Abstract: The exploring of the genetic information carried by DNA has become a major scientific challenge. Routine DNA analysis, such as PCR, still suffers from intrinsic important limitations. Surface-enhanced Raman spectroscopy (SERS) has emerged as an outstanding opportunity for the development of DNA analysis but the application to duplexes (dsDNA) has been largely hampered due to reproducibility and/or sensitivity issues. Here we show a simple strategy to perform ultrasensitive direct label-free analysis of unmodified dsDNA with the means of SERS by using positively-charged silver colloids. Electrostatic adhesion of DNA promotes nanoparticle aggregation into stable clusters yielding intense and reproducible SERS spectra at nanogram level. As potential applications, we report the quantitative recognition of hybridization events as well as the first examples of SERS recognition of single base mismatches and base methylations (5-methylated cytosine and N6-methylated Adenine) in duplexes.

DNA is the custodian of the memory of life. Although very stable, it is subject to modifications which are associated with evolution but more frequently with genetic diseases or cancer. Due to the small content of DNA in cells and its structural complexity, its analysis requires denaturation (heat), fragmentation (restriction endonucleases), separation (electrophoresis), amplification (polymerase chain reaction, PCR) and detection (quantitative PCR or microarray techniques). Besides the cost both in time and money, during these processes samples are subject to alterations and losses of epigenetic information. The advent of nanophotonics offers a unique opportunity for the development of direct, fast and ultrasensitive methods for DNA analysis. In the broad field of plasmonics, surface-enhanced Raman scattering (SERS) spectroscopy has arisen as a powerful analytical tool for detection and structural characterization of biomolecules. The large majority of SERS-based DNA detection strategies rely on the selective recognition between two complementary strands to identify a specific sequence, and the use of extrinsic Raman labels for SERS readout. However, labeling of the DNA strands requires complex and costly chemistry, and does not provide any chemical-specific information. A second approach is based on the direct detection of the distinctive SERS signal from DNA strands directly adsorbed onto the nanostructured surface. This strategy shows outstanding potential in terms of sensitivity, selectivity and chemical-specific information. To date, however, direct SERS analysis of unmodified DNA is mainly restricted to single-stranded DNA sequences (ssDNA) since the direct contact of double-stranded sequences (dsDNA) with nanostructured metallic surfaces (negative at the physiological pH) is largely hindered by the negative charge of the phosphate backbone, unless relatively high DNA concentrations are employed. As a result, dsDNA SERS spectra traditionally suffer from inherent poor spectral reproducibility and/or limited sensitivity hampering the extended application of label-free SERS strategies for the study of unmodified duplexes. Thus, successful translation of the analytical potential of SERS to the direct study of dsDNA would represent a great leap forward for the full exploration of the genetic information.

