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Switchable target-responsive 3D DNA hydrogels as a signal amplification strategy combining with SERS technique for ultrasensitive detection of miRNA 155

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Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.7b02321 • Publication Date (Web): 26 Jul 2017

Switchable target-responsive 3D DNA hydrogels as a signal amplification strategy combining with SERS technique for ultrasensitive detection of miRNA 155

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Abstract

Usually, SERS technology need labeling of Raman reporter to obtain characteristic spectrum for detection of biological samples. However, the number of labeled Raman reporters is often limited, resulting in the restricted improvement for sensitivity of SERS biosensor. In this work, switchable target-responsive 3D DNA hydrogels were introduced to precisely control trapping and releasing of Raman reporter toluidine blue (TB), which not only avoid labeling signal molecule but also improve the sensitivity of miRNA detection due to immobilization of abundant TB. In the absence of target miRNA, the DNA hydrogel presented a weak Raman signal because TB was far away from SERS substrates with an "OFF" status. However, the DNA hydrogel can be opened by the target miRNA to release TB producing strong Raman signal with an "ON" status. Based on this sensitive strategy, this switchable DNA hydrogel-based SERS platform can achieve quantitative detection of miRNA 155. Simultaneously, flexible SERS substrate (leaf@nafion@Ag) and target

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miRNA-induced duplex-specific nuclease signal amplification strategy were employed to significantly improve the sensitivity of the SERS platform. As a result, the as-proposed SERS platform can sensitively and selectively detect miRNA 155 with a wide linear range of 0.1 fM to 100 pM and low detection limit of 0.083 fM, which indicated that the platform has great potential to be applied in miRNA-related clinical diagnostics and biochemical researches.

Key words: surface enhanced Raman spectrum; DNA hydrogel switch; leaf@nafion@Ag; duplex-specific nuclease signal amplification

Introduction

MicroRNA (miRNA) as small non-coding RNAs[1] plays crucial roles in regulatory pathways including development, apoptosis, cell proliferation and differentiation, organ development and cancer.[2-6] There are a lot of methods for detection of miRNA such as electrochemistry[7], fluorescent[8,9], colorimetric[10,11], electrochemiluminescence[12,13] and so on. Surface enhancement Raman spectrum (SERS) technology possesses a good deal of superiorities like high sensitivity, narrow spectral band[14] and abundant fingerprint information[15]. Therefore, this ultra-sensitive technique can be widely applied for detection of miRNA.[16-18]

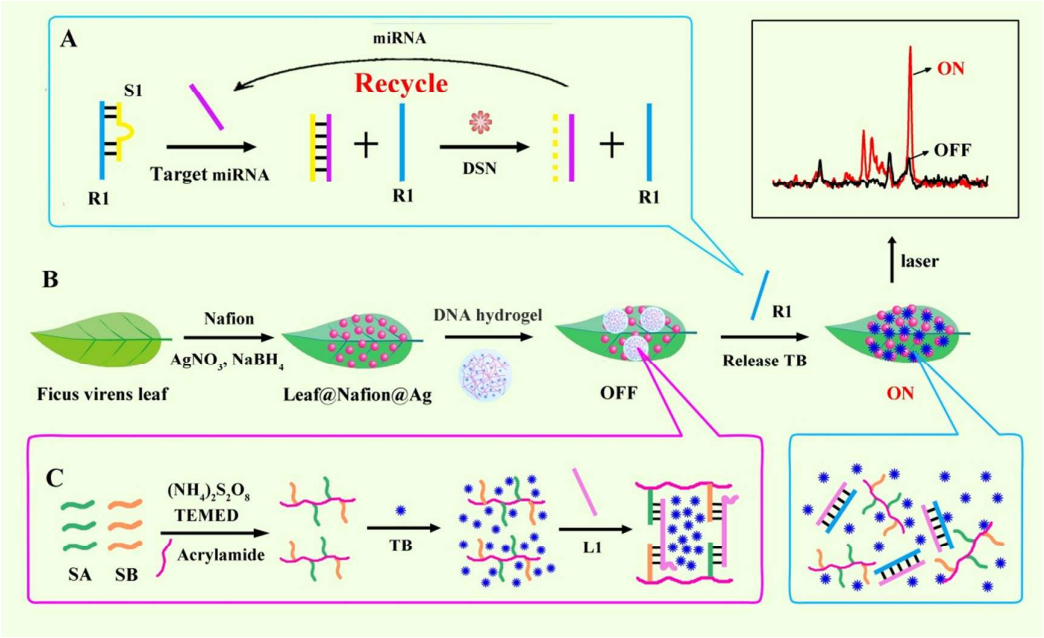
In reality, SERS substrates play the crucial role in SERS assay which decide the sensitivity and stability of the detection. Over the past decades, great deals of high-performance SERS-active substrates have been designed for detection of analytes. Different noble metal-based nanostructures[19-26] and their complexes such as magnetic Fe_3O_4@Au@Ag[27] and NiFe@Au[28] were employed to act as substrates for

