The sonication time for polymer-based DNA delivery systems was fixed at 1 min and the probe tip was cleaned between samples by sterile ddH_2O.

2.4. Agarose gel electrophoresis

After ultrasound exposure, the resulting samples (10 + 2 μl Type IV loading buffer/well) were then loaded onto a 0.7% agarose gel containing ethidium bromide in TAE buffer (pH = 8.0) and electrophoresed at 100 V for 1 h. After electrophoresis, DNA was examined by UV-irradiation. For cationic polymers, polyaspartic acid solution (25 mg/ml) was used to dissociate the cationic polymer–DNA complexes at ambient temperature. The resulting compound was incubated for 24 h and then analyzed by gel electrophoresis.

2.5. In vitro transfection

NIH/3T3 cells (mouse fibroblast, kindly provided by Dr. H.-S. Liu, Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC) were cultured in Dulbecco's modified Eagle medium (DMEM, high glucose, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA) and 100 U/ml penicillin/100 μg/ml streptomycin (Sigma, USA). Cells were seeded into 24-well cell culture plates at a density of 3×10^4 cells/well and grown overnight (60–75% confluence) at 37 °C and 5% CO_2. Immediately prior to transfection, cells were rinsed with PBS and supplemented with 1 ml fresh DMEM per well. The plasmid DNA (2 μg) without/with ultrasound exposure and PEI (10 μg) were each diluted into 50 μl of DMEM solution. PEI (25 μl) was added to plasmid DNA solution and allowed to incubate at room temperature. After 10 min, the resulting solution was vortexed for 30 s and then spun down. The resulting solution was sat for 30 min and then the rest of 25 μl of PEI was added to the resulting solution following the above described preparations. After 10 min, cells were exposed to transfection mixtures for 2 h and then supplemented with 10% FBS and 1% antibiotics. β-Galactosidase gene expression was analyzed 24 h later by using combined β-Gal Assay kit (Invitrogen, USA) and BCA Protein Assay Reagent Kit (Pierce, USA). Results were reported as specific activity ± S.D. for triplicate samples.

3. Results and discussion

To circumvent the stability issue raised by shear stress, we first studied the influence of ultrasound on the structure changes of plasmid DNA (Fig. 1). Without ultrasonication, plasmid DNA exists as a mixture of both SC and OC forms on the agarose gel electrophoresis (Fig. 1, lane 1). Sonication of plasmid DNA for exposure time as low as from 1 to 5 s resulted in some DNA fragmentation (Fig. 1, lanes 2–4), although some SC and OC forms still presented on the agarose gel electrophoresis. Furthermore, only fragments of plasmid DNA were revealed from 10 to 60 s, as shown in Fig. 1, lanes 5–10. The observation is consistent with the previous studies that the induced shear stress by ultrasound may result in the structural degradation of plasmid DNA (Elsner and Lindblad, 1989; Adami et al., 1998).

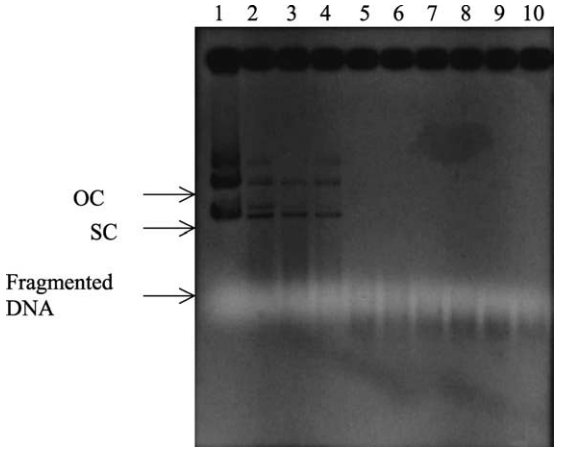

Fig. 1. Agarose gel electrophophoresis of sonicated plasmid DNA at various exposure times. Lane 1: control plasmid DNA (without sonication); lanes 2–10: sonicated plasmid DNA at exposure times of 1, 3, 5, 10, 20, 30, 40, 50, and 60 s, respectively. The band position for SC, OC, and fragmented DNA is indicated with straight lines.

