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Detection of noncovalent interactions of hairpin oligonucleotide with stilbazolium ligands by MALDI TOF mass spectrometry

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Abstract

Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectroscopic analysis was carried out to study the noncovalent complexes of aryl stilbazolium ligands with short deoxyoligonucleotide, 5′CGCTTTGCG-3′ (sT_3). The method enables to assess rapidly stoichiometries of complexes. The number of peaks associated with complexes and their relative abundances depended on the structure of aryl moiety of the ligand and reflected DNA-binding mode and affinity of the ligand. The most potent ligands, 9-[2-(*N*-methylpyridinium-4-yl) vinyl]anthracene and 2-[2-(*N*-methylpyridinium-4-yl)vinyl]naphthalene formed 1:1, 1:2 and 1:3 complexes with both hairpin and bulged duplex structures of sT_3 oligonucleotide.

Keywords: MALDI TOF mass spectrometry; Noncovalent interactions; Oligonucleotides; Stilbazolium ligands

1. Introduction

The binding of small molecules (ligands) to DNA continues to be a subject of considerable interest. It was evidenced that noncovalent interactions is the molecular basis of many antiviral, antitumor and antibiotic drugs [1]. On the other hand, many fluorescent ligands were successfully used as fluorescent labels in analysis of DNA. Small molecules can interact with nucleic acids in a variety of ways, such as surface binding to their minor or major grooves, intercalation between adjacent base pairs, covalent attachment to the double helix, or territorial (electrostatic) binding [1]. It is widely agreed that DNA intercalation does not contribute much sequence discrimination, while most minor groove DNA-binding ligands, such as netropsin, Hoechst 33258, and DAPI, bind with significant preference to AT-rich sequence [2–5]. On the other hand, it is clear from the broad literature in this field, that both a DNA sequence and ligand structure characteristics exert pronounced effects on binding mode selection [5–7]. Small duplex oligonucleotides have served as appropriate models for assessing DNA-binding properties of ligands, and the results allow further improvement of ligand design. A wide variety of physical, chemical and biochemical techniques have been proposed for determining binding mode, stoichiometry, affinity and selectivity of these noncovalent complexes. The methods include UV-Vis absorption and fluorescence spectroscopies, circular and linear dichroism, FT-IR, NMR, X-ray crystallography, gel electrophoresis, and viscosity titration [8–13].

Recently, mass spectrometry has been introduced as a valuable tool in investigations of biopolymers. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) techniques were used in these applications, due to the gentle nature of ionization that allows a wide range of biopolymers to be introduced intact into the gas phase. Mass spectrometry allows analysis of samples at micromolar concentration, which together with short spectrum acquisition time, make this technology more amenable to high-throughput analysis. During the past years, considerable work has been done to apply mass spectrometry to nucleic acid analysis. The applications range from sizing and sequencing DNA to identifying genetic changes and polymorphisms, to study noncovalent interactions, including enzyme-inhibitor complexes, oligonucleotide duplexes and tetramers [14–20]. The studies of noncovalent interactions by mass spectrometry have been the domain of

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ESI until recently. Lin at al. demonstrated that MALDI TOF MS could be used to study short oligonucleotides/peptides complexes [21]. Yamashita et al. succeeded in detecting complexes of duplex oligonucleotides with classical intercalators and minor groove binders using this technique [22]. They showed that binding stoichiometry for complexes of ligand with both single- and double-stranded DNA could be revealed by MALDI TOF MS. The finding that biospecific noncovalent complexes can be detected and analyzed by MALDI TOF MS needs more proofs and extensions. Since formation of clusters or aggregates that do not reflect solution-phase noncovalent complexes was also reported [23,24], a question about the specificity of the complexes in gas-phase obtained by laser desorption should be addressed.

In this paper, we report MALDI TOF MS studies of complexes of hairpin-forming oligonucleotide with a series of stilbazolium ligands, which are known to interact with DNA by intercalation and groove binding [25–28]. By comparing mass spectra for structurally very similar ligands we have tried to answer the question whether nonspecific aggregation plays a significant role and whether quantitative binding data can be retrieved from relative ion abundances.

