Optical Methods for the Detection of 

*Neisseria meningitidis*-Specific DNA

A thesis submitted in fulfilment of the requirements for  
the degree of Doctor of Philosophy  

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Declaration

I certify that except where due acknowledgement has been made, the work described in this thesis is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Signed: ………………………………………. (Sapna G. Thoduka)

Date: ………………………………………..
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<th>Description</th>
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<tr>
<td>$\lambda_{\text{max}}$</td>
<td>Wavelength with maximum absorbance</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Fluorescence lifetime</td>
</tr>
<tr>
<td>Abu</td>
<td>2-Aminobutyric acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Nucleotide base pairs</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CI</td>
<td>Chloroform/Isoamyl alcohol</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTAB</td>
<td>Hexadecyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DAPA</td>
<td>2,3-Diaminopropionic acid</td>
</tr>
<tr>
<td>DCED</td>
<td>Direct current electrical detection</td>
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<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-Dithio-bis (2-nitrobenzioc acid)</td>
</tr>
<tr>
<td>EDAC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FAM</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>MSA</td>
<td>Mercaptosuccinic acid</td>
</tr>
<tr>
<td>PCI</td>
<td>Phenol/chloroform/isoamyl alcohol</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal microbalances</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SERS</td>
<td>Surface enhanced Raman scattering</td>
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<tr>
<td>SET</td>
<td>Surface energy transfer</td>
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<tr>
<td>ss</td>
<td>Single stranded</td>
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<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris-(2-carboxyethyl) phosphine hydrochloride</td>
</tr>
<tr>
<td>TCSPC</td>
<td>Time-correlated single-photon counting</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature</td>
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<tr>
<td>TNF-α</td>
<td>Tissue necrosis factor-α</td>
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Summary

Meningococcal disease is a global problem and is a major cause of childhood morbidity and mortality and causes epidemics in Asia and Africa (Pollard and Maiden, 2001). The mortality reaches 100% if the disease is untreated and remains at 7-10% even if treated with the morbidity about 20% (Goering et al., 2008). The causative agent, *Neisseria meningitidis* is a pathogenic Gram negative diplococcus that can cause diseases such as meningitis and sepsis which progress rapidly and may result in death within 24 hours of the first symptoms. Rapid diagnosis and identification of *N. meningitidis* in clinical samples is essential for the management of the disease and the administration of appropriate and effective treatment. Current methods for detection of *N. meningitidis* include direct microscopy, culture, polymerase chain reaction (PCR) and serology methods that have limitation in sensitivity or time required (Zollinger and Boslego, 2002; Gray et al., 2006; Nadel and Kroll, 2007).

The main aim of this thesis was to evaluate two methods, which use the unique properties of gold nanoparticles, as rapid and simple detection methods for the identification of *N. meningitidis*-specific DNA sequences. These methods utilise the properties of gold nanoparticles such as the dipole plasmon resonance and their ability to quench the fluorescence of dyes in close proximity to their surface (Mirkin et al., 1996; Elghanian et al., 1997; Li and Rothberg, 2004b; Ray et al., 2005). Both methods monitor the hybridisation of probes to a meningococcal-specific DNA sequence and were used as a means to confirm the presence of meningococcal DNA and consequently the bacteria.

A *N. meningitidis*-specific DNA sequence (porA4) was selected from the *porA* gene that is only expressed in *N. meningitidis* (Unemo et al., 2005). The probes designed against this
sequence were conjugated to gold nanoparticles using conditions optimised in this study and were then employed in the two detection methods.

The first detection method utilised the changes in the plasmon resonance of gold nanoparticles when associated with DNA and was first conceptualised by Mirkin and colleagues (1996). This plasmon resonance shift of gold nanoparticles may be produced by the target DNA-directed aggregation of nanoparticles and can be seen by a change in colour of the solution. Conditions optimised for hybridisation of DNA to nanoparticle-bound probes were used to achieve the greatest plasmon resonance or colour shift. Meningococcal genomic DNA could not be detected by this method due to limitations in the sensitivity. However, the porA gene PCR product was detected within 1 hour when a minimum of 2 nmole of the target DNA was present. The PCR required at least $10^3$ copies of template genomic DNA to amplify the target gene, which is similar to what has been reported in the literature (Saunders et al., 1993).

The second detection method used in this study involved the use of DNA probes labelled with fluorophores and monitored the hybridisation of target DNA to the probes by associated changes in fluorescence. In these schemes, the fluorophore label was quenched by the gold nanoparticles in the absence of target DNA (Dubertret et al., 2001; Li and Rothberg, 2004b). When target DNA was present, the DNA probes hybridised to it and the fluorescence of the label could be detected. Time-resolved fluorescence measurements gave an understanding of the interaction of the probes with gold nanoparticles and steady-state fluorescence was evaluated as a method for the detection of meningococcal DNA. Two different fluorophore-labelled probes were assessed for their ability to detect a specific DNA sequence at a
sensitivity level of less than 1 pmole. Both the probes used in this study could detect the presence of synthetic target DNA. When the fluorescence method of detection was employed on meningococcal genomic DNA, both the probes were able to detect meningococcal DNA in the order of 0.1 pmole. The sensitivity of this method was greater compared with the plasmon resonance shift method which required a minimum of 2 nmole of target DNA. Another advantage of this method over the colour shift method was the shorter time required for detection, which was in the order of minutes, when an unmodified DNA probe was used. However, this method requires specialised instrumentation for the detection of fluorescence while the colour shift may be detected by the naked eye.

The concentration of meningococcal cells in blood and cerebrospinal fluid can vary from 0 to $10^7$ cfu/mL (Olcen and Fredlund, 2001) therefore, any method that is used in the diagnosis of meningococcal disease would require sensitivity to detect low numbers of organisms in clinical samples. In order for either of the detection methods used in this study to be clinically applicable, the samples need to undergo a form of DNA amplification such as PCR to enhance the sensitivity prior to detection. This would mean that if these methods were further optimised and used in combination with PCR, a result could be obtained within a few hours, which is necessary when diagnosing the rapidly progressing meningococcal disease.

The sensitivity, specificity and speed of these detection methods suggest that the use of DNA-gold nanoparticle conjugates can have a wide range of applications, both for the detection of other pathogens as well as for genetic screening.
Chapter 1

Introduction
1.1 Gold Nanoparticles

Colloidal gold has long been used for its brilliant colours as pigments for staining glass, pottery, porcelain and enamel, without its exact nature being understood. The famous Lycurgus cup manufactured in the 4th or 5th century attributes its colour to colloidal gold as do the red stained glasses in Gothic churches. In 1659, Johann Rudolf Glauber discovered a way to prepare colloidal gold by reduction of gold salts by tin chloride. This product was called “Purple of Cassius” after Andreas Cassius who published the recipe in De Auro in 1685 (Hunt, 1976). The first methodical study of gold nanoparticles in solution was done by Michael Faraday, who published a paper that described a method to synthesise colloidal gold or “divided metal” as he called it, by the reduction of gold salt in the presence of stabiliser (Faraday, 1857). But it was not until around 1897 that Richard Zsigmondy showed that this solution consisted of gold nanoparticles, defining nanoparticles as between 1 and 100 nm in size. He was awarded the Nobel Prize in Chemistry in 1925 for his contribution (reviewed in Zsigmondy, 1926). Since then, methods for the synthesis of different sizes of gold nanoparticle in both aqueous and organic phases have been developed. There has been significant progress in the understanding of their unusual size-dependent optical and electrical properties. This has contributed to basic material science as well as opened up a wide range of possible applications. Some of the areas in which gold nanoparticles are currently being used include catalysis (Haruta et al., 1987), creation of nano-structured materials by self assembly (Niemeyer et al., 2003), medical diagnostics (Elghanian et al., 1997; Storhoff et al., 1998) and in biosensors (Mo et al., 2005). They have also been used in biological systems for drug delivery, and as labels in transmission electron microscopy.
1.1.1 Synthesis

In recent years, many systematic methods for the synthesis of gold nanoparticles of various shapes and sizes have been developed. Microorganisms such as fungi (Ahmad et al., 2005) and bacteria (He et al., 2007) with the ability to convert gold salts into nanoparticles have also been used for the synthesis of “green” and biologically compatible particles. There are two basic synthetic approaches that are used for gold nanoparticles; “top-down” and “bottom-up” synthesis (Richards and Bonnemann, 2005).

Top-down synthesis involves the use of bulk metal that is reduced in size to give nano-sized particles. This could involve disintegration of metal rods by an electric arc as developed by Bredig in 1898 (Turkevich, 1985a) or mechanical crushing of metal (Gaffet et al., 1996), in the presence of stabiliser. Although these methods appear crude, they are useful in the large scale synthesis of nanoparticles. Electrochemical synthesis uses bulk metal as the sacrificial anode, which is oxidised. The ions migrate to the cathode where they are converted into metal atoms that form nanoparticles, whose size can be adjusted by varying the amount of stabiliser present (Richards and Bonnemann, 2005). Other top-down methods include photolithography and electron beam lithography (Rechberger et al., 2003).

Bottom-up synthesis is an enlargement approach that involves the chemical reduction of gold salts to create nanoparticles. In general, a reducing agent is used to donate electrons to the gold ions in solution to produce atomic gold. Reducing agents such as citrate, phosphorous and sodium borohydride as well as radiation such as UV (Mallick et al., 2001; Sau et al., 2001), near-IR laser (Niidome et al., 2000), ultrasonic (Okitsu et al., 2001), laser and pulse
(Kurihara et al., 1983) and γ-radiation (Gachard et al., 1998) have been used to synthesise nanoparticles.

Faraday published the earliest method for the synthesis of nanoparticles by the reduction of gold salt using phosphorus in the presence of a stabilising agent (Faraday, 1857). However, the first reproducible method for synthesis, by reduction of \([\text{AuCl}_4]^-\) using citrate was established by Turkevich and his colleagues (1951), and is discussed in detail in the next section. More recently, Brust and his colleagues used a two-phase system for the reduction of \([\text{AuCl}_4]^-\) with sodium borohydride where an alkanethiol was used as a stabilising agent. This resulted in 1 to 3 nm particles that were soluble in non-polar solvents and very stable due to the strength of the gold-thiol bond (Brust et al., 1994).

There has also been considerable interest in the development of techniques to synthesise nanoparticles of various shapes. By the selection of different techniques, reducing agents, time and stabilising agents shapes such as spheres (Jana et al., 2001), rods (Kim et al., 2002) and prisms (Millstone et al., 2005) have been synthesised in a range of sizes.

These approaches for the control of the size and shape of nanoparticles are helpful in fine-tuning optical and other properties, which depend on these aspects.

**Turkevich Method**

The Turkevich method for the synthesis of gold nanoparticles gives mono-dispersed (standard deviation is less than 20% of average size) particles that are very stable. As mentioned above, this method uses citrate to reduce gold salt in the form of \([\text{AuCl}_4]^-\) to give citrate-stabilised gold nanoparticles. An advantage of this method is that by altering the
amount of citrate, the size of the particles can be controlled (Turkevich et al., 1951). The reduction of gold salt to give nanoparticles occurs in three steps; nucleation, growth and coagulation (Turkevich et al., 1953).

