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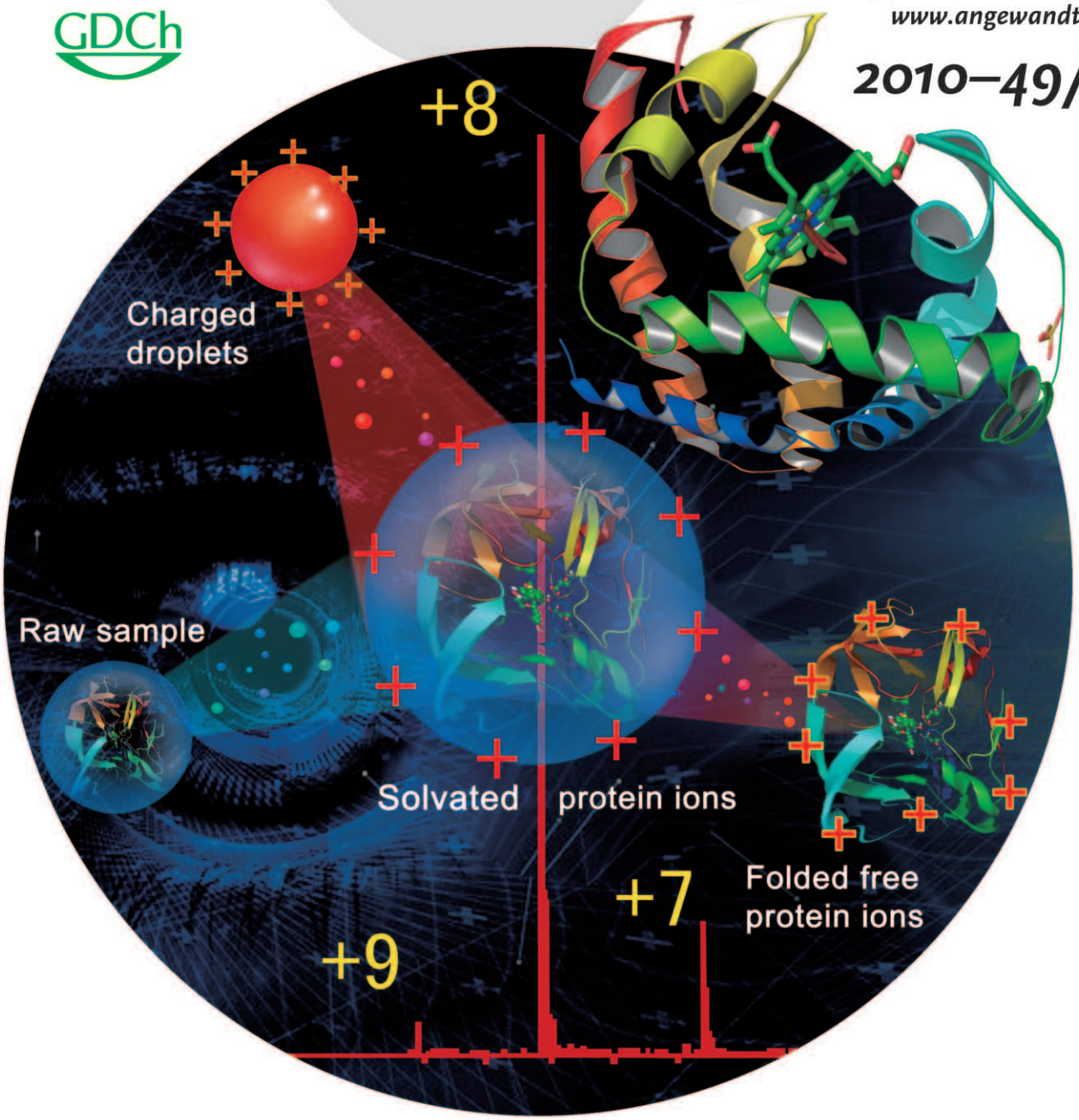

Characterization of native proteins ...

... can be undertaken with extractive electrospray ionization mass spectrometry (EESI-MS). As H. Chen and co-workers describe in their Communication on page 3053 ff., EESI deposits charges on native proteins, which are separated from any high electric field. This method facilitates mass spectrometry, even from raw biological samples, without significant conformational changes or reduction in activity.

