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Remnant cationic dendrimers block RNA migration in electrophoresis after monophasic lysis

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Abstract

Cationic dendrimers such as poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) have attractive characteristics for the delivery of nucleic acid and various biomedical applications. Most studies have focused on cationic dendrimer-based intracellular delivery, and very few studies have focused on the non-specific interaction of remnant cationic dendrimers with total RNA after isolation directly from cells *in vitro*. We examined RNA isolation using the common method of monophasic lysis from human macrophage-like cells (U937) and mouse fibroblast cells (NIH/3T3) that had been exposed to dendrimers and DNA/dendrimer complexes using gel electrophoresis. We found that PAMAM and PPI dendrimers strongly altered the mobility of RNA in the gels. In addition, the extent of dendrimer-induced alteration in RNA mobility was directly dendrimer-generation-dependent: the alteration was greater with higher-generation dendrimers. We also found that DNA/dendrimer complexes at higher dendrimer to DNA ratios interacted with RNA after isolation while gene expression was maintained. The interactions between RNA and remnant dendrimers after isolation were caused by electrostatic bindings, and we recovered total RNA using high ionic strength solvents (2 M NaCl solution) to disrupt the electrostatic forces binding dendrimers to RNA. Because RNA isolation is routinely used for biological applications, such dendrimer-induced alteration in RNA mobility should be accounted for in the further processing of RNA-related applications.

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Keywords: Cationic dendrimer; RNA isolation; DNA/dendrimer complexes; Monophasic lysis; Gel electrophoresis

1. Introduction

Dendrimers are a family of highly branched polymers with radial symmetry characterized by their unique structures and properties, such as dense end groups at the surface, a spherical shape in solution, biocompatibility, and the precise control of size (Boas and Heegaard, 2004; Esfand and Tomalia, 2001; Svenson and Tomalia, 2005). These attractive nano-sized architectures of dendrimers make them suitable for targeted delivery systems, for example, delivery of DNA and oligonucleotides into eukaryotic cells, solubilization of sparingly soluble drugs, nano-carriers for transepithelial transport, and platforms for cancer therapeutics (Cloninger, 2002; Dennig and Duncan, 2002; Kitchens et al., 2005; Kobayashi and Brechbiel, 2005). One of the most exciting research fields has been that of gene delivery

using cationic dendrimers such as poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) (Dufes et al., 2005). Cationic dendrimers interact with anionic DNA to form highly condensed complexes for increased binding to the cell surface, enter the cell via endocytosis, protect DNA from enzyme degradation, and lead to efficient gene expression (Ruponen et al., 2003).

Because most cationic dendrimers used are non-biodegradable, their non-specific binding to anionic molecules such as RNA may occur during or after intracellular trafficking. Cationic dendrimers interact with anionic synthesized RNA as well as deliver RNA molecules into cells (Bielinska et al., 1996; Wu et al., 2005; Zhao et al., 2004). RNA has been regarded as a highly informative molecule involved in diverse cellular processes such as transcriptional regulation, post-transcriptional RNA processing, retroviral replication, and protein synthesis, among others (Bielinska et al., 1996). The isolation and purification of total RNA are basic methods for a variety of biomedical applications and analyses, and

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obtaining high quality intact RNA is important because this is the first and the most crucial step in performing fundamental molecular biology research. While most efforts have focused on cationic dendrimer-based intracellular delivery, few studies have focused on the interaction of remaining dendrimers with total RNA after isolation directly from cells *in vitro*. Among the methods of isolation and purification of total RNA from cells, monophasic lysis is the most frequently used method that allows the simultaneous recovery of RNA, DNA, and protein from cultured cells. The monophasic solution composed primarily of guanidine isothiocyanate, surfactant, and phenol has lysed cells and inhibited RNase. RNA in the aqueous supernatant was then separated from DNA and protein by adding chloroform, and recovered using precipitation with isopropyl alcohol (Sambrook et al., 2001).

We hypothesize that non-specific interactions between RNA and remnant dendrimers may interfere with the RNA purification process from cells treated with cationic dendrimers alone or nucleic acid-dendrimer complexes. In the present study, we tested this hypothesis by investigating the interaction of cationic dendrimers (PAMAM and PPI) with total RNA isolated from cells using monophasic solution. We evaluated RNA isolation from human macrophage-like cells (U937) and mouse fibroblast cells (NIH/3T3) exposed to dendrimers and DNA/dendrimer complexes. We found that the strong interactions occurring between these remnant dendrimers and RNA were reflected in gel electrophoresis. These results provided direct experimental support for our hypothesis.