**Nucleation**

Nuclei are created during the nucleation step. Citrate is a weak reducing agent, and as such it is unable to reduce gold ions at room temperature. When heated, the citrate oxidises to give acetone dicarboxylate, which reduces [AuCl₄]⁻ (Figure 1.1). A lag period is observed while the citrate ion is being oxidised. As acetone dicarboxylate is produced, it forms a complex with gold ions and forms a polymer. When the polymer reaches its critical mass, it irreversibly decomposes to give a stable nucleus which is less than 1 to 3 nm in size, containing about 1000 gold atoms.

The number of nuclei that are formed depends on the concentration of citrate. The final number of nanoparticles in the solution is determined by the number of nuclei formed at the beginning and this in turn, determines the size of each nanoparticle. By increasing the molar ratio of citrate to [AuCl₄]⁻, the number of nuclei formed can be increased to give smaller, more mono-dispersed nanoparticles. If a reducing agent with a greater reducing potential such as sodium borohydride is used, the number of nuclei formed increases.
Figure 1.1: Reduction of gold salt by citrate and formation of nuclei.

Citrate is oxidised to acetone dicarboxylate which in turn reduces chloroaurate to form nuclei.
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**Growth**

Once a sufficient number of nuclei are formed, the nucleation process slows down and the growth step begins. During this step, excess gold ions are reduced on the surface of the existing nuclei until all the ions are consumed (Figure 1.2).

**Coagulation**

Coagulation is the formation of larger gold particles by the joining of several nuclei (Figure 1.2). The citrate prevents this aggregation by acting as a stabilising agent and giving the nanoparticle surface a negative charge, which is electrostatically repulsive to other nanoparticles with similar layers, aiding the nanoparticles to remain in solution as a stabilised colloidal suspension.

**Stabilising Agents**

Stabilising agents bind to gold atoms at the nanoparticle surface to avoid coagulation during the synthesis and stabilise them to prevent aggregation. In general, these stabilisers contain a functional group such as thiol, phosphine or amine groups that have high affinity for gold nanoparticles (Grabar et al., 1996a). However, in some instances a weakly binding molecule such as citrate is used to create a layer that can later be substituted with molecules of interest.

There are two types of stabilisation; steric and electrostatic. Steric stabilisers use large organic molecules to shield the nanoparticles, whereas electrostatic stabilisers use the Coulombic repulsion of the charged layer around each particle to keep them apart (Richards and Bonnemann, 2005).
Figure 1.2: Schematic diagram of the formation of gold nanoparticles.

After the nucleation stage, gold atoms are added to the existing nuclei during the growth stage. The particles coagulate to generate nanoparticles stabilised by citrate as represented by “-”.
When hydrophobic stabilising agents such as alkanethiols are used, the nanoparticles are soluble in organic media, but hydrophilic agents such as citrate make the nanoparticles water-soluble (Richards and Bonnemann, 2005).

In addition to having a stabilising agent that can be replaced with other molecules, citrate-stabilised nanoparticles have another advantage. They are water-soluble rather than hydrophobic and therefore compatible with biological systems. The citrate ions confer the nanoparticles an anionic charge whose repulsive force is stronger than the attractive van der Waals force between the particles (Turkevich, 1985b). The negative charge is balanced by the loosely bound sodium ions making the overall solution neutral (Turkevich, 1985b).

**1.1.2 Properties of Gold Nanoparticles**

Gold nanoparticles have unusual properties that are intermediate between those of bulk metal and individual atoms. It is only recently that the properties of structures at this interface are being understood. These properties depend on the size of the particle rather than simply the nature of the material. There appears to be two main properties that can be attributed to size; one due to the large fraction of atoms that are found on the surface and another due to the band structure.

**Effect of Greater Fraction of Atoms at Surface**

When compared with bulk metal, a very high fraction of atoms are found on the surface of nanoparticles compared with the interior. The atoms at the surface of nanoparticles have more freedom to move and interact with other molecules compared with internal atoms.
because they have fewer neighbours and consequently fewer coordination bonds. Therefore more electrons are available to form chemical bonds or be involved in redox reactions.

**Melting Point**

Atoms at the surface are less strongly bound to the rest of the atoms in the nanoparticle because they have fewer neighbouring atoms to form bonds with. As such, they have greater energies and therefore melt at a lower temperature. The melting point decreases with the size of the nanoparticle. The melting point for bulk metal is 1337 K, which becomes less than 1300 K for 20 nm particles and then drops sharply as the size decreases to less than 5 nm, to about 300 K for 2 nm particles (Buffat and Borel, 1976).

**Catalysis**

Bulk gold is regarded as a noble metal, being very unreactive. On the other hand, gold clusters, or small nanoparticles embedded in a support matrix have been shown to be reactive and are able to catalyse reactions such as the oxidation of carbon monoxide (Haruta et al., 1987), the epoxidation of propylene (Hayashi et al., 1998), hydrogenation of unsaturated hydrocarbons (Haruta and Date, 2001) and nitrogen oxide reduction (Bond and Thompson, 1999). The crystal structure of gold appears to play an important role in the activity, and it is very small clusters having atoms projecting out from the surface that have the greatest activity. The catalytic activity of the clusters are also dependent on the type of the support used as well as the method of synthesis (Valden et al., 1998; Davis, 2003).
Adsorption of Functional Groups

Due to uncoordinated electrons in the atoms on the surface, gold nanoparticles can bind to a range of molecules with particular functional groups. It is well known that molecules with thiol, phosphine, or amine functional groups are able to bind due to the lone unpaired electrons of the S, P and N atoms respectively to form coordinate bonds with gold atoms (Grabar et al., 1996a). There is considerable interest in functional groups that bind to gold surfaces since they can be used to stabilise as well as functionalise gold nanoparticles.

Effect of Band structure

Some of the properties of gold nanoparticles such as metal-to-insulator transition, fluorescence and non-linear optics are dependent on the space in which the electrons of the structure are restricted (El-Sayed, 2001). In atoms, where the electrons are most restricted, there are limited types of motion with discrete energy levels or orbitals in which they can exist (Roduner, 2006a). The more the electrons are confined, the more specific the amounts of energy required for them to move from one energy level to another. This gives sharp lines in the absorption spectra.

When bulk metal is formed, the bonds between the atoms cause the energy levels of all atoms to combine, resulting in bands where the electrons have motion with energies that are not quantised (Roduner, 2006a). The density of states or the number of energy levels packed into a band is very high. Electrons in the lower energy bands are able to move to higher bands by the absorption of a continuous range of energies determined only by the upper and lower energy limits of the band, resulting in broad absorption bands rather than discrete lines.
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(Fox, 2004). This is termed inter-band absorption. In bulk gold, the energy gap between the 5d-bands and the partially filled 6s-bands provides its yellowish colour.

Electrons fill the bands up to Fermi energy ($E_f$), which is the highest occupied energy level at absolute zero. Each band is able to hold $2 \times N$ electrons, $N$ being the number of atoms present in the bulk (Fox, 2004). For a metal such as gold that has an odd number of electrons per atom, the highest occupied band is only half filled causing the $E_f$ to be positioned in the centre of the band (Fox, 2004). Therefore there is no energy gap between the highest occupied level (valence band) and the lowest unoccupied level (conduction band) i.e. the Kubo gap is zero (Roduner, 2006b). This allows the electrons below the $E_f$ in this band to occupy the empty states above by gaining an immeasurably small amount of energy. When the electrons are not confined to their energy level and are able to position themselves around any atom in the metal, they are considered delocalised.

When electrons are confined in a small space such as a nanoparticle, there are discrete energy levels, as found in atoms and molecules (Kittel, 2005). The edge of the valence and conduction bands divide into discrete energy levels as seen in Figure 1.3 (Rao et al., 2002). Therefore, in nanoparticles electrons need energy in the form of heat or light to move from the valence to the conduction band.
Figure 1.3: Schematic energy level diagram of a gold atom, nanoparticle and bulk metal.

The atom and nanoparticle have discrete energy levels, while bulk metal has bands. The black levels/band indicate electron occupancy. The grey levels/band indicate allowed energies.
Size-induced Metal-insulator Transition

In bulk gold, the development of energy bands allow electrons in the valence band to rise to the conduction band because the energy gap is insignificant. This makes gold a very good conductor of electrical current. As the particle size decreases, the average energy spacing between energy levels or the Kubo gap increases. Provided that the energy difference is higher than thermal energy, the valence electrons are able to move to the conduction band and the nanoparticle acts as a conductor, but when the Kubo gap is lower than thermal energy, the particle acts as an insulator. This size-induced metal-insulator transition occurs at about 2 nm for gold nanoparticles (Edwards et al., 2001).

Intrinsic Fluorescence

Bulk metals have very weak intrinsic fluorescence with a quantum yield of about $10^{-10}$, due to inter-band transition of electrons (Mooradian, 1969). More recently, gold nanoparticles have been shown to have fluorescence with high quantum yields that increase from $10^{-4}$ for a 31-atom cluster to $7 \times 10^{-1}$ for a 5-atom cluster (Zheng et al., 2004). Since the inter-band gap is dependent on nanoparticle size, the emission can be tuned from the UV to IR region by increasing the number of atoms in the cluster (Zheng et al., 2004). These clusters are an attractive alternative to the traditionally used fluorescent molecules due to their inherent stability.

Optical Absorption

The most prominent property of gold nanoparticles is the intense absorption in the visible region that is absent in the absorption spectrum of bulk metal. The absorption coefficient is
orders of magnitude higher than conventional dyes. This strong interaction with light gives the nanoparticles their characteristic colours. Red light is transmitted because the gold nanoparticles absorb in the green region. Particles less than 50 nm do not scatter a significant amount of light. However, at greater particle size light scattering occurs in addition to absorption. With a decrease in size, particles tend to absorb light at shorter wavelengths and the absorption coefficient decreases (Palpant et al., 1998).

For nanoparticles less than 2 nm, the inter-band transitions of electrons lead to the characteristic absorption spectrum (Alvarez et al., 1997). However, for larger particles the observed red colour is mainly attributed to surface plasmons (Roduner, 2006a).

**Dipole Plasmon Resonance**

A gold nanoparticle consists of a positively charged atom core surrounded by a negative surface which forms the conduction band. When a beam of light is incident on the nanoparticle, the electric field of the light interacts with the surface electrons, exciting them and displacing them in relation to the core. A restoring force originating from the attraction to the positive core causes them to oscillate (Figure 1.4). This electron cloud oscillates causing an electric field at a certain frequency, though out of phase, producing secondary radiation to be dispersed in all directions. A quantum of this longitudinal plasma oscillation is called surface plasmon (Kittel, 2005). Accordingly, this overall phenomenon is called dipole plasmon resonance (Kelly et al., 2003).

