Advantages and Drawbacks of Nanospray for Studying Noncovalent Protein-DNA Complexes by Mass Spectrometry

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Abstract

The noncovalent complexes between the BlaI protein dimer (wild type and GM2 mutant) and its double-stranded DNA operator were studied by nanospray mass spectrometry and MS/MS. Reproducibility problems in the nanospray single-stage MS are emphasized. The relative intensities depend greatly on the shape of the capillary tip, and on the capillary-cone distance. This results in difficulties in assessing the relative stabilities of the complexes simply from MS spectra of protein-DNA mixtures. Competition experiments are a better approach to determine relative binding affinities. A competition between histidine-tagged BlaIWT (BlaIWTHis) and the GM2 mutant revealed that the two proteins have similar affinities for the DNA operator, and that they co-dimerize to form heterocomplexes. The low sample consumption of nanospray allows MS/MS spectra to be recorded at different collision energies for different charge states with 1 μL of sample. The MS/MS experiments on the dimers reveals that the GM2 dimer is more kinetically stable in the gas phase than the wild type dimer. The MS/MS experiments on the complexes shows that the two proteins require the same collision energy to dissociate from the complex. This indicates that the rate-limiting step in the monomer loss from the protein-DNA complex arises from the breaking of the protein-DNA interface rather than the protein-protein interface. The dissociation of the protein-DNA complex proceeds by the loss of a highly charged monomer (carrying about two thirds of the total charge and one third of the total mass). MS/MS experiments on a heterocomplex also show that the two proteins BlaIWTHis and BlaIGM2 have slightly different charge distributions in the fragments. This emphasizes the need for better understanding the dissociation mechanisms of biomolecular complexes.

Introduction

Electrospray ionization mass spectrometry has proven to be extremely useful for the study of noncovalent complexes. Several reviews have been dedicated to studies of this kind.[1-9] It is now commonly accepted that, provided that some experimental precautions are taken to minimize non-specific aggregation, the electrospray mass spectra reflect the composition of the solution regarding the stoichiometries of the observed complexes. It is however more tricky to assess whether the relative intensities of the peaks reflect the relative abundances of the corresponding species in solution. Different studies on small model systems have been conducted in electrospray ionization to test this hypothesis, and several authors have shown that the equilibrium binding constants could be determined.[10-13] Others have proceeded by competition experiments to determine relative binding affinities that correlate well with solution data.[14-16]

The present work is part of a project which aims to study the complexation of the BlaI repressor with its DNA target. The BlaI repressor is a prokaryotic regulator that, in the absence of β-lactam antibiotic, prevents the transcription of the blaP gene, encoding the *Bacillus licheniformis* 749/I β-lactamase BlaP.[17] BlaI is a protein with 128 amino acids composed of two functional domains: a DNA-binding domain located in its N-Terminal end and a dimerization domain located in its C-Terminal region.[18,19] In *Bacillus licheniformis*, BlaI specifically recognises 3 regulatory regions. These operators present a symmetry dyad of 23 bp long with the following consensus sequence[20]: 5'-AAAGTATTACATATGTAAGNTTT-3'. Cross linking experiments revealed that BlaI is present in the bacterial cell as a dimer, and gel filtration experiments revealed that BlaI binds the DNA as a preformed dimer.[21]

Several papers have already described the study of protein-DNA complexes by electrospray mass spectrometry, either in the negative[22-25] or in the positive ion mode.[26-29] In all these papers the stoichiometries of the complexes were found to match the solution-phase behavior. Competition experiments between the specific DNA sequence and mutant DNA's have also shown that only the specific complexes were detected by ESI-MS.[22-24,26,29] The stability of the complexes in solution as a function of the ammonium acetate concentration has been monitored by ESI-MS.[22,29] The stability of the complexes in the gas phase has been studied by in-source CID.[26,29] Kapur et al.[29] have addressed the problem of the relative response factors in the ESI-MS spectra of their complexes, and found that, in their experimental conditions, the response of the free protein and the response of the complex were the same within experimental error. However, this finding is not supposed to be a general case.