To overcome this major challenge, in this study we use positively-charged silver nanoparticles coated with spermine molecules (AgNP@Sp). Spermine bound to AgNPs acts as stabilizer and, upon addition of negatively-charged DNA, promotes nanoparticle aggregation into stable particle clusters. This has important consequences for the direct SERS analysis of dsDNA. Firstly, the DNA adsorption occurs via non-specific electrostatic interaction of the phosphate groups rather than by base-specific nucleoside-metal binding. Secondly, DNA promotes the nanoparticle aggregation removing the need of external aggregating agents. On the one hand, this significantly simplifies the sensing scheme, reducing the number of variables to be controlled (type and concentration of the aggregating agent, time and order of addition, etc.). On the other hand, DNA sequences are selectively sandwiched between interparticle junctions (i.e. hot-spots) thus maximizing the intensification of the Raman signal. Thirdly, the nanoparticle aggregation occurs fast and independently of the base composition, generating long-term stable clusters in suspension, as demonstrated by the extinction spectra illustrated in Fig. 1b. This also allows us to perform the SERS measurements after several hours upon the analytical addition (i.e. when the process of DNA adsorption has reached equilibrium). Finally, SERS spectra are acquired in colloidal suspensions with a long working distance objective. In this experimental set-up, the recorded SERS spectra are the statistically-averaged result of a large ensemble of nanoparticle/DNA clusters in continuous Brownian motions within the scattering volume. Such averaged bulk SERS regime yields good-quality spectra with well-defined average band centers, bandwidths and relative intensities. As a result, dsDNA electrostatic adhesion onto the AgNP@Sp nanoparticles allows for the ultrasensitive...
The overall dsDNA concentration was kept constant throughout the entire study at ca. 630 ng/mL (corresponding to less than 3.0 ng in the probed volume, see Section S2) and appropriately selected to yield long-term stable clusters in suspension with reproducible and unvaried SERS spectral profiles (Section S2). Vibrational assignment of dsDNA was based on literature references and comparative spectral analysis with homo- and bi-polymeric sequences (Section S3). In contrast with previous results on negatively charged colloids,[13] when the individual ss units hybridize into the ds a large reshaping of their SERS spectra is observed (Fig. 1c), influencing peak position, bandwidths and relative intensity. Notably, if ss1 and ssc spectra show small changes in relative intensity that are associated with the base composition, the stacking and pairing of the nucleobases into the hybridized native structure is revealed by several characteristic features of the Raman melting profiles.[14] Among others, we highlight the marked intensity decrease and peak-shifting of the ring breathing modes (700-800 cm⁻¹) and the carbonyl stretching modes (1653 cm⁻¹), the latter one extremely sensitive to the disruption of Watson-Crick hydrogen bonding.[14] We also observe a general narrowing of the Raman features in the 1150–1600 cm⁻¹ spectral region, containing many overlapping bands mainly arising from in-plane vibrations of base residues.[15] Such spectral modification can be ascribed to the adoption of a more rigid and well-defined conformation by the two individual ss when they self-organize into the rigid duplex geometry. The investigation of a set of solutions containing different ds1 and ssc molar ratios, R_{hybr} = [ds1]/([ds1] + [ssc]) (Fig. 1d) shows a progressive shift of the ring breathing bands of adenine (from 734 cm⁻¹ to 730 cm⁻¹) when the DNA population is enriched with ds1. The spectral subtraction of the SERS spectra allows us to fully disclose the spectral alterations associated with changes in the DNA structure. In particular, the difference spectrum ds1-ssc (dotted line, Fig. 1d) reveals a minimum at 724 cm⁻¹ and a maximum at 738 cm⁻¹. The corresponding ratiometric peak intensities \( \frac{I_{724}}{I_{738}} \) were then selected to monitor the relative ds1/ssc populations in the samples, and plotted against \( R_{hybr} \) (Fig. 1e). Data shows that ds sequences can be clearly identified within the mixture even when present at values < 1%, whereas SERS spectra are fully dominated by ds1 contributions for duplex populations ≥ 20%. Such result is consistent with the higher affinity of ssDNA to AgNP@Sp due to the larger availability of negatively-charged phosphate groups as compared to ssDNA. Linear correlation (\( r^2 > 0.99 \)) is observed in the 0.01-0.19 \( R_{hybr} \) range.

To test the possibility of detecting single-base mismatches in DNA duplexes, SERS spectra of the full-complementary ds1 and the heteroduplexes ds2, ds3 and ds4 were acquired and compared (Fig. 2). These heteroduplexes contain one adenine base, A, in place of: (ds2) one guanine, G, (ds3) one cytosine, C, (terminal position) and (ds4) one cytosine (internal position). Subtraction of the SERS spectra of ds1 from the other samples generates difference spectra containing vibrational signatures associated with the additional (positive features) and removed (negative features) nucleobase. Positive A bands emerge in all difference spectra at 730 and 1507 cm⁻¹, whereas negative G features are observed at ca. 620, 675 and 1354 cm⁻¹ in the ds2-ds1. In this case, negative bands also appear at 1487 and 1577 cm⁻¹, ascribed to purine modes (mainly G contribution) while the other purine feature at 1325 cm⁻¹ (mainly A contribution) does not suffer from a marked change in the relative intensity. These results, together with the lack of significant changes in SERS intensity of the pyrimidine bases, clearly indicate that the spectral alterations are ascribed to the A→G base-mismatch in the ds2. On the other hand, ds3-ds1 and ds4-ds1 difference spectra show very similar spectral patterns where, in addition to the positive

