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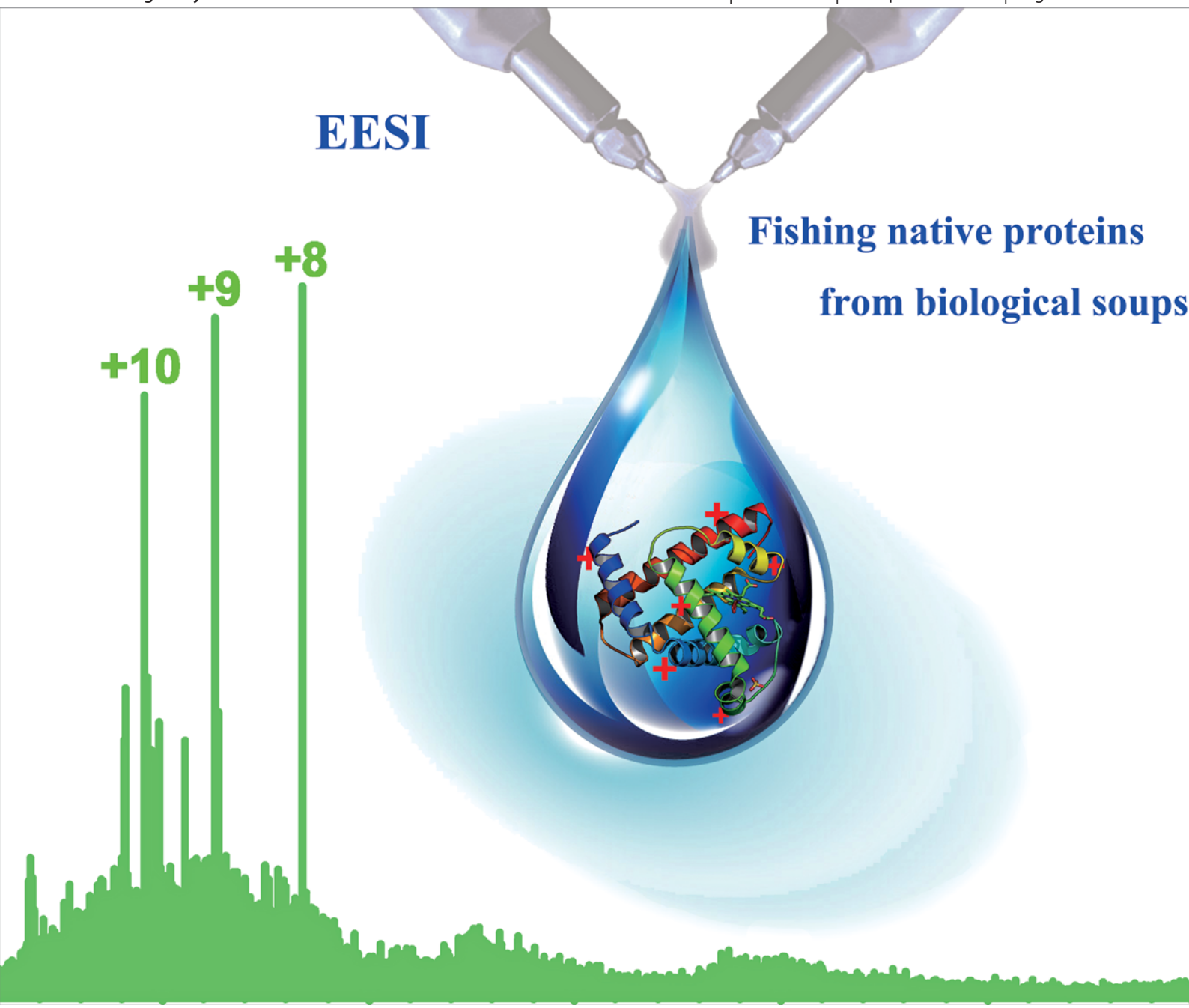

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COMMUNICATION

Direct detection of native proteins in biological matrices using extractive electrospray ionization mass spectrometry†

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The high-throughput and sensitive characterization of native proteins in biological samples is of increasing interest in multiple disciplines. Extractive electrospray ionization (EESI) forms ions of native proteins including lysozyme, α-chymotrypsin, myoglobin, human serum albumin, RNAse A and blood hemoglobin in extremely complex biosamples or PBS buffer solutions by softly depositing charges on the protein molecules. This method produces no significant conformational changes of the proteins in the ion formation process, and features direct detection of trace proteins present in biological matrices. The detection limit of low pmol L^{-1} for lysozyme in untreated biological liquids such as human urine and tears was demonstrated using EESI mass spectrometry (MS), showing an attractive MS platform for the direct analysis of native proteins in actual biological samples.

