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From Synthetic DNA to PCR product: Detection of Fungal Infections using SERS

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ABSTRACT

We report the use of silver hydroxylamine nanoparticles functionalised with single stranded monothiolated DNA for the detection of fungal infections. The four different species of fungi that were targeted were *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Aspergillus fumigatus*. Rational design of synthetic targets and probes was carried out by carefully analysing the 2-D folding of the DNA and then by global alignment of the sequences to ensure specificity. The effects of varying the concentrations of the DNA and dye surrounding the nanoparticles on the resultant surface enhanced Raman scattering (SERS) signal was also investigated to ensure compatibility of the probes in a multiplexed environment. Using principal components analysis (PCA) it was possible to detect the presence of the individual presence of each target and group them accordingly. The move to detect the *C. krusei* single stranded PCR product (ssPCR) was significant to demonstrate that the methodology could be employed for the detection and diagnosis of invasive fungal infections (IFDs) within a clinical setting. Initially the PCR product was subjected to an alkali shock method in order to separate the strands ready for detection using the nanoparticle probes system. This time 18 base probes were employed to enhance hybridisation efficiency and dextran sulfate was found to have a vital role in ensuring that detection of the C. *krusei* target was achieved. This demonstrated the use of DNA functionalised silver nanoparticle for detection of clinically relevant DNA relating to a specific fungal infection and offers significant promise for future diagnostic applications.

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INTRODUCTION

Candida and *Aspergillus* fungi are ubiquitous within the environment yet are amongst the most well known pathogens responsible for causing fungal infections.[1] The pathogenic fungi from the *Aspergillus* genus is most often found growing on decomposing organic material, whilst *Candida* is a genus of yeasts, hence its presence in humans being diagnosed as a yeast infection.

Both types of fungi present little risk to healthy individuals, however invasive fungal infections (IFDs) have been identified as important causes of morbidity and death in chronically immunosuppressed patients whose weakened immune systems are unable to fight the fungal infection.[2] Patients at high risk of infection from *Aspergillus* species include those with acute myelogenus leukemia or myelodysplatic syndrome during remission induction chemotherapy, patients undergoing allogenic haemotopoietic stem cell transplantation, recipients of solid organ transplants and those with other conditions of severe and prolonged immunosuppression.[3] *Candida* also poses a risk to the immunocompromised, but defining patients who are at high risk for invasive candidiasis (IC) is much more difficult. Patients at highest risk from IC include those who have a central venous catheter, people being treated in intensive care units, those with kidney failure, people being treated with broad spectrum antibiotics, recipients of gastrointestinal surgery and diabetics.[4]

Candida infections account for 70% to 90% of all IFDs.[5] Invasive aspergillosis (IA) is less prevalent, however it is estimated to affect 5-13% of patients who undergo bone marrow transplants, 5-25% of solid organ transplant recipients and 10-20% of people with leukaemia undergoing intensive chemotherapy. Mortality rates are also particularly high in people with IA where rates can rise as high as 90% under certain circumstances. [6]

Diagnosis of both types of invasive infections is difficult mainly because the patients are already ill from other medical conditions. The most common symptoms are fever and chills, but it is often not until unsuccessful treatment with antibiotics that other treatments are pursued. Antifungal drugs for the treatment of the pathogens can generally be placed into three categories; polyenes,[7, 8] azoles[7-9] and echinocandins.[8, 10-12]

Some types of *Candida*, most prominently *C. glabrata* (studied here) has been shown to be increasingly resistant to antifungal treatments such as fluconazole (7% resistance) and echinocandins (1%). *Candida* is not alone in developing resistance,[13] *Aspergillus* has also been shown globally to be resistant to 3-6% of marketed azole drugs.[14] Alternative treatment options are available but these are uneconomical and have toxic side effects, which are undesirable for patients who are already ill. It is becoming increasingly evident that for effective treatment and reduced resistance that discrimination of fungi at a species level is needed to ensure targeted treatment and to ensure resistance to the existing drugs does not reach the current levels of antibiotic resistance in bacteria which is now a major global issue.

One of the most readily used methods of infection diagnosis alongside blood testing is culturing.[15, 16] Culturing is the most common means of identifying fungi to allow selection of the most appropriate drug for treatment, however it is not without flaws. The main issue is that diagnosis can take anywhere from days to weeks and another issue is the susceptibility of the method to false positives. False diagnosis can be down to a number of factors including human collection error, specimen delay in reaching the laboratory and harmless colonisation due to the ubiquitous nature of the fungi.[17] Other non-invasive diagnostic tools involve searching for serum biomarkers such as galactomannan[18-20] and *β-D*-glucan,[20, 21] but these tests are not species specific therefore neither is the treatment given to the patient.

