ELECTROSPRAY MASS SPECTROMETRY TO STUDY DRUG-NUCLEIC ACIDS INTERACTIONS

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Abstract

We present here a tutorial review on the electrospray mass spectrometry technique and its applications to the study of drug-nucleic acid noncovalent complexes. Particular emphasis has been made on the basic principles of the technique, to allow even the non-specialist to design fit-for-purpose mass spectrometry experiments and interpret the results. Standard applications will be described in detail, including the determination of stoichiometries and equilibrium binding constants of noncovalent complexes, the study of binding kinetics, and the development of ligand screening assays. We also outline the potentials of more advanced and/or more recent MS-based techniques (tandem mass spectrometry, ion mobility spectrometry and gas-phase spectroscopy) for the study of the nucleic acid-ligand complexes.

Keywords: Electrospray mass spectrometry, ligand, nucleic acid, noncovalent complex, binding constant

1. Introduction

All mass spectrometers determine the mass-to-charge ratio of ions in vacuum, but there are various ways of ionizing molecules and transferring them from the solution to the mass spectrometer. Electrospray ionization [1; 2] is a commonly used ionization method for the analysis of biomolecules like peptides, proteins, and nucleic acids. The major feature of electrospray ionization mass spectrometry is that the analytes of interest can be transferred from the sample solution to the mass spectrometer with minimal fragmentation. Soon after the development of the first electrospray mass spectrometers, it was demonstrated that even noncovalent complexes could be detected intact [3]. This seminal paper in 1991 was the starting point of a whole field of research, namely the analysis of complexes of biological interest by ESI-MS [4-6].

The observation of intact DNA duplexes by ESI-MS was made in 1993 [7; 8], and the first reports on the observation of duplex-ligand interactions appeared soon thereafter [9; 10]. Electrospray mass spectrometry analysis of noncovalent complexes including for DNA or RNA-targeting drugs has now found important applications as a screening tool in drug discovery [11-15]. Two comprehensive reviews appeared in 2001, describing the analysis of various types of noncovalent DNA complexes (nucleic acid multi-stranded structures, nucleic acid-ligand complexes, and nucleic acid-protein complexes) by ESI-MS [16; 17]. Since then, the number of papers reporting ESI mass spectra of nucleic acid-ligand complexes has continued growing, and as the availability and ease of operation of ESI-MS mass spectrometers increases, the techniques is more and more commonly used among the panel of more traditional spectroscopic techniques.

2. The basics of electrospray ionization mass spectrometry (ESI-MS)

2.1. Electrospray ionization (ESI)

The electrospray mechanism

In electrospray, the sample consists of an aqueous solution of the analyte. The sample is infused at atmospheric pressure with a syringe or from a liquid chromatograph. The electrospray mechanism has been described in several review papers [18-20], and a thorough description can be found in these references. Here we will just outline the major stages of the mechanism, which is generally divided in three steps: droplet formation, droplet fission and production of desolvated ions. The electrospray capillary containing the solution is maintained at a potential of a few kilovolt, and is located a few millimeters from the entrance of the mass spectrometer, which is generally at ground. The strong electric field causes an electrophoretic movement of the ions inside the liquid, and charged droplets are emitted at the tip of the capillary. The droplets are charged because they contain excess of ions of one polarity. The polarity of the droplet depends of the sign of the applied potential. For nucleic acids, negative ion mode is used because nucleic acids are naturally negatively charged in solution.

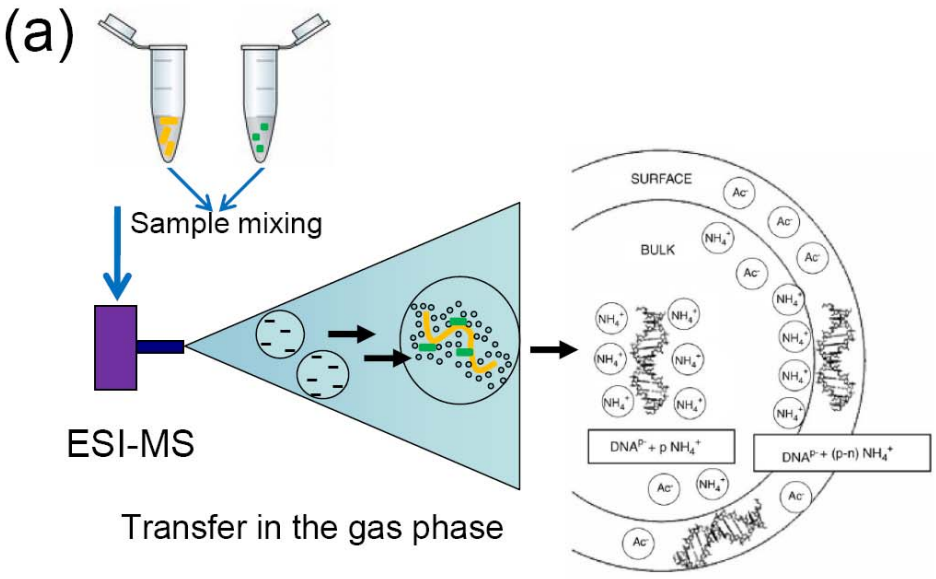
The next step is droplet fission. As the droplets travel from the capillary to the mass spectrometer, they undergo collisions with the ambient gas, and the solvent evaporates. The radius of the droplets decreases at constant charge until the Coulomb repulsion between the charges becomes greater than the cohesive forces. At a critical radius called the Rayleigh limit, the droplets explode asymmetrically, producing a series of small daughter droplets from the surface of the mother droplet. The daughter droplets are therefore enriched with the ions that were at the surface of the mother droplet. The daughter droplets then undergo evaporation and

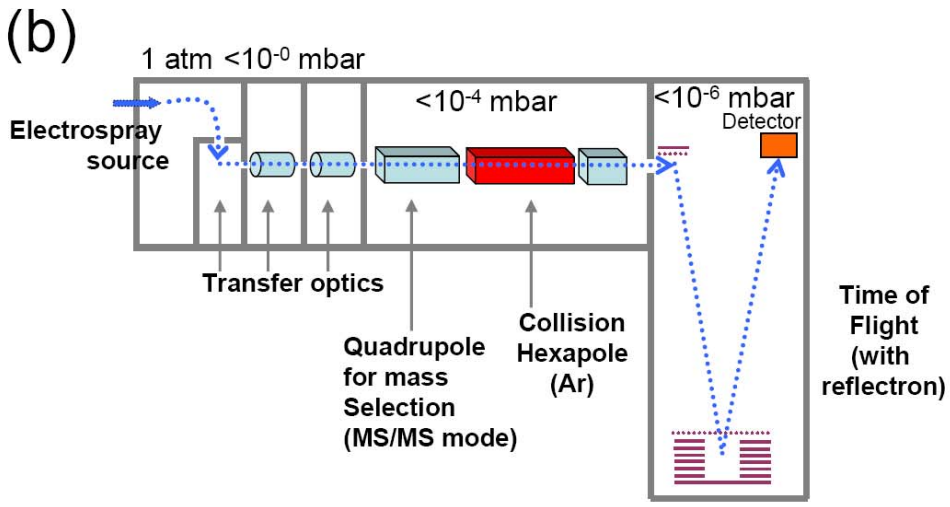
fission themselves. In about a hundred of microseconds, the size and charge of the droplets decreases to a point where single ions are isolated, surrounded by residual counterions and solvent molecules. The last step of the production of desolvated ions in the gas phase, and the most commonly accepted mechanism for large ions like the complexes described here is the "charge residue model": a final droplet with containing only one analyte ion (here a DNA ion or a noncovalent complex) evaporates until the last solvent molecule is lost.