2. Materials and methods

2.1. Materials

Starburst PAMAM dendrimers (Generation 5 (PAMAM 5.0) and 2 (PAMAM 2.0)) and PPI dendrimers (DAB 3.0 and 2.0) were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Starburst PAMAM dendrimers were supplied in methanol, which was removed using vacuum evaporation, and stored with phosphate buffered saline (PBS) at 4 °C. All other chemicals were of the highest grade available. The pDNA (pSG5*lacZ*, 8 kb), which encodes the *lacZ* gene for β -galactosidase, was driven by an SV40 promoter to assess gene expression. The pSG5*lacZ* was amplified in *E. Coli* and purified using a Pure Yield Plasmid Midiprep System (Promega Corp., Madison, WI, USA). The purity of pSG5*lacZ* was established using UV spectroscopy (an A_{260}/A_{280} ratio ranging from 1.80 to 1.89 was used).

2.2. Cell lines

A U937 (human macrophage-like) cell line was maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin/100 μ g/mL streptomycin (Sigma). NIH/3T3 (mouse fibroblast) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (penicillin and streptomycin) and 10% FBS.

2.3. Cationic dendrimer treatment of cells

Cells (1×10^7) were subjected to cationic dendrimers in serum-free medium for 3 h of incubation at 37 °C, washed with PBS, and then analyzed for RNA isolation. For DNA/cationic dendrimer complexes, cells (1×10^7) were treated to a standard transfection protocol. Briefly, DNA/dendrimer complexes at various weight ratios were formed in serum-free medium for 10 min at room temperature before adding them to the medium of cells. Transfection was performed for 3 h in serum-free medium and then supplemented with 10% FBS and 1% antibiotics. Cells were harvested 48 h after transfection, washed with PBS, and then analyzed for gene expression of β -galactosidase and RNA isolation. The transfection activity was quantified using a combined β -Gal Assay kit (Invitrogen, Carlsbad, CA, USA) and BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) (Rosenthal, 1987).

2.4. Isolation of RNA from cells

Total RNA from cells, untreated or treated with cationic dendrimers and DNA/cationic dendrimer complexes, was extracted using TRIZOL reagent consisting of phenol and guanidine isothiocyanate (Invitrogen, Gaithersburg, MD, USA). Briefly, the cell pellet was lysed and homogenized by adding 1 mL TRIZOL reagent. After 5 min of incubation at 25 °C, phase separation was achieved by adding 0.2 mL chloroform to the homogenized samples. After 10 min of centrifugation at $11,000 \times g$ at 4 °C, RNA from the aqueous phase was precipitated by mixing it with 0.5 mL of isopropyl alcohol. The RNA precipitate was washed three times with 1 mL of 75% ethanol in diethylpyrocarbonate (DEPC)-treated water and air-dried for 10 min. The RNA was dissolved in RNase-free water and stored at –80 °C for further analysis. The purity of extracted total RNA was confirmed using the A_{260}/A_{280} ratio.

2.5. Agarose gel electrophoresis

The integrity of the isolated RNA was assessed using agarose gel electrophoresis. One microgram of isolated RNA was electrophoresed at 100 V for 20 min on a 1% agarose gel containing formaldehyde and then stained with ethidium bromide.

2.6. Cytotoxicity assay

Before isolating the RNA, the activity of dehydrogenases (an indicator of cell viability) in the cells (untreated or treated with cationic dendrimers and DNA/dendrimer complexes) was simultaneously assessed. Negative control cells contained no cationic dendrimers. To measure cell viability, 10 μ L of a cell-counting kit solution, a tetrazolium salt that produces a highly water-soluble formazan dye upon biochemical reduction in the presence of an electron carrier (1-methoxy PMS) (Cell-Counting Kit-8; Dojindo Laboratories, Tokyo, Japan), was added to a 100 μ L culture medium and incubated for 1–4 h. The amount of yellow formazan dye generated by dehydrogenases in cells is directly proportional to the number of viable cells in a culture

medium. The absorbance at 450 nm was obtained using an ELISA reader with a reference wavelength of 595 nm. Results are reported as cell viability percentage (average OD/average negative control OD) ± standard deviation (S.D.).

2.7. Recovering the RNA from the dendrimer–RNA pellets

After checking retarded RNA migration using agarose gel electrophoresis, the dendrimer–RNA pellets were precipitated by mixing them with 0.5 mL of isopropyl alcohol. The RNA precipitate was washed three times with 1 mL of 75% ethanol, air-dried for 10 min, and then dissolved in 1 mL of 2 M NaCl solution for 12 h. After it had been precipitated and washed, the RNA was restored in RNase-free water at –80 °C.

3. Results

3.1. Cell viability assays

To assess the cytotoxic effect of cationic dendrimers, cells before RNA isolation were incubated with cationic dendrimers and DNA/dendrimer complexes. Dehydrogenase activity was

then analyzed. An ideal carrier for biomedical applications should be non-toxic to cells. Thus, our study focused on the concentration ranges within which cationic dendrimers did not elicit toxic effects to cells. For the concentration ranges of the cationic dendrimers and their complexes with DNA used in the following experiments, cell viability was greater than 90%.