Currently there is a lack of information on the true potential for dual infection as none of the current techniques look for both *Candida* and *Aspergillus* infections simultaneously (e.g. real-time PCR for *Aspergillus* or *Candida* species, Roche Septifast – *Candida*, Galactomannan – *Aspergillus*). However, recent examples of dual infection have been seen in clinical evaluations carried out by Renishaw Diagnostics Limited (unpublished). Given the low prevalence of disease, the true benefit of a higher order multiplex is confidently saying that someone doesn't have one of the disease targets so as to not give treatment unnecessarily (the negative predictive value). The other benefit is that when someone does have a disease target present, being able to diagnose and target the treatment appropriately, which is the reason we purposefully detect C. *glabrata*, C. *krusei* and A. *terreus* as these are treated with the use of different drugs. Diagnosis made on the basis of DNA extraction and PCR amplification can be made specific, targeting at a species level and when coupled with surface enhance Raman scattering (SERS) it offers excellent analytical sensitivity and multiplex capabilities as discussed in this paper.

SERS is a vibrational spectroscopy that uses plasmonic materials to enhance Raman scattering by several orders of magnitude yielding an ultra-sensitive and selective analytical technique.[22, 23] In this paper we make use of silver nanoparticles and use a biologically specific event to exploit the fact that aggregated nanoparticles give rise to an increase in SERS. In our original demonstration of this we used DNA functionalised silver nanoparticles with a Raman reporter to detect a specific DNA sequence.[24] Follow up papers investigated the different geometries and a variety of Raman tags[25] but to date biological relevant, PCR product DNA has not been investigated using this approach. In addition, the multiplexing capability of the system has not been pushed beyond two model sequences.[24]

Here, detection of single stranded DNA (ssDNA) specific to the fungal infection through aggregation of nanoparticle probes induced by sequence specific hybridisation is reported. We initially start with synthetic targets and demonstrate a multiplex of four before demonstrating the ability to detect a specific fungal DNA sequence from PCR product.

EXPERIMENTAL

Synthesis and Characterisation of Silver Nanoparticles (AgNPs)

Silver nanoparticles were synthesised according to a protocol developed by Leopold and Lendl.[26] Initially all glassware was soaked in fresh *aqua regia* to remove contaminants, the clean glassware was then washed with copious amounts of deionised water and left to dry before synthesising the nanoparticles. Hydroxylamine hydrochloride (1.67×10^{-3} M) and sodium hydroxide (3.33×10^{-3} M) were added to 450 mL of deionised water. This solution was then stirred vigorously whilst silver nitrate (50 mL, 1×10^{-2} M) was added immediately, turning the solution a dark brown colour. The sol was left to stir for a further 15 min until a colour change to golden brown signified successful formation of AgNPs. A λ_{max} of 410 nm and NP concentration of ~480 pM (extinction coefficient = 2.87×10^{-10} M) was determined using spectrophotometry, and SEM images verified the spherical nature and size (53 ± 5 nm) of the AgNPs. Prior to DNA functionalisation, the concentration of the particles were diluted to 200 pM using deionised water and the SERS activity/signal reproducibility was assessed using a range of isothiocyanate dyes to ensure their validity over a three month time period.

Functionalisation of AgNPs with Single-Stranded Monothiolated DNA and Dye

All thiolated DNA was purchased from ATDbio (Southampton, UK), treated with DTT then HPLC purified to remove di-thiols and other potential contaminants prior to use. Whilst the site of thiol functionalisation could be differed depending on the hybridisation orientation being achieved (discussed in results section), the modified oligonucleotide always consisted of the same basic moieties; DNA sequence for specific detection, three hexaethylene glycol (HEG) units to give the oligonucleotides flexibility away from the NP surface and a thiol for tethering to the NPs. For fast attachment of the thiolated-DNA to the NP surface a pH-assisted methodology was employed.[27] Spectrophotometry measurements of the functionalised nanoparticles were carried out to elucidate their concentration. The average yield of the particles was around 72% (~144 pM). Once re-suspended in PBS (phosphate buffered saline solution), the dye was added. The amount added differed from dye to dye but isothiocyanates generally varied from 1000 to 5000 molecules/NP. After dye addition the nanoparticles were left to equilibrate for 18 h, then centrifuged and re-suspended in 0.1M PBS. Yields of the NP probes after addition of dye were around 64% (~128 pM).

