Cationic Polysaccharides for Gene Delivery

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ABSTRACT: Cationic polysaccharides based on spermine−dextran conjugates were synthesized and tested as vectors for gene transfection. Dextrans of 10−380 kDa were oxidized under mild conditions by potassium periodate to obtain the respective polyaldehydes in 90% overall yield. The oxidized dextrans were reacted by reductive amination with increasing amounts of spermine, and the efficacy of conjugation between the oligoamine and polysaccharides was studied as a function of spermine/aldehyde mole ratio, pH, and temperature of medium. The optimal conjugation yields were obtained at 1.25 mole ratio (spermine/aldehyde groups) and pH 11 at room temperature. Under these conditions, ∼2 μmol/mg (spermine/polysaccharide) conjugation was achieved with 25−30% of the spermine moieties were conjugated in both sides to form branched polymers. The water-soluble polymers obtained were interacted with pCMV-GFP plasmid to form nanoparticles that were introduced to HEK293 and NIH3T3 cells in vitro for transfection efficacy assessment. Out of about 50 different polymer structures, only spermine−dextran of 6000−8000 Da, spermine content of ∼2 μmol/mg, and degree of branching of 25−30% was active in transfecting about 50% of the cells while all other polymers were significantly less active.

Introduction

Gene therapy has been progressively developed with the hope that it will be an integral part of medical modalities.[1] The ultimate goal of gene therapy is to cure both inherited and acquired disorders in a straightforward manner by removing their cause, that is, by adding, correcting, or replacing gene.[2] This notion gave birth to a wide variety of possibilities for true therapeutic approaches for treating human pathological conditions.[3] Gene-delivery vectors are classified routinely as viral and nonviral, and the advantages and disadvantages of each category are well-documented.[4] In designing efficient delivery vehicles for DNA to the nucleus, one should consider the way in which a plasmid becomes active in the cell and tissue. The DNA first has to be protected from DNA-degrading enzymes in the extracellular medium. Then it penetrate the cell membrane and is protected from degrading systems, i.e., lysosome and enzymes, in the intracellular medium until it is internalized in the nucleus, allowing for the insertion of the genetic material in its active form. Finally, it is biodegraded and eliminated from the cell and tissue without causing toxicity.[5−6]

Polycations are a leading class of nonviral gene-delivery systems in part because of their molecular diversity that can be modified to fine-tune their physicochemical properties.[7,8] These polycations are able to condense a large gene into smaller and compact structures and to mask the negative DNA charges, necessities for transfecting most types of cells.[9−12] Polycations used for gene complexation are polyamines that become cationic at physiologic conditions. All polymers contain primary, secondary, tertiary, or quaternary amino groups capable of forming electrostatic complexes with DNA under physiological conditions.[13] The highest transfection activity is obtained usually at a 1.1−1.5 charge ratio of polycation to DNA, respectively.[14] The most studied polycations used for gene complexation and delivery include linear and branched structures, block and graft copolymers. The linear polycations category include diethylaminoethyldextran (DEAE−dextran),[15] poly(vinylpyridine),[16] linear poly(ethylenimine),[17,18] Chitosan,[19] and poly(dimethyl aminoethyl methacrylate).[20−22] Branched backbone polycations include pAMAM dendrimer[23,24] and branched poly(ethylenimine).[18] Block polycations include poly(ethylene glycol)−poly(ethylenimine) of various molecular weights and poly(hydroxypropyl methacrylate)−poly(trimethyl aminoethyl methacryale) copolymers.[25] The last category (i.e., graft copolymers) include a copolymers of various molecular weights poly(L-lysines) and poly(ethylene glycol),[26] dextran,[27] hyaluronic acid,[28] poly(hydroxypropyl methacrylate),[26] or poly(dimethyl aminoethyl methacrylate).[29] All of the above are polycations with a random distribution of the cationic sites along the polymer chains. This randomness is probably the reason for the fact that these polymers may work for some nucleotides and cell types and not for others. Most of these polymers are toxic to cells and nonbiodegradable, while the polymers based on amino acids such as poly(lysines) are immunogenic.[30] More advanced polymeric gene delivery systems employ macromolecules with a very high cationic charge density that act as endosomal buffering systems, thus suppressing the endosomal enzyme activities and protecting the DNA from degradation. The high cationic charge mediates both DNA condensing and buffering capacity that

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diminish the requirement for the addition of endosomolytic agent.[31−33]

In a recent publication,[34] we reported on a new class of biodegradable polycations capable of complexing and administering various genes to many cell lines in relatively high yields. More than 300 different polycations were prepared starting from various polysaccharides and oligoamines having two to four amino groups. These polycations were prepared by reductive amination reaction between primary amines and periodate-oxidized polysaccharides. Although most of these conjugates formed stable complexes with various plasmids as determined by turbidity experiments and ethidium bromide quenching assay, only the dextran−spermine-based polycations were found to be active in transfecting cells in vitro. The purpose of this study was to determine the conditions for the synthesis of dextran−spermine-based conjugates as a function of the following factors: type of conjugation (imine/amine), aldehyde content of the starting dextran, degree of conjugation and molecular weight of conjugates.