In contrast to this whole body of literature describing the use of the electrospray technique (with flow rates from 0.3 to 10 μL/min), only one paper was found to report a study of a protein-DNA complex by nanospray mass spectrometry.[30] These authors used the method to detect a previously unexpected complex stoichiometry. The main advantage of nanospray is the low sample consumption. The use of nanospray to study noncovalent biomolecular complexes has been pioneered by Robinson's group.[31-38] Nanospray is believed to be more gentle than electrospray for the transfer of the ions from the solution to the mass analyzer:[34,35] there is no gas flow coaxial to the spray, and therefore there are fewer activating collisions in the nanospray source. Nanospray has allowed very large assemblies,[33,39,40] and even a virus capsid of 2.5 MDa,[41] to be detected intact. Like electrospray, nanospray allows specific complexes to be detected.[31-38] A competition experiment between peptides for SH2 protein also shows a good agreement between calorimetric and nanospray-MS results.[34] The

association or dissociation of protein complexes has also been monitored by nanospray-MS.[37,42] In particular, the relative proportions of tetramer and monomers of transthyretin and mutants are in agreement with the biological activity.[31]

The present paper describes a nanospray-Q-TOFMS and MS/MS study of the complexes between the repressor protein BlaI (wild type and GM2 mutant: Met97Ile/Val98Leu) and its DNA operator. To our knowledge, this is the first MS/MS study on protein-DNA complexes.

Experimental

Plasmids and DNA manipulations

PET (Novagen) containing the T7 promotor under the control of the lacI repressor, was used as a vector for the overexpression of the BlaIWT, BlaIGM2 and BlaIWTHis products. The construction of the plasmids PET22BBlaIWT and PET22BBlaIGM2 was described elsewhere.[21] Plasmid PET22BBlaIWTHis was constructed with the following protocol: a 400 bp fragment covering the blaI gene was amplified by polymerase chain reaction (PCR) using PET22BBlaIWT as template and the following oligonucleotides as primers: 5'-ATACATATGAAAAAAATACCTCAAATCTCTG-3' (BlaINdeI) and 5'-TTACTCGAGTTCCTTCTTTCTGTTCTTATG-3' (BlaIXhoI). The amplified fragment was ligated to the PGEM-T-Easy plasmid to give the PGEM-T-Easy BlaIWT. The identity of the amplified DNA segment was confirmed by determination of its nucleotide sequence. PGEM-T-EasyBlaIWT was digested with NdeI and XhoI restriction enzymes, and the fragment corresponding to BlaIWT was purified by agarose gel electrophoresis and ligated to the PET22B digested with the same endonucleases to give the PET22BBlaIWTHis. In this construct, an inframe fusion was obtained between the 3' end of the blaI gene and the PET22B sequence coding for the histidine tag, to give BlaI protein with six additional histidine residues at the C terminal end.

Protein overexpression and purification

BlaIWt, BlaIWTHis and BlaIGM2 proteins were prepared by growing *Escherichia Coli* Bl21DE3plys containing the corresponding recombinant PET22B plasmid on LB