Hybridisation to Synthetic DNA Target

In a general hybridisation experiment, 10 pM of each probe conjugate (P1 and P2) was added to a plastic PCR tube along with 10 nM of synthetic target (final concentration). The volume was then made up to 60 uL using 0.3 M PBS and left to hybridise for 30 min. 6 samples were created for each probe set; 3 consisted of a complementary synthetic target, whilst the other 3 contained a non-complementary synthetic target (control). Successful hybridisation of the nanoparticle probes to the target was signified by the loss of yellow colour (although this can often be difficult to observe). After 30 min, the solutions are transferred to a 96 well plate and a further 90 uL of 0.3 M PBS was added to each replicate

SERS analysis

SERS analysis was carried out on a Ren-DX Raman plate reader (Renishaw Diagnostics Ltd) equipped with a 532 nm laser with maximum laser power of 50 mW, and 20x objective. Samples were deposited in a black 96 well plate for interrogation. Unless stated, all sample volumes were 150 uL and spectra were collected using 5% laser power at sample (~2.5 mW) and a 1 s accumulation. The system was calibrated using silicon (520 cm^{-1}) and the distance between the sample and objective (z-axis) optimised using ethanol (150 uL) before sample interrogation.

RESULTS AND DISCUSSION

The basic principle of the assay is based on the specific nanoparticle assembly of silver nanoparticles by DNA hybridisation. This changes the intensity of the SERS signal from a Raman reporter attached to the nanoparticle indicating which DNA sequence has been involved in the hybridisation. Multiple combinations of DNA functionalised nanoparticles can be added together and the exact sequence match determined by examining the change in intensity of the Raman reporter coding for that sequence. The basic components of this approach are shown in Figure 1. The head-to-tail conformation was used for the 12 base probes because it enabled sufficiently quick hybridisation to the target DNA whilst maintaining an acceptable distance between the nanoparticles for sufficient SERS enhancement of the reporter (dye) (B) is presumed to be a compromise between all three orientations available. Whilst tail-to-tail (C) hybridises the quickest to the target, the larger nanoparticle separation reduces the plasmonic coupling so the SERS signal tends to be lower than the other orientations. Head-to-head (C) produces a large SERS response, yet the reduced distance between the nanoparticles causes steric hindrance reducing their ability to bind effectively to the target. When moving to longer overall DNA sequences steric influences become more prevalent therefore we investigated the use of both 12 and 18 bases probes as well as examining the different geometries. The majority of the synthetic probes were examined in a head to tail arrangement (B) unless otherwise stated and tail to tail was used for the PCR product detection.

