Single and double stranded DNA detection using locked nucleic acid (LNA) functionalized nanoparticles

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ABSTRACT

Gold and silver nanoparticles functionalized with oligonucleotides can be used for the detection of specific sequences of DNA. We show that gold nanoparticles modified with locked nucleic acid (LNA) form stronger duplexes with a single stranded DNA target and offer better discrimination against single base pair mismatches than analogous DNA probes. Our LNA nanoparticle probes have also been used to detect double stranded DNA through triplex formation, whilst still maintaining selectivity for only complementary targets. Nanoparticle conjugates embedded with suitable surface enhanced resonance Raman scattering (SERRS) labels have been synthesized enabling simultaneous detection and identification of multiple DNA targets.

1. INTRODUCTION

There has been significant interest in utilising oligonucleotide functionalised nanoparticles for DNA sequence detection. Noble metal nanoparticles interact very strongly with light, giving rise to bright colours which can be seen by the naked eye. Incident photons of a resonant frequency with the collective excitation of conduction electrons (surface plasmons) are responsible for nanoparticles exhibiting a maximum optical extinction; a phenomenon that does not occur in the bulk metal. It is the sensitivity of the surface plasmon resonance (SPR) to changes in the immediate environment surrounding the nanoparticles that makes metallic nanoparticles ideal candidates for biosensing.

Hybridisation of a split nanoparticle modified oligonucleotide to a single stranded DNA target will bring nanoparticles in close proximity to one another, increasing the dielectric constant experienced by each nanoparticle.

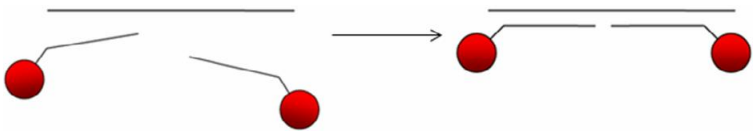

Figure 1. Concept of nanoparticle probe hybridisation to single stranded DNA

The change in dielectric results in a red-shift of the plasmon resonance wavelength, accompanied by a dramatic colour change from red to blue; hence a colorimetric test can be employed for simple determination of DNA hybridisation status. Another advantage is the narrowing of the temperature range over which double stranded DNA is denatured therefore allowing assignment melting temperatures with increased precision. The narrow melting transition is the result of the oligonucleotide-gold conjugates melting in a cooperative manner.[1] It should be noted that several oligonucleotides are conjugated to the gold surface, 100-200 strands per nanoparticle,[2] and hence the duplex systems are part of a much larger three dimensional network.

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It is necessary to be able to discriminate between DNA sequences that contain base mismatches, that is, when only partial hybridisation occurs between DNA strands. This is important for identifying specific gene variants that may correlate to a genetic disease. DNA sequences containing base pair mismatches will be less stable than fully complementary strands resulting in lower melting temperatures. There have been several reports utilising the sharp melting transition and colour change associated with gold oligonucleotides conjugate to allow accurate identification of non-complementary strands and single base pair mismatches .[3-5]

It is desirable to be able to target double stranded DNA for diagnostic applications. Nanoparticle-modified oligonucleotide probes utilised at present can only target single stranded DNA *via* Watson-Crick base pairing and as such double stranded DNA needs to be denatured first so the probes can access the target region. Double stranded DNA-seeking oligonucleotides that form triplexes *via* Hoogsten hydrogen bonds with the region of diagnostic interest would not require prior denaturation of the double stranded DNA and hence greatly simplifies the method of detection. It should also be noted that the oligonucleotide probes will not be in competition with denatured complementary strand as either addition to or strand displacement of the duplex occurs.

