Direct detection of lysozyme in viscous raw hen egg white binding to sodium dodecyl sulfonate by reactive wooden-tip electrospray ionization mass spectrometry

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Abstract

Direct characterization of native protein binding to ligands in raw biological sample is a challenging task, because ligand solution might induce proteins to aggregation, flocculation and denaturation. In this work, we developed a reactive wooden-tip electrospray ionization mass spectrometry (ESI-MS) for formation and characterization of protein-ligand complexes upon rapid mixing in electrospray droplets. Raw viscous hen egg white (HEW) was directly loaded onto a wooden tip to induce spray ionization, and sodium dodecyl sulfonate (SDS) solution was directly loaded into the HEW spray by a pipette tip, and thus lysozyme-DS complexes were then formed in the electrospray droplets and were detected subsequently by mass spectrometry. The new approach was successfully applied to investigate interaction of SDS and native lysozyme in electrospray droplets of standard solution and raw egg white. Our results showed that wooden-tip ESI-MS is a promising method to form and characterize protein-ligand complexes.

Keywords: Egg White, Native Lysozyme, Protein Complex, Native Protein, Electrospray Droplet, Wooden-Tip Electrospray Ionization
Lysozyme is one of the important proteins in living organisms. Lysozyme is mainly produced from hen egg white (HEW) that is of importance to biological properties, pharmaceutical use, and nutritional values in daily life and food industry.\textsuperscript{1-3} Sodium dodecyl sulfonate (SDS) is an anionic detergent commonly used in biochemical studies. Lysozyme/SDS system is typical model molecules frequently used for investigation of globular protein and anionic surfactant in physical chemistry, analytical chemistry and interfacial chemistry.\textsuperscript{4-6} There are very few studies on the lysozyme/SDS system in raw biological samples that contain native lysozyme such as egg white. Raw HEW is a highly viscous liquid that contains complicated biological matrices, and therefore direct MS detection of native lysozyme from raw HEW is quite difficult. Furthermore, under such viscous condition, direct observation of native lysozyme in raw hen egg white binding to SDS is still a challenging task, because SDS solution could induce native proteins to aggregation, flocculation and denaturation.

Various analytical techniques, \textit{e.g.}, mass spectrometry (MS), cryo-electron microscopy, X-ray crystallography and nuclear magnetic resonance spectroscopy, are frequently used for investigation of protein structures, conformations and interactions.\textsuperscript{7-10} Compared with other techniques for characterization of analytes, MS has significant advantages in speed, sensitivity and specificity.\textsuperscript{11-16} Electrospray ionization (ESI) is one of soft ionization techniques for MS analysis and ESI-MS has been widely used to characterize protein.\textsuperscript{17} Under ESI-MS condition, protein molecule is typically detected as multiple-charged ions, and thus charge state distribution (CSD) of protein could be related to protein conformation. Considerable efforts on developments and applications of ESI
have been made to further improve protein analysis. For example, nanoESI
technique allows softer ionization, lower consumption, and tolerant to more salts
in protein samples.\textsuperscript{18-20} However, direct detection of native proteins and protein
complexes from real-life raw biological samples still poses a big challenge to MS,
mainly due to ion suppression effect and a fact that conventional MS-based
approach cannot directly analyze protein samples in untreated biological samples
such as raw biofluid and biological tissue.

In the last decade, ambient MS has been developed for direct analysis of
complex samples with no or little sample pre-treatment under atmospheric
condition.\textsuperscript{21} Direct characterization of proteins by ambient MS has made great
progress in recent years.\textsuperscript{22} Some of those ambient MS techniques have also been
used for direct detection of native proteins and protein complexes from raw
biological samples.\textsuperscript{22} Particularly, ambient substrate spray ionization techniques
using different substrates,\textsuperscript{23} e.g., probe,\textsuperscript{19} paper,\textsuperscript{24} wooden tip,\textsuperscript{25} biological
tissue\textsuperscript{26} and living organism\textsuperscript{27} has been successfully developed, and among these
techniques have been paid much attention for protein analysis.\textsuperscript{28} Unlike
conventional capillary ESI, these substrate-based ESI can be used for direct
loading and ionization of various raw samples such as powder, viscous and bulk
solid samples.\textsuperscript{28} Particularly, protein ions could be directly generated under
ambient condition when protein sample and optional solvent were loaded on
substrate under an electric field.\textsuperscript{20,25,29,30} Owing to porous structure of wooden
surface, protein solution in complicated matrices and salt solutions could also be
detected using wooden-tip ESI-MS,\textsuperscript{31} e.g., direct detection of native protein in
untreated biological samples, such as raw viscous egg white using wooden tip.\textsuperscript{32}
Our previous work has demonstrated that wooden-tip ESI enabled rapid detection
of analytes in complex samples with the advantages and merits, including simple fabrication, easy operation, rapid analytical speed, high sensitive and quantitation capacity.\textsuperscript{4,25,31-34}

