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Label-Free Surface-Enhanced Raman Spectroscopy Detection of DNA with Single-Base Sensitivity

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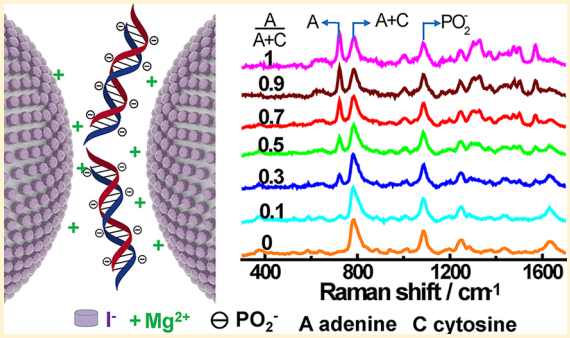
Supporting Information

ABSTRACT: Direct, label-free detection of unmodified DNA is a great challenge for DNA analyses. Surface-enhanced Raman spectroscopy (SERS) is a promising tool for DNA analyses by providing intrinsic chemical information with a high sensitivity. To address the irreproducibility in SERS analysis that hampers reliable DNA detection, we used iodide-modified Ag nanoparticles to obtain highly reproducible SERS signals of single- and double-strand DNA in aqueous solutions close to physiological conditions. The phosphate backbone signal was used as an internal standard to calibrate the absolute signal of each base for a more reliable determination of the DNA structure, which has not been achieved before. Clear identification of DNA with single-base sensitivity and the observation of a hybridization event have been demonstrated.



INTRODUCTION

Detection of specific DNA sequences in nucleic acids is indispensable for genomic screening and diagnostic purposes and is now becoming increasingly important in different areas, including medical diagnosis, forensic analysis, and environmental monitoring.[1] Traditional DNA analysis methods, such as polymerase chain reaction and microarray techniques, usually need fluorescent reporters and complicated procedures and suffer from high cost, complex analytical procedures, and a loss of the intrinsic chemical structure information on DNA molecules.[2]

Surface-enhanced Raman spectroscopy (SERS) has recently emerged as a powerful analytical tool for rapid detection and structural characterization of DNA, by providing ultrahigh sensitivity and intrinsic chemical fingerprint information.[3] Various detection strategies have been proposed, and single-base mismatch has been detected.[3−11] The SERS-based DNA detection can be classified into the three categories. The first is a label method, which is to modify DNA with an extrinsic Raman label. After recognition, the Raman intensity of the label will change.[4] A much lower detection limit than for the fluorescence method has been achieved.[5] This method requires modification of DNA and synthesis of Raman labels and does not take full advantage of the chemical information on the DNA itself. The second method is to utilize the different chemical or physical properties of the DNA chain or signal transduction system before and after hybridization, resulting in the change of the Raman intensity of the label.[6] This method does not require the modification of DNA, and the SERS signal is still from the label, not the DNA. The third is a truly label free method. DNA molecules are brought to the enhanced electromagnetic field of plasmonic nanostructures to generate SERS signals of the DNA molecules themselves.[3a,7] This method takes full advantage of the fingerprint information of Raman spectroscopy over other methods. However, a direct mixing of DNA with SERS nanoparticles does not produce a sufficiently strong and reproducible signal. Instead, usually the signals are just too weak to obtain. The following two points may account for that: (1) the synthesized nanoparticles usually have a layer of surfactant or capping agents inherited from synthesis processes, which are unstable and will lead to the variation and complication of SERS signals shown as impurity signals, and (2) the interaction of negatively charged DNA with the metal surface of the same charge is usually very weak,[8] which prevents the effective electrostatic interaction with the metal surface necessary for the enhancement. Thiolation of DNA or the thermal annealing method has been developed to promote the surface binding of DNA to obtain reproducible signals of DNA (dominated by adenine).[3a,9] There was also an attempt to obtain the SERS signal of unmodified DNA molecules in the dried state on Ag nanorods combined with the least-squares chemometric method.[10] There is increasing interest in detection of DNA molecules without any modification and in their native state, by introduction of an aggregation cation and surface modification with positively

