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Detecting Chemically Modified DNA Bases Using Surface Enhanced Raman Spectroscopy

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Abstract

Post-translational modifications of DNA- changes in the chemical structure of individual bases that occur without changes in the DNA sequence- are known to alter gene expression. They are believed to result in frequently deleterious phenotypic changes, such as cancer. Methylation of adenine, methylation and hydroxymethylation of cytosine, and guanine oxidation are the primary DNA base modifications identified to date. Here we show it is possible to use surface enhanced Raman spectroscopy (SERS) to detect these primary DNA base modifications. SERS detection of modified DNA bases is label-free and requires minimal additional sample preparation, reducing the possibility of additional chemical modifications induced prior to measurement. This approach shows the feasibility of DNA base modification assessment as a potentially routine analysis that may be further developed for clinical diagnostics.

Keywords

Epigenetic; SERS; DNA; DNA methylation; DNA oxidation

The chemical modification of DNA bases has become a topic of rapidly increasing interest in the assessment of human disease. There are two major types of DNA base chemical modifications of interest in this context. The first occurs in epigenetics, the study of alterations in gene expression induced by changes other than modifications in the genetic code. Chemically modified bases can alter phenotype without changing DNA sequence. (1) Epigenetic markers are thought to arise due to the influence of environmental factors influential in the onset of diseases such as cancer. In eukaryotic cells, methylated cytosine (mC) is the most common epigenetic marker.(2) In addition to its role in controlling gene expression, DNA methylation has been shown to strongly correlate with cancer in humans. Hypomethylation of DNA in human tumors,(3) hypermethylation of tumor-suppressor genes(4, 5) and the inactivation of microRNA genes by DNA methylation(6) are all strong

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evidentiary factors of a relationship between the presence of chemically modified DNA bases and various human cancers. Hydroxymethylcytosine (hmC) is a stable DNA modification of great interest as a new epigenetic marker that has recently been discovered. (7, 8) Hmc is highly abundant in the brain and believed to be important in neuronal function. It is currently accepted that hmC performs a fundamentally different epigenetic function than mC, making discrimination between hmC and mC extremely important; however, distinguishing hmC from mC is very challenging. Common methods such as enzymatic approaches and bisulfite sequencing have proven unable to distinguish mC from hmC in DNA.(9)

A second type of chemically modified DNA that is also extremely important is oxidized DNA. DNA oxidation occurs as a result of the interaction of reactive species of oxygen or nitrogen with genomic DNA.(10) The most common form of DNA oxidation in eukaryotes is guanine oxidation,(11) resulting most commonly in G to T transverse mutation.(12) Despite the absence of a firm correlation between guanine oxidation and cancer, there is strong evidence that the level of guanine oxidation in genomic DNA is a relevant biomarker for assessing antioxidant status and cancer risk.(13)

Typical methods for detecting DNA base modifications include single cell gel electrophoresis assay(13) for guanine oxidation detection, and bisulfite-based (14)-(15, 16) or enrichment-based methods(15, 16) for DNA methylation. All these methods require multiple-step sample preparation with chemicals that may induce additional chemical modifications in the DNA or interfere with the detection of modified DNA. In addition, most of these methods fail to distinguish between hmC and mC. The low throughput of these methods make them unreliable strategies for the development of clinical diagnostic assays. Therefore, the need for robust, streamlined methods for detecting chemically modified DNA bases is an important goal for analytical research, with the promise of clinical applications and technological impact.

Surface enhanced Raman spectroscopy (SERS) has been extensively explored to detect different types of biomolecules using broad range of nanostructures.(17-20) In particular, Au nanoshells has shown great success as an active SERS substrate.(21-23) In this article, we report a simple and direct method for detecting the DNA base chemical modifications of adenine and cytosine methylation and hydroxymethylation, and guanine oxidation, using SERS. This method is based on identifying SERS spectral variations due to DNA base modifications. No chemical treatments are needed, which eliminates the interaction of DNA with other chemicals, minimizing unwanted chemical modifications due to sample preparation. Applying this method would make detection of DNA base modifications straightforward.