2. Experimental

The deoxyribonucleotide d(CGCTTTGCG) abbreviated as sT_3 was synthesized and HPLC-purified by TaKaRa Inc. (Tokyo, Japan) and was used without further purification. The aryl stilbazolium derivatives (**1**–**7** in Fig. 1) were prepared as for previous works by standard procedures [25–28].

MALDI TOF mass spectrometry experiments were carried out with a Voyager DE Spectrometer (Applied Biosystems Japan Ltd.). One μl of a solution containing 15.4 μM d(CGCTTTGCG) oligonucleotide, 7.7 g/l 3-hydroxypicolinic acid (3-HPA) as a matrix, and 77 μM ligand was placed on the sample plate and dried slowly in the air to leave a crystalline residue. It was then ionized by a laser beam (intensity 1800) at 5×10^{-7} Torr and mass spectra were recorded by the negative mode at 25 °C and acceleration voltage of 20 kV.

3. Results and discussion

Fig. 1 shows the structures of sT_3 oligonucleotide and DNA-binding ligands. Oligonucleotide is able to form hairpin and bulged duplex structures, but the former is the predominant species in solution under ordinary conditions (pH 3–7, 0.1 M NaCl) [29,30]. The hybridization of hairpin-forming oligonucleotides is concentration-independent thus it is expected to exhibit diminished abundance of single-stranded species in gas phase (collision of duplexes during desorption–ionization–acceleration facilitates duplex dissociation [31–33]). Matrix properties play an important role both in duplex stabilization, fragmentation and complex desorption efficiency. ATT (6-aza-2-thiothymine) was reported to be a good matrix choice for studying DNA/peptide noncovalent complexes due to its neutral pH that preserves ionic interactions between peptide and DNA [21]. This feature of ATT seems to be disadvantageous for our studies since ionic interactions may produce nonspecific aggregates. Moreover, GC-rich oligonucleotides (like sT_3 hairpin) undergo poor desorption when ATT is used as a matrix [21]. Therefore, conventional 3-HPA matrix with weak acidic properties (pH 3.2) was used in all experiments. As reported by Little et al., upon low acceleration voltage and slow drying procedure, the 3-HPA matrix allows detection of a double strand in gas phase [33].

Mass spectra of oligonucleotide alone and its complexes with stilbazolium ligands, obtained by the negative mode, are shown in Fig. 2. Two diagnostic peaks appeared at 2704 and 5409 mass-to-charge ratio in the mass spectrum of oligonucleotide alone (Fig. 2A). They correspond to the intact $[M-H]^-$ and $[M_2-H]^-$ species, respectively. The former corresponds to mass of hairpin structure and/or single-stranded oligonucleotide (they have the same mass-to-charge ratio) and the later, much less intense, to the bulged double-stranded DNA. This observation is consistent with the preferential formation of very stable hairpin structure (T_m = 60 °C [30]). This intramolecular process is concentration-independent and should minimize the content of single-stranded (ss) random-coil conformation of oligonucleotide. The presence of large amounts of unwanted ss species in mass spectra is commonly observed for self-complementary oligonucleotide samples [19–22]. It should be noted that in addition to the signals at 2704 and 5409 Da, there are additional satellite peaks at around 2400 and 5100 Da. These peaks can be ascribed to the fragments with terminal nucleotide missing: hairpin $[M - dCMP/dGMP]^-$ and bulge duplex $[M_2 - dCMP/dGMP]^-$, respectively. The presence of these fragmentation peaks do not disturb in observation of noncovalent complexes with ligands since they appear at lower *m*/*z* than main peaks.