medium supplemented with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. Cells were grown at 37 °C to an A_{600nm} of about 0,7-0,8 and isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM. After 3h, cells were collected by centrifugation (at 9000 g for 20 min) and resuspended in the washing buffer (10 mM Tris-HCl pH = 7,5 – NaCl 1%). Cells were then collected by centrifugation and resuspended in the lysis buffer (15 mM Tris-HCl pH = 8 – 10mM $MgSO_4$ – 100 mM KCl – 2.5 mg/L Pefabloc). The cells were disrupted by passage through a desintegrator (INCELTECH Z, MODEL) at 20 kpsi. DNA was digested by an endonuclease (benzonase, Eurogentec, 5µl/4l cultivation) for 30 min at 4 °C. Insoluble materials were removed by centrifugation at 14000 rpm for 40 min and the resulting supernatant was dialyzed two times against 2l of buffer A (50 mM HEPES pH = 7.6 – 1 mM EDTA – glycerol 5 %). The solution was first applied to a S-Sepharose Fast Flow equlibrated in buffer A and then eluted at 5 ml/min using buffer B (50 mM HEPES pH = 7.6 – 1 mM EDTA – 0.6 M NaCl - glycerol 5 %). The BlaI-containing fractions were pooled, dialysed two times against 2 L of buffer C (50 mM HEPES pH = 7.6 – 1 mM EDTA – 0.2 M NaCl – glycerol 5 %) and then loaded onto Hitrap-Heparin-S Sepharose equilibrated with buffer C and eluted at 1mL/min using a gradient of 0.2 – 2 M NaCl. The major protein fractions were pooled and prepared for MS studies. The final yield of purified (95 % pure) protein was approximately 15 mg per litre of cell culture.

MS sample preparation

Protein. Purified BlaI proteins were prepared for MS analysis by dialysis against a buffer containing 150 mM ammonium acetate (NH_4OAc) and then concentrated to 25 µM by using a centricon (Amicon, Brussel, Belgium). Finally, protein solutions were

desalted by filtration on a Micro Bio-Spin 6 microcolumn (Bio-Rad, Hercules, CA, USA) conditioned with ammonium acetate (150 mM).

DNA Operator. The DNA sequence of the operator duplex used in this work represents the perfect palindromic operator recognized by BlaI. Oligonucleotide 5'-AAAGTATTACATATGTAATACTTT-3' (M = 7348 Da) was obtained from Eurogentec (Liège, Belgium). The duplex is a dimer of this self-complementary oligonucleotide (M = 14696 Da). The duplex is formed by heating the single strand to 95 °C, followed by overnight cooling.

Complexes. The complexes were formed by mixing the protein and the DNA operator in equimolar (protein/duplex) amounts, and incubating for 2h at room temperature.

Mass Spectrometry

The nanospray tips were gold-coated glass capillaries (Protana, Odense, DK) that are cut manually. The spectrometer was a Q-TOF2 (Micromass, Manchester, UK) equipped with the Z-Spray nanoflow source. The source block was heated to 80 °C. Note that only the cone was heated and that the nanospray capillary remained cold. A voltage of 850 to 1050 V (see text) was applied to produce the spray. The cone voltage was set at an optimal value of 40 V for all experiments. This is well below the dissociation onset for the complex, and high enough to achieve good ion transmission. In the MS/MS mode, argon was used as collision gas.

Results and Discussion

MS^1 of protein-DNA mixtures

Initially, our goal was to investigate whether nanospray-MS could give a reliable measure of the relative binding affinities of different proteins for DNA, and eventually a measure of the absolute binding constants. Therefore it was necessary to measure the relative intensities of complex and free DNA or protein in mixtures. We first tested the reproducibility of the mass spectra (MS^1) for the wild type (BlaIWT) mixed with equimolar amounts of DNA operator (double-stranded). Several capillaries were made with the same mixture, and spectra were recorded over several days. It rapidly appeared that the reproducibility regarding the relative intensities of the peaks was poor. To illustrate this point, the two most different spectra among all those obtained are displayed in Figure 1. Except for the sodium, content which is obviously higher in spectrum (a) than in spectrum (b), the mixtures were prepared identically. Spectrum (a) shows a signal corresponding to the free double-stranded DNA operator (ds) and to the protein dimer (D), but these peaks do not appear in spectrum (b). The MS/MS results (see below) show that these two species cannot arise from the dissociation of the complex. The species present in solution are clearly sampled in a different way in the two nanospray experiments.