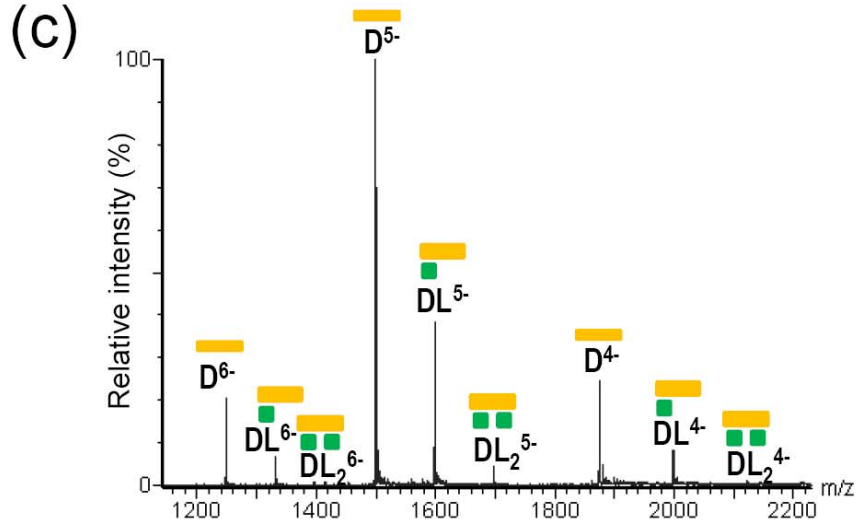
Electrospray of nucleic acids

ESI-MS investigations of nucleic acids are carried out using negative ion polarity. This follows logically from the knowledge that the phosphodiester backbone of the DNA has a pKa < 1, and is therefore fully deprotonated in solution. In order to preserve native nucleic acid structures, solutions with an ionic strength corresponding to ~150 mM monovalent cation should be studied. However, a major limitation of electrospray mass spectrometry is its low salt tolerance, because of the counterion condensation on the nucleic acid during droplet evaporation. Even minute amounts of sodium or potassium result in the detection of a wide distribution of adduct stoichiometries on the DNA. Using ammonium acetate circumvents this salt adduct problem. In negative ion mode electrospray, the droplets carry excess negative charges consisting of DNA polyanions and acetate anions (Figure 1a). After complete solvent evaporation, further activation of the DNA with its ammonium cation counterions results in proton transfer reactions from NH_4^+ to PO^-, hence neutralization of phosphates by protons. When using 150 mM ammonium acetate, only a small fraction of phosphates remain negatively charged (on average, 5 out of 22 in a 12-mer duplex DNA).

Figure 1 (next page): Generic electrospray mass spectrometry experiment on drug-nucleic acid complexes. (a) Sample is prepared by mixing DNA (D; yellow) and ligand (L; green), and the sample is injected in the mass spectrometer via the electrospray source. The right side of the panel is a schematic view of the electrospray process at the molecular level in negative ion mode (see text for details). (b) Schematic representation of a hybrid quadrupole-time of flight mass spectrometer. The ion trajectory is in blue. The ions are produced in the electrospray source, and pass through different transfer optics where desolvation and focusing is completed. The quadrupole is used as transfer optics in simple MS mode or as a mass selective device for MS/MS experiments. The collision hexapole is used as transfer optics in simple MS mode or for collisional activation in MS/MS experiments. Finally ions are analysed according to their mass-to-charge ratio using the time-of-flight analyzer and the number of ion of each m/z is counted on the detector. The differential pressures (from atmospheric pressure to high vacuum) inside the mass spectrometer are indicated. (c) Typical electrospray mass spectrum of a DNA-ligand mixture, showing three species of different masses *m* corresponding to the free DNA (D), 1:1 and 2:1 ligand-DNA complexes (DL and DL_2), each at three different charge states ($z = 6$, 5 and 4).


(a)
Sample mixing
ESI-MS
Transfer in the gas phase
SURFACE
BULK
DNA^p- + p NH4+
DNA^p- + (p-n) NH4+


(b)
1 atm <10-0 mbar
<10-4 mbar
<10-6 mbar
Detector
Electrospray source
Transfer optics
Quadrupole for mass Selection (MS/MS mode)
Collision Hexapole (Ar)
Time of Flight (with reflectron)


(c)
Relative intensity (%)
100
0
m/z
1200
1400
1600
1800
2000
2200
D5-
D6-
DL6-
DL2 6-
DL5-
DL2 5-
D4-
DL4-
DL2 4-

Finally, even though most recent mass spectrometers allow recording ESI mass spectra from aqueous solutions in the negative ion mode, the signal is usually much enhanced when some methanol is added to the solution prior to injection. This is because methanol decreases the surface tension of the droplets and favors the droplet formation, fission, and evaporation processes. Usually 15-20% methanol is added to the samples just prior to infusion. This methanol concentration gives significant signal enhancement, minimizes risks of conformational changes in solution (as tested by circular dichroism spectroscopy), and was not found to induce major changes in the relative peaks intensities.

2.2. Mass spectrometers (MS) [21]

A legitimate question here is: which mass spectrometer to choose? The short answer is: any mass spectrometer can be used provided that it has an electrospray source! From home-made to sophisticated ultra-high resolution machines, all mass spectrometers can be tuned to observe nucleic acid-ligand complexes. The key is to choose instrumental settings that allow proper evaporation of the droplets and desolvation of the ions to obtain reasonably large ion signals, while minimizing extra internal energy uptake by the ions to avoid disruption of the noncovalent interactions between the nucleic acid and the ligand. Critical parameters are therefore source or capillary temperatures (kept as low as possible), and all acceleration voltages in the transfer optics (all cone, skimmer, and lens voltages along the ion path must be kept low) (Figure 1(b)).

The resolution of the mass spectrometer will only influence the complexity of the mixtures that can be resolved in a single spectrum. It will also determine if the isotopic distribution of a given species can be resolved. Distinguishing the isotopic distribution can be very helpful to assign the charge of a peak (isotopes are separated by 1 Da, so on the *m/z* scale the spacing between isotopic

peaks is equal to the inverse of the charge: *1/z*). Once the charge is known, the mass is obtained by multiplying the *m/z* of the peak by the charge. There are nevertheless other tricks to interpret mass spectra even if the isotopes are not resolved. Usually in nucleic acid-ligand investigations the masses of the nucleic acids and ligands mixed are known, and the easiest is to calculate the theoretical *m/z* for all possible complexes at different charges.

The sensitivity determines how much sample is required to record the data. In any case, recording a single mass spectrum requires < 50 µL of sample at a nucleic acid concentration of 1-10 µM, hence less than a picomole of nucleic acid per spectrum. Figure 2 shows typical ESI mass spectra recorded on a Q-TOF Ultima Global mass spectrometer (Micromass, now Waters, Manchester, UK) from 30 µL of solution containing 5 µM of 12-mer duplex DNA $d(CGCGAATTCGCG)_2$ and 5 µM of ligand MMQ1 [22; 23]. The spectra are shown at two different "RF lens" voltages (the RF lens is accelerating the ions just after the ESI source). When using ammonium acetate solutions, a good indication of the softness of source conditions is the detection of a few remaining ammonium adducts on the nucleic acid anions [24]. The relative intensity of adducts decreases as the RF Lens1 voltage increases from 60 V to 100 V, but the relative intensities of free duplex vs. complexes does not change. However, if too high acceleration voltages are applied, dissociation of the duplex and/or the complexes can occur.