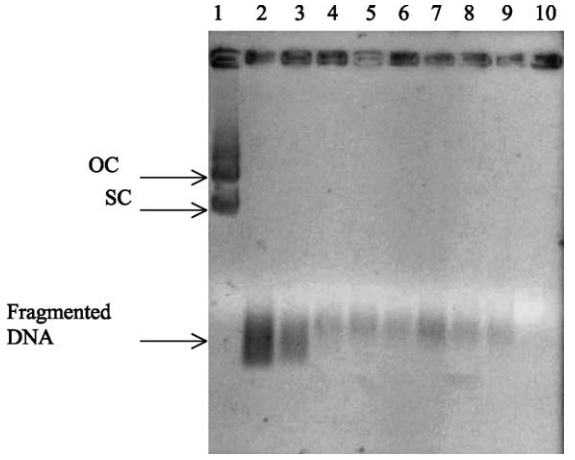

Fig. 3. Agarose gel electrophophoresis of plasmid DNA under the protection of interactive polymers after ultrasound exposure for 60 s. Lane 1: control plasmid DNA (without sonication); lanes 2–6: sonicated plasmid DNA in 10, 20, 30, 40, and 50% PVP solutions, respectively; lanes 7 and 8: sonicated plasmid DNA in 1 and 10% Pluronic F-68 solutions; lanes 9 and 10: sonicated plasmid DNA in 1 and 10% Pluronic F-127 solutions.

We then investigated the effect of topological forms of DNA on the in vitro transfection efficiency. Plasmid DNA under ultrasound at various exposure times was formed complexes with PEI and then used to transfect NIH/3T3 cells (Fig. 2). The results demonstrated no change in transfection efficiency for sonicated plasmid DNA from 1 to 5 s relative to unsonicated DNA. Whereas plasmid DNA with ultrasound exposure from 10 to 60 s showed negligible specific activity of β-galactosidase. As previously reported, the transfection efficiency in several cell lines was much higher for SC/OC plasmid DNA than for linearized plasmid DNA (Xie and Tsong, 1993; Adami et al., 1998). The much decreased transfection efficiency of linearized plasmid DNA was due to its instability in the host cell, where linearized plasmid DNA was rapidly degraded by the enzyme (Xie et al., 1992). Indeed, treatment of plasmid DNA with ultrasound from 1 to 5 s remained some SC/OC forms from the agarose gel and maintained the ability to transfect NIH/3T3 cells after complexation with PEI. In contrast, fragmented DNA by treatment with sonication from 10 to 60 s resulted in the absence of gene transfer for NIH/3T3 cells.

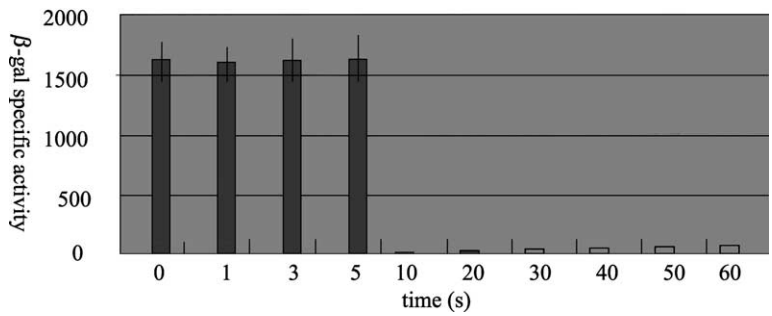

Fig. 2. β-Galactosidase gene expression levels following in vitro transfection of NIH3T3 cells with either control plasmid DNA (without sonication)/PEI complexes or sonicated plasmid DNA (1, 3, 5, 10, 20, 30, 40, 50, and 60 s, respectively)/PEI complexes. The data are shown as means ± S.D. ($n = 3$).