This is further illustrated in Figure 2. The two spectra were acquired from the same capillary, containing equimolar amounts of the BlaI mutant GM2 and DNA. The spray was induced, and spectrum (2a) was recorded. Then the spray was stopped, the capillary was re-cut, and the spray was restarted. Spectrum (2b) was acquired with this second spray from the same capillary. The data obtained clearly show that the way in which the capillary is cut is a crucial parameter in the appearance of the mass

spectrum. In the course of our systematic studies, we also found that two other parameters have a dramatic influence on the relative intensities in the spectra: the spacing between the capillary and the cone (in the Z-spray configuration), and the capillary voltage. These two parameters are linked: the larger the capillary-cone distance, the larger the capillary voltage must be in order to maintain a stable spray. We found that the larger the capillary-cone distance, the more favored are the species of high *m*/*z* (data not shown).

Such a dependence of relative intensities on the position where the plume is sampled has already been demonstrated in electrospray.[43,44] The present results show that a dramatic dependence on the capillary position also occurs in nanospray. They also illustrate that in nanospray the mass spectra depend on parameters that are difficult to control, like the shape of the capillary tip, which have a large influence on the formation of a stable spray in the cone-jet mode. Moreover, it is well known that achieving a stable cone-jet mode is extremely difficult for aqueous solutions.[45] These problems are avoided in electrospray, in which the flow rate is controlled and fixed, and a sheath gas regulates the spray formation. It is obvious, by comparing Figure 1 and Figure 2 that the relative binding affinities of the two variants of BlaI can not be reliably ascertained by comparing the relative intensities of the complexes in the nanospray mass spectra (MS^1).

Competition between wild type and mutant BlaI

The only way to avoid the reproducibility problem between different capillaries is to perform competition experiments between species of similar masses. Here we mixed equimolar amounts of BlaIWT and BlaIGM2 with the DNA operator. As BlaIWT and BlaIGM2 cannot be distinguished by their masses (4 Da difference), BlaIWT was

replaced with a mutant bearing a histidine mass tag at the C-terminal-domain, BlaIWTHis. Such a modification is supposed not to affect the DNA binding. The mass spectrum of the equimolar mixture is shown in Figure 3. Not only the homodimer complexes can form, but also a heterodimer complex (WTHis + GM2 + dsDNA) forms, despite the fact that the mutation is located in the dimerization domain. Furthermore, the relative intensities of the homodimer peaks show that BlaIWT has only a slightly higher affinity for the DNA than BlaIGM2. This was impossible to assess from the simple mass spectra of the protein-DNA mixtures only. In nanospray mass spectrometry, the competition experiment is an approach that allows the confident determination of the relative binding affinities, with not too many experimental constraints.

MS/MS

It is obvious that the great advantage of nanospray is the low sample consumption, which allows us to perform MS/MS experiments with only a few picomoles of material. To probe the gas-phase stability of the complexes, we performed MS/MS experiments on the WT and GM2 protein dimers (Figure 4) and on their complexes with the DNA operator (Figures 5 and 6). In all cases, MS/MS was performed by automated data acquisition. For each charge state, eight different collision energies were used (the cone voltage was maintained at 40 V), and the total duration of acquisition was 40 min. For three hours of data acquisition, usually less than 1 μL of sample is consumed.

A great advantage of MS/MS over in-source CID in nanospray is that, in contrast to full scan MS^1, the MS/MS spectrum does not depend on the spray conditions (shape of the capillary, capillary-cone distance, capillary voltage). Sometimes the spray had

to be re-initiated during the data acquisition, with no effect on the relative intensities measured in the MS/MS spectra.

For BlaI dimers, MS/MS was performed on a 25 μM protein solution, on charge states 12+ and 11+. For charge state 12+, the relative intensity of the dimer peak did not decrease to zero at high energies, but rather levelled off, indicating that either some monomer 6+ is isolated together with the dimer^{12+}, or that the dimer^{12+} dissociates into monomer^{6+}. The former hypothesis is more plausible than the latter, due to the uneven charge partitioning that is observed for the charge state 11+ (see below). We therefore have chosen to discuss the MS/MS behavior of the non-even charge state 11+, which is unambiguously a dimer. Two fragmentation pathways are observed: (1) dimer^{11+} → monomer^{7+} + monomer^{4+} and (2) dimer^{11+} → monomer^{8+} + monomer^{3+}. The uneven charge separation of these dimers recalls that already observed by Versluis et al.[46] For both the wild type and the mutant BlaI dimers, the percentage of dimer as a function of the collision energy was calculated as follows:

$$\% \text{ Dimer} = I(\text{dimer}^{11+}) \times 100 / (I(\text{dimer}^{11+})+I(\text{mono}^{8+})+I(\text{mono}^{7+})) \quad (1)$$