Experimental Section

All solvents and reagents were of analytical grade and were used as received. Dextrans with a wide range of molecular weights (9−500 kDa) were obtained from Sigma Chemical Co. (St. Louis, MO). Spermine, potassium periodate and sodium borohydride were obtained from Fluka Chemie (Buchs, Switzerland). A Sage metering pump model 365 (Orion, NJ) was used for slow and reproducible addition of reactants. IR spectra were recorded on a Perkin-Elmer System 2000 FT-IR. NMR spectra were recorded on a Varian 300 MHz instrument using D_2O as solvents. Values were recorded as (ppm) relative to internal standard (TMS). Molecular weights of starting polymers and conjugates were determined on GPC−Spectra Physics instrument (Darmstadt, Germany) containing a pump, column (Shodex KB-804 or KB-803) and refractive index (RI) detector. Average molecular weights were determined according to pullulan standards (PSS, Mainz, Germany) with molecular weights between 5800 and 404 000. Eluents used were 0.05 M $NaNO_3$ for the uncharged polymers and 5% (w/v) Na_2HPO_4 in 3% (v/v) acetonitrile (pH 4) for the cationic polymers. Elemental microanalysis (% N) of the polymers was performed on a Perkin-Elmer 2400/II CHN analyzer.

Oxidation of Polysaccharides. Dextran (10 g, 62.5 mmol of glucose units) of 9 to 500 kDa in average molecular weight, was dissolved in 200 mL of double deionized water (DDW). To this solution was separately added potassium periodate at 1:1, 1:3 or 1:5 mole ratio (IO_4^-/saccharide), and the mixture was vigorously stirred in the dark at room temperature until a clear yellow solution was obtained (6−8 h). The resulting polyaldehyde derivatives were purified from iodate (IO_3^-) and unreacted periodate (IO_4^-) by Dowex-1 (acetate-form) anion exchange chromatography, followed by extensive dialysis against DDW (12 000 cutoff cellulose tubing) for 2 days and at 4 °C. Purified polyaldehyde derivatives were freeze-dried to obtain a white powder in 90% average yield.

$$\text{FT-IR (KBr)} = 1724\ \text{cm}^{-1}\ (\text{C}{=}\text{O})$$

Aldehyde content was determined according to the literature.[35] In brief, 100 mg of oxidized polysaccharide was dissolved in 25 mL of freshly prepared hydroxylamine hydrochloride water solution (0.25 M, pH 4). The mixture was gently mixed overnight at room temperature and titrated with standardized sodium hydroxide solution (0.1 M) to the end point as recorded on a digital pH meter (Hanna, model H8424).

Dextran—Spermine Conjugates (General Method). A solution of oxidized dextran (4−11.25 mmol/g aldehyde groups) in 100 mL of DDW was slowly added during 5 h (sage metering pump) to a basic solution containing 1.25 equimolar amount of spermine (to aldehyde) dissolved in 50 mL of borate buffer (0.1 M, pH 11). The mixture was gently stirred at room temperature for 24 h and dialyzed against DDW (5 × 5 L) at 4 °C, applying 3500 cutoff cellulose tubing (Membrane Filtration Products, Inc., San Antonio, TX) followed by lyophilization to obtain a deep yellow-red imine-based conjugate. The amine-based conjugates (reduced) were obtained after reducing the imine conjugates with excess $NaBH_4$ (1 g, 4 equiv) in water at room temperature for 48 h. The reduction was repeated with additional portion of $NaBH_4$ (1 g) and stirring for 24 h at the same conditions. The resulting light-yellow solution was dialyzed against DDW (5 × 5L) at 4 °C for 2 days followed by freeze-drying to obtain a yellowish reduced amine-based conjugates in 30% overall yield.

FT-IR (KBr, imine-based conjugates): 1640 (C═N) and 3300 cm^{-1} (N−H). FT-IR (KBr) of the amine-based conjugates (reduced) showed the disappearance of the peak at 1640 cm^{-1} (C═N) indicating a complete hydrogenation.

^{1}H NMR (D_2O): 1.645 (m, 4H, dextran−CH_2 NH $(CH_2)_3$ NH $CH_2CH_2CH_2CH_2$ NH $(CH_2)_3$ NH_2), 1.804 (m, 4H, dextran−CH_2 NH $CH_2CH_2CH_2$ NH $(CH_2)_4$NH CH_2CH2CH_2 NH_2), 2.815 (m, 14H, dextran−CH_2 NH $CH_2CH_2CH_2$ NH $CH_2CH_2CH_2CH_2$ NH CH_2CH2CH_2 NH_2), 3.52−4.19 (m, glycoside hydrogens), and 5.02 (m, 1H, anomeric hydrogen).

Determination of Primary Amines by the TNBS Method. The primary amine content was determined according to standard protocol with a slight modification.[36] In brief, a total of 20 μL of freshly prepared aqueous TNBS solution (15 mg/mL) was separately added to marked tubes containing up to 0.2 μmol spermine (or other soluble oligoamine) dissolved in 600 μL of DDW. The mixtures were separately diluted with 200 μL of sodium bicarbonate buffer (0.8 M, pH 8.5), vortexed for 1 min, and incubated for 2 h at 37 °C. Then, 600 μL of 1.0 N HCl was added to each tube, vortexed for 1 min and gently sonicated for 2 min to remove bubbles. Absorbances of samples were recorded at 410 nm. A sample containing the same composition (without the spermine) was used as reference in the absorbance measurements. Weighed conjugates (100−500 μg, depending on the degree of conjugation) were treated as above and the primary amino content was calculated according to the calibration curve.