There are several limitations associated with the formation of triplex DNA. At present, only polypurine DNA sequences can be targeted. Cytosine and thymine have only one hydrogen bonding site in the major groove and hence makes targeting a polypyrimidine region of duplex DNA, or strand of mixed purine and pyrimidine bases, problematic. Accessibility of thymine bases is also hampered due to the steric clash provided by the methyl group at the 5 position. A triplex-forming oligonucleotide (TFO) will bind to a polypurine strand in the major groove of the DNA duplex through Hoogsten hydrogen bonds. A polypurine TFO will bind anti-parallel to the purine strand; a polypyrimidine TFO will bind parallel. Anti-parallel TFOs are known to form less stable triplexes[6] and guanine-rich oligonucleotides can be inhibited by physiological concentrations of monovalent cations.[7, 8] A limitation associated with parallel TFOs is the requirement of acidic conditions to ensure protonation of cytosine, necessary for hydrogen bonding to a GC base pair. This can be overcome by modification of the cytosines present in the oligonucleotide sequence to yield 5-methyldeoxycytosines. These have an increased pKa and therefore are protonated at physiological pH. Triplexes involving three DNA strands are highly unstable, presumably due to a largely unfavourable entropic term and often require intercalating binders for stabilisation.[9] LNA chimeras have found many benefits as TFOs due to their conformationally-restricted structure, resulting in a decreased loss of entropy on hybridisation, and increased base-stacking interactions to stabilise the triplex structure. [10, 11]

Further to detecting double stranded DNA it is also advantageous to detect several oligonucleotides simultaneously. Oligonucleotide-labelling with nanoparticles provides an effective method for determining if a complementary target sequence is present but as a technique it is not information-rich. We have previously demonstrated that surface-enhanced resonance Raman scattering (SERRS) can be employed for simultaneous detection of five oligonucleotide sequences by exploiting the resonance selectivity of SERRS, thus allowing multiple dye labels to be used with reduced interference from each other.[12] Surface-enhancement may also be exploited for detection of DNA sequences since strong SERRS intensities are often reliant on aggregated nanoparticles due to formation of hot-spots at nanoparticle junctions, where surface enhancement is at its most effective. Graham *et al*. has illustrated the use of dye-labelled DNA silver nanoparticle conjugates for hybridisation to complementary DNA. Hybridisation-induced aggregation of the nanoparticles led to large increases in SERRS intensities allowing selective detection of complementary sequences over non-complementary.[13]

This paper details the functionalisation of gold nanoparticles with LNA-DNA chimeras. The stability of the resultant duplex is shown to have significantly increased upon incorporation of the LNA modifications, compared to analogous DNA nanoparticle conjugates, and an improved degree of discrimination against single base pair mismatches was observed. The LNA nanoparticle conjugates were also used to target double stranded DNA whilst still maintaining selectivity. Efforts have been made towards introducing dye labels into oligonucleotide sequences for multiplex analysis by SERRS.

2. METHODOLOGY

Oligonucleotide nanoparticle conjugations and hybridisations

DNA oligonucleotides were purchased from ATD Bio, Southampton. LNA/DNA chimera oligonucleotides were purchased from Eurogentec, Belgium.

5' thiolated and 3' thiolated oligonucleotides were conjugated to citrate-reduced gold nanoparticles using a slightly modified standard protocol. A thiol-modified oligonucleotide solution was added to colloidal gold (37.8 nM, 1 mL) to yield a final oligonucleotide concentration of 3 μM. The samples were incubated overnight at room temperature, followed by the addition of 60 mM pH 7 phosphate buffer giving a final concentration of 10 mM phosphate. Following 24 hours at room temperature, the salt concentration was brought up to 0.1 M by successive additions of NaCl over 48 hours. The gold conjugates were then centrifuged at 7000 rpm for 10 minutes and resuspended in 0.3 M PBS, pH 7. The concentration of the gold conjugates was determined by UV-Visible spectroscopy using the extinction coefficient at 520 nm of 2.7×10^8 L mol^{-1} cm^{-1}.