The results are displayed in Figure 4. The GM2 mutant dimer is found to be unambiguously more kinetically stable in the gas phase than the WT dimer. The same qualitative result was found for the charge state 12+ (not shown). This indicates that the mutation in the dimerization domain of the protein increased the gas-phase kinetic stability of the GM2 dimer. This probably means that favorable electrostatic interactions are created by the mutation in GM2. This does not, however, necessarily reflect the relative stabilities in solution, which can be partly due to hydrophobic interactions.

The MS/MS experiment was then conducted on the complexes (dimer + dsDNA) at charge states 14+ and 13+. The MS/MS spectra of the complex^{14+} at 40 eV for the

WT and the GM2 protein are shown in Figure 5. The major fragment is the monomer^{9+}. The complementary fragment (monomer + dsDNA)$^{5+}$ could be detected at high *m*/*z*, but with a low relative intensity. The shape of the peak also indicates some simultaneous neutral loss, which could not be resolved from the (monomer + dsDNA)$^{5+}$ species. This could be due to depurination of the dsDNA.[26,29] For the charge state 13+ the two major fragments are the monomers 9+ and 8+. The loss of one highly charged monomer (much lower *m*/*z* than the parent ion) parallels a feature commonly encountered for protein complexes.[46-48] This is the first time such behavior has been reported for protein-DNA complexes, to our knowledge.

In order to compare the gas-phase kinetic stability of BlaIWT and BlaIGM2 complexes with DNA, the percentage of surviving complex, calculated with equation 2, is plotted against the collision energy.

$$\% \text{ Complex} = I(\text{complex}) \times 100 / \{I(\text{complex}) + \Sigma I(\text{mono})\} \quad (2)$$

The results are plotted in Figure 6 for the charge states 14+ and 13+. In contrast with the MS/MS results for the dimers, there is no difference in the relative collision energy dependence of the percentage of surviving complex. As the mutation is located in the dimerization domain of BlaI, this indicates that the rate-limiting step in the dissociation is likely to imply the opening of the complex at the protein-DNA interface rather at the protein-protein interface (dimerization domain).

We also performed a MS/MS experiment on the heterocomplex (WTHis + GM2 + dsDNA)$^{14+}$. The spectrum recorded at 45 eV is shown in Figure 7. It was hoped that the proportion of BlaIWTHis and BlaIGM2 monomers could give, by analogy with the kinetic method,[49,50] an estimation of the relative affinities for the DNA. The results in Figure 7 show that the situation is not so simple: depending on the charge state, the relative abundances of the two proteins differ. The BlaIWTHis has a greater

tendency to accommodate more charges than the GM2 mutant. This was also observed in the MS/MS experiments on the heterocomplex at charge state 13+ (not shown). This further illustrates the importance of studying the hows and whys of charge partitioning upon collision-induced dissociation of multiply charged biomolecular complexes.

Conclusion

In summary, we have studied the complexes between BlaI protein dimers and their DNA operators by nanospray mass spectrometry. Reproducibility problems in the relative intensities of the complexes were emphasized in MS^1 experiments. These are attributed to the low spray stability in aqueous solutions. As a consequence, the sampling of the ions emitted by nanospray is highly influenced by parameters like the shape of the spray tip, the capillary-cone distance, etc… However, MS/MS experiments were highly reproducible (the relative intensities did not depend on the spray stability), and could be conducted with minimal sample consumption. MS/MS allows us to probe the contribution of intermolecular interactions (hydrogen bonds, etc…) to the stability of the complexes.

