Bioconjugate Chemistry

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Preparation of Aptamer Responsive DNA Functionalized Hydrogels for the Sensitive Detection of α-Fetoprotein Using SERS Method

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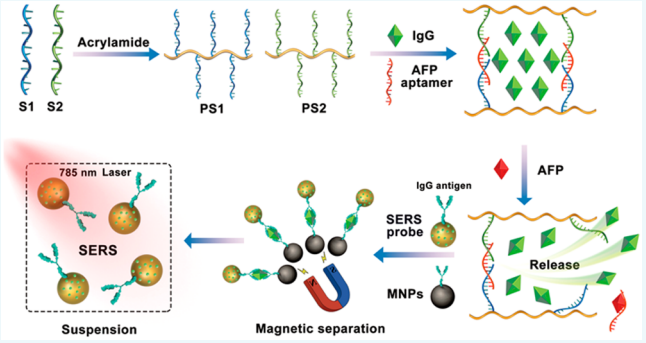
Cite This: *Bioconjugate Chem.* 2020, 31, 813–820 Read Online

ABSTRACT: Hepatocellular carcinoma (HCC), which is one of the three major cancers, has attracted growing attention due to its high mortality, health care cost, and circumscribed therapeutic methods. Hence, the development of a fast, accurate, and flexible method to detect α-fetoprotein (AFP), the specific marker of HCC, is significant for diagnosis and treatment of cancer. Here, we constructed a novel SERS biosensing platform combining the target-responsive DNA hydrogel for the sensitive detection of AFP. The linker strand in DNA hydrogel is an aptamer that can specifically recognize AFP and accurately control the release of immunoglobulin G (IgG) encapsulated in hydrogel. In the presence of AFP, the hydrogels were disentangled and the IgG was released. Thereafter, the released IgG was captured by SERS probes and biofunctional magnetic beads through formation of sandwich-like structures, resulting in the signal of Raman tags decreasing in the supernatant after magnetic separation. Due to the ultrahigh sensitivity of the SERS biosensor, the proposed method has a wide detection linear range (50 pg/mL to 0.5 μg/mL) and a detection limit down to 50 pg/mL. Moreover, the sequence of the linker strand in the DNA hydrogel can be specifically encoded into a new aptamer that responds to other cancer markers. This convenient and inexpensive detection method provides a new strategy for the detection of tumor markers.



INTRODUCTION

α-Fetoprotein (AFP) is an embryo-derived glycoprotein,[1] which is an important biomarker for fetal defects and hepatocellular carcinoma (HCC).[2] The concentration of AFP in human serum is related to the stage of HCC.[3] It is usually produced by the liver during fetal development and decreases after birth.[4] The concentration of AFP is barely detectable with a concentration less than 25 ng/mL in the serum of healthy adults.[5] However, AFP concentration in the serum of patients with various neoplastic diseases is significantly higher than normal levels, especially in nearly 75% of patients with liver cancer (HCC), where the concentration of AFP is significantly increased to 500 ng/mL.[6] Therefore, it is in great demand to develop a rapid, sensitive, reliable, and low-cost platform to detect AFP.

In recent years, various assays have been applied to detection of AFP, such as enzyme-linked immunosorbent assay,[7] fluorescence immunoassay,[8] electrochemical assay,[9,10] and surface plasmon resonance (SPR).[11] Not only did all these methods obtain reliable results for AFP detection, but they also generally require complicated sample pretreatment and dedicated equipment with high cost and special technical skills. Surface-enhanced Raman spectroscopy (SERS) is considered as an attractive tumor marker detection and analysis technology for its advantages of convenient operation, high sensitivity, fast detection speed, and good reproducibility. However, most traditional SERS assays are based on the reaction between antigen and antibody of biomarker,[12−15] which requires expensive antibodies due to the long experimental period and complicated procedures. In order to develop a low-cost, convenient, rapid, and sensitive detection method, some specific aptamers have introduced into the detection systems in recent years. Aptamers are a series of short single-stranded DNA or ribonucleic acid molecules with high affinity, specificity, and selectivity.[16,17] They can recognize and combine various target molecules such as target proteins,[18] polypeptides,[19] metal ions,[20] and even living cells[21] by its different secondary or tertiary structures. Meanwhile, compared with AFP antibody, AFP aptamer has the advantages of cheap and easy availability, stability, easy storage, and nonimmunogenicity. Therefore, we replaced antibodies with aptamers for specific recognition of AFP in this paper.