**Figure 1.** (a) Schematic diagram of dsDNA sandwiched between two positively charged AgNP@Sp colloids. (b) Extinction spectra of pure AgNP@Sp colloids and in the presence of ds1 (final conc. 630 ng/mL) acquired 2h and 24h upon DNA addition (the AgNP@Sp-ds1 mixture was sonicated for few seconds before the spectral acquisition). (c) SERS spectra of ss1 and ssc (3.15 μg/mL), and ds1 (630 ng/mL) on AgNP@Sp. SERS spectra were normalized to the PO labile phosphate group (630 nm) before 2 h and 24 h before the spectral acquisition). (d) SERS spectra of ss1 and ssc (3.15 μg/mL), and ds1 (630 ng/mL) on AgNP@Sp. SERS spectra were normalized to the PO labile phosphate group (630 nm) before 2 h and 24 h before the spectral acquisition). (e) Ratiometric peak intensities \( \frac{I_{724}}{I_{738}} \) vs. \( R_{hybr} \) (Fig. 1e). Data shows that ds sequences can be clearly identified within the mixture even when present at values < 1%, whereas SERS spectra are fully dominated by ds1 contributions for duplex populations ≥ 20%. Such result is consistent with the higher affinity of ssDNA to AgNP@Sp due to the larger availability of negatively-charged phosphate groups as compared to ssDNA. Linear correlation (\( r^2 > 0.99 \)) is observed in the 0.01-0.19 \( R_{hybr} \) range.

The average SERS spectra of two complementary single-stranded sequences (ss1 and ssc) and their corresponding double-helix structure (ds1) on AgNP@Sp colloids are shown in Figure 1c.
A (730 and 1507 cm\(^{-1}\)) and the negative C (1250 and 1528 cm\(^{-1}\)) contributions, a consistent intensity increase of the purine bands (1325, 1487 and 1577 cm\(^{-1}\)) is observed. The pyrimidine ring breathing (787 cm\(^{-1}\), C+T) undergoes a drastic intensity decrease whereas thymine marker bands do not reveal significant alterations. These spectral changes can therefore be associated with the A-C base mismatch in ds3 and ds4. Minor differences between ds3 and ds4 difference spectra (Fig. 2) can be ascribed to the different position of the base mismatch within the sequence.\(^{[10]}\)

The potential application of AgNP@Sp in SERS analysis of 5-methylated cytosine bases (\(^{\text{mC}}\)) and 6-methylated adenine bases (\(^{\text{mA}}\)) within ds structures was examined by acquiring the SERS spectra of ds\(^{\text{A}}\) and ds\(^{\text{mA}}\) and comparing to their unmethylated analogues ds1. In ds\(^{\text{A}}\) and ds\(^{\text{mA}}\), the C or A nucleobases of one of the strands were all replaced by their respective 5-methylated and N6-methylated counterparts (see sequence structures in Fig. 3 and Fig. 4). All the spectral changes are in good agreement with the normal Raman studies of the substitution of the methyl group into the 5C and N6 atom in cytosine\(^{[8]}\) and adenosine\(^{[11]}\) nucleosides. The major spectral markers of the cytosine methylation can be easily recognized through the whole difference spectrum (Fig. 3a). Among others, we observe a red-shift and relative intensity decrease of the pyrimidine ring breathing band at 787 cm\(^{-1}\), together with the appearance of a new weak feature at ca. 753 cm\(^{-1}\). In the 1200-1450 cm\(^{-1}\) spectral region, the normal Raman of unmodified cytidine aqueous solution contains two main bands at 1242 and 1292 cm\(^{-1}\), whereas the 5-methyl substituted shows a very different spectral signature with three discernible bands at 1224, 1258 and 1302 cm\(^{-1}\) and a new very intense feature at 1362 cm\(^{-1}\).\(^{[8]}\) Accordingly, the difference spectrum in Fig. 3a reveals two negative bands at ca. 1244 and 1287 cm\(^{-1}\); three positive contributions broadly centered at 1218, 1258 and 1315 cm\(^{-1}\), and a very strong and sharp band at 1362 cm\(^{-1}\). Moreover, the C ring band at ca. 1510 cm\(^{-1}\) also undergoes a marked red-shift and intensity decrease while the broad carbonyl stretching band centered at 1653 cm\(^{-1}\) shows a large red-shift (well highlighted in the difference spectrum by the negative feature at ca. 1622 cm\(^{-1}\) and the positive one at ca. 1662 cm\(^{-1}\)).

