Polysaccharide−Oligoamine Based Conjugates for Gene Delivery

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This work describes a versatile and universal polycation system based on oligoamines grafted on natural polysaccharides that is capable of complexing various plasmids and administering them into various cells in high yield to produce a desired protein. These polycations are expected to better meet the requirements for effective complexation and delivery of plasmid or an antisense and to biodegrade into nontoxic components at a controlled rate. The developed biodegradable polycations are based on spermine, a natural tetramine, conjugated to dextran or arabinogalactan. These polycations were prepared by reductive amination of oxidized polysaccharides with the desired oligoamines. The Schiff base conjugates thus obtained were reduced to the stable amine conjugates by sodium borohydride. Over 300 different polycations were prepared starting from various polysaccharides and oligoamines, mainly oligoamines of two to four amino groups. Although most of these conjugates formed stable complexes with various plasmids as determined by turbidity experiments, only a few polycations were found to be active in transfecting cells. This work indicates that the structure of the polycation plays a significant role in the transfection activity of polycations.

Introduction

Gene therapy can be broadly defined as the introduction of genetic material (genetically engineered cells, gene, DNA) into a cell or tissue for the production of a needed peptide or protein. The aim is enhancement of gene expression or inhibition (by antisence approaches), repair of a defective gene, or replacement of a disease gene by a healthy gene for the ultimate purpose of preventing or altering a particular disease state.[1] Gene therapy has potential applications in the treatment of many genetic disorders such as cystic fibrosis, hemophilia, many forms of cancer, and chronic infectious diseases such as allergy and AIDS.[2]

Over the past 10 years a number of synthetic[3] and viral vectors[4] have been developed for the delivery of transgenes, which have potential therapeutic effects in treating malignant tumors in the central nervous system (CNS). Several gene transfer systems have been described for the local treatment of cancer, including ex vivo gene transfer with cytokines or major histocompatibility genes to induced immune-mediated tumor regression[5−7] and drug susceptibility genes to induce "suicide" of tumor cells.[7] Among the gene delivery systems currently used, the retroviral-mediated gene transfer and the adenovirus vectors are the most common techniques.[8] Although most research in vivo in gene therapy has focused on the use of recombinant virus vectors,[9] progress has been made toward developing nonviral formulations of gene for in vivo human gene therapy.[10,11] The advantages of nonviral vectors are that they can introduce DNA into nondividing cells, do not integrate into the chromosome, do not possess infective risk, and are potentially less expensive than viral vectors.[12] The principle underlying nonviral gene delivery is that the problem of delivering DNA in vivo is not significantly different from the problem of delivering conventional drugs or biological products to intracellular compartments in the body. Nonviral gene therapies involve known drug delivery methods for the administration and targeting of genes to selected cells in vivo, where they express therapeutic products.[13]

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 physiologic conditions. The highest transfection activity is obtained usually at a 1.1−1.5 charge ratio of polycation to DNA, respectively.[14] The most studied polyamines for gene transfer include poly-L-(lysine) and its derivatives,[15] polyamidoamine starburst dendrimers,[16] polyethyleneimines,[17] natural and modified polysaccharides,[18] and acrylic cationic polymers.[19] Polycations may be more versatile for use than the liposomes and other conventionally used spherical gene carriers. Several polycations have been reported to induce gene expression, for example, diethylaminoethyl dextran and other cationized polysaccharides.[14,18] These polymers have little structural similarity with each other except possessing cationic groups.

The polymers described in the literature[10−19] include linear or dendrimeric polymers with a random distribution of amino groups, which are part of polymer backbones such as poly(ethyleneimine),[17] poly(amidoamine) dendrimers,[16] and poly(alkylaminoglucaramide)[20] or

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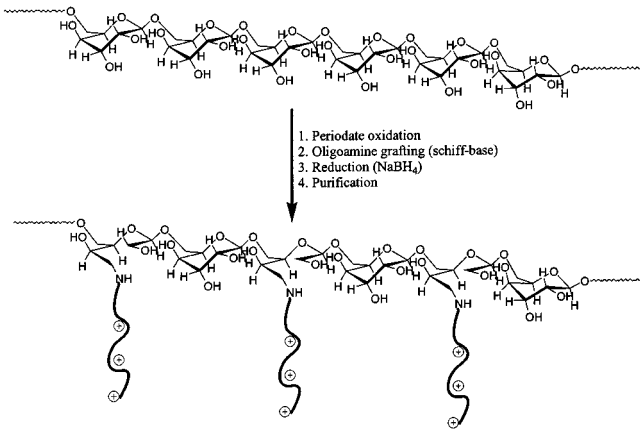
Scheme 1. Grafting Concept of Various Oligoamines on Polysaccharide



part of linear polymers with a single primary, secondary, or tertiary amino group attached to polymer units such as poly(dimethylaminoethyl methacrylates),[19] dimethylaminodextran,[18] and poly(L-lysines).[15] 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.[21]

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 enzymes activity and protecting the DNA from degradation. The high cationic charge mediates both DNA condensing and buffering capacity that diminish the requirement for the addition of endosomolytic agent.[22]