As a new biocompatible and intelligent-responsive material, DNA hydrogel has been widely used in the field of the biosensing system and biomedical analysis.[22−26] For instance, the DNA hydrogel encapsulating Raman dyes for constructing

Received: December 27, 2019
Revised: January 24, 2020
Published: January 24, 2020

https://dx.doi.org/10.1021/acs.bioconjchem.9b00874
Bioconjugate Chem. 2020, 31, 813–820

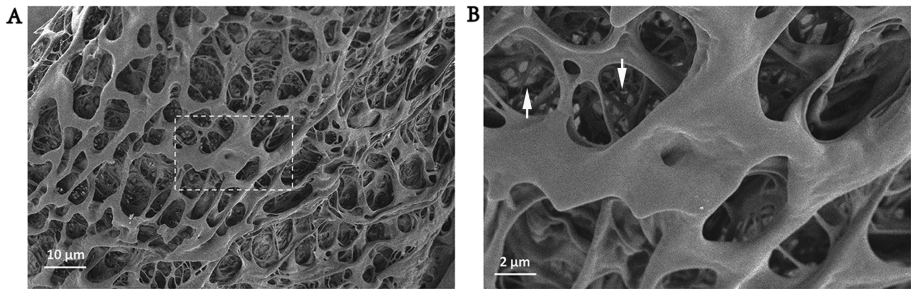
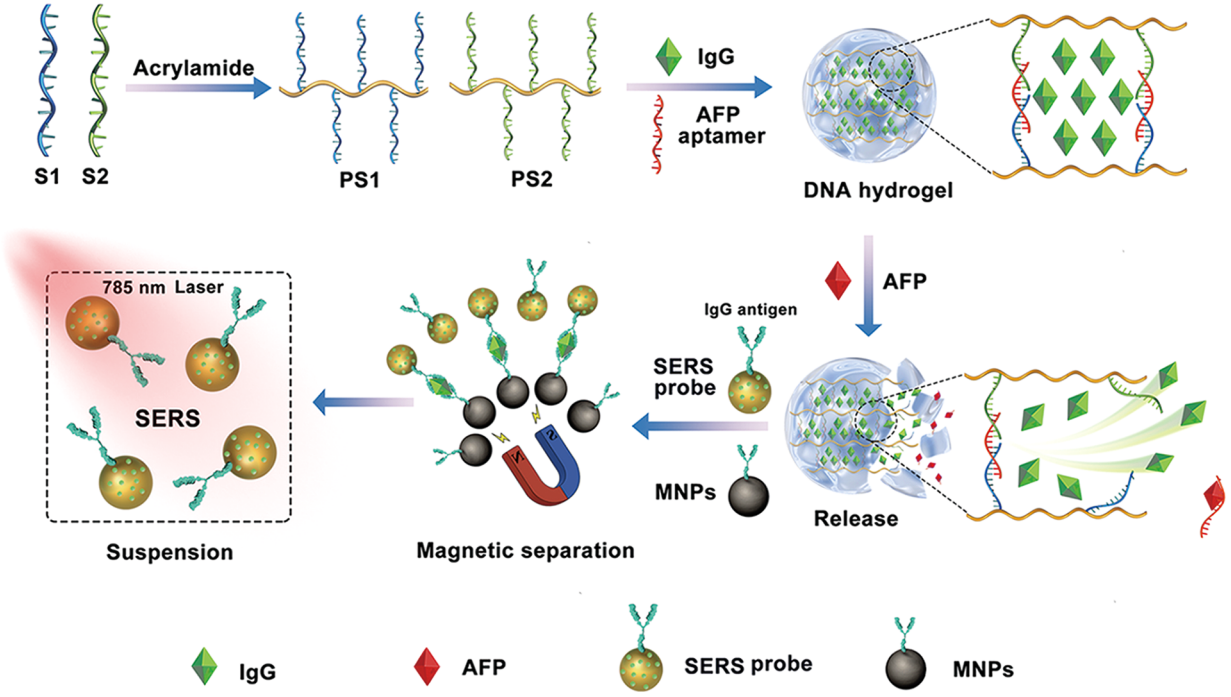


Figure 1. SEM image of the freeze-dried hydrogels with different magnification.