3.2. The interaction between cationic dendrimers and RNA molecules

We isolated total RNA from cells untreated or treated with cationic dendrimers using monophasic lysis. The integrity of the isolated RNA was assessed using agarose gel electrophoresis and then ethidium bromide staining (Fig. 1). Untreated cells revealed sharp typical rRNA bands with more RNA in the 28S band than the 18S band. Cationic dendrimers exhibited dose- and generation-dependent blocks on the mobility of RNA in the gels. For U937 cells, high-generation dendrimers at high concentrations (PAMAM 5.0 (≥7.5 μg/mL) and DAB 3.0 (≥20 μg/mL)) completely prevented the mobility of RNA in the gels. PAMAM 5.0 (7.5 μg/mL) required less concentration than

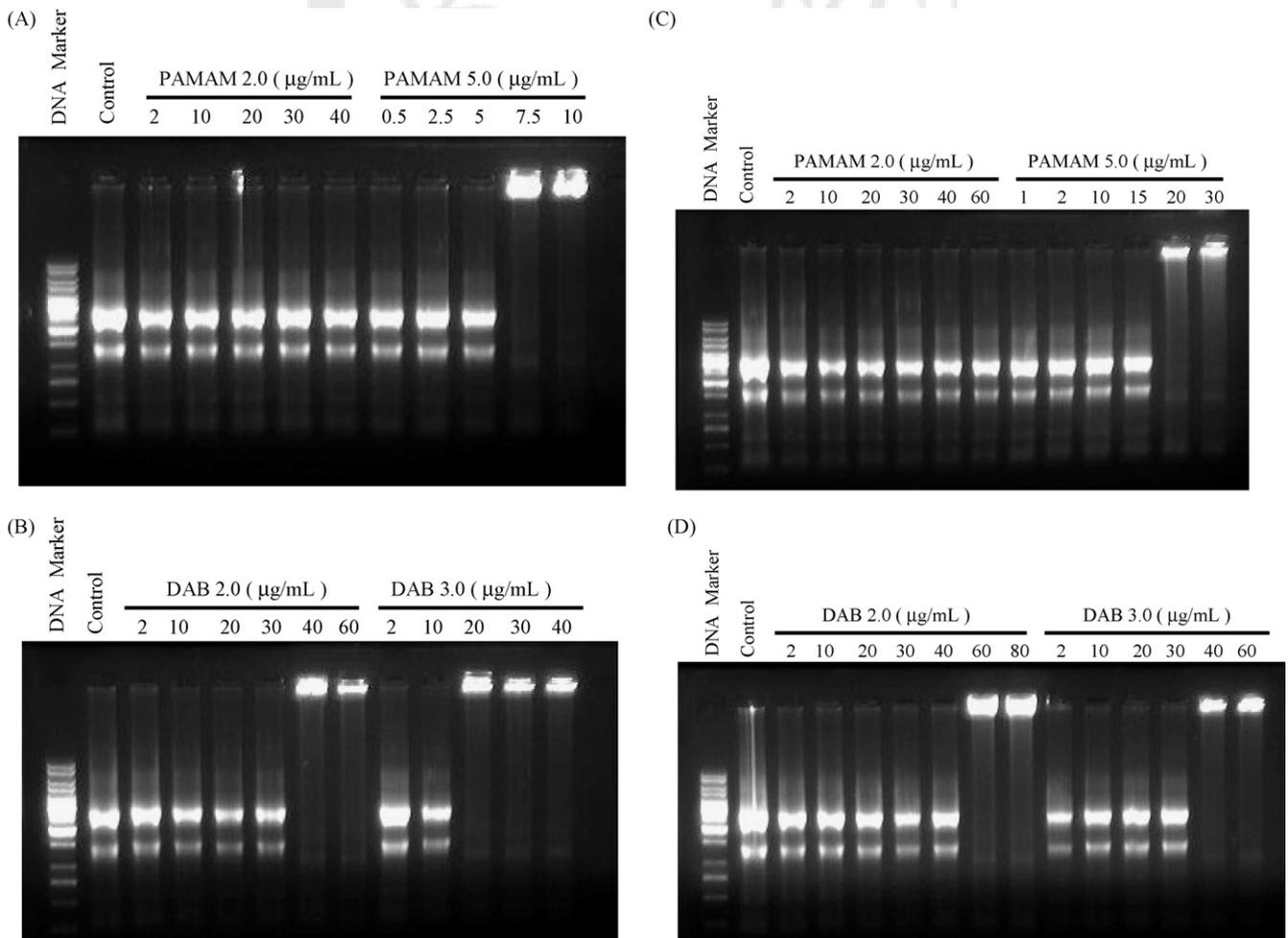


Fig. 1. Electrophoresis of isolated RNA from (A, B) U937 and (C, D) NIH/3T3 cells treated with PAMAM (PAMAM 2.0 and 5.0) and PPI (DAB 2.0 and 3.0) with an increasing concentration of dendrimers. Control samples were isolated RNA from cells without adding cationic dendrimers.