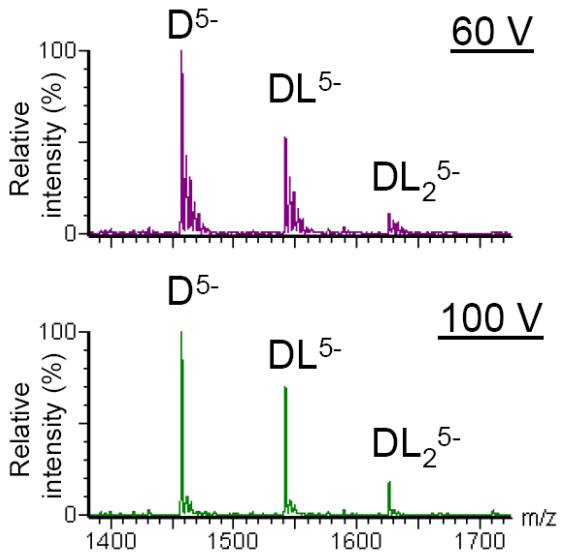


Figure 2: ESI mass spectra of a equimolar solution of d(CGCGAATTCGCG)$_2$ duplex (molecular weight = 7292.86 Da) and MMQ1 (MW = 422.56 Da) collected at two different acceleration voltage (RFLens1). The desolvation is increased by using higher acceleration values. If too large voltage is applied, dissociation of the species will occur.

2.3 Tandem mass spectrometry (MS/MS) and collision-induced dissociation (CID)

In simple MS mode, all ions produced in the electrospray source travel to the analyzer and the instrumental parameters are chosen so as to keep fragmentation minimal. However, most mass spectrometers also offer the possibility to perform tandem mass spectrometry (MS/MS) experiments. A common MS/MS experiment consists in recording a product ion spectrum. In that case, ions of a given mass-to-charge ratio are isolated, fragmented, and the resulting fragments are analyzed. In a Q-TOF mass spectrometer (shown in Figure 1B), the ions are first selected in a quadrupole, and then accelerated into a hexapole filled with argon at low pressure. At each

collision of the ion with an argon atom, a fraction of the relative kinetic energy is converted to vibrational energy of the ion (also called internal energy). When the ions have accumulated enough internal energy they can fragment in the mass spectrometer. This process is called collision-induced dissociation (CID). The mass spectrum that is recorded after CID is the product ion spectrum. In the last part of the article, we will describe some applications of MS/MS in the field of nucleic acid-ligand studies. However, the most important information obtained on the composition of the solution is found in the source mass spectrum.

3. Stoichiometry determination

The major strength of mass spectrometry is its ability to resolve complex mixtures. As opposed to other spectroscopic techniques, mass spectrometry gives one signal for each species differing by mass. Therefore, the stoichiometry of each complex present in a given sample, even minor products, can be read directly from the mass spectrum. From the mass of a complex, one can calculate the number of DNA strands involved, the number of bound cations if present, and the number of bound ligands.

3.1. Detecting intact nucleic acid assemblies

One of the first noncovalent complexes ever detected by ESI-MS was a DNA duplex. Now, several kinds of assemblies like duplexes, triplexes [25-27], and G-quadruplexes [27-35] have been successfully analyzed by ESI-MS. The key in sample preparation is to form the desired structure while minimizing the sodium and potassium contaminations. This is usually achieved by using ammonium acetate in replacement for NaCl or KCl. Similarly, pH adjustments are done with acetic acid or ammonia. Thermal denaturation and fluorescence ligand titrations in solution

have shown that duplex stability and ligand-duplex binding constants were very similar in NH_4OAc and NaCl [36].

The case of G-quadruplexes is a little more peculiar, because G-quadruplexes are stabilized by cations bound between the G-tetrads. Fortunately, most G-quadruplex forming sequences adopt a similar structure in the presence of ammonium ions as in the presence of potassium, with ammonium cations coordinated between tetrads. In the case of tetramolecular quadruplexes like $[d(TG_nT)]_4$, the inner ammonium cations are so tightly bound that they remain inside the G-quadruplex even after complete evaporation of the solvent and of the outer counter-ions. This particularity of ESI-MS has been exploited to determine the number of ammonium ions embedded in parallel tetramolecular quadruplex structures. For the unmodified sequence $[d(TG_nT)]_4$, *(n-1)* ammonium ions are found in the quadruplexes, as shown in Figure 3 for $[d(TG_5T)]_4$. When one guanine is replaced by 7-deazaguanine (^{7}**G**), the quadruplex $[d(T^7GGGGGT)]_4$ is detected with only three ammonium ions, suggesting that this modified tetrad do not forms a sufficiently stable architecture to keep the coordinated ammonium ion included between adjacent tetrads. The number of ammonium ions is therefore indicative of the number of effective tetrads present in the tetramolecular G-quadruplexes [34].

However, there are particular cases where the structure in potassium differs from the structure in ammonium. For example, the telomeric sequence GGGTTAGGGTTAGGGTTAGGG adopts a mostly parallel structure in potassium [37; 38], and an antiparallel structure in ammonium [33; 35] (as in sodium). A remaining challenge is therefore to find experimental conditions that mimic the native structure while remaining compatible with ESI-MS. A recent paper describes an ethanol precipitation and washing procedure that allows detecting $[d(TG_nT)]_4$ with *n-1* potassium cations inside [39]. This is showing the way towards resolving that challenge.

