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Elevation of Transfection Efficiency by Conjugation of Poly(amindoamine)-diethylenetriamine (PAM-DET) with Dexamethasone

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A number of modifications and functionalizations of dendrimers as non-viral vectors of gene delivery system have been attempted by many researchers in order to solve the general problem associated with these structures, *i.e*., the high cytotoxicity of polymers such as the 25-kDa poly(ethylenimine) (PEI) and poly(amidoamine) dendrimer (PAMAM).[1-5] As a result of these attempts, PAMAM-R and PAM-DET, which are respectively arginine- and diethyltriamine (DETA)-modified PAMAM dendrimers, were synthesized by our group and have shown high transfection efficiency and low cytotoxicity. In this study, we tried to conjugate the nontoxic and hydrophobic group, dexamethasone (DX) with the ends of PAM-DET and synthesized PAM-DET-DX. We introduced DX group specially due to both its nuclear localization effect in gene-transfection and its biocompatibility as a glucocorticoid analog.[9,10] In comparison with PAM-DET, this conjugated PAM-DET dendrimers showed polyplexes (gene-polymer complexes) with better and longer size-stability in aqueous solutions with high ion strength. The PAM-DET-DX gene carriers showed higher transfection efficiency than PAM-DET and cytotoxicity level equivalent to that shown by PAM-DET in HeLa and U87MG Cell lines.

PAM-DET was synthesized as previously described.[6] The conjugation reaction was performed as previously reported with some modification.[7] The overall synthesis scheme is displayed in Figure 1(a). Briefly, dexamethasone coupling to PAM-DET was performed with 4 equivalents of Traut's reagent and 4 equivalents of dexamethasone mesylate in anhydrous DMSO for 4 h at room temperature. An equal volume of water was added to the reaction mixture, and it was dialyzed against pure water and filtered through a 0.45-μm syringe filter to remove insoluble impurities. The product was then obtained after freeze-drying. The resulting PAM-DET-DX product was analyzed by ^{1}H NMR at 40 °C to calculate the actual degree of conjugation (Figure 1(b)). Each PAM-DET-DX conjugate was named based on its actual degree of DX conjugation (Table 1).

The pDNA/PAM-DET and pDNA/PAM-DET-DX polyplexes were prepared in 10 mM HEPES buffer (pH 7.4) by incubation at 25 °C for 30 min. To prepare each polyplex sample with PAM-DET and PAM-DET-DX (2), (5), and (10) (their charge ratio of cationic polymer/pDNA was commonly 16) as the first group, 1 mg/mL of pDNA was used. For the second group, pDNA/PAM-DET and pDNA/PAM-DET-DX (10) polyplexes with charges of 4, 8, and 16 were also prepared, taking into consideration the molecular weight and actual degree of conjugation, as shown in Table 1.

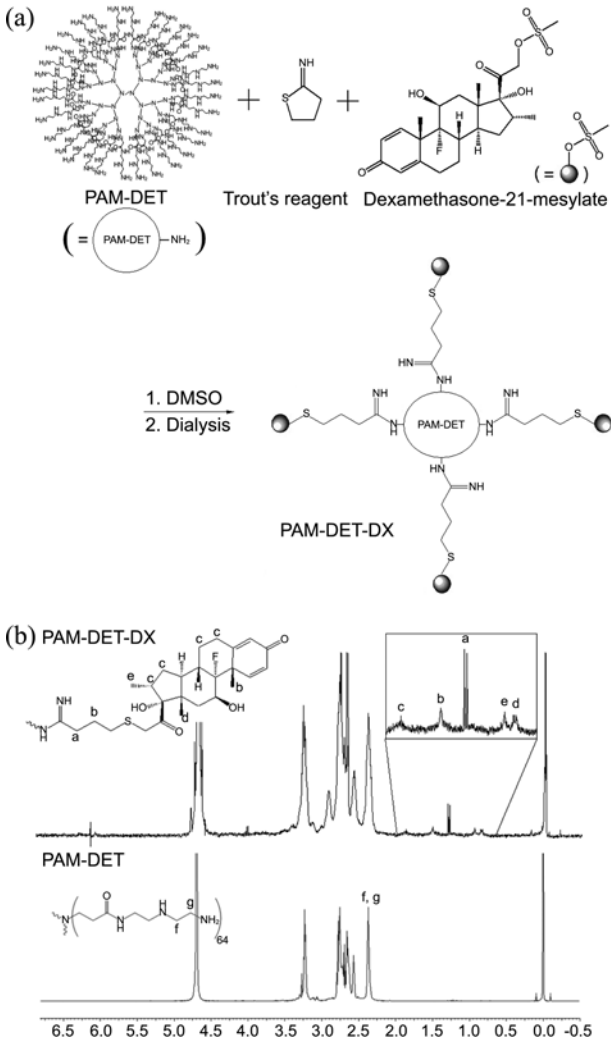

Figure 1. (a) Synthetic scheme for PAM-DET-DX, (b) ^{1}H-NMR spectrum of PAM-DET and PAM-DET-DX.

