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Ultrasensitive CRISPR/Cas12a-Driven SERS Biosensor for On-Site Nucleic Acid Detection and Its Application to Milk Authenticity Testing

Ruiyuan Pan, Jianghua Liu, Panxue Wang, Di Wu, Jian Chen, Yongning Wu, and Guoliang Li*

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ABSTRACT: An ultrasensitive surface-enhanced Raman scattering (SERS) biosensor driven by CRISPR/Cas12a was proposed for on-site nucleic acid detection. We tactfully modified single-strand DNA (ssDNA) with a target-responsive Prussian blue (PB) nanolabel to form a probe and fastened it in the microplate. Attributed to the specific base pairing and highly efficient trans-cleavage ability of the CRISPR/Cas12a effector, precise target DNA recognition and signal amplification can be achieved, respectively. In the presence of target DNA, trans-cleavage towards the probe was activated, leading to the release of a certain number of PB nanoparticles (NPs). Then, these free PB NPs would be removed. Under alkali treatment, the breakdown of the remaining PB NPs in the microplate was triggered, producing massive ferricyanide anions ($Fe(CN)_6^{4-}$), which could exhibit a unique characteristic Raman peak that was located in the "biological Raman-silent region". By mixing the alkali-treated solution with the SERS substrate, Au@Ag core−shell NP, the concentration of the target DNA was finally exhibited as SERS signals with undisturbed background, which can be detected by a portable Raman spectrometer. Importantly, this strategy could display an ultralow detection limit of 224 aM for target DNA. Furthermore, by targeting cow milk as the adulterated ingredient in goat milk, the proposed biosensor was successfully applied to milk authenticity detection.

KEYWORDS: *nucleic acid detection, surface-enhanced Raman spectroscopy, CRISPR/Cas12a, biosensor, food authenticity, goat milk*

INTRODUCTION

Nucleic acids, which are responsible for storing and transmitting the genetic information of life, have been utilized as a unique marker in various fields.[1] For example, the detection of miRNA is significant for cancer screening at an early stage, and the identification of pathogenic bacteria genes is important for food safety monitoring.[2−4] Therefore, developing efficient, sensitive, and on-site strategies for nucleic acid detection is critical to human health. Conventional methods for nucleic acid detection are basically polymerase chain reaction (PCR) methods, such as common PCR, quantitative PCR (qPCR), etc.[5,6] These PCR methods are equipped with high selectivity, specificity, and sensitivity; however, the demands for sophisticated equipment and strict laboratory environment preclude their deployment in the field.[7,8] Notably, the surface-enhanced Raman scattering (SERS) technique, an effective vibrational spectroscopic technique for quantitative analysis,[9] has attracted tremendous attention. Attributed to its advantages, including a portable device, convenient operation, and ultrahigh sensitivity, the SERS technique is qualified enough to work as an on-site detection tool.[10] Nonetheless, due to the complexity of the biological sample, strong background interference becomes the main limitation for the SERS technique to be applied in nucleic acid detection. Moreover, as far as we know, there is less research directly utilizing the SERS technique to analyze DNA to obtain reliable results with high selectivity. Hence, the exploration of pathways that can effectively convert the target nucleic acids into a distinguishable SERS signal with low background noise is of tremendous benefit for ultrasensitive and on-site DNA detection.

The clustered regularly interspaced short palindromic repeats and its associated proteins (CRISPR/Cas) systems are existing in nature as a part of the microbe's adaptive immune system against viral pathogens.[11] As a powerful genome-editing technology, CRISPR/Cas systems have taken center stage in biotechnology, and CRISPR/Cas9 has won the 2020 Nobel Prize in Chemistry because it provides a tool for rewriting the code of life.[12] Furthermore, CRISPR/Cas effectors have been deployed in novel biosensors for nucleic acid detection in recent years.[13] Especially, LbCpf1 (known as CRISPR/Cas12a), a type V-A CRISPR-associated enzyme guided by a single-stranded guide CRISPR RNA (crRNA),[14] has been considered as a rising star in nucleic acid biosensor fabrication. The CRISPR/Cas12a system is equipped with not only precise cis-cleavage towards target DNA but also indiscriminate collateral trans-cleavage towards single-stranded nontarget DNA. To stimulate the trans-cleavage activity of the CRISPR/Cas12a effector, the activator target DNA must have a binding sequence for crRNA as well as a protospacer adjacent

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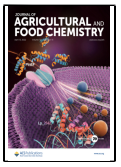

motif (PAM) site nearby.[15] By combining with the trans-cleavage function of CRISPR/Cas12a, various kinds of signal readouts, including fluorescence, color, electrochemistry, and so on, have been reported to detect nucleic acids in the biosensing platform.[16−20]

