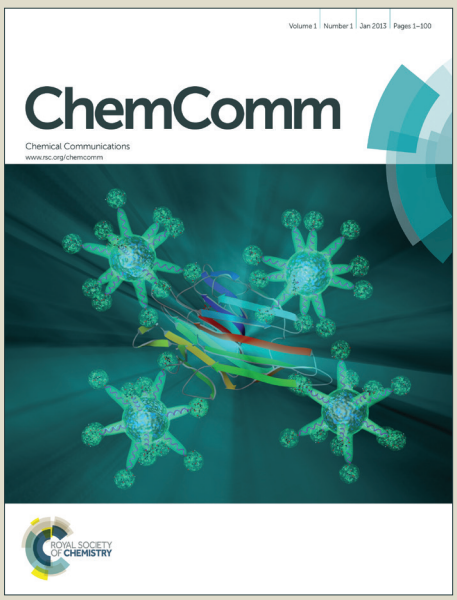
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ARTICLE TYPE www.rsc.org/xxxxxx | XXXXXXXX

Sensitive SERS Assay of Protein and Nucleic Acid using Triple-Helix Molecular Switch for Cascade Signal Amplification

Sujuan Ye,[ab] Yanying Wu,[b] Wen Zhang,[a] Na Li[a] and Bo Tang*[a]

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X
First published on the web Xth XXXXXXXXX 200X
DOI: 10.1039/b000000x

A novel surface-enhanced Raman scattering (SERS) detection system is developed for protein and nucleic acid based on triple-helix molecular switch for multiple cycles signal amplification, achieving high sensitivity, universality, rapid analysis, and high selectivity.

Protein and nucleic acid have been regarded as the key biomarkers and therapeutic targets in cancer diagnosis and treatment.[1] It has been generally recognized that, the concentrations of relevant biomarkers are usually on a relatively low level in the early stage of disease. Thus, highly sensitive detection of protein and nucleic acid are of great importance in the early diagnosis of cancers and major diseases. Up to date, various methods have been developed for the detection of nucleic acid and protein, including electrochemistry,[2] fluorescence,[3] colorimetry,[4] chemiluminescense,[5] and surface-enhanced Raman scattering (SERS).[6]

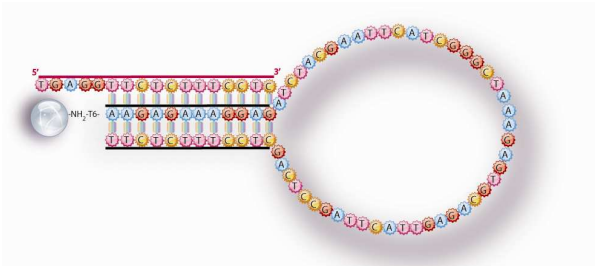
Among these detection methods, SERS has emerged as a specific, rapid and sensitive tool for biological analysis.[7] It can provide molecular-level identification of sample, easy operation without complicated sample preparation, nondestructive detection in a wide variety of matrices.[8] Therefore, SERS has been employed as an efficient biosensing method for the detection of small bioactive molecules,[9] DNA,[10] proteins[11]. For obtaining more enhanced Raman signals, Signal amplification methods based on DNA recycling have been introduced in the SERS bioassay recently.[12] A SERS detection system was designed to detect DNA and cell with DNA machine amplification. [12a] The bifunctional SERS assay based on strand-displacement polymerization was for the detection of small molecule or protein in parallel.[12b] Although various types of SERS amplification methods for the detection of bioanalytes have been developed, there are some limitations affects the sensitivity and universality toward target detection. Such as, the target recognition is often restricted to the steric hindrance effect of the DNA-aptamer complex,[12] and only specific DNA (the sequences containing the recognition site of nicking endonuclease) could be detected. [12a] Additionally, the signal amplification efficiency of two-cycle mode is still not high enough. [12]

[a] *College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Normal University, Jinan 250014, P. R. China. Fax: +86-531-86180017; E-mail: tangb@sdnu.edu.cn*
[b] *College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, China.*

† Electronic Supplementary Information (ESI) available: Experimental details, characterization, optimization ,selectivity, real sanple analysis and the comparison of different methods are included in the supporting information.and additional figures. See DOI: 10.1039/b000000x/