In Vitro Transfection. Transfections with polycations were performed and analyzed using expression plasmid pCMV-GFP containing GFP gene under the control of cytomegalovirus long terminal repeat enhancer/promoters. 0.5 μg of purified plasmid (Qiagen kit) per well of transfected cells was mixed with dextran−spermine at a variety of charge ratios, from 1 to 0.025 ∓ (phosphate/nitrogen, respectively). The required amount of a polycation for the complexation with 0.5 μg of DNA (1.54 nmol, phosphate groups) at a certain charge ratio was calculated according to the formula

$$V\ (\mu\text{L}) = 1.54 \times 14 \times 100/(\%\ \text{N} \times \text{ratio}(\mp)C)$$

where V (μL) is the volume of polycation solution (in μL) needed for the complexation, % N, the amino content of a particular polycation as found by elemental microanalysis (expressed in percentage numbers), ratio(∓), the complex charge ratio (phosphate/nitrogen) and C, the concentration of polycation in double-deionized water. Here 1000 ng/μL concentrations were applied for all polymers.

The DNA/polycation complexes at a particular charge ratio were diluted to a final volume of 200 μL and allowed to stand at room temperature for 30 min. Then, 24-well plates were seeded 24 h before the transfection with 1.5×10^3 cells per well (NIH3T3 and HEK293), and were washed with serum-free medium (SFM), and 200 μL of the complexes containing 0.5 μg of DNA in SFM were added to each well of cells and incubated for 4 h at 37 °C. The medium containing the complexes was then replaced with standard growth medium (10% FCS). The cells were incubated in culture for 48 h and the cell wells were analyzed for gene expression using fluorescence microscope instrument (model Axiovert 35, Zeiss, Jena, Germany). The yield of transfection (% transfection) was calculated by counting the fluorescent cells in a certain area

Table 1. Aldehyde Content and Average Molecular Weight of Representative Polysaccharides and Their Aldehyde Derivatives

polysaccharide[a]	M_w(starting)[b]	KIO_4:glucose[c]	% aldehyde (mmol/g)[d]	M_w(oxidized)[e]
dextran (9300)	9845	1:1	11.25 ± 0.50	6780
dextran (18300)	15 665	1:1	9.38 ± 0.60	10 890
dextran (39100)	35 480	1:1	6.88 ± 0.60	24 960
dextran (39100)	35 480	1:3	2.75 ± 0.38	28 430
dextran (39100)	35 480	1:5	1.50 ± 0.40	29 960
dextran (70000)	52 610	1:1	4.63 ± 0.50	48 250
dextran (500000)	380 000	1:1	4.00 ± 0.63	365 000

[a] Dextrans (Sigma) and their average molecular weights (in parentheses). [b] Average molecular weight of starting polysaccharides as determined by GPC. [c] Mole ratio of reactants (periodate to anhydrous glucose units). [d] % Aldehyde content (mmol/g) as determined by the hydroxylamine hydrochloride method ($n = 3$). [e] Average molecular weight of oxidized derivatives (GPC).

of a particular well, and dividing the number of fluorescent cells by the number of total cells in the same field.

Transfast lipid was used as control reference applying the manufacturer's instructions and can be found at *www.promega.com* (product E2431).

For cell-transfection with calcium phosphate, 0.5 μg of pCMV-GFP was diluted with 450 μL of DDW and 50 μL of 2.5 M $CaCl_2$ was added. This solution was added dropwise to 500 μL of 20 mM HBS (hepes buffer saline) with gentle air bubbling. The solution was allowed to stand at room temperature for 20 min and added to the cell cultures without medium. Then, 6−18 h post transfection, cells were washed with PBS and 1 mL of complete growth medium was added to each cell well. Transfection efficacy was evaluated by fluorescence microscope as described earlier.

For the transfection of cells in glycerol shock, we used the following procedure: on the day of transfection, the cell medium in wells was removed, and 50 μL of 20% glycerol solution in PBS was added to each cell well. After 1 min, the glycerol solution was removed, and cell wells were washed twice with SFM. After this, complexes were added and the transfection was continued as described earlier for the polycation−DNA complexes.

Results and Discussion

Chemistry. Dextran polysaccharides with various molecular weights were separately oxidized under similar conditions using potassium periodate to obtain the corresponding dialdehyde derivatives. These activated polymers were purified first by anion exchange chromatography (DOWEX-1, acetate form, pH 7) following extensive dialysis against double-distilled water and finally lyophilization to obtain a white powder in 90% overall yields. Table 1 shows the aldehyde content and average molecular weights of starting and oxidized dextrans. Low molecular weight dextrans (i.e., 9.3 and 18.3 kDa) resulted in high aldehyde content (11.25 and 9.38 mmol/g, respectively) with marked decrease in average molecular weights. Dextran with starting average molecular weight of 36 kDa was similarly oxidized at 1:1 to 1:5 mole ratios (KIO_4/saccharide units). At 1:1 mole ratio, 6.88 mmol/g of aldehyde groups were obtained with a decrease to 25 kDa average molecular weight. The lower ratio of KIO_4 (i.e., 1:3 and 1:5 mole ratio) oxidized the polymer as expected to lesser extent (2.75 and 1.5 mmol/g, respectively) with minimum chain scission. High molecular weight dextrans (70 and 500 kDa) gave a lower degree of oxidation (4.63 and 4 mmol/g, respectively) with minimum chain scission. These results indicate that dextrans with high range molecular weight are less accessible to oxidation probably due to entanglements in polymer structure.[37]