In the presence of all stilbazolium ligands **1**–**7**, additional peaks appeared in the range of 3050–3100 and 5750–5800 Da but their number and relative intensities differ for particular ligands. Except for ligands **2** and **4**, mass spectra of other systems show only peaks that correspond to the 1:1 stoichiometry of DNA/ligand complexes as it can be inferred from the mass increments corresponding to the molecular weights of the ligands. The 1:1 complexes are formed by both hairpin (around 3100 Da) and bulged duplex structures (around 5800 Da). Such a binding stoichiometry is consistent with intercalation since the hairpin stem possesses three paired base pairs, but there is only one intercalation site if one considers the nearest neighbor exclusion principle [1]. Although bulged duplex has potentially two intercalation sites, the low abundance of the duplex and insufficient binding affinity hamper detection of a 1:2 duplex/ligand species. It should be stressed

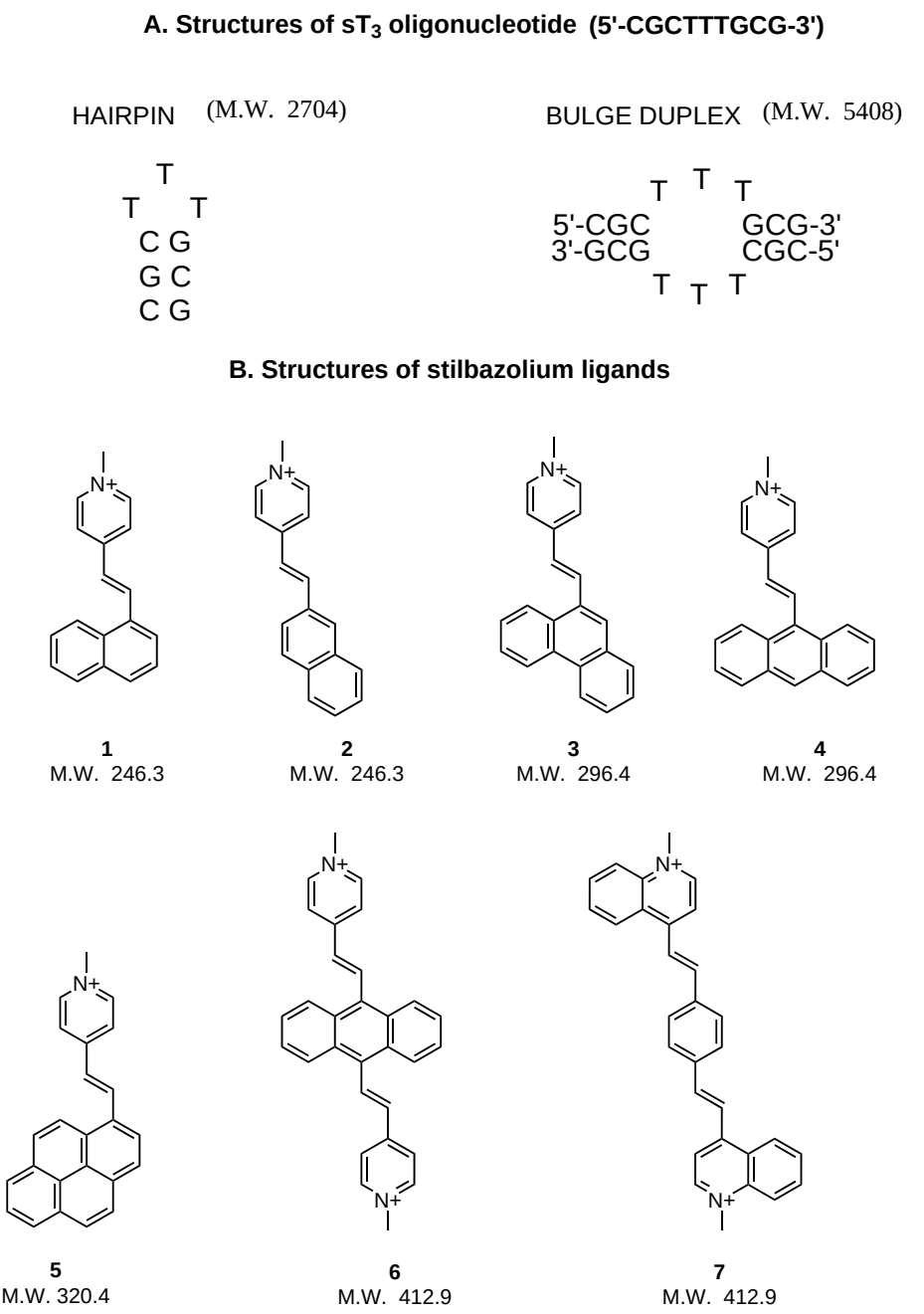

Fig. 1. Structures of oligonucleotide sT_3 (A) and stilbazolium ligands **1**–**7** (B) used in MALDI TOF MS experiments.

that intercalation has been proven for these planar ligands by independent methods including circular dichroism and viscosity titration [25,27,28].

