DNA Detection

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Quantitative Simultaneous Multianalyte Detection of DNA by Dual-Wavelength Surface-Enhanced Resonance Raman Scattering**

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Quantitative identification of specific DNA sequences in a mixture without separation and at clinically relevant concentrations is a crucial requirement for the development of more effective and simpler molecular diagnostic assays. By using dual-wavelength surface-enhanced resonance Raman scattering (SERRS), we have developed a quantitative 5-plex DNA-detection system with detection limits of 10^{-12} mol dm^{-3}. Five dye-labeled DNA sequences were identified by eye without separation. Calibration graphs are linear, there is no interaction between labels, indicating a high degree of multiplexing is possible. The combination of sensitivity, reliable target detection in solution, and multiple analyte detection without separations provides a new dimension for the development of more effective multiplex analytical methods for targets such as disease identification and toxicological screening.

Many methods exist for the detection of specific DNA sequences and traditionally they have focused on gel electrophoresis or, more recently, quantitative polymerase chain reaction (QPCR) based methodologies involving in situ fluorescence measurements.[1–3] Although gel electrophoresis is accurate and widely used, it suffers from being a time-consuming and laborious process and still relies on chromatographic separation of different strands. QPCR offers many advantages over gel electrophoresis for sequence identification as most methods use homogeneous closed-tube assays, which offer the ability to detect several sequences simultaneously through deconvolution of the fluorescence emission spectra. Recently we proved that surface-enhanced resonance Raman scattering is a more sensitive technique than fluorescence for DNA-sequence detection by three orders of magnitude when using routinely available laboratory equipment.[4] To further increase the attractiveness of SERRS as a technique to be used in molecular biology for DNA analysis, the ability to multiplex has been examined and is reported herein.

SERRS relies on the adsorption of a colored molecule onto a roughened metal surface.[5,6] In our case we prefer to use silver nanoparticles, as the dye adsorbed on the surface gives a resonance contribution as well as a surface enhancement from the nanoparticle surface-enhanced resonance Raman scattering is produced.[7] This output is molecularly specific as a result of the vibrational nature of the spectroscopy, and a fingerprint spectrum of the dye label adsorbed on the surface is obtained.[8] In a previous study we demonstrated how we could exploit the necessity for surface adsorption of specific dyes to measure enzyme activities and specificities.[9] Excitation of a SERRS label by using a frequency close to that of the absorption maximum of the label has a very significant effect on the scattering efficiencies. Herein we demonstrate how we can exploit the resonance contribution from the label adsorbed on the metal surface to improve the multiplexing capability of SERRS for DNA sequence analysis by using a range of labels with different chromophores and two laser excitation frequencies.

The 5-plex described was carried out by means of five synthetic oligonucleotide probes, each of which incorporated a different dye label at the 5′ end. The sequences used corresponded to a range of different targets. FAM, Cy5.5, and BODIPY TR-X were used to label a universal reverse primer, rhodamine 6G (R6G) was used to label a probe for Human papillomavirus (HPV), and ROX to label a probe to the VT2 gene of *E. coli* 157. The labels were chosen because of their different absorbance maxima (Table 1) and unique SERRS spectra.[10,11] The DNA sequence has minimal effect on the SERRS, which indicates that these combinations of labels could be used for any 5-plex analysis. Since the FAM label is negatively charged, the oligonucleotide probe was modified to contain four positively charged modified nucleosides, 5-aminopropynyl-2′-deoxyuridine. The positively charged bases

Table 1: DNA sequences used in the multiplex study.

Target	5′ Label	Sequence (5′→3′)[a]	λ_{max} [nm]
universal reverse	FAM	XCX CXC XCT CCA CGT TTT CCC AGT CAC GAC GT	494
HPV	R6G	TGC TTC TAC ACA GTC TCC T	524
E. coli 157 VT2	ROX	GCG TCA TCG TAT ACA CAG GAG CAG	585
universal reverse	BODIPY TR-X	TCC ACG TTT TCC CAG TCA CGA CGT	588
universal reverse	Cy5.5	XCX CXC XCX CXC TCC ACG TTT TCC CAG TCA CGA CGT	683

[a] X = 5-(3-aminopropynyl)-2′-deoxyuridine.