In designing universal polycation systems for gene delivery, one should consider the way in which a plasmid becomes active in the cell and tissue. The plasmid first has to be protected from DNA-degrading enzymes in the extracellular medium. Then it penetrates the cell wall 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.[23]

We now report on a range of biodegradable polycations based on grafted oligoamine residues on natural polysaccharides, which are effective in delivering plasmids for a high biological effect. The grafting concept where side chain oligomers are attached to either a linear or a branched hydrophilic polysaccharide backbone (Scheme 1) allows two- or three-dimensional interaction with an anionic surface area typical of a double- or single-strand DNA chain. This type of flexible cationic area coverage is not available with nongrafted polycations or low molecular weight cations.[24] Low molecular weight cations and their lipid derivatives such as the lipofectin and lipofectamine have a localized effect on the DNA, in which the degree of complexation is dependent on how these small molecules organize around the anionic DNA.[24] Each molecule has to be synchronized with the other molecules at all times of the transfection process, whereas when the oligoamines are grafted on a polymer, they are already synchronized and each side chain helps the other side chain to be arranged to fit the anionic surface of a given DNA.[25] Grafting the functional groups as an average distribution along a polymer chain at a certain distance from each other (for example, grafting an oligoamine chain every one, two, three, or four monomer units) may provide optimal complexation with various DNAs. The use of biodegradable polysaccharide carriers is especially suitable for transfection and biological applications because they are water soluble, they can be readily transported to cells in vivo by known biological processes, and they act as an effective vehicle for transporting agents complexed with them.[26]

Chemistry

Oxidized arabinogalactan, dextran, and pullulan at various degrees of oxidation were obtained by reacting the desired polysaccharide in water with potassium periodate at 1:1, 1:3, and 1:5 (KIO_4/anhydrous glucose) mole ratios. The resulting polyaldehydes were purified from iodate and unreacted periodate ions by DOWEX-1 anion exchange chromatography (acetate form, pH 7), followed by extensive dialysis against DDW (12 000 cutoff cellulose tubing) and lyophilization to dryness. The aldehyde content of oxidized polymers was determined by the hydroxylamine hydrochloride method.[27] Average molecular weights of starting polymers and derivatives were determined by GPC using pullulan standards (Table 1).

Table 1 shows that oxidation with periodate at a 1:1 mole ratio of dextran to pullulan yielded a 50−60% oxidation of the saccharide units with a marked decrease in molecular weight. The lower ratio of KIO_4 oxidized the two polymers to a lesser extent with minimum chain scission. Oxidation of arabinogalactan under similar conditions yielded less oxidation with minimal change in the molecular weight, as observed. The difference in the degree of oxidation between the polymers relies on the polysaccharide structures. Dextran and pullulan are linear chains with glucose units connected by 1,6-[26] and 1,4-glycoside bonds, respectively.[27] These bonds allow oxidation of the polymer backbone, resulting in high dialdehyde formation with some chain session. Arabinogalactan units are connected by 1,3-glycoside bonds,[29] which are stable to oxidation; thus, oxidation takes place only in the branched chains.

An aqueous solution of the desired polyaldehyde was added dropwise to a basic solution (pH 11) containing 0.5−2 equimolar amount of diamine (commercial oligoamines of two to four amino 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 diamine moieties onto the polymer backbone (Scheme 2).

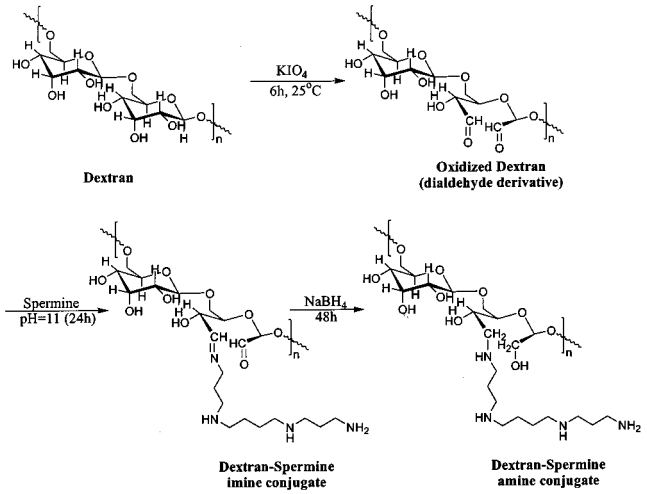
After the conjugation was completed, polymers were reduced by the addition of excess borohydride with stirring at room temperature for prolonged periods (48−72 h). In some cases, additional amounts of borohydride were added to ensure sufficient reduction. Oxidized polymers having different molecular weights and alde-

Table 1. Aldehyde Content and Average Molecular Weights of Oxidized Dextran, Arabinogalactan, and Pullulan

	dextran[a]		arabinogalactan[b]		pullulan[c]	
KIO_4/glucose units (mole ratio)	% oxidation	M_w (GPC)	% oxidation	M_w (GPC)	% oxidation	M_w (GPC)
1:1	48	24 960	32	21 960	62	10 410
1:3	20	28 430	12	20 540	26	16 080
1:5	12	29 960	6	20 800	13	29 620
initial polysaccharide	0.4	37 460	0.6	22 000	0.4	46 000

[a] Commercial dextran with initial molecular weight of 40 kDa. [b] Arabinogalactan (19 kDa). [c] Pullulan (50 kDa). Molecular weights of starting polysaccharides and derivatives were determined by GPC using 0.05 M $NaNO_3$ as eluent. % Oxidation was determined by the hydroxylamine hydrochloride method as described in Experimental Section. Values were recorded as percentages of dialdehyde groups per 100 glucose units.