providing high sensitivity and easy operation. However, it is difficult to avoid their aggregation. To realize the uniform SERS substrates, metal nanoparticles can be *in situ* deposited on the nanocarriers to obtain homogeneous hot spots[29]. Reports show that natural materials such as lotus leaf[29] and rose petal[30] exhibited large surface which can be used as sensitive and reproducible SERS substrates. Based on this, a low-cost, sensitive, reproducible SERS substrate was proposed based on *in situ* generation of silver nanoparticle on the surfaces of ficus virens leaves.

Hydrogel, often as network of polymer chains, is water-insoluble and superabsorbent which possess a degree of flexibility similar to the natural tissue.[31] It was also reported that it can sensitively respond to pH, temperature, electric fields and saccharides.[32-35] Three-dimensional DNA-based hydrogels, not only owns the characters of the polymer hydrogel, also possesses remarkable features such as stability, flexibility, precise programmability and so on.[36] Consequently, DNA hydrogel technology has been widely applied in the field of fluorescence[37,38], colorimetric[39], electrochemistry[40] and so forth. Acrydite-modified DNA can be conveniently incorporated to form gel which can be designed as DNA-switchable structures to switch "ON" or "OFF" in the presence or absence of released DNA. And due to the three-dimensional structure, if Raman reporter (TB) is added prior to the gel formation, they will be trapped inside the 3D network of the hydrogel in the presence of linker DNA. Once hydrogel switch was open by released DNA, the Raman reporter would be released from the 3D structure. Based on this principle, we designed one DNA hydrogel without labeling which can be open by miRNA.[41-43] This

target-responsive DNA hydrogels as a signal switch was applied in field of SERS for detection of miRNA. And this DNA hydrogel amplification technology was firstly applied in SERS biosensor which can remarkably improve the sensitivity and selectivity.

Herein, switchable target-responsive 3D DNA hydrogels were designed to control the trapping and releasing of Raman reporter for sensitively and selectively detection miRNA 155. In this strategy, flexible ficus virens leaves as SERS substrates were modified with nafion, on which AgNPs were *in situ* produced. This substrate owns the advantages of high sensitivity, good uniformity and easy production. During the process, firstly, target miRNA 155 was completely complementary with ssDNA (S1) which can take S1 away from released DNA (R1). Then, a duplex-specific nuclease (DSN) was introduced to cleave DNA-RNA hydrid (miRNA-S1) duplexes, releasing miRNA to induce the next cycle for obtaining numerous R1 for further use. Then 3D DNA hydrogel structure were constructed to capture lots of Raman reporters (TB), resulting in a weak Raman signal with an "OFF" status. Nextly, lots of R1 were added in the DNA hydrogel to open the 3D structure which would release abundant TB to produce strong Raman signal ("ON" status). Based on this label-free and high-sensitive principle, the proposed SERS platform can achieve sensitively detecting miRNA 155 with wide linear range and low detection limit, holding great potential in biomedical research and clinical diagnostics.



Scheme 1. (A) Target miRNA 155-induced duplex-specific nuclease signal amplification possess; (B) Schematic diagram of the construction of the SERS platform; (C) The preparation of TB trapped 3D DNA hydrogel.

