Articles

Analysis of Interaction between Dendriplexes and Bovine Serum Albumin

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Dendrimers are new nanotechnological carriers for gene delivery. Short oligodeoxynucleotides (ODNs) are a new class of antisense therapy drugs for cancer and infectious or metabolic diseases. The interactions between short oligodeoxynucleotides (GEM91, CTCTCGCACCCATCTCTCTCCTTCT; SREV, TCGTCGCTGTCTCCGCTTCTTCCTGCCA; unlabeled or fluorescein-labeled), novel water-soluble carbosilane dendrimers, and bovine serum albumin were studied by fluorescence and gel electrophoresis. The molar ratios of the dendrimer/ODN dendriplexes ranged from 4 to 7. The efficiency of formation and stability of the dendriplexes depended on electrostatic interactions between the dendrimer and the ODNs. Dendriplex formation significantly decreased the interactions between ODNs and albumin. Thus, the formation of dendriplexes between carbosilane dendrimers and ODNs may improve ODN delivery.

Introduction

Short oligodeoxynucleotides (ODNs) are a new class of antisense therapy drugs for cancer and infectious or metabolic diseases.[1] However, because of their anionic charge, ODNs can interact with serum albumins (major transport proteins that bind many anionic and cationic ligands[2]). The bioavailability of ODNs is decreased by such interactions with serum proteins, necessitating higher doses to achieve the desired therapeutic effect. In some cases, nonspecific binding to proteins can induce toxic effects, especially for phosphorothioate ODN derivatives.[3] It has been shown that ODNs bound to serum albumins (or other proteins) do not enter the extravascular space.[3−5] To reduce the interactions of ODNs with serum proteins, drug carriers such as liposomes or dendrimers have been proposed.[6−7] However, lipoplexes (liposome/ODN complexes) are of limited use because liposomes bind nonspecifically to anionic serum proteins.[8−9] Dendrimers have proved to be an alternative to liposomes and other polymeric systems for drug delivery. Their major advantages are uniform structure, multiple sites of attachment and the versatility with which their skeletons and surfaces can be modified, allowing the dendrimer/ drug interaction to be characterized precisely. Dendritic carriers based on polyamidoamine[10] and phosphorus-containing[11] dendrimers show transfecting activity even in the presence of serum, despite possible interactions with serum albumins.[12−14]

Water-soluble carbosilane dendrimers containing ammonium or amine groups at their peripheries have recently been described by our group as biocompatible molecules with good potential as ODN carriers. We have studied the interactions of different carbosilane dendrimers with an ODN sequence corresponding to an antisense sequence of the HIV mRNA polypurine tract element (5-fluoresceine-AAT TTT CTT TTC CCC CCT-3).[15−16]

In the present work we describe the interactions of bovine serum albumin (BSA) with the dendriplexes formed between ODNs (GEM91, CTC TCG CAC CCA TCT CTC TCC TTC T; SREV, TCG TCG CTG TCT CCG CTT CTT CCT GCC A; unlabeled or fluorescein-labeled) and ammonium-terminated carbosilane dendrimers with two different types of peripheral units based on neutral $-OCH_2CH_2NMe_2$ or $-OCH_2CH_2N(Me)CH_2CH_2NMe_2$ fragments.

Materials and Methods

BSA and ethidium bromide (EB) were purchased from Sigma-Aldrich (USA). BSA labeled with Alexa Fluor 488 (BSA-AF) was obtained from Invitrogen (USA). Fluorescein, FluorePrime fluorescein phosphoramidite and Sephadex G-25 were purchased from Amersham Biosciences (USA). A 100 bp DNA ladder was purchased from Gibco BRLTM (USA). Blue Paragon was purchased from Beckmann-Coulter (USA). Buffer solutions were obtained from POCh (Poland) and Sigma-Aldrich (USA). For fluorescence measurements, 0.15 mol/L Na−phosphate buffer (pH 7.4) was used.