Polycations (dextran−spermine-based conjugates) were prepared by reacting the naturally occurring tetramine (spermine) with oxidized dextrans in aqueous medium by means of a reductive-amination reaction. An aqueous solution of the corresponding oxidized dextran at known average molecular weight and aldehyde content was added dropwise to a buffered aqueous solution (0.1 M borate, pH 11) containing spermine at 1.25 equiv excess to aldehyde groups. A sage-metering pump was used to maintain a slow and reproducible rate of addition. The purpose of the slow addition was to minimize cross-linking and to facilitate grafting of oligoamine moieties onto the polymer backbone (Scheme 1). After the reaction was completed (24 h), conjugates were either dialyzed and lyophilized to obtain the imine-based conjugates or in parallel reduced by the addition of excess borohydride and stirring at room temperature for 48 h. Additional amounts of borohydride were added, and the reduction was continued for another 24 h at the same conditions. The reduced amine-based conjugates were similarly dialyzed extensively against double-distilled water and lyophilized to dryness.

Oxidized dextran having different molecular weights and aldehyde content were reacted under similar conditions with spermine (Table 2). Each particular conjugate was isolated and purified in unreduced (imine) and reduced (amine) form. All synthetic polycations were characterized by nitrogen elemental analysis, primary amino content (TNBS) and average molecular weights utilizing the GPC technique. Table 2 shows that dextran polysaccharides with relatively low molecular weights (6.8−24.9 kDa) and high aldehyde content (7−11 mmol/g) resulted in a high degree of conjugation (11.2−11.8% N) in both imine- and amine-based conjugates. High molecular weight polymers (53 and 380 kDa) having relatively low aldehyde content (4.63 and 4 mmol/g, respectively) resulted in low degree of conjugation (5.75 and 7.85% N, respectively). Reductive-amination between oxidized dextran and oligoamines are considered to be nonreproducible due to random branching of the oligoamine moieties onto the polymer backbone chains. The degree of branching was determined according to the following equation:

$$\%\ \text{branching} = [(\text{spermine(total)} - \text{spermine(graft)})/\text{spermine(total)}] \times 100$$

where spermine(total) refers to the total spermine content calculated from the % N (elemental analysis) and spermine(graft) refers to the grafted spermine content calculated from the primary amino content (TNBS). At high aldehyde content (6.88−11.25 mmol/g), high branching spermine (30−37%, Table 2) moieties were obtained in complement with 63−70% of grafted spermine. A lower aldehyde content (4 and 4.63 mmol/g) resulted in low branching spermine (0−20%) moieties