On the other hand, 2-naphthyl and 9-anthryl derivatives (structures **2** and **4** in Fig. 1), showed additional molecular peaks corresponding to complexes with higher DNA:ligand stoichiometry. It is unclear why these two ligands prefer multiple binding. Formation of 1:2 complexes with hairpin structure cannot be accounted for the intercalation exclusively and the explanation should involve other binding modes. There are three alternative processes: (i) binding of a second molecule to the minor groove of hairpin, (ii) groove binding of a dimeric form of ligand without intercalation, (iii) interaction of a second ligand with the loop-forming thymines. The first case involving mixed binding with one ligand intercalated and a second one bound to minor groove should be rejected since the minor groove of sT_3 oligonucleotide containing GC sequences is unable to accommodate a ligand molecule. The second binding mode was previously observed for typical minor groove binders [19,22,34], therefore ligand **2**, which binds also to the minor groove at AT sequences [28], could be suspected of interacting according to this mechanism. Although oligonucleotide sT_3 does not contain AT base pairs, this binding mode cannot

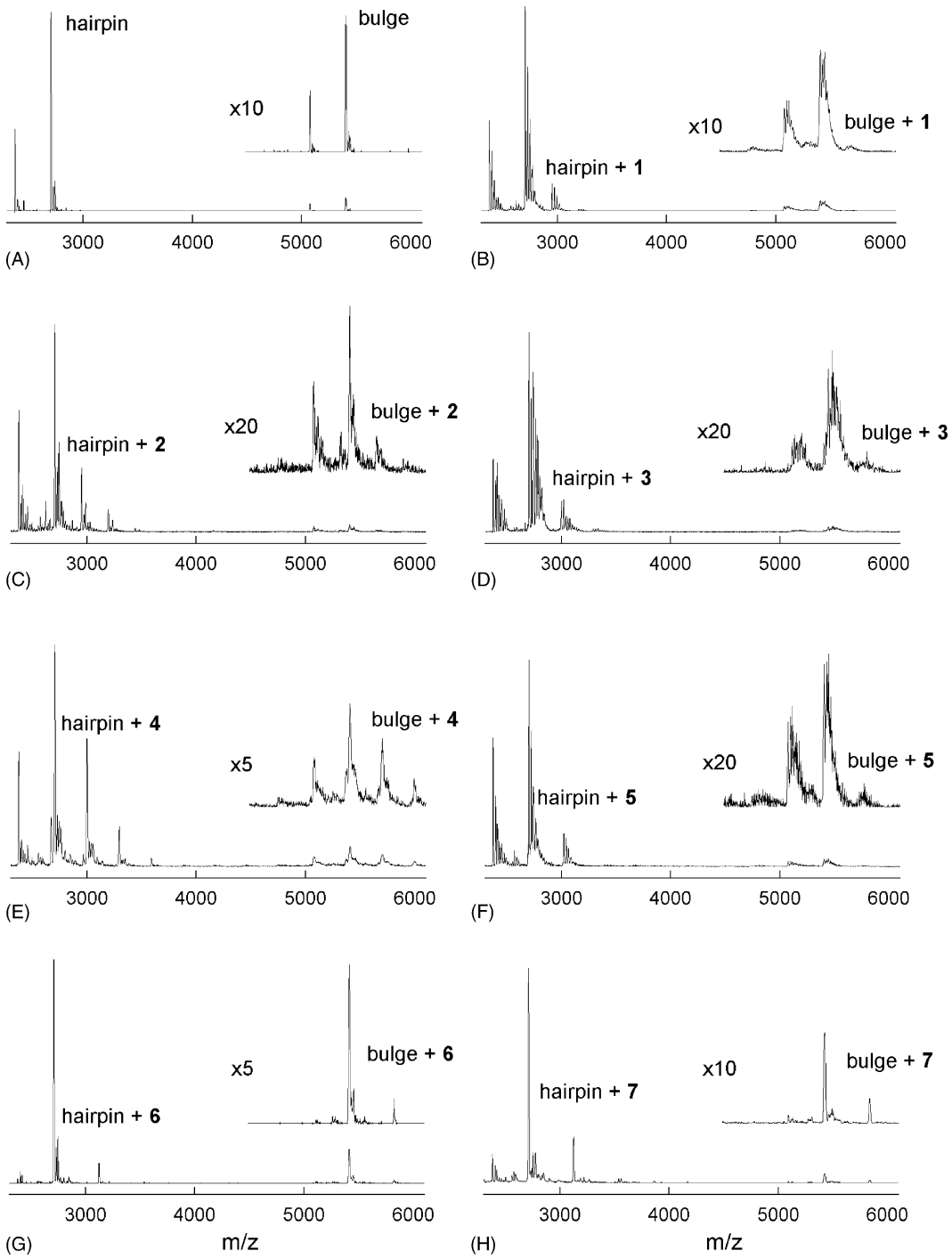

Fig. 2. MALDI TOF mass spectra of sT3 oligonucleotide in the absence (A) and the presence of stilbazolium ligands **1**–**7** (B–H).

be excluded since it may operate in the major groove of DNA. This groove is wider than the minor groove in B-DNA (limited van der Waals contact with drug molecule) but can easily accommodate two aromatic molecules stabilized by stacking interactions. Electron donating properties of amine group of cytosine located in the major groove could further stabilize the complex. The groove binding ability of the ligand seems to play an important role since typical groove binder, Hoechst 33258, bound to mismatched DNA duplexes showed also higher stoichiometry complexes in MALDI TOF spectra [22]. The third process, an affinity of the ligands