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Analytical Methods

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Sensitive Detection of Native Proteins Using Extractive Electrospray Ionization Mass Spectrometry**

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Protein analysis provides abundant information crucial to understanding mechanisms and giving insight into life on the molecular level. Because of its unparalleled sensitivity and specificity, mass spectrometry[1–4] is the premier method for protein analysis. The native protein conformation, key to understanding the structure and function of proteins,[2,5–7] is difficult to determine by conventional electrospray ionization mass spectrometry (ESI-MS)[1–4] owing to multiple steps required for matrix cleanup.[8–10] The charge state distribution (CSD) of protein ions recorded using ESI-MS provides a highly sensitive tool to probe the overall compactness of a protein in solution,[11] and thus it is being used increasingly to gauge protein conformations.[11–13] Real-world protein samples such as cell materials contain intrinsically complex mixtures including liquids, salts, and almost every type of species involved in biological processes. Prior to protein analysis, specific sample pretreatments (separation, preconcentration, digestion, etc.) are usually required for most analytical techniques.[2–4] High-throughput protein analysis has been demonstrated using ambient ionization techniques such as desorption electrospray ionization (DESI),[14,15] electrospray laser desorption/ionization (ELDI),[16] and ambient matrix-assisted laser desorption/ionization (MALDI).[17] These techniques are suitable for the characterization of proteins on solid surfaces with minimal sample pretreatment. However, conformational changes occur rapidly (10^{-12}–10^{2} s)[9] when the molecular environment of the native proteins is changed dramatically.[2,11,18] The use of drying, crystallization, and energic desorption/ionization processes usually results in protein denaturation, aggregation, and subunit dissociation. Micelles have been used successfully to protect protein complexes while they are transferred from solution to vacuum for mass analysis.[10] However, the specific sample pretreatment required compromises the high-throughput analysis of proteins.

Herein we report a novel method for the rapid detection of native proteins in various matrices and untreated biological samples using extractive electrospray ionization mass spectrometry (EESI-MS).[19–22] In EESI (Figure 1), another "Cooks method", neutral molecules are neutrally sprayed into the charged plume generated by electrospraying the reagent solvent (e.g. methanol/water solution). The sample is isolated from any high voltages during the EESI ionization process,[19–22] for maximum safety and to minimize changes in the physiological/pathological states of the biological samples. EESI tolerates highly complex matrices, and has been applied for the direct detection of various small molecules with intricate matrices.[19–22] Thus, untreated biological samples can be applied directly to EESI for rapid characterization at the molecular level and under native conditions.

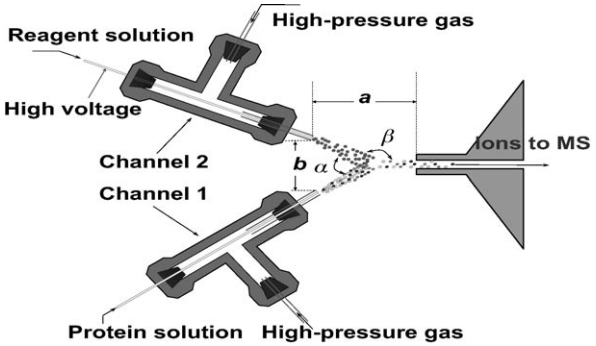

Figure 1. Illustration of the EESI setup attached to the LTQ-XL mass spectrometer for direct protein analysis. The charged reagent plume was created using ESI in channel 2, and the untreated biological samples were infused for EESI ionization through channel 1. The analyte ions produced at atmospheric pressure were then guided to the ion trap mass analyzer for further analysis. The distance *a* between the EESI and the ion entrance of the LTQ instrument was 10 mm; the distance *b* between the tips of channels 1 and 2 was 2–3 mm. The angle α formed between channels 1 and 2 was 60°; the angle β formed between the channel 1 and the heated capillary of the LTQ instrument was 150°. The temperature of the ion entrance capillary was maintained at 200°.