1. Dendrimers and ODNs Synthesis. The dendrimers 2G-[Si($OCH_2CH_2NMe_3^+I^-$)]8 (**1**) and 2G-[Si($OCH_2CH_2NMe_3^+I^-$)$_2$]8 (**2**), containing

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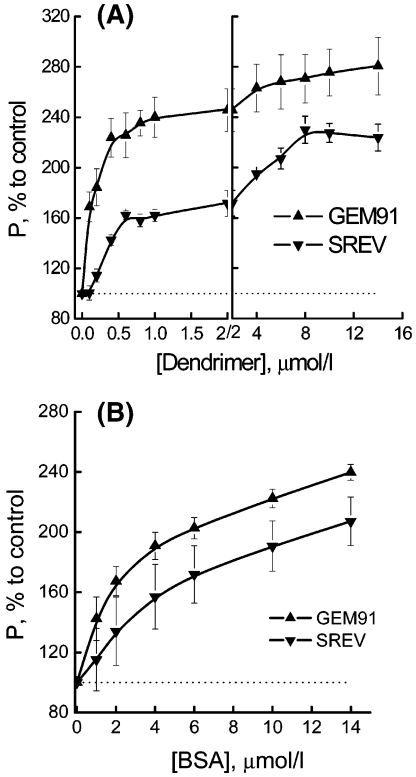

Figure 1. (A) Changes in GEM91 and SREV fluorescence polarization on addition of four carbosilane dendrimers. Data are presented on two X axis scales: 0−2 and 2−14 μmol/L. The dotted line indicates 100% polarization and corresponds to the fluorescence polarization of ODNs in the absence of dendrimers. (B) Changes in GEM91 and SREV fluorescence polarization on addition of different concentrations of BSA. The dotted line indicates 100% polarization and corresponds to the fluorescence polarization of ODNs in the absence of BSA. (A, B) [ODN] = 0.1 μmol/ L, λ_{ex} = 485 nm, λ_{em} = 516 nm. 37 °C; 0.15 mol/L Na−phosphate buffer, pH 7.4.

8 and 16 peripheral units, respectively, are of the same type, whereas 22G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_3^+I^-$}]8 (**3**) and 2G-[Si{$O(CH_2)_2N(Me)_2^+(CH_2)_2NMe_3^+(I^-)_2$}]8 (**4**), containing 8 groups of singly and doubly methylated outer fragments, respectively, have a different external architecture. Dendrimers **1**−**3** were prepared according to previously reported methods.[15−16] Dendrimer **4** was prepared by a similar method, adding 16 equiv of MeI to the amine-terminated dendrimer 2G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_2$}]8.[15−16] ODN sequences were prepared using standard solid-phase 2-cyanoethyl phosphoramidites[17] in a 392 DNA synthesizer (Applied Biosystems, USA). Phosphorothioate linkages were introduced by replacing the iodine solution with a solution of 3H-1,2-benzodithiol-3-one-1,1-dioxide.[18] Fluorescein was added to the 5′-end using FluorePrime fluorescein phosphoramidite. After the sequence was assembled, the ODN was stained in 32% aqueous ammonia at 55 °C for 16 h. The ammonia solution was evaporated to dryness and the product was desalted on a NAP-10 column (Sephadex G-25) and eluted with water. The length and homogeneity of the ODNs were confirmed by denaturing polyacrylamide gel electrophoresis. Finally, the following ODNs were used: GEM91 (25 bases; FW 8112.5 g/mol; sequence CTC TCG CAC CCA TCT CTC TCC TTC T; labeled with fluorescein and non-labeled) and SREV (28 bases; FW 9086 g/mol; sequence: TCG TCG CTG TCT CCG CTT CTT CCT GCC A; unlabeled or fluorescein-labeled).

2. Fluorescence Measurements. Fluorescence was measured with a Perkin-Elmer LS-50B spectrofluorometer. The excitation and emission wavelengths were 485 and 516 nm for ODNs labeled with fluorescein; 295 and 350 nm for intrinsic BSA fluorescence; and 487 and 520 nm for BSA-AF. All measurements were made at 37 °C. The excitation and emission slit widths were set to 10 and 5 nm, respectively.