Scheme 1. Working Principle of the AFP-Responsive DNA hydrogel[a]



[a]IgG was embedded in the hydrogel, after introducing AFP, the hydrogel dissociated and IgG released, leading to a change of Raman signal in the supernatant.

a sensitive SERS biosensor to detect miRNA 155 was proposed by Chai's research group,[27] and Yang's group reported a target-responsive hydrogel for visual quantitative detection of ochratoxin A.[28] These studies are attributed to the excellent properties of gel. In other words, DNA hydrogel are networks of DNA strands cross-linked in an aqueous solution. It not only maintains the original biological characteristics of DNA, such as biocompatibility, molecular recognition, and specific coding, but also has the stability and encapsulation ability of gel. DNA hydrogel combines biomaterial and biotechnology well. By introducing specific groups or DNA sequences into DNA hydrogels, the functions of DNA hydrogel can be further improved and achieve intelligent and sensitive responses to different stimuli, such as pH,[29,30] temperature,[31] light,[32] and biomolecules.[33] Therefore, as an intelligent responsive material with good flexibility and stability, DNA hydrogel can be used in the detection of small molecules,[34] nucleic acids,[35] proteins,[36] enzymes,[37] metal ions[38−40] (copper ions, silver ions, mercury ions), and other substances. Based on these advantaged properties of DNA hydrogels, target responsive hydrogels with the simple assembly for sensitive detection of AFP has been synthesized.

Herein, a novel SERS biosensing platform combining aptamer-responsive DNA hydrogel was designed and constructed for the sensitive detection of AFP. First, the IgG was embedded by utilizing the encapsulation capability of DNA hydrogel. In the presence of AFP, AFP-aptamer can specifically recognize AFP and form a target−aptamer complex, which leads to the dissociation of reticular structure of hydrogels and the release of IgG embedded in the hydrogels. Then, the released IgG combined with the antibody attached on the nanoprobes and capture probes. Finally, the Raman signal in the supernatant is changed after magnetic separation, and the low-cost, rapid, sensitive detection of AFP is achieved. With this approach, the limit of detection of AFP down to 50 pg/mL and the wide detection linear range from 50 pg/mL to 500 ng/mL are obtained. Since it is easy to change the sequence of the DNA strand constituting hydrogels, the hydrogel can be modified into other targeted responsive hydrogels as long as the linker strand can be specifically identified with the target tumor markers. Therefore, this strategy is so versatile and convenient that it can be applied to the detection of a variety of tumor markers.

https://dx.doi.org/10.1021/acs.bioconjchem.9b00874
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RESULT AND DISCUSSION

Characterization of DNA Hydrogels. The sizes of AuNPs were characterized by transmission electron microscope (TEM) and UV−vis spectrometer. It can be clearly seen from Figure S1 that the diameter of AuNPs is about 30 nm. As shown in Figure 1, the surface morphology of DNA hydrogels was characterized by scanning electron microscopy (SEM). In Figure 1A, we can clearly observe the dense pore structure of the lyophilized hydrogels. The results indicate that it is a porous, dense three-dimensional network polymer with high cross-linking property. In Figure 1B, the IgG embedded in the DNA hydrogel can be observed.[41] (Before SEM imaging, the prepared DNA hydrogels need to thoroughly freeze-dried under vacuum and sprayed with gold to improve conductivity.) To validate the formation of AFP-responsive DNA hydrogels, the three complementary DNA strands are analyzed by native PAGE in Figure S2.

Principle of SERS Biosensor Based the AFP-Responsive DNA Hydrogels. Scheme 1 showed the process of the DNA hydrogel-based SERS biosensing platform for AFP detection in details. Two short DNA sequences (S1 and S2) are grafted onto linear polyacrylamide polymers by copolymerization of acrylic acid DNA and acrylamide monomers to form polymer chains PS1 and PS2. The linker strand is complementary to S1 and S2 in the adjacent region. After adding the linker strand, PS1 and PS2 are cross-linked with each other to form hydrogels, and the IgG added in advance is embedded in hydrogels stably. Note that the linker strand in the hydrogels is AFP-aptamer, which can be specifically identified by AFP. In the absence of AFP, IgG was stably embedded in hydrogels, the SERS probe signals in the supernatant were not affected and presented a strong original signal (black curve in Figure 2). In the presence of AFP