Probe name	Oliognucleotide sequence (5' to 3')
5' thiol LNA	Thiol C_6 AAA AAA AAA AAA AAA AAA AAt ^{m}CT^{m}c T^{m}cT
3' thiol LNA	^{m}c^{m}C^{m}C tTt TAA AAA AAA AAA AAA AAA AAA C_3 Thiol
5' thiol DNA	Thiol C_6 AAA AAA AAA AAA AAA AAA AAT CTC TCT
3' thiol DNA	CCC TTT TAA AAA AAA AAA AAA AAA AAA C_3 Thiol
Target DNA A	5'AAA AGG GAG AGA GA 3'

Table 1. Probe sequences used for nanoparticle modification. ^{m}C is 5-methyldeoxycytidine. Lowercase letters denote LNA modifications.

5' thiolated and 3' thiolated probe sequences were mixed together with target DNA A in 0.3 M PBS (0.3 M NaCl, 10 mM pH 7 phosphate buffer). Non-modified oligonucleotides were used at a final concentration of 100 nM; oligonucleotide nanoparticle probes were 1 nM. The target DNA corresponds to a region of the mouse NOS gene.[11]

Duplexes containing LNA-functionalised nanoparticles were monitored at 520 nm and 260 nm, over a range of temperatures, typically between 10 °C and 70 °C using a *Varian Cary Bio 300* spectrophotometer. Non-modified oligonucleotide based assays were only monitored at 260 nm.

To determine the effect of base mismatches on the melting temperature of the duplexes, other target DNA sequences were utilised exhibiting base mismatches at the probe head (closest to the nanoparticle), probe tail (furthest from the nanoparticle) and mid-probe. The sequences used are shown in Table . Oligonucleotides were used at the same concentrations; 1 nM for nanoparticle conjugates and 100 nM for non-modified oligonucleotides. UV melting curves were obtained at 520 nm between 10 °C and 80 °C.

Target DNA B	5’ AAA AGG CAG AGA GA 3’
Target DNA C	5’ TAA AGG GAG AGA GA 3’
Target DNA D	5’ AAA TGG GAG AGA GA 3’
Target DNA E	5’ AAA TGG GAC AGA GA 3’

Table 2. Mismatch oligonucleotide sequences used

The thiolated LNA sequence nanoparticles were added to double stranded DNA to assess triplex formation. The following target sequences (0.1 μM) were added to the nanoparticle conjugates (1 nM) in 0.3 M PBS.

dsTarget DNA F	5’ AGA GAG AGG GAA AA 3’
	3’ TCT CTC TCC CTT TT 5’
dsTarget DNA G	3’ AGA GAG AGG GAA AA 5’
	5’ TCT CTC TCC CTT TT 3’
dsTarget DNA H	5’ AGA GAG ACG GAA AA 3’
	3’ TCT CTC TGC CTT TT 5’

Table 3. Double stranded DNA target sequences used

A 5’ thiol Cy5-labelled DNA oligonucleotide: 5’ thiol C_6 Cy5 $(A)_{10}$TCT CTC TCT C (ATDBio, Southampton) was conjugated to citrate-reduced silver nanoparticles (1 mL) to yield a final oligonucleotide concentration of 10 μM. The samples were incubated overnight at room temperature, followed by the addition of 60 mM pH 7 phosphate buffer giving a final concentration of 10 mM phosphate. Following 24 hours at room temperature, the salt concentration was brought up to 0.1 M by successive additions of NaCl over 48 hours. The silver conjugates were then centrifuged at 7000 rpm for 10 minutes and resuspended in 0.3 M PBS, pH 7. The Cy5 DNA conjugates (25 pM) were mixed together with the 3’ thiol LNA conjugates (25 pM) and the complementary target, 5’ AAA AGG GGA GAG AGA GA 3’ (2.5 nM), in 0.3 M PBS. UV-visible melts were obtained at 415 nm between 15 and 70 °C.