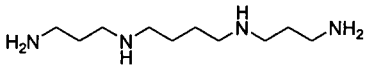
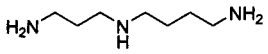
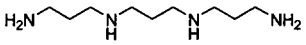
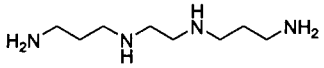
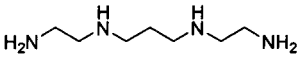
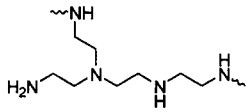
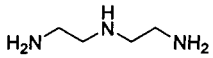
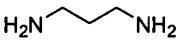
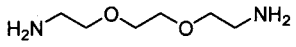
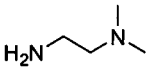
Scheme 2. General Synthesis of Polysaccharide−Oligoamine Conjugates (Dextran−Spermine)



hyde content were reacted under similar conditions with various oligoamines (Table 2). When reactants were directly mixed, insoluble gels were frequently obtained because of high cross-linking between the polyaldehdyes and the diamines. All synthetic polycations were characterized by nitrogen elemental analysis, primary amino content (TNBS), ^1H NMR (D_2O), and average molecular weights (GPC). Reduced conjugates were purified by extensive dialysis against DDW and lyophilization. The resulting polymers were found to be highly soluble in water and in a mixture of water and several organic solvents (i.e., DMF, DMSO, and THF). Group A in Table 3 represents three different conjugates prepared under similar conditions starting with dextran (40 kDa) having different aldehdye content. As expected, a high degree of oxidation resulted in a high yield of conjugation (10.78% N and 1.25 μmol/mg primary amine), and a lower degree of oxidation (D(1:3) or D(1:5)) resulted in decreasing nitrogen and primary amino content. Conjugates prepared from dextran (40 kDa, 50% oxidation) and increasing amounts of spermine are summarized in group B (Table 3), which indicates that high molar ratios (oligoamine/aldehyde, 1.25−2.0) resulted in high nitrogen (11.2−12.78% N) and primary amino content (1.45−1.60 μmol/mg). Differences in nitrogen and primary amino content above the 1.25 molar ratio (oligoamine/aldehdye) were found to be negligible, indicating full saturation of the aldehyde groups. On the basis of these results, 1.25 (oligoamine/aldehdye) was chosen for all optimal syntheses. In group C (Table 3), a wide range of molecular weights of dextran polymers (18−500 kDa) was separately oxidized under similar conditions by an equal amount of periodate followed by reaction with spermine at 1.25 mole ratio (spermine/aldehye groups). Oxidized derivatives of dextrans with relatively low molecular weights (9.3−40 kDa) resulted in high aldehdye content and therefore in a high degree of conjugation (11.2−11.8% N). High molecular weights polymers (75 and 500 kDa) resulted in relatively low aldehdye content (32 and 38%, respectively) and therefore in low conjugation (5.75 and 7.85% in N content). These results indicate that dextrans with high-range molecular weights are very stable to oxidation probably because of entanglements in polymer structure.[30]

Conjugates containing dextran polysaccharide (M_w = 40 kDa) and various oligoamines were similarly prepared under the same conditions (Tables 2 and 4). Reaction between oxidized dextran (∼50% dialdehdye) and natural oligoamines (i.e., spermine or spermidine) resulted in similar structures with a nitrogen content of 10.05−10.77% and 1.40−1.48 μmol/mg (primary amines). A comparison between the nitrogen (% N) and primary amino content (TNBS) of most conjugates indicates that more than 80% of the oligoamine moieties are being grafted. The remaining moieties (<20%) are probably connected with both terminuses leading to slightly branched structures. When spermine analogues were used as the grafted oligoamines (i.e., G7-TA-24, LS-15, and G7-TA-23; Table 4), similar values of nitrogen and primary amino content were obtained. When polyethyleneimine (600 Da) was used for grafting (TA1-38-4, Table 4), an insoluble gel was obtained probably because of the formation of a three-dimensional structure between PEI oligomer and the highly oxidized polysaccharide chains. Under similar conditions, dextran with milder oxidation degree (D(1:3) and D(1:5)) formed soluble polymers with PEI oligomer (data not shown). Simple diamines with two, three, or four methylene groups (G7-TA-2, G4-TA-132, or G7-TA-3, respectively; Table 4) resulted in polymers with relatively high primary amino content (>2.1 μmol/mg). This could be explained by the fact that small diamines enhance grafting and minimize branching to lesser extent. Diamines with six or eight methylene groups (G7-TA-5 and G7-TA-6; Table 4) resulted in polymers with low water solubility and high solubility in aprotic solvents (i.e., DMSO and DMF). This behavior is expected because of an increase in hydrophobicity of the oligoamines, which is caused by the loss of at least one primary amino group during the Schiff base reaction. Oligoamines containing primary amino and tertiary amino groups in both terminuses were also conjugated to the representative polysaccharides (G7-TA-20 and LS-16; Table 4) under similar conditions. The primary amino content of these polymers were found to be

