

Analysis of Short Tandem Repeats by Using SERS Monitoring and Electrochemical Melting**

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The analysis of short tandem repeats (STRs) is commonly used to determine genetic profiles for forensic applications. We have used the techniques of surface enhanced Raman spectroscopy (SERS) and electrochemical melting to discriminate between different polymorphic repeat sequences of the D16S539 locus. We were able to distinguish between 10, 11, 12, 13, and 14 repeats of this commonly employed STR on the basis of the measured electrochemical melting potentials. Both SERS and electrochemical melting are approaches which can be incorporated into a portable technology for DNA analysis.^[1,2]

STRs are short repeating sequences of DNA (2–6 base pairs). They can be found at many loci within the human genome and those which are polymorphic have been used in the identification of individuals.^[3] The biology of STRs has been studied extensively and STR analysis has seen application in several fields including forensic analysis, paternity/kinship testing, and disease related linkage analysis.^[4–6] The current method of STR analysis is laboratory-based. A typical protocol involves DNA extraction, PCR amplification with 5'-labeled primers, electrophoretic separation, and analysis.^[7,8] HyBeacon probes have recently been used for interrogation of STRs.^[9,10] They are single-stranded oligonucleotides labeled with fluorescein on some of the heterocyclic bases. HyBeacons are being developed for rapid STR analysis at the scene of crime or in custody suites. They have the potential to be used with portable analyzers as they do not rely on laborious electrophoretic separation of STRs of different lengths. The disadvantage of this fluorescence-based approach is that the emission spectra of only a small number of different fluorescent dyes can be resolved in a single assay, thus limiting the number of STRs that can be analyzed simultaneously. This constraint does not apply to SERS labels, the spectra of which are far more information-rich.^[11]

D16S539 is a polymorphic locus commonly employed in STR analysis. Its repeating sequence is GATA which does so between 5 and 15 times. In this study, the D16S539 locus was employed as a model STR in order to determine whether analysis by SERS monitoring and electrochemical melting

was possible. SERS analysis of STRs has a number of potential advantages over traditional methods of DNA analysis. The technique is potentially more sensitive than fluorescence and the narrow spectral lines of a SERS spectrum would allow for simultaneous multiplex detection of labeled DNA sequences.

In earlier work we showed that we could use SERS together with electrochemical melting to discriminate single-nucleotide polymorphisms (SNPs) in short DNA strands.^[12,13] This method relies on the use of sphere segment void (SSV) surfaces to provide reproducible, stable SERS enhancements. The SSV surfaces are made by electrodeposition around closely packed monolayers of sub- μm polystyrene spheres. After electrodeposition the spheres are dissolved in a solvent, leaving a sculpted metal surface (Figure 1a) capable of

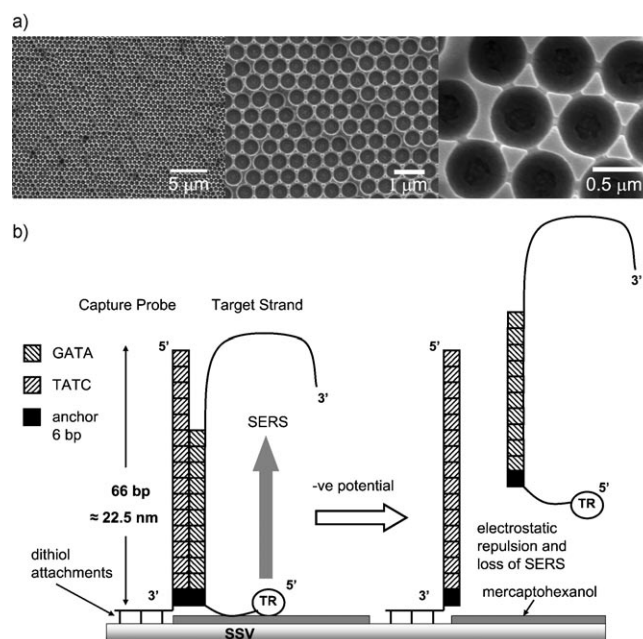


Figure 1. a) Scanning electron microscope (SEM) images at different magnification of the SSV electrodes used for the SERS measurements. b) Model representation of SERS and electrochemical melting of STR sequences on SSV substrates.

supporting both localized and propagating plasmons.^[14] These surfaces are thus excellent substrates for SERS.^[15–19] The optical properties of SSVs can be tuned^[20,21] by using different template sphere sizes and different film thicknesses. For suitably structured gold SSV surfaces SERS enhancements of 10^6 can be obtained.^[22] With the use of resonant dyes, where an electronic transition in the molecule is in resonance

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with the wavelength of the laser, an additional enhancement of 10^3 can be achieved.^[23] Because the SERS effect is localized to the metal surface (ca. 20 nm) there is no significant signal from molecules in the bulk solution; only those at the surface contribute. Compared to SNPs, STRs are longer sequences (often over 100 bp) with much smaller differences between their melting curves and T_m values. Successful STR discrimination, particularly between STR sequences of similar length is therefore significantly more challenging than SNP detection.

