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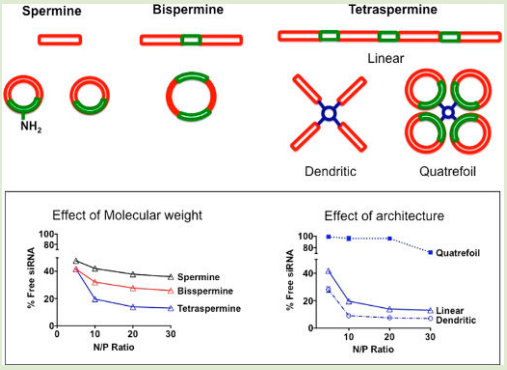
Oligospermines and Nucleic Acid Interaction: A Structure Property Relationship Study

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Supporting Information

ABSTRACT: A variety of delivery vehicles use spermine as a polycationic component to form complexes with nucleic acids. Thus, we investigated the influence of molecular architecture, amine density, and molecular weight of oligospermines on its binding to nucleic acids. We report the synthesis of mono, bis, and tetraspermines with linear, cyclic, dendritic, and quatrefoil architecture. The effect of molecular weight was more pronounced in linear oligospermines than their cyclic counterparts. Oligospermines with similar amine density but different molecular architectures exhibited different binding profiles. Among all oligospermines evaluated, dendritic tetraspermine exhibited the highest binding affinity. Atomistic molecular dynamics simulations also indicated higher affinity for dendritic tetraspermine to siRNA than its linear counterpart suggesting the importance of spermine geometry in binding to nucleic acids. Importantly, dendritic tetraspermine was less toxic than linear tetraspermine, suggesting its potential in nucleic acid delivery.



Nucleic acids such as siRNA, microRNA, or cDNA have tremendous potential to be developed as therapeutic modalities.[1] However, delivering nucleic acids is challenging due to their inherent characteristics such as lower cellular uptake, susceptibility to nucleases, and low half-life.[2] Polycationic polymers are often used to overcome these barriers.[3] Spermine (SP) is an important component of a variety of nucleic acid delivery vehicles including polymeric systems because of its high affinity toward nucleic acids that enables formation of stable complexes within nanometer size range.[4] Spermine is often grafted on polymers to improve their affinity toward nucleic acids.[5] Because of a high interest in using spermine as a polycationic component we examined the interaction of spermine with nucleic acids. The number of amines, amine density, and polymer composition are considered important parameters in the design of polycationic nucleic acid carriers,[6] whereas parameters such as polyplex morphology and molecular architecture get less importance.[3b,7] Thus, we designed our studies to place the emphasis on examining the effect of molecular architecture on nucleic acid binding. We report the synthesis of oligospermines having one, two, or four spermines arranged in a linear, cyclic, dendritic, and quatrefoil architecture (Scheme 1) and examine their intrinsic toxicity, and affinity toward siRNA and plasmid DNA.