![Figure 2. SERS spectra of ds1 and ds2; and digitally subtracted SERS spectra ds2-ds1, ds3-ds1 and ds4-ds1. For the sake of clarity, the digitally subtracted spectra were multiplied by 3 for ds2-ds1 and 4 for ds3-ds1 and ds4-ds1. Frequency positions of characteristic nucleotide bands are highlighted in: blue (Adenine), yellow (Cytosine), orange (Guanine) and green (Thymine). The overall ds concentration was kept constant at 630 ng/mL.](image)

The spectral modifications of the SERS spectral profile of the normal DNA upon methylation of six of their eleven the A bases are also striking (Fig. 4a, difference spectrum). The A ring breathing band at 731 cm\(^{-1}\) undergoes a drastic 11 nm up-shift together with a notable intensity decrease, whereas the pyrimidine stretching of the adenine at 1509 cm\(^{-1}\) almost disappears from the spectrum. Furthermore, we observe up-shifts of the phosphate stretching mode at 1090 cm\(^{-1}\) (together with a marked intensity increase); the v(CN) imidazole feature at 1487 cm\(^{-1}\) and the 1577 cm\(^{-1}\) band ascribed to base ring modes (mainly G+A).

Quantification of relative methylated base populations within the sample was investigated by monitoring the ratiometric peak intensities \(I_{\text{1528}}/I_{\text{1328}}\) for \(^{\text{mC}}\) and \(I_{\text{1487}}/I_{\text{1328}}\) for \(^{\text{mA}}\) at different molar ratios 
\[
R_{\text{mA}} = \frac{[^{\text{mA}}]/[^{\text{A}}]}{[^{\text{A}}]/[^{\text{mA}}]},
\]
and 
\[
R_{\text{mC}} = \frac{[^{\text{mC}}]/[^{\text{C}}]}{[^{\text{C}}]/[^{\text{mC}}]}.
\]
Details of the ring breathing spectral regions are illustrated in Fig. 3b and 4b. In the case of ds\(^{\text{A}}\) (Fig. 3b), we highlight the progressive intensity decrease of the C+T ring breathing band at 787 cm\(^{-1}\) upon increasing of the relative \(^{\text{mA}}\) populations. Differently, for ds\(^{\text{mC}}\) (Fig. 4b), the A ring breathing band at 731 cm\(^{-1}\) suffers a dramatic
intensity decrease and large peak red-shift as the $R_{\text{on}}$ value becomes larger. Linear correlations were obtained in both cases ($r^2$ >0.99 for $A$ and >0.94 for $C$) with detection limits of one $^1C$ per 22 total cytosine bases and one $^3A$ per 10 total adenine bases in the sample (Fig. 3c and 4c, respectively).

In summary, we have demonstrated the potential application of SERS for the development of a fast and affordable high-throughput screening method for gaining detailed genomic information on DNA duplexes. Importantly, due to the low amount of DNA required (comparable to only 10-100 cells), the analysis can be performed without the necessity of amplification thus providing realistic direct information of the DNA in its native state. Due to the direct nature of this sensitive and selective assay, we anticipate these findings of key importance in a number of different fields including diagnosis, genetic engineering, biotechnology, drug discovery (antibiogram in microbiology), agriculture and forensic science.


Acknowledgements

This work was funded by the Spanish Ministerio de Economía y Competitividad (CTQ2011-23167), the European Research Council (CrossSERS, FP7/2013 329131, PrioSERS FP7/2014 623527) and Medcom Advance SA. We are grateful to Dr. Matteo Masetti (Department of Pharmacy and Biotechnology, Università di Bologna, Bologna, Italy) for the dsDNA drawings.

Keywords: DNA • SERS • hybridization • single-base mismatch • methylated bases
COMMUNICATION

Accessing genomic information. Fast and affordable high-throughput screening method based on SERS as a low-cost and ultrasensitive genotyping strategy for gaining detailed genomic information on DNA duplexes: recognition of hybridization events, single-base mismatch and base methylation.

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Direct Surface-enhanced Raman Scattering analysis of DNA duplexes