Scheme 1. Grafting of Spermine Moieties on Dextran Polysaccharide

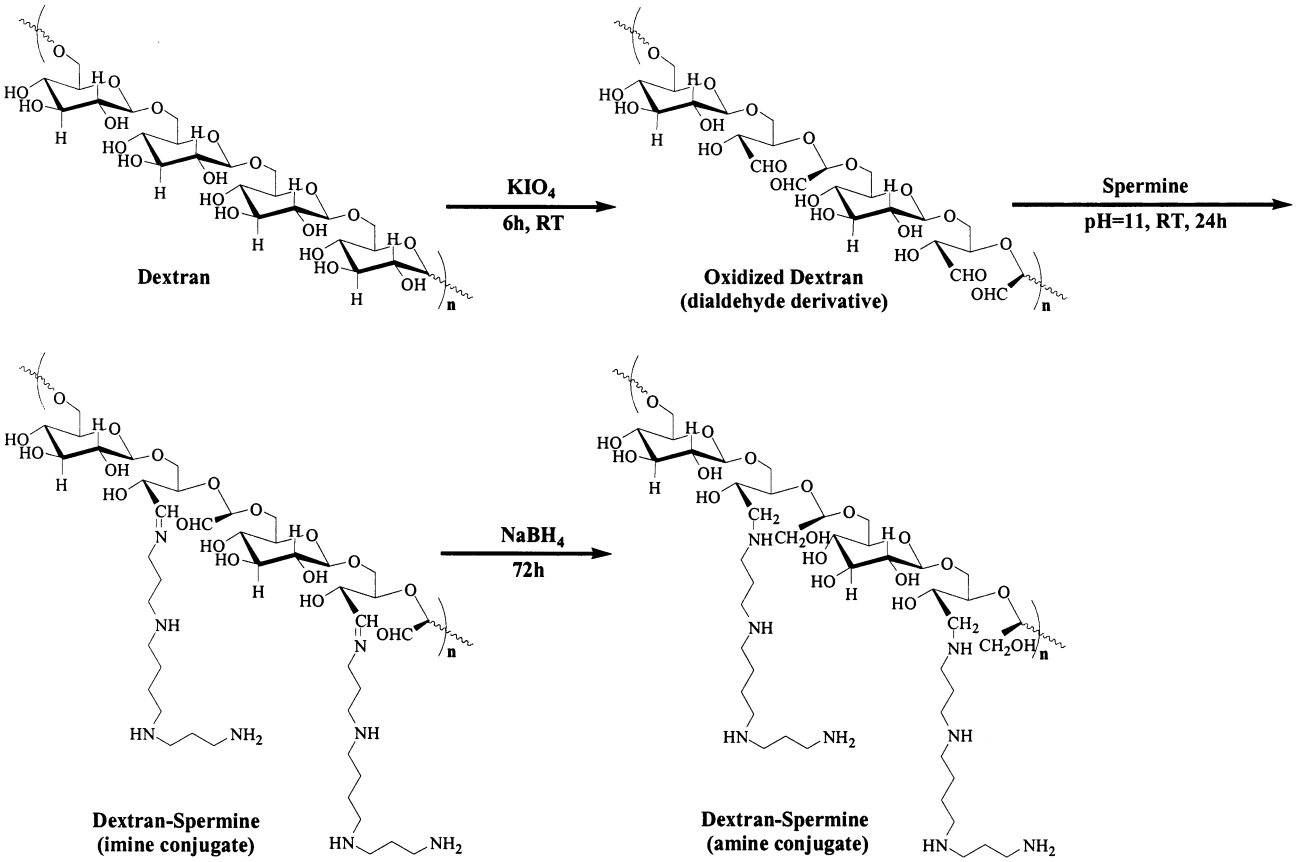

Table 2. Grafting of Spermine on Oxidized Dextran of Various Molecular Weights[a]

dextran (oxidized),[b] kDa	% aldehyde (mmol/g)	type of conjugate	tot. spermine content		grafted spermine content (μmol/mg)[d]	% branching[e]
			% N	μmol/mg[c]		
6.8	11.25 ± 0.50	amine	11.52	2.05	1.35 ± 0.20	34
		imine	11.76	2.10	1.46 ± 0.10	31
10.9	9.38 ± 0.60	amine	11.80	2.10	1.47 ± 0.14	30
		imine	11.96	2.13	1.36 ± 0.20	36
24.9	6.88 ± 0.60	amine	11.19	2.00	1.25 ± 0.20	37
		imine	11.34	2.02	1.30 ± 0.05	36
48.3	4.63 ± 0.50	amine	7.85	1.40	1.20 ± 0.16	14
		imine	7.95	1.42	1.12 ± 0.08	21
365	4.00 ± 0.63	amine	5.75	1.03	1.05 ± 0.16	3
		imine	5.85	1.04	1.01 ± 0.10	0

[a] Dextran−spermine were obtained from the reaction of oxidized dextran with spermine (1.25 mole ratio, spermine/aldehyde) in buffer borate pH 11 at room temperature for 24 h. Hydrogenation of imine to the stable amine conjugates was achieved by reacting the imine with large excess of $NaBH_4$ at room temperature for 72 h. [b] Starting average molecular weights of oxidized dextran (GPC). [c] Calculated amount of total spermine content determined from % N. [d] Amount of grafted spermine content determined from the primary amino content (TNBS, $n = 3$). [e] % Branching is the calculated percentage of spermine moieties reacting with two aldehyde groups (cross-linked).

in complement with 80−100% grafting. This could be explained by the fact that high aldehyde-content polysaccharides are statistically more accessible for branching than in low aldehyde-content polymers, which result in one spermine reacting with two aldehyde groups.

In an experiment oxidized dextran (M_w = 24.9 kDa, 6.88 mmol/g of aldehyde groups) was allowed to react under similar conditions with increasing amount of spermine ranging from 0.5/1 to 2/1 mole ratios (spermine/aldehyde, respectively). Table 3 shows that at large excess of spermine (G1-43A to G1-43C), high % N was obtained with relatively low branching spermine (25−30%) moieties. Lower spermine ratios (G1-43D and G1-43E) resulted as expected in low % N (7.68 and 6.84%, respectively) and in high branching spermine (~70%). This also could be explained by the fact that high spermine content allow saturation of the majority of the aldehyde groups and therefore in high degree of grafting. Differences in % N and primary amino content above the 1.25 mole ratio (spermine/aldehyde) were found to be negligible, indicating full reaction of aldehyde groups. On the basis of these results, 1.25 mole ratio (spermine/aldehyde) was chosen for future synthesis.

The degree of conjugation was tested also as a function of pH and temperature of medium. In an experiment, conjugates were similarly prepared starting with oxidized dextran (M_w = 24.9 kDa, 6.88 mmol/g of aldehyde groups) and spermine at 1.25 mole ratio (spermine/aldehyde). Three different pH values (7.4, 9 and 11) and two different temperatures (4 and 25 °C) were used for all synthesis. Table 4 summarizes the % N and primary amino content of conjugates obtained under these variable conditions. At neutral pH (7.4)

Table 3. Effect of Mole Ratio (Spermine/Aldehyde) on the Degree of Conjugation and Branching[a]

code	mole ratio[b]	tot. spermine content		grafted spermine content	M_w[c]	% branching
		% N	μmol/mg	(μmol/mg)		
G1−43A	2/1	12.77	2.27	1.60 ± 0.22	5980	30
G1−43B	1.5/1	11.40	2.03	1.52 ± 0.15	6100	25
G1−43C	1.25/1	11.2	2.00	1.45 ± 0.12	6800	27
G1−43D	0.75/1	7.68	1.37	0.48 ± 0.05	11 890	65
G1−43E	0.5/1	6.84	1.22	0.37 ± 0.10	23 450	70

[a] Dextran−spermine were obtained from the reaction of oxidized dextran (24.9 kDa, 6.88 mmol/g aldehyde groups) with increasing amount of spermine at 2:1 to 0.5:1 mole ratio (spermine/aldehyde, respectively) in borate buffer (0.1 M, pH 11) at room temperature for 24 h. Hydrogenation of all conjugates was achieved by reacting the conjugates with excess of borohydride as previously described. [b] Initial reactant mole ratios (spermine/aldehyde groups). [c] Average molecular weights of dextran−spermine conjugates as determined by GPC for the polycationic materials (Experimental Section).