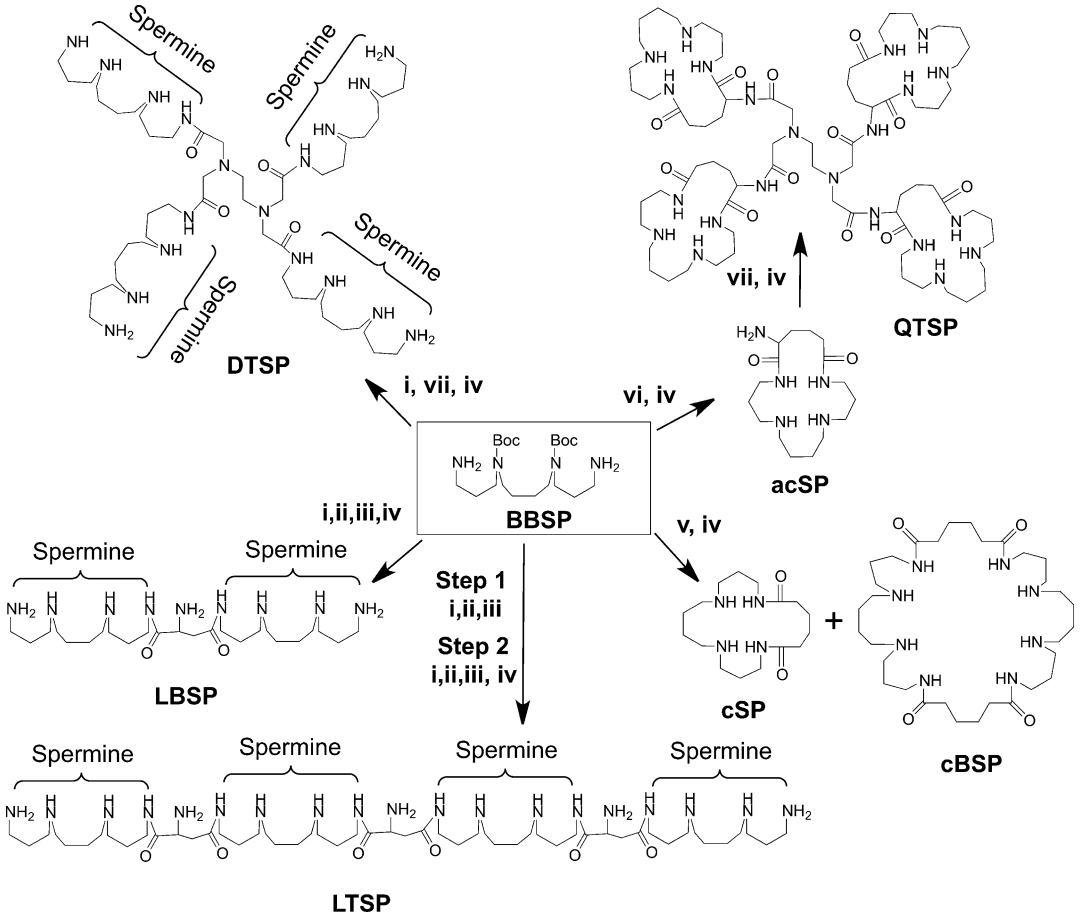
Bisbocspermine (BBSP), a derivative of spermine in which two secondary amines are protected with a *tert*-butyloxy (Boc) group, was synthesized using standard protection and deprotection chemistry (Supporting Information, Scheme 1 (SI-S1)). BBSP served as a key starting material for the synthesis of oligospermines with varying molecular weight, molecular architectures, and amine densities. Four- and six-carbon chain amine reactive bi- and tetra-functional linkers were synthesized as *p*-nitrophenyl esters of dicarboxylic acids (boc-aspartic acid, adipic acid, and DL-α-amino-adipic acid) and tetracarboxylic acid (EDTA) using DCC as a coupling agent (SI-S2). A schematic representation for the synthesis of all oligospermines is depicted in Scheme 1, and their characteristics are reported in Table 1. Detailed synthetic procedures and LC/MS profiles for all oligospermines (S1-F1) are provided in the SI.

Linear tetraspermine (LTSP) and dendritic tetraspermine (DTSP) synthesized in this report differ in their molecular architecture but have a fairly similar number of amines (13 vs 14 total amines, 5 vs 4 primary amines, 8 vs 8 secondary amines) and amine density (1.2 vs 1.4, Table 1). This allowed us to examine the effect of molecular architecture on nucleic acid interaction irrespective of the number of amines and amine density. We synthesized three cyclic oligospermines (cyclic spermine (cSP), cyclic bisspermine (cBSP), quatrefoil tetraspermine (QTSP)) lacking primary amines and having same amine density (0.6) but differing in the number of secondary and tertiary amine groups. This allowed us to examine the influence of number of secondary amines irrespective of amine density. Conjugating one, two, and four

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Scheme 1. Synthesis of Oligospermines[a]



[a](i) CF_3COOEt; (ii) Boc Asp$(ONp)_2$; (iii) NH_4OH, MeOH; (iv) TFA; (v) adipic $(ONp)_2$; (vi) aminoadipic acid $(ONp)_2$; (vii) EDTA $(ONp)_4$. cSP = cyclic spermine, acSP = amino cyclic spermine, LBSP = linear bisspermine, LTSP = linear tetraspermine, cBSP = cyclic bisspermine, DTSP = dendritic tetraspermine, QTSP = quatrefoil tetraspermine.