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Figure Legends

Figure 1.

Nanospray mass spectra (MS^1) of an equimolar mixture (25 μM) of BlaIWT and double-stranded (ds) DNA. Spectra A and B were recorded with different capillaries. C = (WT_2 + dsDNA); ds = double-stranded DNA ; ss = single-stranded DNA.

Figure 2.

Nanospray mass spectra (MS^1) of an equimolar mixture (25 μM) of BlaIGM2 and double-stranded (ds) DNA. Spectra A and B were recorded with the same capillary. Spectrum B was recorded after re-cutting the capillary that was used to record spectrum A. C = ($GM2_2$ + dsDNA); ss = single-stranded DNA; M = GM2 monomer; D = GM2 dimer.

Figure 3.

Nanospray mass spectrum (MS^1) of an equimolar mixture (25-25-25 μM) of BlaIGM2, BlaIWTHis and double-stranded DNA (competition experiment). Only the region of the complexes (protein dimer + dsDNA) is shown for clarity. Two white circles represent the homocomplex ($GM2_2$ + dsDNA); two black circles represent the homocomplex ($WTHis_2$ + dsDNA); a white and a black circle represent the heterocomplex (GM2 + WTHis + dsDNA).

Figure 4.

Relative intensity of the dimer as a function of the collision energy in nanospray-MS/MS experiments on the protein dimers (black = WT; white = GM2). The relative intensities were calculated using equation (1).

Figure 5.

Nanospray-MS/MS spectra of the complexes (A) $(WT_2 + dsDNA)^{14+}$ and (B) $(GM2_2 + dsDNA)^{14+}$ at the same collision energy (40 ev). C = complex; M = protein monomer; ds = double-stranded DNA. Note the magnification factor between *m*/*z* 5300 and 6300.

Figure 6.

Relative intensity of the complex as a function of the collision energy in nanospray-MS/MS experiments on the protein-DNA complexes (black = WT; white = GM2; circles = charge state 14+; triangles = charge state 13+). The relative intensities were calculated using equation (2).

Figure 7.

Nanospray-MS/MS experiment on the heterocomplex $(GM2 + WTHis + dsDNA)^{14+}$ at 45 eV collision energy. C = complex; G = GM2 monomer; W = WTHis monomer.