to bind to a single-stranded DNA region (thymine loop), seems to be also plausible since it can explain the formation of even higher stoichiometry (1:3) complexes. Such conclusion is consistent with UV and calorimetric studies of Marky and co-workers, who observed two different binding sites for ethidium derivatives with a hairpin oligonucleotide d(GCGCT_5GCGC): a high affinity site in a stem and a lower

affinity site located at the thymine loop [35]. One can assume that these specific interactions depend on the structural peculiarities of the ligand, but probably do not reflect simply the extent of planarity of its aromatic moiety. It should be noted that in case of 9-anthryl derivative **4**, the structural peculiarities might be important since only this ligand possesses nonplanar structure [26]. Interestingly, distilbazolium ligands **6** and **7** possessing double positive charges could be expected to interact with single-stranded loops of hairpin and bulged duplex (electrostatic attraction), but surprisingly, they formed only 1:1 complexes. Presumably, steric hindrance and size of molecule may be responsible for the behavior of these ligands. Concluding, both processes: the groove binding of dimeric form of the ligand and the interaction of a second ligand with thymine loop can be responsible for higher stoichiometry complexes observed in case of ligands **2** and **4**.

On the other hand, diverse binding characteristics for such similar ligands provide solid support that formation of nonspecific complex (trivial aggregation) can be ruled out in the experimental conditions used here. If the detected complexes correspond to nonspecific interactions one can expect the superior complexation for more hydrophobic derivative, e.g., 1-pyrene. Moreover, two isomeric ligands, 1-naphthyl and 2-naphthyl derivatives, should exhibit comparable affinities. Because such trends are not reflected in the experimental results, there is strong suggestion that specific noncovalent complexes are present in the gas phase.