Inspired by the merits of SERS and CRISPR/Cas12a, we proposed a CRISPR/Cas12a-driven SERS biosensor to detect trace-level target nucleic acids. In this strategy, poly T ssDNA fragments with PB NPs and biotin double-end conjugation serve as the substrate for the collateral trans-cleavage of the Cas12a enzyme under target DNA activation. Through specific biotin−streptavidin affinity, the PB NP-tagged ssDNA was anchored on the microplate, and with the engagement of target DNA, the activated CRISPR/Cas12a system would efficiently cleave the linker ssDNA, making a corresponding number of PB NPs suspended in the solution. Notably, the PB NPs in this assay have advantages like regular shape, easy modification, simple synthesis procedure, and a great target-responsive function.[21] Under alkali condition, the breakdown of PB NPs would be triggered, releasing massive $Fe(CN)_6^{4-}$. The special C≡N structure of $Fe(CN)_6^{4-}$ exhibits a unique characteristic Raman peak that is located in the "biological Raman-silent region" (1800−2800 cm^{-1}).[22] By combining with the SERS substrate, the core−shell structure Au@Ag NP, the number of broken PB NPs can be transformed into distinctive and strong SERS signals with ultralow background noise, leading to trace-level DNA identification. Notably, goat milk is getting popular now due to its lower allergenic properties and better digestibility than cow milk.[23] However, the lower milk yield per goat but higher price per liter of goat milk than cow milk has incurred food fraud by intended adulteration with cow milk.[24] To prevent the potential deceit to customers, unhealthy commercial competition, and risk of adulterant allergies,[25] effective adulteration detection methods are being widely explored. By targeting a specific cow gene as the model analyte, the adulteration information can be dramatically amplified as an anti-interference SERS signal in this biosensor. Furthermore, this strategy can potentially provide insights into various applications such as clinical diagnosis, food safety detection, and environment monitoring.

MATERIALS AND METHODS

Materials and Chemical Reagents. The Magnetic Blood Genomic DNA Kit was purchased from TIANGEN Biotech Co., Ltd. (Beijing, China). Chloroauric acid ($HAuCl_4·4H_2O$), trisodium citrate dihydrate ($Na_3C_6H_5O_7·2H_2O$), silver nitrate ($AgNO_3$), Tween 20, ascorbic acid, $FeCl_3$, $K_4[Fe(CN)_6]$, streptavidin, bovine serum albumin (BSA), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich (Shanghai, China). Recombinant CRISPR/Cas12a protein was provided by Beijing KEXIN Biomedical Technology Co., Ltd. (Beijing, China), and Bst 3.0 DNA polymerase and deoxyribonucleoside triphosphate (dNTP) mix were purchased from New England BioLabs Ltd (Beijing, China). All of the primers of LAMP (loop-mediated isothermal amplification) were referenced to SN/T 4419.21-2016. The optimal crRNA of target DNA was designed and selected in http://crispor.tefor.net/. The above primers and crRNA were synthesized from Genscript Biotech Corporation (Nanjing, China). The oligonucleotides used in this study are listed in Table S1. All of the other reagents were of analytical grade and the aqueous solutions were prepared by using deionized water (DI).

Apparatus and Characterization. The morphological images of the materials were characterized via transmission electron microscopy (TEM) using the TEM instrument (FEI11688Tecnai G20, FEI Company, USA). The amplification of target DNA was verified by nucleic acid electrophoresis (PowerPac Basic, BIO-RAD, USA) as well as a gel imager system (JY04-S3C, JUNYI, China). Dynamic light scattering (DLS) measurements were finished by a Zetasizer (NANO-ZS90, Malvern, U.K.). The Raman spectra were collected using a portable Raman spectrometer (BWS465-785, B&W Tek. Inc., USA) equipped with a785 nm laser as the excitation source. The ultraviolet−visible (UV−vis) absorbance measurements were carried out using a microplate reader (Multiskan GO, Thermo Scientific, USA).

DNA Isolation and Amplification. The DNA used for the detection process was extracted from cow milk sample using a Magnetic Blood Genomic DNA Kit according to the manufacturer's instruction with slight modification: a 400 μL sample was put in a 1.5 mL centrifugal tube containing 20 μL of proteinase K. With the addition of 300 μL of buffer GHL, the mixture was incubated in a water bath at 65 °C for 60 min after vibration. After 5 min of setting at room temperature, 350 μL of isopropyl alcohol and 20 μL of Mag Attract Suspension were added in sequential order. After 1 min of vibrating and 2 min of setting in room temperature (repeat 3 times), the tube was placed on a magnet frame so that the useless solution could be easily removed. Then, 700 μL of buffer GDA (GDA/ethanol (70 wt %) = 1:4) was added into the magnetic beads-containing tube, and the tube was vibrated for 5 min and set for 30 s on the magnet frame. After carefully moving the supernatant, the magnetic beads were fully washed with 700 μL of washing buffer (PWD/ethanol = 1:1) twice. The precipitate was dried in room temperature for 15 min. Finally, 50 μL of TB buffer was added into the tube and incubated at 56 °C for 10 min, and thus, the nucleic acids of the sample milk were obtained. Then, LAMP was performed for 10 min by following the protocol in Table S2. The obtained amplified target DNA was stored at 4 °C.