Table 1. Characteristics of Oligospermines

polymer	M_w calcd	No. of amines 1°, 2°, 3°	amine density[a]	CE_{85}[b]	ret.[c] time (min)
spermine	202.3	2, 2, 0	2.0	>60	7.2
cSP	312.2	0, 2, 0	0.6	>60	6.6
acSP	327.4	1, 2, 0	0.9	>60	7.4
LBSP	501.7	3, 4, 0	1.4	7.45	8.5
cBSP	624.5	0, 4, 0	0.6	>60	8.2
LTSP	1100.5	5, 8, 0	1.2	5.23	9.4
DTSP	1029.5	4, 8, 2	1.4	5.11	9.3
QTSP	1529.0	0, 8, 2	0.6	>60	8.7

[a]Amine density is calculated as number of amines per 100 Da. [b]CE_{85} is the N/P ratio at which compounds show 85% complexation with siRNA in water. [c]Ret. time is the elution time obtained after reversed phase chromatography. Details described in SI.

spermines in a linear fashion allowed us to examine the effect of molecular weight on binding.

Interaction of oligospermines with siRNA was investigated using SYBR gold assay to detect the presence of free siRNA as indicated by quenching of the fluorescence.[3c] Higher affinity of oligospermines indicates lower percentage of free siRNA in the complex. For quantitative analysis, affinity of oligospermines for siRNA was calculated and reported in terms of N/P ratio required to quench fluorescence by 85% (C_{85}; Table 1). The value of 85% was chosen so that we could find high affinity oligospermines.

Spermine and all other monomeric spermines had C_{85} of more than 60 (Table1), suggesting relatively low affinity; however, some important information was obtained from their binding profiles. Although spermine exhibited good binding to siRNA, protection of any two amines in spermine led to a substantial decrease in binding despite the presence of other two free amines in the structure (SI-F2). Thus, BBSP, irrespective of having two free primary amines, was ineffective in binding to siRNA. Similarly, cSP exhibited very low binding affinity, despite having two free secondary amines (Figure 1A). Reinsertion of one primary amine in cSP to form acSP restored the binding affinity. Thus, acSP and SP had similar binding affinity (26 vs 31% free siRNA for acSP and SP, Figure 1A) despite a more than 50% difference in amine density. These findings from binding profiles of monomeric spermine indicate that both primary and secondary amines contribute substantially in binding to siRNA, and three out of four amines in spermine are sufficient to achieve the required binding. Moreover, these results also provide the rationale for linking two spermines together using one of the four amines without adversely affecting the binding.

Figure 1A demonstrates an increase in binding affinity with the corresponding increase in the molecular weight for oligospermines. Interestingly, an inverse relationship existed

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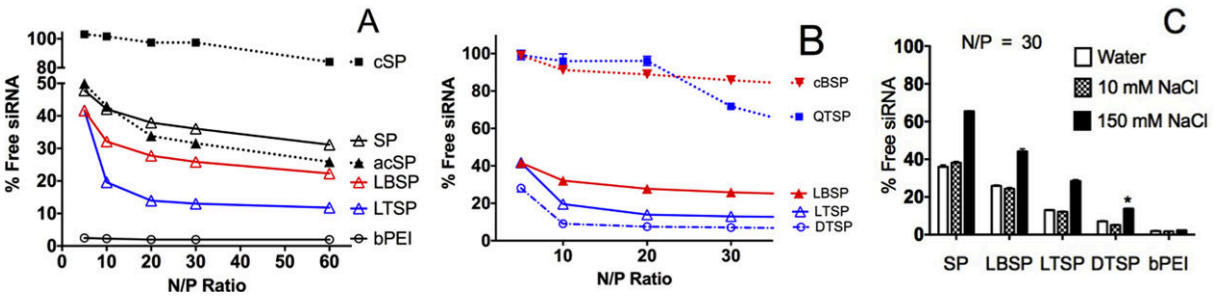


Figure 1. Effect of (A) molecular weight, (B) molecular architecture, and (C) salt concentration on binding of oligospermines to siRNA. Binding assays were performed at least twice in triplicate.

between amine density and binding affinity. Thus, LTSP with the lowest amine density of 1.2 exhibited the highest affinity (C_{85} = 5.23) followed by LBSP (amine density 1.4, C_{85} = 7.45) followed by spermine (amine density 2.0, C_{85} > 60). Higher affinity of LTSP to siRNA despite its lower amine density demonstrates the importance of multivalency effect. In contrast to linear oligospermines, only a modest increase in binding affinity was observed with the increase in the molecular weight of cyclic spermines. This could be because of the absence of primary amines in the cyclic amines examined.