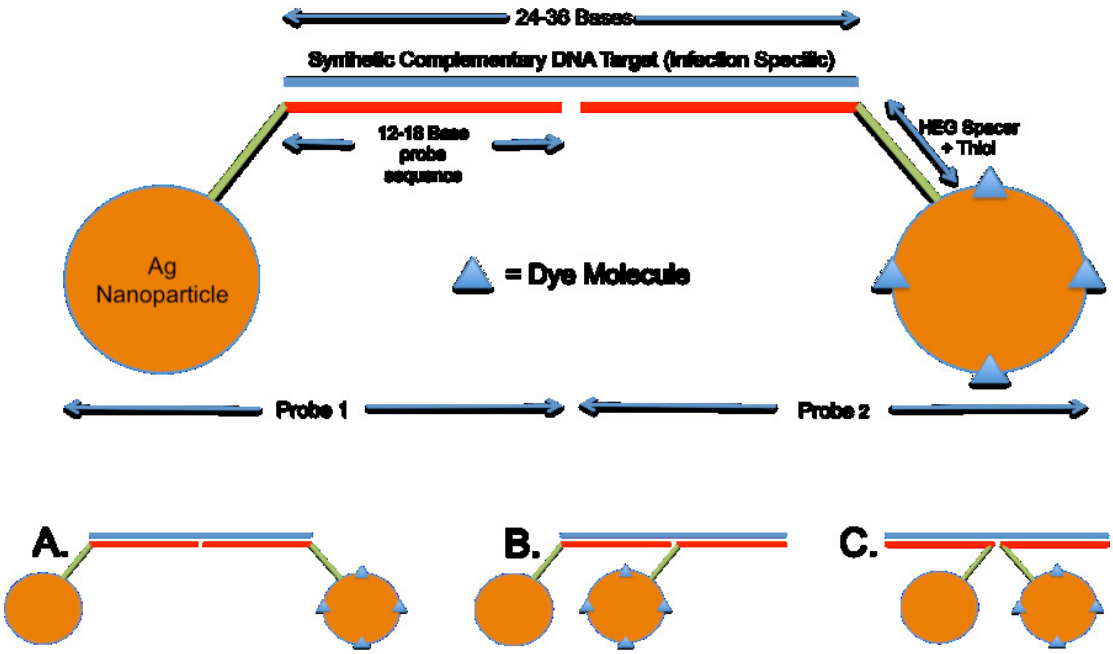

Figure 1. The top scheme shows the hybridisation of nanoparticle probes to complementary single stranded DNA. The length of the probe sequence can be varied to increase discrimination and stability. The Raman reporter (Dye) is added to the nanoparticle surface along with the DNA probe sequence to create a unique code for this particular DNA sequence. Schematics A, B and C represent the three different orientations that the probes can take when attaching to a complementary single strand of DNA due to the stereochemistry induced by the deoxyribose sugar on DNA. A) Tail to tail, B) Head to tail, C) Head to head.

Multiplexed DNA Sequence Detection

Sequences for each of the species were acquired from the basic local alignment search tool (BLAST) and unique sections of the fungal DNA were selected for specific targeting. Careful selection of the DNA target and hence the probes used to functionalise the nanoparticles is of paramount importance for target specificity and to achieve multiplexed detection. However, choosing the DNA with which to modify the nanoparticles is not trivial. Single stranded DNA probes are not only capable of hybridising extremely well to their targets, but also to themselves or other probes within a multiplex, rather than the target if a degree of complementarity is present. Hybridisation of probes to themselves is termed homodimerisation whilst probes which hybridise to each other are called heterodimers. In an attempt to overcome this problem, 2-D folding models generated from amplified sequences were analysed at 25 ^{o}C and 0.3 M (basic hybridisation conditions) to identify regions that were not involved in the formation of loops or base pairing, thus allowing for more direct targeting using the probe sequences. Alongside the 2-D structure formation, global alignment of each of the species amplified regions was carried out to highlight unique bases for targeting. After carrying out a thorough assessment of the sequences and highlighting target regions, twelve base probes were developed. The main goal of this DNA assessment was to ensure that probes used to detect synthetic DNA could be easily translated for the detection of single stranded PCR product (ssPCR).

Nanoparticle Functionalisation

Modification of the oligonucleotide sequences with a thiol (for attachment of the probe to the silver surface) and HEG groups (allowing extension of the DNA away from the nanoparticle) can take place on either the 5' or 3' ends, but changing the modification site changes the conformation with which the nanoparticle probes bind to the target. Figure 1 A-C highlights the different orientations of the probes that can be achieved, depending on the site of thiol modification. The 12 base probes were all modified at the 5' termini therefore they hybridise to the target DNA in a head to tail conformation. The 18 base probes however feature thiol modification on opposite ends. Probe 1 (P1) has a thiol attached to the 3' end whilst probe 2 is modified on the 5' site, giving a tail to tail orientation upon hybridisation with a complementary single strand of DNA. Functionalisation of the hydroxylamine reduced nanoparticles was carried out using the same pH reduction method as reported by Liu *et al*.[27] Initial stability testing of the DNA functionalised nanoparticles (conjugates) was carried out by subjecting the particles to a high concentration of salt solution where it was found that the DNA coating on the nanoparticles made them stable in PBS solutions up to 0.7 M.

Selection of Raman reporters (normally resonant dyes for maximum intensity) is also an important factor in realising a SERS multiplex. The DNA alone does not have a sufficiently large Raman cross-section making it inappropriate to use as a Raman reporter for identification of the target DNA. Fortunately, there are a range of fluorescent dyes commercially available that are capable of tethering to the nanoparticles surface. The dyes used in this work were all isothiocyanates (ITC) that attach to the nanoparticles via the sulphur. The dyes used for developing the multiplex were substituted rhodamine isothiocyanate (XRITC), tetramethylrhodamine isothiocyanate (TRITC), rhodamine B isothiocyanate (RBITC) and BODIPY-ITC (structures shown in Figure S1).

Each of the probe sets were tested separately by adding the complementary sequence to ensure that they showed sufficient discrimination between the presence of complementary and non-complementary target (Figure 2A).