Nanoparticle plasmons generate enhanced electromagnetic fields that affect the environment about them. These fields are much stronger than those generated by bulk gold. These fields
Figure 1.4: Schematic diagram of dipole plasmon resonance.

(a) Gold nanoparticle with positively charged atom cores surrounded by an electron cloud.

(b) Displacement of light-excited electrons in relation to the core out of phase with the electric field vector. (c) Electrostatic restoring force.
greatly enhance the Raman scattering and fluorescence of adsorbed molecules as discussed later in this section.

Gold nanoparticles have a characteristic red colour due to the fact that the frequency of light with which their plasmons resonate is in the visible region. In 1908, Gustav Mie used Maxwell’s equations in classical electromagnetic theory modified to suit spherical particles using appropriate boundary conditions, and explained the absorption spectrum as well as the light-scattering of gold nanoparticles (Mie, 1908). Although the simplest explanation of the optical properties of nanoparticles, this theory cannot provide the relationship between the wavelength at maximum absorbance ($\lambda_{\text{max}}$) and the size of the nanoparticle.

Factors Influencing Dipole Plasmon Resonance

The size and shape of nanoparticles influence the plasmon resonance because they change the electron density and their distribution (Kelly et al., 2003). Chemical effects such as the dielectric constant of the surrounding media and changes in the nanoparticle due to adsorption of molecules to its surface also influence the dipole plasmon resonance (Mulvaney, 1996). The electromagnetic coupling that occurs when particles are sufficiently close together to change the frequency at which the plasmons oscillate, also alters the spectrum.

Size

Mie theory predicts that below a certain size (less than $1/10 \lambda$), the absorption band should remain constant, i.e. independent of size. However, experiments have shown that there is a slight red-shift in the absorption peak with increase in size, and scattering increases
(Turkevich, 1985b). Therefore, other theories have been proposed to explain the shift in absorption peak, as reviewed by Kreibig and Genzel (1985). The mean free path for the conduction electrons in gold is ~50 nm and therefore nanoparticles smaller than 50 nm do not scatter light (Eustis and El-Sayed, 2006). Only the plasmon resonance contributes to the absorption of these nanoparticles (Link and El-Sayed, 1999). The position of the absorption peak has also been shown to depend on particle size, contrary to the Mie theory. As the particle size decreases, the absorption peak is blue-shifted and the intensity decreases (Palpant et al., 1998).

**Distance Between Nanoparticles**

Since gold nanoparticles act as electric dipoles when excited by light, they interact with each other electromagnetically in two ways. When the distance between them is larger than the wavelength of light, far-field dipolar interactions dominate, but when the nanoparticles are more closely spaced, it is the near-field coupling that is observed (Maier et al., 2002).

When gold nanoparticles begin to aggregate, the colour turns from red to blue and this absorption is accompanied by light scattering (Turkevich, 1985b). The coupling of the oscillating dipole moments in each nanoparticle modifies the plasmon resonance of the system and shifts the resonant wavelength to higher wavelengths. The shift in wavelength depends on the distance between the particles in relation to their size. It has been shown to be negligible when the space between the particles is greater than 1.5 times their diameter however, it increases exponentially as they are brought closer together (Rechberger et al., 2003; Su et al., 2003).
Surface Enhanced Raman Scattering (SERS)

Raman spectroscopy is a vibrational spectroscopy method which has been used to study the absorption of molecules at solid-gas, solid-liquid interfaces (Fleischmann et al., 1974). Although spontaneous Raman scattering is very weak and difficult to detect, nanoparticles have been found to enhance Raman scattering of molecules adsorbed to them by up to 6 orders of magnitude due to their plasmon oscillations (Michaels et al., 1999). Although, this is only observed when the frequency of the molecular excitation frequency is resonant with the surface plasmon band of the nanoparticle (Blatchford et al., 1982). The Raman scattering signal increases even further when the nanoparticles aggregate due to the change in the plasmon resonance (Blatchford et al., 1982).

Enhancement of the Raman scattering is believed to be due to chemical enhancement, which depends on the nature of the molecule (Moskovits, 1985) and electromagnetic enhancement by the fields around a nanoparticle generated by plasmon resonance (Moskovits and Hülse, 1978). SERS is highly selective to molecules directly adsorbed to the surface of nanoparticles in preference to the molecules in solution, eliminating the need for washing steps in such analyses (Sokolov et al., 1998).

Fluorescence of Surface-bound Fluorophores

The electromagnetic field generated around a nanoparticle either enhances or quenches the fluorescence of fluorophores depending on the distance between the fluorophore and the nanoparticle surface (Thomas and Kamat, 2003). The change in lifetime of a fluorophore is influenced by both radiative decay rate (photon emission) and non-radiative decay rate (loss
of energy to the environment) (Anger et al., 2006). Depending upon which decay rate dominates, there is either enhancement or quenching of fluorescence. When the distance between nanoparticle and the fluorophore is in the range of 10-20 nm, the radiative decay rate is increased and the fluorescence enhanced up to a 100-fold, but when the distance is less than 5 nm the non-radiative decay rate is increased and the fluorescence is quenched (Eustis and El-Sayed, 2006).

Enhancement

The electromagnetic field close to nanoparticle surfaces is very strong because of plasmon resonance. This concentrates the incoming electric field, increasing the incident excitation intensity (Gryczynski et al., 2002). In addition to altering the electric field, the presence of the metal surface also increases the rate of radiative decay by emission, which shortens the lifetime of the fluorophore and increases the quantum yield or photons emitted (Lakowicz et al., 2002). This results in enhanced fluorescence. The difficulty in using this property is that the molecule to be analysed must be positioned at a carefully defined distance from the nanoparticle surface so as not to allow energy to be transferred from the fluorophore to the nanoparticle (Lakowicz et al., 2004).

An advantage of using nanoparticles in combination with fluorophores is that they decrease the time that the molecule remains in its excited state, during which they can be photochemically degraded. Shortening the lifetime allows the fluorophore to undergo more excitation-de-excitation cycles before it is photobleached (Lakowicz et al., 2004).
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Quenching

When the fluorophore is within the Förster radius (distance at which rate of energy transfer is equal to the decay rate of the fluorophore in the absence of the nanoparticle) from the nanoparticle surface, surface energy transfer (SET) takes place. This is the consequence of non-radiative energy transfer from the fluorophore to the nanoparticle (Sokolov et al., 1998), which results in a shortening of the lifetime (Sen et al., 2007). Factors that affect the quenching are location of the fluorophore around the particle, distance from surface and molecular dipole orientation with respect to the surface (Hernandez et al., 2005).

1.1.3 Nanoparticles in Biological Systems

Gold nanoparticles are increasingly being used in biological systems because (a) their size is comparable to biological molecules, (b) they are chemically stable, and (c) they are biocompatible.

Gold nanoparticles conjugated to antibodies have long been used in transmission electron microscopy as probes to visualise biomolecules because of their high electron density (Hayat, 2000).

The biocompatibility of gold nanoparticles have made them an attractive vector for delivery of drugs and therapeutics to cells in vivo and their ability to bind to biological species even allows targeting to specific cells such as cancer cells. Oligonucleotide-modified gold nanoparticles that target certain genes have been used for intracellular gene regulation because of their ability to increase cellular uptake, decrease nuclease activity and increase the affinity of the oligonucleotide for complementary target nucleic acids (Rosi et al., 2006).
Intracellular gold nanoparticles can be heated using an external radio frequency field and are able to destroy an individual cell thermally and will be particularly useful if specifically targeted to tumour cells (Gannon et al., 2008).

Electrochemical methods that are based on gold nanoparticle-labels have been used for the detection of mainly DNA in sensors (Castañeda et al., 2007). The ability of gold nanoparticles to specifically catalyse the reduction of silver on their surface has been applied to direct current electrical detection (DCED) for the recognition of specific target DNA (Burmeister et al., 2004).

The sensitivity of quartz crystal microbalances (QCM) can be increased to enable them to detect DNA hybridisation by using probes conjugated to gold nanoparticles, which have high specific mass (Zhao et al., 2001; Liu et al., 2004; Mo et al., 2005). Using this technique, target DNA corresponding to as little as $2 \times 10^3$ cfu/mL of bacterial cells can be detected (Wang et al., 2007). Antibody-conjugated nanoparticles were used for the enhanced detection of human carcinoma cells captured on the surface of the QCM, allowing detection of 100 cell/mL (Ma et al., 2002).

The unusual size-dependent properties of gold nanoparticles have also been exploited. The highly specific recognition properties of biomolecules such as DNA and antibodies have been combined with the optical properties of gold nanoparticles for various applications.

One of the properties that make gold nanoparticles particularly useful is their colour responses, particularly its changes when associated with biological species. This phenomenon has led to the development of different techniques for the detection of a wide
range of analytes. DNA-conjugated gold nanoparticle probes have been developed for the detection of complementary sequences (Elghanian et al., 1997; Storhoff et al., 1998). Other analytes that can be detected by the colour change associated with the aggregation of nanoparticles mediated by biomolecular interactions include lectin (Otsuka et al., 2001), bacterial pili (Lin et al., 2002a), antibodies (Thanh and Rosenzweig, 2002), potassium ions (Lin et al., 2002b) and adenosine (Liu and Lu, 2004).

The ability of nanoparticles to enhance fluorescence can be used for the enhancement of weak intrinsic fluorescence from molecules such as DNA (Lakowicz et al., 2001). The ability to quench fluorescence of closely linked fluorophores has a great potential for application in diagnostics. Gold nanoparticles used in a molecular beacon system have displayed a quenching efficiency that is 100 times greater compared to traditionally used organic quenchers (Dubertret et al., 2001). This method may be used in the detection of specific DNA sequences. SET has also been applied in the monitoring of DNA cleavage (Ray et al., 2006).


1.2 Meningococcal Disease

Bacterial meningitis is less common but more severe than viral meningitis. More than 75\% of cases are caused by *Neisseria meningitidis*, *Haemophilus influenzae* or *Streptococcus pneumoniae* leading to acute meningitis (Mims *et al.*, 1998). The occurrence of disease caused by different bacteria in different age groups and the accompanying symptoms are shown in Table 1.1.

Since the introduction of a vaccine against *H. influenzae* type B, *N. meningitidis* has been the leading cause of bacterial meningitis and septicaemia, both serious infections that could lead to rapidly progressing fatal shock of otherwise healthy people (Tzeng and Stephens, 2000). This disease is a worldwide problem which can cause epidemics or sporadic disease. The mortality reaches 100\% if the disease is untreated and remains about 7-10\% even if treated (Goering *et al.*, 2008).

In severe meningococcaemia, problems occur in blood circulation as a result of capillary leak syndrome, depletion of intravascular volume and vasodilation (Pathan *et al.*, 2003). Disseminated intravascular coagulation (DIC) also occurs. This is a result of over-activation of coagulation and down-regulation of fibrinolysis (Stephens *et al.*, 2007). This leads to meningococcal sepsis. Inflammatory cytokines increase the permeability of the blood brain barrier and allow the bacteria to invade the meninges and cause meningitis.