Synthesis of PB NPs. PB NPs were prepared in a simple one-step aqueous solution route. According to a previous report,[26] 0.5 mmol of citric acid was first added to 20 mL of 1.0 mM aqueous $FeCl_3$ solution under stirring at 60 °C. Then, this solution was added to 20 mL of 1.0 mM aqueous $K_4[Fe(CN)_6]$ solution that contained 0.5 mmol of citric acid under stirring at 60 °C. After that, the mixture solution was stirred continuously at 60 °C for 1 min, and then allowed to cool down to room temperature with continuous stirring for another 5 min. Then, this blue PB NP colloid received centrifugation at 9500 rpm for 20 min. The as-produced precipitate was washed several times with acetone and ultrapure water in equal volumes. Subsequently, the precipitate was dried in an oven at 50 °C overnight to obtain the PB NP powder.

Preparation of the ssDNA-PB NP Probe. Conjugation of the amino-modified ssDNA with PB NP was performed according to a previously reported method.[27] Briefly, 1.0 mg of PB NP powder was redispersed in 4.0 mL of phosphate-buffered solution (PB buffer, pH 7.4) by sonication, and 0.2 M HCl was then titrated into the mixed solution to adjust the pH to 5.0. Then 4.0 mg of EDC and 8.0 mg of NHS were added to the PB NP solution, which was followed by reacting on a shaker for 2 h. After that, 300 μL of ssDNA (1 μM) was added, the solution was stirred gently until fully mixed, and received continuous mechanical oscillation for 3 h at room temperature. Then the mixture was reacted at 4 °C overnight. Finally, 0.2 M NaOH solution was titrated into the mixed solution to adjust the pH to 7.0. After 30 min, the reaction solution was centrifuged at 9500 rpm for 10 min and the obtained precipitate was resuspended in PB buffer (pH 7.4). After washing three times, the obtained precipitate was redispersed in PB buffer (600 μL) and stored at 4 °C before use.

Preparation of the SERS Substrates. The SERS substrate, Au@Ag core−shell NPs, was synthesized according to a previously reported method with some modifications.[28] Briefly, 0.75 mL of 1% sodium citrate trihydrate solution was added to 50 mL of boiling 0.25 mM $HAuCl_4$ solution under vigorous stirring and heated for 10 min to get a wine solution. After cooling at room temperature, the Au NPs (i.e., Au seeds) solution was prepared. To obtain the Au@Ag core−shell NPs, 3 mL of 0.1 M ascorbic acid was added to 20 mL of the above seeds solution under moderate stirring. Then, 5 mM $AgNO_3$

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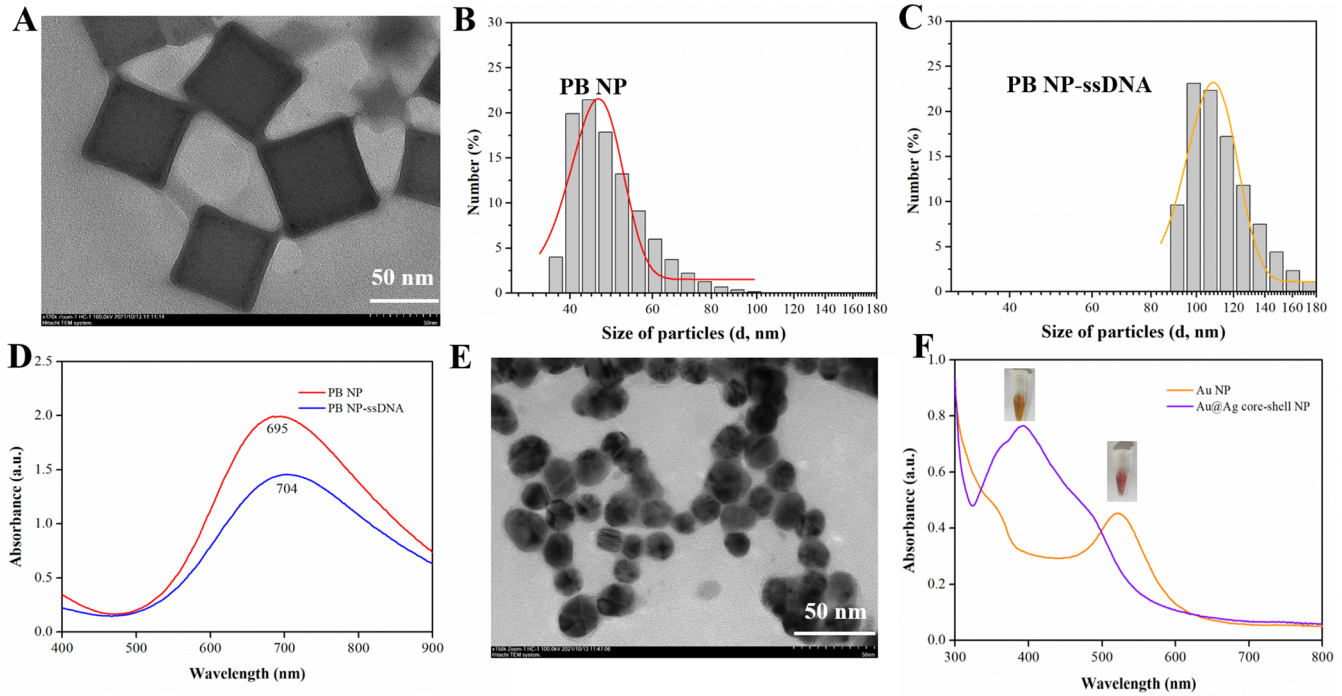
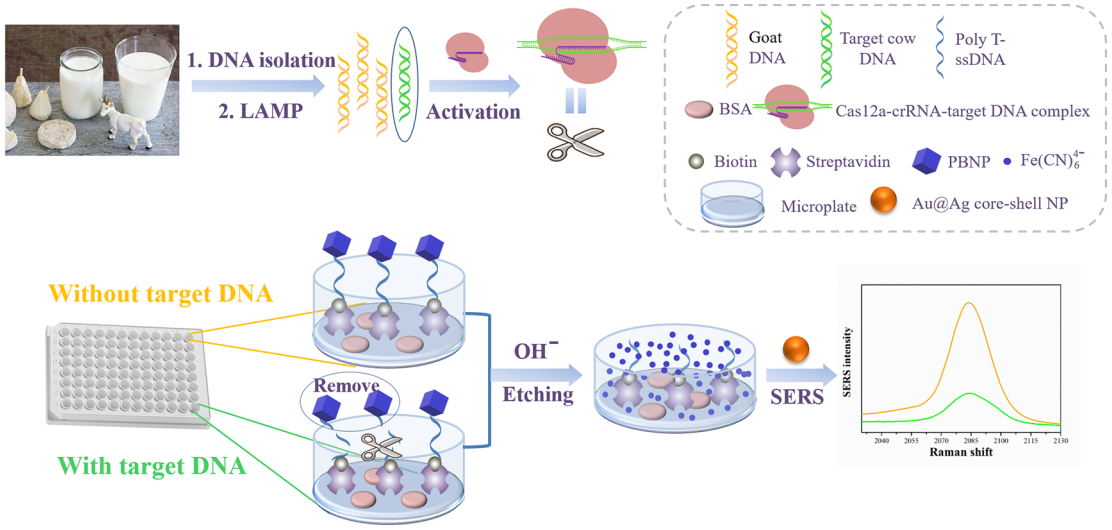


