Quantitative Enhanced Raman Scattering of Dye-Labeled Phosphorothioate Oligonucleotides

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Abstract

For the diagnosis and treatment of genetic diseases and infections the detection of specific DNA sequences is important. New techniques are continually being designed to improve selectivity, sensitivity reliability and multiplexing of current DNA detection methodologies.

One of these methods is surface-enhanced resonance Raman spectroscopy (SERRS) which has been used successfully as an ultrasensitive technique for the direct analysis of dye-labelled oligonucleotides using aggregated silver and gold nanoparticles. Key to the sensitivity and reproducibility is the affinity of the DNA towards the enhancing surface. This paper investigates the detection of phosphorothioate modified dye-labelled oligonucleotides whereby a non-bridging oxygen atom in the phosphodiester backbone is replaced by sulfur. TAMRA-labelled phosphorothioate oligonucleotides with the same base pair sequence but different number of modified phosphate units were compared to un-modified TAMRA-labelled oligonucleotides using silver nanoparticles as SERRS substrates using a 532 nm excitation wavelengths and different aggregation agents. This study demonstrates the importance of choosing the right experimental setup for DNA SERRS analysis to maximise sensitivity and reproducibility when oligonucleotides are modified for greater surface affinity.

Introduction

Molecular biodiagnostics are evermore relying on nanotechnology to improve sensitivity capabilities and the ability to multiplex *i.e.* to identify more than one analyte at a go. For example, gold nanoparticles have become a general tool for such biodiagnostic techniques whether monitoring their colorimetric (Han et al., 2006), scattering (Storhoff et al., 2004) or electrical properties (Park et al., 2002). These diagnostic tools can potentially have a huge impact in medical diagnosis, whereby results for various diseases and infection can be immediately determined at the point-of-care or at home. These diagnostic tools would have an added advantage of being non-invasive hence the person seeking medical attention can do so in a less stressful environment.

A case in point of improvement in diagnostics by nanomaterials is the detection of DNA. Routinely this is done by fluorescence labelling of DNA whereby the common disadvantage encountered apart from photostability issues is having a broad spectral response making it an inefficient multiplexing technique. Photostability and sensitivity for the detection of DNA was greatly increased with the use of quantum dots (Alivisatos, 1996, Nirmal and Brus, 1998) whereby the excitation occurs at a single wavelength however the emissions from such systems are still broad (25 nm). The solution for multiplexing lies in the use of spectroscopical techniques, especially in conjunction with metal nanoparticles in surface enhanced Raman scattering (SERS) as it gives narrow spectral lines (Vo-Dinh et al., 1984). The sensitivity is further enhanced by labeling the DNA with chromophores (the same used in fluorescence) to have resonance Raman scattering (Cunningham et al., 2006), which reportedly can go down to single-molecule detection (Nie and Emory, 1997; Kneipp et al., 1997). Surface enhanced resonance Raman scattering (SERRS) has been effectively used as a quantitative technique for the direct analysis of dye-labelled oligonucleotides (Graham et al., 1997; Graham et al., 2000; Brown et al., 2001; Fruk et al., 2002; Graham et al., 2003; Faulds et al., 2004a; Faulds et al., 2004b; Faulds et al., 2005b; Faulds et al., 2005a). It has been shown that SERRS can have limits of detection of at least three orders of magnitudes lower than those obtained by a fluorescent technique (Faulds et al., 2004a). For SERRS analysis of dye-labelled oligonucleotides it is important not only for the biomolecule to adsorb onto a suitable metal surface (Rodger et al., 1996), but also that the chromophore attached coincides with the laser excitation wavelength. The metal substrate used was a silver colloidal solution, which gives high electromagnetic enhancement in the visible region. In direct SERRS analysis of dye-labelled oligonucleotides using negatively charged silver nanoparticles, spermine a tetramine molecule, is used which reduces the negative charge of the DNA backbone and induces formation of silver nanoparticle aggregates creating appropriate SERRS conditions in solution. Furthermore, if a dye that has a net negative charge in solution is used the oligonucleotide can be modified using propargylamine (Faulds et al., 2007) which will provide a positive charge facilitating adsorption to the silver surface. Backbone and sugar modifications of DNA are becoming cheaper and easier to make. A modification that is gaining interest due to its properties are phosphorothioate linkages whereby a non-bridging oxygen atom of a phosphodiester is replaced by a sulfur atom (Eckstein, 1979). Phosphorothioate oligonucleotides can be easily prepared through solid phase phosphoramidite synthesis where the oxidation step is replaced with a sulfurization step. Phosphorothioates modified oligonucleotides have been used in antisense applications (Kurreck, 2003) as the backbone modification infers strand resistance to both exo- and endonucleases. This is particularly useful for clinical applications whereby the enhanced stability is ideal when used in cells and sera. Another useful