In this work, we further developed a reactive wooden-tip ESI to investigate interaction of native lysozyme from raw egg white binding to SDS. A reaction between native lysozyme from standard solution or raw viscous hen egg white (HEW) and sodium dodecyl sulfonate (SDS) in electrospray droplets could be characterized using reactive wooden-tip ESI. Upon short time of mixing process of lysozyme and SDS in spray ionization at microsecond (\textmu s) level, lysozyme-DS complexes were rapidly formed and detected using the presented reactive wooden-tip ESI. Direct analysis of the mixture of lysozyme and SDS by MALDI-MS was also compared. Overall, our results showed a promising method for rapid formation of and characterization of protein complexes from raw biological samples.

**Experimental**

*Chemical and materials*

Lysozyme, sodium dodecyl sulfonate (SDS), ammonium acetate, and \(\alpha\)-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma (St. Louis, Missouri, U.S.A.). Wooden tips (common wooden toothpick) were purchased from Parknshop (Hong Kong). Pipette tips were purchased from Eppendorf (Hamburg, Germany). Fresh hen eggs were purchased from local supermarket (Guangzhou, China). Water used in this study was Milli-Q water (Millipore, Billerica, MA, U.S.A.).

*Reactive wooden-tip ESI-MS*

As shown in Figure 1, reactive wooden-tip ESI was constituted by a wooden tip
and a pipette tip. The slim cylindrical wooden tip (diameter: 2 mm) has a optional coniform tip-end and a shape tip-size at 0.2 mm. The wooden tip was directly mounted on a nanoESI device (Thermo Fisher Scientific, Bremen, Germany). 1.0 μL of protein sample (raw viscous HEW or lysozyme standard solution) was directly loaded on the tip-end. And then, 1.0 μL of SDS solution at different concentrations was directly loaded on the Taylor cone of HEW spray by a gel loading pipette tip with a slow flow rate. The distance between the wooden tip-end and the MS inlet was 10 mm. An angle between wooden tip and pipette tip was 90 º. The temperature of ion entrance capillary was maintained at 150 ºC. Initial ionization voltage, cone angle of coniform tip-end of wooden tip and sample volume was set at + 3.5 kV, 15 º, and 1.0 μL, respectively, and was further optimized by direct detection of lysozyme standard (10 μM) in buffer solution (50 mM ammonium acetate). Therefore, protein ions were directly generated from wooden tip-end and detected by an Orbitrap mass spectrometer (Q-Exactive, Thermo Fisher Scientific, Bremen, Germany).

**MALDI-MS**

Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (CMI 6100, Guangzhou Hexin Instrument Co., Ltd, Guangzhou, China) was also used for direct analysis of protein samples. 1 μL of protein sample, i.e., lysozyme standard, mixture of standard lysozyme/SDS (1/1, vol/vol, 100 μM for lysozyme and SDS), raw HEW, and the mixture of HEW/SDS (1μL, 0.5 μL for SDS at 100 μM, 0.5 μL for raw HEW), was directly loaded on MALDI plate in which 1 μL of matrix solution of CHCA (saturated solution in acetonitrile) was pre-loaded and was then drying in the air. All the experiments were repeated at least three times and thus error bars were calculated accordingly.