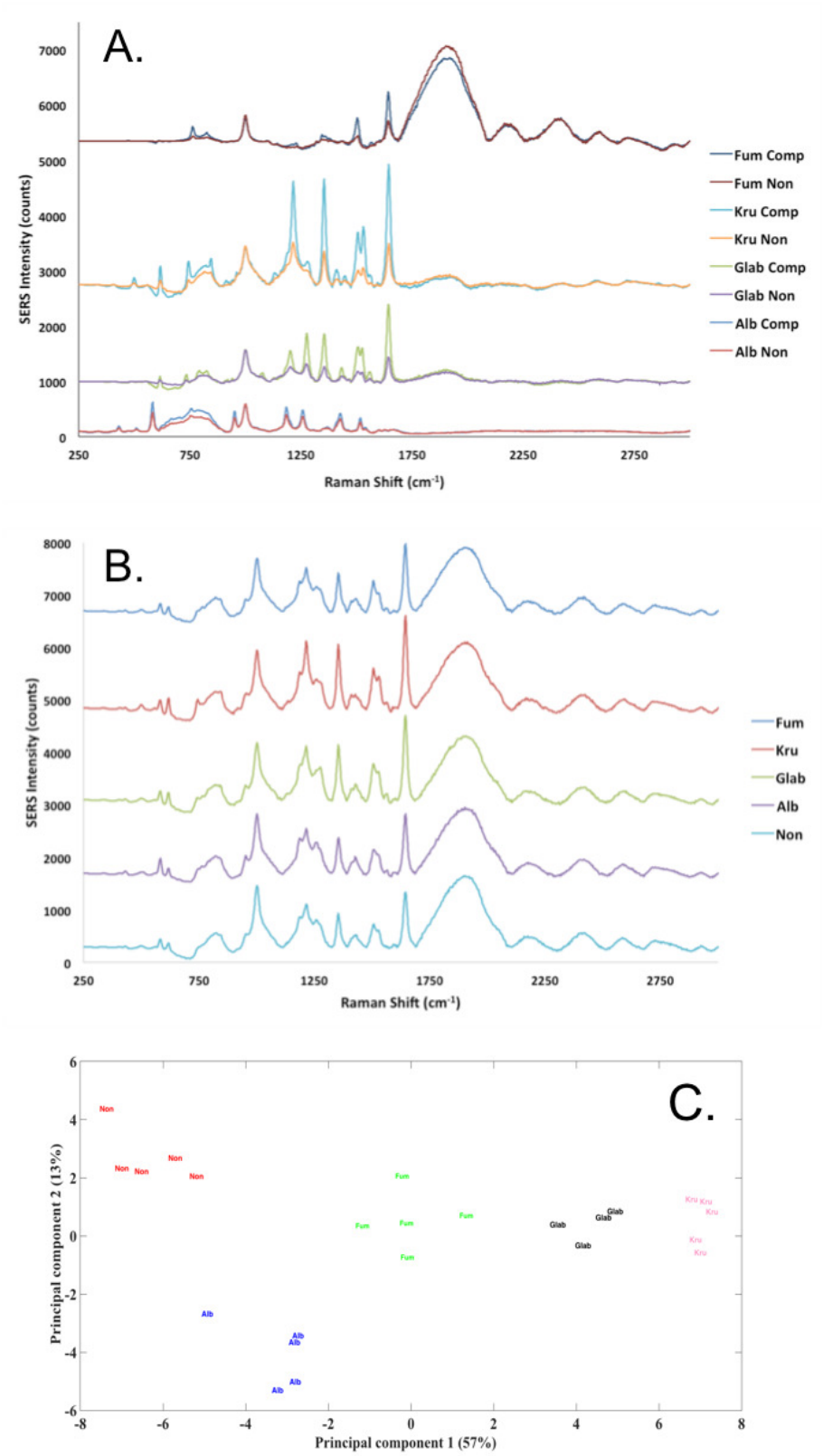

Figure 2. SERS data from the nanoparticle probes with the target alone and then as a multiplex. Spectra have been baseline corrected and offset for clarity. The four probes *A. Fumigatus*, *C. Krusei*, *C. Glabrata* and *C. Albicans* have been abbreviated to Fum, Kru, Glab and Alb respectively in all plots whilst the nonsense is represented by Non. A) Shows the discrimination achieved by each of the fungal

probes when hybridised alone in the presence of complementary and then non-complementary single stranded synthetic DNA. B) Shows the spectral signal arising from a multiplex, consisting of all the fungal probes together with the individual targets. Discrimination of these spectra is difficult by eye. C) Discrimination of the multiplexed targets is possible when PCA is applied to the dataset.