Materials and Methods

Materials and reagents

HPLC-purified microRNA was synthesized by Takara Biotechnology Company Ltd. (Dalian, China). The DNA probes used in this experiment were obtained from Sangon, Inc. (Shanghai, China). The corresponding oligonucleotide sequences are listed in Table 1. Duplex-specific nuclease (DSN) was purchased from Newbornco Co., Ltd (Shenzhen, China). Nafion were purchased from Sigma-Aldrich (St. Louis, MO)). Ammonium persulfate ($(NH_4)_2S_2O_8$), N, N, N', N'-tetramethylethylenediamine (TEMED), ethylene diamine tetraacetic acid (EDTA), acrylamide, sodium chloride (NaCl), magnesium chloride ($MgCl_2$), silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), sodium citrate were obtained from ChengDu Kelong Chemical Reagent

Company (Chengdu, China).

Table 1. Sequence information for the nucleic acids used in this study

Name	Sequence (5'-3')
miRNA 155 (target)	UUA AUG CUA AUC GUG AUA GGG GU
ssDNA (S1)	ACC CCT ATC ACG ATT AGC ATT AA
Released DNA (R1)	TTA ATC GAA ATC GTG ATA GGG GTA AGA GTG CTT CAC ACG GAG GCT TCA GGC CG
Link DNA (L1)	CGG CCT GAA GCC TCC GTG TGA AGC ACT CTT ACC CCT ATC ACG ATT TCG ATT AA
Acrydite-modified DNA (SA)	Acrydite-TTA ATC GAA ATC GTG ATA GG
Acrydite-modified DNA (SB)	Acrydite-GGT AAG AGT GCT TCA CAC GG
Single-base mismatch (sRNA)	UUA AGG CUA AUC GUG AUA GGG GU
miRNA 21	UAG CUU AUC AGA CUG AUG UUG A
miRNA 141	UAA CAC UGU CUG GUA AAG AUG G

Apparatus

The SERS substrates were characterized by a scanning electron microscope (SEM, S-4800, Hitachi, Japan). During the whole experimental process, SERS spectra were tested with a Raman spectrometer (Renishaw Invia Raman spectrometer, Invia, UK) with 50-objective. 633 nm excitation wavelength was chosen to detect SERS platform with a single 10 s accumulation. And the Raman spectrometer was calibrated by a silicon wafer at 520 cm^{-1} Raman shift before SERS measurement.

Preparation of SERS substrates

Fresh leaves of ficus virens were picked from tree and washed with ultrapure water and ethanol separately. Nextly, clean and dry leaves were immersed in the 1% nafion ethanol solution for 30 min to form a thin film. After that, the nafion modified

leaf was put into 1 mL 10 mM $AgNO_3$ for 30 min and then 1 mL 2 mM sodium citrate and 1 mL 10 mM $NaBH_4$ were slowly dropped into the mixture for 20 min. In this way, AgNPs can be uniformly reduced on the leaf@nafion to form high sensitive SERS substrates leaf@nafion@Ag.

Preparation of DNA hydrogel

Stock solutions of acrydite-modified DNA strand SA and SB were prepared with concentration of 10 μM. The stock solution contained: 10 mM Tris, 1 mM EDTA (pH = 8.0), 200 mM NaCl, 4% acrylamide, 10 μM DNA strand SA or SB and ultrapure water. After directly mixing SA and SB to form mixed solution A, nitrogen was bubbled through solution A for 5 min. Nextly, 0.05 g $(NH_4)_2S_2O_8$ and 25 μL TEMED were added into 0.5 mL H_2O to form solution B. After that, solution A and solution B were mixed with a volume ratio of 1:0.014, and nitrogen was bubbled through the mixture for an additional 5 min while polymerization proceeded. Then 0.1 mM TB was added into the mixture and the mixture was shaken well followed by bubbling nitrogen for additional 5 min. To crosslink the polyacrylamide solution, 10 μM linker strands L1 were added and incubated at 25 ^oC for 10 min to form DNA responsive hydrogel.