A question arises if the gas-phase species observed in MALDI TOF spectra correspond to solution-phase noncovalent complexes and whether peak intensities can be regarded as relative binding affinities of the ligands, similarly as it was observed in ESI mass spectra [19,20]. The relative abundances of particular complexes, calculated on the basis of peak ratios (intensities of free DNA peaks were taken as 100%), are collected in Table 1. Taking into account the relative abundances of 1:1 complexes as representative, the DNA-binding affinities of ligands increase in the following order: **6** < **1** < **5** < **3** < **7** < **2** < **4**. Larger binding affinities (higher relative abundances) of ligands **2** and **4** are consistent with their ability to form 1:2 and 1:3 complexes with both hairpin and bulged duplex structures of sT_3 oligonucleotide. Since binding constants of ligand/sT_3 complexes are not known, we compared the relative abundances with the binding parameters of stilbazolium ligand complexes with calf thymus DNA or $[poly(dG\text{-}dC)]_2$ [25,27,28]. Bisquinolinium ligand **7** exhibits superior DNA affinity with a binding constant in the range of 1×10^6 to $7 \times 10^6\ M^{-1}$ (depending on salt concentration and DNA sequence) [27]. Ligand **6** has slightly lower binding affinity (binding constant ca. $2 \times 10^6\ M^{-1}$) and ligands **1**–**5** bind weaker to DNA with comparable binding constants in the range of 0.4×10^5 to $3 \times 10^5\ M^{-1}$ [25,28]. The overall order of binding constants for ligands is **5** < **2** < **1** < **4** < **3** < **6** < **7**. The discrepancy between the relative abundances of complexes in MALDI TOF spectra and their stability constants is obvious, especially if one compares ligands **1**–**5** with a single charge and ligands **6**, **7** possessing two positively charged sites. Trying to explain this discrepancy one cannot exclude some specific effects connected with matrix crystal growth in the presence of particular ligands that in turn may affect desorption of the resulting complexes. Such effects could be critical for ligands with different charges. Indeed, for a pair of complexes **6** and **7** the abundances of gas-phase ions do reflect the true solution-phase order **6** < **7**, but the monocationic ligands **1**–**5** still exhibit unexpected order (high abundances of complexes of ligands **2** and **4)**. Possible explanation should involve different conditions and different oligonucleotides used in MS experiments and in binding studies. This factor may be of particular importance for ligands **2** and **4**, which exhibit peculiar affinity for hairpin oligonucleotide. Yamashita et al. also observed significant differences in complex abundances between a classical intercalator (ethidium), a threading intercalator (naphthalenediimide derivative) and a groove binder (Hoechst 33258) [22]. As pointed out by Gross and co-workers in their ESI MS studies [19], the binding affinity and selectivity may depend on the size and sequence of oligonucleotide used and do not necessarily reflect those determined for synthetic polymers or native DNAs. Concluding, the binding affinity can be inferred from relative abundances of gas-phase complexes provided that ligands bind to DNA by the same binding mode. It looks as the binding parameters commonly reported for calf thymus DNA or synthetic polynucleotides (AT- or GC-type) have a limited value for such purpose. To clarify this point, the binding studies of stilbazolium ligands with hairpin oligonucleotide should be carried out.

In summary, we have shown here that analysis of noncovalent complexes of organic ligands with hairpin and bulged structures of short oligonucleotide can be accomplished by using MALDI TOF mass spectrometry. The binding stoichiometry can be easily established, but the relationship between relative abundances of complexes in gas phase and their binding constants is aqueous solution is more complex especially for ligands that interact with DNA by different binding modes. The technique needs to be developed further so that it can be utilized routinely in

Table 1
The relative abundances of complexes of stilbazolium ligands **1**–**7** with hairpin and bulged duplex structures of sT_3 oligonucleotide calculated from MALDI TOF spectra

	sT_3:ligand stoichiometry	Relative abundance of complex/%						
		1	**2**	**3**	**4**	**5**	**6**	**7**
Hairpin	1:1	14	31	16	61	15	9	21
	1:2	N.D.	11	N.D.	18	N.D.	N.D.	N.D.
	1:3	N.D.	2	N.D.	4	N.D.	N.D.	N.D.
Bulged	1:1	6	25	18	67	17	14	27
Duplex	1:2	N.D.	12	N.D.	30	N.D.	N.D.	N.D.
	1:3	N.D.	3	N.D.	8	N.D.	N.D.	N.D.

N.D.: not detectable.

analysis of ligand/DNA complexes. Systematic study of the influence of experimental parameters (matrix compound, organic solvent additives, pH, laser fluence, oligonucleotide sequence and size) are needed to provide further evidence that observed stoichiometries are true solution-phase equilibria and do not correspond to nonspecific phenomena intrinsic to the desorption/ionization process.

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