In view of the above limitations, a sensitive and universal cascade signal amplification SERS sensing platform is proposed based on triple-helix molecular switch. Compared to conventional signal amplification methods, the strategy is unique in some characteristics as follows: (i) Triple-helix molecule (THM) as a multifunctional probe is firstly introduced into DNA cascade signal amplification, which including target recognition region, triggering region and signal carrier region. In especial, the THM leave the free single aptamer strand to bind with the target analyte, for the suitable hybridization without hindering the affinity of an aptamer toward its target. (ii) Using the unique structure switch of the THM, three-cycle mode is developed to amplify the SERS signal. The amplification efficiency is enhanced. The DNA multiple recycling reaction is only involved in the THM, hairpin DNA, and SERS active bio barcode (SERS signal probe). (iii) It is potentially universal because the triple-helix molecule can be easily designed for other target analytes by changing the corresponding loop sequence. Given the unique and attractive characteristics, a simple and universal strategy is designed to accomplish ultrasensitive detection of protein and nucleic acid.

As proof-of-concept, lysozyme and p53 gene are used as model analytes in our experiments. The current strategy exhibits not only high sensitivity with the detection limits of lysozyme and p53 target DNA as low as 0.54 fM and 21 aM, respectively, but also excellent performance in real human serum assay. To the best of our knowledge, the THM mediated multiple recycling strategy has never been reported, not only in SERS assay, but also in any other detection methods. This work promises to open an exciting new avenue for future development of cancer and major disease early diagnosis.



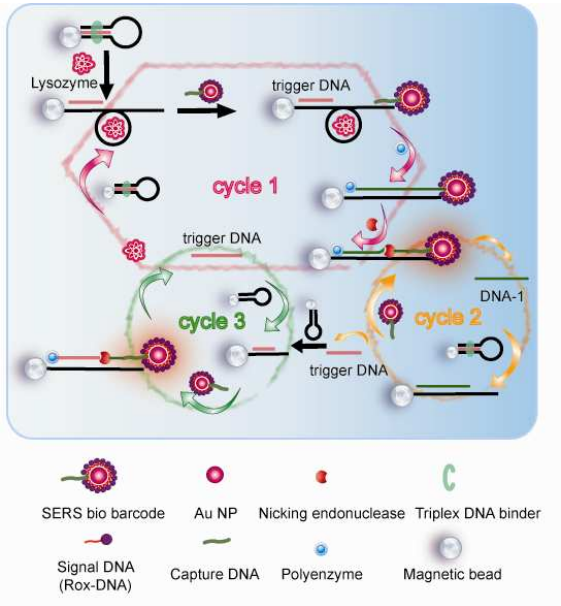
Scheme 1 The structure and sequences of THM immobilized on MB.

In Scheme 1, the THM adopts a hairpin structure by intramolecular DNA hybridization immobilized on magnetic bead (MB). The loop of the hairpin structure is designed to contain lysozyme aptamer for target recognition. The trigger DNA strand is bound to the stem sequence of the hairpin DNA by Watson-Crick and Hoogsteen base pairings.[13] A THM structure is formed and thereafter maintained by a triple helical stem region. The

THAM has three functional regions, the aptamer loop region for target analyte recognition and the trigger DNA region and the template of the strand-displacement amplification (SDA). The SERS-THM system consists of the THM, hairpin DNA template and SERS signal probe. The SERS signal probe is composed of capture DNA and Raman dyes labeled DNA (Rox-DNA) immobilized on gold nanoparticles (AuNPs). The capture DNA is complementary to the opened stem of hairpin DNA. Rox-DNA as the signal probe is immobilized on AuNPs to generate intense SERS signal.

As illustrated in Scheme 2, in the presence of lysozyme, the hairpin structure of the THM is unfolded via aptamer-target binding event. The SERS signal probe is attached to the unlocked stem of THM through capture DNA, which is the primer for DNA strand polymerization. The SDA is initiated with the cooperation of Klenow Fragment polymerase (KF polymerase) and nicking endonuclease (Nb.BbvCI). With the progress of the primer extension, lysozyme and the trigger DNA strand are replaced and released. Thus three-cycle working mode is initiated, including target recycling-oriented reaction (cycle 1), triple-helix hairpin DNA assisted SDA (cycle 2) and double-helix DNA assisted SDA (cycle 3)

In cycle 1, lysozyme is replaced with the extension of the primer on the THM template strand. The released lysozyme is then bound to another THM, which is known as target recycling-oriented reaction. As a result, more SERS signal probes are anchored to MBs for cycle 2, and many trigger DNA strands are released to the solution for cycle 3.