**Results and Discussion**
**Formation and characterization of lysozyme-DS complexes**

Figure 2a shows wooden-tip ESI mass spectrum of lysozyme standard (10 μM in ammonium acetate). The main peaks corresponded to lysozyme ions at +7, +8, and +9, respectively. It is noted that signal intensity ratio of for +7, +8, and +9 was 40:100:2. The strong preference of formation of lysozyme ions at +8 can be explained because the presence of eight basic amino acid residues, e.g., lysine and arginine, exposed on the outer surface of compact lysozyme molecule. Therefore, signal of lysozyme ions at +8 was investigated under different experimental parameters. For example, lysozyme signal was found to be linearly increased with the sample volume until the volume reached 10 μL (Figure 3a). Unlike sample loading on a foil surface, large volume could be dropped from the slim wooden tip. Considering rapid spray ionization and protein separation on surface of wooden tip, 1.0 μL of protein sample was used in this work. Ionization voltage and cone angle of wooden tip were optimized at 3.5 kV (Figure 3b) and 30 ° (Figure 3c), respectively.

Observation of narrow CSDs indicated that lysozyme molecule maintained a folded conformation at native state in ionization process. When lysozyme solutions (10 μM in ammonium acetate) was electrosprayed out from wooden tip-end and SDS solution (1.0 μL, 100 μM) was immediately injected into the electrospray using pipette tip, a wider CSD from +7 to +10 was obtained, as shown in Figure 2b. Unlike single wooden-tip ESI case, wide CSDs indicated that compact lysozyme was inducted to be partially unfolded by SDS under the reactive wooden-tip ESI condition. Meanwhile, lysozyme-DS complexes were also clearly observed in reactive wooden-tip ESI spectrum (Figure 2b). Multiple SDS bound lysozyme can also be observed (Figures 2b). In the electrospray
droplets of lysozyme solution, formation of lysozyme-DS complexes can be explained by interaction between presences of active amino acid residues on lysozyme and DS anion. To better understanding the formation of lysozyme-DS complex, signal ratios of lysozyme-DS complex at different charge states over the unbinding lysozyme against concentration ratios of SDS/lysozyme were plotted, as shown in Figure 2c. Because lysozyme binding to multiple DSs could not be formed at low concentration, single DS (n=1) binding to lysozyme was plotted in this work. With increasing concentration of SDS solution, the signals of protein complexes were increased. It is reasonable that more DS anions were complexed by lysozyme in electrospray droplets. These linear plots also suggest that the formation and response of lysozyme-DS complexes is relative stable against the free lysozyme in the droplets. For example, the signal ratio of lysozyme-DS/lysozyme at +8 was found to be 0.025 when concentration ratio of lysozyme/SDS at 1.0 (both at 10 μM).

To better understanding the interaction between lysozyme and SDS, the same concentration ratios for lysozyme/SDS at higher concentration levels were further investigated. As shown in Figure 2d, the same pattern of native lysozyme was observed when lysozyme reached to 100 μM. Similarly, lysozyme-DS complexes were also clearly observed with high signal-to-noise at a high concentration of SDS (1000 μM) using reactive wooden-tip ESI-MS (Figure 2e). The plots of signal ratios of lysozyme-DS/lysozyme against concentration ratios of SDS/lysozyme confirmed that interaction of lysozyme and SDS is also increased with the concentration ratios (Figure 2f). The similar signal ratios indicated that formation and response of lysozyme-DS complexes is also relatively stable against free lysozyme in the droplets under high concentration.
level, e.g., the signal ratio of lysozyme-DS/lysozyme at +8 was found to be 0.021 when the concentration ratio of lysozyme/SDS at 1.0 (both at 100 μM).

According to the literature, in the case of the protein complex, the stoichiometry of complex between protein and ligand and the equilibrium constant $\beta$ of lysozyme and SDS can be described as follows:

$$\text{lysozyme} + n\text{DS} \overset{\beta}{\iff} \text{lysozyme} - n\text{DS}$$

Where $n$ is the stoichiometry and lysozyme-DS is complex, the $\beta$ of lysozyme and SDS in electrospray droplets can be obtained as $\beta = [\text{lysozyme-DS}]/[\text{lysozyme}][\text{DS}]^n$. For the $\beta$, a possible explanation is that the interaction of SDS and lysozyme electrospray plume maintain at equilibrium state. Therefore, the signal ratio (R), $R = I_{\text{lysozyme-DS}}/I_{\text{lysozyme}}$, could be expected to be a constant value when the same concentration ratio of lysozyme/SDS was used in this equilibrium state under a right concentration range.