*N. meningitidis* infection can also lead to the development of a number of other human diseases such as pneumonia, otitis media, epiglottitis, sinusitis, conjunctivitis, septic arthritis,
Table 1.1: Clinical features and incidence of bacterial meningitis.

(Howlett, 2004; Goering et al., 2008)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Patients</th>
<th>Important Clinical Features</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. meningitidis</em></td>
<td>Children and adolescents</td>
<td>Acute onset (6-24 hours) skin rash</td>
<td>25-60</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>Children &lt; 5 years of age</td>
<td>Onset often less acute (1-2 days)</td>
<td>1-2</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>children &lt;2 years of age and elderly</td>
<td>Acute onset may follow pneumonia and/or septicaemia in elderly</td>
<td>13-50</td>
</tr>
</tbody>
</table>
urethritis and purulent pericarditis (Tzeng and Stephens, 2000; Cartwright, 2001; Rosenstein et al., 2001).

1.2.1 Neisseria meningitidis

The genus *Neisseria* is a member of the family *Neisseriaceae*. Although this genus contains non-pathogenic bacteria, it is the main group of Gram negative cocci associated with human disease as it includes *N. meningitidis* and *N. gonorrhoeae*.

The bacteria *N. meningitidis* was first isolated from patients with meningitis by a Viennese doctor called Weichselbaum in 1887 (Cartwright, 2001). It has never been isolated from other animals and therefore is considered an obligate human pathogen. This may be due to the fact that the bacteria can only acquire iron from humans (Schryvers and Gonzalez, 1990; Cartwright, 2001). They do not survive long outside the host due to their fastidious nature and fragility and therefore they do not have any environmental reservoirs.

**Microbiology**

*N. meningitidis* is an aerobe and grows best on blood or chocolate agar which contains blood. It is a Gram negative diplococcus and like other Gram negative organisms, has an outer membrane. In addition to this *N. meningitidis*, unlike the closely related species *N. gonorrhoeae*, has a capsule attached to the outer membrane at certain stages in its life cycle. The outer membrane is comprised of a phospholipid bilayer with outer membrane proteins partially embedded in it and lipoooligosaccharides (LOS) and pili extending out (Figure 1.5).
Figure 1.5: Schematic representation of the structure of *N. meningitidis*.
The porins PorA and PorB are major outer membrane proteins that are involved in host-cell interactions. Other important outer membrane proteins include Opa and Opc which help in the adherence of the bacterium to host cells. The LOS, known as endotoxin, consists of three short oligosaccharides and is a major virulence factor. Pili are complex outer membrane organelles that extend several microns from the cell surface. They assist the bacterium in colonisation by movement through the mucous layer and initial attachment to the epithelial cells of the nasopharynx.

The capsule that envelopes the outer membrane consists of polysaccharide. The structural differences in the capsule allow classification of *N. meningitidis* into serogroups. The polysaccharide is either a homopolymer in serogroups such as B and C or a heteropolymer in serogroups such as W135 and Y and the main serogroups associated with disease, except for serogroup A, are composed of sialic acid derivatives (Vogel *et al.*, 2001). The role of the capsule is to protect the bacterium from desiccation under harsh environmental conditions.

The genome sequences of the *N. meningitidis* strains MC58 (serogroup B), Z2491 (serogroup A), FAM18 (serogroup C) and isolate 053442 (serogroup C) have been reported (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000; Bentley *et al.*, 2007; Peng *et al.*, 2008). The genome is approximately 2,200 kilobases and codes for around 2000 genes. It has several repetitive sequences that are thought to be involved in genetic recombination and antigenic variation (Parkhill *et al.*, 2000). The antigenic variation is very important in the virulence of meningococci as phase variation is necessary for colonisation and invasion by the bacteria.
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Classification and Distribution

Traditional classification of *N. meningitidis* is based on specific antibody recognition. There are 13 serogroups based on capsular polysaccharide structure (A, B, C, D, 29E, H, I, K, L, W135, X, Y and Z), 20 serotypes based on the major outer membrane porin protein PorB, 10 subtypes based on another outer membrane protein PorA and 12 immunotypes based on LOS (Tzeng and Stephens, 2000). The current classification scheme used is [serogroup]: [serotype]: [serosubtype]: [immunotype] (Kuipers *et al.*, 2001).

Meningococcal disease is a global problem, although there is a variation in the serogroups that cause disease in different continents (Table 1.2). Serogroup B is responsible for up to two-thirds of cases and serogroup C accounts for nearly all the rest of the cases (Howlett, 2004). However, since the introduction of the conjugated serogroup C vaccine in 1999, the incidence of disease caused by serogroup C has fallen by 75 per cent (Howlett, 2004). Serogroup A causes large epidemics in Africa (Howlett, 2004).
Table 1.2: Distribution of disease-causing meningococcal serogroups.
(Stephens et al., 2007)

<table>
<thead>
<tr>
<th>Continent</th>
<th>Serogroups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td>A, W135, C, X</td>
</tr>
<tr>
<td>Asia</td>
<td>A, C</td>
</tr>
<tr>
<td>Australasia</td>
<td>B, C</td>
</tr>
<tr>
<td>Europe</td>
<td>B, C</td>
</tr>
<tr>
<td>North America</td>
<td>B, C, Y</td>
</tr>
<tr>
<td>South America</td>
<td>B, C</td>
</tr>
</tbody>
</table>
1.2.2 Pathogenesis

Carriage

The human nasopharyngeal mucosa is the only natural reservoir of *N. meningitidis*. About 10% of the total population in endemic areas are asymptomatic carriers (Cartwright *et al.*, 1987). The rate of carriage is dependent upon age. In children younger than 4 years, the carriage is less than 3%, which increases with age up to 24-37% between ages 15 and 24 years and decreases to less than 10% in older populations (van Deuren *et al.*, 2000). However, approximately 90% of these are considered to be non-pathogenic since they are not known to cause invasive disease (Bevanger *et al.*, 1998).

Transmission

Since *N. meningitidis* cannot survive for long outside the host, the only means by which it is transmitted from person to person is through respiratory secretions or saliva either by direct contact or less effectively, by droplets. Transmission is more widespread in areas where there is crowding such as university dormitories (Neal *et al.*, 2000) and among military recruits (Tyski *et al.*, 2001).

Colonisation

Once transmitted, *N. meningitidis* has to attach itself to epithelial cells of the respiratory tract, utilise accessible nutrients and evade the immune system to successfully colonise the nasopharynx (Yazdankhah and Caugant, 2004).
The pili are important in the movement of meningococci through the mucosal surface of the nasopharynx by twitching motility (Tzeng and Stephens, 2000). When meningococci reach the epithelium, the type IV pili anchor the bacteria to the cells by attaching to the membrane cofactor protein (MCP) or CD46 receptor (Källström et al., 1997). This allows the outer membrane protein Opa to bind to the CD66 receptor (Dehio et al., 1998; Virji et al., 1999) and Opc to bind to proteoglycan receptors (de Vries et al., 1998), to adhere tightly to the epithelium.

*N. meningitidis* expresses iron acquiring proteins including HmbR which is able to acquire iron from human haemoglobin, TbpA and TbpA B, which acquire iron from transferrin, HbpA and HbpA B, which acquire it from lactoferrin and HpnA and HpnA B, which have the ability to acquire it from the haemoglobin-haptoglobin complex (Stephens et al., 2007).

The immunoglobulin (Ig) A1 is found in human mucosal tissue which normally prevents the attachment and colonisation of bacteria. *N. meningitidis* expresses an extracellular IgA1 protease that cleaves the immunoglobulin (Vitovski et al., 1999). It also produces factors that inhibit ciliary activity (Stephens and Farley, 1991). In this manner, the bacteria are able to evade the human immune system.

Colonisation may result in carriage for a time period of several days to months, or in invasive disease that develops within 1-14 days of acquisition (Stephens et al., 2007). It is not known clearly what causes invasive disease with no way of predicting disease from the carriage rate, although some factors such as previous infections, inhalation of dry, dusty air and smoking have been linked with the disease (Pathan et al., 2003).
Carriage of meningococci is an immunising process that allows the body to generate protective antibodies against the bacteria and therefore carriers do not normally fall ill. However, invasion occurs almost immediately after colonisation in around 1% of people infected with virulent strains (Caugant et al., 1994).

**Invasion**

Although epithelial cells are not phagocytic, their interaction with the meningococcal Opa and Opc proteins results in the ingestion of the bacteria (Meyer, 1999). This process is hindered by the LOS and the bacterial capsule and so at this stage there is down-regulation of expression of the LOS and capsule to promote phagocytosis (van Deuren et al., 2000). The bacteria are then able to pass through the epithelial tissue in phagocytic vacuoles by modulating the cells to reach the bloodstream (Meyer, 1999).

The ability of *N. meningitidis* to survive and multiply in the blood stream again depends on its ability to evade the immune system, particularly the humoural and phagocytic defences (Tzeng and Stephens, 2000).

There is an inverse correlation between the titre of specific antibodies and susceptibility to meningococcal disease. This relationship provides the reason for a greater incidence of the disease among children aged between 6 and 24 months, when the maternal antibodies are gradually lost (Goldschneider et al., 1969a, 1969b). However, people who have been newly colonised will take at least a week to generate specific antibodies (Harthug et al., 1986) and therefore have to rely on the innate immune responses involving complement pathways for protection (Pollard and Frasch, 2001). The polysaccharide capsule of the bacteria prevents
Chapter 1: Introduction

phagocytosis and complement-dependent bacteriolysis (Klein et al., 1996). Certain outer membrane proteins also obstruct phagocytosis by neutrophils (van Deuren et al., 2000).

If *N. meningitidis* is not killed by factors in the bloodstream, multiplication occurs rapidly to titres that are reported to be the highest for any bacterium (Hitchcock et al., 1999).

### 1.2.3 Pathophysiology

The meningococci circulating in the blood can result in infection of the meninges and other sites in the body that are normally sterile. During multiplication, *N. meningitidis* undergoes lysis, releasing outer membrane components as well as forming and over-synthesising outer membrane vesicles containing LOS (Brandtzaeg et al., 2001). The severity of meningococcal disease has been directly linked to the amount of endotoxin in circulation (Brandtzaeg et al., 1989).

Damage to the host tissue in meningococcal disease is caused by the activation of immune mechanisms (Pathan et al., 2003). The LOS binds to endotoxin binding protein and activates macrophages and other inflammatory cells (Pathan et al., 2003). This causes pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tissue necrosis factor-α (TNF-α) to be released (Tzeng and Stephens, 2000). Activated neutrophils release inflammatory proteins, proteases and other enzymes that damage epithelial tissues. This leads to increased coagulation and inflammation and decreased fibrinolysis which can account for the symptoms observed (Pathan et al., 2003).