An effect of molecular architecture was clearly observed among tetraspermines. Although, LTSP and DTSP had similar amine density and number of amines, DTSP exhibited higher affinity than LTSP (Figure 1B). As described later, this difference was also maintained at higher ionic strength and confirmed by atomistic molecular dynamics (MD) simulation studies. Both DTSP and LTSP had significantly higher affinities than QTSP. But this could be a mixed effect of architecture, amine density, and steric parameters. All cyclic spermines (cSP, cBSP, QTSP) exhibited very low binding affinity compared to their linear or dendritic counterparts (Figure 1A,B). Among all the oligospermines evaluated, DTSP exhibited highest binding to siRNA (CE_{85} of 5.11) and less than 10% free siRNA was detected in its complexes at N/P ratio of 10 (Figure 1B).

Next, we evaluated the effect of salt concentration on (10 and 150 mM NaCl) binding affinity. No significant differences in binding were observed between water and 10 mM NaCl, whereas binding affinity for most oligospermines substantially decreased in the presence of 150 mM NaCl (Figure 1C and SI-F3). Once again, DTSP exhibited highest affinity due to the lowest loss in binding at higher salt concentration.

The effect of salt concentration on binding of LTSP and DTSP to siRNA was further explored using atomistic MD simulations. We examined the binding of LTSP and DTSP to siRNA at two different salt concentrations by modeling four systems; one molecule of siRNA with either 10 molecules of LTSP or 8 molecules of DTSP at 10 mM and 150 mM ionic strengths. All systems were ionized to the appropriate ionic strength using sodium chloride and then solvated in TIP3 water using the VMD solvate plugin. Each system was simulated using the NAMD package,[8] using periodic boundary conditions, particle mesh Ewald (PME) method, and a Langevin damping constant of γ_{Lang} =1.0 ps^{-1}. All systems were visualized and analyzed using VMD.[9]

The average energies of binding between the tetraspermines and siRNA calculated (in vacuum) from the actual configurations of all the systems (in water) are LTSP, 10 mM (−635 kcal/mol); LTSP, 150 mM (−585 kcal/mol); DTSP, 10 mM (−788 kcal/mol); DTSP, 150 mM (−615 kcal/mol). These results show that DTSP has a greater binding affinity than LTSP, and the binding is weaker at higher ionic strengths. We observe that four DTSPs are bound to the siRNA at 10 mM, whereas only three DTSPs are bound at 150 mM (Figure 2A,B). This DTSP loss is a direct result of the lowered binding

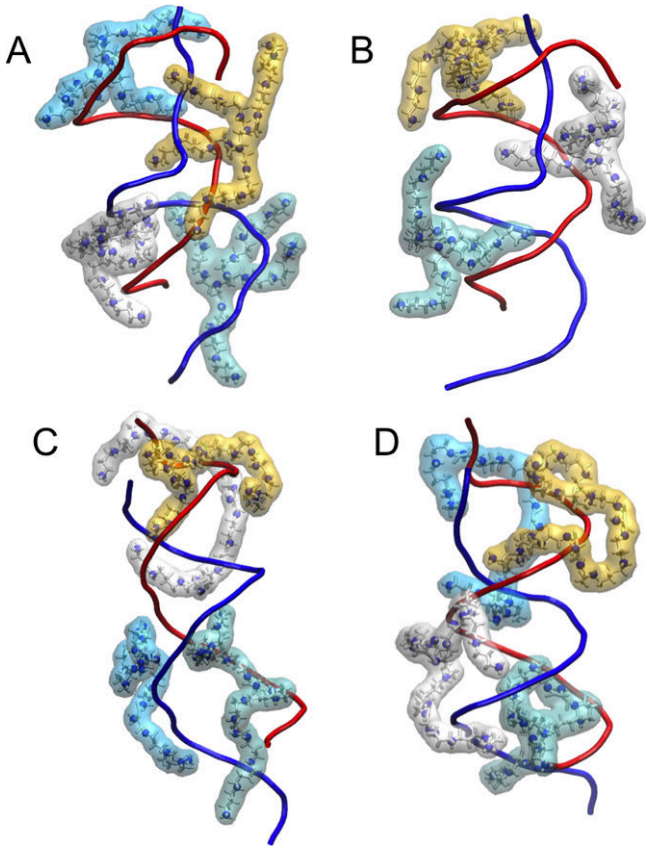


Figure 2. Model of tetraspermine bound to siRNA: (A) DTSP, 10 mM; (B) DTSP, 150 mM; (C) LTSP, 10 mM; (D) LTSP, 150 mM. Two strands of siRNA are represented as ribbons and each tetraspermine molecule is represented with a different color.