Adenine Methylation

Adenine methylation is common in microbial genomes. N⁶-methyl-adenine is found in the genomes of fungi, bacteria, and protists.(24) The role of DNA base methylation in prokaryotes is still under investigation. It is believed that adenine methylation is involved in genome defense, DNA replication and repair, gene regulation and expression, and host-pathogen interaction in some groups of Proteobacteria.(25-27) All these functions are based on regulating the interaction between DNA and DNA-binding proteins as a result of introducing the adenine base methylation.(28, 29) Since adenine methylation is a reversible modification it can be easily used to determine when and where certain protein binding occurs at a specific DNA site of the genome. The detection of adenine methylation is becoming the main interest for modern genomic technologies owing to its potential applications in fields such as basic biology and clinical diagnosis.

The spectrum of thermally pretreated 12 bases single stranded DNA sequences (TCAAGCTGTGAC) is shown in Figure 1a. As expected, the SERS spectrum is dominated by the 736 cm^{-1} peak (adenine breathing mode).(21) When all adenine bases are substituted by 6-methyladenine (TCA*A*GCTGTGA*C) two new peaks appeared on the SERS spectrum, at 390 , 1100 and 1197 cm^{-1} (Fig. 1b). The 625 cm^{-1} adenine peak disappears, and the 665 cm^{-1} peak (guanine breathing mode) has higher intensity. The appearance of these new peaks is a confirmative marker indicating the methylation of adenine bases.

The studied methylated adenine DNA sequence is synthetically pure with all adenine bases substituted with methylated adenine; therefore, spectral changes were substantial. Real DNA samples will be mixtures of modified and unmodified sequences. An Ideal detection technique should be able to detect adenine methylation in the presence of all DNA bases including unmodified adenine. Therefore, a mixture of modified (TCA*A*GCTGTGA*C) and unmodified (TCAAGCTGTGAC) DNA sequences was investigated. It is worth to note that DNA mixture contains the same number of modified and unmodified DNA molecules. Our previous work on SERS of DNA showed that the DNA spectra depend only on base composition (ratio of different bases with respect to each other) especially for bases with higher cross sections (adenine and guanine) regardless the base order.(21) Thus, having modified and unmodified adenine on different sequences should be similar to having them on the same sequence. The same reasoning is valid for all modified DNA bases. Figure 1c shows SERS spectrum of the DNA mixture. The same spectral features that appeared on pure modified DNA are still present in the case of the DNA mixture indicating the possibility of adenine methylation detection even when modified and unmodified DNA sequences are combined. The adenine methylation indicative peaks appear less intense due to the dilution of methylated adenine as well as the difference in cross sections. The relative dominance of the SERS spectrum of the DNA mixture by the unmodified DNA strongly suggests that modified bases have a lower cross section. However, the spectral change is still confirmative enough to prove the presence of methylated adenine bases. Because the ultimate goal is to apply SERS to determine the adenine methylation patterns on genomic DNA, a technique such as ligationmethylated PCR(30) could be used to amplify highly methylated genomic regions prior to SERS detection, since the occurrence of adenine methylation can be relatively low (out of 100 adenine residues, 1.4 to 2 are methylated in *E coli*)(31).

Cytosine Methylation

The cytosine methylation is the most abundant epigenetic marker in eukaryotes. The occurrence of cytosine methylation ranges from almost undetectable to very abundant.(32, 33) In mammals, cytosine methylation changes during development and is strongly related to gene expression.(34) In humans, the lack of methylation causes diseases such as Immunodeficiency Craniofacial Syndrome and Rett Syndrome.(35) In addition, irregular DNA methylation is a general marker of cancer. For example, epigenetic silencing of tumor suppressor genes causes sporadic human cancers.(36) Unfortunately, cytosine bases possess a significantly weak Raman cross section compared to adenine and guanine.(21) Cytosine SERS features can hardly be distinguished in DNA SERS spectrum. Therefore, it is expected that the methylation of cytosine bases would not introduce large spectral variations in DNA spectrum. Figure 2b shows that the substitution cytosine by 5-methylcytosine (TC*AAGC*TGTGAC*) causes a slight increase in the 786 cm^{-1} intensity (cytosine breathing mode). For the DNA mixture (cytosine methylated DNA and normal DNA), the increase of the 786 cm^{-1} is still detectable and can be considered a marker for cytosine methylation as shown in Figure 2c.

Cytosine methylation of many eukaryotes frequently occurs at the CpG nucleotide sequences and 60 to 90 % of all CpGs in mammals are methylated.(37, 38) Given this high degree of cytosine methylation, mC SERS detection in biological DNA samples is still possible without the need of cytosine methylated DNA amplification. Although changes in the SERS DNA spectrum of this base are relatively minor, detection is still possible if DNA regions are carefully chosen for SERS analysis.