SERS strategy assay process

Scheme 1 showed the procedure of the DNA hydrogel-based SERS platform for target miRNA detection. Firstly, 5 μL 100 μM DNA strands S1 partly hybridize with 5 μL 100 μM strands R1 in 10 μL phosphate buffer solution (PBS) (pH = 7.4) containing 0.25 mM $MgCl_2$ for 1 h with 37 ^oC. Then, different concentrations of

target miRNAs were added in the above solution to completely complement with S1. At the same time, 5 μL 0.05 U DSN were put into the solution at 60 ^{o}C for 40 min to get lots of R1. Then the products obtained (R1) were added in 3 μL DNA hydrogel on the SERS substrate for 20 min at 37 ^{o}C to open the DNA hydrogel. Finally, the biosensor was measured by Raman microscope with a 633 nm He-Ne laser.

Results and Discussion

Characterization of Materials

Figure 1A was the photograph of ficus virens leaf and Figure 1B was the corresponding SEM image, from which we can see the obvious reticulate structure. After AgNPs *in-situ* reduced on the leaf, we can find that lots of white AgNPs were uniformly distributed on it (Figure 1C).

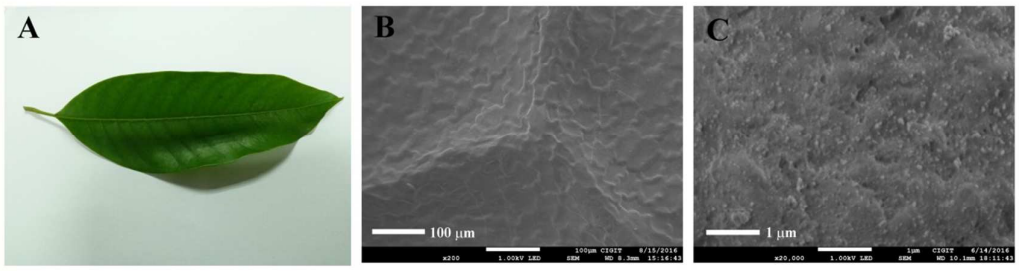


Figure 1. (A) Photograph and (B) SEM image of ficus virens leaf; (C) SEM of ficus virens leaf@Ag.

The principle of DNA hydrogel-based SERS platform

The fabrication process of DNA hydrogel-based SERS platform was characterized by Raman spectrometer with a 633 laser. As shown in Figure 2, when the DNA hydrogel which trapped lots of TB was dropped on the leaf@nafion@Ag SERS substrate, a weak Raman peak (1627 cm^{-1}) belonged to TB appeared (red curve

of Figure 2). This phenomenon may be due to that TB was trapped inside the 3D DNA hydrogel which made TB far away from SERS substrate, resulting in an "OFF" status. After the released DNA (R1) were added into the DNA hydrogel to open the DNA switch, TB molecules released to the SERS substrate directly, with a strong Raman signal ("ON" status) (Figure 2, black curve). During this signal amplification process, the number of R1 was positively correlated with the amount of target miRNAs, so this SERS platform can quantitatively detect miRNA. Based on this DNA hydrogel amplification strategy, the proposed SERS platform would achieve significant improvement for detection miRNA.

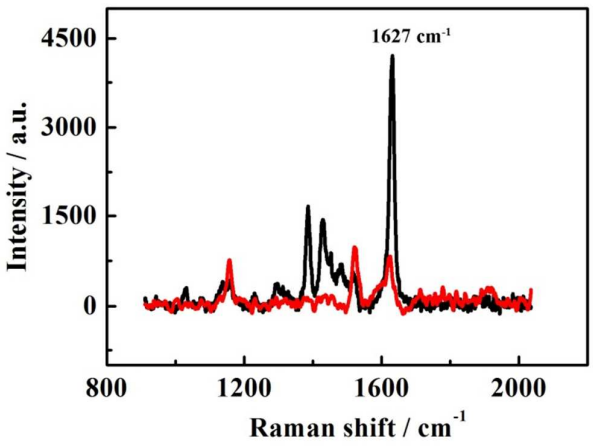


Figure 2. Raman characterization of different SERS substrates: DNA hydrogel before added R1 with status of "OFF" (red line); DNA hydrogel after added R1 with the status of "ON" (black line).