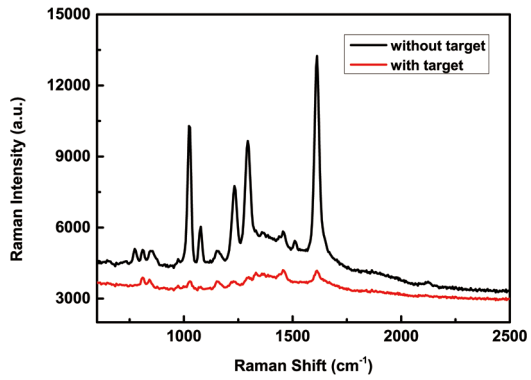

Figure 2. Feasibility of AFP-responsive DNA hydrogel for detection of AFP. Comparison of the Raman signal in the presence (red line) or absence (black line) of AFP.

combined with the linker strand of hydrogels to form a complex, which caused the hydrogels to rupture and release a large amount of IgG. The released IgG is captured with the antibody conjugated on the SERS probes and the magnetic capture probes to form the sandwich-like structures. After magnetic separation, the signal of SERS probes remaining in the supernatant decreased and presented a weak signal (red curve in Figure 2).

Due to the concentration of AFP being related to the amount of IgG released, the higher the concentration of AFP added, the more IgG released to attach both the SERS probes and the magnetic capture probes simultaneously. After magnetic separation, the residual SERS probe signals in the supernatant became weaker. Thus, the concentration of AFP is negatively related to the Raman signal of SERS probes within a certain linear range. The peak at 1612 cm^{-1} was chosen as the standard to quantitatively detect AFP. The linear detection range of this SERS detection platform for AFP can reach 50 pg/mL to 500 ng/mL.

Optimization of SERS Assay Condition. In order to obtain high sensitivity, reproducibility, and selectivity of the detection results, the volume and concentration of IgG encapsulated in DNA hydrogel and the incubation time need to be optimized. When the DNA hydrogel could be preliminarily synthesized, 2, 4, 6, 8, 10, 12, 14, and 16 μL of the antigen were embedded in DNA hydrogel, respectively. It was found in Figure S3 that the linear range of the responsive signal was too narrow if the volume of the embedded antigen was too small, which is not convincing in the experiments. As shown in Figure S4, if the volume of the embedded antigen was too large, the degree of cross-linking was too low to be stable. After slightly adjusting the proportion of various reagents for synthesizing DNA hydrogel, it was verified that the best degree of cross-linking in the DNA hydrogel could be obtained by adding 10 μL of antigen. After determining the antigen volume of 10 μL, different concentrations of antigen were incubated with SERS tags and capture probes in the experiments of magnetic separation. As shown in Figure 3A, the signal of SERS probes remaining in the supernatant was lower after magnetic separation with the increase of antigen concentration. When the concentration of antigen was 0.5 mg/mL, the signal decreased drastically and almost could not be detected. It indicated that almost all antigens were attached with the antibody labeled on the nanoprobe; thereby, the widest linear range was obtained. Therefore, we chose 10 μL and 0.5 mg/mL as the optimal volume and concentration for the IgG embedded in DNA hydrogel. Figure 3B showed that the signal remained unchanged when the incubation time between IgG and antibody was more than 2 h, which suggested that the optimal incubation time was about 2 h. (The error bar represents the standard deviation of three measurements.)

Performance of SERS Biosensor Based the AFP-Responsive DNA Hydrogels. After optimizing the experimental conditions, AFP with different concentrations was added to the hydrogels, and the results are shown in Figure 4A. As the concentration of AFP increases (50 pg/mL to 0.5 μg/mL), the DNA hydrogel is dissociated and Raman signal is decreased. Theses result can also be verified by the visual method (Figure S5). In the process of quantitative analysis, the experimental data were analyzed by using the intensity ratio of 1612 cm^{-1} (the characteristic Raman peak of DP) to 520 cm^{-1} (the Raman peak of silicon wafer). The experiments were repeated six times with the same concentration of AFP to calculate the error bar. Figure 4B shows a good linear relationship between the value of I_{1612}/I_{520} and the logarithm of AFP concentration. The regression equation is $y = 5.728 - 1.029x$, and the square of correlation coefficient (R^2) is 0.9769.