After an incubation period of 1-3 days, there is a sudden onset of symptoms (within 6-24 hours). These include sore throat, headache, drowsiness; signs of meningitis include fever,
irritability, neck stiffness, and photophobia and signs of sepsis include haemorrhagic rash (Goering et al., 2008).

1.2.4 Diagnosis

A diagnosis of meningococcal sepsis or meningitis is usually suspected from clinical examination of the patient. The main features are headache, fever, and haemorrhagic rash. Laboratory identification is essential to identify *N. meningitidis* so that other bacteria can be ruled out and the correct antibiotic (penicillin, ceftriaxone or chloramphenicol) treatment can be administered (Goering et al., 2008).

Clinical specimens that can be collected are blood, cerebrospinal fluid (CSF), swabs of throat and skin lesions, joint fluid and other normally sterile sites depending on the symptoms. Concentration of meningococci in blood can be less than 1 cfu/mL; while in CSF the concentration can vary from 0 to $10^7$ cfu/mL (Olcen and Fredlund, 2001).

Initial laboratory tests include direct microscopy and culture. However, patients with suspected meningococcal disease are administered with antibiotics before admission to prevent mortality or morbidity caused by delays in diagnosis (Richardson et al., 2003). Hence, there is low sensitivity with these methods in patients already undergoing treatment due to lower numbers of the pathogen. The CSF is sterile within 2 hours of starting antibiotic treatment (Kanegaye et al., 2001), but results from biopsy of skin lesions are not affected by antibiotic treatment for up to 45 hours (van Deuren et al., 1993). Culture is useful for the confirmation of diagnosis, grouping of the strain and allows for antibiotic-susceptibility testing. However, it takes 12-24 hours to confirm the diagnosis.
There have been many non-culture methods developed for the diagnosis of meningococcal disease. They are being increasingly used in laboratories since they provide greater speed and sensitivity than direct microscopy or culture, especially in cases when antibiotic treatment has been administered before admission to hospital. These techniques generally use specific antigen or nucleic acid sequence detection.

Latex agglutination assays that use antibody-coated latex beads for the detection of meningococcal antigens in CSF, blood, serum or urine are being used to give results in less than 10 minutes (Williams and Hart, 1988; Clarke et al., 2001; Zollinger and Boslego, 2002). The antibodies are generally against group-specific capsular polysaccharides and can be used for serogrouping also. However, there are problems with false positives (Clarke et al., 2001) and although this method can be used for screening purposes, the results need to be confirmed by other methods such as culture or polymerase chain reaction (PCR). There are also problems with sensitivity when the number of bacteria in the blood or CSF is low (Zollinger and Boslego, 2002).

PCR, which amplifies specific nucleotide sequences, is increasingly being used for diagnosis of meningococcal disease, sometimes even without culture (Gray et al., 2006). This technique does not need live organisms and has been found to be useful even in patients who have begun receiving antibiotic treatment (Nadel and Kroll, 2007). Some of the genes targeted include porA (Saunders et al., 1993), nspA (de Filippis et al., 2005), saiD (Carrol et al., 2000) and ctrA (Guiver and Borrow, 2001). PCR can also be used for serogrouping by using 16S rDNA (Baethgen et al., 2003). PCR assays require the PCR product to be analysed by agarose gel electrophoresis or enzyme-linked immunosorbant assay (ELISA).
(Borrow et al., 1997). Results can be obtained within 2 hours of DNA extraction from the sample (Richardson et al., 2003). However, this sensitive assay is not available at many hospitals and is too expensive to be used on a regular basis in developing countries.

1.3 Overview of Thesis

Meningococcal disease is characterised by the sudden onset of symptoms and rapid progression of the disease. This can result in permanent damage to the central nervous system or death within hours. Rapid diagnosis is essential for commencing appropriate and effective treatment.

The primary aim of this thesis was to optimise and evaluate rapid and simple methods for the detection of meningococci using gold nanoparticles by targeting a meningococcal-specific DNA sequence. The high specificity of Watson-Crick base-pair interactions in double stranded (ds) DNA can be exploited in the diagnosis of disease and detection of pathogens. The hybridisation of DNA probes with the specific target sequence was detected by utilising different physical properties of gold nanoparticles in order to confirm the presence of meningococcal DNA and consequently the bacteria.

In Chapter 2, a meningococcal-specific DNA sequence was selected as a target and complementary DNA probes were designed to recognise it. The presence of the chosen sequence in the genomic DNA of the bacterial strain used in this study was confirmed and its specificity ensured by searching a nucleotide database for matches. The binding properties of different functional groups including thiols, amines and hydroxyl groups were studied using model compounds in order to determine the optimal conditions and functional group
for the directional conjugation of the DNA probes to the nanoparticles. The synthetic DNA probes were modified with a thiol group in order to bind them directionally to the gold nanoparticles. Buffer conditions such as ionic strength and pH were examined to find the optimum conditions to conjugate the modified probes to gold nanoparticles. These DNA-nanoparticle conjugates were then used in two different methods for the detection of the target DNA in the later chapters.

The first detection scheme used was the target DNA-mediated aggregation of the probe-conjugated gold nanoparticles. This can lead to a colour change in the nanoparticle solution that may be detected semi-quantitatively by spectrophotometry that confirms the presence of target DNA in the solution. Chapter 3 describes studies on the effect of probe density on the surface of gold nanoparticles as well as the effect of salt concentration in the solution on the hybridisation efficiency of the probes. These results were used to maximise the hybridisation of the probe to target DNA and therefore leading to increased colour changes. The colour-shift studies were initially conducted on synthetic DNA target sequences and subsequently on meningococcal derived DNA, both genomic DNA as well as a PCR product containing the selected target sequence.

In Chapter 4, DNA labelled with fluorophores were used as probes. Two different probes were used. The first possessed a thiol group to directionally bind it to the nanoparticles surface and the other without a functional group that relied on its free bases to bind to the nanoparticles. The fluorescence of both of these probes was shown to be quenched by gold nanoparticles in the absence of target DNA. When the probes hybridised to target DNA, the fluorescence was observed to increase and can be used to detect meningococcal DNA. The
interaction of the probes with the nanoparticles was examined using time-resolved fluorescence spectroscopy. Steady-state fluorescence measurements were also assessed as a method to detect *N. meningitidis*-specific DNA sequences.

The two DNA detection methods used in this study are rapid relative to existing diagnostic tests and have been shown to effectively detect meningococcal DNA at varying degrees of sensitivity. Therefore these methods may, with further optimisation, be used on clinical samples and prove to be valuable in the diagnosis of meningococcal disease.
Chapter 2

Conjugation of DNA to Nanoparticles
Chapter 2: Conjugation of DNA to Nanoparticles

2.1 Introduction

2.1.1 Binding of Functional Groups to Gold Nanoparticles

There is considerable interest in functional groups that bind to gold surfaces and their use for the conjugation of different molecules to gold nanoparticles. Thiol, phosphine, amine and cyanide groups are known to have high affinities for gold nanoparticles because of the availability of electron lone pairs from the sulphur, phosphorus, and nitrogen atoms respectively (Freeman et al., 1995; Grabar et al., 1996b).

Thiol groups are the most commonly used functional group to bind to gold nanoparticles. Although several studies have examined the nature of the thiol-gold bond, it remains poorly understood. It is generally believed that the hydrogen atom of the thiol group leaves when the sulphur atom binds to the gold (Hasan et al., 2002). However, it has been shown that the bond does not have the characteristics of gold sulphide and therefore is not a covalent bond (Brust et al., 1994). The thiol-gold bond is thermodynamically stable and the monolayers formed on the surface are very stable. However, the thiols bound to gold nanoparticles are in equilibrium with free thiols in solution and dynamic ligand exchange occurs (Schroedter and Weller, 2002).

Recently there has been growing interest in amine groups as a means to functionalise gold nanoparticle surfaces (Leff et al., 1996; Selvakannan et al., 2003; Aslam et al., 2004). Although amines bind weakly to planar gold surfaces, they form monolayers that are almost as stable as thiol monolayers on gold nanoparticle surfaces (Leff et al., 1996). However the amine monolayers are not as not as closely packed as thiol monolayers (Leff et al., 1996).
Both thiols and phosphines are replaced by amines by ligand exchange, however the opposite does not occur (Brown and Hutchison, 2001; Kumar et al., 2003).

Phosphine groups also bind to gold nanoparticles, although competitive binding experiments have shown that there is stronger adsorption with thiols and amines (Warner et al., 2000). Cyanide groups have a much lower rate of binding compared with thiols and amines and are therefore employed less frequently (Freeman et al., 1995). Alcohols have not been shown to have an interaction with gold nanoparticles through the oxygen atom, despite it being a Group VI element like sulphur.

### 2.1.2 Binding of DNA to Gold Nanoparticles

Double stranded (ds) deoxyribonucleic acid (DNA) does not bind to citrate stabilised gold nanoparticles. This occurs due to the fact that when complementary strands of DNA hybridise, the ds-DNA forms a double helix with the bases involved in base-pairing and buried in the structure while the negatively charged sugar-phosphate backbone is exposed. The phosphate groups of the backbone, which are negatively charged, are repelled by the negative charged of the citrate on the gold surface. In contrast, despite the negatively charged backbone, single stranded (ss) DNA is able to adsorb to gold nanoparticles due to the fact that it can unwind and allow the hydrophobic bases to lie flat on and interact with the gold surface (Storhoff et al., 2002; Sandström et al., 2003).

The affinity of the DNA bases for gold nanoparticles varies with the functional groups that are involved in binding. The mechanism by which the bases bind to gold nanoparticles is thought to be different among the four bases. Surface enhanced Raman scattering (SERS)
studies suggest that in the case of adenine, it is the nitrogen atom of the imidazole ring that
binds to the gold surface while in the case of both cytosine and guanine, nitrogen atoms of
the pyrimidine ring interact with the gold (Jang, 2002). The oxygen of the carbonyl group of
the pyrimidine ring of thymine has been shown to interact with the gold surface (Jang, 2002).
Cytosine has been found to have the highest affinity to gold surfaces whilst thymine has the
lowest (Storhoff et al., 2002; Gourishankar et al., 2004).

There is growing interest in the conjugation of ss-DNA to gold nanoparticles, which can act
as probes in biosensors to monitor the hybridisation of complementary sequences. Although
ss-DNA is able to bind unassisted to nanoparticles, it is important to directionally conjugate
the DNA probes so that the target has access to the entire sequence attached to the
nanoparticle. This is normally achieved by the functionalisation of the probe with a thiol
group at one end. The thiol group preferentially binds to the gold surface and anchors the
DNA strand which remains free to hybridise with the target.