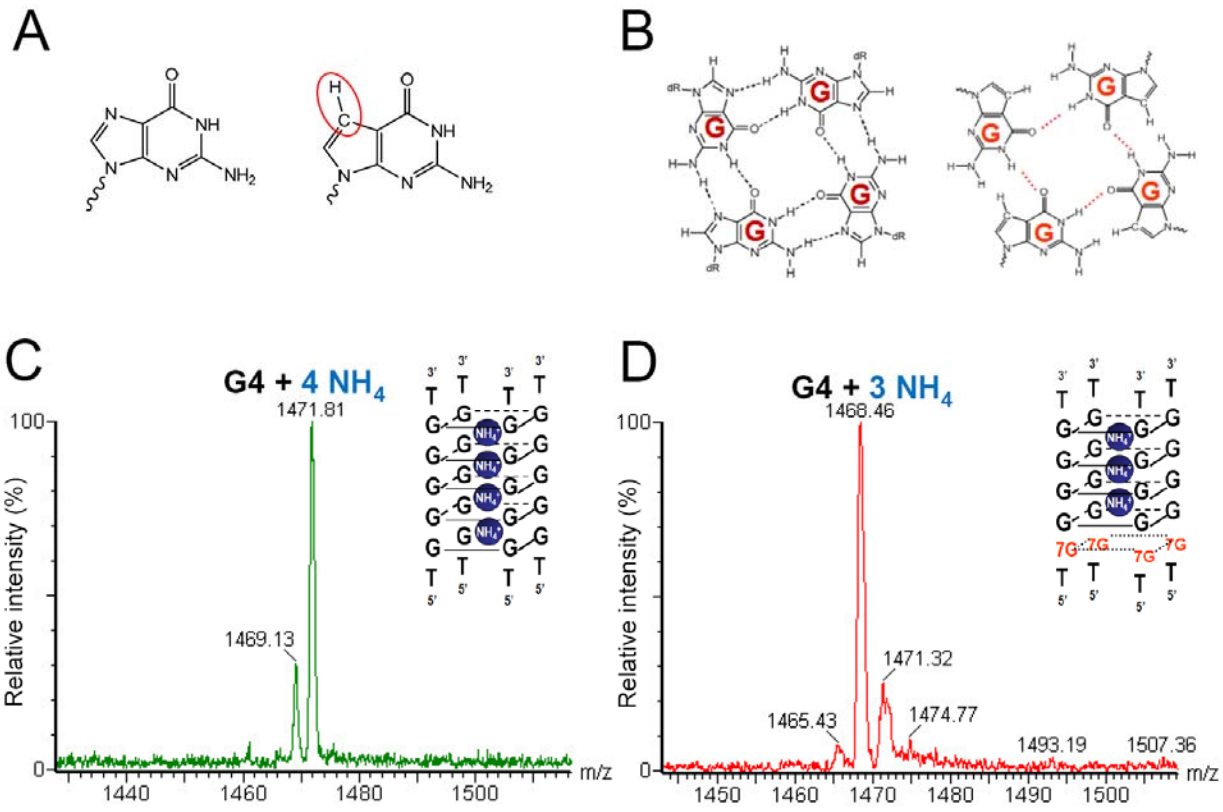


Figure 3: (A) Structure of guanine (G) and 7-deazaguanine (^{7}G) derivative. (B) Structure of the guanine tetrad and the hypothetical 7-deazaguanine tetrad. (C-D) Zooms of the ESI mass spectra of the quadruplexes (C) $[d(TGGGGGT)]_4$ with predominantly 4 ammonium ions bound, and (D) $[d(T^{7}\mathbf{G}GGGGT)]_4$ with predominantly 3 ammonium ions bound. The quadruplex concentration was 5 µM. Spectra were recorded in 150 mM ammonium acetate, in negative ion mode on a Q-TOF Ultima Global mass spectrometer.

3.2. Stoichiometry of nucleic acid-ligand complexes

Since 1994, results on intercalator and minor groove binders suggested that ESI-MS could be an effective analytical technique for the detection of specific noncovalent drug-DNA complexes and

that the stoichiometries of the complex observed in ESI-MS reflect the solution [9; 10; 40-43]. Our group also made several test experiments on drug-DNA systems where no binding is expected, and indeed no binding is detected using ESI-MS [43], but most of these (non)results are of course unpublished. This is however a convincing indication that the complexes detected by ESI-MS are indeed representative of the species present in solution (no false positive).

Figure 4 shows the relative intensities of the different species detected by ESI-MS for solutions of different concentration of drugs DAPI, Hoechst 33342 and distamycin A (from 0 to 10 or 20 µM), added to the 5 µM duplex $(GGGGATATGGGG•CCCCATATCCCC)_2$ solution. For DAPI and Hoechst 33342, a small amount of 2:1 complex is detected, only once the AATT binding site is saturated. For distamycin A, the 2:1 complex becomes rapidly predominant as the drug concentration increases. This illustrates the utility of ESI-MS for stoichiometry characterization: binding cooperativity is detected unambiguously, and the contribution of minor species can also be detected (like the low abundance 2:1 complex in Figures 4A and 4B).

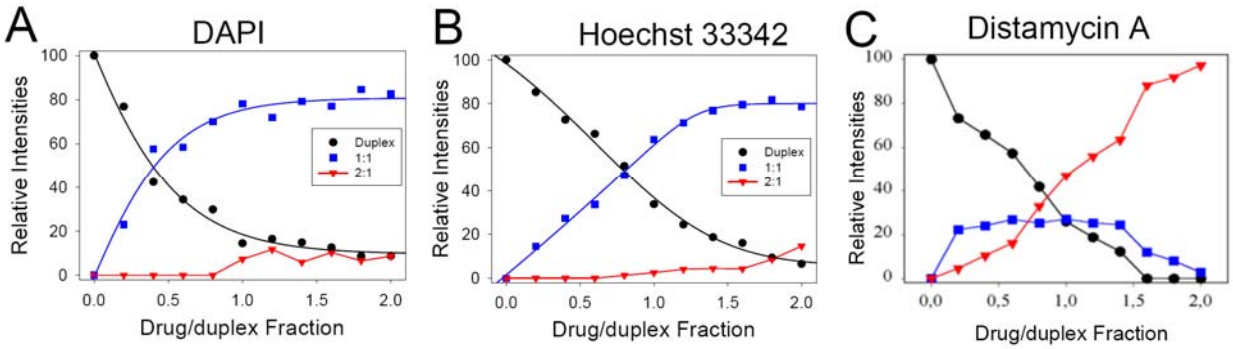


Figure 4: Graphics representing the relative abundances of the different species as a function of the drug molar fraction added to a 10 mM duplex solution for (a) DAPI, (b) Hoechst 33342, (c) Distamycin A. (●) Abundance of the duplex; (▼) abundance of the 1 : 1 complex; (■) abundance

of the 2 : 1 complex. The lines have been added only to guide the eye. Spectra were recorded using a LCQ mass spectrometer.

4. Quantitative aspects

The position of the peaks in the ESI-mass spectrum allows determination of the stoichiometries of the complexes that are present in a sample. In addition, the relative intensities of the peaks can be used to quantify the complexes. This section explains how to determine the concentration of each complex, perform binding assays, determine equilibrium binding constants, and monitor reaction kinetics using electrospray mass spectrometry.

4.1. Determination of the concentrations from the relative intensities of mass spectral peaks

Data processing allows the determination of the peak areas of the free DNA and each complex formed. The relative concentrations of free nucleic acid (D) and each complex (DL, DL_2, DL_3,…) are then calculated from the total nucleic acid concentration ($[D]_{total}$) and the peak areas (A) using the following equations:

$$[D] = [D]_{total} \times \frac{A(D)}{A(D) + A(DL_1) + A(DL_2) + A(DL_3)} \quad (1)$$

$$[DL] = [D]_{total} \times \frac{A(DL_1)}{A(D) + A(DL_1) + A(DL_2) + A(DL_3)} \quad (2)$$

$$[DL_2] = [D]_{total} \times \frac{A(DL_2)}{A(D) + A(DL_1) + A(DL_2) + A(DL_3)} \quad (3)$$

$$[DL_3] = [D]_{total} \times \frac{A(DL_3)}{A(D) + A(DL_1) + A(DL_2) + A(DL_3)} \quad (4)$$

The total concentration of bound ligand is then calculated from the concentration of each complex (Equation 5), and the concentration of free ligand is equal to the total ligand concentration minus the concentration of bound ligand (Equation 6):

$$[L]_{bound} = [DL] + 2 \times [DL_2] + 3 \times [DL_3] \quad (5)$$

$$[L]_{free} = [L]_{total} - [L]_{bound} \quad (6)$$

4.2. Binding assays

To visually determine the relative affinity of a given ligand for different DNA structures, and therefore determine the ligand's specificity, a convenient procedure is the graphical comparison of the amount of bound ligand (determined using Eq. 5), or of the complex/duplex ratio [44]. Figure 5 shows such graphical comparison obtained for the screening of cryptolepine [45] binding to three duplex DNA with different GC percentage, a triple helical DNA, and several G-quadruplexes. ESI-MS results show that cryptolepine has the highest affinity for triplex DNA, in agreement with equilibrium dialysis experiments [45]. One advantage of ESI-MS over equilibrium dialysis is the very good reproducibility of the results and the rapidity of the experiments (less than 5 minutes per oligonucleotide target). A disadvantage is, as discussed above, the restrictions in the composition of the buffer.