To investigate the reproducibility and stability of this SERS platform, we collected points of SERS spectra at 1612 cm^{-1} 15 times (c_{AFP} = 100 pg/mL). According to Figure 4C, these ten different SERS curves are basically consistent, especially the peak at 1612 cm^{-1} with a relative standard deviation (RSD) of 5.201% (Figure 4D).

https://dx.doi.org/10.1021/acs.bioconjchem.9b00874
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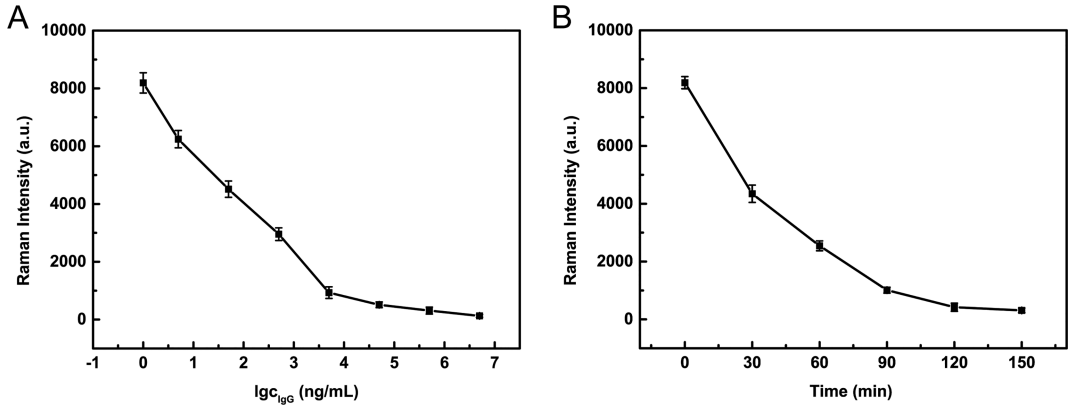

Figure 3. (A) Effects of concentration of the embedded IgG on the signal of SERS platform. (B) Influence of the incubation time of IgG immunoreaction on the signal of SERS platform.