For many applications amplification of the target DNA sequences is an important first step in any assay. In this study, amplification was carried out using 5' SERS labeled primers and by conducting an asymmetric PCR to amplify significant amounts of single-stranded DNA bearing a SERS label. It was found that purification of the PCR reaction volume was not necessary. A distinct SERS signal for our label was easily detected with no interference from the other reagents present in the PCR reaction.

The assay (Figure 1 b) described here was conducted using a DNA target strand labeled at its 5' end with a Texas Red label ($\lambda_{\text{max}} = 590$ nm) partially resonant with the 633 nm laser. For analysis, SSV substrates were prepared by immobilizing a dithiol-modified capture probe on the gold SSV surface and then passivating the remainder of the surface with mercaptohexanol. Following an asymmetric PCR, the complementary target strand bearing the Texas Red label was hybridized with the immobilized capture probe in a high salt concentration (1M NaCl). Application of a negative potential then melts the hybridized probe/target and leads to the disappearance of the SERS signal. DNA and primer sequences are presented in Table 1. Full experimental details are given in the Supporting Information.

Table 1: DNA sequences used in this study.

	Sequence ^[a]
Forward primer	CATCTGTAAGCATGTATCTA
Reverse primer	Texas Red–CCTAGATCAATACAGACAG
D16S539 target	CATTACGTTTGTGTGTCATCTGTAAGCAT
10–14 repeats	GTATCTATCATCCATCTCTGTTTGTCTTTCAATGA (TATC) _n CACCTGTCTGTCTGTCTGTATTGATCTAGG GAAGAGGAAGAGCTTGGGATCTGCCTTTGTTTGTCTCC (TATC) ₁₅ CACCTG–HXHXHX
Capture probe	

[a] H = hexaethyleneglycol spacer; X = dithiol monomer; n = number of repeats (10 to 14).

Figure 2 shows a typical set of raw spectra from a SERS melting experiment. The peaks at 1501 and 1645 cm^{-1} are characteristic of Texas Red and are assigned to aromatic ring stretching and N–H deformation modes, respectively.^[13] Based on our earlier work,^[13] we estimate that spectra are representative of ca. 1500 DNA molecules illuminated by the laser spot. By performing a background subtraction on spectra and noting the intensity at 1501 cm^{-1} it was possible to construct a normalized SERS melting curve as shown in Figure 3 where the background-corrected peak intensities at 1501 cm^{-1} have all been normalized to the maximum value. As the potential is stepped more cathodic (in -100 mV

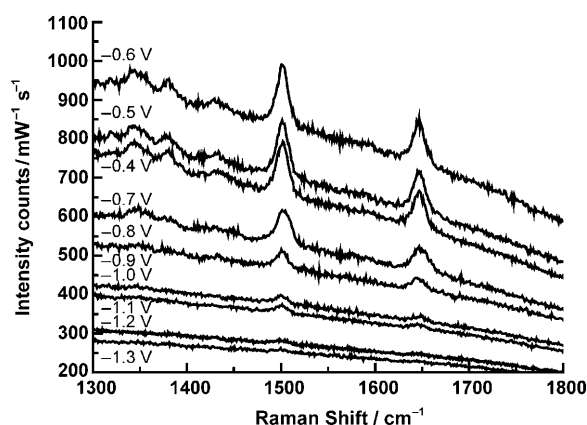


Figure 2. SERS spectra at increasingly cathodic potentials from the Texas Red labeled PCR product of the 10-repeat polymorph of the D16S539 locus in a pH 7.0 measurement buffer consisting of 10 mM Tris + 10 mM NaCl vs Ag/AgCl reference electrode. A ULWD 50x objective was employed on a Renishaw 2000 Raman microscope; acquisition time 5 s, laser power 2.4 mW.

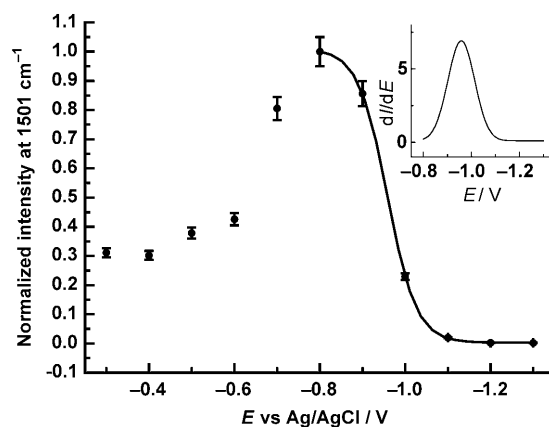


Figure 3. Typical melting curve for the 11-repeat polymorph of the D16S539 locus. The line is the best fit of the Boltzmann curve to the melting transition. The corresponding derivative is shown in the inset.