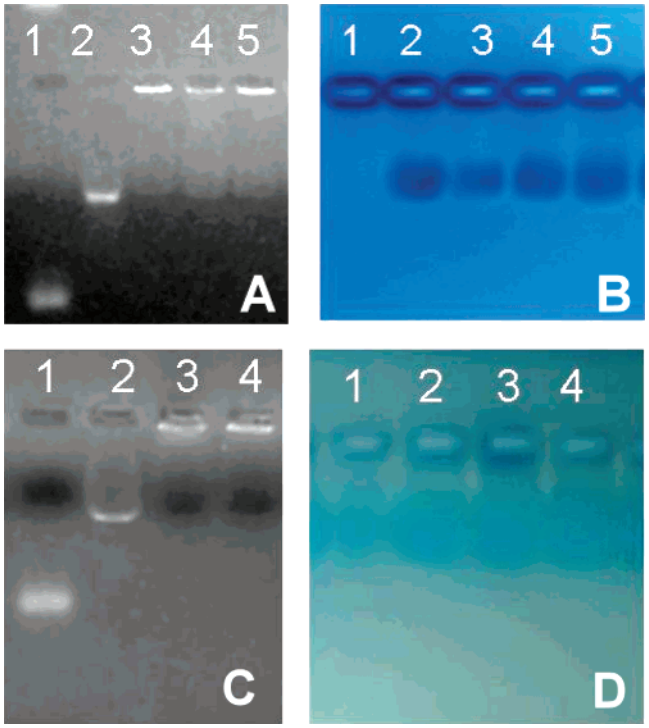

Figure 2. (A) Electrophoresis on a 3% agarose gel (stained with ethidium bromide) of dendriplexes formed by different carbosilane dendrimers and GEM91 in a 2:1 (+):(−) ratio in the presence of BSA. (B) The same gel stained with Blue Paragon showing protein migration. (1) GEM91; (2) GEM91 + 10% BSA; (3) **1**/GEM91 + 10% BSA; (4) **3**/GEM91 + 10% BSA; (5) **2**/GEM91 + 10% BSA; (6) **4**/GEM91 + 10% BSA. (C) Electrophoresis on a 3% agarose gel (stained with ethidium bromide) of dendriplexes formed by different carbosilane dendrimers and SREV in a 2:1 (+):(−) ratio in the presence of BSA. (D) The same gel stained with blue paragon showing protein migration. (1) SREV; (2) SREV + 10% BSA; (3) **3**/SREV + 10% BSA; (4) **2**/SREV + 10% BSA; (5) **4**/SREV + 10% BSA.

The fluorescence polarization (P_{ABS}) of BSA, BSA-AF, and ODNs labeled with fluorescein was calculated as $P_{ABS} = (I_{VV} - G \times I_{VH})/(I_{VV} + G \times I_{VH})$, where I_{VV} is the vertical component, I_{VH} the horizontal component of the light emitted by a fluorophore excited by vertical plane-polarized light, and G the G-factor. The G-factor was calculated as $G = I_{HV}/I_{HH}$, where I_{HV} is the vertical component and I_{HH} the horizontal component of the light emitted by a fluorophore excited by horizontal plane-polarized light. The relative polarization (P, as percentage of control) was calculated as $P = P_{ABS}/P_{ABS}^0 \times 100\%$, where P_{ABS} and P_{ABS}^0 are, respectively, the fluorescence polarization of BSA (or BSA-AF or fluorescein-labeled ODN) in the presence and absence of dendrimer.

Fluorescence quenching of BSA and BSA-AF was analyzed on the basis of the Stern−Volmer equation: $F_0/F - 1 = K_{SV}\cdot[Q]$, where F_0 is the intensity of fluorescence in the absence and F in the presence of the quencher, [Q] is the quencher concentration, and K_{SV} is the quenching constant.

3. Gel Electrophoresis. The solutions were prepared with a sufficient excess of positive charge to be able to interact with the ODN; dendrimers **1**−**3** were added to the ODN solutions at an electrostatic charge ratio (+):(−) of 2:1. The ratio of ODN to dendrimer was based on the calculated electrostatic charge of each component, e.g., the number of S groups in the ODN versus the number of terminal ammonium groups on the dendrimer. As an example, ODN and dendrimer **1** were diluted in sterile distilled water at 1 and 2 mg/mL, respectively; 2.43 μL of ODN solution was mixed with 3.25 μL dendrimer **1** solution in PBS (final volume 60 μL) and incubated for 20 min at room temperature. After this incubation, some of the ODN−dendrimer mixture was exposed to increasing BSA concentrations in a

Table 1. (A) Number of Dendrimers Per Molecule of ODN, (B) Changes of Fluorescence Polarization (*P*, Percentage of Control) of Labeled ODNs on Addition of Dendrimers at Dendrimer/ODN Molar Ratios Taken from Part A, and (C) Quenching Constants of BSA and BSA-AF Fluorescence on Addition of Dendrimers[a]