Table 4. Effect of pH and Temperature of Medium on Conjugation[a]

code	reaction conditions		tot. spermine content		grafted spermine content (μmol/mg)	M_w
	temp (°C)	pH	% N	μmol/mg		
G1-42A	4	7.4	5.85	1.05	0.65 ± 0.05	19 760
G1-42B	25	7.4	6.7	1.19	0.64 ± 0.05	18 950
G1-41A	4	9	7.45	1.33	0.95 ± 0.12	18 650
G1-41B	25	9	9.25	1.65	1.05 ± 0.05	11 280
G1-40A	4	11	8.84	1.58	1.10 ± 0.10	12 430
G1-40B	25	11	10.84	1.93	1.35 ± 0.10	7180

[a] 1.25 mole ratio (spermine/aldehyde) was used in all synthesis. A 0.1 M aqueous boric acid buffer was used, and the pH was adjusted with 1 N NaOH aqueous solution.

relatively low conjugation was obtained both at 4 and 25 °C. At pH 9 moderate increase in conjugation was obtained at 4 °C and significant increase at 25 °C. The highest degree of conjugation was obtained at pH 11 at room temperature (G1-40B, Table 4). Reaction of highly oxidized polysaccharides with multiamino containing molecules is believed to enhance degradation of the polysaccharide chains due to extensive aminolysis of glycoside linkages.[38] Table 4 also shows that all conjugates exhibit lower average molecular weights in comparison to starting dextran. At 25 °C and pH 11, drastic decrease in average molecular weight was observed (G1-40B). In contrast, when moderate temperature and pH were applied less chain scission and slight decrease in molecular weights were observed (G1-41A and G1-42A).

Biological Results. The efficiency of transfection of the synthetic vectors was evaluated as a function of the following factors: type of conjugate, charge ratio, spermine content, and molecular weights of vectors.

Type of Conjugate. Over 50 cationic conjugates were prepared starting from oxidized dextrans of various molecular weight and aldehyde contents and spermine as the oligoamine. These cationic polymers were tested for their transfection activity in HEK-293 cells using pCMV-GFP as the marker gene. Each single polymer was tested at a wide range of charge ratios (∓, DNA/polymer) from 1 to 0.025. Figure 1 summarizes a series of transfection results applying this system (HEK293/GFP). The first limiting barrier for transfection is the penetration of complexes through the cell membrane.[5,6] The purpose of this transfection experiment was to evaluate the efficacy of each conjugate to

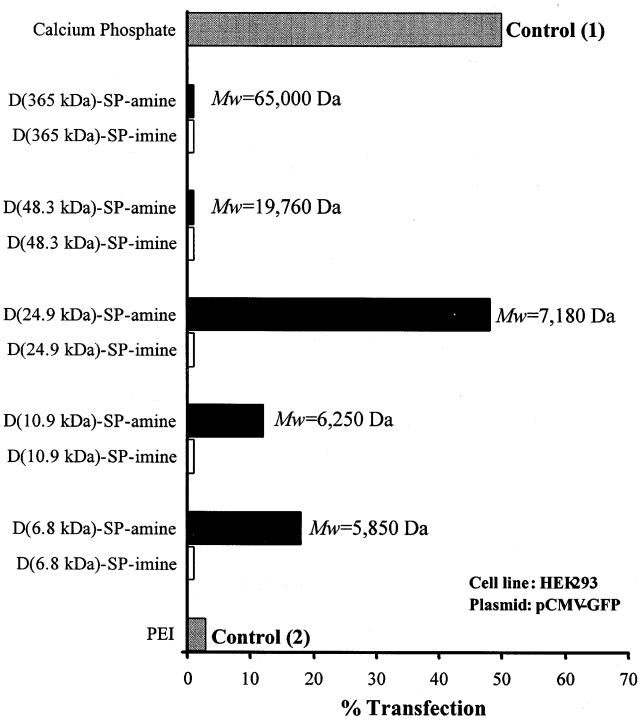

Figure 1. pCMV-GFP transfected HEK293 cells applying various dextran−spermine-based conjugates. Cells were pretreated with glycerol to enhance the transfection as described in experimental. Abbreviations: D(6.8 kDa)-SP-imine, dextran−spermine-based conjugate prepared from oxidized dextran of initial average molecular weight of 6.8 kDa, etc; imine (unreduced) and amine (reduced), to the type of bond between the spermine moieties and the polymer backbone; PEI, polyethylene imine (600 Da, Sigma). Conjugate characteristics (i.e., % N, primary amino content and %branching) are summarized in Table 2. Average molecular weights (M_w) of reduced conjugates were determined by GPC.

