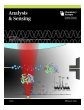

Infrared Laser Desorption and Electrospray Ionisation of Non-Covalent Protein Complexes: Generation of Intact, Multiply Charged Species

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We present a novel method enabling the infrared laser desorption and electrospray ionisation (ESI) of protein complexes in their native state. Using this method, we demonstrate the surprising generation of intact, multiply charged ions of myoglobin, non-covalent haemoglobin complex, and intact immunoglobulin G antibody in their native states. The observation of a surviving population of intact non-covalent complexes is characteristic of the low internal energy build-up experienced during both laser desorption from solution and subsequent ionisation. Compared to conventional nano-ESI, this approach yielded slightly lower average charge states suggesting additional maintenance of tertiary structure during desorption and ionisation, and is more tolerant to salts enabling simpler sample purification procedures. This approach may enable the development of high-throughput native-MS methods capable of analysing the composition and sequence of multiple macromolecular samples per minute.

The study of intact protein complexes by mass spectrometry has gained increased interest over the past few decades.[1] Such methods are now extending MS to the field of structural biology, for example the coupling of MS and cryo-EM or X-ray crystallography data.[2] Native MS utilises nano-electrospray ionisation (nano-ESI) of complexes from aqueous solution containing physiological buffer. Under such conditions quaternary structure is preserved and non-covalent complexes in the MDa range can be ionised and detected in their native confirmation.[3] Typically native MS is performed by manually loading a glass nanospray capillary with desalted and fractionated sample – an approach not well suited to high throughput analysis, e.g. drug-interaction screening. The ability to generate native MS spectra direct from a sample surface would provide a major step towards the localized and higher throughput analysis of macromolecular assemblies.[4]

Laser-based mass spectrometric (MS) strategies – which are extensively used for high throughput sampling approaches[5] – are rarely used under native conditions. For instance, matrix assisted laser desorption/ionisation (MALDI) is frequently employed to analyse intact proteins[6] and peptides[7] from surfaces, but requires organic solvents and acidic matrices. These conditions, combined with the energetics of the desorption/ionisation process, provide a denaturing environment that promotes dissociation and loss of quaternary structure.[8] Additionally, MALDI generates low charge state species (mostly singly and doubly charged) that are not well-suited for structural analysis by tandem mass spectrometry (MS/MS) and result in high mass-to-charge (*m/z*) values that may not be efficiently analysed.

Several studies have developed strategies that utilize laser desorption and/or electrospray to enable top-down analysis of intact proteins.[9] The group of Morgner has developed laser induced liquid bead ion desorption (LILBID), whereby charged and intact protein complexes are liberated from microdroplets in vacuum following IR laser desorption. LILBID shows good sensitivity and high salt tolerances. However, LILBID is still an infusion-based technique and – similar to MALDI – produces mostly singly and doubly charged ions.[10] Desorption electrospray ionisation (DESI) has been successfully applied to non-covalent protein complexes by the group of Robinson, demonstrating desorption of spray-deposited protein complexes from a surface.[11] Additionally, liquid extraction surface analysis (LESA), a nano-ESI based surface sampling method has also been shown to be compatible with native-MS.[12]

Shiea et al.[13] and others[14] demonstrated laser ablation electrospray ionisation (LAESI)-based analysis of various small proteins, such as myoglobin, cytochrome C and hemoglobin sub-units. These results were obtained with traditional electrospray solvents and additives, containing a high organic modifier percentage and high acidity. To the best of our knowledge none have demonstrated analysis of native non-covalent complexes.

Here, we demonstrate that LAESI-MS can in fact generate multiply charged non-covalent protein complexes in their native state. LAESI uses an infrared (IR) 2.94 μm laser to softly desorb analytes from aqueous solution at atmospheric pressure. Desorption is initiated via the coupling of laser energy into OH bonds of the solvent (e.g. water). The plume of

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desorbed material is intersected at some distance from the surface by an orthogonal electrospray (ESI) plume, where ionisation occurs analogous to conventional ESI. Charged analyte molecules then enter the mass spectrometer through the inlet due to the electric field and gas flow gradients.