increments) the spectral intensity of Texas Red bands initially increases between -0.3 and -0.8 V and then decreases sharply as the dsDNA on the surface melts. Note that the initial increase in intensity is reversible and thought to be due to a change in orientation of the label with respect to the SSV surface.^[13] It is also notable in Figure 2 that the background signal follows the intensity of the Texas Red SERS bands showing the two effects are interrelated.^[13]

To establish the ability of the technique to discriminate different STRs the melting curves for five different polymorphic sequences of D16S539 (10–14 repeats) were recorded separately. These sequences ranged in length from 121 to 137 bp, increasing in 4 bp increments bound to a common capture probe containing 15 repeats of GATA and a 6 bp anchor region. Predicted thermal melting values of 55, 56, 57, 58, and 59 °C were calculated based on the nearest-neighbor principle.^[24] Calculations were performed using OligoCalc (www.basic.northwestern.edu/biotools/oligo-calc.html). For the calculations oligonucleotide concentra-

tions were estimated using the DNA surface coverage measurement of 1.6×10^{12} molecules cm^{-2} ^[13] and Na^+ concentrations of 10 mM were used.

Figure 4 shows a set of normalized melting curves for the five different polymorphic repeats of the D16S539 locus. In this case the spectra were recorded during a slow (0.5 mV s^{-1})

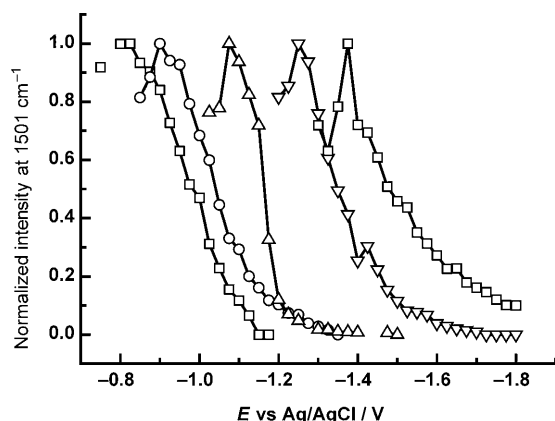


Figure 4. Melting curves for 10 ($\square$, left trace), 11 ($\circ$), 12 (Δ), 13 (∇) and 14 ($\square$, right trace) polymorphic repeats of the D16S539 locus recorded using a linear sweep of potential (0.5 mV s^{-1}). Measurements were performed in pH 7.0 measurement buffer consisting of 10 mM Tris + 10 mM NaCl vs Ag/AgCl reference electrode. A ULWD 50x objective was employed on a Renishaw 2000 Raman microscope; acquisition time 5 s, laser power 2.4 mW. Errors in the normalized intensities are ± 0.05 .

cathodic potential scan. The precise choice of scan rate does not affect the results. The electrochemical melting potentials, E_m , for each STR were obtained by taking the value of the potential at half maximum. On the basis of the measured electrochemical melting potentials (Table 2) we are clearly

Table 2: Melting potentials ($\pm 10 \text{ mV}$).

STR	10R	11R	12R	13R	14R
E_m vs. Ag/AgCl [V]	-0.98	-1.04	-1.15	-1.35	-1.48

able to discriminate the different STRs for the D16S539 locus in the unpurified PCR buffer solution. This is a significant challenge which illustrates the discriminating power of this technique because these are long sequences of DNA with only around 1°C differences in their predicted thermal melting temperatures. PCR products of this length have not previously been discriminated using SERS monitoring and electrochemical melting. Given that the Texas Red spectrum originates from approximately 1500 molecules directly present under the laser spot, SERS and electrochemical melting is an extremely sensitive technique in terms of both detection levels (equal to or better than fluorescence) and its ability to discriminate between long STR sequences with similar T_m values. Analysis times are quick with unoptimized hybridization and melting taking 30 to 60 min. The SSV technology

and electrochemical melting approach in combination with a portable Raman spectrometer offer unique advantages for incorporation into a field-operable DNA analysis device. With narrow spectral lines, the SERS approach also has the advantage of being able to use many more labels than fluorescence detection permits. Preliminary results indicate that the method can be multiplexed to detect more than one STR by co-immobilizing the different capture probes and using different Raman labels (see Supporting Information). This opens up the possibility for multiplex STR analysis where simultaneous electrochemical melting and monitoring should allow multiplex STR analysis.

It has been shown that using SERS monitoring and electrochemical melting it is possible to distinguish between polymorphic repeat sequences of the STR locus D16S539. Under illumination with a 633 nm laser, PCR products ranging in length from 121–137 bp with 4 bp increments could be differentiated against each other using a common capture probe immobilized on the SSV substrate. The sequences investigated had 1°C differences in their predicted thermal melting temperatures and it was possible to distinguish their melting profiles using this approach. This method should be applicable to the analysis of polymorphic tetranucleotide repeat sequences. SERS and electrochemical melting has significant potential in the application of portable technologies for STR analysis. Significant room for optimization also exists with the number of PCR cycles, potential assisted hybridization, immobilization conditions and the use of novel Raman labels remaining unexplored. We consider analysis times of less than one hour to be a realistic possibility using the methods detailed here.

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