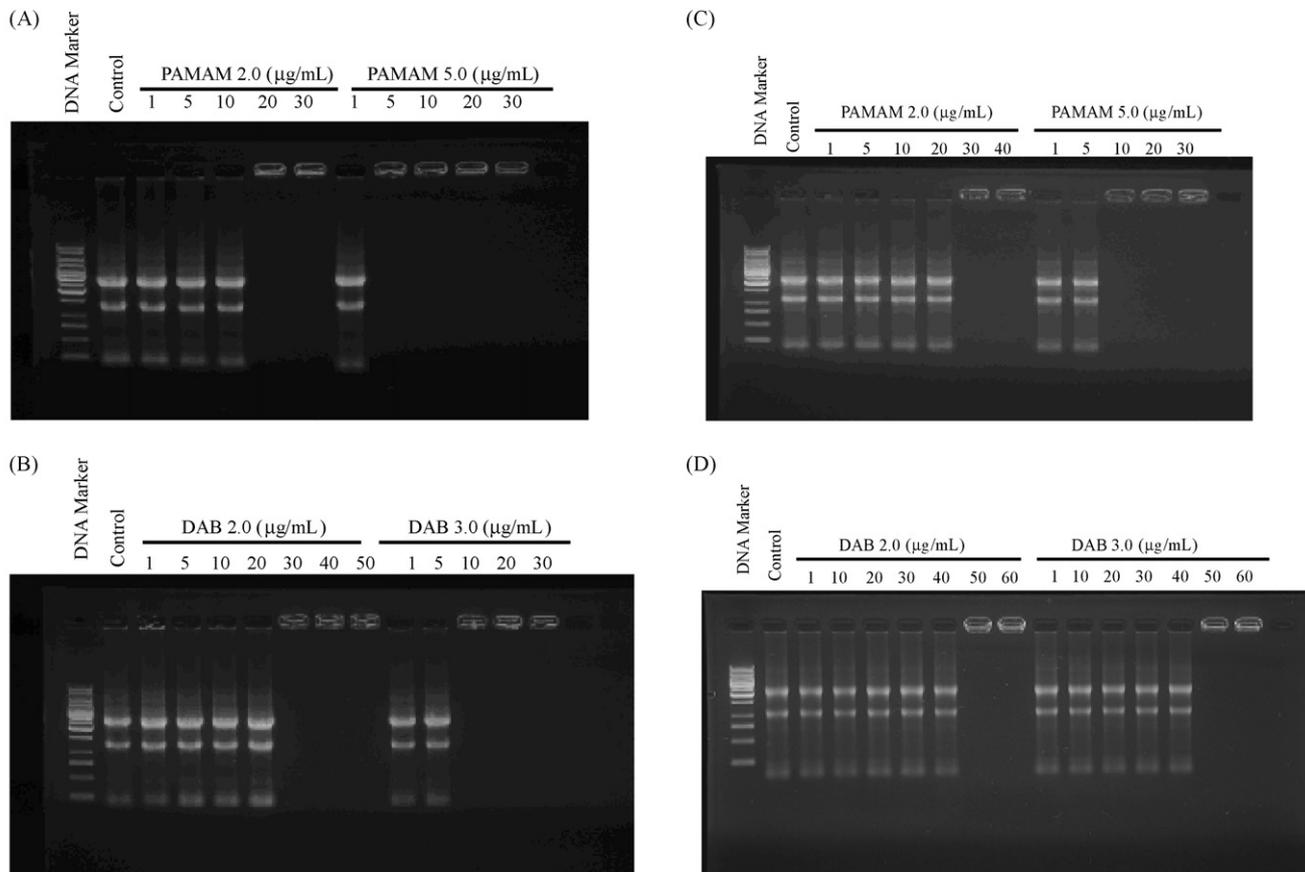


Fig. 2. Electrophoresis of mixing purified RNA from (A, B) U937 and (C, D) NIH/3T3 cells with PAMAM (PAMAM 2.0 and 5.0) and PPI (DAB 2.0 and 3.0) with an increasing concentration of dendrimers. Control samples were isolated RNA from cells without adding cationic dendrimers.