Cytosine Hydroxymethylation

Hydroxymethylcytosine is an oxidized form of methylcytosine and its function is still under rigorous investigation.(39) hmC was found in DNA samples from neurons, brain(7) and embryonic stem cells.(8) The abundance of hmC in genomic DNA is still unknown. The hydroxymethylation of cytosine introduces a significant change in the DNA SERS spectrum. It is worth noting that this particular DNA sequence has only one hmC due to synthesis limitations (TGAC*AGTTGTGATAG). Figure 3b shows a significant intensity increase of the 665 cm^{-1} mode as well as the 963 cm^{-1} feature. Most importantly, the 1397 cm^{-1} is greatly suppressed as a result of the presence of the hmC base in the DNA sequence. The spectral variations between normal DNA and hydroxymethylated DNA are significant and dramatic, making verification of the presence of hmCs in a DNA sequence using SERS very straightforward. This data clearly indicates that a discrimination between mC and hmC is possible using SERS. The significant increase of the 665 cm^{-1} peak is an obvious marker of the presence of hmC. The relative increase of the 786 cm^{-1} peak intensity for cytosine methylated DNA is still larger than the same peak increase for hydroxymethylated cytosine, rendering the detection of mC in the presence of hmC possible as well.

When hydroxymethylated DNA sequence was mixed with normal DNA, all spectral changes became indistinguishable (Figure 3c). The SERS spectrum of the DNA mixture is very similar to pure unmodified DNA. This is expected since the modified sequence contains only one hmC. The abundance of hmC in particular genomic DNA regions will determine whether a complementary amplification technique is needed.

Guanine Oxidation

The SERS spectrum of guanine oxidized DNA (where all guanine bases are substituted with 8-oxo-guanine, (TCAAG*CTG*TG*AC) was acquired (Figure 4). Here there are two significant markers indicating guanine oxidation: the appearance of a new mode at 1079 cm^{-1} and the disappearance of the guanine breathing mode (665 cm^{-1}). A 390 cm^{-1} peak has also appeared similar to the peak with adenine methylation. Since the same peak appears with adenine and guanine with completely different chemical modifications, we speculate that this peak is due to the interaction of the modified bases with the Au surface. When oxidized guanine DNA sequence was mixed with unmodified DNA the 1079 cm^{-1} new mode is still present but less intense (Figure 4.c). However, the remarkable decrease of the 665 cm^{-1} is still a strong marker indicating the presence of 8-oxo-guanine. It is clear that the 8-oxo-guanine base does not possess a peak at 665 cm^{-1} and the small peak that appears on Figure 4.c is due to the guanine bases from the unmodified DNA.

SERS of DNA mixture

To examine the capability of SERS to simultaneously detect multiple DNA base modifications, a mixture of all modified DNA sequences previously describes was prepared. The mixture was prepared such that the final concentration of each sequence is the same. The SERS spectrum (Figure 5) shows that three out of 4 modifications were discernable. There are spectral proves indicating the presence of methyladnine, methycytosine and 8-

oxo-guanine. For the same reason previously described, the detection of hydroxymethylcytosine was not possible.

In light of these initial, proof-of-concept studies, more detailed experimental investigations coupled with theoretical analysis are needed to identify the specific structural origin of these observed changes in the SERS spectrum. It is possible that the presence of modified DNA bases may affect the overall DNA spectrum by introducing new modes as well as suppressing or enhancing existing modes related the modified base itself. Also, modified bases may affect the DNA/ surface interaction causing enhancement and/or suppression of modes not related to the modified base itself. While the scope of this initial study was limited to the experimental demonstration of SERS detection and the identification of spectral markers for specific DNA base modifications, further investigations, and protocols combining SERS with amplification techniques, may prove to be a useful analytical tool for the identification of chemical modifications in DNA sequences.