transfect cells with a corresponding gene independently of their ability to penetrate the cell membrane. Therefore, all cell wells were pretreated before transfection with 50 μL of 20% glycerol in PBS for 1 min following rapid wash with SFM. The purpose of the glycerol shock was to facilitate the uptake of complexes to cell cytoplasm.[39] Calcium phosphate precipitating technique (control 1, Figure 1) and polyethylene imine (PEI) of 600 Da in average molecular weight (control 2, Figure 1) were used as references in this transfection experiment. Figure 1 shows that all imine-based conjugates (unreduced) gave no transfection at all tested charge ratios. PEI600 (control 2) gave a low and negligible transfection yield (∼2%) in comparison to the calcium phosphate reagent (control 1), which caused transfection of approximately 50% of the cells. Amine-based conjugates (reduced) prepared from oxidized dextran of low molecular weights (6.8 and 10.9 kDa) showed relatively medium transfection yields (14 and 18%, respectively). The highest transfection was obtained with the spermine conjugate prepared from oxidized dextran of starting molecular weight of 24.9 kDa (Figure 1). Applying this polycation as gene vector resulted in similar transfection yield (∼50%) to the calcium phosphate control. Amine-based conjugates of high molecular weight dextrans (i.e., 48.3 and 365 kDa) gave no transfection at all tested charge ratios. Average molecular weights of the active conjugates were determined by GPC and found to be in the range of 7−10 kDa. Higher molecular weight conjugates (i.e., >20 kDa)

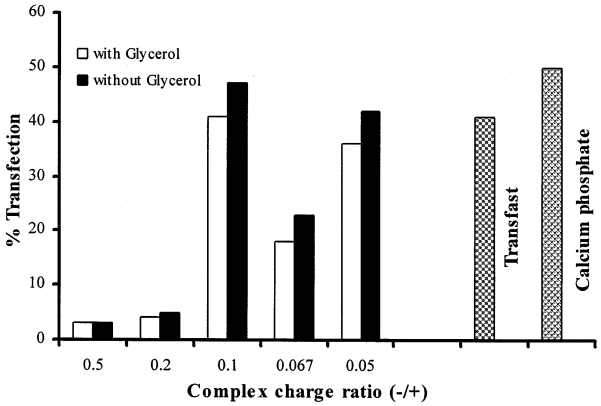

Figure 2. Effect of charge ratio (∓) and glycerol on the transfection efficiency of active dextran−spermine-based conjugate. HEK293 cells and pCMV-GFP marker gene were used for the transfection experiment.

resulted in low and negligible transfection yields (Figure 1).

Effect of Charge Ratio (∓) and Glycerol Shock on the Transfection Yield. According to the transfection results shown in Figure 1, it was decided to focus on the most active conjugate (D(24.9 kDa)-SP-amine) for further transfection experiments. This polycation was tested in similar transfection experiment (pCMV-GFP/HEK293) with glycerol shock to the cells and without glycerol shock (Figure 2). Low transfection yields were obtained at 0.5 and 0.2 charge ratios (∓) both in the presence of glycerol or without glycerol. At 0.1−0.05 charge ratios, high GFP expression was monitored similarly to the control reagent, calcium phosphate. Transfast, a cationic lipid-based transfecting agent, was also used as second control and showed a similar transfection activity to the active polycation (dextran−spermine) and calcium phosphate reagent. It should be noted that no significant differences in transfection efficiencies were obtained with or without glycerol shock, which indicates that the polycation has the capability of delivering the plasmid into untreated cells probably by endocytosis mechanism.

Figure 3 shows a typical fluorescence imaging of pCMV-GFP transfected HEK293 and NIH3T3 cells, which shows that Transfast (part A) and dextran−spermine-based conjugate (parts B and D) possess a strong expression of GFP (∼50% transfection) in HEK293 cells and ∼30% transfection in NIH3T3 cells similarly to calcium phosphate control (part C).

Effect of Spermine Content on Transfection. Dextran−spermine-based conjugates were prepared from dialdehyde dextran (M_w = 24.9 kDa, 6.88 mmol/g of aldehyde groups) and an increasing amount of spermine (Table 3). Figure 4 shows a series of in vitro transfection experiments applying pCMV-GFP as the marker gene both in HEK293 and NIH3T3 cells. At 1:2 to 1:1.25 mole ratios (spermine/aldehyde), similar transfection yields were obtained which were similar to the control, calcium phosphate. Lower mole ratios (1:1 to 1:0.5, spermine/aldehyde) resulted in gradual decrease in transfection yields both in HEK293 and NIH3T3 cell lines. Differences in the transfection efficiencies above the 1.25:1 mole ratio (spermine/aldehyde) were found to be negligible at all tested charge ratios. On the basis of these results, 1.25:1 (spermine/aldehyde) was used as the optimal mole ratio for the conjugate synthesis.