Figure 2a shows an EESI mass spectrum recorded with a high signal-to-noise ratio ($S/N = 10^4$) when a chicken egg lysozyme solution (pH 5.3, 1 μmol L^{-1}, NH_4Ac aqueous solution) was introduced directly to the EESI source. The signal intensity ratio for the most abundant peaks detected at *m/z* 2042 (+7), 1787 (+8), and 1588 (+9) was 16:100:2. The strong preference for formation of +8 ions can be explained by the presence of only eight basic groups (e.g., arginine, lysine) exposed on the outer surface of the compact lysozyme molecule.[23] Very narrow CSDs were observed in all EESI mass spectra (see Figure S1 in the Supporting Information) obtained using lysozyme solutions of various acidities (pH 5–

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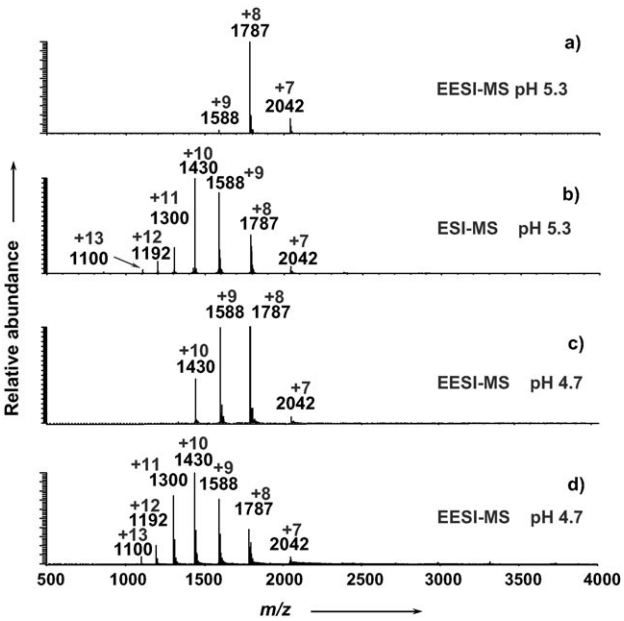

Figure 2. Mass spectra of a lysozyme solution ($M_r = 14290$) from chicken eggs detected in various matrices and at various pH values. a) EESI mass spectrum recorded at pH 5.3 which shows a narrow CSD; b) ESI mass spectrum recorded at pH 5.3 which displays signals corresponding to charges of +7 to +13; c) EESI mass spectrum recorded at pH 4.7 which displays signals corresponding charges of +7 to +10; d) EESI mass spectrum recorded at pH 4.7 with a methanol/water solution (8:1, v/v) which displays signals corresponding to charges of +7 to +13.

11). This suggests that the lysozyme maintained a folded conformation[23] throughout the entire analysis process and demonstrates how "soft" EESI conditions are for protein analysis. Surprisingly, when the same lysozyme solutions (pH 5.3, 1 μmol L^{-1}) were electrosprayed using a commercial ESI source, a much wider CSD was obtained, with signal ratios of 2:10:35:100:85:40:5 corresponding to the charge states of +13 to +7 (Figure 2b). The wide CSD indicated that unlike the EESI case, the compact lysozyme was partially unfolded under the ESI conditions. Comparable CSDs were obtained for more basic lysozyme solutions (pH 8–11) using ESI; however, the signal intensities were 2–3 orders of magnitude lower (see Figure S2 in the Supporting Information) than those obtained with EESI. Note that all the CSD patterns recorded using ESI were unaffected by the application of a neutral solvent spray to the electrospray plume. This confirmed that the unique EESI-MS patterns could not be simply produced by intersecting the ESI plume with a solvent spray.

The CSD of a lysozyme (Figure 2c) recorded by EESI-MS with relatively strongly acidic lysozyme solutions (pH 4.7) differed from that recorded using less acidic lysozyme solutions (pH 5.3), because the lysozyme was partially unfolded in the low pH medium. When a denaturant such as methanol was added into the lysozyme solution (pH 4.7), the lysozyme was further unfolded and the CSD shifted to higher charged states (+7 to +13) in the EESI mass spectrum (Figure 2d). However, the same solution generated a much wider CSD (+7 to +14; see Figure S3 in the Supporting Information) when it was subjected to the ESI process. These findings suggest that EESI maintains the structure of proteins in solution phase at the maximal degree. Interestingly, the EESI-MS patterns were not changed when different reagent solvents (Table S1 in the Supporting Information) were electrosprayed with the same lysozyme solution (either pH 4.7 or 7.3); this suggests that under the operating conditions the composition of the electrospraying reagent solution does not affect the conformation of proteins.