property that such modification brings about is its affinity towards silver and gold surfaces. As thiol modified molecules (Brust et al., 1994; Brust et al., 1995; Brust and Kiely, 2002) and thioctic acid modified DNA (Dougan et al., 2007), phosphorothioate modified DNA are being used to functionalise quantum dots (Ma et al., 2009), gold nanoparticles (Lee et al., 2007), as a cross-linking agent for gold nanoparticles to form rigid structures (Kumar et al., 2003) and fabrication of bimetallic core-satellite nanoclusters (Pal et al., 2009).   In this paper the limit of detection of phosphorothioate modified dye-labeled oligonucleotides are compared to an unmodified dye-labeled oligonucleotide using SERRS with different aggregating agents and the mechanism by which the DNA attaches to the nanoparticle surface using poly-adenine oligonucleotides is determined.

Experimental

Materials

Oligonucleotides were purchased from Eurofins (Germany) and purified by HPLC. All other materials were purchased from Sigma-Aldrich and used without further purification. All water used was doubly distilled (18.2 mΩ cm).

Nanoparticle preparation

The citrate reduced silver colloid was prepared using a modified version of the Lee and Meisel method (Lee & Meisel, 1982). Water (500 mL) was heated up to 45 ºC in a three parallel-necked round bottom flask (1 L) and silver nitrate (90 mg in 10 mL H_2O) was added. The solution was heated up to 98 ºC and 1% w/v sodium citrate solution (10 mL) was added. The temperature (98 ºC) was maintained stable for 90 minutes with continuous stirring throughout the flask. The necks were covered with aluminium foil to prevent excessive water evaporation.

Instrumentation

Spectra were recorded at 532 nm excitation wavelength using an Avalon Instrument Ramanstation R3. The laser power was 100 mW and the spectra were recorded with an exposure time of 5 x 1s in a Poly(methyl methacrylate) (PMMA) cuvette.

Aggregation Study

Aggregation studies were carried out on the modified and unmodified carboxytetramethylrhodamine (TAMRA)-labelled oligonucleotides. The samples were prepared for SERRS analysis using 7 µL of TAMRA-labelled oligonucleotide, 10 µL of spermine, 175 µL of water and 175 µL of silver nanoparticles. The following initial concentration of spermine tetrahydrochloride diluted in water were used: 0.1, 0.01, 0.001, 0.0001, and 0.00001 mol dm^{-3}, followed by an analysis with no spermine present. The aggregation was monitored by taking a spectrum of the dye every 30 s for 30min. It is to note that he addition of analyte, spermine, water and nanoparticles in this particular order gave the highest peak counts.

Limit of Detection Studies

Concentration studies were carried out on the modified and unmodified oligonucleotides by diluting them with water to various concentrations. The samples were prepared for SERRS analysis as before by adding 7 µL of TAMRA-labelled oligonucleotide, 10 µL of 0.1 mol dm^{-3} spermine, 175 µL of water and 175 µL of silver colloid. The samples were analysed 10 min after the addition of the silver colloid. Each concentration was analysed 5 times, with a 2 s exposure and 5 accumulations. The average peak height of the strongest peak in the spectrum was plotted against the concentration of the dye-labelled oligonucleotide. This was repeated with 0.01 M initial spermine concentration and 2M initial concentrations of NaCl, KNO$_3$ and MgSO$_4$ as aggregating agents.