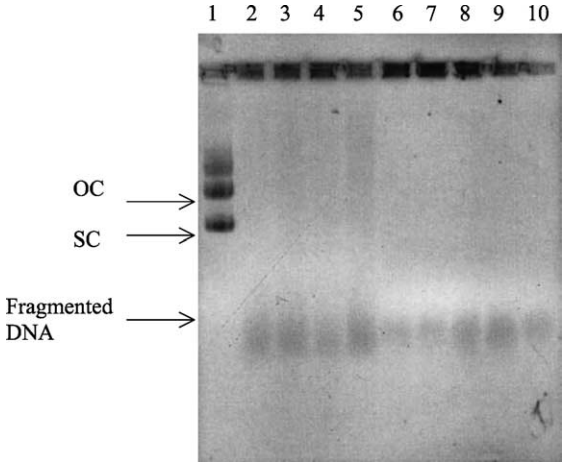

Fig. 4. Agarose gel electrophophoresis of plasmid DNA under the protection of PEGs after ultrasound exposure. Lane 1: control plasmid DNA (without sonication); lanes 2 and 3: sonicated plasmid DNA in 40 and 50% PEG 200 solutions; lanes 4 and 5: sonicated plasmid DNA in 40 and 50% PEG 1000 solutions; lanes 6 and 7: sonicated plasmid DNA in 40 and 50% PEG 6000 solutions; lanes 8–10: sonicated plasmid DNA in 30, 40, and 50% PEG 20,000 solutions, respectively.

Since polymer-based delivery systems are necessary to provide the protection of plasmid DNA from ultrasonic degradation and maintain optimal gene expression, we then analyzed the stability profile of interactive polymer-based DNA delivery systems after ultrasound exposure. Interactive polymers such as PVP and poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide), have been reported to increase plasmid DNA bioavailability to muscle or ocular tissues (Mumper et al., 1996; Lemieux et al., 2000; Liaw et al., 2001). Fig. 3 shows the effect of interactive polymers on the stability of plasmid DNA after ultrasound exposure for exposure time 60 s. A similar profile of fragmented DNA was observed on the agarose gel electrophoresis for various concentration conditions. This evidence demonstrates that the protective effect of interactive polymers was not sufficient to protect the plasmid DNA from structural change. Therefore, the attempt to minimize damage to DNA by ultrasound may be to introduce stronger interactions between DNA and polymers in the solution.

Published evidence suggests that the stabilization afforded by condensation with a peptide or a cationic lipid protects plasmid DNA after ultrasound exposure (Wasan et al., 1996; Adami et al., 1998). To examine whether DNA condensation alone could stabilize the plasmid DNA during sonication, PEG at various molecular weight was used to verify this hypothesis. Accordingly, DNA condensation by neutral crowding polymers such as PEGs, through an excluded volume mechanism, may contribute to the minimized DNA

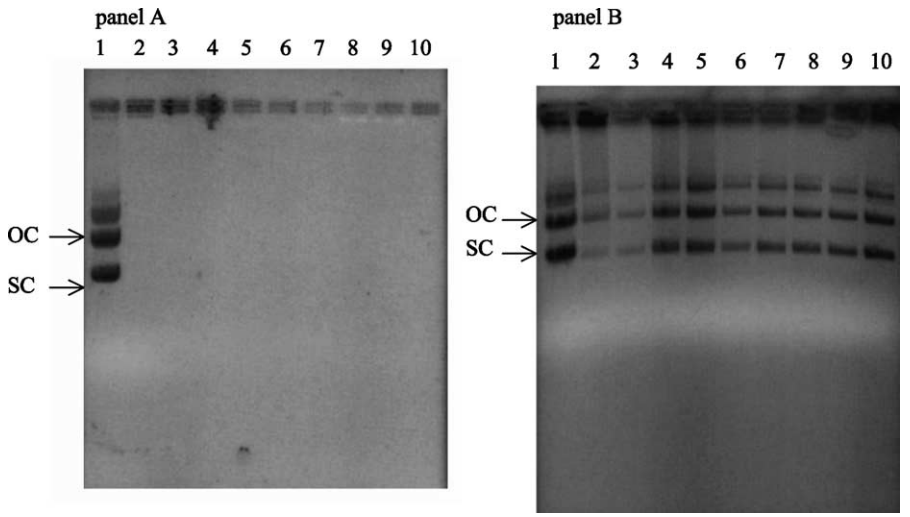

Fig. 5. The stability of PEI-based plasmid DNA delivery systems after ultrasound exposure. Panels A and B: PEI/DNA complexes prepared in aqueous solutions and treated with excess polyaspartic acid; plasmid DNA without sonication was analyzed in lane 1 for panels A and B. Lanes 2–10 in above panels: PEI/DNA weight ratios at 1/10, 3/10, 1/2, 3/4, 1/1, 3/2, 2/1, 5/2, and 3/1, respectively.