The detection of protein–ligand binding provides critical information about protein conformation and bioactivities, and is thus of great significance in drug discovery and the life sciences. However, protein complexes held together by noncovalent binding may undergo subunit dissociation during detection. For example, when an aqueous myoglobin solution (pH 7.3, 100 nmol L^{-1}) was electrosprayed under the standard ESI conditions, a series of apoglobin ions and the heme group (m/z 616) were detected with abundant signal intensities (Figure 3a). In contrast, almost no signal corresponding to apoglobin ions or heme group was evident in the EESI spectrum (Figure 3b), where the narrow CSD of the myoglobin ions confirmed that the myoglobin molecules maintained a compact conformation at the physiological pH conditions. These data provide experimental evidence that EESI is a much "softer" ionization technique than ESI for protein analysis. A possible explanation is that proteins for EESI are sprayed into the charged reagent plume. Here, the proteins do not have direct contact with the ESI high voltage (≥3 kV), which might contribute remarkably to the conformation changes and/or subunit dissociation of proteins observed in the ESI mass spectra.

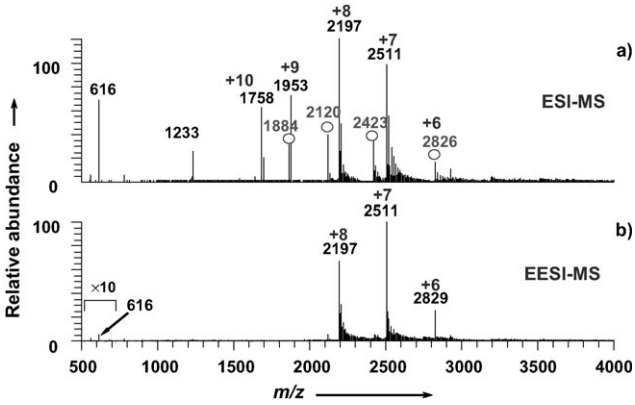

Figure 3. Detection of native myoglobin ($M_r = 17570$; apomyoglobin $M_r = 16954$). a) ESI mass spectrum of myoglobin. A series of apoglobin ions (labeled with circles) and the heme group (m/z 616, at 50% abundance) were detected which were formed by dissociation of subunits from myoglobin during the ESI process. b) EESI mass spectrum of myoglobin recorded using a methanol/water (1:1) mixture as the electrospray solution.

In the ESI mass spectrum of α-chymotrypsin in solution (pH 5, 0.5 M NH_4Ac methanol/water solution), the α-chymotrypsin ions were detected at high charge states (+8 to +13; Figure 4a). The dissociated α-chymotrypsin ($M-90$) and the

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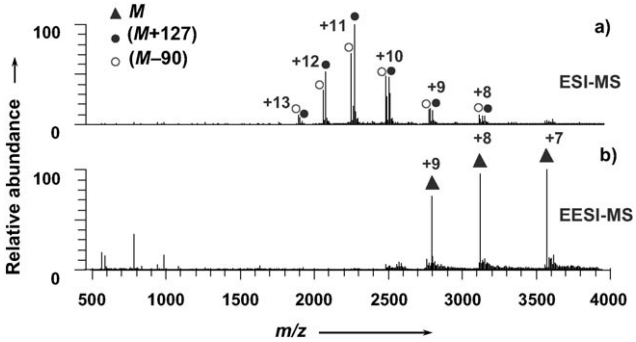

Figure 4. Detection of α-chymotrypsin ($M_r = 25\,326$). a) ESI mass spectrum recorded using α-chymotrypsin solution (pH 5.0), showing a wide CSD. Subunit dissociation and complex formation were observed. b) EESI mass spectrum of α-chymotrypsin solution (pH 5.0) using pure water as the electrospraying solvent; only native α-chymotrypsin was detected. The molecular weight of the α-chymotrypsin was deduced from the EESI data.