Conclusions

This work describes the formation of water-soluble polycations and their effectiveness as gene transfection

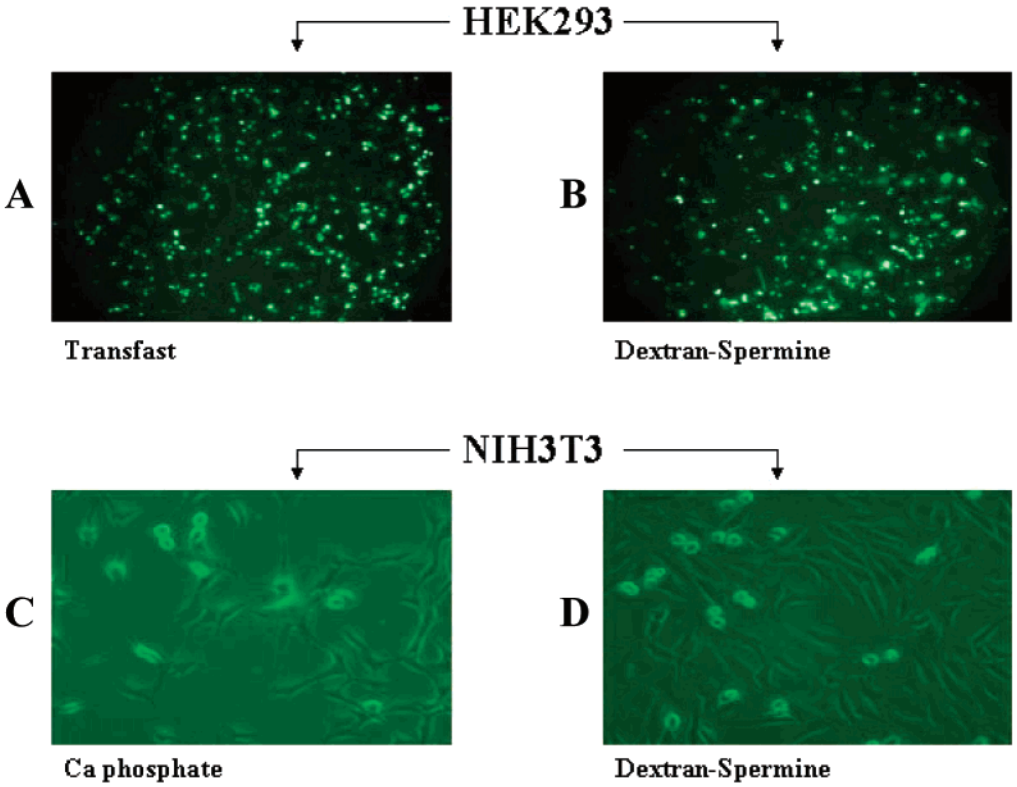

Figure 3. Inverted fluorescent microscope of pCMV-GFP transfected HEK293 and NIH3T3 cells. Parts A and B represent the Transfast control and dextran−spermine (respectively) transfected HEK293 cells. Parts C and D represent the calcium phosphate precipitating technique and dextran−spermine (respectively) transfected NIH3T3 cells.

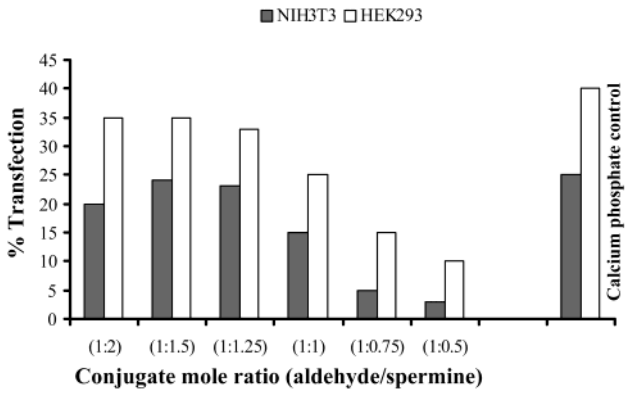

Figure 4. In vitro transfection of dextran−spermine-based conjugates prepared with increasing amount of spermine (0.5/1 to 2/1 mole ratios, spermine/aldehdye). Transfected cells were NIH3T3 (■) and HEK293 (□) applying the pCMV-GFP marker gene. Conjugates characteristics are summarized in Table 3.

vectors. Dextrans of molecular weight of 10−380 kDa were oxidized by KIO_4 at various degrees to obtain the respective polyaldehydes. The oxidation was under mild condition resulting in minimal changes in molecular weights. The degree of oxidation was correlated with the ratio of periodate to saccharide units along the dextran molecules. Also, it was found that the degree of oxidation decreases with the increase in molecular weight probably due to entanglement in polymer structures of high molecular weight dextrans. Spermine was reacted with the oxidized dextran at different spermine/aldehyde mole ratios under different temperatures and pH of the buffer solutions. The obtained imine conjugates were hydrogenated to the amine bond by $NaBH_4$ at room temperature. Spermine was conjugated at high yields with over 55−60% of the aldehyde reacted.

All polymers were water-soluble and formed a complex with DNA plasmids of particle size ranging from 150 to 300 nm (data not shown). The imine derivatives as well as high molecular weight polymers were inactive as gene transfection carriers. Only the polycation of 6000−8000 Da with high degree of spermine conjugation showed high transfection yields (∼50% of the cells) which was similar to the references, calcium phosphate and Transfast transfecting reagents. The transfection was efficient without the need for pretreatment of the cells with glycerol to improve cell-membrane penetration.

This study indicates the specificity of the polymeric transfection agent, where only oligoamines of four amino groups conjugated to a short chain dextran (5−10 kDa) at a high grafting yield are effective. Thus, the imine derivatives were inactive because the side chain is of three cationic groups as the imine bond is not cationic. Similar indications were reported in our previous publication.[34] Current studies focus on understanding the specificity found in the compounds by means of physical characterization of the polycation−DNA complex and trafficking studies.

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