DAB 3.0 (20 $\mu\text{g}/\text{mL}$) to block the mobility of RNA in the gels. No retarded RNA migration was observed with low-generation PAMAM 2.0, even at higher concentrations (up to 40 $\mu\text{g}/\text{mL}$). However, low-generation PPI dendrimers (DAB 2.0) at a higher concentration (≥ 40 $\mu\text{g}/\text{mL}$) retarded RNA migration in the gels. Also, fibroblasts (NIH/3T3) required a greater concentration of dendrimers than did macrophages (U937) to retard RNA migration in the gels. To further confirm electrostatic interactions between RNA and cationic dendrimers, electrophoresis by mixing purified RNA with cationic dendrimers was performed. All cationic dendrimers strongly bonded to RNA molecules in both U937 and NIH/3T3 cells (Fig. 2). These findings provided direct evidence that electrostatic interactions occurred between cationic dendrimers and RNA molecules. As the generation and concentration of cationic dendrimers became higher, stronger bonding occurred between RNA and cationic dendrimers. These findings correlated well with previous results obtained using synthesized RNA (Wu et al., 2005; Zhao et al., 2004).

3.3. The interaction between DNA/dendrimer complexes and RNA molecules

Because cationic dendrimers bind with DNA and mediate gene transfer into eukaryotic cells, the effect of complexing DNA with cationic dendrimers on the isolation of total RNA

from cells was also tested. Complexes between DNA and high-generation dendrimers (PAMAM 5.0 and DAB 3.0) blocked RNA migration in the gels at higher ratios of dendrimer to DNA (Fig. 3). No significantly preventive effects on RNA migration were observed for complexes between DNA and low-generation PAMAM dendrimers (PAMAM 2.0), but DNA/DAB 2.0 complexes at higher ratios of dendrimer to DNA also blocked RNA migration. Due to attenuated cationic charges by anionic DNA, complexes between DNA and dendrimers required a greater concentration of dendrimers than did dendrimers alone to block the mobility of RNA in the gels. Also, fibroblasts (NIH/3T3) required a greater concentration of DNA/dendrimer complexes than did macrophages (U937) to retard RNA migration in the gels. As nitrogen/phosphorus (N/P) ratios of complexes became higher (except for PAMAM 2.0), stronger bonding occurred between RNA and cationic dendrimers. These results further confirmed that electrostatic interactions between RNA and cationic DNA/dendrimer complexes occurred during or after intracellular trafficking. We also simultaneously examined the *in vitro* gene expression of DNA/dendrimer complexes (Fig. 4) and found significantly higher gene expression for DAB 3.0 and 2.0 than for PAMAM 5.0 and 2.0. These findings revealed that the transfection activity of DNA/dendrimer complexes was not altered by the interaction between dendrimers and intracellular RNA.