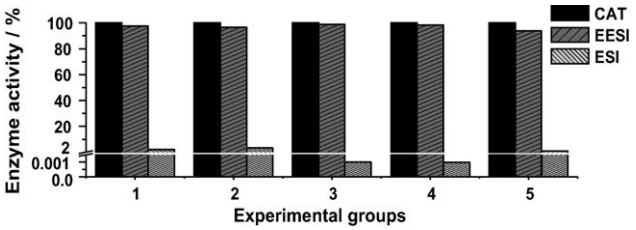

Figure 5. Enzyme activities of CAT protein ions recovered at ambient pressure. Data show that the CAT ions produced by EESI maintain the significantly high level of enzyme activities. Break region: from 0.0015 to 0.01. Each column represents the averaged results of five measurements.

protein–solvent complexes (M+127, water + methanol + NH_4Ac) were simultaneously detected by ESI for each charge state. The same solution generated α-chymotrypsin ions of low charge states (+7 to +9) when it was directly infused to EESI (Figure 4b). Signals corresponding to subunit dissociation or the protein–solvent complex were not detected in the EESI mass spectrum. The strong electric field in ESI probably facilitates the unfolding of the native protein. This allows appropriate small species to bind in the interior of the protein molecule such that they cannot be removed during the desolvation process; this results in unexpected protein–solvent complexes, especially in cases where proteins are analyzed with complex matrices or multiple ligands.

Compact protein ions may maintain their bioactivities better than the unfolded ones. Catalase bovine liver (CAT, $M_r = 240$ kDa), a protein with the highest turnover numbers of all enzymes, was examined to determine whether the ions maintain their enzyme activities for the decomposition of hydrogen peroxide. Amazingly, in comparison with the blank experiments where the CAT proteins were neutrally sprayed without any voltage (for details see the Supplementary Information), we found that the catalase molecules that had undergone the EESI process maintained high enzyme activity (94–99 %; Figure 5); CAT molecules that had been recovered from the ions generated by ESI retained only 0.001 %–3.4 % of the original activity, probably because the CAT ions had lost heme groups during ESI. These data show that protein ions created by EESI indeed maintain high bioactivities. In such a case, the native conformation of proteins should be preserved during the EESI process.

The narrow CSD in EESI mass spectra is a feature of the sensitive detection of trace amounts of proteins in raw biological samples. Low detection limits such as 0.32 pmol L^{-1} and 36 fmol L^{-1} were found for lysozymes in samples of saliva and human tears, respectively (for details see the Supporting Information). The lysozyme content was found to be 2–7 mg mL^{-1} and 20–260 μg mL^{-1} in tear samples from five healthy volunteers and three patients with pink eye, respectively. This indicates that EESI-MS could be used to diagnose conjunctivitis (pink eye caused by lack of lysozyme), for which the symptoms and signs are relatively nonspecific,[24] by measuring the lysozyme levels in tears. Following a "top-down" strategy,[25–28] proteins detected in the EESI mass spectrum can be identified by multiple-stage mass spectrometry, further extending this method for broad application.

Unlike the typical ESI process, charges are deposited "softly" on the neutral analytes, and the samples are isolated from high voltages during the EESI process. This allows maximum safety, and minimizes changes in the physiological/pathological state of the proteins during the sample analysis. Protein ions are created gently at ambient conditions, with minimal conformation changes and/or reduction in bioactivity. Owing to its unique design, EESI is suitable for highly complex matrices. Thus, untreated biological samples can be supplied to EESI for rapid mass spectrometric characterization at the molecular level and under close to native conditions. Our data show that EESI-MS is a sensitive tool for the rapid detection of trace amounts of native proteins in biological matrices such as tears and saliva; the method thus has potential in many applications in proteomics, clinical diagnosis, and other cases involving protein analysis.

Experimental Section

Experiments were carried out using a commercial LTQ-XL mass spectrometer (Finnigan, San Jose, CA) equipped with a homemade EESI source (Figure 1). Human tear or saliva samples collected from healthy volunteers were used directly. Myoglobin (90 % purity), and α-chymotrypsin (85 % purity) were obtained from Sigma–Aldrich (St. Louis, MO). Chicken egg lysozyme was purchased from Yuanju Bio-Tech CO. Ltd. (Shanghai). Collision-induced dissociation (CID) experiments were performed to the precursor ions isolated using a mass-to-charge window width of 1.0–4 Da, with 10–25 units of collision energy and a duration time of 30–50 ms. For further information on other reagents and the procedure, see the Supporting Information.

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