All of the DNA probe sets used with the isothiocyanate dyes showed discrimination between the complementary and non-complementary single stranded DNA targets. The next step was to employ the conjugates in a multiplexed environment. To set up the multiplex 10 pM of each probe set was added to a PCR tube along with 10 nM of fungal target. Each fungal target along with a non-complementary sequence was added in turn to the mixture of probes to see whether discrimination was possible. The average spectra from each probe set in response to the complementary target presence is displayed in Figure 2B. Spectra changes caused by the presence of the different complementary fungal sequences are very difficult to differentiate by eye therefore chemometric methods had to be applied. Principal components analysis (PCA) was used to evaluate the data and showed that the probes could be effectively separated depending on which target was present in the multiplexed environment (Figure 2C). Prior to evaluation using PCA, all data from 1700 cm^{-1} to 3000 cm^{-1} was removed as background, the remaining data were then baseline corrected and scaled. This is a significant result demonstrating that the DNA specific hybridisation of the appropriate probe set was capable of displaying a discernible change in the SERS in a background of the other combinations. However, this data were acquired using synthetic target sequences that were the same length as the combined probe sequences and to translate this into a clinically useful measurement, longer, biologically relevant sequences must be used. Therefore, measurement of a fungal target sequence of DNA generated from a plasmid using PCR was attempted, however due to the target now being a much longer sequence (200-400 bases) this required significant further development and optimisation.

Progression towards PCR target discrimination

We have demonstrated that short, synthetic DNA targets can be identified, however this represents an idealised system, whereby the probes (12 bases each) form a perfect overlap with the 24 base target derived from the fungal BLAST sequence. In reality the amplified sequences are much longer, consisting of between ~200-400 bases. This presents challenges as the hybridisation of the probes to the longer sequences is much more difficult. The longer sequences are prone to forming 2-D folded structures when subjected to physiological buffer conditions at 25^{o}C. In order to demonstrate the diminished hybridisation of the 12 base probes (C. *krusei* with MGITC functionalised nanoparticles), longer synthetic sequences consisting of 48, 96 and 144 bases were used. The results shown in Figure S2 demonstrates how the SERS signal decreases as the length of target increases. The reduced 'off to on' ratio in comparison with the non-complementary target signal therefore presents an issue in discrimination of PCR product sequences.

In an attempt to address the reduction in signal an exclusion buffer (dextran sulfate) was employed.[28] The main action of the dextran sulfate has been reported to enhance hybridisation by denying volume to the DNA-NPs and targets, thereby minimising the effects of diffusion. The effects of the buffer addition on the SERS signal when using a 144 base target can be seen in Figure S3.

Increasing the w/v percentage of dextran sulfate present in the hybridisation buffer results in an increase in the SERS intensity for longer targets. Without dextran the longer targets show only a small increase in SERS intensity compared with the non-complementary control. The SERS signal increases until a plateau is reached at 3 % final concentration. However, it should be noted that the addition of the exclusion buffer increases the background observed but it is not significant enough to affect the ability to discriminate complementary and non-complementary targets.

18 base probes and ssPCR detection

Initial attempts to detect ssPCR product was carried out using 12 base probes, however this was not successful with a number of possible reasons postulated; the long length of the ssPCR target impedes hybridisation, the potentially low concentration of the ssPCR and that the alkali shock method employed did not produce viable DNA. By incubating the double stranded DNA in a NaOH solution at room temperature, the production of single stranded DNA was hoped to be quicker than subjecting the DNA to urea during multiple heat/cool cycles to denature the double strand. To potentially circumvent these problems longer oligonucleotide probes were examined. By extending the sequence conjugated to the nanoparticle from 12 to 18 this would address the length and concentration concerns. Using DNA-NPs specific for *C. krusei* the SERS response of the 18 base DNA-NPs were tested with an exact length complement synthetic target (36 bases) and ssPCR target (Figure 3). Figure 3 shows that the 18 base targets gave a similar off-to-on SERS increase when using the synthetic complement, but no change in intensity was observed when using ssPCR (*C. krusei* = 380 bases). To further push the conjugates to hybridise to the ssPCR the concentration of dextran sulfate used was increased from 3% to 10% in the assay. This is close to the maximum concentration of dextran sulfate possible as the solution becomes saturated at 15-20%.