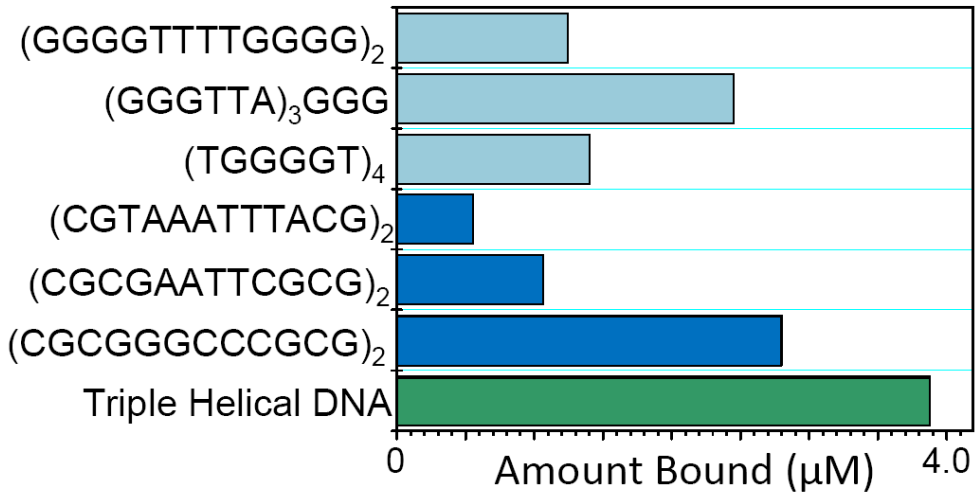


Figure 5: ESI-MS binding assay. Concentration of bound ligand deduced from the ESI mass spectra of mixtures of 10 μM cryptolepine and different oligonucleotide structures: the antiparallel quadruplex $[d(GGGGTTTTGGGG)]_2$, the human telomeric intramolecular quadruplex $d(GGGTTA)_3GGG$, the parallel tetramolecular quadruplex $[d(TG_4T)]_4$, three self-complementary duplexes and a triplex sequence d(CCTTTTCTCTTTCC)•d(GGAAAGAGAAAAGG)•d(CCTTTCTCTTTTCC). Each DNA assembly was tested at a concentration of 5 μM. ESI-MS spectra were recorded using the Q-Tof Ultima Global.

In the experiments reported above, one nucleic acid-ligand mixture is tested at a time. However, provided that mass spectral peaks do not overlap, competition experiments using mixtures of several drugs for the same nucleic acids target [42; 46; 47] or even for several oligonucleotides at the same time can be performed [47-49]. In the latter case, very careful sodium or potassium

elimination must be achieved and high resolution mass spectrometers help reducing potential peak overlaps.

4.3. Determination of equilibrium binding constants

4.3.1. Equations

The concentrations of all species at equilibrium allow the calculation of the equilibrium binding constants. The stepwise binding constants are defined in Equations 7 to 9.

$$K_1 = \frac{[DL]}{[D]\times[L]_{free}} \tag{7}$$

$$K_2 = \frac{[DL_2]}{[DL]\times[L]_{free}} \tag{8}$$

$$K_3 = \frac{[DL_3]}{[DL_2]\times[L]_{free}} \tag{9}$$

Alternatively, the cumulative binding constants K'_2 and K'_3 can also be calculated (Eq. 10-11).

$$K'_2 = \frac{[DL_2]}{[D]\times[L]^2_{free}} = K_1 \times K_2 \tag{10}$$

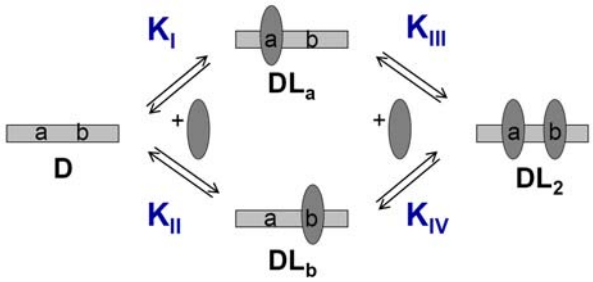
$$K'_3 = \frac{[DL_3]}{[D]\times[L]^3_{free}} = K_1 \times K_2 \times K_3 \tag{11}$$

The order of magnitude of binding constants that can be determined using ESI-MS depends on the limit of quantification of the mass spectrometer (concentration of species giving a signal-to-

noise ratio ≥ 10). K_1 association constants from 10^3 M^{-1} [24] to 10^8 M^{-1} [36] have been determined using ESI-MS.

4.3.2. Interpretation of the data: site equivalence or cooperative ligand binding

Because the mass analyzer is sensitive to the total mass of the complex but not to the nature of the binding site, the binding constants calculated as described above are not equal to the microscopic equilibrium constants at each binding site. However they are mathematically related, as shown below in the simple example of the formation of a 2:1 complex. The microscopic equilibrium binding constants K_I, K_{II}, K_{III} and K_{IV} are defined in Scheme 1 and in Equ. (12-15).



Scheme 1.

$$K_{\mathrm{I}} = \frac{[DL_a]}{[D]\times[L]_{free}} \tag{12}$$

$$K_{\mathrm{II}} = \frac{[DL_b]}{[D]\times[L]_{free}} \tag{13}$$

$$K_{\mathrm{III}} = \frac{[DL_2]}{[DL_a]\times[L]_{free}} \tag{14}$$

$$K_{\mathrm{IV}} = \frac{[DL_2]}{[DL_b]\times[L]_{free}} \tag{15}$$

Taking into account that the total amount of DL measured in the mass spectrum is the sum of all complexes containing one ligand per DNA target, whatever the binding site (Eq. 16), the constants defined in Eq. (7-8) can be related to the microscopic constants defined in Eq. (12-15), as shown in Equations (17-18).

$$[DL] = [DL_a] + [DL_b] \quad (16)$$

$$K_1 = K_I + K_{II} \quad (17)$$

$$\frac{1}{K_2} = \frac{1}{K_{III}} + \frac{1}{K_{IV}} \quad (18)$$

If the two ligand binding sites are equivalent and independent, i.e. if $K_I = K_{II} = K_{III} = K_{IV}$, then $K_1 = 4 \times K_2$. So a four-fold ratio between the constants K_1 and K_2 strongly suggests independent binding sites. If the ligand binding sites are not equivalent ($K_I \neq K_{II}$ and $K_{III} \neq K_{IV}$) or if they cooperative negatively ($K_{III} < K_{II}$ and $K_{IV} < K_I$), then $K_1 > 4 \times K_2$. On the contrary, if the ligands bind with positive cooperativity ($K_{III} > K_{II}$ and $K_{IV} > K_I$), then $K_1 < 4 \times K_2$.

A consequence of the equations above is that the DNA targets used in the ESI-MS screenings must bear a limited number of binding sites in order to be able to interpret the ESI-MS binding constants in terms of binding mechanism or binding sites. Another reason for using oligonucleotides rather than long DNA is the higher sensitivity of the ESI mass spectrometers for smaller molecules. Note however that very large DNA strands can in principle be analyzed using ESI-MS [50-52].

4.3.3. How reliable are equilibrium constants determined by ESI-MS?

The binding constants are determined from a single mass spectrum. It does not require any titration. However, it is highly recommended to verify the binding constants by repeating the measurement with at least one different concentration of ligand. When the DNA and ligand concentrations are carefully determined, equilibrium binding constants are the same whatever the ligand concentration. A single mass spectrum is actually a sum of several scans, to obtain good statistics on the peak intensities. To give a feeling of the scan-to-scan variability and of the time required to record an exploitable mass spectrum, we calculated the equilibrium association constant for each 1-second scan, during the recording of the ESI mass spectrum from a sample containing 4 µM duplex d(CGCGAATTCGCG)$_2$ and 4 µM netropsin ligand. The standard deviation of the binding constant value does not exceed 3.7 % of the mean value.