In this work all experiments were performed using a LAESI ion source coupled to a Orbitrap UHMR mass spectrometer optimised for native-MS and high mass transmission.[15] First, we developed a method to analyse small proteins with LAESI-MS in their native state. We chose myoglobin as a target molecule because it has a clear transition from a native to a denatured state characterised by loss of the heme group and the creation of ions with higher charge states. The key optimization parameters were the LAESI electrospray solvent composition and the analyte solution composition. The optimal solvent composition that we found – and that was used for all native LAESI experiments presented here – was 100 mM of ammonium acetate in water, with 10% of methanol as organic modifier. We formulated a sample matrix of 200 mM of ammonium acetate in water with 10% ethylene glycol, which we will refer to as "native matrix" for the remainder of this text. Addition of an ammonium acetate buffer ensures a constant pH in solution, a requirement to retain native protein confirmation. Ethylene glycol was found to improve desorption repeatability without inducing heme-myoglobin dissociation when added to the sample matrix. The full experimental description is provided in the Supporting Information.

Figure 1 shows the mass spectrum of a myoglobin standard under optimized native LAESI conditions (Figure 1a) and that produced under conventional, denaturing LAESI conditions (Figure 1b). In both experiments the sample was 10 µl of 180 µM myoglobin solution in native matrix, which was spotted on a vendor-supplied plastic well plate. The native electrospray solvent composition dramatically lowers the amount of charges that attach to myoglobin, and dissociation of heme is largely avoided, both hallmark spectral features of native myoglobin. For comparison spectra generated with native-ESI and conventional microflow ESI are provided in Figure 1c, d.

The insets in Figure 1a and c show that fewer sodium and potassium adducts are observed in native LAESI compared to in native nano-ESI of myoglobin, even though the nano-ESI sample was dialysed prior to analysis and the LAESI sample was not. Adduct formation is identified as a key limitation in native mass analysis of large proteins.[16] The spray needle tip has been identified as the location where adduct formation in ESI can occur, which explains the observed differences.[17] Alternatively, the glass nano-ESI capillary, as common in native MS, could be a source of sodium. The LAESI sample solution did not come into contact with glass.

Next, we analysed a non-covalently bound protein complex. We chose hemoglobin, one of the most common and essential protein complexes in human biology.[18] This tetrameric complex consists of two α and two β subunits, each containing a heme group, that are non-covalently bound. Figure 2a shows the spectra obtained from native LAESI-MS on 10 µl of 200 µM hemoglobin in native matrix. Monomeric units with and without heme are detected, as well as heme-containing dimeric and tetrameric ions. Tetrameric hemoglobin A species were found between *m/z* 3600 and 4315 (*z* = 17 to 14), with sufficient mass