The highly organized quaternary protein structures provide essential information to investigate protein topology and dynamics, and offer insights into the mysteries of life. The native structures of proteins in biological states are sensitive to their molecular environment, and thus native proteins are difficult to be detected using methods involving substantial molecular environment changes (*e.g.*, organic solvent extraction, *etc.*). Remarkable progress has been achieved in probing the native protein structures using mass spectrometry (MS),[1–5] although these methods have not been routinely used for practical sample analysis. A smart strategy using micelles to protect protein complexes preserves the native conformation of membrane proteins for ESI-MS detection.[6] Protein complex isoforms and sub-complexes can be separated by using native capillary isoelectric focusing[7] before mass spectrometric analysis. However, the dedicated sample pre-treatment steps compromise the high-throughput analysis of proteins. Techniques including desorption electrospray ionization (DESI),[8] electrospray-assisted laser desorption/ionization (ELDI)[9] and laser-induced acoustic desorption electrospray ionization (LIAD-ESI)[10] tolerate biological matrices in native protein detection. These techniques require drying protein solutions on solid surfaces or adding light absorbents (*e.g.*, carbon powders, gold nanoparticles) to protein solutions. Recently, Venturi easy ambient sonic-spray ionization (V-EASI) has been reported for the direct detection of both liquid (V_L-EASI) and solid (V_S-EASI) samples.[11] A continuous flow-extractive desorption electrospray ionization (CF-EDESI) can be used to manipulate the charge states of proteins.[12] In this study, a straightforward method based on extractive electrospray ionization (EESI)[13] was developed for fishing native proteins from a highly complex biological soup for mass spectrometric analysis without any sample pre-treatment.

All mass spectra were recorded in the positive ion mode using a Thermo Finnigan LTQ mass spectrometer (San Jose, CA) equipped with an extractive electrospray ion source which was previously described elsewhere.[13–18] Briefly, biological samples were directly introduced into the EESI source (maintained at 25 °C), which was configured by two separated sprays. In the EESI experiments, primary charged droplets were generated by electrospraying a methanol–water (1 : 5) solution, and the sample plume was created by nebulising sample solutions without applying any electric voltage. Numerous soft collisions between the neutral analytes and the charged particles, which were generated by the ESI, produced ions of proteins under the ambient conditions. The distance (*a*) between the EESI and the ion entrance of the LTQ instrument was 10 mm; the distance (*b*) between the tips of two sprays was 2∼3 mm. The angle (α) formed between the ESI spray and the neutral sample spray was 60°; the angle (β) formed between the ESI spray and the heated capillary of the LTQ instrument was 150°. The temperature of the ion entrance capillary was maintained at 200 °C. For tandem mass spectrometry, the precursor ions were isolated using a window of 1.5 *m/z* units (full-width) and dissociated by collisional activation with helium buffer gas for 30 ms using a 20∼30% collision energy setting (Thermo LTQ arbitrary unit). The lysozyme quantification was based on a selected reaction monitoring type of experiment,[19,20] in which the fragments generated from the multiply charged lysozyme molecules were monitored to create the calibration curves. All the mass spectra were recorded using an average time of 1.5 min. The reagents and materials used are detailed in the Supporting Information.†

Fig. 1a shows the EESI mass spectrum of chicken egg lysozyme (100 μL, 10 μmol L^{-1}) added into an undiluted human urine sample§ (10 mL). Three most abundant peaks corresponded to the lysozyme

