Effect of Cation Content of Polycation-type Gene Carriers on *in vitro* Gene Transfer

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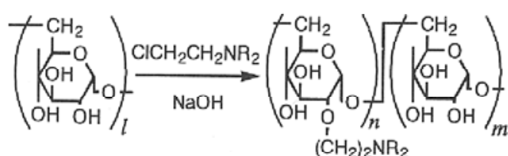
Dextran-based polycations having different cation structures and contents were evaluated as carriers for transferring genes to mammalian cells *in vitro*. 2-(*N,N*-Dimethylamino)ethyl dextran having tertiary-amine-type cations induced very high transient gene expression in the COS-1 cells, while 2-aminoethyl dextran having primary-amine-type cations did not induced it at all. The cation content of the former was also found to affect the transient gene expression and the cytotoxicity of the polycations.

Water soluble polycations have recently been attracting great attention as nonviral gene carriers in the field of gene therapy and biology[1], because they may be more versatile for *in vivo* usage than the liposomes and other conventionally used spherical gene carriers. Until now, several polycations have been reported to induce gene expression, for example, diethylaminoethyl dextran and other cationized polysaccharides,[2] polyethylenimine,[3] polybrene,[4] and polyamidoamine cascade polymers.[5] These compounds have little structural similarity with each other except possessing cationic groups. On the other hand, it has been reported that gene introduction efficiency of poly-L-lysine (PLL) can be enhanced by modification with various bioactive moieties like transferrin or asialoglycoprotein,[6] while PLL itself shows low gene transfection efficiency.[7] In addition, the relatively high cytotoxicity of these polycations has interfered with their practical use as gene carriers. Since the structural requirements needed for the polycation carriers have not yet been well understood, polycation-type gene carriers with optimized chemical structure can not be well designed at present.

Recently, we reported on an important role of the hydroxyl groups involved in the carrier polycations in the gene expression.[8] This finding implicated a definite direction of the carrier design. In the present paper, 2-(*N,N*-diethylamino)ethyl (D-dex(x)) and 2-aminoethyl dextrans (A-dex(x)) with different cation contents ("x" in the parentheses represents the percent cationic groups relative to the glucose units) were synthesized and evaluated as gene carriers in order to clarify how the gene transfection and cytotoxicity should be influenced by the cation structure and content of the fully hydroxylated polycations.

Thus, both D-dex and A-dex were prepared according to Scheme 1.[9] The cation content of D-dex was adjusted to 22, 31, and 55% while that of A-dex was to 27%. A given amount of each of these samples was mixed with 0.1 μg of pCH110 plasmid DNA[10] to form the polyion complexes at various C/A ratios where C and A indicate the molar quantities of the cationic groups of the polycation and the phosphate groups of the DNA, respectively. Figure 1 shows the ethidium bromide-stained agarose gel electrophoreses of the mixtures. For D-dex(55) and D-dex(31), the migrating bands due to the free DNA are shown in addition to the band staying at the slots at C/A=0.5. The latter band is attributed to the DNA/polycation complexes. For D-dex(22) and A-dex(27), the migrating bands are stronger and their migration distance is shorter with increasing C/A. The band migration is detected even at C/A=1.0. The bands migrating in a short distances are attributed to the DNA partly complexed with the polycations, which have a larger size or a less charges than free DNA depending on their cation content. The higher the cation content, the shorter the migration distances. At C/A > 1.5 a bright band is shown on the anode (upper) side wall of the slots in every case, indicating that the complexes are positively charged, involving an excess polycation relative to DNA. This suggests that all the D-dex and A-dex, whose pKa values were above the physiological conditions, can form positively charged complexes with DNA at C/A >1.5.



Scheme 1. Syntheses of cationic dextrans. The substituents are probably introduced into the each of the three hydroxyl groups of the sugar unit.

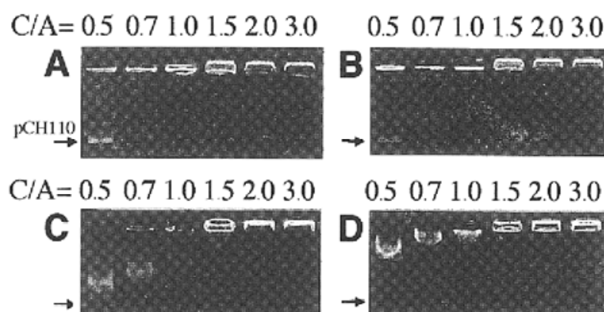


Figure 1. Agarose gel electrophoresis (0.8%) of the complexes of DNA and (A) D-dex(55), (B) D-dex(31), (C) D-dex(22), and (D) A-dex(27) at different C/A ratios indicated.

In the presence of these carriers, a pCH110 plasmid DNA containing the *lacZ* gene coding β-galactosidase was transfected into the COS-1 cells by the osmotic shock procedure.[12] The percent transient expression of *lacZ* was then evaluated by X-gal staining method.[11] Figure 2 shows the dependencies of the transient expression on the C/A ratio for the four polycations. At C/A < 1.0, no gene expression was observed for all the polycations because of the negative charges of the complexes as indicated by the DNA bands on the cathode side walls of the gel slots (Figure 1). At C/A > 1.5 where the polyion complexes have cationic feature, each D-dex induced high transient

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expression. D-dex with higher cation content led higher transient expression. The maximum expression of 70 % was attained at C/A =3.0 for D-dex(55). On the other hand, A-dex(27) did not induce gene expression at all, although its DNA complex has cationic feature at C/A>1.5. The fluorescence observation of a FITC-labeled DNA complexed with A-dex indicated that the DNA is internalized in the cells as that complexed with D-dex. Therefore, A-dex(27) can be a carrier for introducing DNA molecules into cells but lose an ability of leading gene expression in the cell. This reason has been unknown yet.