Scheme 2 Schematic representation of the THM-SERS assay for lysozyme.

In cycle 2, the hairpin DNA assisted SDA is initiated. The hairpin DNA serves as not only the template of strand-displacement amplification (SDA) reaction, but also the signal carrier through capturing the SERS signal on MB. A double strand is formed that binds Nb.BbvCI upon the progress of the primer extension. Then the strand segment with cleavage site is cleaved, which triggered new strand polymerization. Since the SDA can be recycled for multiple rounds, hundreds of DNA-1 are released, then, hybridize with the loop region of the THM. More THMs are unfolded, which stimulates the binding of abundant SERS signal probes with THM on MBs.

Moreover, in cycle 1 and cycle 2, many trigger DNA strands are released. That is, cycle 3 is triggered simultaneously, which is the hairpin DNA assisted SDA similar to cycle 2. As a result, a large number of SERS signal probes could be immobilized on the surface of MBs. After the execution of the whole network of THM-SERS detection system, lysozyme as target analyte could be identified by measuring the Raman signal of SERS signal probes anchored on MBs upon magnetic separation. The problem of background induced by excess bio-barcodes is circumvented using MB as the carrier. It is conceivable that the detection sensitivity could be significantly improved through accomplishing three-cycle amplification.

To assess the feasibility of the system for lysozyme detection, a series of control experiments were carried out. In Figure 1, the laser excitation of the samples provided discrete vibrational peaks at 1344, 1499, and 1645 cm^{-1}, which could be attributed to the ring C–C stretching vibrations of Rox, consistent with that of Rox dye immobilized gold nanoparticles.[14] The strongest Raman band at 1499 cm^{-1} was chosen as the characteristic peak for the following assay of lysozyme, and the corresponding Raman intensity was utilized to quantitatively evaluate the SERS response to lysozyme. In the absence of lysozyme (curve a), the SERS signal probes could not be anchored on the MBs. Not surprisingly, the Raman signals were very low due to the nonspecific adsorption of the SERS signal probe on the surface of MBs.[13b,e] In the presence of lysozyme but without KF polymerase or dNTPs (curve b), a small number of SERS signal probes were anchored on MBs. It is understandable that the Raman intensity was still low. In the presence of lysozyme, polymerase but no Nb.BbvCI (curve c), cycle 1 and cycle 3 were initiated with polymerization, but nicking reaction was not activated, the further amplification was not initiated, so the peak intensity was only improved than those of curve b (curve c). In the presence of lysozyme, KF polymerase and Nb.BbvCI, but the absence of duplex hairpin DNA on MB (curve d), cycle 3 was not carried out. But cycle 1 and cycle 2 can be carried out. Hence, the signal intensity was improved over those of curve c. When all reagents coexisted in the system, the Raman intensity was greatly raised, which confirmed the feasibility of this system.

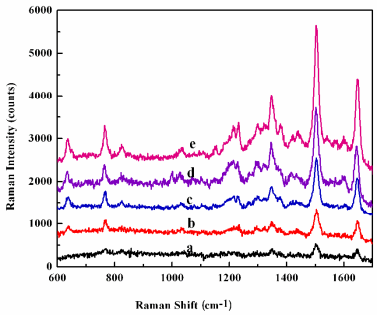


Fig. 1 SERS spectra obtained from serials of controlled experiments: (a) in the absence of lysozyme; (b) in the presence of lysozyme but the absence of KF polymerase or dNTPs; (c) in the presence of lysozyme, KF polymerase and dNTPs but without Nb.BbvCI; (d) in the presence of lysozyme, KF polymerase and dNTPs but without hairpin DNA; (e) lysozyme, Klenow polymerase, dNTPs, hairpin DNA and Nb.BbvCI coexisting in the reaction system. The concentration of lysozyme, 10^{-13} M. SERS spectra were measured with excitation at 633 nm, 5 mW laser power and the acquisition time for each spectrum was 10 s.