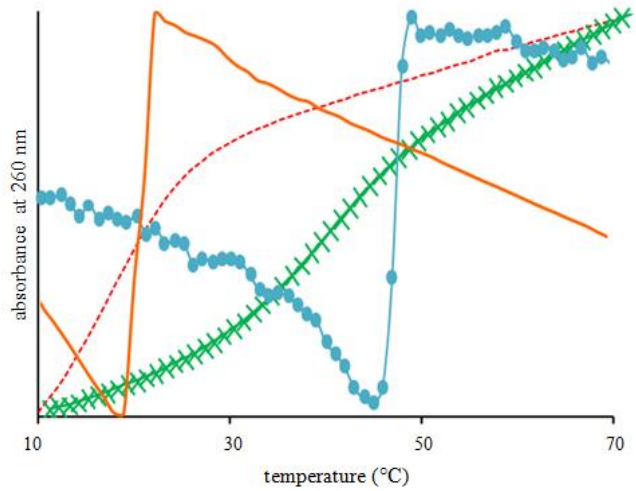
SERRS analysis

All SERRS analyses were recorded using a 632.8 nm He Ne laser coupled to *Renishaw System 2000* Raman spectrometer. Back-scattered light was collected using a *Leica DMLM* microscope equipped with a long-working distance objective (20x/0.4). Scattered light was analysed using a grating of 1200 lines/mm and a *RenCam* CCD.

3. RESULTS AND DISCUSSION

Initially, the melting properties of LNA modified nanoparticles hybridised with a single stranded DNA target were investigated.[14] 3' and 5' LNA/DNA chimeras were modified with gold nanoparticles for preparation of a tail-to-tail hybridisation mode assay. In addition to incorporating LNA ribose sugars, the cytosine residues were methylated at the 5 position. 5-methyldeoxycytosine has been reported to improve duplex stability by increasing the enthalpic component of hybridisation.

a)



b)

Duplex type	T_m (°C)
DNA-DNA	19.0
LNA-DNA	38.5
Nanoparticle-modified DNA-DNA	24.5
Nanoparticle-modified LNA-DNA	47.2

Figure 2. (a) UV melting curves obtained at 260 nm. Red dashed line: unmodified DNA-DNA duplex; green crosses: LNA-DNA duplex; orange solid line: nanoparticle modified DNA-DNA duplex; blue circles: nanoparticle modified LNA-DNA duplex. (b) Melting temperatures for DNA and LNA/DNA duplexes. The melting temperature for the nanoparticle-modified duplex was deduced from analysis at 260 nm and 520 nm.

Incorporation of LNA nucleotides into the oligonucleotide probes results in an increase of 19.5 °C. This is consistent with claims that LNA increases duplex thermal stability.[15] It is thought that this is attributable to less loss of entropy as the probes hybridise with the target DNA and increased base stacking interactions.[16] Interestingly, modification of the oligonucleotides with gold nanoparticles stabilises the duplex furthermore, as illustrated by the 8.7 °C increase in melting temperature, relative to the non-nanoparticle modified LNA duplex. The magnitude of this increase in stability was unexpected and therefore identical melting experiments were

conducted for DNA-nanoparticle probe systems to establish whether the nanoparticle modification was responsible for the observed increase in melting temperature. An increase in 5.5 °C stabilisation for the DNA conjugates compared with the unmodified DNA duplex was observed. This suggests nanoparticle modification confers a degree of stability towards the resultant duplex. It has been suggested by Mirkin and co-workers that the duplex DNA interconnects are stabilised by an increased dielectric produced by the nanoparticles.[1] Comparison of the analogous DNA and LNA conjugates illustrates there is still 3.2 °C unaccounted for. At this stage, it may be assumed that together the LNA and gold nanoparticles exhibit a synergic effect, that is, the combination of these two modifications yields a greater stability than that which would be expected by addition of the individual modifications.