Poly-adenine study

SERS spectra were obtained for modified and unmodified poly-adenine oligonucleotides (PO1, PO2 & PO3). The DNA was prepared for SERS analysis by heating them up to 95 ºC for 15 minutes after which they were immediately cooled down in an ice bath. Following this 7 µL of the oligonucleotide was added to 10 µL of 0.1 M spermine, 175 µL of water and 175 µL of AgNP solution, giving a final DNA concentration of 100 nM. The same procedure was used when 1% (w/v) NaCl solution was used as aggregating agent, however a final DNA concentration of 1.9 µM was used.

Results and Discussion

Phosphorothioate modified oligonucleotides are known to have a greater affinity towards silver surface due to the sulfur atoms availability for coordination with the metal surface. This is the first report to the best of our knowledge of phosphorothioate dye-labelled oligonucleotides used in direct DNA SERRS analysis. Increasing the affinity of the oligonucleotide towards the nanoparticle should in practice increase the Raman enhancement and give lower detection limits of detection if affinity was the only the key factor and not considering the mechanism by which the aggregating agent work. To examine such hypothesis three oligonucleotides with an identical 20 base pair sequence but with different backbone linkages were used as listed in Table 1.

	5'-dye label	oligonucleotide sequence
O1	TAMRA	5'GGTTCATATAGTTATAATAA3'
O2	TAMRA	5'*G*G*T*T*C*A*T*A*T*A*G*T*T*A*T*A*A*T*A*A3'
O3	TAMRA	5'*G*G*T*T*C*A*T*A*T*AGTTATAATAA3'
* is a phosphorothioate linkage		

Table 1 Dye-labeled oligonucleotide sequence used in the study

The first oligonucleotide (O1) has normal phosphodiester linkages, the second one (O2) has all phosphorothioate linkages and the last sequence (O3) was a chimeric DNA whereby the first ten linkages starting from the 3' are normal phosphodiester bonds, while the other half, the remainder ten linkages are phosphorothioate bonds. The dye-label attached at the 5' end of the oligonucleotide used is TAMRA which has an overall positive charge, facilitating the adsorption to the substrate. The absorption of the dye is at 544 nm which makes it in resonance with the laser excitation wavelength used (532 nm).