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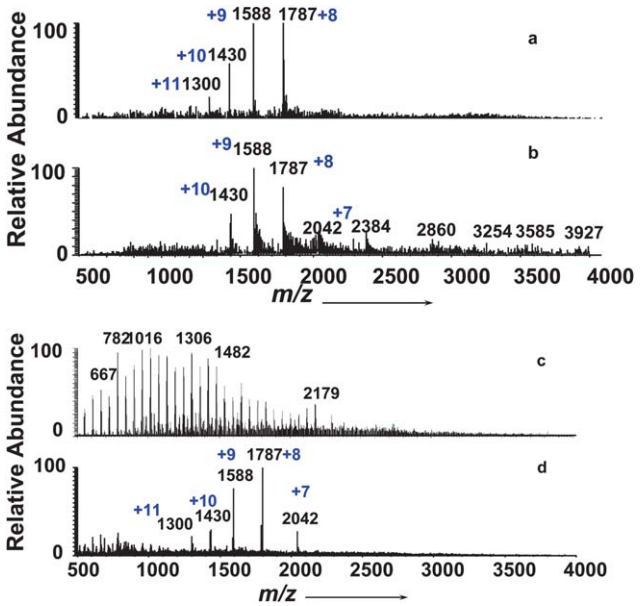

Fig. 1 Direct detection of native proteins in untreated biological samples by EESI-MS: (a) mass spectrum of chicken egg lysozyme spiked in undiluted male human urine; (b) EESI-MS spectrum recorded from an *Escherichia coli* mixture spiked with chicken egg lysozyme; (c) ESI-MS spectrum recorded using a lysozyme solution (10 μmol L^{-1}, pH = 8, controlled by 2 M buffer solution) containing sodium chloride (1%, w/w); (d) EESI-MS spectrum recorded using lysozyme solutions (70 nmol L^{-1}, pH = 8, controlled by NH_3HAc buffer solution) containing a high sodium chloride concentration (~15%, w/w).

ions with 10, 9 and 8 protons, respectively. The EESI-MS/MS data (Fig. S1a–d, Supporting Information†) confirmed the successful detection of trace amounts of lysozyme in the undiluted urine samples. The charge state distribution (CSD) observed using the urine samples (pH = 5.5) is slightly different from that detected from the lysozyme in PBS buffer solution (pH = 5.5) without urine matrices (Fig. S2, Supporting Information†). As demonstrated previously,[21] EESI softly charges proteins with minimal alteration of the original protein conformations in solutions. It is possible that the urinary ingredients such as urea accounted for the CSD changes since urea is a common denaturant which promotes protein conformation changes.

A narrow CSD of lysozyme (Fig. 1b) was detected from a slashed *E. coli* cell mixture spiked with chicken egg lysozyme (5 μmol L^{-1}). The *E. coli* mixture is an intrinsically complex biological sample which is widely involved in many typical biological studies. Dedicated sample pre-treatments (separation, pre-concentration, digestion, *etc.*) are required prior to the ESI-MS analysis of proteins in *E. coli* mixtures. Although more biological species were detected in the EESI-MS spectrum, the lysozyme signals maintained almost constant (from 2440 cps to 2030 cps) for the whole lysing process (>18 h), showing that the lysed *E. coli* soup, with gradually increased complexity, had no serious matrix effect on the native lysozyme detection. In addition, the direct EESI-MS detection of the native lysozyme added to the *E. coli* mixture showed that the matrix itself caused no conformation alteration during the extractive ionization step.

Most buffer solutions used in structural biology are incompatible with ESI-MS as the salts present interfere or even obstruct the protein ionization process. In comparison with ESI, the unique configuration of EESI allows the matrix to be dispersed in a relatively large spatial section rather than in the small capillary of ESI, resulting in high tolerance to extremely complex matrices for protein analysis. Fig. 1c shows no satisfactory ESI signal detected from a lysozyme solution (10 μmol L^{-1}) containing sodium chloride (≤1%, w/w). In contrast, lysozyme solutions (70 nmol L^{-1}) containing a high sodium chloride concentration (~15%, w/w) generated typical EESI-MS lysozyme spectra with clean background and easily recognized peaks (Fig. 1d). The CSD of lysozyme ions was slightly wider than that shown in Fig. S2,† which indicates that the unusually high salt content may partly unfold rigid proteins in the solution. The limit of detection (LOD) for lysozyme in the sodium chloride solution (3 mol L^{-1}) was 67 pmol L^{-1}. Similarly, proteins including human milk lysozyme, α-chymotrypsin, myoglobin, human serum albumin, RNAse A and blood hemoglobin in the PBS buffer solution were sensitively detected at nM levels (Table 1). The CSD of these proteins (Fig. S3–S6,