Figure 1

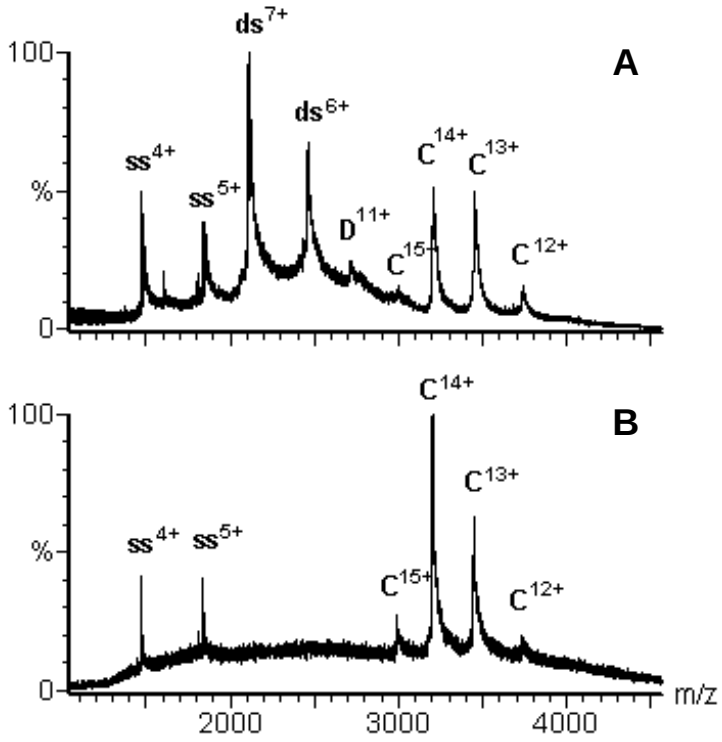

Figure 2

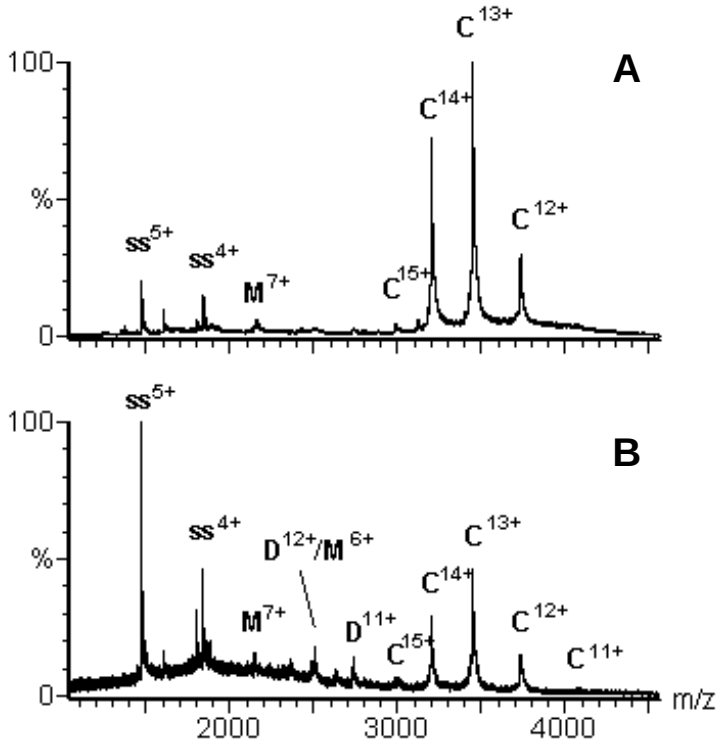
A
100
%
0
C13+
C14+
C12+
ss5+
ss4+
M7+
C15+
B
100
%
0
ss5+
ss4+
D12+/M6+
M7+
D11+
C15+
C14+
C13+
C12+
C11+
2000
3000
4000
m/z

Figure 3

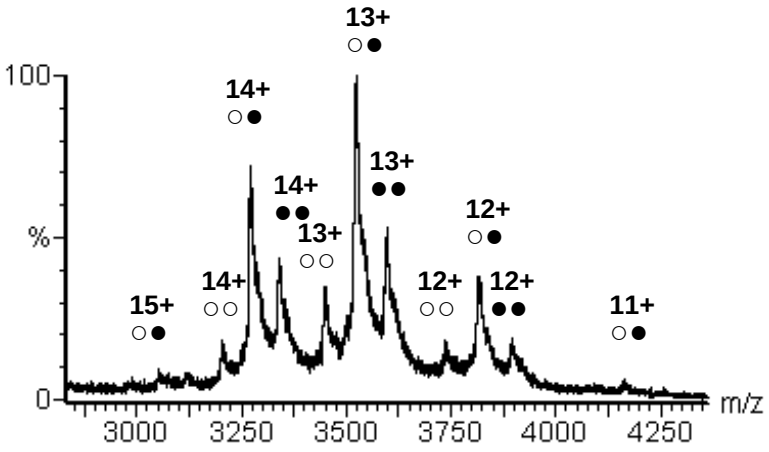
13+
○●
100
14+
○●
14+
●●
13+
●●
12+
○●
%
13+
○○
14+
○○
15+
○●
12+
○○
12+
●●
11+
○●
0
m/z
3000
3250
3500
3750
4000
4250