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provide a site of attraction for the probes to the negatively charged silver nanoparticles used to provide the surface enhancement.[12] The probe labeled with Cy5.5 was also modified in a similar manner as this had been shown to improve the detection limits in previous studies.[11]

To obtain optimal SERRS, spermine was used to neutralize the phosphate backbone of the probes and also aggregate the silver nanoparticles.[13] The spectra obtained from each of the individual labels with two different laser excitation frequencies are shown in Figure 1. Each label gave a distinctive spectrum, thus allowing the labels to be easily distinguished from each other. However, because of the different absorbance maxima of the dyes, they will not all be in resonance at the same laser excitation frequency and this property can be exploited to produce a very sensitive and selective method for detecting each of these labels within a mixture of the others by using two different laser excitation frequencies. Figure 1a shows the spectra of the five labeled oligonucleotides when an excitation wavelength of 514.5 nm was used. Only three of the dye-labeled oligonucleotides (R6G, FAM, and ROX) gave an intense spectrum at this wavelength, which is because they are in resonance with this wavelength. Figure 1b shows the spectra of the same five dye-labeled oligonucleotides with an excitation wavelength of 632.8 nm. Again, only three of the labels gave an intense spectrum at this wavelength. In this case the labels were Cy5.5, BODIPY TR-X, and, again, ROX. This is because

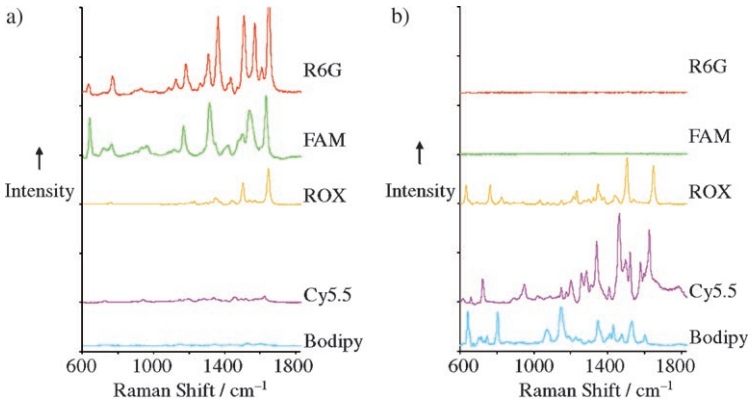

Figure 1. The individual SERRS spectra of each dye-labeled oligonucleotide at a concentration of 1.82×10^{-9} M with excitation at a) 514.5 nm and b) 632.8 nm.

Cy5.5 and BODIPY TR-X are closer to molecular resonance with the 632.8-nm than with the 514.5-nm laser light. ROX could be detected at both excitation wavelengths as, even though its absorbance maximum is at 585 nm, it also has an absorbance peak at around 530 nm which will be in resonance with the 514.5-nm laser light. Thus, it can be easily detected at both excitation wavelengths.

The multiplex spectra obtained at each excitation frequency are shown in Figure 2. Figure 2a shows the multiplex spectrum with 514.5-nm excitation; the two identifying bands from FAM and rhodamine 6G are marked on the spectrum. At 514.5 nm it was impossible to identify a band that was uniquely due to ROX, although a good spectrum was

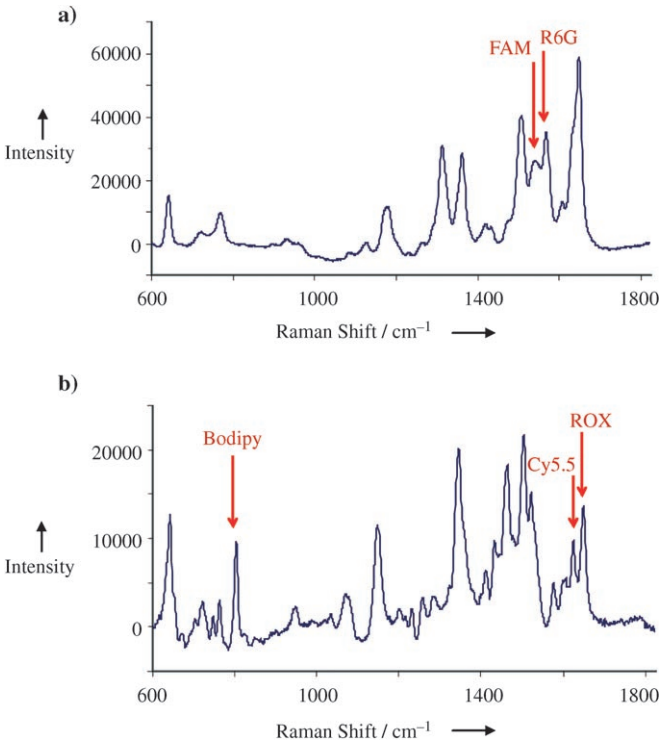

Figure 2. Multiplexed SERRS spectra of the 5-plex at a concentration of 1.82×10^{-9} M and excitation at a) 514.5 nm and b) 632.8 nm.