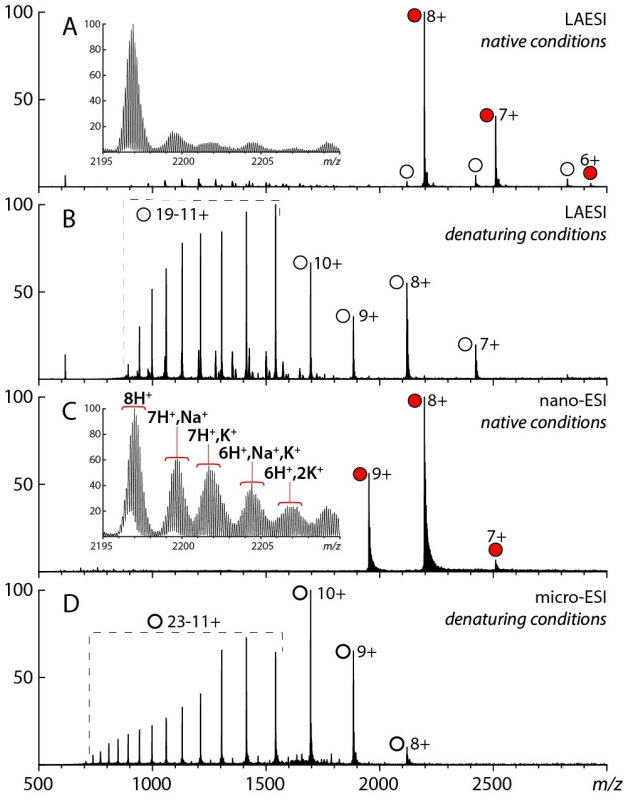

Figure 1. Mass spectra recorded from solutions of myoglobin standard in native matrix with (A) LAESI under native spray conditions (10% MeOH, 100 mM NH_4OAc), (B) LAESI under denaturing spray conditions (50% MeOH, 1% FA), (C) nano-ESI using native conditions (100 mM NH_4OAc) and (D) micro-ESI using denaturing conditions (50% MeOH, 1% FA). Multiple charge states of myoglobin are observed, either as native – with the heme group still attached (red-filled circle) or as dissociated (empty circle).

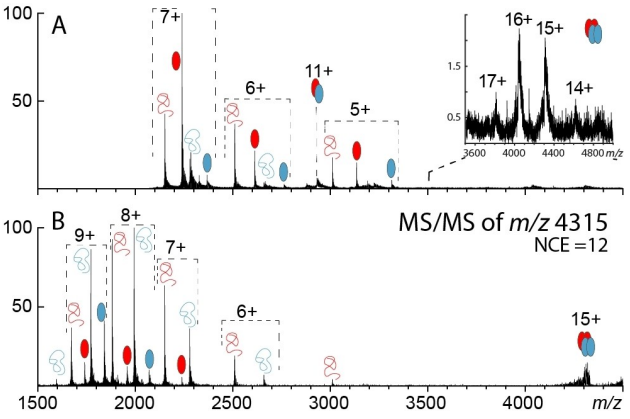

Figure 2. LAESI-MS spectra of 200 µM hemoglobin standard in native matrix, with heme bound α-monomers (red oval), heme bound β-monomers (blue oval), denatured monomer (string), dimer (blue and red oval) and native tetrameric hemoglobin (two blue and two red ovals). (A) full LAESI spectrum, whereas (B) HCD-MS/MS spectrum (NCE = 12) of the native hemoglobin species around *m/z* 4315 (*z* = 15), with a 30 Th wide isolation window. The spectra represent 100 shots, averaged from 40 scans over a period of 20 seconds. NCE = normalised collision energy. Peak were assigned within a mass error tolerance of 5 ppm.

resolving power and sensitivity to deconvolute them to an intact mass of 64,660 Da (Figure S1 in the Supporting Information).

To unequivocally demonstrate the generation of a population of tetrameric hemoglobin we dissociated the *m/z* 4315 ($z=15$) tetrameric precursor, as shown in Figure 2b. Both α- and β-monomers were detected with and without a heme group. This confirms the identity of tetrameric hemoglobin A. The bulk of the detected monomers after HCD fragmentation have a charge state between 6+ and 9+, arising from well-known asymmetric charge partitioning between the ejected monomer and remaining trimer.[19] Supporting Figures S2 and S3 show the LAESI MS/MS fragmentation spectra of hemoglobin subunit α (HBA) and subunit β (HBB), respectively, to confirm their identity.

Next, we investigated the detection of Immunoglobulin G (IgG) antibodies using native-LAESI. Figure 3a shows a native LAESI-MS spectrum from 200 μM IgG solution. This sample was prepared simply by dissolving the commercially bought IgG in matrix solution. The corresponding native spectrum produced from SEC fractionated IgG using nano-ESI is shown for comparison in Figure 3b. Similar to the results above native-LAESI gives comparable, although slightly lower, charge state distribution as nano-ESI, supporting the hypothesis that that the tertiary structure of IgG is preserved during IR-laser desorption. The average IgG species that was detected for both LAESI and nano-ESI has a mass of approximately 148 kDa, as is expected for a glycosylated IgG sample. The broad peaks are indicative of the different glycosylation states of IgG. Figure 3b shows the native nano-ESI spectrum which was obtained from the same – but purified through SEC-fractionation – human IgG standard (see the Supporting Information for details). For LAESI, the sample preparation was limited to dissolving an unfractionated sample in our native matrix solution.