Figure 1. TEM image of (A) as-prepared PB NPs. DLS spectra of (B) PB NPs and (C) PB NP-ssDNA probes. (D) UV−vis absorption spectra of PB NPs and PB NP-ssDNA probes. (E) TEM image of the Au@Ag core−shell NPs. (F) UV−vis absorption spectra of Au NPs and Au@Ag core−shell NPs.

Scheme 1. Schematic Illustration of the CRISPR/Cas12a-Driven SERS Biosensor for Goat Milk Adulteration Detection



solution was dropwise added to the mixture until the solution color turned to orange yellow. Finally, after 30 min of continuous stirring, the SERS substrates solution was prepared and stored at 4 °C.

Grafting PB NP-ssDNA on a 96-Well Plate. First, 100 μL of streptavidin at the concentration of 10 μg/mL in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) was added to each microplate well and left overnight at 4 °C. The wells were washed 3 times with phosphate-buffered saline (PBS) buffer (10 mM, pH 7.4) containing 0.05% Tween 20 (PBST). Second, the microplate wells were blocked with blocking buffer (20 mg/mL BSA in 0.01M PBS buffer) for 1 h at room temperature to prevent non-specific adsorption, following PBS buffer wash for 3 times. Then, the microplates were incubated with 100 μL of 1 μM biotinylated ssDNA-PB NP probe at 37 °C for 1 h and then washed with PBS buffer for 3 times.[29]

CRISPR/Cas12a-Mediated ssDNA Cleavage and Signal Output. The Cas12a-mediated cleavage assay was conducted in a 96-well plate with DNA grafting. Forty microliters of the Cas12a enzyme digestion reaction system containing 0.5 μL of 8 μM Cas12a nuclease, 0.5 μL of 10 μM crRNA, 33 μL of 1× NEB buffer 2.1, and 6 μL of the target DNA was incubated at 37 °C for 70 min. After incubation, the supernatant was removed, and the wells were washed three times with PBS buffer. Later, 100 μL of NaOH (1 M) was directly added in each well and reacted at 43 °C for 22 min. Then, 10 μL of the above solution was mixed with 100 μL of SERS substrate solution. After being gently stirred for 2 min, 8 μL of the mixed solution was carefully dropped on a tinfoil paper-wrapped glass slide (and setting for 10 min) for laser scanning. The SERS spectra were recorded with 10 mW of laser power and 20 s of acquisition time. The obtained SERS data were analyzed using the Origin 8.5 software.