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charged functional groups.[5,7b,8,11] Although the phosphate backbone is abundant in DNA, up to now, most reports can only give the signal of the bases.[3a,7a,11a] Only recently, two works from the same group reported spectra showing the signal of the phosphate backbone.[11e,d]

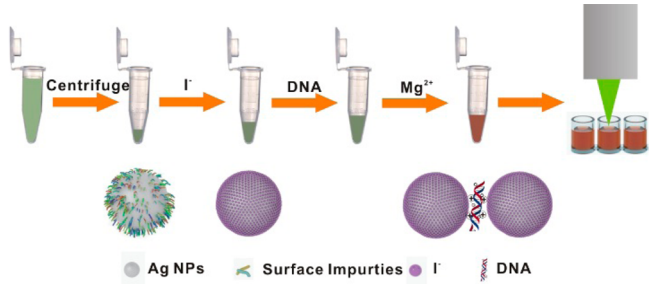
It should be noted that most previous SERS studies relied on the strong interaction of molecules with the substrate, with a few exceptions using the electrostatic or hydrophobic interactions between the surface immobilized molecule and DNA.[12] Such a strong interaction will remarkably modify the spectral feature of the molecule, and the resulting spectral feature (especially the relative intensity) will highly depend on the interaction strength, the coadsorbed species, and the microenvironments. Therefore, even the same molecule will produce dramatically different spectral features on different metal surfaces and under different conditions.[13] This problem makes the SERS identification (even qualitative) of DNA extremely challenging, because DNA molecules are formed by the four bases adenine (A), thymine (T), cytosine (C), and guanine (G), with different relative contents. Therefore, one would only expect to observe the difference in the relative intensity for DNA molecules with different numbers of bases. However, the use of relative intensities of different bases to identify different DNA is almost impossible for the SERS method for the reason mentioned above.

Recently, we proposed a simple SERS detection protocol using iodide-modified Ag nanoparticles (Ag IMNPs).[15] The method involves coating a monolayer of iodide ions on chemically synthesized Ag nanoparticle surfaces, which not only cleaned the surface and modified the interfacial structure of nanoparticles but also avoided the strong chemical interaction between the metal surface and the biomolecules and reduces the risk of denaturation. We successfully used it for the reliable and reproducible SERS detection of proteins. The obtained SER spectra resembled the normal Raman spectra of proteins but with much improved detection sensitivity. Since the detection was performed in aqueous media, it not only retained the native state of biomolecules but also improved the photostability of samples. Herein, the Ag IMNPs method was modified and extended for the label-free detection of DNA. $MgSO_4$ was added to neutralize the surface charge and enhance the interaction of DNA with the Ag IMNPs, so that strong and reproducible SERS signals could be detected. High-quality SERS signals of different single-stranded DNA (ssDNA) were obtained, which allows a clear assignment of the characteristic peaks of the four bases. We further used the SERS signal of the phosphate backbone as an internal standard to calibrate the SERS signal of each base, so that the content of each base in the DNA sequences could be estimated. Such a concept was supported by the SERS measurements of DNA sequences with different A and C ratios, with which the single-base detection sensitivity was convincingly demonstrated. We further extended this method to the detection of double-stranded DNA (dsDNA) and the hybridization of DNA.

EXPERIMENTAL SECTION

The detailed experimental procedure is given in Scheme 1 for clarity. Ag IMNPs were prepared according to previously published procedures.[15] Briefly, 4.5 mL of silver colloid reduced by sodium citrate was centrifuged (5000 rpm, 10 min) and the supernatant was removed. The final concentration of Ag nanoparticle in the concentrated colloid was about 23.55 nM. A 50 μL portion of the concentrated colloid was mixed with 50 μL of 1 mM potassium iodide,

Scheme 1. Schematic Diagram for the SERS Detection of DNA with Ag IMNPs



which was incubated for 20 min at 25 °C to ensure a complete modification of the surface of Ag NPs with iodide. After that, the Ag IMNPs were mixed with 50 μL of DNA aqueous solution at room temperature, and then 20 μL of 0.01 M $MgSO_4$, used as aggregation agent, was added to force the aggregation of the Ag IMNPs. The color of the colloids turned from yellow-green to chocolate immediately. Finally, the sample was added to 96-well plates for SERS measurements.