Table 2. Structures of Various Oligoamines Used in the Preparation of Polycations

Oligoamine used for conjugation	Oligoamine structure	^{1}H-NMR (D_2O) of conjugate (ppm)
Spermine		1.645 (m), 1.804 (m), 2.815 (m), 3.52-4.19 (m) and 5.019 (m)
Spermidine		1.685 (m), 1.89 (m), 2.942 (m), 3.51-4.25 (m) and 5.076 (m)
N,N′-bis(3-aminopropyl)-1,3 propanediamine		1.84 (m), 2.871 (m), 3.49-4.29 (m) and 5.056 (m)
N,N′-bis(3-aminopropyl) ethylenediamine		1.862 (m), 2.885 (m), 3.49-4.29 (m) and 5.056 (m)
N,N′-bis(2-aminoethyl)-1,3-propanediamine		1.83 (m), 2.798 (m), 2.873 (m), 3.39-4.45 (m) and 5.068 (m)
Polyethyleneimine, 600 Da (Random branching)		Insoluble (crosslinked)
Diethylenetriamine		2.654 (m), 2.883 (m), 2.918 (m), 3.57-4.22 (m), 4.308 (m) and 5.074 (m)
1,3-Propane diamine		1.863 (m), 2.407 (m), 2.805 (m), 2.946 (m), 3.52-4.24 (m) and 5.058 (m)
Triethylene glycol diamine		2.41-3.31 (m), 3.46-4.47 (m), and 5.08 (m)
N,N-dimethylethylene diamine		2.26 (m), 2.51 (m), 3.104 (s), 3.2-4.25 (m) and 4.831 (m)

negligible because of the presence of tertiary amino functionalities instead of primary amino groups.[28]

Transfection Results

Transfection experiments were performed using three different cell types: HEK-293, NIH-3T3, and EPC. Plasmids used for these studies were pCMV-GFP encoding to green fluorescence protein, pLNC-luciferase encoding to luciferase protein, and pCMV-hGH encoding to the human growth hormone. Stock solutions of DNA and polycations at various charge ratios were mixed, diluted with HBS, and left to stand at room temperature for at least 30 min. The complex solutions containing a constant amount of DNA (2 μg) were added separately to each cell well. Replacement of serum-free medium with a fresh complete medium was performed after 4 h, and incubation was performed for a prolonged period of time to complete transfection (24−72 h). In the case of GFP protein, the transfection yield was quantified by visual counting of fluorescent cells in a certain field using a fluorescence microscope. HGH and luciferase were quantitatively assayed using commercial elisa kits according to standard protocols. DOTAP/Chol (Avanti) and Transfast (Promega) cationic lipids were used as controls according to the manufacturers' protocols. In many cases, the calcium phosphate precipitating technique was also used as a control.

Over 300 cationic conjugates were prepared starting from various polysaccharides and oligoamines. All these cationic polymers were tested for their transfection

Table 3. Effect of Oxidation Degree (Group A), Mole Ratio (Group B), and Initial average molecular weight of dextran (Group C) on Conjugation (Spermine Was Used as a Model Oligoamine)

code	initial polyaldehde[a]	% dialdehyde	spermine/aldehyde[b]	% N[c]	μmol/mg ($-NH_2$)[d]
		Group A			
TA1-129A	D(1:1), 40 kDa	48	1.25	10.78	1.25 ± 0.10
TA1-129B	D(1:3), 40 kDa	20	1.25	4.91	0.80 ± 0.06
TA1-129C	D(1:5), 40 kDa	12	1.25	2.65	0.45 ± 0.08
		Group B			
G1-TA-43A	D(1:1), 40 kDa	48	2	12.77	1.60 ± 0.22
G1-TA-43B	D(1:1), 40 kDa	48	1.5	11.40	1.52 ± 0.15
G1-TA-43C	D(1:1), 40 kDa	48	1.25	11.2	1.45 ± 0.12
G1-TA-43D	D(1:1), 40 kDa	48	0.75	7.68	0.48 ± 0.05
G1-TA-43E	D(1:1), 40 kDa	48	0.5	6.84	0.37 ± 0.10
		Group C			
D500-SP	D(1:1), 500 kDa	32	1.25	5.75	1.05 ± 0.16
D75-SP	D(1:1), 75 kDa	37	1.25	7.85	1.20 ± 0.16
D40-SP	D(1:1), 40 kDa	48	1.25	11.19	1.05 ± 0.20
D18-SP	D(1:1), 18 kDa	52	1.25	11.80	1.47 ± 0.14
D9.3-SP	D(1:1), 9.3 kDa	58	1.25	11.52	1.35 ± 0.20

[a] Abbreviation: D(1:1), 40 kDa: oxidized Dextran with 1:1 mole ratio (KIO_4/anhydrous glucose units) starting with initial molecular weight of 40 kDa, etc. [b] Mole ratio of reactants (spermine/aldehyde groups). [c] Found nitrogen content (elemental analysis). [d] Amount of primary amine (μmol/mg) determined by the TNBS method ($n = 3$).