The nanoconjugate melting profile exhibits a much sharper transition, due to the oligonucleotides melting in a cooperative manner. This is attributable to the highly structured three-dimensional network of oligonucleotide functionalised nanoparticles.[1] Free oligonucleotide melting profiles are often quite broad and it can be difficult to discriminate between oligonucleotides perfectly complementary to a target and those containing base pair mismatches. Utilisation of oligonucleotide-nanoparticle conjugates is advantageous when base mismatch discrimination is required and there are reports detailing the differentiation between complementary hybridisation and a single base pair mismatch.[4, 5]

Melting experiments of the LNA chimera probes with non-complementary DNA were conducted to investigate the ease at which mismatches could be identified.

	T_m (°C)	
	LNA Conjugate	DNA Conjugate
Complementary strand	47.2	24.5
Probe tail mismatch (A)	27.1	24.2
Probe head mismatch (B)	45.1	19.3
Mid-probe mismatch (C)	42.2	15.3
Double mismatch (D)	19.0	

Table 4. Melting temperatures of mismatch oligonucleotide duplexes

The presence of a base pair mismatch causes a reduction in the thermal stability of the duplexes. This is expected as there are less stabilising hydrogen bond interactions. There are several different factors that contribute to the extent of the destabilisation, as apparent by the range of melting temperatures for mismatch sequences (19.0 °C to 27.1 °C) An additional hydrogen bond exists between CG base pairs compared with AT, therefore greater destabilisation is expected when a mismatch involves either cytosine or guanine. This may partly explain the difference between the melting temperatures observed however both the probe head and mid-probe mismatch sequences incorporate a mismatch between AT and therefore mismatch location must also affect thermal stability. It is possible that the small difference in thermal stability observed when a probe head mismatch is present is attributable to partial denaturation of the duplex close to the nanoparticle surface and therefore any base mismatches will have negligible effect on the melting temperature, as even a complementary sequence will not be fully hybridised to the probes. It is possible that secondary electrostatic interactions between the oligonucleotide and the gold surface are responsible for this denaturation[5] or steric crowding[2] from the gold nanoparticle or adjacent oligonucleotide strands, limiting the accessibility of incoming target sequences.

The LNA modified nanoparticle conjugates were then employed for targeting double stranded DNA.[17] Target DNA addition to a solution containing the nanoparticle probes at room temperature resulted in a dramatic colour change from red to blue, indicative of nanoparticle aggregation arising from probe hybridisation through

Hoogsten hydrogen bonds to the target sequence. The thermal stability of the nanoparticle modified triplex derived from thermal melting studies was found to be 37 °C. The melting temperature of the unmodified triplex could not be ascertained due to broad, overlapping melting transitions. It has previously been suggested that nanoparticle modification of triplexes provides a method for characterising triplexes by UV-visible spectroscopy which would otherwise not be possible for many sequences.[6]

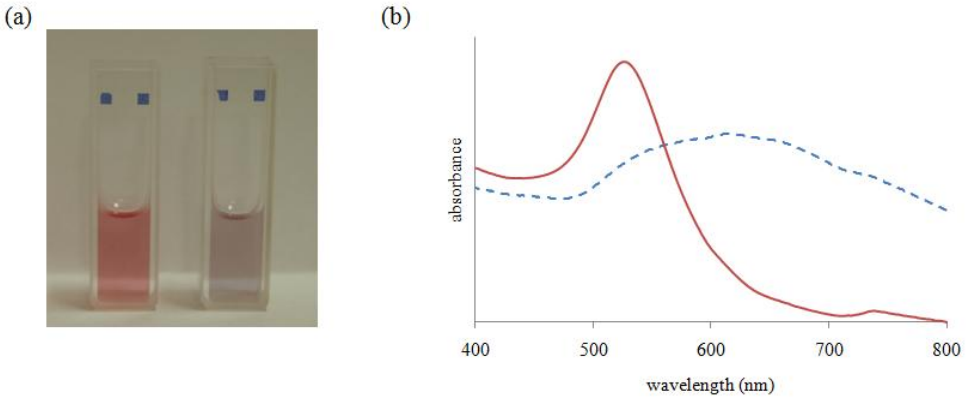


Figure 3a) photograph of LNA nanoparticle conjugates. Left cuvette: prior to double stranded DNA addition; right cuvette: after double stranded DNA addition. b) UV-visible spectra of nanoparticle modified triplex. Red solid line and blue dashed line: prior to and after double stranded DNA addition, respectively.