The names and corresponding sequences of all the DNA used in this work are listed in Table 1. All of the ssDNA were purchased from

Table 1. Names and Sequences of the Single-Stranded DNA

name	sequence (5′−3′)
A_{12}	AAA AAA AAA AAA
T_{12}	TTT TTT TTT TTT
C_{12}	CCC CCC CCC CCC
$(TG)_{12}$	TGT GTG TGT GTG TGT GTG TGT GTG
A_{40}	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A
T_{40}	TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T
C_{40}	CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC C
X	ACA ACC CCC A
Y	TCA ACC CCC A
SN5	GAC TGC GAC CAA CCT AGC CTG CTA TGA TGT
SN5C	ACA TCA TAG CAG GCT AGG TTG GTC GCA GTC
SN6	AAC AGG ACA ATA TAT GTC CTG TTT TTT TTT TTA ACA GGA CAT ATA TTG TCC TGT T
SN6C	AAC AGG ACA ATA TAT GTC CTG TTA AAA AAA AAA ACA GGA CAT ATA TTG TCC TGT T

Bio Basic Inc. The dsDNA used in our experiments was prepared by incubating the 1:1 mixture of the two complementary single-stranded sequences at 95 °C for 10 min followed by cooling down to room temperature. Unless otherwise stated, the final concentration of ssDNAs and dsDNA was 7 and 3.5 μM, respectively. High-purity water (Milli-Q, 18.2 MΩ·cm) was used throughout the study.

Normal Raman and SER spectra were acquired using a confocal Raman system (Xplora, Horiba) using a 532 nm laser as the excitation light. In the SERS measurements of the solution samples, the laser beam was directly focused into the solution in the 96-well plates and a 10× objective (NA = 0.25) was used. In the normal Raman measurements of the solid DNA, a 50× objective (NA = 0.50) was used. The laser power was around 5 and 20 mW for SERS and normal Raman measurements, respectively. The typical exposure time for DNA measurements was 10 s. All the spectra were present only after baseline correction by the polynomial fitting method.

RESULTS AND DISCUSSION

The main challenge in the direct SERS analysis of unmodified DNA is the interference from the surface impurities[14] and the negatively charged phosphate backbone. For example, when

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mixing A_{12} (a DNA sequence containing 12 identical adenines, AAA AAA AAA AAA) directly with Ag NPs, the signal of citrate dominates the spectra, and the negatively charged DNA cannot displace the surface citrate (Figure 1a). However, when $MgSO_4$

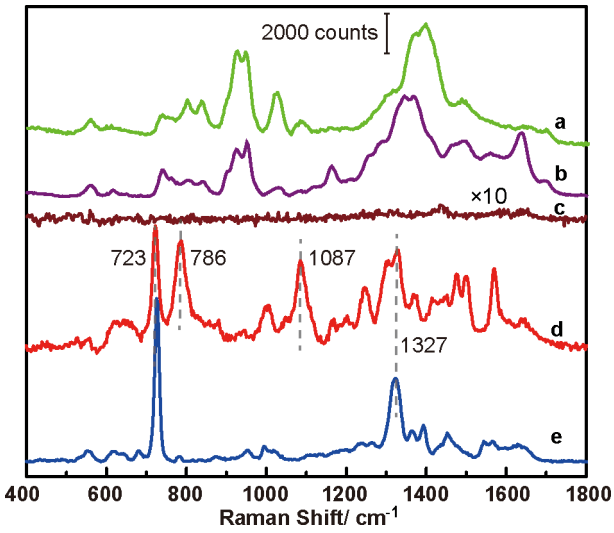

Figure 1. SER spectra of A_{12} on Ag NPs without (a) and with (b) $MgSO_4$, A_{12} on Ag IMNPs without (c) and with (d) $MgSO_4$ and (e) adenine with Ag IMNPs.