Initial experiments were conducted using spermine as to understand if the phosphorothioate modifications change the interaction of the oligonucleotide with the spermine. Spermine has the advantage over inorganic ions or acids as aggregating agents in DNA analysis since apart from causing the aggregation of colloid by the reduction of the nanoparticle surface charge, it also interacts with the DNA to modify its charge (Graham et al., 1997). This was found to give a controlled aggregation of the nanoparticles. For our study, varying the concentration of spermine for the three different oligonucleotides show a decrease in SERRS intensity of the TAMRA dye peaks (see Figure 1).
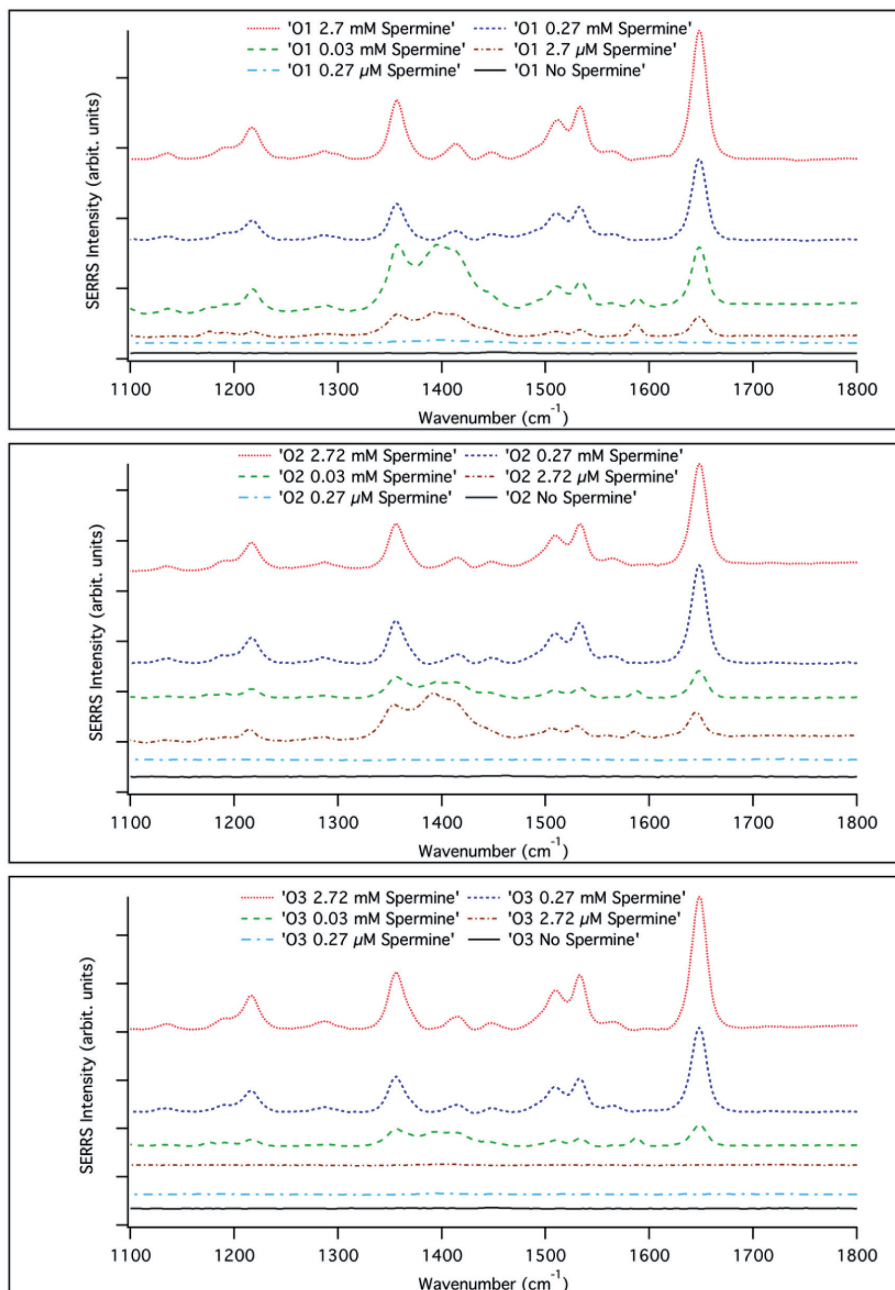
Figure 1 Comparison of SERRS spectra containing 1 nM TAMRA-labeled oligonucleotide (O1, O2, & O3) at different spermine concentration and no spermine with excitation at 532 nm.

No dye peaks could be observed at very low spermine concentrations. When no aggregation agent was added, no dye spectrum could be obtained, however when the oligonucleotides were left to incubate with the colloid for 24 hours the TAMRA peak at 1650 cm^{-1} could be seen for O2 (see Figure 2).
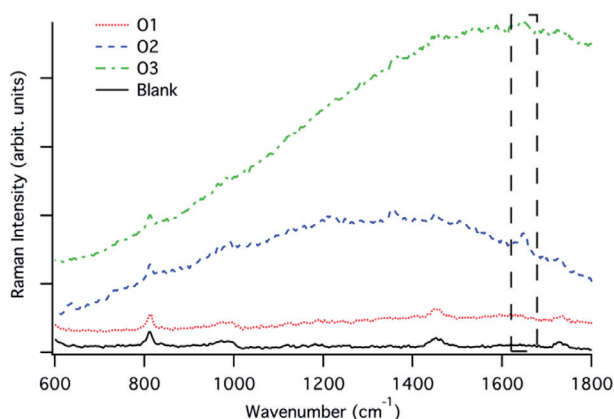

Figure 2 Monitoring the 1650 cm^{-1} peak of the three TAMRA-labelled oligonucleotides at 1 nM concentration against time after 24hrs of incubation with AgNP using a 532 nm excitation wavelength.