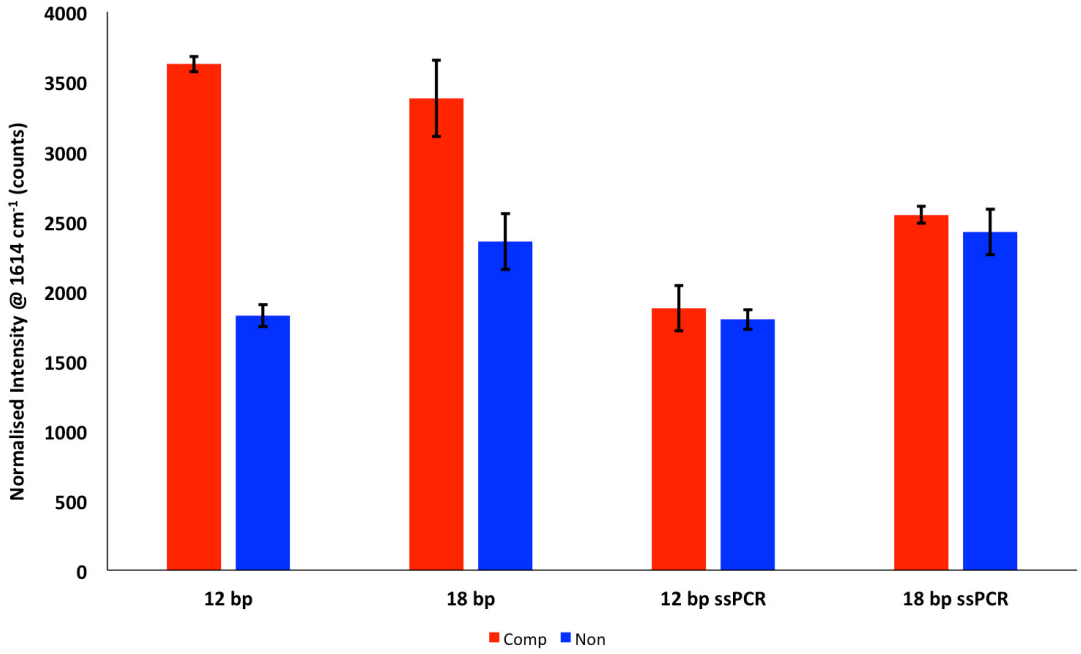

Figure 3. The 12 and 18 base probes (12 bp and 18 bp respectively) show comparable performance in complementary and non-complementary discrimination when hybridised to exact complements (24 bases and 36 bases). However, the levels of discrimination between the complementary and non-complementary ssPCR product is much lower.

Figure 4 shows the results when 18 base DNA-NPs were used in conjunction with a high concentration of dextran sulfate and the ssPCR. The SERS spectra show a clear increase in intensity when the complement (*C. krusei*) is used when compared to that of a non-complementary target (*A. fumigatus*). This successfully demonstrated the potential application of the SERS methodology described here in the interrogation of clinical samples based on PCR products.

After the successful detection of ssPCR target the focus shifted to ascertaining the concentration dependence. After treatment of the double stranded DNA with NaOH ~125 µL of ssPCR product is generated from 10 µL of 1000 input copy number dsPCR (double stranded PCR) product this approximately corresponds to 0.04 µL or 40 nL of the original dsPCR target, illustrating the sensitivity possible using this method. Figure 4 shows that using lower volumes of the target it was still possible to detect a clear off-to-on SERS results. The parameter used to provide the most accurate discrimination of the different concentrations was the ratio between the off and on signal. This is shown in figure 4B and C and indicates that although the absolute intensities vary, the ratio of 'off to on' follows a concentration dependence. Therefore, we have clearly demonstrated that these nanoparticle conjugates can be used to detect a specific PCR product relating to a fungal infection and shows promise for future development into a multiplexed based measurement for multiple infections.