was added into a mixture of A_{12} and Ag NPs, the signal is still dominated by citrate (Figure 1b). After iodide modification, the citrate can be remarkably removed, as evidenced by the broad featureless spectrum, and no signal of DNA can be obtained (see Figure 1c). Although the surface impurities can be removed, the surface charge is still negative and the repulsion between the negatively charged surface of Ag IMNPs and the DNA backbones prevents the binding of DNA to the surface. To solve this problem, $MgSO_4$ was introduced to the system to neutralize the surface charge.[11b] In this case, Mg^{2+} played dual roles: a bridge between Ag IMNPs and DNA, so that DNA could be electrostatically adsorbed onto the Ag IMNPs, and an aggregation agent to induce the aggregation of Ag IMNPs. Thereby, more "hot spots" were produced, DNA molecules were naturally trapped in the hot spots, and the signal of the DNA could be tremendously enhanced. Indeed, a high-quality SERS signal of A_{12} was obtained using Ag IMNPs (see Figure 1d). The SERS feature of A_{12} is much different from that of the literature, in which the adenine signal dominated the SERS spectra.[3a,7a] To see the difference clearly, the SERS signal of adenine adsorbed on Ag IMNPs is also given in Figure 1e. In addition to the signals at 723 and 1327 cm^{-1} assigned to the breathing and the ring skeleton vibration modes of adenine (see Figure 1e), we can also detect many more features that were not observed in the SERS spectrum of adenine, which will be discussed later on.

Direct SERS analysis requires a clear assignment of all the characteristic peaks related to the four DNA bases. Therefore, we selected 12-base homopolymeric DNA sequences for a clear assignment of the experimentally observed peaks to each nucleotide. As shown in Figure 2a,c,e, SER spectra of A_{12}, C_{12}, and T_{12} (C_{12} and T_{12} have a similar meaning to A_{12}) display distinctive fingerprints, respectively. They show a common peak at 1087 cm^{-1} (assigned to the symmetric stretching vibration of phosphodioxy PO_2^-).[16] The peaks at 786 cm^{-1},

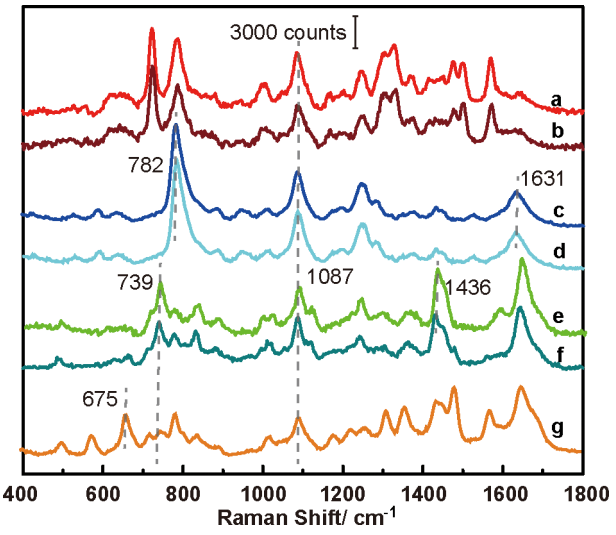

Figure 2. SER spectra of (a) A_{12}, (b) A_{40}, (c) C_{12}, (d) C_{40}, (e) T_{12}, (f) T_{40}, and (g) $(TG)_{12}$ on Ag IMNPs normalized with the PO_2^- signal.