Figure 4

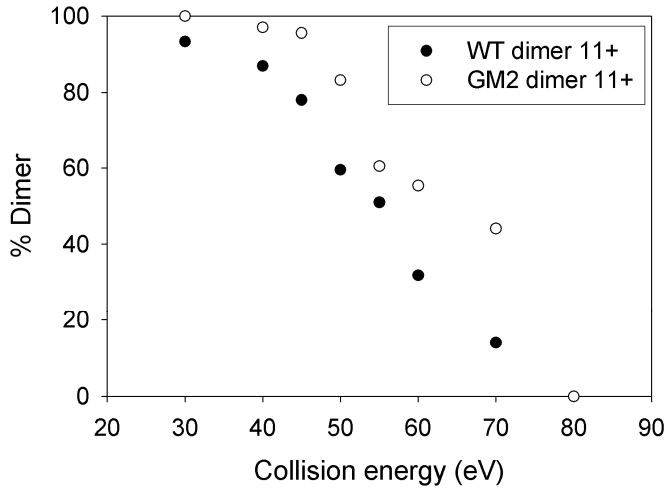
100
80
60
40
20
0
% Dimer
20
30
40
50
60
70
80
90
Collision energy (eV)
WT dimer 11+
GM2 dimer 11+

Figure 5

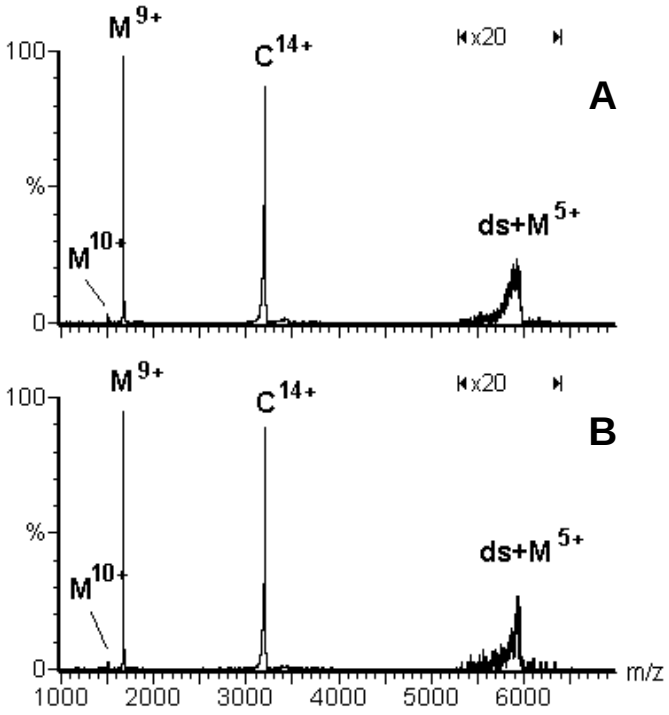
M 9+
C 14+
x20
A
100
%
0
M 10+
ds+M 5+
M 9+
C 14+
x20
B
100
%
0
M 10+
ds+M 5+
m/z
1000
2000
3000
4000
5000
6000

Figure 6

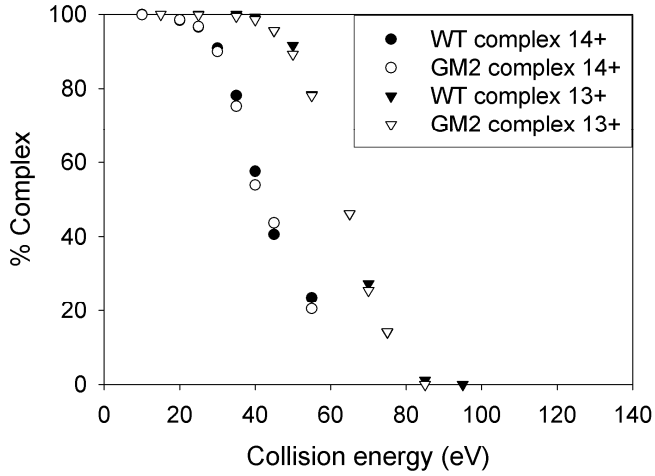
100
80
60
40
20
0
% Complex
0
20
40
60
80
100
120
140
Collision energy (eV)
WT complex 14+
GM2 complex 14+
WT complex 13+
GM2 complex 13+

Figure 7