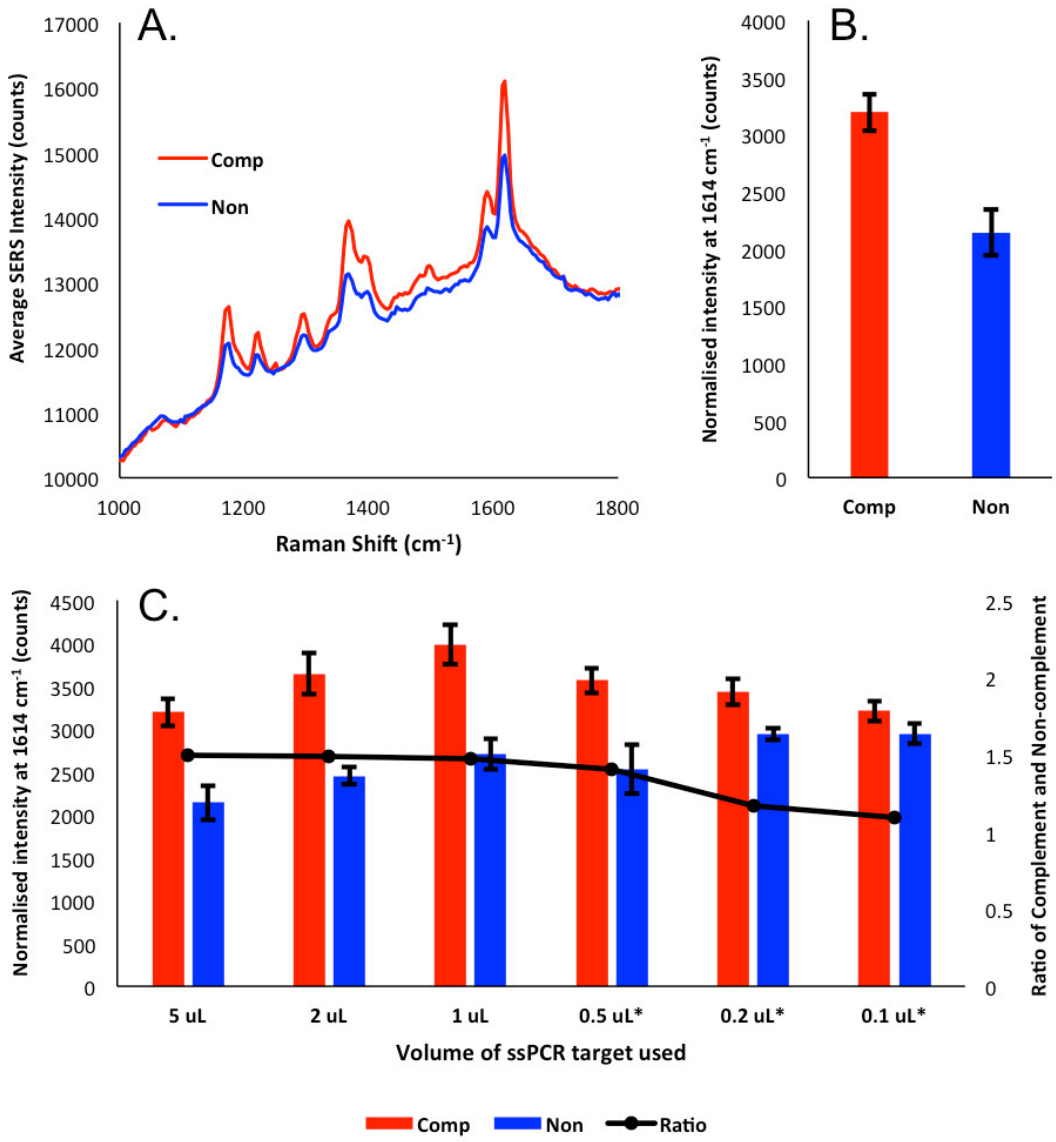

Figure 4. This figure shows the discrimination of *C. krusei* using ssPCR target. The plot in A shows the spectral discrimination between the complementary and non-complementary (*A. Fumigatus*), whilst the bar chart in B demonstrates the mean level of discrimination and accompanying standard deviations (4 replicates for both complementary and non-complementary targets) using the intensity at 1614 cm^{-1}. The mean signals and standard deviations in chart C shows that by lowering the volume of ssPCR target used that discrimination is still possible to around 0.1 µL (4 replicates for complementary and non-complementary targets).

CONCLUSION

Successful detection of both synthetic and ssPCR targets has been demonstrated using DNA functionalised nanoparticles and SERS. Careful design of the probes at the initial stages was of paramount importance, not only to allow specific detection of the fungal targets but to ensure that unwanted hybridisations between the probes themselves was avoided. The rational design also allowed sequences to be easily extended for use in detection of longer single strands as seen with the ssPCR target. Steric issues with the longer PCR based target were overcome by using 18 base probes and dextran sulfate in the buffer system.

Concentration dependence in the SERS signal was observed and this preliminary set of experiments indicates promise for this approach in combination with end point PCR measurement in a multiplexed manner. The work carried out here lays important foundations for a move towards specific detection of fungal species within a clinical setting using this nanoparticle assembly approach.

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