Au nanoshells were synthesized according to previously published procedures.(40, 41) The dimensions of the silica core (120 nm colloidal silica, Precision Colloids LLC, Cartersville GA) and the Au shell were chosen such that the peak plasmon resonance in aqueous suspension was 785 nm, corresponding to the excitation wavelength used in this experiment. Nanoshell-based SERS substrates consisting of dispersed nanoshells bound to glass substrates were prepared as following. A fused quartz microscope slide (Piranha cleaned) was incubated overnight in an (1%) ethanolic solution of poly(4-vinylpyridine) (MW = 160,000 from Sigma-Aldrich) and dried with nitrogen gas. Subsequently, 100 μ l of a concentrated, aqueous nanoshell suspension was deposited onto the substrate. The substrate was then allowed to sit at room temperature for 3-4 h to allow nanoshells immobilization onto the wafer forming a dense layer of nanoshells that will be used as the SERS active substrate. To remove non-immobilized nanoshells, the substrate was rinsed with Milli-Q water (Millipore, Billerica, MA), followed by drying with a gentle flow of nitrogen.

All DNA sequences used in this study were custom synthesized (Integrated DNA Technology, Inc). Modified bases were inserted during the sequence synthesis such that the number and position of modified bases is well determined. DNA sequences were used as received, having been HPLC-purified by the vendor. DNA uncoiling was achieved by heating the DNA solutions in a water bath to 95°C for 10-15 min, followed by rapid cooling in an ice bath. To bind DNA to the nanoshell SERS substrates, 100 μ L of thermally uncoiled DNA sequence (100 μ M) was deposited onto a freshly made nanoshell SERS substrate. In case of DNA mixture, equal volumes of different thermally uncoiled sequences (all at the same concentration, 100 μ M) were mixed to a final volume of 100 μ L and deposited onto freshly made SERS substrate. After overnight incubation, the excess DNA solution was removed by rinsing with Milli-Q water.

SERS spectra were recorded while substrates were immersed in water using a Renishaw inVia Raman microscope (Renishaw, U.K.) with 785 nm excitation wavelength. Backscattered light was collected using a 63x water immersion lens (Leitz, Germany), corresponding to a rectangular sampling area of 3 μ m $\times$ 30 μ m. All the SERS spectra in this work were obtained with an integration time of 20 seconds and a laser power of 0.57 mW before the objective. All SERS spectra are average of at least 5 individual spectra acquired from different spots on the same substrate to insure spectral reproducibility. All SERS spectra have a Γ (spectral correlation function) closer to 1.(21, 42)

In conclusion, we demonstrated that SERS can be used to identify chemically modified DNA bases, including methylated adenine, methylated and hydroxymethylated cytosine, and oxidized guanine in purely synthesized DNA. When modified DNA sequence was mixed

with unmodified DNA sequence, the identification of three base modifications was still valid. Since modified DNA sequence contains only one hmC, the SERS detection of hmC was not possible when modified DNA was mixed with unmodified DNA. All identified markers are unique and evident for discrimination of the specific modification. No chemical pretreatment was used, maintaining the integrity of the DNA sequence and making this a potentially promising approach for diagnostic applications.

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Figure 1. SERS spectra of (a) normal DNA, (b) adenine-methylated DNA, and (c) mixture of normal and adenine-methylated DNA. Inset: molecular structures of (top) adenine, and (bottom) 6-methyladenine. Dashed green lines are a guide to the eye used to highlight the main changes in SERS spectra.

Figure 2.

SERS spectra of (a) normal DNA, (b) cytosine-methylated DNA, and (c) mixture of normal and cytosine-methylated DNA. Inset: molecular structures of (top) cytosine, (bottom) 5-methylcytosine. Dashed green line is a guide to the eye used to highlight the appearance of a new peak in case of cytosine-methylated DNA.

Figure 3. SERS spectra of (a) normal DNA, (b) cytosine-hydroxymethylated DNA, and (c) mixture of normal and cytosine hydroxymethylated DNA. Inset: molecular structures of (top) cytosine, (bottom) 5-hydroxymethylcytosine. Dashed green lines are a guide to the eye used to highlight the main changes in SERS spectra.

Figure 4. SERS spectra of (a) normal DNA, (b) guanine-oxidized DNA, and (c) mixture of normal and guanine-oxidized DNA. Inset: molecular structures of (top) guanine, and (bottom) 8-oxo-guanine. Dashed green lines are a guide to the eye used to highlight the main changes in SERS spectra.

Figure 5.

SERS spectra of (a) normal DNA and (b) mixture of adenine methylated, cytosine methylated, cytosine hydroxymethylated and guanine-oxidized DNA sequences. 1, 2 and 3 represent spectral probes of the presence of 8-oxo-guanine, methylcytosine and methyladenine respectively. Dashed green lines are a guide to the eye used to highlight the main changes in SERS spectra.