The capacity to open the DNA hydrogel of DSN enzyme was also explored in this experiment. We know that DSN can cleave the DNA/RNA duplex.[44] In Figure 3, black curve represented SERS spectrum after the DSN enzyme directly added into TB trapped DNA hydrogel, and the results indicated that no obvious Raman signal of TB

appeared. When the DSN were added in the DNA cycle process (c_{miRNA} = 100 fM) to produce abundant R1, the R1 can sensitively open the hydrogel releasing TB to obtain strong Raman signal (red curve). This phenomenon indicated that DSN only work in the cleave process to induce signal amplification, which cannot destroy DNA hydrogel.

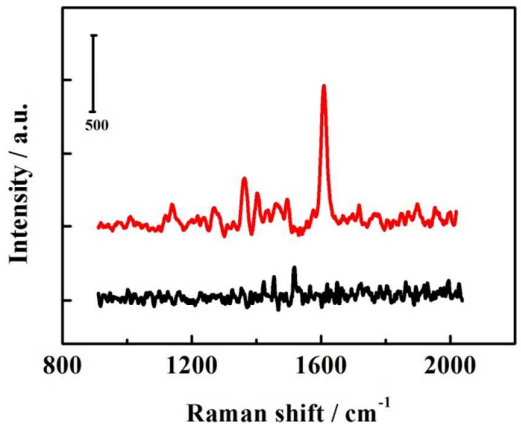


Figure 3. Capacity of DSN enzyme to open DNA hydrogel: DSN enzyme was directly added into DNA hydrogel with a weak Raman intensity (black curve); the products obtained from the DSN induced DNA cycle were added into DNA hydrogel with a strong Raman intensity (red curve).

Optimization of assay conditions

In order to develop the SERS platform with high sensitivity, several parameters such as the concentration of DSN and the incubation time of miRNA were investigated. With the increasing concentration of DSN, the Raman intensity at 1627 cm^{-1} reached the plateau at 0.05 U (Figure 4A) (c_{miRNA} = 100 fM). Therefore, 0.05 U DSN was chosen in the cleave-cycle process to obtain large amounts of R1. Figure 4B revealed the relationship between incubation time of miRNA 155 (c_{miRNA} = 100 fM)

and Raman intensity. When the incubating time is 40 min, the Raman intensity reached maximum which indicated that 40 min was the optimum condition.

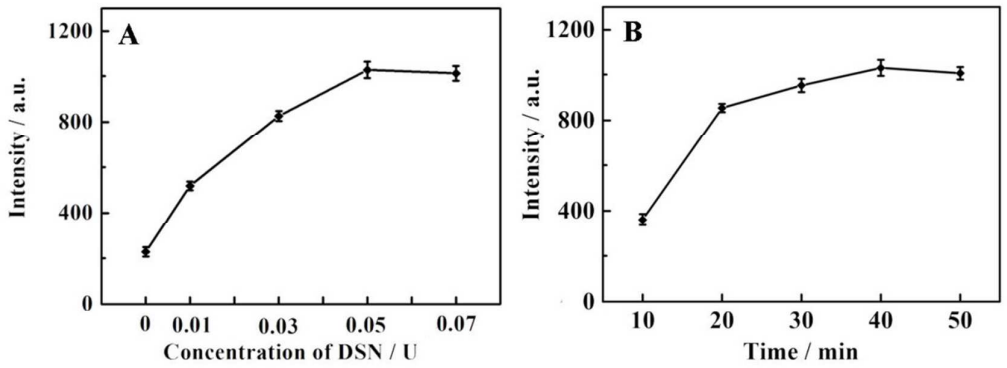


Figure 4. (A) Effects of concentration of DSN on the signal of SERS platform; (B) Influence of the incubation time of target miRNA on the signal of SERS platform. (c_{miRNA} 155 = 100 fM)

Analytical performance of the DNA hydrogel based-SERS platform for detection of miRNA 155