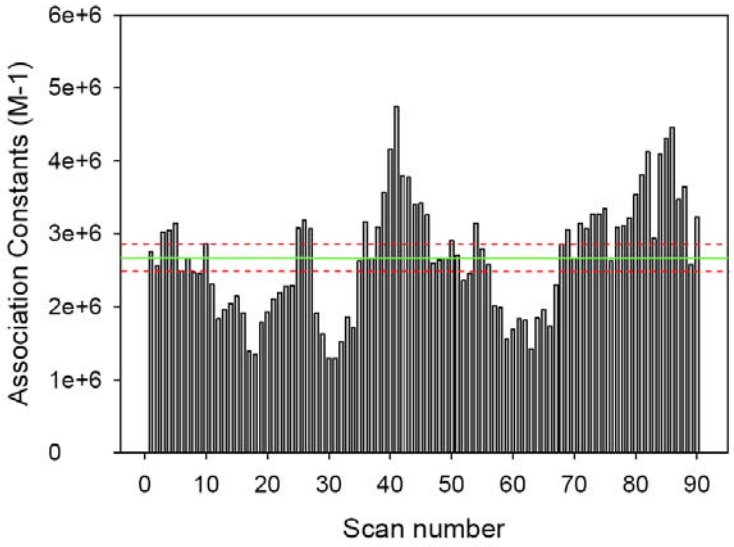


Figure 6: Scan to scan evolution of the MS-determined equilibrium association constant K_1 for an equimolar solution (4 µM) of netropsin drug and the dodecamer (CGCGAATTCGCG)$_2$ (only

a 1:1 complex is observed). The green line shows the mean value. The red lines show the 95% confidence interval.

All equations described above are based on the assumption that the intensity ratios determined in the ESI mass spectra are equal to the concentration ratios in solution. It is therefore assumed that free and complexed nucleic acid ions have the same response factors. What is the validity of this assumption?

Response factors are affected by all parameters affecting ionization efficiency, transmission efficiency, and detection efficiency in the mass spectrometer. Parameters like the mass spectrometer's transmission and detection efficiencies depend on the instrument, not on the system under study. Usually, species with similar *m/z* transmit equally well, and species with the same charge *z* are detected with the same efficiency. When investigating complexes between nucleic acids and small molecules, the peaks of the free nucleic acid and its complexes at a given charge state are therefore not subjected to large differential response due to the mass spectrometer. However, when comparing assemblies of different size, like single strand and duplex, the relative intensities in the mass spectra are most probably not proportional to the relative abundances.

Another factor playing a role when analyzing noncovalent complexes is the possible disruption of complexes on their way from the source to the mass analyzer (several μs). If the complex is more fragile than the free nucleic acid (this is the case for loosely bound ligands), then the binding constants would be underestimated (the complex is partially dissociated). If however the free nucleic acid is more fragile than the complex (this can happen for example when the nucleic acid

is itself a noncovalent complex like a triplex DNA), then the binding constants would be overestimated (the free DNA is partially dissociate). It is usually good practice to determine the binding constants by using different source parameters to determine how collisional activation in the source influences the relative intensities. In any case, the binding constants recorded at low voltages (soft condition) should always be preferred.

The most unpredictable factor is however the electrospray response factor, i.e. the efficiency of production of the ions from the species in the charged droplets. In the ideal situation, all species used for quantification would have the same the ionization efficiency. Mechanistic studies of the electrospray process established that the electrospray response depends mainly on the analyte partitioning between the core of the droplet and its surface [53]. More hydrophobic analytes tend to move to the droplet surface while hydrophilic analytes tend to stay in the bulk of the droplet [54; 55]. When analyte concentrations are low compared to the amount of charges on the droplet surface (i.e. when using low analyte concentrations and low flow rates), all analytes can efficiently compete with the droplet surface and can become ionized, and there is no marked difference of response factors between analytes [56; 57]. However, when analyte concentrations are higher compared to the available charges on the surface, competition for ionization is biased towards the most hydrophobic analytes.

What is meant by "low analyte concentration and low flow rate"? Flow rates down to a few nL/min can be attained with nanoelectrospray emitters [58], but these thin needle can not be used at physiological ionic strength (150 mM salt) because they clog rapidly. ESI-MS measurements are therefore typically done with conventional electrospray sources, with a syringe pump and assisting gas flow, at flow rates from 150 nL/min [59] to a few µL/min. In our experience, when performing ESI-MS determination of equilibrium binding constants at 4 µL/min injection flow

rates from solutions containing maximum 10 μM nucleic acid, with duplex minor groove binders, good agreement is obtained between ESI-MS binding constants and those determined by other methods [36], with a two-fold difference in response factor between free duplex and [duplex + minor groove binder] complex [60]. The case of minor groove binders is supposed to be particularly favorable because only slight distortion of the duplex DNA is associated with ligand binding, and hence only slight changes in hydrophilicity is anticipated. In contrast, studies of ligand bound to RNA aptamers that undergo conformational rearrangement upon binding showed significant discrepancies between abundances in ESI-MS and binding constants in solution [61].

Another intriguing question is: with positively charged ligands, why do free DNA and complexed DNA nevertheless appear with the same total charge? Actually the reason for that is not clear, and would warrant further fundamental studies, but the experimental facts are that the charge state or distribution of charge states observed in ESI-MS depends more on the total size of the complex than on the spatial distribution of charges within a complex. When a slight shift of the charge state distribution is observed for the complexes with some ligands, as it is impossible to know at which charge state the relative intensities most closely mirror the relative abundances in solution, good practice would be to determine the binding constant separately for each charge state, and then calculate the average binding constant and the error inherent to the method. For example, from 60 binding constants determined for MMQ ligands (see companion paper [Monchaud et al]) and several duplexes and quadruplexes, the average standard deviation on $\log(K_1)$ is equal to 0.2 (with average $\log(K_1) = 5.0$).

In conclusion, even if the absolute values of binding constants might be taken with caution for the reasons outlined above, the error inherent to the ESI-MS method remains modest compared to

the selectivities that are expected for specific ligands. Furthermore, the *relative* affinities determined by ESI-MS usually match closely the ranking obtained by other methods [45; 62-64], thereby validating ESI-MS as an approach for screening a series of ligands for a given target or for determining ligand selectivity for various targets. As the main advantage of ESI-MS is its rapidity (2 min per spectrum is enough to obtain binding constants!), and the absence of false positives, it is a very attractive method for finding hits that are worth further more labor-intensive investigation by more traditional methods.