Specific conditions are required to conjugate functionalised DNA to nanoparticles and
prevent non-specific binding of the bases, which occurs at low ionic strengths. Methods such
as increasing salt concentration, heat and sonication have been used to reduce the non-
specific binding and allow only the thiol group to interact with the gold nanoparticle surface
(Hurst et al., 2006). This increases the probe density and makes the nanoparticles more
stable but it can also lead to steric hindrances in the hybridisation of the target DNA
(Castelino et al., 2005). Therefore it is important to be able to establish an optimal probe
density which will allow hybridisation to occur while keeping the nanoparticles stable.
2.1.3 Overview of Chapter

*N. meningitidis* is the only known *Neisseria* species that expresses the *porA* gene, which encodes for the class 1 outer membrane protein PorA (Unemo *et al.*, 2005). The protein is found to consist of the variable regions VR1, VR2 and VR3 that are used to serosubtype strains (Clarke *et al.*, 2003). These regions are contained in surface-exposed loops of the protein and are under evolutionary pressure (Sacchi *et al.*, 1998). These sequences are distributed between highly conserved regions (Barlow *et al.*, 1989).

In this chapter, the sequence of the *porA* gene was examined and a target designed to a conserved region in the gene. A Southern blot analysis was carried out to establish if the target sequence recognises the gene in the *N. meningitidis* genome, while the theoretical specificity of the target was established using the BLAST (Basic Local Alignment Search Tool) database.

Binding isotherm studies using model molecules were carried out to compare the binding of thiol and amine groups to gold nanoparticle. The effects of ligand concentration and pH conditions on the binding of thiol, amine and hydroxyl groups were studied using the dipole plasmon resonance properties of gold nanoparticles to monitor the binding. Fourier Transform Infrared (FTIR) studies were performed to confirm which functional groups of the compounds bind to the gold nanoparticles. These studies on model compounds were done to compare the interaction of the different functional groups to gold nanoparticles because although several studies have been done previously, there still is uncertainty.
Using information on the binding of different functional groups, a pair of probes was designed for the detection of \textit{N. meningitidis} DNA and the effect of salt concentration and pH on their binding to gold nanoparticles during their construction was examined.

\section*{2.2 Methods}

\subsection*{2.2.1 Design of Target/Probes}

The nucleotide sequence of the \textit{porA} gene from \textit{N. meningitidis} strain MC58 (Genbank accession number AF226344) (Pizza et al., 2000) was examined to select a suitable target.

\textbf{Sequence BLAST Search}

The nucleotide sequence of the selected 24 base target was used to perform a BLAST search (February 1, 2008). The MEGABLAST algorithm used the NCBI non-redundant database to search for matches. The expect threshold was set at 10 and the scoring parameters consisted of a gap cost with existence of 5 and extension of 2. Low complexity regions were filtered.

\textbf{Analysis of Specificity and Binding of DNA Probes}

The genome sequence of \textit{N. meningitidis} strain MC58 (Genbank accession number AE002098) was analysed using Vector NTI version 10 (Invitrogen, USA) to generate a map of restriction sites. Southern blotting using the synthetic target sequence as the probe was performed to verify the presence of the target area in the genome.
Preparation of Probe

A digoxigenin DNA labelling kit (Roche Diagnostics, Germany) was used to label the probe in preparation for Southern blot hybridisation. The probe (porA4) was labelled with digoxigenin (DIG)-11-dUTP using the random primed labelling reaction. Three micrograms of porA4 was diluted to 15 µL with Milli-Q water and denatured at 100°C for 10 minutes and then chilled on ice. The sample was then mixed with 2 µL of 10× dNTP labelling mixture, 2 µL of 10× hexanucleotide mixture and 1 µL of Klenow enzyme (100 U/mL) and incubated at 37°C for 20 hours. The reaction was stopped by the addition of 2 µL of 0.2 M ethylenediamine tetraacetate (EDTA).

The concentration of the labelled probe was determined with a spot test by comparison to DIG-labelled control DNA. Serial dilutions of the probe and control DNA were spotted on a piece of Immobilon positively charged nylon membrane (Millipore, USA) according to the manufacturer’s instructions (Roche Diagnostics, Germany). The membrane was then cross-linked under UV light for 5 minutes. The membrane was washed briefly in 2× SSC wash buffer containing 0.1% SDS, 0.3 M NaCl, 30 mM trisodium citrate pH 7, and then incubated for 30 minutes at room temperature in blocking solution (1% (w/v) Blocking Reagent (Roche Diagnostics, Germany) in maleic acid buffer containing 100 mM maleic acid, 150 mM NaCl pH 7.5). The membrane was then incubated with anti-DIG Fab fragment-alkaline phosphatase (Roche Diagnostics, Germany) (diluted 1:5,000 in blocking solution) for 30 minutes at room temperature. Unbound anti-DIG was removed with two 15 minute washes in maleic acid wash buffer containing maleic acid buffer, 0.3% (v/v) Tween 20. The membrane was briefly rinsed in Milli-Q water before colour was developed using Western
Blue substrate (Promega, USA). Colour development was stopped by washing the membrane with Milli-Q water. The experimental probe spot intensities were compared with the standard DIG-Labelled control DNA spots to estimate the probe concentration. The probe was diluted in standard hybridisation solution to give a final concentration of 8.4 ng/mL for use in Southern blot hybridisation.

**Extraction of Meningococcal Genomic DNA**

*N. meningitidis* was cultured in Meuller-Hinton broth (Oxoid, UK) and incubated at 37°C for 24 hours. The meningococci were harvested from 50 mL of culture by centrifugation at 5000 × g for 15 minutes. The bacterial pellet was resuspended in 10 mL of lysis buffer (refer to Appendix A) and mixed thoroughly. After a 2 hour incubation at 37°C, 1.8 mL of 5 M NaCl and 1.5 mL of CTAB/NaCl solution (refer to Appendix A) were added, mixed and the mixture was incubated for 10 minutes at 65°C. Genomic DNA was extracted from the cell lysate using an equal volume of chloroform/isoamyl alcohol (24:1). The phases were separated by centrifugation at 5000 × g for 10 minutes, the upper aqueous phase collected and mixed with an equal volume of phenol/chloroform-isoamyl alcohol (25:24:1). The phases were separated as before. The DNA was precipitated from the aqueous layer with 6 mL of isopropanol and washed with 70% (v/v) ethanol. It was then air dried and dissolved in 10 mM Tris, 1 mM EDTA pH 8.0 (TE buffer) overnight at room temperature. The genomic DNA was stored at -20°C.

The DNA was diluted in Milli-Q water and its concentration and purity estimated by light absorption at wavelengths 260 and 280 nm using a Hitachi U-2000 spectrophotometer (Hitachi, Japan). The concentration of ds-DNA was calculated from the absorbance at 260
nm. The purity was determined using the absorbance ratio 260/280 nm; a ratio between 1.8 and 2.0 was considered acceptable.

Restriction Digests

Restriction enzyme reaction conditions used were in accordance with the manufacturer’s specification (Promega, USA). The enzymes *Bam*HI and *Eco*RI were employed to digest the DNA separately. Ten micrograms of genomic DNA was digested with 40 U of enzyme, in 1× buffer supplied by the manufacturer. Acetylated BSA, also supplied by the manufacturer, was added at a concentration of 100 µg/mL in a total volume of 20 µL for stabilisation and the reaction was incubated at 37°C overnight.

Agarose Gel Electrophoresis

A 0.8% (w/v) agarose gel in 1× Tris-Acetate-EDTA (TAE) electrophoresis buffer (refer to Appendix A) was used to separate the DNA fragments. The genomic DNA restriction digests were mixed with 1× loading buffer (refer to Appendix A) and electrophoresed against a 1kb DNA ladder (Promega, USA) in an adjacent lane. Electrophoresis was performed at a constant voltage of 50 V for 6 hours. The gel was stained with 3 µg/ml of ethidium bromide before destaining in running tap water. The DNA products were visualized with an UV illuminator and photographed using a Biorad Geldoc imaging system running Quantity One software (Biorad, USA).
Southern Blot and Hybridisation

The DNA fragments in the gel were depurinated by immersion in 0.2 M HCl for 10 minutes. After rinsing the gel in Milli-Q water, the DNA was denatured by immersing and gently shaking it in 0.5 M NaOH, 1.5 M NaCl twice for 15 minutes each. The gel was rinsed briefly in Milli-Q water, and neutralised in 0.5 M Tris-HCl, 3 M NaCl pH 7.5 for 30 minutes during which time the neutralisation solution was changed once. The DNA fragments were transferred from the gel to a pre-wetted nylon membrane using 3 M NaCl, 300 mM trisodium citrate pH 7 (20× SSC) overnight. The membrane was then cross-linked under UV light for 5 minutes and rinsed briefly in Milli-Q water.

The membrane was pre-hybridised at 55°C in 20 mL per 100 cm² of membrane of standard hybridisation solution (5× SSC, 0.1% N-lauroylsarcosine, 0.02% SDS , 1% Blocking reagent diluted in Milli-Q H₂O) for 2 hours. The probe was diluted in 20 mL per 100 cm² of membrane of standard hybridisation solution and heated for 5 minutes at 100°C, cooled on ice, then added to the membrane and incubated overnight at 55°C. The membrane was washed twice with 2× SSC wash buffer (refer to Appendix A) for 5 minutes at room temperature, then washed twice in 0.5× SSC wash buffer (refer to Appendix A) for 15 minutes at 55°C. Following these stringency washes, the membrane was equilibrated in maleic acid wash buffer for 1 minute then incubated in blocking solution for 1 hour at room temperature. The blocking solution was discarded and replaced with anti-DIG solution and incubated for 30 minutes at room temperature. Unbound anti-DIG was removed with two 15 minutes washes in maleic acid wash buffer. The membrane was briefly rinsed in Milli-Q
water before colour was developed using Western Blue substrate (Promega, USA). Colour development was stopped by washing the membrane with H₂O.

### 2.2.2 Synthesis and Characterisation of Gold Nanoparticles

Monodispersed 15 nm nanoparticles were synthesised by reduction of hydrogen tetrachloroauration by trisodium citrate using a modification of the method described in Turkevich et al. (1951). All glassware and the stirring bar were cleaned in freshly prepared aqua regia (3 parts HCl:1 part HNO₃), washed well with Milli-Q water and dried before use. Ten millilitres of 5 mM HAuCl₄·3H₂O was added to 180 mL Milli-Q water and brought to boil in a two-neck round bottom flask fitted to a condenser. Ten millilitres of 0.5% (w/v) trisodium citrate was rapidly added while the solution was being stirred vigorously. After the colour of the solution underwent a series of changes and finally changed to red, it was boiled for another 10 minutes before the flask was allowed to cool to room temperature while being stirred. If necessary, the final volume was adjusted to 200 mL. This resulted in a nanoparticle solution containing 0.25 mM gold. The solution was filtered through a 0.45 µm micropore polypropylene membrane (Alltech, USA) and stored at 4°C away from light to prevent oxidation.