	A		B		C	
dendrimer	GEM91	SREV	**n**/SREV	**n**/GEM91	BSA	BSA-AF
1	0	0			0	0
2	3	6	129.1 ± 4	169.3 ± 10.9	0	0
3	4	7	159.6 ± 6.8	183.1 ± 14.3	0	0.07
4	4	7	142.3 ± 4.3	223.7 ± 15.4	0.0192	0.0017

[a] $P = 100\%$ corresponds to fluorescence polarization of ODNs in the absence of dendrimers. B: $\lambda_{ex} = 485$ nm, $\lambda_{em} = 516$ nm. C: [BSA] = 5 μmol/L, $\lambda_{ex} = 290$ nm, $\lambda_{em} = 350$ nm. [BSA-AF] = 0.1 μmol/L, $\lambda_{ex} = 485$ nm, $\lambda_{em} = 516$ nm. A−C: 37 °C; 0.15 mol/L Na−phosphate buffer, pH 7.4.

Table 2. (A) Changes of Fluorescence Polarization (*P*, Percentage of Control) of Dendriplexes (**n**/ODN) on Addition to BSA Solution at a BSA/dendrimer/ODN Molar Ratio 10/7/1 and (B) Changes of Fluorescence Polarization (*P*, Percentage of Control) of BSA/ODN Complex on Addition of Dendrimers at a Dendrimer/BSA/ODN Molar Ratio 7/10/1[a]

	A		B	
dendrimer	**n**/GEM91 + BSA	**n/**SREV + BSA	**n** + BSA/GEM91	**n** + BSA/SREV
1	75 ± 6	72 ± 12	107 ± 6	106 ± 8
2	51.3 ± 2	75 ± 6	165 ± 12	123 ± 6
3	100 ± 3	100 ± 5	153 ± 11	135 ± 6
4	104 ± 8	97 ± 4	177 ± 3	112 ± 5

[a] (A) $P = 100\%$ corresponds to fluorescence polarization of dendrimer/ODN dendriplex in absence of BSA. (B) $P = 100\%$ corresponds to fluorescence polarization of BSA/ODN complex in absence of dendrimers. A, B: [ODN] = 0.1 μmol/L, $\lambda_{ex} = 485$ nm, $\lambda_{em} = 516$ nm. 37 °C; 0.15 mol/L Na−phosphate buffer, pH 7.4.

final volume of 100 μL PBS. Complex formation and stability were assessed by the retardation of fluorescein-labeled ODN migration during electrophoresis on 3% agarose gels. A 100 bp DNA ladder was used as reference. Fluorescein enhanced the ODN signal significantly, facilitating visualization of the dendriplex during electrophoresis. In addition, the gel was polymerized in the presence of EB so that ODN migration was more evident. After electrophoresis, the EB-stained gel was digitally photographed; the same gel was subsequently stained with Blue Paragon to show protein migration. Finally, the gel was washed with 10% glacial acetic acid and a second digital photograph was taken.

Results and Discussion

1. Interaction between ODNs and Dendrimers. The interaction between ODNs and dendrimers was estimated by monitoring changes in the fluorescence polarization of fluorescein attached to the ends of the ODNs when increasing concentrations of dendrimers were added (Figure 1A, Table 1). These interactions depended on both the type of ODN and the type of dendrimer. Two binding processes were observed: first, specific binding between the dendrimer and the ODN (strong direct binding of dendrimer to ODN regions) when the dendrimer:ODN molar ratio was (2−7):1; second, micellation (weak binding/aggregation) of dendrimer/ODN complexes by dendrimer molecules when the dendrimer:ODN molar ratio was (10−40):1. This was confirmed by studies on the interaction between carbosilane dendrimers and the hydrophobic anionic fluorescent probe ANS (ref 19 and unpublished data). The micellation was characteristic for dendrimer **1**, while **2**−**4** showed specific binding. Dendrimers **3** and **4** bound ODNs more effectively, perhaps because of differences in the end groups.[15−16] Both types of dendrimer, **1**−**2** and **3**−**4**, have positively charged NMe_3^+ groups, but dendrimer **3** also has neutral $-NR_2$ groups capable of quaternization, forming cationic $-NR_3^+$ groups as in **4**. The presence of two positive charges in **4** or two possible positive charges in **3** close to the surface increases the efficiency of binding. GEM91 binds effectively to practically all dendrimers, while SREV interacts with lower efficiency. These differences can be explained as follows. First, the ODNs differ in self-complementarity (docking), i.e., the maximal number of C−G and A−T pairs that may form in solution between two molecules of the same type of ODN. GEM91 has 3 pairs (2 pairs C−G + 1 pair A−T) and SREV has 6 pairs of C−G. Therefore, there are more possible ODN−ODN hydrogen bonding interactions for SREV than for GEM91.[20]