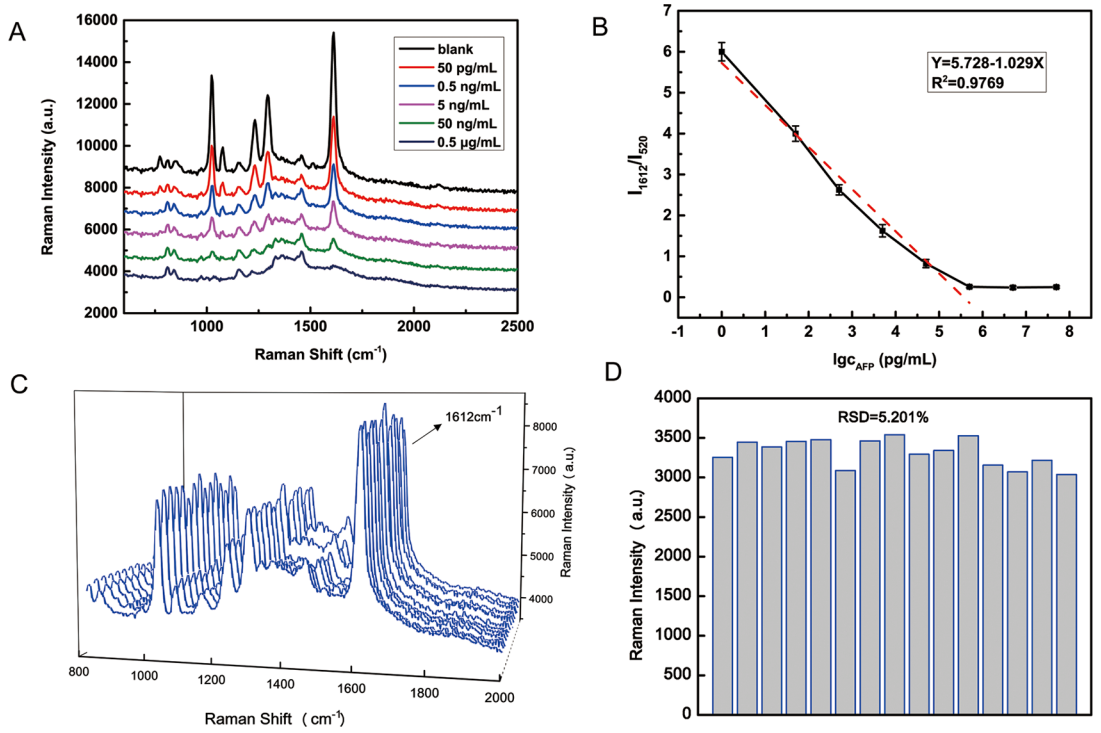

Figure 4. Performance of the SERS platform for the detection of AFP. (A) SERS spectra of this detection platform collected with various concentrations of AFP. (B) Linear relationship between the value of I_{1612}/I_{520} and the logarithm of AFP concentration. (Error bars indicate the standard deviations experiments repeated six times.) (C) SERS spectra measured at spots of 1612 cm^{-1} 15 times (c_{AFP} = 100 pg/mL). (D) Corresponding histogram for the RSD of peak intensity at 1612 cm^{-1}.

Moreover, the responsive DNA hydrogel SERS biosensing platform was compared with other methods of AFP detection (Table 1).[42−45] In terms of detection limit, this SERS platform covered the range of AFP concentration in serum from healthy adults to patients with advanced liver cancer (25 ng/mL to 500 ng/mL). Compared with other detection methods, it has the advantages of wider linear range, lower cost, and good reproducibility. Thus, this SERS platform has broader application prospects in various types of biological detection.

Table 1. Comparison of This SERS Platform with Other Methods of AFP Detection

analytical method	detection limit	linear range	ref
surface plasmon resonance	0.65 ng/mL	1.0−200 ng/mL	42
fluorescent	0.474 ng/mL	10−100 ng/mL	43
electrochemical	0.003 ng/mL	0.01−100 ng/mL	44
SERS	0.5 ng/mL	0.5−1000 ng/mL	45
SERS	50 pg/mL	0.05−500 ng/mL	This work

Selectivity of SERS Biosensor Based the AFP-Responsive DNA Hydrogels. In order to evaluate the specificity of the SERS platform under the same experimental conditions, IgG, CEA, PSA, and Thrombin were served as the four parallel groups in our experiments. Additionally, there were another series of control experiments without adding any antigen, only the signal probes and capture probes remaining in the solution. As shown in Figure 5, only samples with AFP showed significant decrease in Raman signal, while the samples

https://dx.doi.org/10.1021/acs.bioconjchem.9b00874
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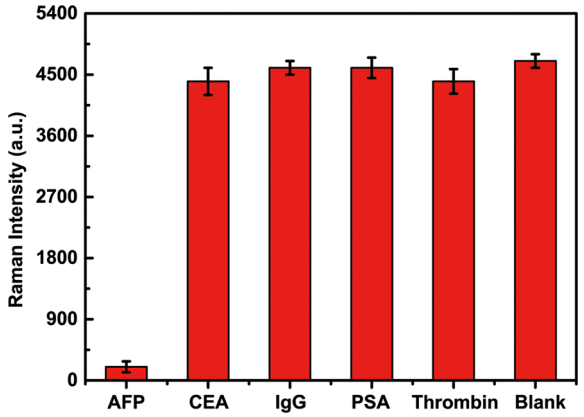

Figure 5. Selectivity of the SERS platform for detection of AFP and another antigen.

with IgG CEA, PSA, and thrombin have no significant change (error bar was calculated by 3× detection). The results were consistent with the colorimetric method which was shown in Figure S6. The linker strand in the hydrogel can only specifically identify AFP; therefore, the SERS biosensing platform has excellent selectivity and sensitivity for AFP detection.

Application of SERS Biosensor Based on the AFP-Responsive DNA Hydrogels in Real Sample. In order to verify the reliability and accuracy of the SERS platform based on DNA hydrogel in the real samples, we performed AFP detection of human serum samples, which can be regarded as a simple simulation of the real samples. First, serum samples were centrifuged and diluted to reduce the matrix effect, and then AFP with different concentrations was added to serum samples. Finally, the AFP in the serum was detected on this SERS platform. The recovery rates and RSD of AFP with different concentrations were calculated and shown in Table 2.

Table 2. Recovery of the AFP of This SERS Platform in Human Serum

sample number	theoretical values (I_{1612}/I_{520})	measured values (I_{1612}/I_{520})	recovery (%)	RSD (%)
1	5.728	6	104.7	0.9
2	3.980	4	100.5	1.1
3	2.951	2.625	89.0	1.7
4	1.922	1.625	84.5	1.4
5	0.893	0.825	92.4	0.9

The recovery rate of the SERS platform is between 84.5% and 104.7%, and RSD is between 0.9% and 1.7%, which exhibits good repeatability. These results show the application potential of DNA hydrogel-based target-responsiveness of AFP detection in actual sample.