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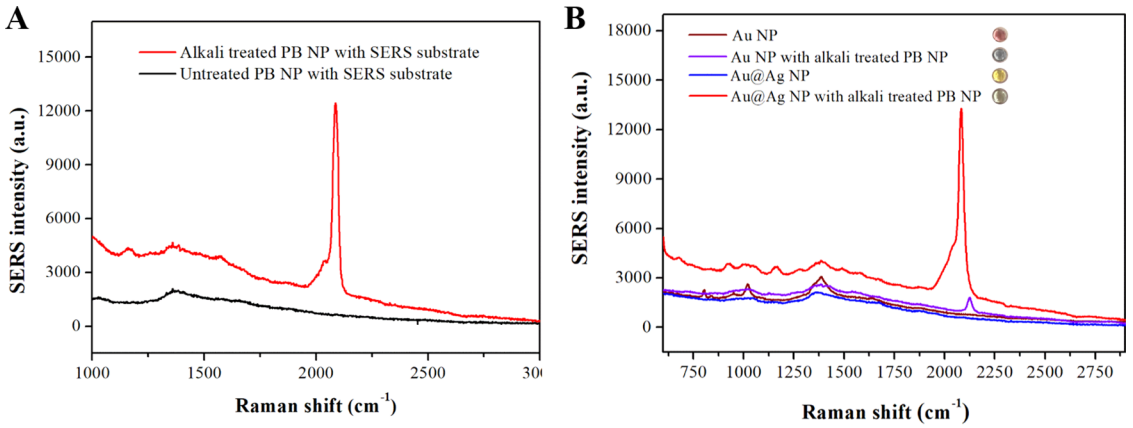

Figure 2. SERS spectra of (A) alkali-treated and untreated PB NPs with the SERS substrate, and (B) bare Au NPs, Au NPs with alkali-treated PB NPs, bare Au@Ag NPs, and Au@Ag NPs with alkali-treated PB NPs.

RESULTS AND DISCUSSION

Material Characterization. The PB NPs employed in this work were synthesized using one-step synthesis and the PB NP-ssDNA probes were fabricated via EDC/NHS reaction on the surface of the PB NPs. The morphology of the PB NPs and the particle distribution of PB NPs and PB NP-ssDNA probes were characterized by TEM and the DLS test, respectively. As shown in Figure 1A, the PB NPs were well-formed cubes with diameters of ∼50 nm. The DLS studies revealed that the as-formed PB NP-ssDNA probes were endowed with increased diameter via modification of ssDNA, and both PB NPs and PB NP-ssDNA probes were finely synthesized without aggregation (Figure 1B,C). The UV−vis absorption spectra are shown in Figure 1D; both the PB NP and the PB NP-ssDNA displayed a strong absorption peak around 700 nm, which was attributed to the charge transfer transition between Fe(II) and Fe(III) in the PB NPs.[30,31] Also, compared with PB NP, PB NP-ssDNA exhibited a clear shift towards the longer wavelength in the spectra (from 695 to 704 nm), further confirming that the ssDNA successfully attached on the surface of PB NP. Besides, the TEM images of the prepared SERS substrate Au@Ag core−shell NP are shown in Figure 1E. It could be seen that the Au NP was wrapped by a thin shell and was well dispersed. According to the UV−vis spectra of bare Au NPs and Au@Ag NPs (Figure 1F), the plasmonic band shifted from 520 to 394 nm after the addition of $AgNO_3$ and the reductant to the Au NP solution. The band shift could be exclusively ascribed to the collective nature of the surface plasmon resonance of Au and Ag NPs.[32] Based on these facts, it could be reasonably inferred that the core−shell structure of the Au@Ag composite NP is formed.

Working Principle. Scheme 1 shows the principle of the developed CRISPR/Cas12a-driven SERS biosensor for on-site nucleic acid detection. The target DNA was first amplified by LAMP, a simple and efficient isothermal amplification method, to facilitate the following activation of the CRISPR system. In the meantime, we designed a 96 bp-long poly T ssDNA with double-ended modification of biotin and NH_2 in the 5′ and 3′ ends, which was the substrate for trans-cleavage of the Cas12a enzyme. Via robust EDC/NHS coupling chemistry and biotin−streptavidin affinity, the pH-responsive PB NP-labeled ssDNA probes were fabricated and grafted on the streptavidin-coated 96-well plate. Next, the CRISPR/Cas12a reaction system was added. In the absence of target DNA, the trans-cleavage activity of Cas12a was silenced, retaining the whole PB NP-ssDNA probes in the microplate. In the presence of target DNA, highly efficient trans-cleavage of Cas12a towards the ssDNA probes was initiated under crRNA-target DNA recognition, leading to a decreased concentration of fastened PB NPs. After completely removing the supernatant, PB NPs that were labeled on the unbroken ssDNA were remaining in the well, and would receive further treatment to produce the SERS signal. For SERS measurement, the analyte−surface distance should be close enough (∼1 to 10 nm) to obtain a distinctive signal enhancement.[33] However, the substrate particle cannot directly touch the surface of the PB NPs, let alone the inner cyano group in PB NPs; therefore, no representative peak of the cyano group was exhibited in the Raman spectra (Figure 2A). Fortunately, upon addition of NaOH, the structure of the PB NPs was broken and numerous hydrophilic $Fe(CN)_6^{4-}$ were released in the solution; the reaction is as shown below.

$$12OH^- (aq) + Fe_4[Fe(CN)_6]_3 (s) \rightarrow 3Fe(CN)_6^{4-} (aq) + 4Fe(OH)_3 (s)$$

Then, by mixing the SERS substrate with the above alkali-treated solution, a distinctive SERS signal at 2083 cm^{-1} was obtained in the "biological Raman-silent region", where the signal interference of biological endogenous molecules was effectively circumvented.[34]