This shows that the phosphorothioate is actually seeking and interacting with the silver nanoparticle surface. To confirm that the phosphorothioate modified oligonucleotides are attaching to the surface through the sulfur on the DNA backbone when spermine is used as an aggregating agent, poly-adenine oligonucleotides modified analogously as the TAMRA-labelled oligonucleotides but without dye were used (Table 2).

oligonucleotide sequence	
PA1	5'AAAAAAAAAAAAAAAAAAAA3'
PA2	5'A*A*A*A*A*A*A*A*A*A*A*A*A*A*A*A*A*A*A*A3'
PA3	5'A*A*A*A*A*A*A*A*A*AAAAAAAAAAA3'
* is a phosphorothioate linkage	

Table 2 Poly-adenine oligonucleotides

Poly-adenine oligonucleotides were used as to facilitate the assignment of peaks. No dye was used, as its enhanced Raman spectra would overshadow the weaker peaks coming from the actual DNA. From the spectra obtained a 620 cm^{-1} peak which corresponds to the P-S vibration on to a metal surface (Jiang et al., 2005) can be seen for the modified DNA (O2 & O3) (see Figure 3).
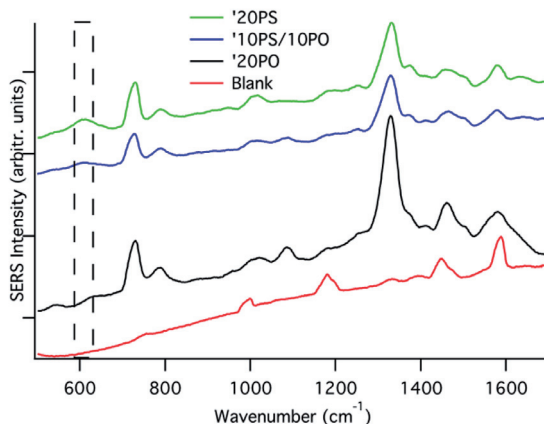
Figure 3 SERS spectra of 100nM poly-A oligonucleotides at 633 nm excitation wavelength using 2.72 mM spermine as aggregating agent. Peaks at 620 cm^{-1} are present for poly-A phosphorothioate modified oligonucleotides (10PO/10PS & 20PS).

These preliminary results show that phosphorothioate oligonucleotides have an affinity to the silver nanoparticle surface therefore in theory an increase in the enhancement from the dyes is expected as more DNA particles would be found in SERRS hot spots. From the limit of detection studies carried out it is shown that oligonucleotide affinity when using spermine as aggregating agent is not the only factor that contributes to the detection. When using a 2.72 mM final spermine concentration the unmodified TAMRA-labelled oligonucleotide (O1) gives a limit-of-detection (LOD) of 1.1×10^{-12} mol dm^{-3} a whole order of magnitude lower to the modified ones (O2 & O3) (see Figure 4 & Table 3).
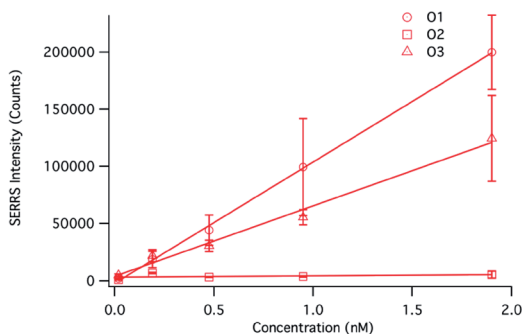

Figure 4 Concentration dependence of the signal (1650 cm^{-1}) graph obtained for the three different TAMRA-labelled oligonucleotides (O1, O2, & O3) using a final concentration of 2.72 mM spermine at 532 nm excitation wavelength. Each point is the average of five repeat samples and the error bars are their corresponding standard deviation.

