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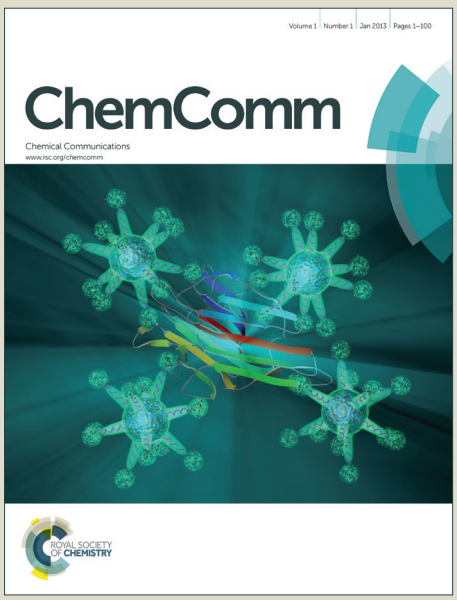

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Ultrasensitive SERS Assay of Lysozyme using a Novel and Unique Four-Way Helical Junction Molecule Probe for Signal Amplification

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A unique four-way helical junction molecule (FHJM) probe was ingeniously designed, and firstly introduced into DNA signal amplification as a novel Surface-enhanced Raman scattering (SERS) probe. Significantly, a new FHJM-SERS method was successfully developed for the lysozyme detection with high sensitivity and specificity.*

Surface-enhanced Raman scattering (SERS) spectroscopy has become one of the most valuable tools in chemistry, biology, and materials science.[1] It can facilitate molecular-level identification of samples, nondestructive detection in various matrices, and easy operation without complicated sample preparation.[2] So, SERS as an efficient biosensing method has been successfully applied to analyze small bioactive molecules,[3] DNA,[4] cells,[2a] and protein.[5] To garner further signal enhancement, DNA amplification techniques have been introduced into the SERS bioassay recently.[6] A SERS detection system was designed to detect target DNA by a hybridization chain reaction, and a detection limit of 45 pM was achieved.[7] A SERS detection strategy based on a triple-helix molecular switch was developed for nucleic acids and proteins, achieving high sensitivity and selectivity.[5a] In spite of considerable progress, various types of SERS amplification methods still have some limitations for practical applications. For instance, the detection efficiency was not high, the detection universality was restricted, and the target recognition was often confined through the steric hindrance effect.[5a] Hence, it has attracted considerable attention to develop the novel, simple, and efficient SERS detection approaches for assaying biological samples.

Recently, the properties of four-way DNA junctions have attracted an increasing interest, especially DNA Holliday Junctions (HJ).[8] DNA HJ is a branched structure with four double-helical arms.[9a] The four-way DNA junction was found to be an intermediate in genetic recombination, and it was structurally polymorphic.[9a] In the absence of divalent metal ions, the center is unfold and the four helical arms are pointed to the corners of a square.[9b] Nevertheless, in the existence of magnesium ions, the structure folds into the stacked X-structure via coaxial pairwise stacking of helical arms.[9c] In synthetic X-structure DNA, component DNA sequences can be reasonably projected that the branch point is fixed and the structure is stabilized.[9] Inspired by the above reports, a novel four-way helical junction molecule (FHJM) was designed with distinctive sequence of HJ-aptamer for the first time (Fig. 1), might act as an active recognition capable. Combined with DNA amplification methods, this FHJM was fabricated including target recognition region, triggering region and the template region.

Lysozyme, as a prevalent protein, is widely distributed in most living organisms, which can damage the bacterial cell wall to kill bacteria. It plays an important role in the science of human health.[10] The concentration of lysozyme is low under normal physiological conditions in body secretions and tissues. However, the changes of the lysozyme concentrations in serum, cells, urine and saliva, were connected with many diseases, such as leukemia, meningitis and renal diseases.[10a] Thus, sensitive and specific determination of lysozyme in fundamental research and clinical practice has attracted an increasing interest.