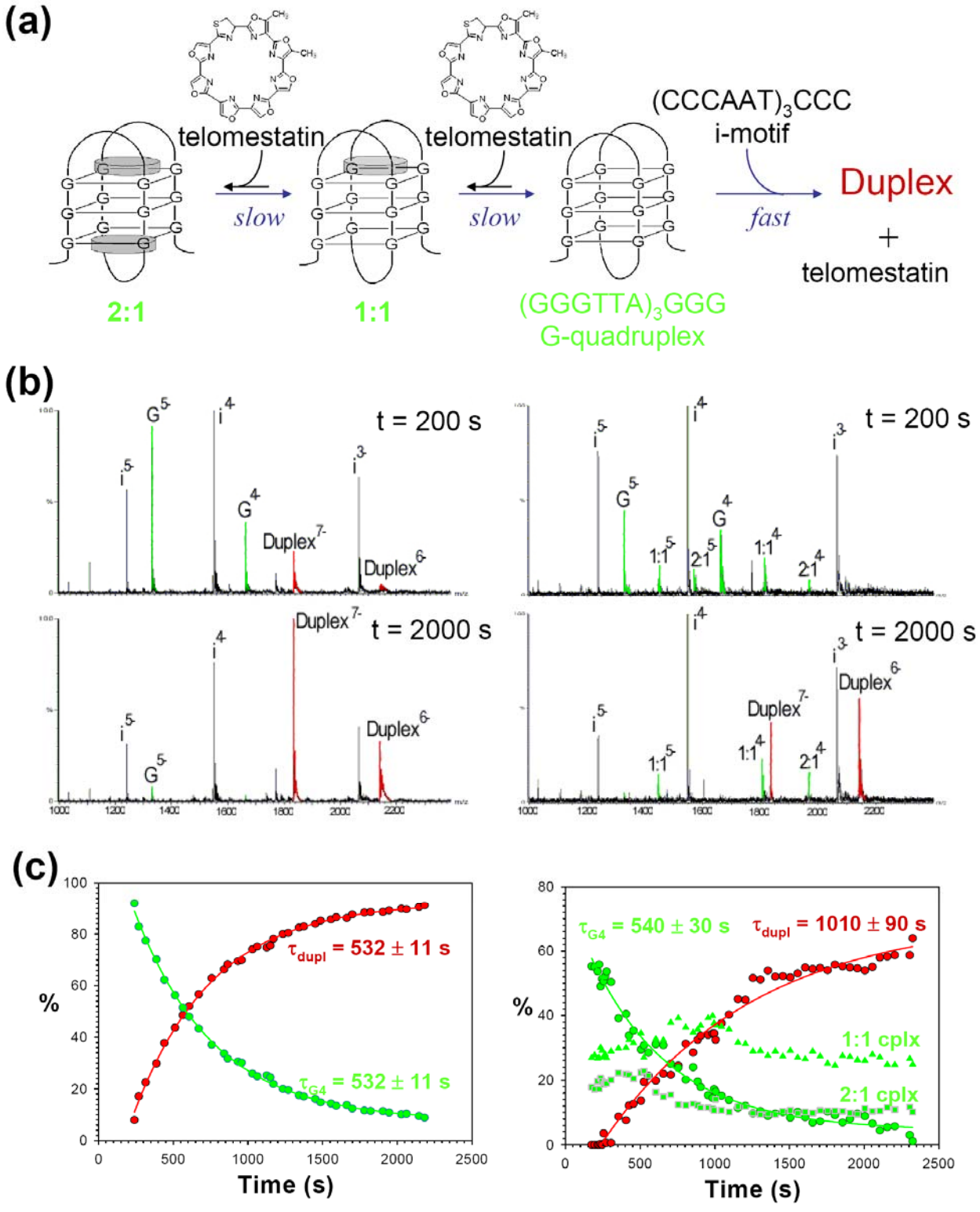
4.4. Monitoring reaction kinetics using ESI-MS

The use of ESI-MS to characterize ligand binding to DNA is not limited to the characterization of the equilibrium state. As only a few seconds of acquisition are necessary to obtain good statistics on the ion signals, ESI-MS can therefore be used to study slow kinetics (reactions occurring on a time scale of minutes to hours), by monitoring the relative intensities of the different peaks as a function of time. In the following example, ESI-MS was used to study the kinetics of hybridization of the human telomeric sequence by its complementary strand [65], mimicking the binding to the RNA template of telomerase, and to test the influence of a ligand (telomestatin) on this reaction kinetics. The telomeric G-rich strand $d(GGGTTA)_3GGG$ is folded into a G-quadruplex in the experimental condition (50 mM NH_4Ac pH 6.5). Mixing of the quadruplex with the complementary strand $d(CCCAAT)_3CCC$ sets the starting time of duplex formation, and the disappearance of the free G-quadruplex and appearance of the duplex are monitored by ESI-MS, as shown in Figure 7.

Traditional spectroscopic methods (UV spectrophotometry, circular dichroism or fluorescence resonance energy transfer) also allow studying the reaction kinetics of nucleic acids

hybridization, but they are not able to sort out the contribution of all different complexes on the kinetics pathway. ESI-MS has the great advantage of monitoring each species separately, which is of prime importance for study of the effect of drug binding on the reaction kinetics. When telomestatin is added to the G-quadruplex before addition of the complementary strand, 1:1 and 2:1 complexes between telomestatin and the G-quadruplex can be distinguished. Furthermore, ESI-MS demonstrates that telomestatin is binding neither to the C-rich strand, nor to the duplex. The ESI-MS kinetic data therefore not only provide information on the reaction kinetics, but also on the reaction mechanism.

Figure 7 (next page): (a) Schematic representation of the different equilibrium present in solution between the G-rich DNA strand, the drug Telomestatin, the C-rich DNA strand. (b) ESI mass spectra of a mixture of 5 µM d(GGGTTA)$_3$GGG ("G") and 5 µM d(CCCAAT)$_3$CCC ("i") after 200 s (top) and 2000 s (bottom). (B) ESI mass spectra of a mixture of 5 µM "G", 5µM telomestatin, and 5 µM "i" after 200 s (top) and 2000 s (bottom). "Duplex" stands for "G·i"; "1:1" stands for "telomestatin·G"; "2:1" stands for "2 telomestatin·G". The G strand and the complex telomestatin.G are colored in green. The resulting duplex is colored in red. (c) Relative abundances of the different forms of the G-strand as a function of time. The complementary strand (5 µM) is added to a solution (5 µM) of preformed (GGGTTA)$_3$GGG quadruplex alone (left) or in the presence of 5 mM telomestatin (right). ● duplex; ● free G-strand; ▲ 1 : 1 complex with telomestatin; ■ 2 : 1 complex with telomestatin.


(a)
telomestatin
telomestatin
(CCCAAT)3CCC
i-motif
slow
slow
fast
Duplex
+
telomestatin
2:1
1:1
(GGGTTA)3GGG
G-quadruplex
(b)
t = 200 s
t = 200 s
t = 2000 s
t = 2000 s
Duplex7-
Duplex6-
(c)
τdupl = 532 ± 11 s
τG4 = 532 ± 11 s
%
Time (s)
τG4 = 540 ± 30 s
τdupl = 1010 ± 90 s
1:1 cplx
2:1 cplx
%
Time (s)

5. Energetics: probing intermolecular interactions without solvent

We briefly outlined in section 2 the principle of tandem mass spectrometry experiments. MS/MS experiments are performed on the nucleic-acid ligand complexes, so they probe the charged complexes isolated in the vacuum of the mass spectrometer, in complete absence of solvent. Although these experiments do not seem relevant to solution-phase studies, they can provide information that is difficult to determine from solution data [66-68]: the contribution of intermolecular interactions to ligand binding, free of any solvent contribution. Mass spectrometry is the only experimental technique that allows probing experimentally the intermolecular interactions in the gas phase.

MS/MS experimental data are useful when compared to molecular modeling of the complexes *in vacuo* to ascertain which structural model fits the experimental data [24; 69]. With minor groove binding [43; 70] and intercalating complexes [43] with double-stranded DNA, we consistently found that MS/MS data were reliable with the structure of the complexes in solution being preserved in the gas phase ions. MS/MS data are also useful when compared to the solution-phase binding constants to detect significant solvent contribution to the ligand selectivity. What is generally found is that, even though the main contribution to the binding free energy in solution may come from hydrophobic interactions, what usually fine tunes ligand selectivity among a given ligand family is short-range electrostatic contributions, and these small differences can be probed very sensitively by MS/MS.

For those interested in learning more on the theoretical aspects of MS/MS, and how energetic information can be extracted from tandem mass spectrometry data, the following tutorial reviews are recommended: [71-73]. We also recently reviewed the do's and don'ts of using CID MS/MS

to obtain meaningful information on ligand-duplex complexes [43]. The main guidelines can be summarized as follows. When interpreting MS/MS data, it is important to know that the extent of fragmentation must be interpreted in terms of reaction kinetics (as opposed to an equilibrium in the gas phase): the fragmentation extent depends the amount of internal energy given to the parent ion by collisions, and on the time scale left for the parent ion to fragment before the product ion spectrum is recorded. The dissociation kinetics depends on an activation enthalpy term and an activation entropy term. Comparing activation enthalpies is what we are interested in, because this parameter is proportional to the interaction energy between the partners that dissociate.