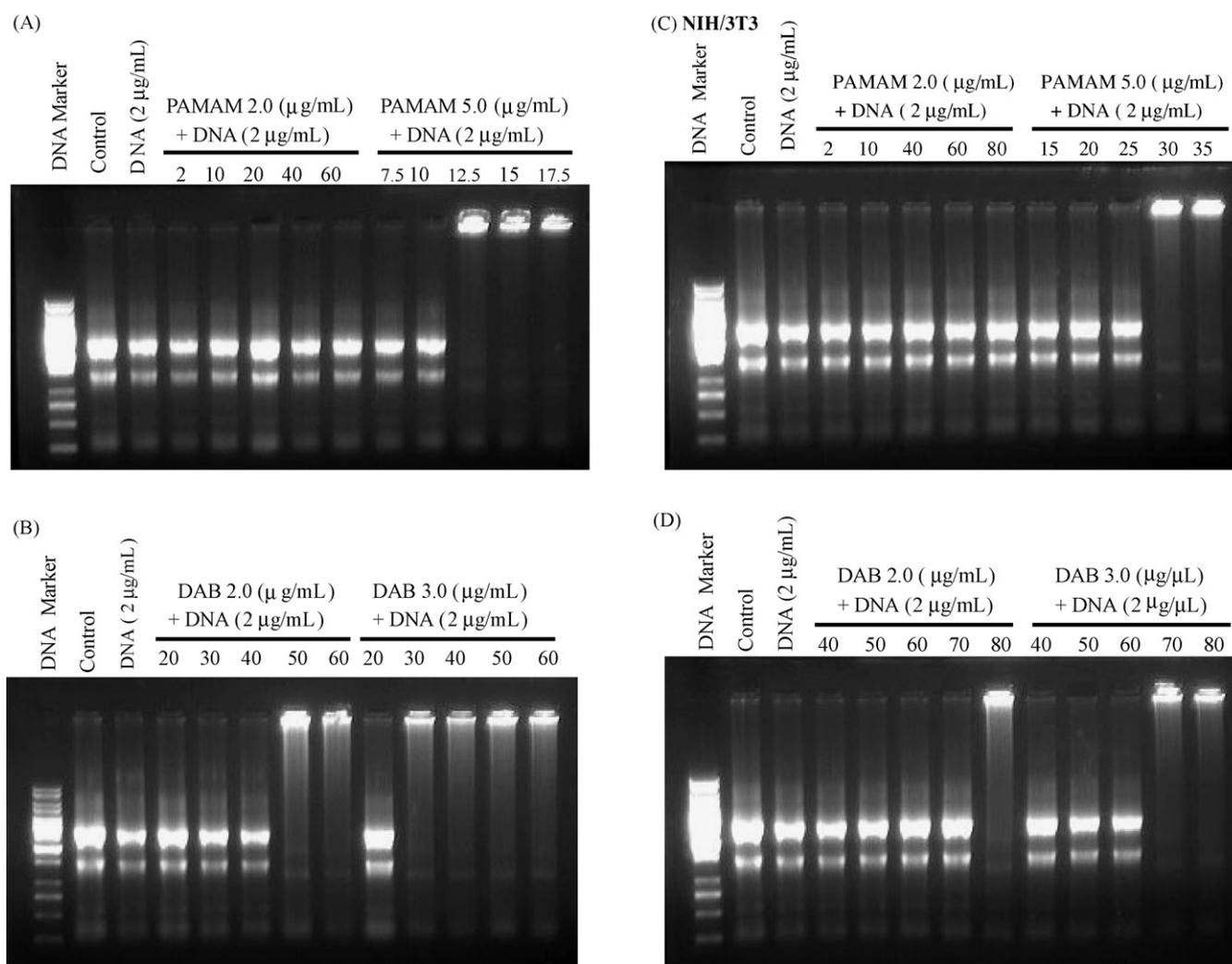


Fig. 3. Electrophoresis of isolated RNA from (A, B) U937 and (C, D) NIH/3T3 cells treated with complexes between dendrimers (PAMAM (PAMAM 2.0 and 5.0) and PPI (DAB 2.0 and 3.0)) and DNA (2 $\mu\text{g/mL}$) with an increasing concentration of dendrimers. Control samples were isolated RNA from cells without adding cationic dendrimers. Lane 3 in each panel presents isolated RNA from cells after adding 2 $\mu\text{g/mL}$ of DNA.

3.4. Recovering the RNA from the dendrimer–RNA pellets by using NaCl solution

The electrostatic forces binding cationic molecules to nucleic acid can be disrupted by high ionic strength solvents (Dahle and Macfarlane, 1993; Tang and Szoka, 1997). We recovered RNA from the dendrimer–RNA pellets using 2 M NaCl solution for 12 h to reduce the electrostatic bonding between the isolated RNA and cationic dendrimers (PAMAM 5.0, DAB 3.0, and DAB 2.0). The integrity of the recovered RNA was then assessed using agarose gel electrophoresis and then ethidium bromide staining (Fig. 5). We found that the isolated RNA was totally recovered for cells treated with PAMAM 5.0, DAB 3.0, and DAB 2.0. Total RNA was also recovered from cells treated with DNA/dendrimer (PAMAM 5.0, DAB 3.0, and DAB 2.0) complexes.

4. Discussion

The successful isolation of total RNA from cells is fundamentally important for molecular biology research. Cationic

molecules interact with anionic RNA in cells. Cationic surfactant (Catrimox-14) has been successfully used to isolate total RNA from cells in culture and clinical samples because it lyses cells and protects RNA from degradation (Macfarlane and Dahle, 1997; Dahle and Macfarlane, 1993). PAMAM dendrimers bind *trans*-acting responsive element (TAR) synthesized RNA and inhibit the catalytic activity of *Candida* ribozymes by forming RNA/dendrimer complexes (Wu et al., 2005; Zhao et al., 2004). In addition to the delivery of nucleotides and small interfering RNA (siRNA), PPI dendrimers alter the expression of many endogenous genes (Omidi et al., 2005). However, no study has indicated that RNA electrophoresis after the isolation of total RNA from cells using monophasic lysis is blocked by remnant cationic dendrimers.

Dendrimer-induced alteration in RNA mobility after isolation is difficult to detect solely by checking the A_{260}/A_{280} ratio (typically greater than 1.9) using UV spectroscopy, because PAMAM and PPI dendrimers also contribute to the intensity of the absorbance band (data not shown). However, we found, using agarose gel electrophoresis, that PAMAM and PPI den-