Oligonucleotide	2.72 mM Spermine LOD/mol dm^{-3}	0.27 mM Spermine LOD/mol dm^{-3}
O1	1.1×10^{-12}	6.2×10^{-12}
O2	3.7×10^{-11}	4.1×10^{-11}
O3	3.0×10^{-11}	3.5×10^{-12}

Table 3 Limit of detection of the 3 labelled oligonucleotides with different spermine concentration

This suggests that spermine interacts with the DNA to give a better detection limit when phosphodiester linkages are present since O2 gave a high LOD and the peak intensity at 1650 cm^{-1} did not increase as concentration of DNA is increased as seen in Figure 4.

When the final spermine concentration is lowered to 0.27 mM the chimeric DNA (O3) now gives a better LOD than the normal oligonucleotide showing that an appropriate balance between the affinity of the sulfur towards the surface and the spermine interaction with the DNA is found (Figure 5).
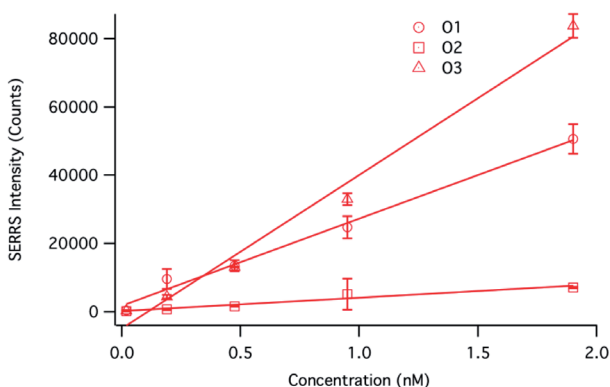

Figure 5 Concentration dependence of the signal (1650 cm^{-1}) graph obtained for the three different TAMRA-labelled oligonucleotides (O1, O2, & O3) using an initial concentration of 0.27 mM spermine at 532 nm excitation wavelength. Each point is the average of five repeat samples and the error bars are their corresponding standard deviation.

The fully modified DNA (O2) however still shows a high LOD hence establishing that phosphorodiester linkages need to be present when spermine is used as an aggregating agent (Figure 5). Also, the results show that when using spermine, modification of the DNA backbone does not give a better LOD unless spermine final concentrations is lower than 2.72 mM.

Further experiments were carried out to show that the results obtained are due to the interaction of spermine with the modified phosphorothioate. The LOD for the three different oligonucleotides were repeated using the same procedure but instead using NaCl, KNO$_3$ and MgSO$_4$ as aggregating agents. Although these inorganic

ions give a high LOD when compared to spermine (see table 4) they do not cause aggregation through the attachment of DNA to the silver surface but only act by reducing the surface charge on the colloid (Munro et al., 1995).

Oligonucleotide	NaCl LOD/mol dm^{-3}	KNO$_3$ LOD/mol dm^{-3}	MgSO$_4$ LOD/mol dm^{-3}
O1	1.3×10^{-10}	8.9×10^{-10}	4.6×10^{-10}
O2	6.1×10^{-11}	4.9×10^{-10}	2.4×10^{-10}
O3	3.1×10^{-11}	4.4×10^{-10}	1.9×10^{-10}

Table 4 Limit of detection of the 3 labelled oligonucleotides with different inorganic salts