Meanwhile, substrate material is also a key factor that determines the signal enhancement. It is well known that nanosized noble metal particles (typically Au NP or Ag NP) are highly SERS-active plasmonic substrates, and have been used to significantly enhance the Raman signal. However, as shown in Figure 2B, bare Au NPs had a relatively poor signal enhancement under Raman laser scanning for $Fe(CN)_6^{4-}$ analysis in this assay. Fortunately, the Au@Ag core−shell nanostructure showed superb performance due to the enhanced localized surface plasmon resonance excitation in the bimetallic composition.[35] Moreover, the multicomponent core−shell structure NPs have better stability and dispersibility.[36] As shown in Figure S1, after being stored at 4 °C for 2 months, the Au NP solution displayed obvious precipitation, while the Au@Ag core−shell NP solution was still clear. Meanwhile, in contrast to Au NPs, the Au@Ag NPs substrate still showed robust SERS signal enhancement for the alkali-

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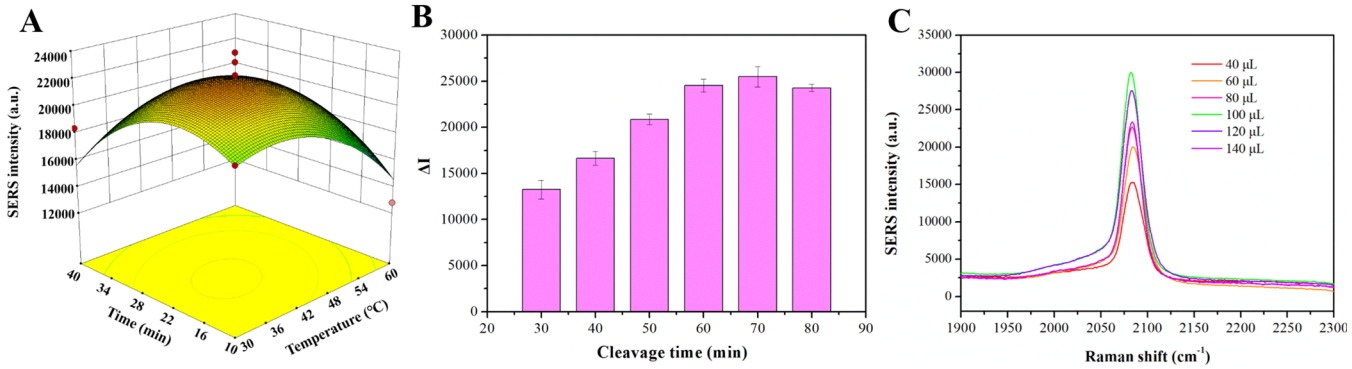

Figure 3. Optimization of (A) the alkali-treated conditions of the PB NP-ssDNA probe through response surface methodology, (B) cleavage time of the activated CRISPR/Cas12a system, and (C) addition volume of the SERS substrate Au@Ag NPs into every 10 μL of the alkali-treated solution.

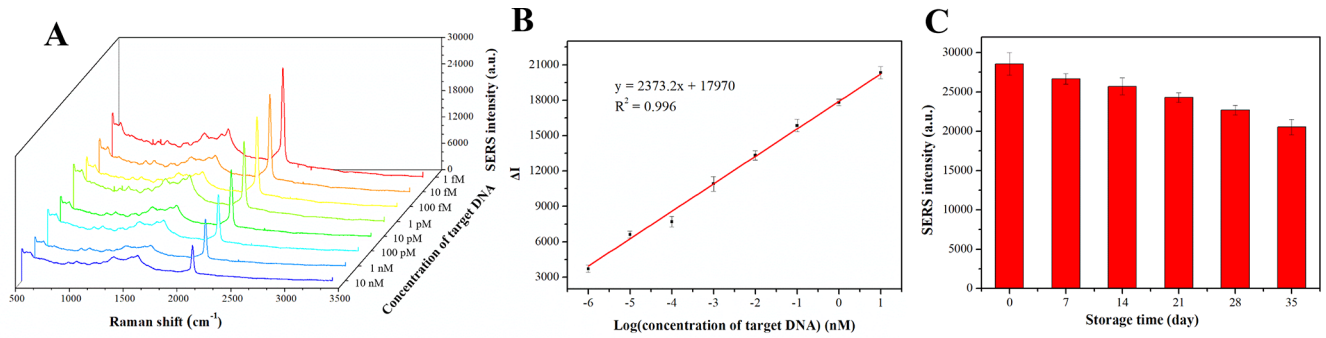

Figure 4. (A) SERS spectra of the detection of target DNA with different concentrations (from 1 fM to 10 nM). (B) Linear calibration curve of ΔI at 2083 cm^{-1} vs the concentration of target DNA. (C) Stability of the PB NP-ssDNA probe stored at 4 °C.

treated PB NP solution after long-term storage. Therefore, we employed Au@Ag core−shell NPs as the SERS substrate in this assay.

The Raman intensity of ΔI ($\Delta I = I_0 - I$, where I_0 and I represent the Raman intensity without and with the target DNA, respectively) at 2083 cm^{-1} was linearly correlated with the concentration of target DNA. Through this strategy, the concentration of target DNA was dexterously transformed into a distinctive Raman signal, achieving ultrasensitive and on-site nucleic acid detection.