which is the most obvious in A_{12}, is assigned to the skeleton stretching vibration of PO_2^- by comparing the SER spectra of A_{12} with that of adenine (Figure 1d,e). The observation of phosphodioxy peaks indicates that the phosphate backbones are very close to the Ag IMNPs surface to generate the enhanced Raman signals. A_{12} is featured by a characteristic peak at 723 cm^{-1} from adenine. It seems that C_{12} has a characteristic band at 782 cm^{-1}. In fact, the correct position of C is at 776 cm^{-1} (as will be shown in the difference spectrum in Figure 5), and it overlaps with that at 786 cm^{-1} of PO_2^- to produce the peak at 782 cm^{-1}. This peak is assigned to the ring breathing mode vibration of C. T_{12} gives a band at 739 cm^{-1}. Different from the case of the protein study, we did see some changes in the spectral feature of the SERS of homopolymeric DNA compared to that of the normal Raman spectra (see Supporting Information, Figure S1). This is reasonable considering the different state of the DNA in SERS (aqueous solution) and normal Raman (solid) measurements. Nevertheless, such changes do not sacrifice the merit of the method. It is particularly interesting to find that the SERS spectra of longer homopolymeric sequences, A_{40}, C_{40}, and T_{40} (Figure 2b,d,f), show exactly the same spectral features as that of A_{12}, C_{12}, and T_{12}, respectively. It demonstrates that the spectra of polynucleotides can really reflect the fingerprint of the basic building blocks of DNA, the mononucleotide containing base and phosphate/sugar backbone, which further proves the high reliability of this method. Unfortunately, we could not obtain reliable SERS spectra for G_{12} because G homopolymers can easily form a quadruduplex structure, which is much different from the state of G in normal DNA sequences. Therefore, we performed the SERS study of $(TG)_{12}$, from which we can easily obtain the characteristic peak of G at 675 cm^{-1} by comparing the spectrum with that of T_{12}. The assignment of principal peaks is listed in Table 2.

In the above study, we have demonstrated that the Ag IMNPs method could reliably obtain the signatures of the four polynucleotides. The further open question is whether it is possible to achieve single-base sensitivity, so that a single-base mutation and hybridization event can be detected. The great challenge of such measurement lies in that DNA are formed by the four bases following a certain sequence. Each DNA

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Table 2. Raman Frequencies of Typical Vibrational Modes of DNA[a]

Raman bands (cm^{-1})	assignments
675	G, ring breathing
723	A, ring breathing
739	T, ring breathing
776[b]	C, ring breathing
786	PO_2^-, skeleton stretching
1087	PO_2^-, symmetric stretching
1240/1248	A, C, or T, ring stretching
1327	A
1436	CH_2, deformation
1570	A, G
1631/1643	T, C

[a]See refs 11e and 16. [b]In the C_{12} spectrum, this peak overlaps with that of 786 cm^{-1} (PO_2^-) to give a peak at 782 cm^{-1}.

repeating unit, i.e., nucleotide, is composed of a nitrogen-containing nucleobase, a monosaccharide sugar, and a phosphate group. Therefore, different DNA molecules will show similar spectral features with the only difference being the relative intensities of the different bases. However, it is known in SERS that the relative intensities of the peaks of the same molecule depend very much on the interaction of the molecule with the surface, which is influenced by the surrounding environment. Nonetheless, for a specific DNA sequence, the number of PO_2^- is the same as the total number of all bases. Therefore, the PO_2^- signal may be used as an intrinsic internal standard for quantitative analysis of the number of each type of bases in DNA; that is, DNA has a natural internal label for quantitative SERS analysis. In this way, we might be able to circumvent the SERS signal fluctuation in the quantitative analysis, and it would be highly desirable if single-base variation could be sensitively discerned by SERS.

To more impressively demonstrate the effect of using the SERS signal of PO_2^- as the internal standard and the possibility of this method for single-base analysis, we synthesized five oligonucleotides with the same length but different A/(A + C) ratios. Figure 3a shows various SER spectra along with that of A_{12} and C_{12}. The spectra were normalized with the intensity of PO_2^-, so that a minor change in the content of each base can be clearly identified. Exhilaratingly, the intensity of the characteristic bands of A showed a progressive increase with the increasing number of A (Figure 3a). It has been shown above that the peak at 723 cm^{-1} is solely contributed by A, and the peak at 1087 cm^{-1} is contributed by PO_2^-. Therefore, if we plot the relative Raman intensity of 723 cm^{-1}/1087 cm^{-1} with the ratio of A/(A + C) for the seven ssDNA in Figure 3a, we may extract some quantitative information. Figure 3b shows an unbelievably linear relationship, with error bars much better than the threshold needed for the single-base discrimination. Such linearity suggests that a quantitative relationship can be established between the relative Raman intensity and the ratio of different bases. In other words, this simple Ag IMNPs SERS method allows us to detect differences between DNA sequences that have different nucleotide contents, which is very important for fast screening.