The UV-visible absorption spectrum of the nanoparticle suspension was recorded using a Hitachi U-2000 spectrophotometer (Hitachi, Japan). Transmission electron microscope (TEM) images of the nanoparticles were taken to determine the quality and the mean diameter. A 10 µL drop of nanoparticle solution was allowed to air-dry on Formvar-coated Cu grids. TEM measurements were carried out using a JEOL 2010 STEM operated at an accelerating voltage of 200 kV.
2.2.3 Studies on Binding of Functional Groups to Gold Nanoparticles

Binding Isotherms

In the binding isotherm studies, mercaptosuccinic acid (MSA) was used as a model compound containing a thiol group whilst 2,3-diaminopropionic acid monohydrochloride (DAPA) was used as a model compound containing an amine group.

Analysis of Binding of Thiol Groups to Gold Nanoparticles

One millilitre aliquots of gold nanoparticle solution were concentrated by centrifugation at 10,000 $\times g$ for 15 minutes. The supernatant fluid was discarded and the nanoparticles were resuspended in 1 mL solutions containing 0 to 20 mM MSA in 10 mM phosphate buffer (pH 6.5, 7.5, 8.5 and 9.5 respectively). Each study was performed in triplicate and allowed to incubate for 40 hours to reach equilibrium after which the nanoparticles were separated from the unbound MSA molecules by centrifugation at 10,000 $\times g$ for 15 minutes. Ellman’s test for thiol groups was used to quantify the amount of MSA in the supernatant (Ellman, 1959). Briefly, 10 $\mu$L of the supernatant liquid was added to 100 $\mu$L of 10 mM DTNB solution (refer to Appendix A) and diluted with 1 mL of 0.1 M phosphate buffer pH 8.0. After the reaction was incubated for 30 minutes at room temperature, absorbance was measured at 412 nm using a Hitachi U-2000 spectrophotometer (Hitachi, Japan). Standard curves were prepared using 0 to 20 mM MSA in 10 mM phosphate buffer (pH 6.5, 7.5, 8.5 and 9.5 respectively). The difference between the amount of MSA added to the nanoparticles and the amount left remaining in solution was used to determine the amount of MSA bound to gold nanoparticles. The results were analysed and the curve was fitted with non-linear regression.
(one-site binding) using GraphPad Prism version 4.02 for Windows (GraphPad Software, USA).

**Analysis of Binding of Amine Groups to Gold Nanoparticles**

One millilitre aliquots of gold nanoparticle solution were concentrated by centrifugation, and the nanoparticles were resuspended in 1 mL solutions containing 0 to 6 mM of DAPA in phosphate buffer (pH 6.5, 7.5, 8.5 and 9.5 respectively). Each study was performed in triplicate and allowed to incubate for 40 hours to reach equilibrium after which the nanoparticles were separated from the unbound DAPA molecules by centrifugation. A ninhydrin assay was used to quantify the amount of DAPA remaining in solution by estimating the free amine content in the solution (Plummer, 1987). One hundred microlitres of supernatant fluid was added to 100 µL of ninhydrin reagent (refer to Appendix A) and incubated in a 100°C water bath for 15 minutes. Once cooled to room temperature, the solution was diluted with 1 mL of 50% (v/v) ethanol and the absorbance at 400 nm was measured. Standard curves were prepared using 0 to 6 mM of DAPA in phosphate buffer (pH 6.5, 7.5, 8.5 and 9.5 respectively). The difference between DAPA added to the nanoparticles and left remaining in solution was used to determine the amount of DAPA bound to gold nanoparticles. The results were analysed as described previously with the MSA studies.

**Plasmon Resonance Studies**

The optical properties of gold nanoparticle solutions were monitored as a function of time, concentration of amino acid and pH using a Varian Cary 500 spectrophotometer (Varian,
Australia), with absorbance recorded at an interval of 1 nm in the range of 400-800 nm. The amino acids and derivatives used in this study were cysteine, 2,3-diaminopropionic acid (DAPA), serine and 2-aminobutyric acid (abu) as illustrated in Figure 2.1. For each determination, 1 mL of gold nanoparticle solution was used. The nanoparticles were harvested by centrifugation at 10,000 \( \times g \) for 15 minutes, the supernatant was discarded and the oily red precipitate was resuspended in the appropriate solution. The solutions used were 0-100 \( \mu M \) of the amino acids and their derivatives at pH 6.5, 7.5, 8.5 or 9.5 respectively. The spectra of the nanoparticle solutions were monitored for 1 hour with wavelength scans recorded every 5 minutes.

**Fourier Transform- Infrared (FTIR) Spectroscopy**

FTIR spectra of the amino acids and their derivatives together with their corresponding gold nanoparticle conjugates were recorded using a Perkin-Elmer AutoIMAGE FTIR microscope (Perkin-Elmer, USA) system in the range of 700 -4000 cm\(^{-1}\). The samples were prepared by air-drying 10 \( \mu L \) of the samples on an aluminium-coated glass slide (Thermo Spectra-Tech, USA). The gold-amino acid conjugates were prepared in 0.1 M ammonium acetate pH 7 so that no precipitates remained when the buffer evaporated.
Cysteine

2,3-Diaminopropionic acid (DAPA)

Serine

2-Aminobutyric acid (abu)

Figure 2.1: Structures of the amino acids and their derivatives used in the binding plasmon resonance studies.
2.2.4 Binding of Thiol-modified DNA to Gold Nanoparticles

The lyophilised DNA probe porA4-A was dissolved in molecular biology grade H₂O (Sigma, USA) to a final concentration of 1 mM. Prior to use, the probe was reduced using tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma, USA). Two microlitres of freshly prepared 10 mM TCEP was added to every 8 µL of probe and incubated at room temperature for 1 hour.

Effect of Salt Concentration

The NaCl concentration of the solution was varied between 0 and 0.5 M while the probe was conjugated to gold nanoparticles to study the effect of salt concentration on the amount of probe bound. Triplicates of 250 µL of gold nanoparticle solution were used for each determination. The nanoparticles were first concentrated by centrifugation at 10,000 × g for 15 minutes and then resuspended in 10 mM phosphate buffer pH 7 containing 1 nmole of reduced probe. The solutions were incubated at room temperature overnight to allow the probe to bind to the gold nanoparticles. The NaCl concentration in the solution was then increased in 0.05 M increments using a 5 M NaCl solution. The solutions were left for at least 1 hour between each addition until the appropriate NaCl concentrations which were varied between 0 and 0.5 M was reached. The final volume of each solution was 250 µL. The reactions were incubated for an additional 16 hours.

The nanoparticles were separated from the unbound DNA molecules by centrifugation at 10,000 × g for 15 minutes. An assay using Hoechst dye 33258 was used to quantify the DNA remaining in the supernatant (Stout and Becker, 1982). Two millilitres of dye solution
(4.5 × 10^7 M Hoechst 33258 in 25 mM phosphate buffer pH 7) was added to 100 µL of the supernatant and the emitted fluorescence was measured at 450 nm during excitation at 360 nm using a Hitachi F-2000 spectrofluorometer (Hitachi, Japan).

A 3% (w/v) agarose gel was used to visualise the relative sizes of DNA-conjugated gold nanoparticles at NaCl concentrations of 0 to 0.5 M. Twenty microlitres of gold nanoparticle samples were mixed with 1× loading buffer (refer to Appendix A) and underwent electrophoresis. The gel was run in Tris-Borate-EDTA (TBE) electrophoresis buffer (refer to Appendix A) at a constant voltage of 80 V for 3 hours.

**Effect of pH**

Two hundred and fifty microlitres of gold nanoparticle solution were first concentrated by centrifugation at 10,000 × g for 15 minutes and then resuspended in 244 µL of 10 mM phosphate buffer (at pH of 5, 6, 7, 8 and 9 respectively) each containing 1 nmole of the reduced probe. The solutions were incubated at room temperature overnight to allow the porA4-A DNA probe to bind to the gold nanoparticles. The NaCl concentration was then increased to 0.1 M by two additions of 2.5 µL of 5 M NaCl, and left for at least 1 hour between each addition. The reactions were then incubated for an additional 16 hours.

The nanoparticle solutions were centrifuged at 10,000 × g for 15 minutes and the amount of DNA remaining in the supernatant was quantified using Hoechst dye 33258 as described previously. Assays were done in triplicate.
Chapter 2: Conjugation of DNA to Nanoparticles

2.3 Results and Discussion

2.3.1 Design of Target/Probes

The nucleotide sequence of the \textit{porA} gene was examined to select a unique target for the detection of meningococcal DNA. In addition to being specific, the target needed to be “relatively” conserved in different strains of \textit{N. meningitidis}. The \textit{N. meningitidis} PorA Variable Region Database (http://neisseria.org/nm/typing/pora/) that is based on the scheme of Suker \textit{et al.} (1994) was used to identify the VR1 and VR2 regions within the PorA protein sequence and the VR3 region was located using the amino acid sequences listed in Clarke \textit{et al.}, (2003). The variable regions VR1, VR2 and VR3 of the porA protein were identified and typed according to the amino acid sequences listed in Table 2.1. These regions were regarded as unsuitable as a target because of their high variability among different strains and the corresponding DNA sequences were not considered when choosing the target region (Figure 2.2).

The length of the target was set at 24 bases which, when acting as a linker between gold nanoparticles, has been shown to produce a greater shift of the plasmon resonance peak at a faster rate compared with longer DNA strands (Storhoff \textit{et al.}, 2000). A pair of probes were designed, each 12 bases long and complementary to one half of the target. The following 24 base sequences could be used as targets since their complementary sequences had 50% or less purine content and had low self-complementarity.

\begin{align*}
\text{porA1} & \quad 5'-\text{TAAAGCCGATAAAACGAGCCGAAAT}-3' \\
\text{porA4} & \quad 5'-\text{TTCAGCCAAGCGCCAGACACGATG}-3'
\end{align*}
Table 2.1: Variable regions of the PorA protein.

<table>
<thead>
<tr>
<th>Variable Region</th>
<th>Type</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VR1</td>
<td>Peptide 7</td>
<td>AQAANGGASGQVKVTKVTKA</td>
</tr>
<tr>
<td>VR2</td>
<td>Peptide 16-2</td>
<td>YYTKNTNNNLTLP</td>
</tr>
<tr>
<td>VR3</td>
<td>Peptide 35</td>
<td>LIGSGSDQ</td>
</tr>
</tbody>
</table>
Figure 2.2: porA gene nucleotide sequence and corresponding amino acid sequence.

The amino acid sequence is in grey aligned below the nucleotide sequence. The variable regions are inverted on a black background. The porA1 region is highlighted in dark grey. The selected porA4 target region is highlighted in light grey. The nucleotide indicated with * is variable in different strains.
The probes corresponding to these possible targets were assessed for suitability through melting temperature analyses and the propensity for dimer formation using Primer Designer 5, Version 5.11 (Scientific & Educational Software, USA) (Table 2.2). Due to the predicted low melting temperatures of the porA1 probes, this target was not considered suitable and porA4 was selected as the target (Table 2.3). The two 12 base long probes against the porA4 target have melting temperatures of 44 and 48°C respectively.