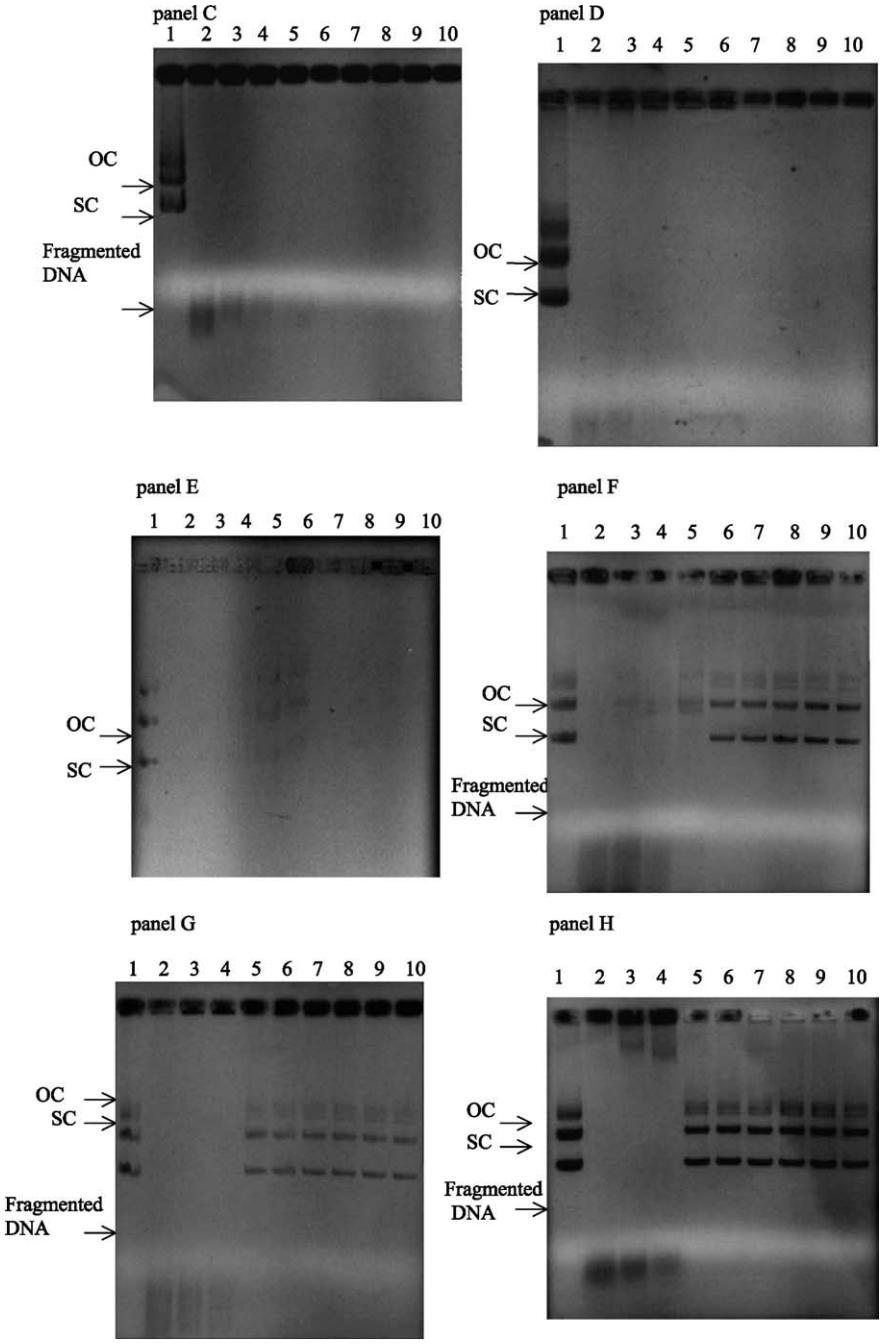

Fig. 6. The effectiveness of PLL with different molecular weight on the DNA stability after ultrasound exposure. Panels C–E: PLLL/DNA, PLLM/DNA, and PLLH/DNA complexes prepared in aqueous solutions, respectively. Panels F–H: relative to panels C–E with polyaspartic acid dissociation. Plasmid DNA without sonication was analyzed in lane 1 for panels C–H. Lanes 2–10 in above panels: cationic polymer (PLLL, PLLM, and PLLH)/DNA weight ratios at 1/10, 3/10, 1/2, 3/4, 1/1, 3/2, 2/1, 5/2, and 3/1, respectively.