To demonstrate this feasibility, we designed two 10-base ssDNA, X (ACA ACC CCC A) and Y (TCA ACC CCC A), and performed the SERS measurement to check the single-base sensitivity. The only difference in X and Y is on the 5′ end and one A in X was substituted by T in Y. The SERS signal of X

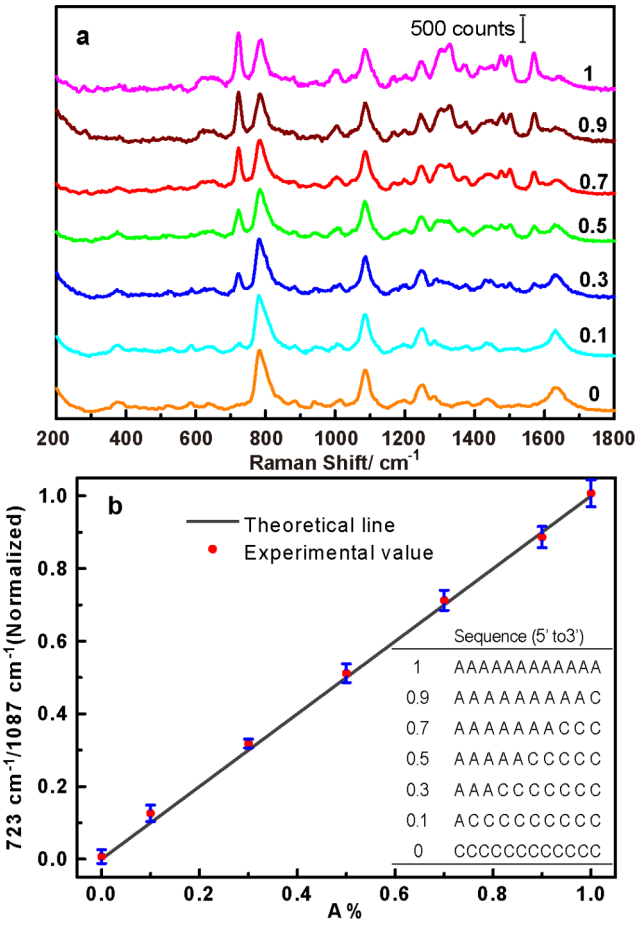

Figure 3. (a) SER spectra of a set of oligonucleotides composed of different numbers of adenine (A) and cytosine (C), in which A% = A/(A + C) = 1, 0.9, 0.7, 0.5, 0.3, 0.1, 0, as indicated in the figure. All the spectra were normalized by the peak intensity of 1087 cm^{-1} of PO_2^-. (b) A plot of the relative peak intensity of 723 cm^{-1}/1087 cm^{-1} with the real ratio value of A in these oligonucleotides. The 723 cm^{-1}/1087 cm^{-1} ratio equals 1 for the pure A sequence and 0 for the pure C sequence. The ratios of the other five oligonucleotides are normalized to within the range of 0 and 1. The inset table lists the sequence of the oligonucleotides.