CONCLUSION

In summary, a method based on SERS and target-responsive DNA hydrogel is proposed in this paper for sensitive detection of AFP, a tumor marker of liver cancer. In this method, we designed a DNA hydrogel that specifically responds and binds to AFP. With the excellent encapsulation properties of the hydrogel, the easily available IgG was embedded in them. Since the dissociation of hydrogel is directly controlled by AFP, the method can be used for quantitative detection of AFP and the detection limit down to 50 pg/mL. The released IgG combined with the corresponding antibody on the nanoprobes, resulting in a good linear range (50 pg/mL to 0.5 μg/mL) of Raman signal changes in the supernatant. At the same time, the combination of AFP and aptamer replaced the immune response of AFP and antibody, which greatly reduced the cost of the experiment. In addition, the aptamer sequences of DNA hydrogel can be specifically encoded, so it was easy to design a new DNA hydrogel that responded to other antigens. In view of these advantages, the SERS biosensing platform proposed in this paper has a wider application range and provides a new strategy for biochemical analysis. Moreover, the concentration of AFP can be easily and intuitively read out by the colorimetric analysis in this paper, which also has great application prospects in rapid analysis and portable detection.

EXPERIMENTAL SECTION

Materials and Reagents. 4,4′-Dipyridyl (DP), trisodium citrate, bovine serum albumin (BSA), and PrEST Antigen AFP were purchased from Sigma-Aldrich, USA. Ammonium persulfate ($H_8N_2S_2O_8$), *N*-hydroxysuccinimide (NHS), NaCl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and carboxyl-functionalized magnetic beads (MBs, average diameter 200−300 nm) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Chloroauric acid ($HAuCl_4$) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 30%Acr-Bis (29:1) was purchased from Beijing Labgic Technology Co., Ltd. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was purchased from Leagene Biotechnology. (Beijing, China). Serum samples from healthy humans were purchased from Amyjet Scientific Inc. complying with the rules of the local ethical committee. The DNA strands were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). All the DNA sequences were purified by HPLC and confirmed by mass spectrometry. According to the previous report,[46] we designed the aptamer sequences as follows (5′−3′):

Acrydite-modified DNA (S1): GATTGGTTCGGACGG-ACAGGACCCACATCAAGACCG

Acrydite-modified DNA (S2): CGAGAACTGCACCTG-AGTCAGCGTTAGGAGCGTCAC

AFP-aptamer: GTGACGCTCCTAACGCTGACTCA-GGTGCAGTTCTCGACTCGGTCTTGATGTGGGT-CCTGTCCGTCCGAACCAATC

The following buffer solutions were used in this study: Borate buffer (BB, pH 8.2), Phosphate-buffered saline (PBS, pH 7.4), and Tris-EDTA buffer (TEB, pH 8.0). The ultrapure water was collected by Millipore water purification system (≥18 MΩ cm resistivity); the other chemical reagents were all of analytical grade or better.

Apparatus. The DNA hydrogels are characterized by a scanning electron microscope (SEM, Zeiss Merlin, Germany). The gold nanoparticles (AuNPs) are characterized by a transmission electron microscope (TEM, JEM-2100HR, Japan) and UV−vis spectrometer (UV-6300, MAPADA, China). Raman spectra were collected from a microscopic Raman spectrometer. The excitation laser (785 nm) was powered from a semiconductor laser (BW & TEK U.S.) with the energy of 400 mW approximately at the sample location; the typical accumulation time was 1 s. Before SERS measurement, the Raman spectra were calibrated by a silicon wafer at 520 cm^{-1}.

Synthesis of AuNPs. The synthesis methods of 30 nm AuNPs refer to the previous literature.[47,48] In brief, magnetic

https://dx.doi.org/10.1021/acs.bioconjchem.9b00874
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stir-bars and glass containers were immersed in aqua regia for 30 min and rinsed thoroughly with ultrapure water. 100 mL of 1 mM $HAuCl_4$ aqueous solution in a three-necked flask was heated to boiling, and then 6 mL of 38.8 mM fresh trisodium citrate solution was added rapidly with vigorous stirring for 20 min. The color of the boiling solution changed from faint yellow to colorless, and became deep red quickly. Then, the solution was stirred continuously until it was cooled down to room temperature. In the resulting red solution, the size of AuNPs was about 30 nm in diameter.