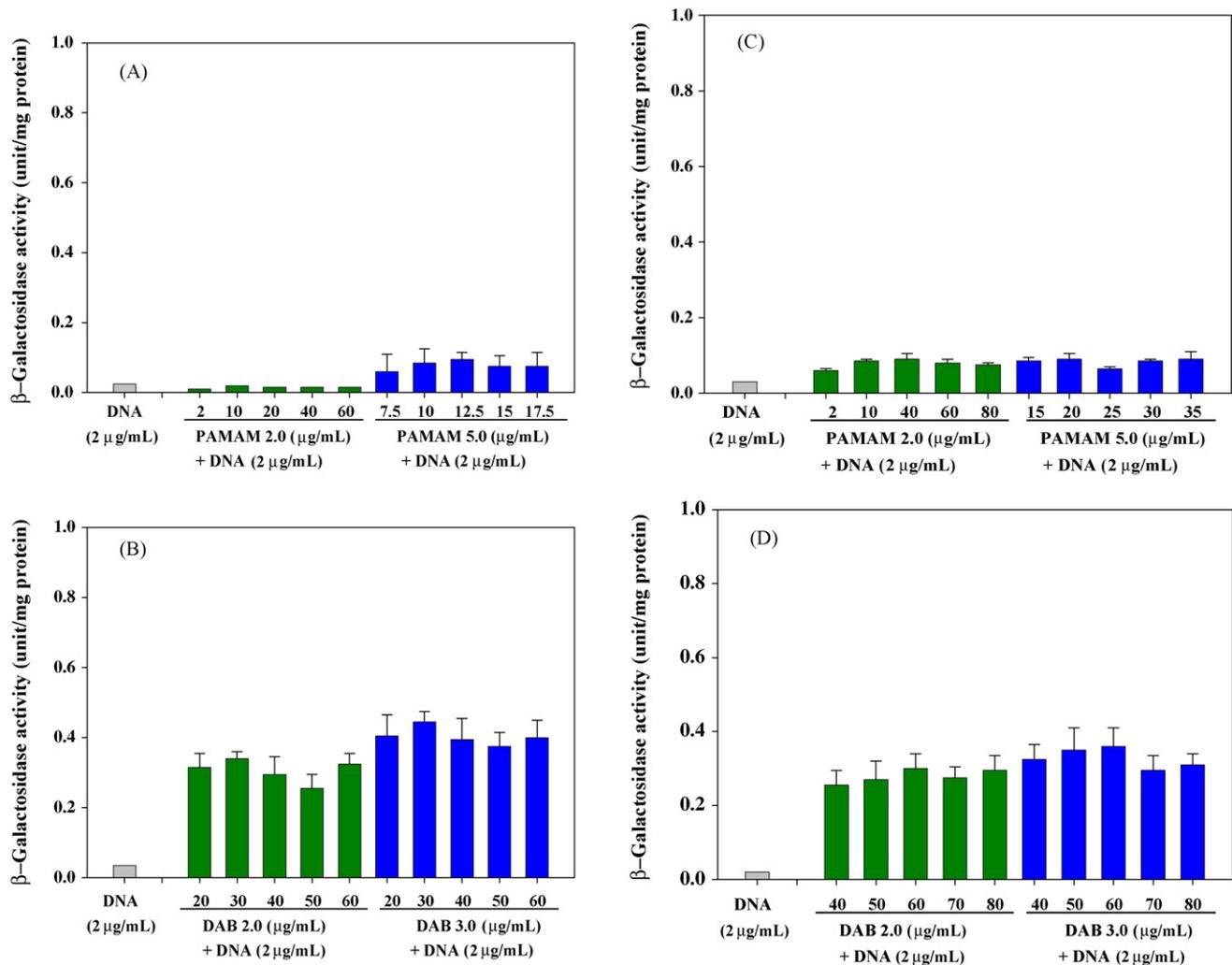


Fig. 4. *In vitro* gene expression after transfection into (A, B) U937 and (C, D) NIH/3T3 cells. Cells (1×10^7) were transfected with 2 μ g/mL of plasmid DNA alone or complexed with increasing concentrations of PAMAM (PAMAM 2.0 and 5.0) and PPI (DAB 2.0 and 3.0) dendrimers. The data are given as the mean \pm S.D. ($n=3$).

drimers strongly block the mobility of RNA after it has been isolated from cells. Therefore, RNA inhibition by dendrimers during isolation might hinder other RNA applications such as Northern blotting, RT-PCR, and microarray target labeling. In the present study, we demonstrated that dendrimer-induced alteration in RNA mobility can be totally recovered using high ionic strength solvents (2 M NaCl solution) to disrupt the electrostatic forces binding dendrimers to RNA. We suggest that washing RNA with 2 M NaCl solution after its isolation from cells treated with cationic dendrimers is necessary to obtain high quality and pure RNA for further biological applications.

Although other RNA isolation methods are available, monophasic lysis is the most frequently used method that allows the simultaneous recovery of RNA, DNA, and protein from cultured cells. No matter of what kind of RNA isolation methods are used, cell lysis using various lysis reagents is the common first step for further purification. After this stage, cytoplasm rRNA is separated from protein in aqueous medium and forms RNA/remnant dendrimer complexes via electrostatic attractions.