**Direct observation of lysozyme-DS complexes in raw HEW**

Reactive wooden-tip ESI then was explored for formation and detection of native proteins from raw viscous HEW. In this work, viscous HEW was directly deposited on wooden tip without any additional solvent, intact lysozyme from HEW was successfully detected, as shown in Figure 4a. Lysozyme ions were predominately observed in the spectrum with a narrow CSD at +7, +8, +9, and +10, this observation was in agreement with the results obtained for detection of native lysozyme in buffer solution (Figure 2a), confirming the observation of native lysozyme from raw biological samples. According to the concentration of lysozyme in fresh HEW 0.38 % (w/w), unambiguous detection of a trace amount of native lysozyme from raw egg white shows the good sensitivity of this method. Interestingly, other proteins, e.g., ovalbumin (45 kDa, 54 %, dry mass),
ovotransferrin (78 kDa, 12 %, dry mass) and ovomucoid (28 kDa, 11 %, dry mass) were not observed, probably mainly due to the their significant differences in concentration, molecular weight, isoelectric point, and ionization response.\textsuperscript{2,3,38} Compared with lysozyme standard (e.g., +8 ions in inset of Figure 2a), it is noted that lysozyme peaks obtained from raw HEW (inset of Figure 4a) had slightly larger m/z values, suggesting that the detected lysozyme from raw HEW has non-covalently bound some small molecules from matrices,\textsuperscript{32} which might reduce the signal of intact lysozyme.

The reactive wooden-tip ESI was further attempted to investigate lysozyme-DS complexes from raw HEW samples. Similarly, 1.0 μL of raw HEW and 1.0 μL of SDS (100 μM) were formed the electrospray droplets. As shown in Figure 4b, mass spectrum of lysozyme-DS complexes shows the CSDs from +7 to +10. Using a higher concentration of SDS, e.g., 1000 μM, higher signals of lysozyme-DS complexes and multiple lysozyme-nDSs complexes were obtained (Figure 4c), confirming that formation of lysozyme-SD complex depended on concentration of lysozyme and SDS. In this work, when 1.0 μL of raw fresh HEW was used (lysozyme concentration is 0.38 % in weight in fresh egg white,\textsuperscript{1} ca. 265.37 μM), signal ratios of lysozyme-DS/lysozyme against different concentration ratios of lysozyme/SDS was plotted as shown in Figure 4d. When the ratio of lysozyme/SDS was at 1.0, signal ratio (R) of lysozyme-DS/lysozyme (+8) was calculated to be 0.11, which is much larger than those (i.e., 0.025 and 0.021) obtained from standard lysozyme/SDS systems when concentration ratios at 1.0. The significant difference should be mainly due to the lysozyme binding to other small molecules in raw HEW\textsuperscript{32} that could reduce the signal of native lysozyme. Another possible reason is that complicated matrices of raw HEW...
suppressed the signal of lysozyme-DS complexes. Both of reasons make the direct quantification of lysozyme from raw HEW to be quite difficult. Although other proteins and small molecules in raw HEW were not detected using wooden-tip ESI, they might make interferences in formation and detection of lysozyme-DS complexes. These results not only demonstrated a new method for rapid characterization of lysozyme-DS complexes in raw egg white, but also gained insightful into differences of protein complexes in standard samples and raw biological samples.