Any difference in LOD between the oligonucleotides could then be attributed to the modification on the DNA. From the results obtained in Figure 6 it shows that the modified DNA gives a lower LOD when compared to the unmodified DNA, showing that the affinity of phosphorothioate DNA in this case helps in the limit of detection.
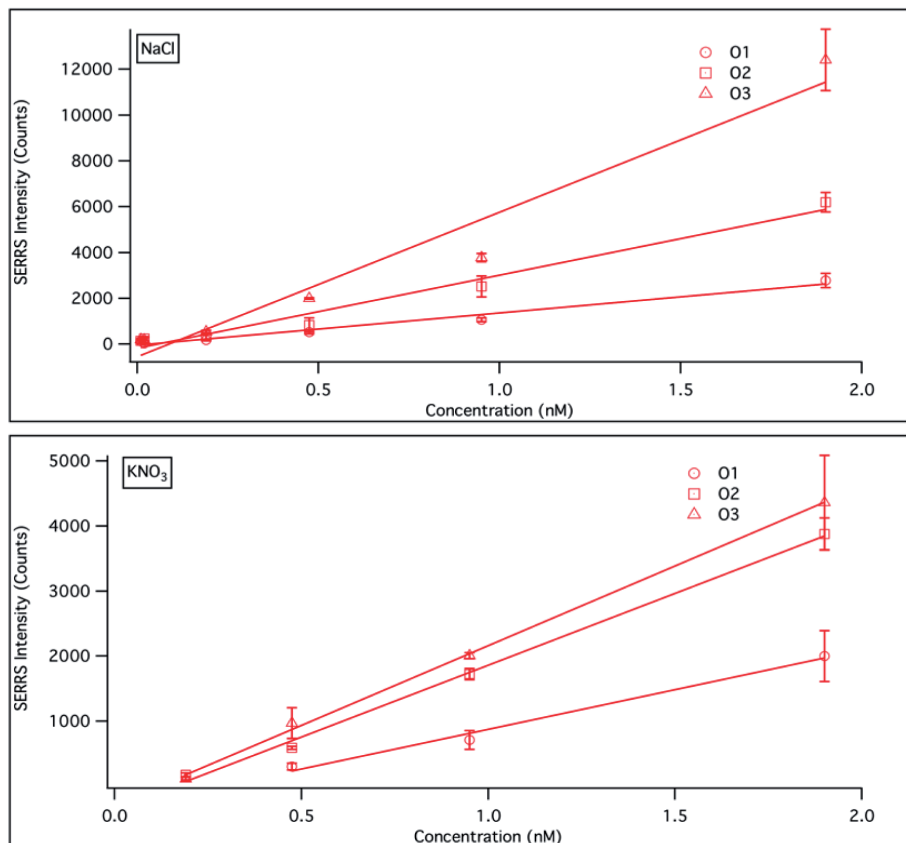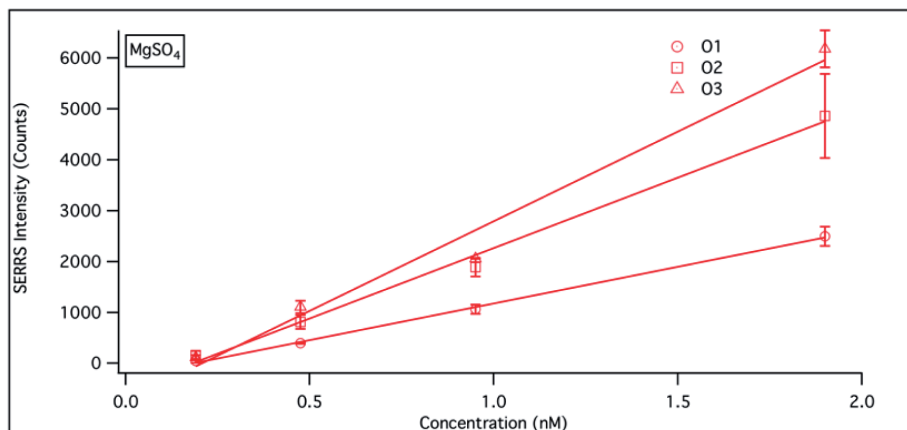
Figure 6 Concentration dependence of the signal (1650 cm^{-1}) graphs obtained for the three different TAMRA-labelled oligonucleotides (O1, O2, & O3) using a final concentration of 54.5 mM inorganic salts (NaCl, KNO$_3$, & MgSO$_4$) as aggregating agent and a 532 nm excitation wavelength. Each point is the average of five repeat samples and the error bars are their corresponding standard deviation.