Under the optimal conditions, (The detail of the optimum experiments in the ESI) According to Figure 2A, the intensity of Raman scattering increased with the increase of the concentration of lysozyme. In Figure 2B, The Raman intensity had a good linear fit to the logarithm of lysozyme in the range from 1.0×10^{-15} to 1.0×10^{-12} M. The regression equation could be expressed as $\Delta I = 3.0105 \lg C + 45.8449$ ($\Delta I = I - I_0$, I is the Raman intensity in the presence of lysozyme, I_0 is the Raman intensity in the absence of lysozyme, C is the concentration of lysozyme), and the corresponding correlation coefficient (R) of calibration curve was 0.9975. A relative standard deviation (RSD) of 7.6% was obtained by 11 replicate measurements of 1.0×10^{-13} M lysozyme, suggesting a good reproducibility of the assay. The obtained detection limit of 0.54 fM demonstrated a higher sensitivity (S/N=3). The comparison of different methods for lysozyme detection is shown in Table S-2 (ESI).

Meanwhile, double-helix molecule (DHM) SERS assay as a control experiment was carried out (ESI, Scheme S-2, Figure S-8). The detection limit of 6.3 fM was obtained (S/N=3). The sensitivity of THM-SERS system was found to be increased about 1 order of magnitude than that of DHM-SERS system. The specificity and the feasibility of the practical clinical analysis of the THM-SERS strategy was investigated (See detail in ESI, Figure S-9, Table S-3).

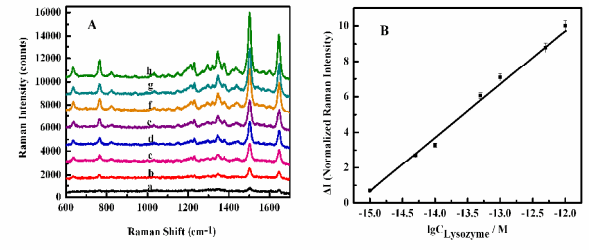


Fig. 2 (A) SERS spectra for increasing concentrations of lysozyme in the THM-SERS system: a) 0, b) 1.0×10^{-15}, c) 5.0×10^{-15}, d) 1.0×10^{-14}, e) 5.0×10^{-14}, f) 1.0×10^{-13}, g) 5.0×10^{-13}, h) 1.0×10^{-12} M. (B) The variation of normalized Raman intensity with the concentration of lysozyme. The average of three spectra was obtained from different spots on the gold slides surface, and three repeated experiments were performed. SERS spectra were measured with excitation at 633 nm, 5 mW laser power and the acquisition time for each spectrum was 10 s. Error bars showed the standard deviation of three experiments. The blank was subtracted for each value.

Highly sensitive detection of p53 gene as cancer biomarker is highly desired in cancer early diagnosis.[15] The THM-SERS sensing system is designed for the detection of p53 target DNA, with changing the loop sequence of the THM to complementary to p53 target DNA. The analysis of p53 target DNA is illustrated in Scheme S-3 (ESI). Three-cycle mode reaction is initiated by the structure switching of THM upon binding to p53 target DNA, After execution of the whole network, p53 target DNA could be detected through the enhanced Raman signal.

The sensitivity of the THM-SERS method was investigated (Figure S-10). The detection limit of 21 aM was obtained (S/N=3). The comparison of different methods for p53 target DNA detection is shown in Table S-4 (ESI). The experiments of selectivity and real blood sample detection are also shown in ESI (See detail in ESI, Figure S-11, Table S-5).

In summary, we report a new SERS cascade signal amplification approach based on the THM for the detection of protein and nucleic acid. The assay exhibits high specificity, sensitivity, and universality. Compared with the double-helix SERS method, this assay significantly improves the sensitivity. We have also demonstrated that the SERS strategy is an effective tool for recognizing target analytes in the human serum-containing samples. More importantly, since various recognition units might be fused in the triple-helix hairpin structure, the new platform can be employed to detect various targets with general applicability. The method not only offers a high sensitive sensing platform in cancer and major diseases early diagnosis, but also opens an exciting new avenue in a wide range of the detection technique.

This work was supported by 973 Program (2013CB933800), National Natural Science Foundation of China (21227005, 21390411, 91313302, 21035003) and Program for Changjiang Scholars and Innovative Research Team in University.

Notes and references

1 (a) N. Risch and K.Merikangas, *Science* 1996**, 273,** 1516; (b) J. J. McCarthy and R. Hilfiker, *Nat. Biotechno.*. 2000, **18,** 505.