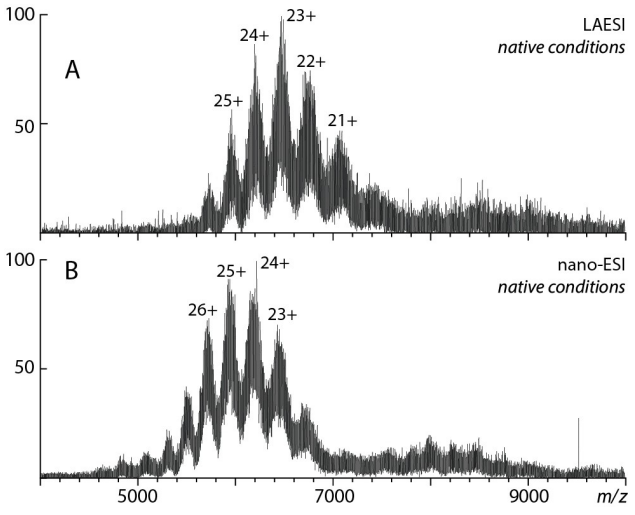

Figure 3. Mass spectra of human IgG measured with (A) LAESI-MS from 10% ethylene glycol and 200 mM NH_4OAc solution at 200 μM, and (B) ESI-MS from 100 mM NH_4OAc solution in a SEC-fractionated sample at 1.5 μM. Both spectra are 38 scans averages (20 seconds/10 LAESI laser shots).

All LAESI spectra shown here are very similar to their ESI counterpart (Figure 1 and Figure 3), except for the lower average charge state observed for LAESI spectra. Low average protein charge states can be attributed to more compact, folded structures, as the degree of unfolding observed in ESI-analysis of proteins has been correlated with higher internal energy values resulting in more extensive denaturing.[20] We surmise that a population of LAESI-generated ions exists that have experienced a lower increase in internal energy than produced under nano-ESI conditions. This is supported by the observation that also under denaturing conditions the LAESI generated charge state distribution is lower than the ESI generated distribution. Two other effects could also contribute to this charge state reduction. First, proteins experience a shorter interaction time between the charge containing electrospray solution and the analyte molecules in LAESI, compared to nano-ESI. Second, the high electric field experienced by the proteins at the nano-ESI tip could contribute to the higher average charge state distribution of the ESI results. Instrument incompatibility is currently preventing us from performing ion-mobility cross-section experiments to further support our hypotheses.

This work demonstrates for the first time the intact laser desorption and ionisation of non-covalent protein complexes using LAESI. Native-LAESI generated ions exhibit slightly reduced charge states compared to those generated from conventional nano-ESI performed under native conditions. These results suggest that neither the IR-desorption nor ESI post-ionisation impart sufficient energy for complete loss of tertiary structure (although we note some populations of ions are the result of dissociate processes, e.g. Figure 2a). Results are consistent with observations made using: (i) LILBID-MS and (ii) the survival yield method demonstrating that LAESI using conventional denaturing solvents results in ions with comparable internal energies as those formed from ESI.[21] The former work demonstrates that in solution very little energy is transferred to solvated analytes. LILBID is also known to form lower charge states than nano-ESI suggesting that the lower charge states seen in our native-LAESI spectra may, in part, arise from a similar ejection of ionised proteins directly from the sample solution rather than purely an ESI-type ionisation process. Given the ease in which multiple samples can be interrogated using LAESI, and the high tolerance to salt contaminants, our results suggest LAESI-based approaches are well suited for rapid analysis (multiple samples per minute) of protein complexes and other macromolecules.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Electrospray ionisation · laser desorption · mass spectrometry · non-covalent complexes · proteins

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