Addition of the double stranded DNA to analogous DNA modified nanoparticles did not result in a plasmon red-shift and colour change, indicating that under these conditions, triplex formation is only achieved if LNA modifications are incorporated. The selectivity of the TFO nanoparticle probes was assessed by addition of double stranded DNA incorporating a base pair mismatch relative to the triplex strand. Extinction spectra were collected over a time period of one hour and compared to the complementary triplex assembly (figure 4).

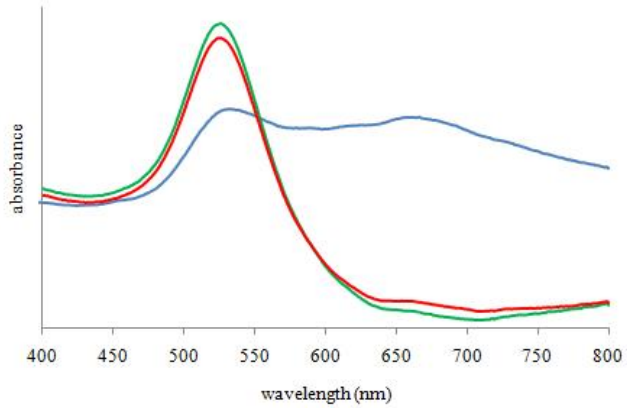


Figure 4. UV-visible spectra of complementary (blue) and mismatch (red) triplex. Green line: prior to target DNA addition.

The nanoparticle probe solution containing the complementary sequence exhibits a plasmon resonance red-shift accompanied by a blue colour change, whereas the solution containing the base pair mismatch displays only a

slight decrease in absorbance at 520 nm and still appears red in colour. This indicates that the LNA probe selectivity for the target has not been compromised when employed in a triple-stranded format.

Nanoparticle labelling provides a simple, colorimetric method for identifying complementary sequences to oligonucleotide probes however this only allows detection of one sequence at a time. SERRS is an information-rich reporter technique with the potential for effective multiple target. Aggregation of the nanoparticles creates regions of strong electric field at that dramatically increase the bulk scattering enhancement in the solution. This is particularly true of reporter molecules that are located at the junctions between nanoparticles. An example of this effect is shown below in figure 5 using an azo reporter dye. Hybridisation-induced aggregation of the nanoparticles as described using the LNA conjugated nanoparticles can be monitored by SERRS analysis, allowing identification of target DNA sequences using molecularly specific dye signals. The combination of unique and narrow SERRS lines for each dye means that several sequences can be identified simultaneously.

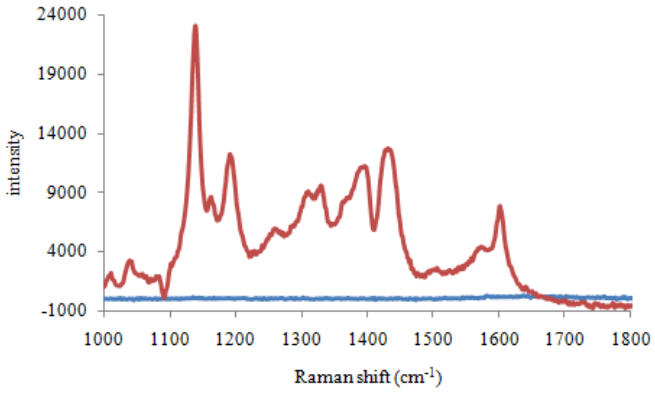


Figure 5. SERRS spectra of aggregated (red) and unaggregated (blue) azo dye labelled nanoparticles.