[a]These authors contributed equally to this work.

Table 1. Linearity, range, LOD, and LOQ. The equations of the calibration curve were obtained from 18 points. LOD and LOQ were defined as the concentrations of at 3×S/N and 10×S/N ratios, respectively

Entry No.	Target degree of conjugation	Actual degree of conjugation	Name based on actual degree of conjugation	M_n
1	4	2	PAM-DET-DX(2)	17,487
2	8	5	PAM-DET-DX(5)	18,865
3	16	10	PAM-DET- DX(10)	21,161

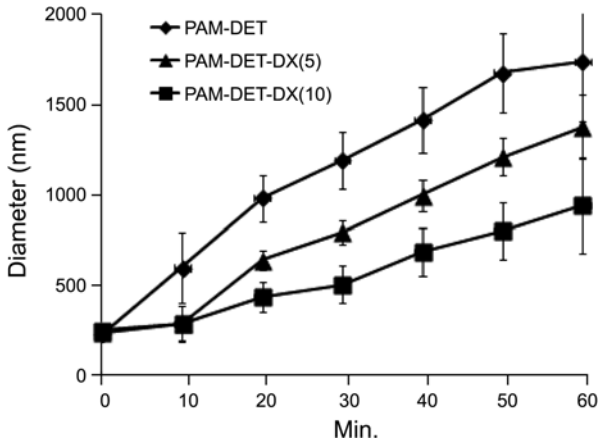

Figure 2. Time-dependent profile of PAM-DET-DX polyplex size under increased ionic strength (50 mM).

After preparing the polyplexes of both groups, the NaCl concentration was adjusted to 50 mM by the addition of 1 M NaCl (20×) to the buffer solution. Changes in the sizes of the polyplexes were measured using a Malvern Zeta sizer 3000HAs (Malvern Instrument Ltd., Worcestershire, UK) at 10 min intervals for 1 h. As results, the size-increase profile of the polyplexes at 25 °C were obtained by a DLS study. Different patterns were observed in the changes in sizes of the polyplexes formed from PAM-DET, PAM-DET-DX (2), (5), and (10) (Figure 2). The polyplexes of pDNA/PAM-DET-DX (2) exhibited almost the same changes in size as PAM-DET, which shows negligible contribution by any hydrophobic group to the stability of this polyplex due to the very low conjugation of the hydrophobic dexamethasone group. The polyplexes of pDNA/PAM-DET-DX (10) showed the slightest changes in size over 1 h compared with the other polyplexes in the groups (Figure 3). This result showed that the degree of conjugation of the hydrophobic moiety is an important factor for controlling the stability of the polyplex against ionic strength. Accordingly, a high degree of hydrophobic group conjugation onto PAM-DET lead to enhanced stability of the polyplex. A similar effect of hydrophobic moiety conjugation on cationic polymers was reported by other groups, thereby supporting our findings.[1]

To confirm the transfection efficiency of the PAM-DET and PAM-DET-DX polyplexes, transfection experiments were performed using HeLa and U87MG cells. Human cervical cancer (HeLa) cells and human glioblastoma-astrocytoma cells (U87MG) were grown in DMEM containing 10% FBS and 1% antibiotic. Cells were incubated in plastic tissue