2. Interactions between Dendrimers and BSA. The changes in ODN fluorescence polarization when different concentrations of BSA were added are shown in Figure 1B. BSA has a negative net charge of −17, but it consists of three domains each carrying different net charges at physiological pH: −11 (domain I), −7 (domain II), and +1 (domain III)[2]. Anionic ODNs can bind to the surface of domain III. Table 1B,C shows the differences in BSA fluorescence quenching when different concentrations of dendrimers were added. Monitoring changes in the intrinsic fluorescence of BSA allows even subtle alterations in BSA structure to be followed.[12−14] The dendrimers affected neither the quenching of fluorescence from the internal and external tryptophanyls of BSA nor the fluorescence polarization (data not shown). Therefore, the dendrimers did not significantly affect the conformation of the protein over the concentration range studied. In contrast, the dendrimers quenched the fluorescence of AF, the label attached to the BSA surface. This indicates that the interactions between carbosilane dendrimers and BSA were weak and occurred preferentially at the protein surface.

3. Dendriplex (Dendrimer/ODN Complex) and BSA. Next, dendriplexes were formed by adding dendrimers to ODN solutions at a 7:1 molar ratio and incubating the mixture for 15 min. Aliquots of the dendrimer/ODN dendriplex solution were added to the BSA solution and changes in ODN fluorescence polarization were monitored. The results are shown in Table 2. In the presence of BSA, the fluorescence polarization of the **1**/ODN and **2**/ODN complexes decreased from 100% to 75%, while that of the **3**/ODN and **4**/ODN complexes did not change significantly. This indicates that BSA destroyed the **1**/ ODN or **2**/ODN micelles by interacting more strongly with either the ODN or the dendrimer. In contrast, BSA did not affect the dendriplexes formed by **3** or **4** with ODNs.

4. Addition of Dendrimers to ODN + BSA Complexes. The interaction between BSA and dendriplexes was also studied by adding BSA to ODN solutions at a 7:1 molar ratio, incubating for 15 min, then adding increasing concentrations of dendrimer and monitoring the changes in ODN fluorescence polarization. The results are shown in Table 2A. Addition of the dendrimer increased the fluorescence polarization of the ODN. This effect was similar to that of the dendrimers in the absence of BSA (see Figure 1A). It is likely that the dendrimer competed with BSA for the ODN; the dendrimer destroyed the BSA/ODN complex and formed dendriplexes with the ODN.

5. Gel Electrophoresis. The fluorescence experiments were confirmed by electrophoresis. Figure 2 illustrates the interactions between dendrimers and ODNs and between BSA and the dendriplexes: dendrimer/GEM91 (A, B) and dendrimer/SREV (C, D). The electrophoregram shows that the migration patterns differ depending on the agent used. ODN migration was retarded in the presence of BSA, indirectly indicating the formation of an ODN-protein complex. It was also altered in the presence of the dendrimers because a dendrimer-ODN complex formed, as described above. It is worth noting that addition of BSA to the dendriplexes did not change the migration pattern; under these conditions, BSA was unable to dissociate the complex.

Conclusions

Carbosilane dendrimers **2**−**4** can bind GEM91 and SREV effectively. The most effective were carbosilane dendrimers containing two N-donor atoms in the outer units. The molar ratios of the dendrimer/ODN dendriplexes ranged from 4 to 7. Dendriplex stability depended on electrostatic interactions between the dendrimer and the ODN. Dendriplex formation protected the ODN from binding to BSA. Thus, carbosilane dendrimers, mainly those containing two N-donor atoms in the outer units, are good candidates as carriers for delivery of ODNs and other anionic drugs.

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