(Figure 4a) clearly shows a simple combination of the spectral feature of A12 (723, 786, and 1570 cm^{-1}) and C12 (781 and 1631 cm^{-1}) nucleotides. This is advantageous in using this method for DNA analysis, because the relative intensity can now be reliably used to analyze the relative number of bases in the DNA, which has never been achieved before. The main SERS feature of Y is still similar to A and dominated by the A and C bands, with some small features appearing at 1436 and 1643 cm^{-1} (Figure 4b). To amplify the minor change in the SERS signal, the SER spectra of X and Y sequences were again normalized with that of PO_2^-, and Y spectrum was subtracted with X spectrum. Surprisingly, the difference spectrum shows very distinct spectral features (Figure 4c), in comparison with the SER spectra of T_{12} (Figure 4d) and A_{12} (Figure 4e). We observed a loss in intensity of the A-related peaks (721 cm^{-1}) and a gain in intensity of the T-related peaks (739 and 1643 cm^{-1}), which faithfully reflects the molecular composition of the bases in the DNA molecules. The result convincingly demonstrates that the method is able to identify the single-base difference in the DNA sequence, which is highly important and meaningful to the detection of single-base mutations and DNA

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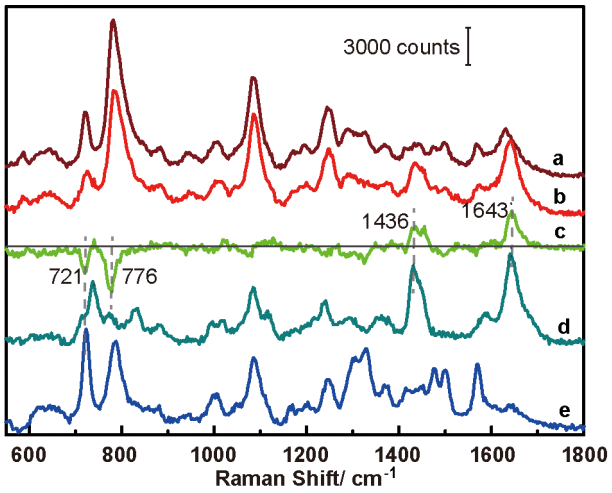

Figure 4. SER spectra of (a) X (ACA ACC CCC A) and (b) Y (TCA ACC CCC A) and (c) the difference spectrum of X − Y. Spectra of (d) T_{12} and (e) A_{12} are also given for a better identification of the peaks.

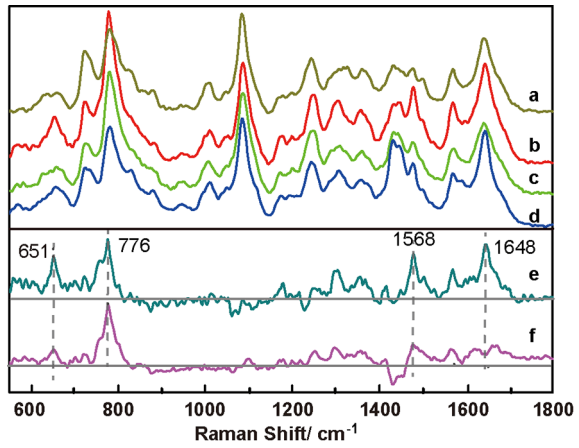

Figure 5. Upper panel: SER spectra of double-stranded DNA (a) dsSN6 and (b) dsSN5 and single-stranded DNA (c) ssSN5 and (d) ssSN5C. The spectra were normalized by the peak intensity of 1087 cm^{-1}. Lower panel: difference spectra of (e) dsSN5 − ssSN5 and (f) dsSN5 − ssSN5C, and the difference spectra were normalized using the intensity at 776 cm^{-1}. The gray solid line is the zero line to guide the eyes.

hybridization. This is also the core of the present work. Although the method is unable to determine the absolute order within the sequence, it is able to rapidly determine the absolute content of each base in the DNA sequence with a known DNA chain length.