In this work, a novel FHJM was tactfully manufactured, and introduced into DNA signal amplification as a unique SERS probe for the first time. Furthermore, a FHJM-SERS method was successfully established to detect lysozyme. The new FHJM-SERS strategy exhibited high sensitivity with the detection limit of 0.5 fM for lysozyme, and fine performance in real human serum assay.

The FHJM probe employed a unique HJ structure via intramolecular DNA hybridization, fastened on magnetic bead (MB) (Fig.1). The FHJM probe with distinctive DNA sequence had three functional regions containing the aptamer loop region for target recognition, the trigger DNA region and the template for the DNA

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polymerization-nicking amplification (PNA). The tentacle DNA-1 strand of the FHJM structure was designed to contain a lysozyme aptamer for target recognition, which was structured for the part of two left-arms sequences of FHJM. The template DNA-2 strand for PNA was constituted the part of two right-arms sequences of FHJM by Watson-Crick base pairings.[11] Its two right-arms were linked by a single-stranded DNA (DNA-b) to form the right circular sector of FHJM, and the triggering DNA-b (Fig. S2) strand made up the four-way sequence of FHJM by Holliday-Junction base pairings,[9d] which could hybridize with the prime DNA-6 in the presence of lysozyme and trigger the polymerization reaction.

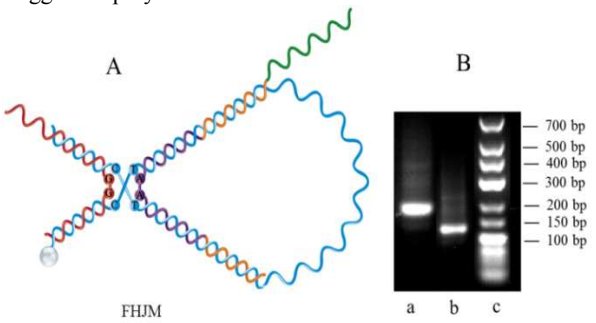
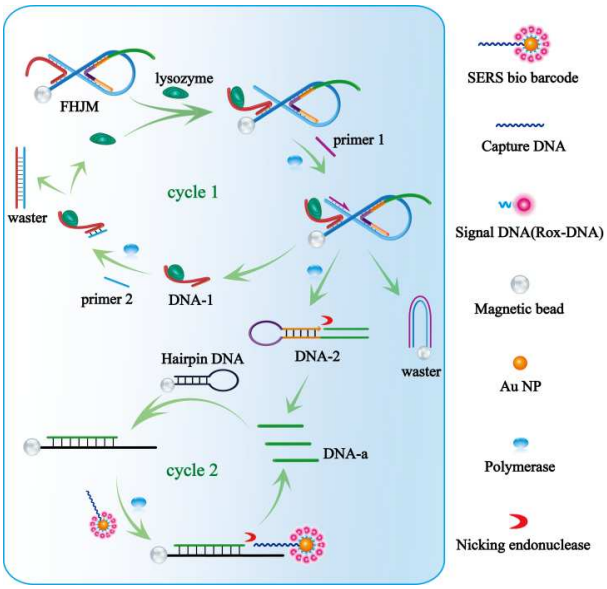


Fig. 1 (A) The structure of the FHJM immobilized on MBs; (B) The FHJM probe was analyzed by 1.0% agarose gel electrophoresis, (a) DNA-b, (b) the FHJM probe, (c) Marker.

The FHJM-SERS system was composed of the FHJM probe, the hairpin DNA template and Raman signal probe. The Raman signal probe consisted of capture DNA and Raman dyes labeled DNA (DNA-Rox) fastened onto gold nanoparticles (AuNPs). DNA-Rox as the signal probe was fastened on AuNPs to produce intense Raman signal.



Scheme 1 Schematic Illustration of FHJM-SERS Strategy for Lysozyme Detection.

Scheme 1 depicted the principle for analyzing the target lysozyme based on a four-way helical junction DNA molecule. The presence of the target lysozyme led to conformational changes of the FHJM probe via an aptamer-target binding event, which created the DNA hybridization sites for hybridizing with the primer. With the extension of the primer, lysozyme and the template DNA-2 strand were substituted and released. So, the two-cycle running mode was activated, containing the target recycling-oriented (TRO) reaction (cycle 1) and PNA reaction based on the template DNA (cycle 2).