Table 4. Chemical Characterization of Dextran Derivatives Grafted with Various Oligoamines[a]

code	oligoamine type	% N (found)	μmol/mg ($-NH_2$)	M_w	M_n	P
G7-TA-21A	spermine	10.77	1.40 ± 0.05	8770	4765	1.84
LS-5	spermidine	10.05	1.48 ± 0.03	6120	4070	1.50
G7-TA-24	*N*,*N*-bis(3-aminopropyl)-1,3-propanediamine	9.58	1.07 ± 0.08	6135	4190	1.46
LS-15	*N*,*N*-bis(3-aminopropyl)ethylenediamine	9.67	1.28 ± 0.05	6950	5190	1.34
G7-TA-23	*N*,*N*-bis(2-aminoethyl)01,3-propanediamine	11.70	1.06 ± 0.03	5480	4530	1.21
TA1-38-4	PEI600	9.77	insoluble			
G7-TA-2	ethylenediamine	10.68	2.2 ± 0.08	6345	4960	1.28
G4-TA-132	propylenediamine	10.18	2.09 ± 0.08	5610	4750	1.18
G7-TA-3	1,4-butanediamine	9.05	2.43 ± 0.12	5700	4680	1.22
G7-TA-5	1,6-hexanediamine	ND	insoluble			
G7-TA-6	1,8-octanediamine	ND	insoluble			
G7-TA-8	diethylenetriamine	12.59	2.21 ± 0.02	11375	5105	2.22
G7-TA-7	triethylene glycol diamine	6.91	1.53 ± 0.04	4240	3680	1.15
G7-TA-20	dimethylethylenediamine	13.85	0.05 ± 0.01	4000	3540	1.13

[a] Reaction conditions: oxidized dextran (~50% dialdehdye, ~28 kDa) and the appropriate oligoamine (1:1.25 mole ratio, respectively) were allowed to react under similar conditions as described in Experimental Section. M_w, M_n, and polydispersity ($P = M_w/M_n$) were determined by GPC as described in Experimental Section for the polycationic materials.

activity (transfectivity) using various cell types and marker genes. Each single polymer was tested over a wide range of charge ratios (−/+, phosphate/nitrogen) from 1 to 0.05. Figure 1 shows a series of transfection results, applying EPC cells and pLNC-luciferase as the transfection system. Transfection results in Figure 1 were accounted as LRUL (luminescence relative unit light) and recorded at the best charge ratio relative to Transfast control. When simple diamines were applied as the grafting oligoamines (i.e., ethylene, propylene, butane, hexane, and octane diamines), no transfection was obtained in both dextran and arabinigalactan based conjugates at all tested charge ratios (Figure 1). PEI600, *N*,*N*′-dimethylethylenediamine, and *N*,*N*′-dimethylpropylenediamine were also grafted on both dextran and arabinogalactan representative polysaccharides. These conjugates were also found to be inactive and showed no transfectivity at all tested charge ratios. On the contrary, when spermine was used as the grafting oligoamine, a slight protein expression was detected in arabinogalactan-based conjugate (~20% to Transfast control). The most active form of these synthetic polymers was a dextran−spermine based conjugate (TA1-129A, Figure 1). Applying this polymer as a gene vector resulted in similar transfectivity to controls (Transfast and DOTAP/Chol, 1:1). Replacement of spermine with spermidine as the grafted oligoamine resulted in a drastic decrease in transfectivity both in dextran and in arabinogalactan based conjugates.

In addition, spermine analogues were grafted in the same manner to both dextran and arabinogalactan based polysaccharides. When *N*,*N*-bis(3-aminopropyl)-1,3-propanediamine (N-3-N-3-N-3; Figure 1) was grafted to dextran, a low protein expression was observed (~20% to Transfast). In contrast, when *N*,*N*-bis(2-aminoethyl)-1,3-propanediamine (N-2-N-3-N-2-N) and *N*,*N*-bis(3-aminopropyl)ethylenediamine (N-3-N-2-N-3-N) were used, no transfectivity was obtained in both representative polysaccharides at all tested charge ratios. Figure 2 shows a typical fluorescence microscope imaging of pCMV-GFP transfected HEK293 cells, which shows that the calcium phosphate precipitating technique (Figure 2A) and TA1-129A (dextran−spermine based conjugate) gave a strong expression of GFP. Thus, the charge ratio (−/+) is a crucial factor in transfection efficiency both in cationic polymers and in liposomes.[14] At 0.1 charge ratio (according to % N), TA1-129A gave a better transfection yield in comparison with the same conjugate complexed at 0.067 (parts B and C of Figure 2, respectively). The commercial cationic lipid (Transfast) was also used in this experiment as a second control and gave a similar GFP expression (Figure 2D).