Optimization of the Condition. In order to achieve the best detection performance of the CRISPR/Cas12a-driven SERS biosensor, the main experimental parameters relating to the alkali treatment of the ssDNA-PB NP probe, CRISPR/Cas12a trans-cleavage system, and the final SERS signal output were optimized. The alkali treatment is a decisive factor for signal conversion. Both the time and temperature of the alkali treatment were already proved to have an obvious influence on the SERS signal output by experiments. Therefore, using 100 μL of 1 M NaOH to treat the PB NP-ssDNA-coated microplate under varied incubating temperatures (30−60 °C) and times (10−40 min), we recorded the SERS intensity at 2083 cm^{-1} of the above treated solution. According to these data, we built a quadratic response surface model, and the analysis of variance for this model is shown in Table S3. The model p value was <0.01, which was significant, and the lack of fit was $p = 0.2108 > 0.05$, which was not significant, indicating that the model was in good quality. The multiple linear regression equation was calculated using the software and is shown below.

$$Y = 22627.60 - 579.80A - 829.48B + 412.25AB - 2666.05A^2 - 2738.80B^2$$

where Y is the SERS intensity in the Raman shift of 2083 cm^{-1}, and A and B are the values of the alkali incubation temperature (A) and time (B), respectively. The three-dimensional response surface curves are presented in Figure 3A. The analysis of variance showed that the fitness was significant at the level of $p = 0.0053$. Also, according to the response surface curves, the optimal conditions were given as an incubation time of 22 min and a temperature of 43 °C. The suitability of the proposed optimal conditions was verified by 6 experiments, and the average SERS intensity was 23 942.5 arbitrary units (a.u.), indicating the feasibility of the response model to reflect the expected optimization.

We subsequently tested the different cleavage times of the CRISPR/Cas12a system (from 30 to 80 min) by monitoring ΔI at 2083 cm^{-1}. Prominent performance was detected at times above 70 min; thus, 70 min was chosen as the work time for CRISPR/Cas12a treatment (Figure 3B). Last, the mixing ratio between the analyte and substrate NP is also important for SERS signal readout. Substrate solutions with varied volumes (from 40 to 140 μL) were mixed with 10 μL of alkali-treated solution in a microplate to obtain the optimal mixing ratio. Satisfyingly, the highest SERS intensity at 2083 cm^{-1} was obtained at the volume of 100 μL of the Au@Ag core−shell NP solution (Figure 3C). So the alkali-treated solution/substrate in 1:10 could well amplify the SERS signal, which was employed as the volume ratio before being submitted to SERS scanning in further study.

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Performance of the CRISPR-Driven SERS Biosensor. To verify the feasibility of the CRISPR/Cas12a-driven SERS biosensor in goat milk authenticity testing, adulterated cow cytochrome b (cytb) gene, a mitochondrial gene, was selected as the target nucleic acid. The partial nucleotide sequence of the cow cytb gene extracted from cow milk was 10-fold serially diluted and then amplified via LAMP. Under the optimized conditions, quantitative evaluation of cow cytb gene was determined by monitoring ΔI at 2083 cm^{-1} in SERS spectrum. Figure 4A shows the concentration-dependent SERS spectra for the detection of cow cytb gene in milk samples with various concentrations, indicating that the SERS intensity at 2083 cm^{-1} concomitantly decreased with the increase in concentration of the cytb gene. As presented in Figure 4B, the ΔI showed a good linear response for quantitative analysis in regard to the concentration of target DNA from 1 fM to 10 nM with a coefficient value (R^2) of 0.996. The linear regression equation was $\Delta I = 2373.2 \lg C_{\text{target DNA}} + 17\,970$, and the limit of detection (LOD) was calculated to be 224 aM according to the 3σ rule (where σ is the standard deviation) for six blank measurements.[37] All of the error bars were calculated by three repeated measurements.

The specificity of this strategy was verified by nucleic acid gel electrophoresis, which was run on 2% agarose gel (Figure S2). It indicated the absence of cross-species binding of the primers and subsequent amplification of other animal species (goat, sheep, and camel), which revealed the primers' sequence specificity towards special cow DNA in practical detection. The stability of the proposed PB NP-ssDNA probe was also evaluated. As shown in Figure 4C, after 35-day storage at 4 °C in the refrigerator, the average SERS signal value of the alkali-treated PB NP-ssDNA still reached 70% of the initial value, verifying the robust stability of this nanoprobe.

We compared a variety of recently published works for nucleic acid detection with the established method to further demonstrate the detection performance. This method combined the cascade signal amplification of CRISPR/Cas12a with the target-responsive PB nanolabel to realize SERS detection of trace-level DNA. Hence, as shown in Table 1, it showed a superior sensitivity than most biosensors and CRISPR/Cas12a-enhanced biosensors for nucleic acid detection.