In cycle 1, lysozyme was captured by the tentacle strand of FHJM probe. With the primer prolonging, lysozyme and the template DNA-2 were freed, which was assisted by Klenow fragment polymerase (KF polymerase) and deoxynucleotide solution mixture (dNTPs). The freed lysozyme by the aptamer-target recognition reaction was then bound to another FHJM probe, which is known as TRO reaction. Thus, more hairpin DNA strands (DNA-2) for SERS signal were released from a mass of FHJM. The strand segment (DNA-2) with cleavage site was cleaved, which generated many trigger DNA strands (DNA-a) by the cooperation of KF polymerase and a nicking endonuclease (Nb.BbvCI). Then, hundreds of trigger DNA-a strands into the system initiated the cycle 2.

In cycle 2, PNA reaction of FHJM-SERS was motivated. The trigger DNA-a strands unfolded many hairpin DNA strands (DNA-8) with MBs. SERS signal probe was attached to the unfolded hairpin DNA-8 via capture DNA (DNA-9). Because the PNA reaction could be repeatedly recycled, a large number of trigger DNA-a were freed, afterwards, hybridized with the loop region of the hairpin DNA. An increasing number of hairpin DNA strands on MBs were unfolded, which stimulated a mass of SERS signal probes to bind with the unfolded hairpin DNA. As a result, abundant SERS signal probes could be fastened on MBs. After accomplished the whole FHJM-SERS assay system, lysozyme as target analyte could be recognized by determining the Raman signal from SERS signal probes fastened on MBs via magnetic separation. It could be avoided by employing MBs as the carrier that the excess bio-barcodes caused the problem of detection background. It is imaginable that the determination sensitivity can be dramatically boosted through achieving two-cycle amplification.

To evaluate the feasibility of the FHJM-SERS method for lysozyme determination, a series of controlled experiments were performed. In Fig. 2, the laser excitation of the samples offered discrete vibrational peaks at 1344 cm^{-1}, 1499 cm^{-1} and 1645 cm^{-1}, which could be due to the ring C-C stretching vibrations of Rox, in accord with that of Rox dye fastened on AuNPs.[12] The strongest Raman band at 1499 cm^{-1} was selected as the characteristic peak for the detection of samples, and the corresponding Raman intensity was used to quantificationally estimate the SERS response to lysozyme. Without lysozyme (curve a), the template DNA-2 could not be released from the FHJM probe to generate the trigger DNA-a, as a result, the SERS signal probes could not be immobilized on the MBs. It's no surprise, Raman signals were extremely weak, which may be owing to the nonspecific adsorption of the SERS signal probe on the surface of MBs.[11b] In existence of lysozyme but no dNTPs or KF polymerase (curve b), the results were the same as predicted. In existence of lysozyme and polymerase but without Nb.BbvCI (curve c), cycle 2 could not be activated, because of lacking the trigger DNA-a without nicking reaction. In existence of lysozyme, Nb.BbvCI and KF polymerase, but without the hairpin DNA on MBs (curve d), cycle 1 could be proceeded, but cycle 2 could not put into effect. When all reagents coexisted in this reaction system (curve e), the Raman signal intensity was significantly enhanced, which verified the feasibility of the FHJM-SERS method.

Under the optimal conditions (The detail of the optimum experiments in the ESI), the Raman intensity enhanced with the adding of the lysozyme concentration in Fig. 3A. The intensity of Raman scattering had a fine linear to the logarithm of lysozyme concentration in the range from 1.0×10^{-15} to 1.0×10^{-12} M in Fig. 3B.