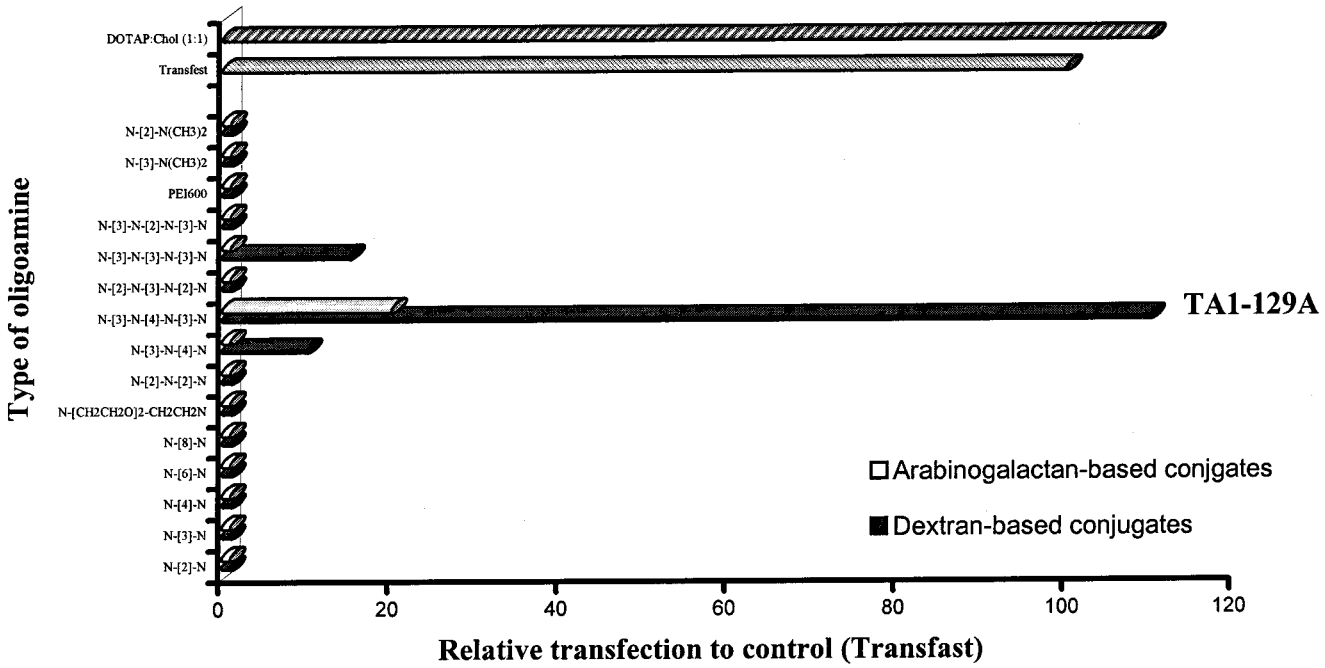


Figure 1. Transfection efficiencies of pLNC-luc in EPC cells applying dextran and arabinogalactan grafted oligoamines as vectors. Abbreviations: N-[2]-N, ethanediamine; N-[3]-N, propanediamine; N-[4]-N, butanediamine; N-[6]-N, hexanediamine; N-[8]-N, octanediamine; N-[CH2CH2O]2-CH2CH2N, triethylene glycol diamine; N-[2]-N-[2]-N, diethylenetriamine; N-[3]-N-[4]-N, spermidine; N-[3]-N-[4]-N-[3]-N, spermine; N-[2]-N-[3]-N-[2]-N, *N*,*N*′-bis(2-aminoethyl)-1,3-propanediamine; N-[3]-N-[3]-N-[3]-N, *N*,*N*′-bis(3-aminopropyl)-1,3-propanediamine; N-[3]-N-[2]-N-[3]-N, *N*,*N*′-bis(3-aminopropyl)ethylenediamine; PEI600, polyethyleneimine with average molecular weight of 600 Da; N-[3]-N(CH3)2, *N*,*N*′-dimethylpropylenediamine; N-[2]-N(CH3)2, *N*,*N*′-dimethylethylenediamine.

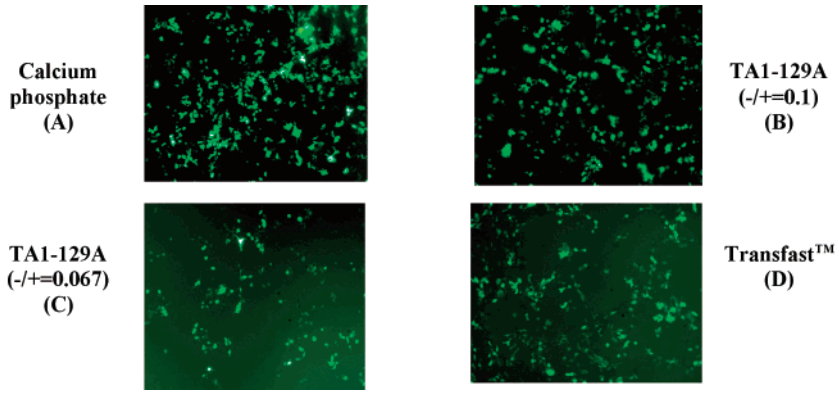


Figure 2. Inverted fluorescent microscope of pCMV-GFP transfected HEK293 cells. Parts A and D represent the calcium phosphate precipitating technique and Transfast controls, respectively. TA1-129A represents the Dextran−spermine optimal conjugate complexed at two charge ratios: 0.1 (B) and 0.067 (C, −/+). The charge content of the conjugate was determined according to % N (elemental analysis).