Based on the optimal conditions, the sensitivity and potentially quantitative application of the DNA hydrogel SERS platform were investigated. Different concentration of target miRNA were tested as shown in Figure 5A, which indicated that the intensity of Raman signals increased with the increasing concentration (0.1 fM to 100 pM) of miRNA. The corresponding linear relationship between the Raman intensity at 1627 cm^{-1} and the logarithm of miRNA 155 concentrations were shown in Figure 5B. The regression equation is $y = 133.6 \lg c + 95.09$ (where y is the Raman peak intensity at 1627 cm^{-1}, c is the concentration of miRNA 155) with a correlation coefficient square of 0.9956. The estimated limit of detection (LOD) was 0.083 fM, which was estimated by LOD = $3m/k$ (m was the standard deviation of the signal in

the blank solution and *k* was the slope of the corresponding calibration curve[45]). Herein, each concentration of miRNA was tested three times to calculate the error bar. In the meantime, this DNA hydrogel SERS platform was compared with other reports which focused on the detection of miRNA 155 (Table 2). From Table 2, our proposed SERS platform showed a higher sensitivity and wider linear range, which illustrated that it would have a wider application in various bioassays. The proposed method also compared with other SERS-based biosensors for detecting miRNA, and the results were shown in Table 3. The good performance of the DNA hydrogel SERS platform may be owing to the following reasons: (1) Lots of released DNAs were obtained through DSN enzyme induced circle amplification strategy which would greatly enhance the sensitivity of the SERS biosensor. (2) The DNA hydrogel switch controlled by target miRNA can release numerous TB to obviously amplify the Raman signal. (3) Ficus virens leaves combined AgNPs as sensitive and uniform SERS substrates can further improve the sensitivity for detecting miRNA 155.

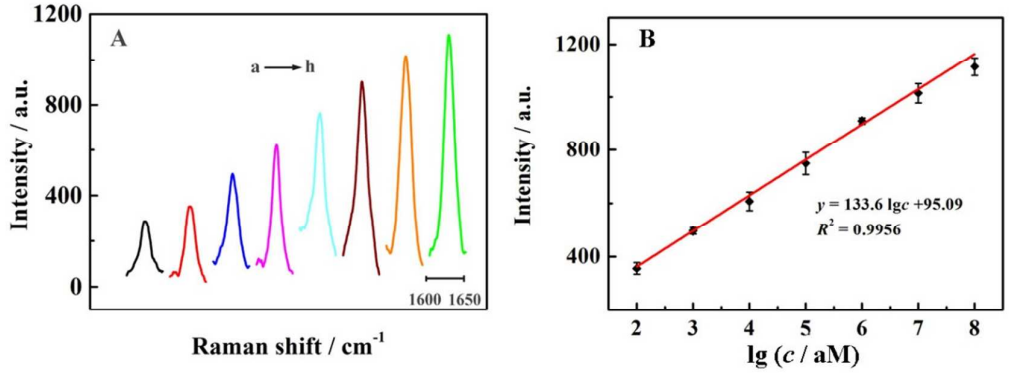


Figure 5. (A) SERS spectra of DNA hydrogel based-SERS platform after incubating miRNA 155 with concentration of (a to h): (a) 0, (b) 10^2, (c) 10^3, (d) 10^4, (e) 10^5, (f) 10^6, (g) 10^7, (h) 10^8 aM; (B) The calibration plot of Raman intensity at 1627 cm^{-1} *vs* lg*c*.

Table 2. The comparison of the proposed DNA hydrogel based SERS platform with other biosensors for miRNA 155 detection.

Analytical method	Detection limit	Linear range	refs
DPV	0.6 fM	2 fM-8 pM	46
Fluorescent	18 pM	0.02 nM-10 nM	47
SWV	12 fM	50 fM-30 pM	48
ECL	0.83 fM	2.5 fM-50 pM	49
Fluorescent	100 pM	0.1 nM-200 nM	50
Nanopore	0.1 pM	0.1 pM-100 pM	51
Amperometric	0.14 fM	1 fM-100 pM	52
SERS	0.083 fM	0.1 fM-100 pM	This work

Table 3. The comparison of the proposed SERS-based platform with other SERS biosensor for miRNA detection.