affinity. Additionally, both LTSP and DTSP are bound more tightly at 10 mM than at 150 mM. At 150 mM the bound tetraspermines were found to be on average 2 Å further away from the siRNA than at 10 mM (SI-Table S3). Both LTSP and DTSP bind predominantly to the siRNA backbone, but LTSP can be found between the two strands (Figure 2C,D). The DTSP seems to bind with the primary amine close to the backbone on each bound arm (on average, one arm in every DTSP molecule is unbound). These differences in binding are

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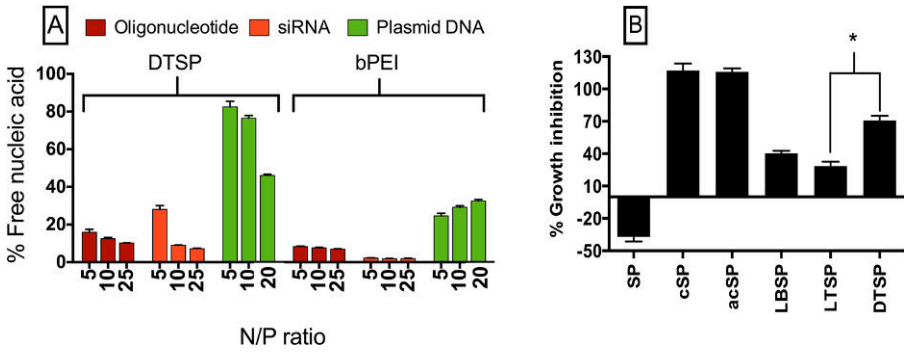


Figure 3. (A) Binding of DTSP and bPEI with oligonucleotide (11-mer), siRNA, and plasmid DNA. Experiments were performed at least two times (n = 3). (B) Growth inhibition of DU-145 after treatment with oligospermines for 48 h.

the result of different spermine geometries and may explain why DTSP has greater binding affinity.

We then evaluated the binding of oligospermines to plasmid DNA (SI-F4). In general binding affinity of oligospermines to plasmid DNA was lower than that of siRNA; however, the trend was similar. Thus, tetraspermines exhibited higher binding affinity compared to their lower molecular weight analogs and among tetraspermines DTSP had higher affinity than LTSP (SI-F4). Although, DTSP exhibited highest binding affinity to plasmid DNA among all oligospermines, its binding affinity to plasmid was low compared to binding to siRNA or single-stranded oligonucleotide (11-mer; Figure 3A). Figure 3A also shows a comparison of binding profile of DTSP with branched polyethylenimine (bPEI). All compounds evaluated in this report exhibited lower binding affinity than that of bPEI (25 kDa). However, a trend of increasing affinity with the increase in the molecular weight suggests that binding affinity similar to bPEI can be reached at much lower molecular weight than 25 kDa for oligospermines. Lower amine density of oligospermines (approximately 1.2) compared to bPEI (approximately 2.3) will be an important beneficial parameter in favor of oligospermines since cytotoxicity is very often directly proportional to amine density.[10]

We have reported previously that DTSP is several folds less toxic than bPEI.[11] In this report, we studied growth inhibition properties of all oligospermines in three cell lines including human embryonic kidney (HEK-293; often used as a model cell line in transfection experiments), a breast cancer (Hs578T), and a prostate cancer cell line (DU-145). We have reported that GI_{50} (growth inhibitory concentration) for DTSP is around 50 μM in DU-145 after 48 h treatment.[11] Thus, we chose 50 μM concentration to examine and compare cytotoxicity of oligospermines after 48 h and 5 days treatment. Cell viabilities of DU-145 after treatment with oligospermines are shown in Figure 3B, whereas cell viabilities of HEK-293 and Hs578T cell lines are reported in SI (SI-F5). Spermine was the most toxic compound in all the cell lines studied, whereas no toxicity was observed for cSP as well as for acSP (Figure 3B and SI-F5). Importantly, DTSP was significantly less toxic compared to its linear counterpart LTSP and LBSP (Figure 3B). The findings demonstrate differential toxicity of oligospermines in different cell lines and also indicate lower toxicity profile for dendritic architecture.

In summary, this report demonstrates that molecular architecture affects binding, and stability as well as cytotoxicity of oligospermines. Dendritic architecture stands out as a favorable architecture for oligospermines. The report also gives insights for the development of next generation oligospermines that will be relatively safer and less toxic than currently available nucleic acid carriers.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis and characterization of the compounds, SYBR gold assay, cytotoxicity study, and modeling methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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