Table 1 Direct detection of proteins in different solutions by EESI-MS

Proteins	MW/kDa	pH	Charge state distribution	Concentration/μmol
Human milk lysozyme	14.6	5.5	8~10[a] 8~10[b]	0.10
Human serum albumin[c]	66	4.8	27~50[a] 29~43[b]	0.015
α-Chymotrypsin	25	5.0	9~12[a] 9~12[b]	0.04
Hemoglobin (subunit)	17	6.8	8~21[a] 7~19[b]	0.06
Myoglobin	16.9	7.0	6~11[a] 7~11[b]	0.06
Rnase A	13.7	4.6	6~9[a] 7~8[b]	0.07

[a] Protein solution prepared using NH_4Ac aqueous solution (1 mol L^{-1}) as the solvent. [b] Protein solution prepared using PBS buffer solution as the solvent. [c] Note that no tetramer ions were detected, probably because that the non-covelently bound tetramers were dissociated into dimers prior to the EESI analysis or the tetramer ions were beyond the mass range of the LTQ instrument in our experiments.

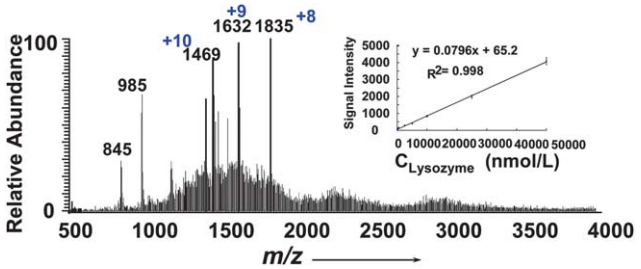

Fig. 2 Direct quantification of human lysozyme in tear samples. The narrow CSD shows that the human lysozyme was detected at native conditions. The inset shows the linear signal responses.

Supporting Information†) were comparable to those obtained from the NH_4Ac aqueous solution (1 mol L^{-1}). The experimental data confirmed that the high content of salts has no significant interference in the EESI-MS protein analysis.

For a given amount of protein, a narrower CSD formed in the EESI-MS spectra indicates that a higher peak height should be detected. This features the sensitive detection of trace amounts of native proteins in raw biological samples. For example, fresh tear samples collected from 3 healthy volunteers and 2 pink eye patients§ (pink eye disease is caused by the lack of lysozyme, for which the symptoms and signs are relatively non-specific[22]) were rapidly analyzed by EESI-MS without further sample cleaning. As shown in Fig. 2, the narrow CSD of human lysozyme and linear signal responses (inset of Fig. 2) were obtained from the tear sample. As a result, lysozyme was found to be 3∼7 mg mL^{-1} in the tear samples donated by the healthy volunteers, while low lysozyme levels (50∼220 μg mL^{-1}) were found in the tear samples collected from the pink eye patients. Low LOD values of 0.36 pmol L^{-1} were found for lysozyme in undiluted human tear samples. The RSD values obtained were in the range between 3.9 and 5.9% (the detailed data seen in Table S2†). These data show the capability of EESI-MS to diagnose conjunctivitis by quantitatively measuring the lysozyme levels in tears.

In summary, by gently depositing charges on protein molecules, EESI softly ionizes proteins present in complex biological samples, with a high tolerance to matrices containing a high content of salts. As demonstrated, trace amounts of proteins spiked into various raw samples such as urine, *E. coli* mixtures and tear samples were directly detected in their native conformations by EESI-MS without sample pre-treatment. The results were comparable to previous findings reported by Prof. Shiea and co-workers using fused-droplet electrospray ionization mass spectrometry.[23,24] This concordance makes EESI potentially attractive for the rapid analysis of proteins in biological samples.

Acknowledgements

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Notes and references

§ The present study was approved by the ethics committee of the Institute and adhered to the tenets of the Declaration of Helsinki. Additionally, the written informed consent was obtained from the volunteer and patients.

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