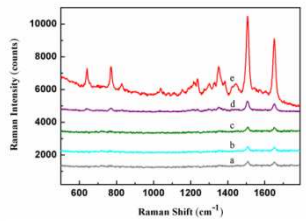


Fig. 2 A series of controlled experiments performed by SERS spectra: (a) without lysozyme; (b) with lysozyme, but no dNTPs or KF polymerase; (c) with lysozyme, KF polymerase and dNTPs, but no Nb.BbvCI; (d) with lysozyme, KF polymerase and dNTPs, but without hairpin DNA; (e) lysozyme, Klenow polymerase, dNTPs, hairpin DNA and Nb.BbvCI coexisting in the system (5.0×10^{-13} M lysozyme). SERS spectra were adopted an excitation laser of 633 nm, the laser power was 5 mW and the acquisition time was 10 s for each spectra.

The regression equation was described as: $\Delta I = 0.296 \lg C + 4.517$ (C is the concentration of lysozyme, $\Delta I = I - I_0$, I is the Raman intensity in the presence of lysozyme, I_0 is the Raman intensity in the absence of lysozyme), the corresponding correlation coefficient (R) of calibration curve was 0.996, and the detection limit of 0.5 fM was garnered (3σ). Compared with most reported methods, the FHJM-SERS method had a higher sensitivity and a far lower detection limit of lysozyme determination (Table S2, ESI†). The reproducibility of FHJM-SERS was examined by 11 successive measurements of 1.0×10^{-13} M lysozyme under the optimum conditions. The relative standard deviation (RSD) was calculated to be 11.7 % for lysozyme, indicating a good reproducibility of the method.

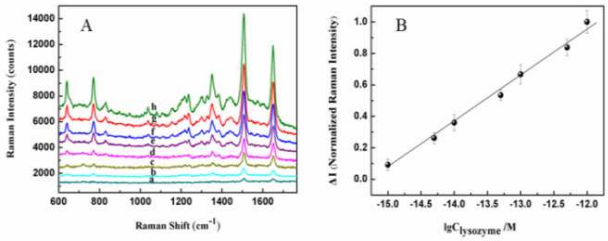


Fig. 3 (A) SERS spectra at different concentrations of lysozyme in the FHJM-SERS system: (a) 0, (b) 1.0×10^{-15} M, (c) 5.0×10^{-15} M, (d) 1.0×10^{-14} M, (e) 5.0×10^{-14} M, (f) 1.0×10^{-13} M, (g) 5.0×10^{-13} M, and (h) 1.0×10^{-12} M. (B) The corresponding calibration curve of normalized Raman intensity *versus* the concentration of lysozyme. The average of three spectra was acquired from different detection spots, and three repetitive experiments were carried out. Error bars displayed the standard deviation of three experiments. The blank was deducted from each value.

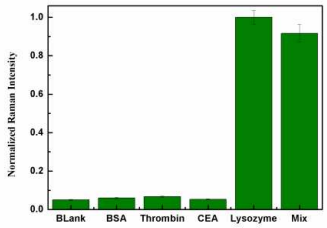


Fig. 4 Specificity for the determination of lysozyme against BSA, thrombin and CEA. The concentration of BSA, thrombin and CEA: 1.0×10^{-10} M, lysozyme concentration: 1.0×10^{-13} M. The blank was deducted from each sample.

To demonstrate the specificity and feasibility of the FHJM-SERS strategy, a series of controlled experiments were implemented. As compared to the blank test, no obvious change of the SERS shift was observed in Fig. 4. However, the target lysozyme led to the noticeable boost of SERS signal. Additionally, corresponding responses of SERS were acquired for lysozyme separately in buffer and human serum environment. The background signals obtained in human serum weakly enhanced compared with those in buffer, which were possibly owing to the interferences of the complicated media. The value of recovery ranged from 87.6 to 106.9% by the standard addition method (Table S3, ESI†), suggesting that this proposed strategy was effective and reliable.

Conclusions

In summary, a novel and unique FHJM was manufactured as a functional probe, and firstly introduced into DNA signal amplification. Significantly, a new FHJM-SERS strategy was successfully developed for the lysozyme detection. The assay exhibits high sensitivity, specificity and universality. The FHJM-SERS approach was also an effective tool for recognizing target analytes in the human serum samples. If various recognition units may be merged into the FHJM structure, the new platforms can be applied to analyze various targets with general applicability. The proposed method provides a high sensitive sensing platform, and exploits an exciting novel horizon for the detection technique.

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