A dye label may be introduced *via* a phosphoramidite during oligonucleotide synthesis. Silver nanoparticle conjugates were prepared using a Cy5 labelled DNA oligonucleotide adjacent to the terminating 5' thiol. The melting temperature of the duplex using the Cy5 DNA conjugate with an LNA nanoparticle conjugate and a single stranded DNA target was found to be 47 °C. The nanoparticle assembly was heated above its melting temperature and the SERRS spectrum was recorded. The SERRS intensity at this temperature is very poor as the oligonucleotide strands have been thermally denatured and therefore the nanoparticles are present in a monodisperse state, meaning little surface enhancement is occurring. Cooling the nanoparticle solution to room temperature will allow the nanoparticle probes to anneal to the target DNA resulting in aggregation of the nanoparticles and surface enhancement of the SERRS signals (figure 6).

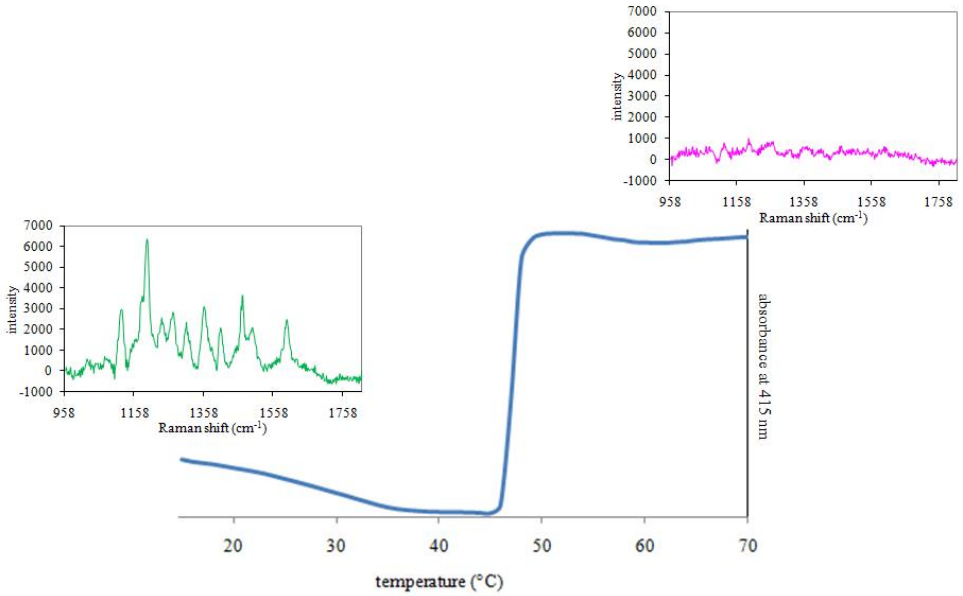


Figure 6. UV-visible melting profile of Cy5 labelled nanoparticle duplex. Green inset: SERRS spectrum of duplex at room temperature; pink inset: SERRS spectrum of duplex immediately following heating to 80 °C.

4. CONCLUSIONS

In summary, we have shown that gold nanoparticles functionalised with 5' and 3' thiol modified LNA/DNA chimeras allowed the preparation of a tail-to-tail hybridisation mode assay which used the surface plasmon shift of the coupling nanoparticles to identify a specific sequence of single stranded DNA. The LNA probes not only formed a highly stable duplex with the target DNA but showed superior selectivity. Melting experiments with non-complementary targets showed significant decreases in thermal stability illustrating superior discrimination over analogous DNA conjugates. The LNA conjugates were also used to target double stranded DNA by triplex formation. The probes were hybridised to the target in a parallel binding mode *via* Hoogsten hydrogen bonds. The resultant triplex was stable at room temperature indicated by the melting temperature of the nanoconjugates. DNA sequence identification *via* triplex formation is advantageous due to the elimination of a denaturation step typically required when targeting single stranded DNA and the non-competitive nature of hybridisation, that is, the probes are not in competition with the complementary DNA strand. Efforts have been made to advance this method of detection by introduction of SERRS labels to the oligonucleotide sequences to allow multiplexed analysis of DNA targets.

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