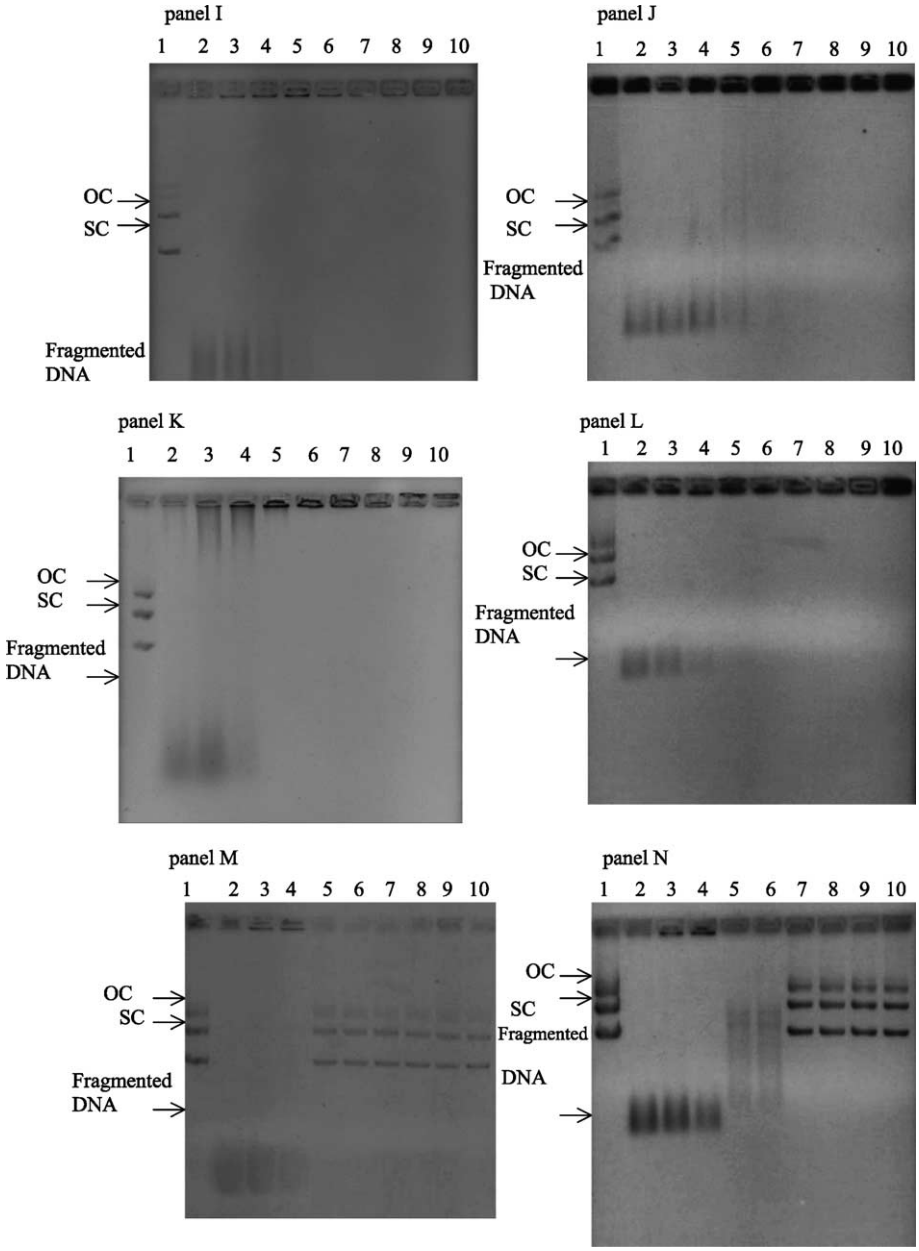

Fig. 7. The protective effectiveness of cationic polymers in the presence of 1 M sodium chloride solution on the DNA stability after ultrasound exposure. Panels I–L: PEI/DNA, PLLL/DNA, PLLM/DNA, and PLLH/DNA complexes prepared in 1 M sodium chloride solutions, respectively. Panels M–P: relative to panels I–M with polyaspartic acid dissociation. Plasmid DNA without sonication was analyzed in lane 1 for panels I–P. Lanes 2–10 in above panels: cationic polymer/DNA weight ratios at 1/10, 3/10, 1/2, 3/4, 1/1, 3/2, 2/1, 5/2, and 3/1, respectively.

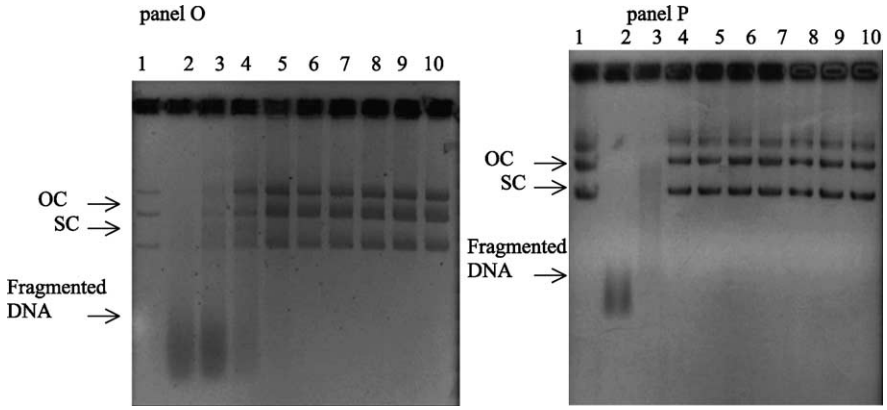

Fig. 7. (*Continued*).

damage by shear stress (Vasilevskaya et al., 1995; Bloomfield, 1996). Results in Fig. 4 show that plasmid DNA protected by PEGs still underwent a complete fragmentation after ultrasound exposure. This evidence demonstrates that the dramatic decrease in plasmid DNA volume provoked by PEG was not sufficient to resist the DNA fragmentation induced by ultrasound exposure.

In addition to DNA condensation, cationic polymer-based gene delivery systems also involve in the attractive bonding between cationic polymer and negative plasmid DNA and this forces may provide the ability to withstand shear stress generated by ultrasound exposure. A number of synthetic cationic polymers such as PEI and PLL have been successfully used to delivery gene into a variety of cell lines (De Smedt et al., 2000). Thus, we selected these cationic polymers as the typical examples to study the effectiveness on the protection of plasmid DNA from structural alternations by sonication. For PEI/DNA complexes, no band was revealed in the agarose gel electrophoresis after ultrasound exposure in aqueous solution from PEI/DNA weight ratios 1/10–3/1 (Fig. 5, panel A). To distinguish DNA from the resulting complexes in the agarose gel, the resulting complex solution was treated with excess polyaspartic acid to dissociate cationic polymers such as PEI and the results were shown in Fig. 5 (panel B). Plasmid DNA in aqueous solution after dissociation remained in SC and OC forms as compared with standard DNA (panel B, lane 1) and no DNA fragmentation was observed. This demonstrates that the DNA condensation utilizing PEI may provide a way to minimize structural damage by ultrasound exposure. These results are in agreement with previous study by peptide/DNA condensates (Adami et al., 1998).