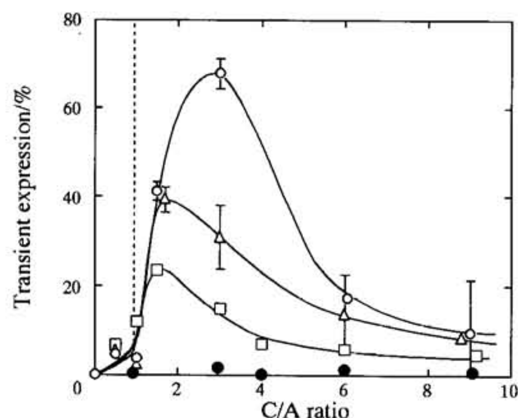

Figure 2. Transient expression of *lac*-Z gene introduced to COS-1 cells using (○) D-dex(55), (△) D-dex(31), (□)D-dex(22) and (●) A-dex(27).

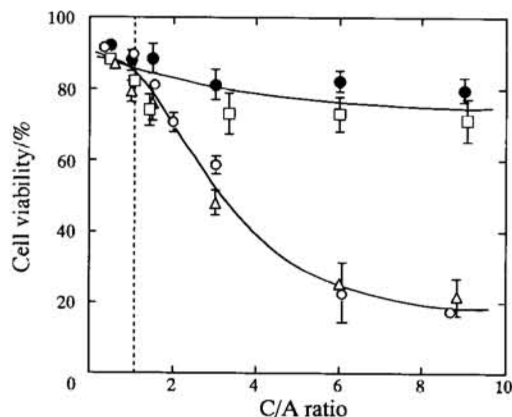

Figure 3. Viability of COS-1 cells after gene transfection with (○)D-dex(55), (△)D-dex(31),(□)D-dex(22), and (●)A-dex(27).

Figure 3 shows the cell viability just after the osmotic shock procedure.[13] D-dex(55) and D-dex(31) showed pronounced cytotoxicity, while D-dex(22) and A-dex(27) did not. The cytotoxicity of the former became stronger with increasing C/A ratio. This may be a reason for the reduced gene expression at the larger C/A ratio (Figure 2). In the case of D-dex(22) the gene expression also decreased at C/A>2.0 in spite of its low cytotoxicity. Its low cytotoxicity was confirmed by the number of cells at 40 h after gene transfection by D-dex(22). Since the cation content of this polycation is low, the polycation/DNA composition should be significantly large at high C/A ratio, and the DNA molecules should be sterically hindered to retard recognition by such intracellular components as transcription factors.

It has been shown that the gene expression and cytotoxicity are greatly affected by the chemical structure of polycations used. The present data may not only lead structural optimization of the dextran-based gene carrier, but also information for the carrier design of other hydrophilic polymers.

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9 Dextran was reacted with 2-(*N,N*-diethylamino)ethyl chloride hydrochloride and 2-aminoethyl chloride hydrochloride in a 5M (1M = 1 mol dm^{-3}) NaOH aqueous solution at 80 °C for 2 h, respectively, dialyzed against double distilled water for 2 days, and lyophilized. These cationic group contents were measured by coloid titration method at pH 2.0 and ^{1}H-NMR. These methods showed a similar cationic contents for each polycation.

10 Takai, T and Ohmori, H. (1990) *Biochemica Biophysica Acta* **1048** 105-109. Two mL of aqueous solution of polycations was added to a solution mixture of 234 μL of RPMI-1640 culture medium, 12.5 μL of 1M Tris-HCl (pH7.3), and 2.5 μL of DNA/Tris-EDTA (1μgDNA/μL, pH 7.5) and incubated at 37 °C for 10 min prior to cell transformation. The C/A ratio was adjusted by changing the concentration of polycation solution. A pellet of 3.75 x 10^4 COS-1 cells was rinsed, resuspended into 250 mL of RPMI-1640 medium containing DNA/polycation complexes, and incubated for 30 min while being mixed gently. At the end of the incubation, a prewarmed high osmotic solution (containing 1M sucrose, 20% PEG with the molecular weight of 4,000, 210 mM NaCl, 70 mM Tris-HCl (pH7.3) and 20% of DMSO) was slowly added to the cell suspension and incubated at 37 °C for another 10 min. The cells were rinsed with 2.5mL RPMI-1640 medium without FCS twice and resuspended into 2 mL of DMEM medium containing 10% FCS.

11 The small aliquot of the cell suspension was mixed with the equivolume of tripan blue solution in order to check the cell toxicity of the polycations and of the osmotic shock procedure. The rest of the cell suspension was plated into each of 6 well culture multiplate (Corning) and cultured for 40 h. After the incubation, the cells were stained with the X-gal dye solution containing 1.2mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.1% Triton X-100, 1 mM $MgCl_2$, 3mM $K_4Fe(CN)_6$, and 3 mM $K_3Fe(CN)_6$, in phosphate buffer saline (pH 7.5) and incubated another 40 h. The number of bright blue cells was counted and the percentage of stained cells to the total cell number at the point after the 40 h was calculated.