Reductive amination reactions between various polyaldehydes and oligodiamines are considered to be nonreproducible because of random branching of the oligodiamine with the polymer backbone chains. Branching was reduced to a lesser extent by the reaction in a relatively high-diluted system and a dropwise addition of the polyaldehydes to the oligoamines. In a typical experiment, 12 dextran−spermine conjugates were prepared in different batches starting with oxidized dextran (∼50% diladehdye, 26 kDa) and spermine at 1:1.25 mol ratios, respectively. The purpose of this experiment was to evaluate the reproducibility of conjugation and transfection efficiencies. Figure 3 shows the % N and primary amino content obtained from these conjugates. Averages of % N and primary amino content in these representative conjugates were found to be 10.86 ± 0.53 and 1.32 ± 0.15 (μmol/mg), respectively. According to elemental analysis of nitrogen and primary amino content, more than 75% of spermine moieties are being grafted while the rest are branched between different polysaccharide chains. The average molecular weight of these conjugates was found to be 8000 ± 2000 Da. The drastic decrease in molecular weights of conjugates in comparison with the starting polymers is explained by the extensive aminolysis of the glycoside linkages during conjugation.[31] Also, transfection efficiencies of these similar conjugates were evaluated in EPC cells applying pLNC-luc as the reporter gene. Average transfection (relative to DOTAP/Chol 1:1, control) was found to be 100 ± 20%, indicating excellent reproducibility in transfectivities.

In vitro transfection of the potential dextran−spermine based conjugates was also tested in different cell lines and reporter genes. Figure 4 shows a typical

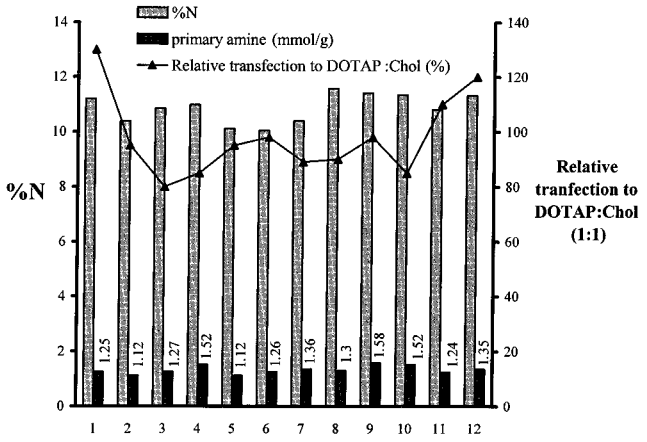


Figure 3. % N, primary amino content, and relative transfectivity of optimal dextran−spermine based conjugates. Transfections values were evaluated in EPC cells applying pLNC-luc and recorded relative to DOTAP/Chol (1:1) control (experimental). Serial numbers along the *X* axis represent the batch number of the particular dextran−spermine based conjugate.

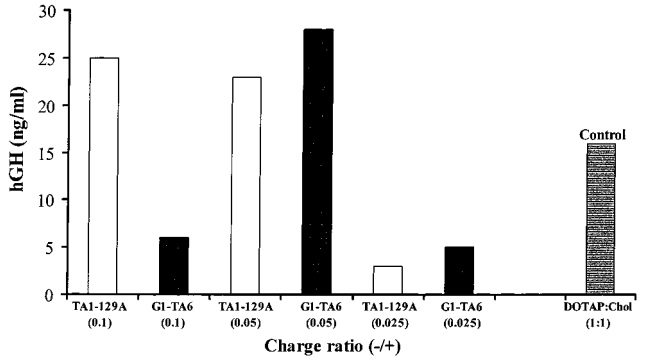


Figure 4. Quantitative transfection efficiencies of dextran−spermine based conjugates (TA1-129A and G1-TA6) applying NIH3T3 cells and pCMV-hGH reporter gene. The protein amount (hGH) was quantified by hGH elisa kit (experimental). DOTAP/Chol (1:1) was used as a control.

transfection system applying NIH3T3 cells and pCMV-hGH as the plasmid. Protein expression was quantified by an hGH elisa kit applying standard protocol. G1-TA6 is similar to the TA1-129A conjugate prepared in a different batch. TA1-129A based conjugate gave the highest transfection yield at 0.1 and 0.05 charge ratios. Higher cation ratio (−/+ = 0.025) resulted in a drastic decrease in hormone expression. G1-TA6 showed a similar protein expression at 0.05. DOTAP/Chol (1:1) (a combination of cationic and helper lipid) was used in this system as a control and gave a protein expression similar to that of the representative polycations (TA1-129A and G1-TA6).

Conclusions

Various polycations were prepared by reductive amination reaction between primary amines and oxidized polysaccharides. The results obtained indicate that all oxidized polymers did not contain the proportional aldehyde content with their initial molecular weights. Higher molecular weight polysaccharide had proportionally less aldehyde content because of high branching in the system. Because of this reason, the polycations of high molecular weight dextrans have less conjugation and less cationic charge densities.