2 (a) C. J. You, S. Wilmes, O. Beutel, S. Lochte, Y. Podoplelowa, F. Roder, C. Richter, T. Seine, D. Schaible, G. Uze, S. Clarke, F. Pinaud, M. Dahan and J. Piehler, *Angew. Chem. Int. Ed.,* 2010, **49,** 4108; (b) K. Hsieh, R. J. White, B. S. Ferguson, K. W. Plaxco, Y. Xiao and H. T. Soh, *Angew. Chem. Int. Ed.,* 2011, **50,** 11176.

3 (a) W. Pan, T. T. Zhang, H. J.Yang, W. Diao, N. Li and B. Tang, *Anal. Chem.* 2013, **85,** 10581; (b) A. R. Connolly and M. Trau, *Angew. Chem. Int. Ed.,* 2010, **49**, 2720; (c) C. H. Lu, F. Wang and I. Willner, *J. Am. Chem. Soc.,* 2012, **134,** 10651.

4 (a) J. Li, H. E. Fu, L. J. Wu, A. X. Zheng, G. N. Chen and H. H. Yang, *Anal. Chem.,* 2012, **84,** 5309; (b) Z. Zhu, C. C. Wu, H. P. Liu, Y. Zou, X. L. Zhang, H. Z. Kang, C. Y. J. Yang and W. H. Tan, *Angew. Chem. Int. Ed.,* 2010, **49,** 1052.

5 S. Shimron, F. Wang, R. Orbach and I.Willner, *Anal. Chem.,* 2012, **84,** 1042.

6 J. Zheng, A. Jiao, R. H.Yang, H. M. Li, J. S. Li, M. L. Shi, C. Ma, Y. Jiang, L. Deng and W. H.Tan, *J. Am. Chem. Soc.,* 2012, **134,** 19957.

7 P. L. Stiles, J. A. Dieringer, N. C. Shah and R. P. Van Duyne, *Annu. Rev. Anal. Chem.,* 2008, **1,** 601.

8 (a) Nie, S. M. and Emory, S. R. *Science,* 1997, 275, 1102; (b) J. F. Li, Y. F. Huang, Y. Ding, Z. L. Yang, S. B. Li, X. S. Zhou, F. R. Fan, W. Zhang, Z. Y. Zhou, D. Y. Wu, B. Ren, Z. L. Wang and Z. Q. Tian, *Nature,* 2010, **7287,** 392; (c) S. J. Ye, Y. N. Mao, Y. Y. Guo and S. S. Zhang, Trends *Anal. Chem.,* 2014, **55,** 43.

9 K. E. Shafer-Peltier, C. L. Haynes, M. R. Glucksberg and R. P.Van Duyne, *J. Am. Chem. Soc.,* 2003, **125,** 588.

10 (a) X. Qian, X. Zhou and S. M. Nie, *J. Am. Chem. Soc.,* 2008, **130,** 14934; (b) T. Kang, S. M.Yoo, I. Yoon, S. Y. Lee and B. Kim, *Nano Lett.,* 2010, **10,** 1189.

11 G. Wang and R. J. Lipert, *Anal. Chem.,* 2011, **83,** 2554.

12 (a) S. J. Ye, Y. X. Yang, J. Xiao and S. S. Zhang, *Chem. Commun.,* 2012, **48,** 8535; (b) S. J. Ye, J. Xiao, Y. Y. Guo and S. S. Zhang, *Chem. Eur. J.,* 2013, **19,** 811; (c) Y. Li, C. C. Lei, Y. Zeng, X. T. Ji and S. S. Zhang, *Chem. Commun.,* 2012, **48,** 10892.

13 (a) J. Zheng, J. S. Li, Y. Jiang, J. Y. Jin, K. M. Wang, R. H. Yang and W. H. Tan, *Anal. Chem.,* 2011, **83,** 6586; (b) T. N. Grossmann, L. Roglin and O. Seitz, *Angew. Chem., Int. Ed.,* 2007, **46,** 5223.

14 A. M. Michaels, M. Nirmal and L. E. Brus, *J. Am. Chem. Soc.,* 1999, **121,** 9932.

15 (a) Y. Yaginuma, H. Westphal, *Cancer Res.,* 1991, **51,** 6506; (b) N. R. Rodrigues, A. Rowan, M. E. Smith, I. B. Kerr, W. F. Bodmer, J. V. Gannon and D. P. Lane, *Proc. Natl. Acad. Sci., U.S.A,.* 1990, **87,** 7555.