The detection of DNA hybridization lays an important foundation for life science research and forensic applications. The grand challenge in the SERS detection of DNA hybridization is that both the target and probe sequences consist of the same four DNA bases. It is extremely challenging to distinguish whether a specific SERS signal originated from the hybridization of the target and probe sequences. In the literature, use of an adenine-free sequence or the combination of SERS and least-squares analysis has been proposed to detect the hybridization event.[7a,10] To detect the hybridization event, the SERS signal of the dsDNA has to be obtained first. It has been pointed out in the literature that it is much more difficult to detect dsDNA, especially unmodified dsDNA, than ssDNA, because the four bases are encapsulated inside the phosphate backbone, which leads to a weak interaction of DNA with the metallic surfaces and renders them from being in a region of a high SERS activity.[3a,7a,10,11e] In the above study, we have demonstrated the single-base sensitivity of our method for the detection of ssDNA, and it would be intriguing if hybridization could also be detected using the present method. The feasibility was tested by measuring the SER spectra of two kinds of dsDNA, dsSN5 and dsSN6 (see Table 1 for the detailed sequences for the related ssDNA). Thiolated SN5 is a 30-base DNA and has been used in ref 3a for the detection of the structural change of DNA after interacting with cisplatin and transplatin. SN6 is a 55-base DNA, has been used in our lab for DNA origami purposes, and can be considered a random DNA. The SER spectra of dsSN6 and dsSN5 are shown in Figure 5a,b. A distinct difference between the SER spectra of dsSN5 and dsSN6 can be observed. For clarity, we again normalized the spectra with the peak of PO_2^- at 1087 cm^{-1}, and we found that dsSN5 produces a stronger C signal at 778 cm^{-1} and weaker A signal at 732 cm^{-1}, but the dsSN6 shows the opposite trends. This trend agrees with a high C content (27%, versus 23% A content) in dsSN5 and a high A content (35%, versus 15% C content) in dsSN6. This result explicitly manifests that the Ag IMNP-based SER method can be reliably applied for the detection of not only ssDNA but also dsDNA. We then measured the two complementary single-stranded sequences (SN5 and SN5C) of dsSN5 to see how the hybridization will affect the molecular signature (shown in Figure 5c,d). There are some obvious differences in the SER spectra of dsDNA with that of SN5 and SN5C. After hybridization, the 781 cm^{-1} band (assigned to C) shifts to 778 cm^{-1}, and the band 659 cm^{-1} (assigned to G) shifts to 654 cm^{-1}. This phenomenon may be attributed to the formation of the strong hydrogen bonds between C and G in duplex DNA. The corresponding difference spectra (dsSN5 − ssSN5 in Figure 5e and dsSN5 − ssSN5C in Figure 5f) reveal the minor difference more clearly. Positive G, C, and A bands were observed both in the dsSN5 − ssSN5 and dsSN5 − ssSN5C spectra at 651, 776, and 1568 cm^{-1}, respectively, whereas negative T features were observed at 1433 cm^{-1} in the dsSN5 − ssSN5C. Another positive band in the dsSN5 − ssSN5 spectrum located at 1648 cm^{-1} is from the carbonyl stretching modes, which is extremely sensitive to the disruption of Watson−Crick hydrogen bonding.[17] Therefore, our method can also detect a hybridization event directly.

CONCLUSION

In summary, we have developed an iodide-modified Ag nanoparticle for use in an important method for the reliable, truly label free detection of DNA in aqueous solutions close to physiological conditions. The iodide layer not only cleans the surface impurities but also prevents the direct interaction of DNA with the surface, ensuring that a highly reproducible signal is obtained. $MgSO_4$ not only neutralizes the surface so that the negatively charged DNA can be adsorbed more effectively but also induces the aggregation of Ag IMNPs to produce a strong SERS signal. The ability to detect the SERS signal from phosphate backbone allows us to use it as an internal standard, against which the SERS spectra of DNA oligonucleotides can be normalized. With this calibration, we are able to determine what fraction of an oligonucleotide is made up of each of the bases and obtain the absolute content of

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each base in a DNA sequence. Clear identification of DNA with single-base sensitivity and the observation of a hybridization event have been successfully demonstrated. The method will have a widespread implication in DNA analysis, especially DNA modification and damage in bioassays.

■ ASSOCIATED CONTENT

Supporting Information

Normal Raman spectra of A_{12}, T_{12}, C_{12}, and G_{12}; SER spectra of X (ACA ACC CCC A) and Y (TCA ACC CCC A) without normalization; and SER spectra of double-stranded DNA dsSN6 and dsSN5 and single-stranded DNA ssSN5 and ssSN5C without normalization. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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