Application to Food Samples. To further validate the practicability, 12 commercially available milk products including 3 goat milk, 3 goat milk powder, 3 goat yogurt, and 3 goat milk tablets were processed by the proposed strategy. Notably, solid samples such as goat milk powder and goat milk tablets were first ground and dissolved with deionized water (7.2 mL for a 1 g sample) to prepare liquid samples for further processing. As a result, 2 real-world samples (1 goat milk and 1 goat milk tablet) were detected positive for cow-derived material: The goat milk sample has condensed milk, as illustrated in its ingredient list. However, the goat milk tablet was detected with 13.39% cow-derived material, which was not mentioned in its ingredient list. Besides, to validate the detection results, recovery tests were performed by spiking 1 and 5% of cow milk in 4 of the 12 samples (one for each type). The recoveries of adulterated cow milk from different spiked samples were calculated as 94.20−106.16%, and the relative standard deviation (RSD) was in the range of 0.89−6.37% (Table 2), indicating the satisfactory detection accuracy. These results demonstrated that the developed sensing method was capable of identifying and detecting target milk adulteration in real samples.

Table 1. Comparison of the Reported Methods for Nucleic Acid Detection

analytical methods	linear range	LOD	references
flow-through colorimetric assay	1 nM to 10 μM	0.6 nM	38
ratiometric fluorescent biosensor	1−9 nM	0.14 nM	39
SPR sensor		10 pM	40
electrochemical biosensor	10 fM to 50 nM	5.1 fM	41
SERS biosensor	1 fM to 10 nM	0.67 fM	42
solid-state CRISPR/Cas12a -assisted nanopores		10 nM	43
isothermal amplification reporting CRISPR/Cas12a assay		45.0 pM	44
fluorescent CRISPR/Cas12a biosensor	0.1−10 nM	1 pM/100 fM	45
electrochemical CRISPR/Cas12a biosensor	10 fM to 0.1 nM	3.5 fM	18
CRISPR/Cas12a-driven SERS biosensor	1 fM to 10 nM	224 aM	this work

Table 2. Recovery Test for Cow Milk Detection in Real Samples

samples	spiked content (%)	detected content (%)	recovery (%)	RSD (%)
goat milk	0	7.29		
	1	8.27	97.68	6.37
	5	12.37	101.70	0.89
goat milk powder	0	ND[a]		
	1	0.94	94.20	3.97
	5	5.03	100.55	2.75
goat yogurt	0	ND[a]		
	1	1.05	104.77	2.24
	5	5.23	104.63	2.88
goat milk tablet	0	13.39		
	1	14.34	95.62	4.78
	5	18.70	106.16	1.37

[a]ND: not detected.

In summary, we have successfully developed a CRISPR/Cas12a-driven SERS biosensing strategy for trace-level DNA identification, and applied it to on-site milk adulteration detection. Attributed to the ingenious probe design, the highly efficient trans-cleavage function of CRISPR/Cas12a could effectively transduce and amplify the signal of target DNA. Based on the target-responsive function of nanolabel PB NP and the bimetal core−shell structure of substrate Au@Ag NP, the SERS signals were dramatically amplified. Meanwhile, the obtained SERS signal exhibited ultralow background noise. In this strategy, the concentration of target DNA, even as low as 224 aM, can be detected by a portable Raman spectrometer. Moreover, because of its excellent performance, this strategy can be a universal platform to be applied to various fields ranging from clinical diagnosis to food safety monitoring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c08262.

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Protocol of LAMP amplification and oligonucleotides used in this work, analysis of variance for SERS intensity response surface model, stability comparison of bare Au NP and Au@Ag NP, agarose gel electrophoresis images of the LAMP product of cow, sheep, goat, and camel (PDF)

AUTHOR INFORMATION

Corresponding Author

Guoliang Li − *School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China;* orcid.org/0000-0001-7951-9750; Email: 61254368@163.com

Authors

Ruiyuan Pan − *School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China*

Jianghua Liu − *School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China*

Panxue Wang − *School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China*

Di Wu − *Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, Belfast BT9 5DL, United Kingdom;* orcid.org/0000-0002-5895-2237

Jian Chen − *School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China*

Yongning Wu − *School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China; NHC Key Laboratory of Food Safety Risk Assessment, Food Safety Research Unit (2019RU014) of Chinese Academy of Medical Science, China National Center for Food Safety Risk Assessment, Beijing 100021, China;* orcid.org/0000-0001-6430-1302

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c08262

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SERS, surface-enhanced Raman scattering; ssDNA, single-strand DNA; PB, Prussian blue; NP, nanoparticle; $Fe(CN)_6^{4-}$, ferricyanide anions; PCR, polymerase chain reaction; q-PCR, quantitative polymerase chain reaction; CRISPR/Cas, clustered regularly interspaced short palindromic repeats and its associated proteins; PAM, protospacer adjacent motif; BSA, bovine serum albumin; sulfo-NHS, N-hydroxysulfosuccinimide sodium salt; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; dNTP, deoxyribonucleoside triphosphate; TEM, transmission electron microscopy; DLS, dynamic light scattering; LAMP, loop-mediated isothermal amplification; crRNA, single-stranded guide CRISPR RNA; UV−Vis, ultraviolet−visible; PB buffer, phosphate-buffered solution; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline buffer containing 0.05% Tween 20; a.u., arbitrary unit; cytb, cytochrome b; R^2, coefficient value; LOD, limit of detection; RSD, relative standard deviation

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