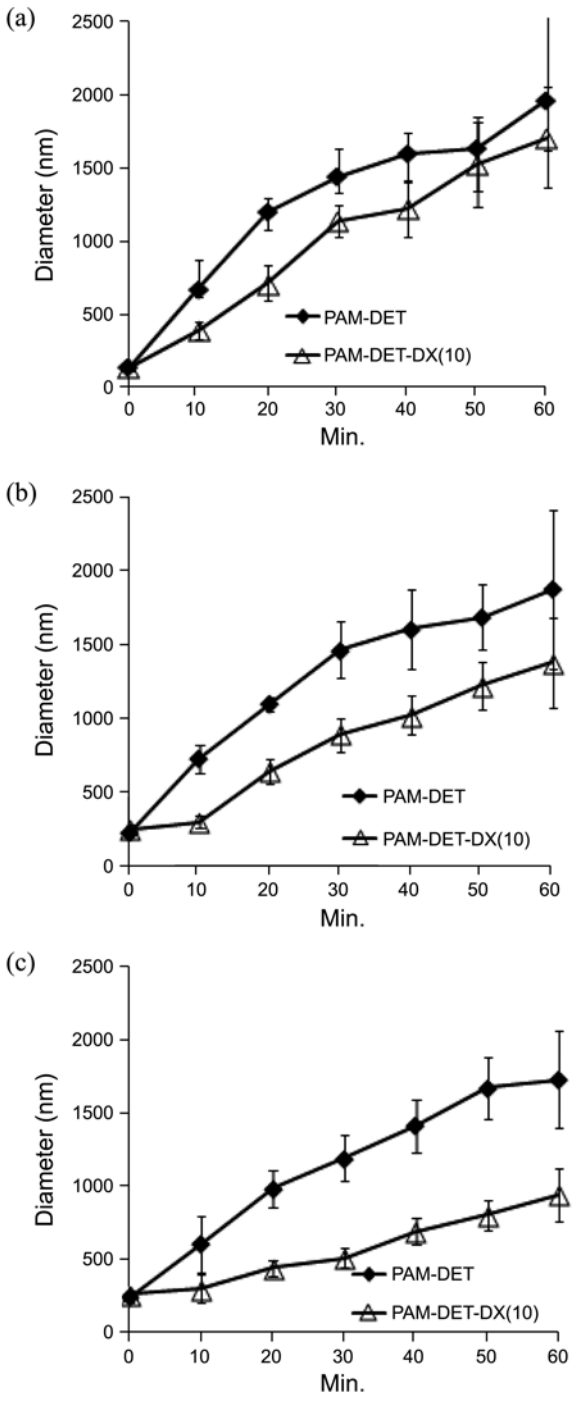

Figure 3. Time-dependent profile of PAM-DET-DX(10) polyplex size with varying charge ratio (+/−; (a) 4, (b) 8, (c) 16) in 50 mM $NaCl_{(aq)}$.

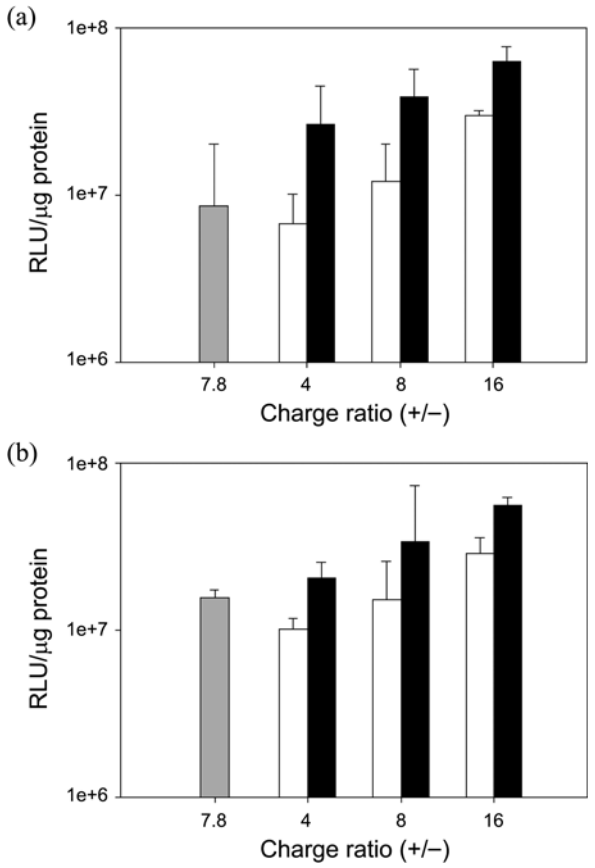

Figure 4. Transfection efficiency of PEI (gray bars), PAM-DET (white bars) and PAM-DET-DX(10) (black bars) against (a) HeLa and (b) U87MG cells.

culture cell binder flasks (Corning) at 37 °C in a 5% CO_2 humidified incubator. Cells were seeded at 30,000 cells/well in 24-well plates in 600 μL of DMEM containing 10% FBS and 1% antibiotic and incubated at 37 °C for 1 day. The cells were treated with a polyplex solution prepared using 2 μg of pDNA and cationic polymer (PAM-DET, PAM-DET-DX (10), and PEI) at different charge ratios in 150 μL of FBS-free DMEM and incubated for 30 min at room temperature. After adding polyplex to each well, the cells were incubated for 2 days. For the assay, the growth medium was removed and the cells were washed with PBS and lysed with 150 μL of Reporter lysis buffer for 30 min at room temperature. The luciferase activity of the transfected cells was measured using an LB 9507 luminometer (Berthold, Germany) using 10 μL of lysate in the luminometer tube and automatic injection of 50 μL of luciferase assay reagent. All experiments were performed in triplicate.