obtained with this excitation frequency. Figure 2b shows the multiplexed spectrum of the same mixture at 632.8-nm excitation. The identifying bands from ROX, Cy5.5, and BODIPY TR-X are marked on the spectrum. The oligonucleotide mixture was at a concentration of 1.82×10^{-9} M and the identifying bands are listed in Table 2. The bands chosen allowed simple and fast identification of the oligonucleotides in the mixture by looking for the presence of these key marker bands. The bands had sufficiently different Raman shifts to allow this multiplexed identification to be done by eye. Clearly, multiplexing could be increased by use of multivariate analysis.

To prove that this was a quantitative multiplex detection method, a series of dilutions of the mixture of the five oligonucleotides were performed and the detection limits determined (Figure 3). Six dilutions of the multiplex were analyzed; each

Table 2: Limits of detection (L.O.D.) for the oligonucleotides in a multiplex and individual assay.

Label	Band [cm^{-1}][a]	L.O.D. (multiplex) [M]	L.O.D. (individual) [M]
FAM	1539	2.91×10^{-12}	2.73×10^{-12}
R6G	1568	3.22×10^{-12}	1.17×10^{-12}
ROX	1647	1.25×10^{-11}	3.30×10^{-11}
BODIPY TR-X	802	3.08×10^{-11}	7.85×10^{-12}
Cy5.5	1625	6.70×10^{-12}	5.52×10^{-12}

[a] The single band used for identification of each oligonucleotide.

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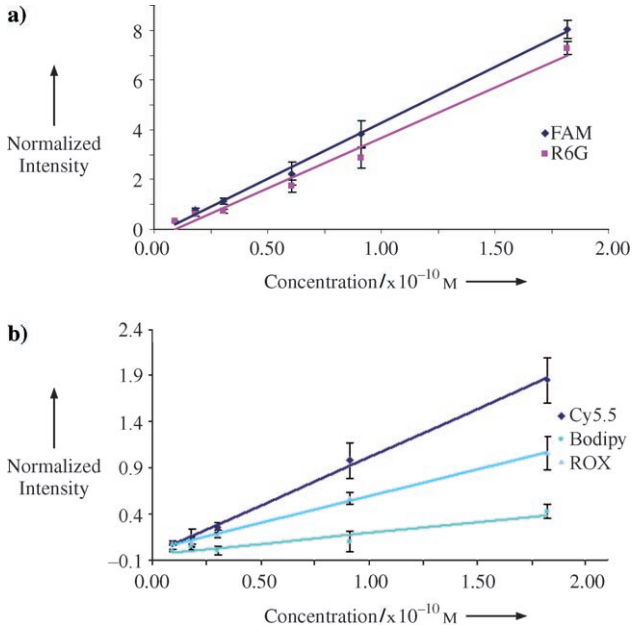

Figure 3. Dilution series of the 5-plex mixture with each dye separately identified. a) 514.5-nm and b) 632.8-nm excitation.

dilution was analyzed five times, and the average was plotted to produce the response shown. Again, two traces are shown for excitation at the two different wavelengths. We found the response to be quantitative for all five oligonucleotides within the multiplexed sampling arrangement. The gradients of the FAM- and R6G-labeled oligonucleotides were almost identical, but the gradients of the labels at 632.8-nm excitation showed differing slopes. This result shows that the sensitivity is different for the three dye-labeled oligonucleotides at this excitation wavelength; however, they could all be detected quantitatively in the mixture. The detection limits obtained for all of the labeled probes in the multiplex are very close to those published previously (Table 2) for SERRS detection of the isolated labeled oligonucleotide probes. This result clearly indicates that there is no reduction in the sensitivity of detection of these probes when used in a 5-plex and shows that through careful choice of the labels and excitation frequencies quantitative multiplexing at ultralow concentrations is possible.

This brief study indicates that it is very simple to multiplex by eye with SERRS by simply choosing the characteristics of the dye-labeled biomolecule, in this case oligonucleotide probes, to match that of the excitation frequencies. The response is quantitative and highly sensitive and offers significant advantages over available technologies for very sensitive quantitative multiplexed analysis of labeled molecules. This has been demonstrated herein as a proof of principle experiment for oligonucleotides. To transpose this method into a molecular biological assay requires further considerations and is the subject of a current study. Further advancements could involve chemometrics to investigate the multivariant changes in spectral intensities across the whole spectrum, which would obviously be more complicated; the elegance of this approach, however, is that it can be done quite simply by eye.

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