Modification of Nanoparticles. *Preparation of SERS Probes.* First, 4,4′-dipyridyl (DP) molecules, as Raman signal reporter, were attached on the AuNPs. 30 μL DP (0.01 mM) was added to 1.0 mL 30 nm AuNPs colloid, amd then the mixture was gently shaken for 15 min. The DP-labeled AuNPs were separated from the solution by centrifugation at 8000 rpm for 12 min and resuspended with 1.0 mL borate buffer (BB, 10 mM, pH = 8.2). After that, 10 μL of IgG antibody (50 μg/mL) was added to the DP-labeled mixture and incubated at room temperature for 2 h. The AuNP colloids were labeled with DP and antibodies were purified by centrifugation at 8000 rpm for 10 min and resuspended with 1.0 mL of BB (pH 8.2). To make sure there were no bare sites on these nanoprobes, 30 μL of BSA (2.5%) was added into the above mixture and incubated at room temperature for 1 h. After centrifugation with 8000 rpm for 8 min and resuspension with 1.0 mL of Tris-EDTA buffer (TEB, pH 8.0), the ultimate SERS nanotag solution was obtained. These SERS nanotags should be stored at 4 °C to retain biological activity and stability.

Preparation of Capture Probes. The synthesis principle of the capture probe can be thought of as the formation of an amide bond between the carboxyl-functionalized magnetic beads (MBs) and the antibody containing amino group.[49] First, 200 μL of carboxyl-functionalized MBs were washed 2−3 times to reduce the matrix effects and then dispersed with 1 mL PBS (pH 7.4). Subsequently, 30 μL EDC (4 mg/mL) and 10 μL NHS (1 mg/mL) as coupling reagent were incubated with the MBs for 30 min with mild shaking. The activated MBs were separated by a magnet and redispersed in PBS (pH 7.4). Then, 20 μL of 50 μg/mL anti-IgG was added to the activated functional MBs to incubate for 2 h under continuous stirring. After redispersion in 1 mL PBS, 30 μL of 2.5% BSA was added in the MBs to block bare sites for 1 h. After separation by a magnet and washing twice with 1.0 mL of TEB (pH 8.0), the ultimate capture probes were produced. (All dosage and experimental conditions have been optimized.)

Preparation of the AFP-Responsive DNA Hydrogels. 4% acrylamide, 1× Tris-EDTA buffer (TEB, pH 8.0), 200 mM NaCl, and ultrapure water made up the stock solutions. 10 μM short DNA strands S1 and S2 in the stock solutions were kept in a vacuum desiccator with pump for 10 min to remove the air separately. After that, the freshly prepared initiator (0.05 g ammonium persulfate dissolved in 0.5 mL H_2O) and catalyst (25 μL TEMED dissolved in 0.5 mL H_2O) were added to the stock solutions at a volume ratio of 0.014:1 in vacuum desiccators. After 20 min polymerization reaction, polymer strand 1 (PS1) and polymer strand 2 (PS2) were obtained. Afterward, PS1, PS2, and 0.5 mg/mL IgG were fully mixed for 10 min in vacuum desiccators. At last, 10 μM of linker strands (AFP-aptamer) were added and incubated for 10 min to form AFP-responsive DNA hydrogels. After washing twice with 1.0 mL of TEB (pH 8.0) to remove the redundant IgG, the ultimate DNA hydrogel was obtained.

Assay Process. Different concentrations of AFP were added in the DNA hydrogel with gentle shaking at room temperature for 10 min, the linker strands combined with AFP and dissociated from the DNA hydrogel, and the embedded IgG released. Then, 100 μL of SERS probes and 100 μL of capture probes were added in the above mixture and incubated for 2 h. After separation with a magnet, the residual SERS probes in the supernatant was measured by 785 nm laser to achieve detection of the AFP.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.9b00874.

Additional experimental figures and procedures—TEM image, PAGE spectra, UV−vis spectra, colorimetric assay (PDF)

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Notes

The authors declare no competing financial interest.

https://dx.doi.org/10.1021/acs.bioconjchem.9b00874
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■ ACKNOWLEDGMENTS

This research has been supported by National Natural Science Foundation of China (NSFC) (Grant nos. 21273083 and U1732146) and the Project under Scientific and Technological Planning by Guangzhou City (Grant nos. 201805010002).

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