Detection method	Detection Target	Detection limit	Linear range	Refs
SERS	miRNA 203	6.3 fM	10 fM -100 n M	53
SERS	Let-7b	0.3 fM	1 pM - 10 nM	54
SERS	miRNA21	5 pM	15 pM - 60nM	55
SERS	miRNA 21	0.3 fM	0.1 fM-100 pM	56
SERS	miRNA 155	0.083 fM	0.1 fM-100 pM	Our work

Reproducibility and uniformity of the DNA hydrogel based SERS platform

In order to investigate the reproducibility and uniformity of the SERS platform, the coefficient of variation of the proposed SERS strategy was investigated. Here, 25 different points of SERS spectra were collected on a SERS platform with the peak intensity at 1627 cm^{-1} ($c_{miRNA155}$ = 100 fM). From Figure 6A we can see that the 25 different spectra were basically consistent, especially the peak at 1627 cm^{-1}. And from

Figure 6B, a coefficient of variation of the intensity at 1627 cm^{-1} was 5.58% which indicated this DNA hydrogel based SERS platform own good uniformity. At the same time, 5 different SERS substrates were separately detect miRNA ($c_{miRNA155}$ = 100 fM) to prove the good reproducibility of SERS substrates with a RSD = 2.41%, and the results were shown in Figure 6 (C and D).

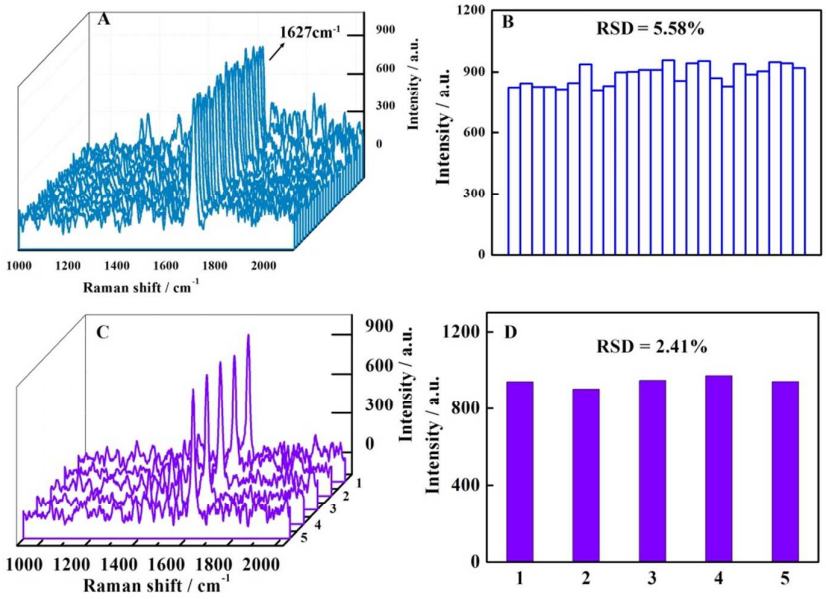


Figure 6. (A) SERS spectra measured from 25 different spots on a single substrate; (B) Corresponding histogram for the peak intensity at 1627 cm^{-1} ($c_{miRNA\ 155}$= 100 fM); (C) SERS spectra measured from 5 different substrates; (D) Corresponding histogram for the peak intensity at 1627 cm^{-1} ($c_{miRNA\ 155}$= 100 fM).

Selectivity of the DNA hydrogel based SERS platform

To investigate the selectivity of the DNA hydrogel based SERS platform, different possible interferences were assessed. Here, 100 pM one-base mismatched DNA, miRNA 21 and miRNA 141 were separately tested with this SERS platform and the corresponding Raman intensity was similar with the blank sample. However,

when 100 fM target miRNA 155 were coexisted with those interferences, the Raman intensity was almost the same with that solution only contain miRNA 155. All these results as shown in Figure 7 demonstrated that this DNA hydrogel based SERS platform possessed high specificity for miRNA 155 assay.