Because the cationic dendrimers we used were highly water soluble and nonspecifically bind to anionic molecules, we expect that dendrimer-induced alteration in RNA mobility still occurs when using other methods of isolation.

The fate of the dendrimer–nucleotide complexes within cells and the mechanism of dendrimer-mediated cellular uptake of nucleotides have not been fully clarified. Recently, PAMAM 5.0 dendrimers were shown to interact with cell membranes by forming holes (Hong et al., 2004). Internalizing DNA/dendrimer complexes was previously shown to be mediated by membrane rafts and escape into the cytosol before trafficking to the nucleus (Manunta et al., 2004). Also, dendrimers may cause endosomal rupture and release the complex or DNA into the cytoplasm by acting as a “proton sponge” (Tang et al., 1996). DNA/dendrimer complexes at higher ratios of dendrimers to DNA have been shown to markedly inhibit transcriptional activity *in vitro* and not affect elongation of the RNA transcript and translation (Bielinska et al., 1997). We found that DNA/dendrimer complexes at higher ratios of dendrimers to DNA also interacted

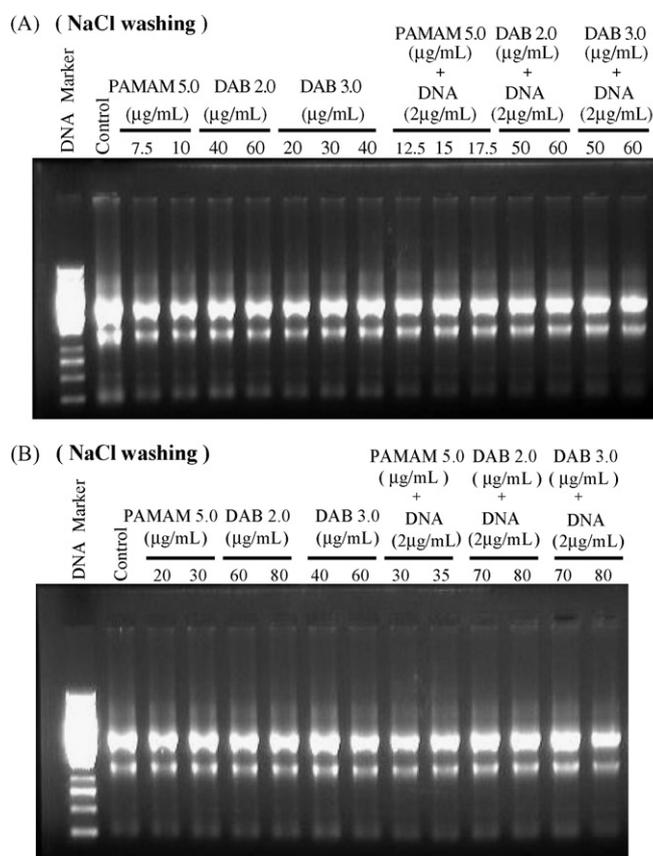


Fig. 5. Using 2 M NaCl solution to recover the isolated RNA from (A) U937 and (B) NIH/3T3 cells treated with cationic dendrimers and DNA/dendrimer complexes.

with RNA during isolation while gene expression was maintained (Figs. 3 and 4). Although various dendrimers (generation or PAMAM/PPI) interacted differentially on a weight-basis, a similarity existed on dendrimer-induced alteration in RNA mobility.

We also isolated RNA from U937 and NIH/3T3 cells using cationic lipid and lipoplexes (50–100 μg/mL, TransFast (Promega)) and found no RNA block in the gels (data not shown). This indicated that cationic dendrimers produced a stronger RNA block than cationic lipids did. We also found that another non-viral vector that is similar to a cationic dendrimer, polyethyleneimine 25 K (Sigma–Aldrich), blocked the mobility of RNA in the gels isolated from U937 and NIH/3T3 cells (data not shown). The preventative effect of dendrimers on the mobility of RNA in other cell types remains to be established.

In conclusion, remnant cationic dendrimers (PAMAM 5.0, DAB 2.0, and DAB 3.0) strongly blocked RNA electrophoresis after the isolation of total RNA from cells using monophasic lysis. These remnant dendrimers strongly blocked the mobility of RNA via electrostatic interactions. The extent of dendrimer-induced alteration in RNA mobility is dependent on the dendrimer generation: alteration in RNA mobility is greater with higher-generation dendrimers. At higher dendrimers-to-DNA ratios, RNA electrophoresis was blocked by DNA/dendrimer

complexes, but gene expression of these complexes was not affected. We also found that dendrimer-induced alteration in RNA mobility was totally recovered using high ionic strength solvents (2 M NaCl solution) to disrupt the electrostatic forces binding dendrimers to RNA. Because RNA isolation is routinely used for biological applications, such dendrimer-induced alteration in RNA mobility should be accounted for when further processing RNA for related applications.

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