**MALDI-MS analysis of the mixture of raw HEW and SDS**

To better understanding the formation of lysozyme-DS complexes in electrospray droplets, images of the mixtures of SDS and lysozyme in standard solution and raw HEW were given in Figure 5. It is can be seen a clean liquid of lysozyme standard (inset of Figure 5a) and lysozyme/SDS standard solution (inset of Figure 5b). Raw HEW is well known limpid, viscous, clear liquid, as shown inset of Figure 5c. However, raw HEW could be immediately changed to be very turbidity when 1.0 μL of SDS (100 μM) was dripped into 1 mL of HEW, as indicated in inset of Figure 5d. Unlike lysozyme standard, native proteins in raw HEW could be aggregated, flocculated and denatured by SDS solution, because native conformation of lysozyme exhibit quite sensitive to its molecular environment in living organisms. It is also noted that such SDS-induced flocculated protein complexes are unable to be sprayed and ionized by wooden-tip ESI-MS and conventional capillary ESI-MS.

To further exam the mixture of lysozyme and SDS in bulk solution, MALDI-MS was further applied for direct comparisons of the responses of lysozyme standard (Figure 5a), lysozyme/SDS standard system (Figure 5b), raw HEW
(Figure 5c), and raw HEW/SDS mixture (Figure 5d). Whatever protein samples were analyzed, lysozyme ions were detected rather than lysozyme-DS complexes. These results indicated that lysozyme-DS complexes are dissociated in MALDI process. Unlike the solid or flocculent state of the mixture in solid or bulk solution, lysozyme and DS was rapidly complexed and ionized in electrospray droplets in which the short time of mixing in electrospray droplets was reported at μs-level. These results further revealed formation and detectability of lysozyme-DS complexes from raw viscous HEW by using reactive wooden-tip ESI-MS.

Conclusions

In conclusion, we developed a reactive wooden-tip ESI to investigate interaction of SDS and lysozyme in standard solution and viscous raw hen egg white. Our work demonstrated native lysozyme from standard samples and raw biological samples binding to SDS in electrospray droplets upon short time using reactive wooden-tip ESI. Compared to conventional ESI and MALDI, successful detection of protein complexes demonstrated the unique feature of reactive wooden-tip ESI upon short time. Overall, this study not only provided new insights into the interaction of native lysozyme and SDS in raw biological samples, but also provided a new strategy to further develop reactive substrate-based ESI-MS.

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Conflict of Interest

The authors declare to no competing interests.

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Figure captions

Figure 1. Illustration of the reactive wooden-tip ESI.

Figure 2. Wooden-tip ESI-MS analysis of lysozyme/SDS system: a) lysozyme standard (10 μM) on wooden-tip ESI, b) lysozyme/SDS (10 μM/100 μM) standard on reactive wooden-tip ESI, c) plots of signal ratios of lysozyme-DS/lysozyme against concentration ratios of SDS/lysozyme (lysozyme at 10 μM), d) lysozyme standard (100 μM), e) lysozyme/SDS (100 μM/1000 μM) standard on reactive wooden-tip ESI, f) plots of signal ratios of lysozyme-DS/lysozyme against concentration ratios of SDS/lysozyme (lysozyme at 100 μM). Inset of a) shows the enlarged mass-to-charge (m/z) range from 1770 to 1830.

Figure 3. Plots of lysozyme signal (+ 8) under different conditions: a) sample volume from 0.5 to 13.0 μL when cone angle of wooden tip at 15 ° and ionization voltage at 3.5kV, b) ionization voltage from 1.0 to 5.0 kV when sample volume at 1.0 μL and cone angle of wooden tip at 15 °, c) cone angle of wooden tip from 10 to 90 ° when sample volume at 1.0 μL and ionization voltage at 3.5 kV.

Figure 4. Wooden-tip ESI-MS analysis of HEW/SDS system: a) raw HEW on wooden-tip ESI, b) raw HEW(1.0 μL)/SDS (1.0 μL, 100 μM) on reactive wooden-tip ESI, c) raw HEW(1.0 μL)/SDS (1.0 μL; 1000 μM) on reactive wooden-tip ESI, d) plots of signal ratios of lysozyme-DS/lysozyme against concentration ratios of SDS/lysozyme in raw HWE. Inset of a) shows the enlarged mass-to-charge (m/z) range from 1770 to 1830.

Figure 5. MALDI-MS analysis of a) lysozyme, b) lysozyme/SDS system, c) HEW, d) HEW/SDS system. Insert of each spectrum in a-d) shows the image of analyzed samples, respectively.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
A color figure for Graphical Index only