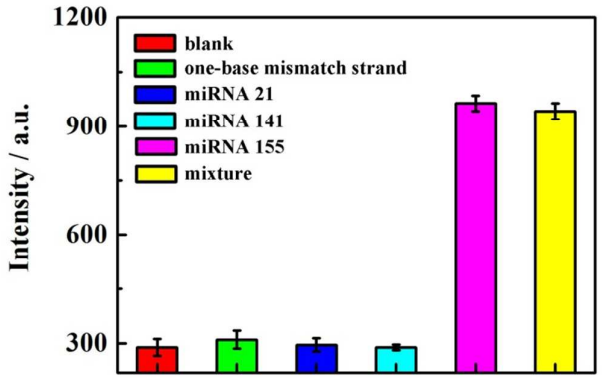


Figure 7. Selectivity of the proposed DNA hydrogel based-SERS platform. The concentration of one-base mismatched strand, miRNA 21 and miRNA 141 were 100 pM. The mixture contain one-base mismatched strand (100 pM), miRNA 21 (100 pM), miRNA 141 (100 pM) and miRNA 155 (100 fM).

Application of the DNA hydrogel based SERS platform

To monitor the reliability of the proposed DNA hydrogel based SERS platform, recovery experiments were carried by adding various concentration of miRNA 155 into the 100-fold diluted healthy human real serum sample (acquired from Xinqiao Hospital of Third Military Medical University, China). Good recoveries were obtained from 97.39% to 107.8% and the RSD values from sample 1 to 5 were shown in Table 4, which indicated the potentiality of this SERS platform for miRNA 155 detection in clinical applications.

Table 4. Recovery results of the proposed method in human serum.

Sample number	Added / fM	Found / fM (n = 3)	Recovery / %	RSD / %
1	1	1.001	100.1	1.8
2	10	10.71	107.1	2.6
3	100	100.6	100.6	1.7
4	1000	1077.5	107.8	0.8
5	10000	97387	97.39	1.6

Conclusions

In summary, a novel switchable DNA hydrogel SERS platform was proposed for sensitive detection of miRNA 155 with DSN enzyme cleaved amplifying strategy. At first, 3D DNA-switchable hydrogel structure can trap toluidine blue inside with the status of "OFF". In the presence of released DNA which was associated with the amount of target miRNA, the 3D structure was destroyed, releasing toluidine blue to produce strong Raman signal ("ON"). By this way, the label-free DNA hydrogel amplification method can improve the sensitivity of SERS platform. And this DNA hydrogel switch can be indirectly controlled by miRNA 155, which lead to quantitative detection of miRNA 155. What's more, DSN enzyme can specifically cleave RNA-DNA hydrid duplexes to release miRNA for inducing the next cycle to obviously enhance the sensitivity of this SERS platform. In addition, flexible ficus virens leaves were chosen as environmental, economic and also uniform substrate to combine with AgNPs for sensitive detection of miRNA 155. In view of these advantages, the proposed SERS platform achieved the detection limit of 0.083 fM and the detection range from 0.1 fM to 100 pM which provide hopes of applying in clinical applications.

Acknowledgements

This project has been financially supported by the NNSF of China (21675129，21575116, 51473136), the Fundamental Research Funds for the Central Universities, China (XDJK2017D049), China Postdoctoral Science Foundation (2015M572427) and Chongqing Postdoctoral Research Project (xm2015019).

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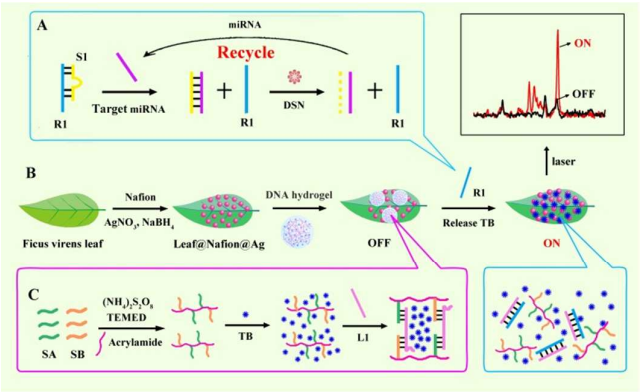
TOC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