As the results of transfection experiment comparing PAM-DET to PAM-DET-DX (10) in both HeLa and U87MG cells, PAM-DET-DX interestingly showed better transfection efficiency than PAM-DET in both cell lines (Figure 4). It was reported that increased transfection efficiency is caused by enhanced stability of the polyplex. Similarly, it seems that the enhanced stability of the polyplex due to conjugation of the hydrophobic moiety contributes to an increase in the

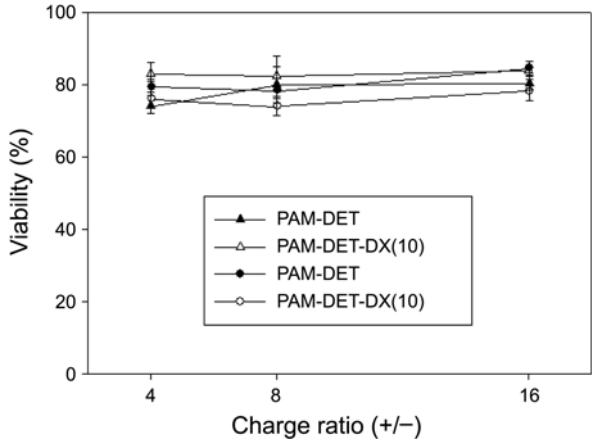

Figure 5. Cell Viability test of PAM-DET and PAM-DET-DX(10) polyplex varying charge ratio against HeLa (▲ and △) and U87MG cell (● and ○).

transfection efficiency due to hydrophobic interaction in aqueous environment. In addition, dexamethasone, a potent glucocorticoid, also has positive properties. Dexamethasone can bind to glucocorticoid receptors after cellular entry, and the receptor/dexamethasone complex is subsequently translocated into the nucleus.[8]

To measure the cell viability of the polyplexes, MTT assays were performed. HeLa and U87MG cells were seeded at 10,000 cells/well in 96-well plates in 120 μL of DMEM with 10% FBS and 1% antibiotic and incubated at 37 °C for 1 day prior to the addition of the polyplexes. PAM-DET and PAM-DET-DX (10) polyplexes containing 0.2 μg of pDNA at the same charge ratio as that in the transfection experiment were prepared in 24 μL of FBS-free DMEM by incubation at room temperature for 30 min. After adding the polyplexes, the cells were incubated for an additional 48 h. Then, the cells were washed with PBS followed by the addition of 26 μL of filtered MTT solution (2 mg/mL in PBS). After incubation at 37 °C for 4 h, the medium was removed from the wells and 150 μL of DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm was measured using a microplate reader (Molecular Device Co., Menlo Park, CA), and cell viability was calculated as a percentage relative to that of untreated control cells. As a result (Figure 5), it suggests that the low cytotoxicity of PAM-DET-DX, is due to the biocompatibility of DX, a glucocorticoid analogue.[8]

In conclusion, we successfully conjugated hydrophobic group, dexamethasone onto the surface of PAM-DET to synthesize PAM-DET-DX to form polyplexes with enhanced stability against ionic strength. We evaluated its stability by measuring the size of its polyplexes; the conjugated PAM-DET polyplex showed decreased growth compared to the PAM-DET polyplex in an environment with increased ionic strength, which implies that the conjugated PAM-DET has enhanced stability against increased ionic strength. Furthermore, conjugation of hydrophobic group caused a slight increase in the transfection efficiency without inducing toxicity. Of course, it isn't a neglectable factor that nuclear localization effect of DX can drive the advanced transfection

efficiency of PAM-DET-DX polyplex. It means that the hydrophobic moieties which have some other positive properties in transfection are good candidates that can be introduced to non-viral polymeric gene delivery carrier. This strongly indicates that the introduction of hydrophobic moiety on PAM-DET is a good method to enhance polyplex stability against ionic strength without diminishing its advantageous properties, such as high transfection efficiency and low cytotoxicity.

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