The differences between cationic polymers on the protective DNA stability after sonication may be due to the structural characteristics such as molecular weight, of cationic polymers. To confirm this hypothesis, we selected PLL with different molecular weight to compare the effectiveness on the DNA stability after ultrasound exposure and the results are shown in Fig. 6. Likewise, PLL with high and medium molecular weight still owns the binding ability to DNA in aqueous solution and no band was detected in the agarose gel after ultrasound exposure in aqueous solution at all PLL/DNA weight ratios (Fig. 6, panels D and E). In contrast, bands of fragmented DNA were observed on the agarose gel electrophoresis under the protection of PLL with low molecular weight at lower PLL/DNA weight ratios (lanes 2–5 in Fig. 6, panel C). After polyaspartic acid dissociation, PLL with low molecular weight failed to maintain DNA in SC and OC forms at PLL/DNA weight ratios (1/10, 3/10, 1/2, and 3/4) as indicated by fragmented DNA whereas plasmid DNA remained protected at higher PLL/DNA weight ratios (1/1, 3/2, 2/1, 5/2, and 3/1) as shown in Fig. 6, panel F. The plasmid DNA stability profile protected by PLL with medium and high molecular weight is identical to PLL with low molecular weight except that DNA fragmentation was shifted to lower PLL/DNA weight ratios (Fig. 6, panels G and H). These evidences support the hy-

pothesis that plasmid DNA are more stable under the protection of cationic polymer with higher molecular weight after ultrasound exposure. Also, the results indicate that the addition of more cationic polymers may resist DNA degradation induced by sonication.

It has been well known that electrostatic forces between cationic substances and DNA decrease when the ionic strength is increased (Schindler and Nordmeier, 1997). In the presence of salt, the attractive and repulsive forces of cationic polymer–DNA complexes were screened by the counterions (Wilson and Bloomfield, 1979; Flock et al., 1995). To circumvent the stability issue raised by ionic strength, we compared the protective effectiveness of cationic polymers in the presence of 1 M sodium chloride solution on the DNA stability after ultrasound exposure and the results are shown in Fig. 7. Even without polyaspartic acid dissociation, the fragmented DNA was clearly observed at lower cationic polymer/DNA weight ratios (Fig. 7, panels I–L). Whereas, except PLLL at lower cationic polymer/DNA weight ratios, no band was detected in the agarose gel after ultrasound exposure in aqueous solution (Figs. 5 and 6). Apparently, the binding between cationic polymer and plasmid DNA was disrupted by raising the ionic strength of the solution and the DNA stability decreased after ultrasound exposure in 1 M sodium chloride solution. However, after polyaspartic acid dissociation (Fig. 7, panels M–P), plasmid DNA remained in SC and OC forms in 1 M sodium chloride solution at higher cationic polymer/DNA weight ratios. The plasmid DNA remains protected by cationic polymers under ultrasound exposure at these higher cationic polymer/DNA weight ratios. Also, plasmid DNA are more stable in 1 M sodium chloride solution under the protection of PLL with higher molecular weight after ultrasound exposure (Fig. 7, panels N and P). These results indicate that electrostatic forces between cationic polymers and plasmid DNA along with molecular weight of cationic polymers play an important role for the stability of plasmid DNA under ultrasound exposure.

In an effort to understand the protective mechanism by polymers, our results demonstrated that the interactions between polymer and plasmid DNA is critical to withstand the shear stress generated by high power ultrasonication. Interactive polymers such as PVP and poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) only provided weak interactions with DNA and failed to stop DNA from degradation under ultrasound exposure. Treatment of plasmid DNA with neutral crowding polymers such as PEG-induced DNA condensation and this kind of interactions with DNA was not sufficient to resist the DNA degradation by ultrasound exposure. In addition to condensation, cationic polymers also generate electrostatic forces with the negatively charged larger piece of DNA and are effective to provide the DNA stability under sonication. Higher molecular weight of cationic polymers and sufficient cationic polymer/DNA weight ratios are essential to prevent DNA from degradation under ultrasound exposure in aqueous or salt solution. From these stability studies, cationic polymer-based gene delivery systems open up opportunities in using ultrasound as a means to reduce particle size or facilitate the transfer of plasmid DNA into cells while still preserving plasmid DNA integrity.

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