More than 300 different polycations were prepared starting from various polysaccharides and oligoamines having two to four amino groups. Although most of these conjugates formed stable complexes with various plasmids as determined by turbidity experiments, only the dextran−spermine based polycations were found to be active in transfecting cells in vitro. The reason for the transfection efficiency of certain polycations (i.e., dextran−spermine) is probably due to the unique complextaion properties formed between the DNA helices and the grafted spermine moieties, which play a crucial role in cell transfections. This work indicates that the structure of the polycation has a significant role in the transfection activity.

Experimental Section

All solvents and reagents were of analytical grade and were used as received. Arabinogalactan with an average molecular weight of 19 kDa was a gift from Larex International (St. Paul, MN). Dextrans with a wide range of molecular weights (20−500 kDa) were obtained from Sigma Chemical Co. (St. Louis, MO). Spermine, spermidine, polyethylenediamine ($M_w = 600$), propanediamine, and butanediamine were obtained from Fluka Chemie (Buchs, Switzerland). *N*,*N*′-Bis(2-aminopropyl)-1,3-propanediamine, *N*,*N*′-bis(3-aminopropyl)-1,3-propanediamine, *N*,*N*′-bis(3-aminopropyl)-1,3-ethylenediamine, *N*,*N*′-dimethylethylenediamine, and *N*,*N*′-dimethylpropylenediamine were obtained from Aldrich (Milwaukee, WI). 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 $CDCl_3$, D_2O, or DMSO-d_6 as solvent. Values were recorded as ppm relative to the internal standard (TMS). Molecular weights of starting polymers and conjugates were determined on GPC-Spectra Physics instrument (Darmstadth, Germany) containing a pump, column (Shodex KB-804 or Kb-803), and refractive index (RI) detector. Average molecular weights were determined according to internal standards (PSS, Mainz, Germany) with molecular weights between 5800 and 212 000. Eluents used were 0.05 M $NaNO_3$ for the uncharged polymers and 5% (w/v) Na_2HPO_4 in 3% (v/v) acetinitrile (pH 4) for the polycationic polymers. Elemental analysis (% N) of polycations was performed on a Perkin-Elmer 2400/II CHN analyzer.

Oxidation of Polysaccharides. The desired polysaccharide (10 g, 62.5 mmol of glucose units) was dissolved in 200 mL of doubly 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. FT-IR (KBr): 1724 cm^{-1} (C=O).

Aldehyde content was determined according to the literature.[27] In brief, 100 mg of oxidized polysaccharide was dissolved in 25.0 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).

Polysaccharide−Oligoamine Conjugates (General Method). A solution of dialdehyde polysaccharide (6.25 mmol of aldehyde groups) in 100 mL of DDW was slowly added over 5 h (sage metering pump) to a basic solution containing 1.25 equimolar amount of diamine (7.82 mmol) dissolved in 50 mL of borate buffer (0.1 M, pH 11). The mixture was gently stirred at room temperature for 24 h, $NaBH_4$ (1g, 4 equimolar) was added, and stirring was continued for 48 h under the same

conditions. The reduction was repeated with an additional portion of $NaBH_4$ (1 g) and with stirring for 24 h under the same conditions. The resulting light-yellow solution was poured into a dialysis membrane (3500 cutoff, Membrane Filtration Products, Inc., San Antonio, TX) and dialyzed against DDW (4 × 5 L) at 4 °C for 2 days. The dialysate was gravimetrically filtered to remove insolubles and lyophilized to dryness. Average yield: 30−40% (w/w). FT-IR (KBr): 1468 ($-CH_2-$, aliphatic), 1653 ($-NH_2$, primary amine), 2935 (C−C, aliphatic), and 3297 (secondary amine and −OH groups) cm^{-1}.

Determination of Primary Amines by the TNBS Method. The primary amine content was determined according to standard protocol[28] with a slight modification. 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 of spermine (or other soluble oligoamine) dissolved in 600 μL of DDW. The mixtures were separately diluted with 200 μL of 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 oligoamine) was used as a reference in the absorbance measurements. Weighed conjugates (50−500 μg, depending on the degree of conjugation) were treated as above, and the primary amino content was calculated according to the calibration curve.

General Transfection Experiments in Vitro. The cell suspension at a concentration of 6×10^5 cells (1.0 mL) per well was preincubated for 24 h in a six-well dish. After adherence, the medium was replaced by 1 mL of SFM. Either polyplexes or lipoplexes were added to the dish for 4 h, then SFM was replaced by 1 mL of complete medium, and the cells were incubated for 24−72 h. Three common cell types were used for the transfection experiments (HEK293, NIH3T3, and EPC). Also, three different kinds of plasmid DNA were used: pCMV-GFP encoding to the green fluorescence protein, pCMV-hGH encoding to human growth hormone, and pLNC-luciferase encoding to luciferase protein. GFP was monitored by fluorescence microscopy, and transfection efficiency was evaluated by counting the fluorescent cells in a certain field. HGH and luciferase were quantitatively assayed by standard protocols using hGH and luciferase kits, respectively. Transfast and DOTAP/Chol (1:1) were used as control vectors according to manufacturer's protocols.

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