In order for the relative fragmentation extent of a series of complexes to reflect the relative interaction energies in the gas phase, all the following parameters must be kept as constant as possible throughout the comparisons [43]: (1) The amount of internal energy. This value is difficult to calculate, but the theory says that ions of similar mass and charge that are given the same collision energy will have the same internal energy. (2) The fragmentation time scale, which can change from instrument to instrument. This explains why the product ion spectra of a very same complex can be very different when recorded on different instruments [74]. Product ion spectra can be meaningfully compared only on a given instrument. (3) Finally, the dissociation rate is proportional to the activation enthalpy only if the activation entropies are the same. This means that all complexes compared must dissociate via the same pathway. We also have demonstrated that the only pathway that can provide direct information on the energetics of drug-nucleic acid intermolecular interactions is the loss of neutral drug from the negatively charged DNA [43; 69].

However, charged molecules represent an important class of compounds, and ligands with the strongest affinities for the negatively charged nucleic acids are generally positively charged in solution. If the positive charges come from protonation, proton transfer(s) from the ligand to the nucleic acid can result in the ligand coming off as a neutral. If the ligand cannot lose its positive charge and remains attached to the negatively charged nucleic acid, then no information on the ligand binding energetics can be obtained, but information on the ligand binding site becomes accessible, as described in section 6.1.

6. Structural characterization of nucleic acid complexes using mass spectrometry-based strategies

Often, comparative ESI-MS experiments on different nucleic acid sequences and different ligand concentrations allow making deductions on the possible binding sites just from the stoichiometries (section 3.2) and the binding constants (section 4.3.2) , but strictly speaking, the mass of a complex tells nothing about its tridimensional structure. There are nevertheless a variety of creative strategies to probe the structure of noncovalent complexes using mass spectrometers, and this is a very active field of research in the mass spectrometry community. Some of these strategies are briefly outlined below.

6.1. MS/MS

Loss of neutral drug is however not the only fragmentation pathway possible. When the ligand is positively charged and does not undergo proton transfer to the nucleic acid, the ligand remains attached to the negatively charged nucleic acid by Coulomb interactions (ion-ion interactions are very much stabilized in the gas phase because the dielectric constant of vacuum is by definition

equal to 1). Other instances where the ligand can remain attached to the nucleic acid is if the covalently reacts at the binding site [75; 76]. In those cases, cleavage of the nucleic acid backbone can become the preferred pathway in MS/MS, and the ligand binding site can be determined from the product ion spectrum like if the ligand was a covalent modification of the nucleic acid. This kind of behavior has been observed in RNA complexes with the aminoglycoside neomycin B [77], and the dissociation of duplex DNA/netropsin complexes [78].

A trick may consist in making the oligonucleotide covalent bonds even weaker. For example, Three adenosine residues were mutated into deoxyadenosine in 16S ribosomal RNA [79]. Fragmentation occurs preferentially at these fragile sites, and upon ligand binding in the vicinity, a decrease in fragmentation efficiency was observed, indirectly indicating ligand binding. Finally, let us mention that there are other fragmentation methods than collision-induced dissociation that are believed to keep noncovalent interactions intact while fragmenting the DNA backbone. These methods include electron detachment dissociation (EDD) [80] and electron photodetachment dissociation (EPD) [81], but the applicability of these methods remains to be firmly established.

Finally, another indirect way to determine ligand binding site by MS/MS is to use covalent chemical probes of the nucleic acid structure in solution, and use MS/MS to determine the location of these probes. In that case, MS/MS needs not be performed on the intact complex, but only on the labeled nucleic acid. Examples can be found in a recent study by Mazzitelli and Brodbelt [82], who used $KMnO_4$ oxidation of thymines to probe thymine accessibility in DNA duplexes and their complexes with ligands, and in papers by Fabris and co-workers who investigated RNA structures and RNA-protein complexes [83-86].

6.2. Ion mobility spectrometry

The strategies outlined above can be implemented in commercial mass spectrometers with MS/MS capabilities, but there are also other instrumental methods that allow obtaining structural information. One such method is ion mobility mass spectrometry [87] [Intermolecular Interactions in Biomolecular Systems Examined by Mass Spectrometry, Thomas Wyttenbach, Michael T. Bowers, Annual Review of Physical Chemistry, Vol. 58: 511-533 (Volume publication date May 2007)]. In the ion mobility spectrometer, ions (for example produced by an electrospray source) are pulsed in a chamber filled with helium gas and where an electric field is applied. The time the ions of a given mass and charge take to travel through the mobility chamber is proportional to the collision cross section of the ions. Ions having more open conformations travel slower than those having more compact conformations.

When the electric field, gas pressure and gas temperature are well controlled, collision cross sections can be determined experimentally, and compared with cross sections calculated for plausible structural models. Bowers and co-workers studied several DNA higher-order structures (duplexes [88-90], triplexes [26], G-quadruplexes [35; 90-92]). and quadruplex-ligand noncovalent complexes [35; 93]. They demonstrated that double-helices were conserved in the gas phase for duplexes containing > 10 base pairs [89], that GC base pairs are more stable than AT base pairs [90], that the conformation of intramolecular G-quadruplexes is the same in the gas phase as in the sprayed solution [35; 92], and that the G-quadruplex ligands were bound via stacking on the tetrads. The ion mobility experiments were crucial for demonstrating that the structure of ions in the gas phase was indeed preserved from the solution after electrospray, and hence that gas-phase methods provide meaningful information for biologically relevant systems.

6.3 The future: spectroscopy of ions inside the mass spectrometer?

A few groups have also developed instrumentation to detect the fluorescence of trapped ions [94-96]. Using FRET probes, Parks and co-workers were able to probe the partial unfolding of a double-stranded DNA in the gas phase [97; 98]. Action spectroscopy (detecting mass spectral fragments) is more easily implemented on commercial mass spectrometers than fluorescence spectroscopy (detecting outgoing photons). Infrared or UV-visible spectra can be recorded by monitoring the fragmentation efficiency as a function of the wavelength. The potential of infrared spectroscopy, that has already proven useful to determine the conformation of small peptides in the gas phase [99; 100], for DNA structural analysis is currently under investigation [101], and the feasibility of recording UV-visible spectra of large DNA [81; 102] and DNA-ligand complexes [102] was demonstrated recently. In the future, spectroscopy of noncovalent complexes with selected stoichiometries (using the mass spectrometer) and conformations (using ion mobility chambers), might therefore become a new approach for probing structure of the complexes.

7. Conclusions

In the last paragraphs we tried to show what mass spectrometry could bring in the future for structural analysis of noncovalent complexes, but let us now summarize what mass spectrometry can do for characterizing ligand-nucleic acid complexes in present time. First, by definition ESI-MS outperforms all other spectrophotometric techniques for the determination of the stoichiometry of noncovalent complexes. ESI-MS can be used to screen ligand for particular targets, to determine ligand selectivity among several possible targets, and even to determine equilibrium binding constants. Its rapidity, low sample consumption, and possibilities of

automation make ESI-MS a method of choice in the arsenal for studying ligand-nucleic acid interactions.

Acknowledgements

The authors thank the FRS-FNRS, the University of Liège, the Walloon Region and the European Community (FEDER) for funding the mass spectrometry facility. The FRS-FNRS is also acknowledged for a post-doctoral fellowship to FR, a research associate position to VG, and FRFC grant 2.4.623.05.

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