The chimeric DNA O3 gives a better LOD over all the three different inorganic salts. This can be attributed to the fact that its affinity (due to the presence of the phosphorothioate linkages) towards the silver surface is greater toward the 5' terminal half where the dye is. For O2 however there is no preference for the 5' or 3' terminal, unless the dye itself is directed away from the surface due to steric hindrance and/or better packing. It was also shown using poly-adenine oligonucleotides that the phosphorothioate DNA attaches to the silver nanoparticle via the sulfur (see Figure 7) when using NaCl as the aggregation agent.
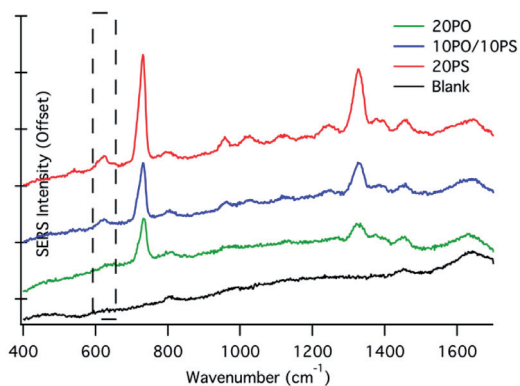

Figure 7 SERS spectra of 1.9μM poly-A oligonucleotides at 633 nm excitation wavelength using 1% (w/v) NaCl as aggregating agent. Peaks at 620 cm^{-1} are present for poly-A phosphorothioate modified oligonucleotides (10PO/10PS & 20PS).

Conclusion

Direct SERRS analysis of dye-labelled phosphorothioate oligonucleotides on aggregated silver nanoparticles is possible as the DNA is coordinated to the nanoparticle surface through the sulfur on its backbone. The LOD for these modified oligonucleotides is higher than that of un-modified counterparts when using spermine. This can be attributed to the reduction in the negative charge on the backbone when oxygen is substituted with sulfur which causes a weaker interaction of the DNA with spermine, hence bringing less DNA to the nanoparticle hotspots. However inorganic salts which do not interact with modified and unmodified DNA but reduce only the negative nanoparticle surface charge give a better LOD for modified DNA. This gives an insight on the factors that affect modified oligonucleotides and that the choice of aggregation agent is as important as the modification. Such studies help out in devising medical diagnostic kits that are more reliable, more efficient, and more sensitive, so that time between diagnosis and treatment is reduced.

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Bio-note

Dr Richard Nicholas Cassar graduated from the University of Malta in 2006 with a B.Sc. (Hons) in Chemistry and Biology. He started a research internship on nanotechnology at the EPFL in Switzerland. He then commenced an M.Sc. on novel materials at the University of Malta which he obtained in 2009. He was awarded a scholarship to pursue a Ph.D. in Chemistry at the University of Strathclyde where he focused on nano-biodiagnostic applications. After graduating in July 2014, Dr Cassar worked as a consultant in the industrial and environmental sectors, whilst lecturing in various institution. In 2017, Dr Cassar joined the Chemistry Department at the Junior College as assistant lecturer. His research interests are nanotechnology for biodiagnostics and bioimaging purposes.

Prof Duncan Graham is based at the University of Strathclyde, Glasgow and is currently Head of Department for Pure and Applied Chemistry. He has a research group of around 35, and over 200 publications to date. Duncan Graham is also a co-founder of the Centre for Molecular Nanometrology and is a co-founder and director of Renishaw Diagnostics Ltd. He serves as Chair of the Editorial Board for Analyst, is president of the Analytical Division of the Royal Society of Chemistry, is on the advisory editorial board for Chemical Science and Chemical Society Reviews, the editorial advisory board for the Journal of Raman Spectroscopy, the editorial board for Biomedical Spectroscopy and Imaging, the advisory board for Analytical Methods, and the advisory board for the new Cell Press journal, Chem.

Prof Karen Faulds is based at the Department of Pure and Applied Chemistry of the University of Strathclyde. Prof Faulds is an expert in the development of surface enhanced Raman scattering (SERS) and other spectroscopic techniques

for novel analytical detection strategies. She has published over 100 peer reviewed publications and has filed 5 patents. In 2013 she was awarded the RSC Joseph Black Award, then in 2016 the Craver Award from the Coblentz Society and was also recently named as one of the Top 50 Women in Analytical Science by The Analytical Scientist. She is the Strathclyde Director of the Centre for Doctoral Training in Optical Medical Imaging, serves on the editorial board of RSC Advances and the editorial advisory board for Analyst and Chemical Society Reviews.