The BLAST search of porA4 revealed more than 250 hits that had 100% identity and an expectation value of $4.0 \times 10^{-4}$ or lower. These matches were all porA genes from various strains of *N. meningitidis*. However, there were 20 other *N. meningitidis* porA gene matches that had expectation values of $9.4 \times 10^{-2}$. Each of these had a single nucleotide difference as highlighted in Figure 2.3, 17 of the sequences had a nucleotide difference at the 12th position as marked with * in Figure 2.2. No other sequences had an expectation value below 5.8, although the *N. gonorrhoeae* porA pseudogene (represented by the sequence with the Genbank accession number AJ223449 in Figure 2.3) and several *Burkholderia* species had high similarity.

Although the BLAST search revealed a high level of conservation in the target region among meningococci strains, total conservation was not observed. A small number of strains possessed a single polymorphism, commonly a substitution of guanine for adenine at position 12 of porA4. If further testing revealed this to be a hindrance in the detection of all meningococcal strains, the incorporation of a degenerate residue within the probe would be required.
Table 2.2: Possible target sequences and complementary probes against them.

<table>
<thead>
<tr>
<th>Targets</th>
<th>Probes</th>
<th>Tm (°C)</th>
<th>Dimers</th>
</tr>
</thead>
<tbody>
<tr>
<td>porA1</td>
<td>ATTTCGGCTCGT</td>
<td>46</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>TTATCGGCTTTA</td>
<td>36</td>
<td>None</td>
</tr>
<tr>
<td>porA4</td>
<td>CATCGTGCTCTGG</td>
<td>44</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>CGCTTGGCTGAA</td>
<td>48</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 2.3: DNA sequence of selected target and corresponding probes.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target</td>
<td>porA4</td>
</tr>
<tr>
<td>Probe A</td>
<td>porA4-A CATCGTGCTCTGG</td>
</tr>
<tr>
<td>Probe B</td>
<td>porA4-B CGCTTGGCTGAA</td>
</tr>
</tbody>
</table>

**porA4 target sequence**: TTCAGCCAAGCGCCAGACACGATG

**N. meningitidis porA (consensus)**: ................................. :RF564254 >250

**N. meningitidis 8050 porA**: ...................................... :RF564249 x1

**N. meningitidis M3087 porA**: ...................................... :AF146588 x17

**N. meningitidis porA**: .............................................. :U92931 x1

**N. meningitidis N103/97 porA**: ...................................... :AF148643 x1

**N. gonorrhoeae porA pseudogene**: ................................. :AJ223449 x8

**Burkholderia spp.**: ................................................... :CP000086 x9

Figure 2.3: Alignment of porA4 target sequence against representative sequences identified using MEGABLAST algorithm.

Differences from the porA4 sequence are highlighted. Genbank accession numbers for representative sequences and number of sequences are included to the right of the alignment.
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The *N. gonorrhoeae* porA pseudogene and a *Burkholderia* species sequence showed high similarity to the target region, with only a two nucleotide difference within the 24 nucleotide sequence. While their hybridisation with probes is possible under less stringent conditions, it is unlikely that the samples such as cerebrospinal fluid (CSF) or blood used for diagnosing meningococcal disease will contain either species.

**Analysis of Specificity and Binding of porA4 Probe to Genomic DNA Target**

The presence of the selected target DNA sequence (porA4) in genomic DNA extracted from the *N. meningitidis* MC58 strain was confirmed by Southern blot hybridisation using porA4 as the probe. The genomic DNA was digested with *BamHI* or *EcoRI* and probed with DIG-labelled porA4 in Southern blot hybridisation (Figure 2.4). As predicted, a 5.2 kb hybridising DNA fragment was observed in the *BamHI*-digested DNA, while a 32 kb DNA fragment was detected in the *EcoRI*-digested DNA.

Since it is now known that the target DNA sequence (porA4) is able to hybridise to meningococcal genomic DNA (coding strand), it may be assumed that the corresponding probes (porA4-A and porA4-B) are able to subsequently bind to the complementary DNA sequence (non-coding strand) in the genome. Therefore these probes may be used for the detection of meningococcal genomic DNA from biological samples by detection of their hybridisation to the DNA.
Figure 2.4: Southern blot analysis.

(a) Agarose gel electrophoresis of genomic restriction digests. (b) Southern blot membrane probed with DIG-labeled porA4. Lane 1: 1 kb DNA ladder; Lane 2: BamHI digest; Lane 3: EcoRI digest.
2.3.2 Synthesis and Characterisation of Gold Nanoparticles

The UV-visible spectrum of freshly synthesised gold nanoparticles showed a dipole plasmon resonance band with a $\lambda_{\text{max}}$ at 518 nm which is characteristic of gold nanoparticle solutions of a nanoparticle size of approximately 15 nm (Figure 2.5). Assuming a 100% yield from the citrate reduction of 0.25 mM HAuCl$_4$·3H$_2$O, the molar absorptivity coefficient for the gold nanoparticles was determined to be 3227.6 M$^{-1}$cm$^{-1}$ at 518 nm.

According to the TEM analysis, the average diameter of the nanoparticles was 14.8 ± 1.2 nm (Figure 2.6). These size measurements were determined by taking the average of the x and y axes of 42 nanoparticles. The standard deviation was 1.2 nm which is less than 20% of the average size, indicating that the nanoparticles can be considered monodisperse (Turkevich et al., 1951).

In order to calculate the number of nanoparticles in solution, two assumptions were made (a) the density of nanoparticles is the same as that of bulk gold (19.30 g.cm$^{-3}$) and (b) all of the gold salt that was added to the reaction was reduced. Using these assumptions, a solution of nanoparticles with a mean diameter of 14.8 nm contains approximately $1.503 \times 10^{12}$ particles/mL of suspension. This nanoparticle concentration results in a molar absorptivity coefficient of $3.222 \times 10^8$ M$^{-1}$cm$^{-1}$ at 518 nm, which is in agreement with data previously reported (Sandström et al., 2003).
Figure 2.5: UV-visible absorbance spectrum of gold nanoparticles prepared by citrate reduction.
Figure 2.6: Representative TEM micrograph of gold nanoparticles prepared by citrate reduction.

(a) TEM micrograph shows well-separated and monodisperse 14.8 nm nanoparticles (scale bar = 20 nm).  (b) Electron diffraction pattern of the particles has a diffraction ring consistent with crystalline gold.
The nanoparticle solution derived from 0.25 mM hydrogen tetrachloroaurate, synthesised by the citrate reduction method was highly stable and was able to undergo centrifugation and resuspension in water or buffer as needed without measurable loss of nanoparticles, allowing concentration of the nanoparticles by this method if necessary.

However, when the concentration of the gold salt was increased together with the corresponding citrate to give a more concentrated nanoparticle solution, it resulted in a solution with highly variable nanoparticle sizes, which could not undergo centrifugation as it caused aggregation of the larger particles while the smaller particles still remained in solution. This may be due to a large number of nuclei forming at different times, resulting in the formation of a range of particles sizes.

**2.3.3 Studies on Binding of Functional Groups to Gold Nanoparticles**

Thiols are the most commonly used functionalising group employed when binding to gold nanoparticles. No definitive study has been done comparing the binding of thiol and other functional groups to gold nanoparticles in aqueous solutions as a function of pH. These studies were therefore carried out to examine this fundamental comparison and determine if thiols are the ideal group to use in this application, or to establish alternate functional groups that would prove more advantageous.

**Binding Isotherms**

Binding isotherms were determined to quantitatively compare the binding of thiol and amine groups to gold nanoparticles so that the functional group with the greater affinity may be used to conjugate the DNA probes to the nanoparticles. Mercaptosuccinic acid (MSA) was used as
a model compound containing a thiol group and 2,3-diaminopropionic acid (DAPA) was used for amine groups.

The concentrations of MSA used in these studies were in the range from 0 to 20 mM. As shown in Figure 2.7, adsorption on the nanoparticles reached saturation at all the pH values examined when the concentration of MSA was increased above 10 mM. The data obtained was then fitted to a one-site binding model and the maximum MSA bound ($B_{\text{max}}$) and kinetic dissociation constant ($K_d$) values for MSA in buffer at different pH values obtained (Table 2.4). The $B_{\text{max}}$ values ranged from 241.0 to 253.4 molecules per nanoparticle and were not significantly different at these different pH values; that is, the maximum number of MSA molecules bound to each nanoparticle remained constant. However, the affinity of MSA for gold nanoparticles increased with increasing pH, as indicated by the decrease in the $K_d$ values with increasing pH values.

The concentrations of DAPA in the solutions used to establish the DAPA adsorption profile were in the range from 0 to 6 mM. As shown in Figure 2.8, adsorption on the nanoparticles reached saturation at all the pH values when the concentration of DAPA was increased above 4 mM. The data obtained was again fitted to a one-site binding model and the $B_{\text{max}}$ and $K_d$ values for DAPA in buffer at different pH values was obtained (Table 2.5). The $B_{\text{max}}$ values ranged from 76.4 to 80.4 molecules per nanoparticle and were not significantly different at different pH values. These $B_{\text{max}}$ values are lower than those observed for MSA, which had an average $B_{\text{max}}$ value of 247 molecules per nanoparticle. There was also no significant difference between the $K_d$ values at different pH values, but they were all significantly lower than those of MSA indicating that DAPA has a greater affinity for nanoparticles.
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Figure 2.7: Binding isotherms of MSA adsorbed to gold nanoparticles.

Table 2.4: Best-fit values for binding isotherms of MSA to gold nanoparticles.

<table>
<thead>
<tr>
<th>pH</th>
<th>$B_{\text{max}}$ (molecules/nanoparticle)</th>
<th>$K_d$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>250.7 ± 3.6</td>
<td>3.59 ± 0.26</td>
</tr>
<tr>
<td>7.5</td>
<td>242.7 ± 7.8</td>
<td>3.18 ± 0.23</td>
</tr>
<tr>
<td>8.5</td>
<td>241.0 ± 5.6</td>
<td>2.95 ± 0.16</td>
</tr>
<tr>
<td>9.5</td>
<td>253.4 ± 6.2</td>
<td>2.66 ± 0.14</td>
</tr>
</tbody>
</table>
Figure 2.8: Binding isotherms of DAPA adsorbed to gold nanoparticles.

Table 2.5: Best-fit values for binding isotherms of DAPA to gold nanoparticles.

<table>
<thead>
<tr>
<th>pH</th>
<th>$B_{max}$ (molecules/nanoparticle)</th>
<th>$K_d$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>80.4 ± 2.2</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td>7.5</td>
<td>80.1 ± 2.6</td>
<td>0.98 ± 0.07</td>
</tr>
<tr>
<td>8.5</td>
<td>78.8 ± 2.5</td>
<td>1.07 ± 0.09</td>
</tr>
<tr>
<td>9.5</td>
<td>76.4 ± 2.1</td>
<td>1.02 ± 0.08</td>
</tr>
</tbody>
</table>
A comparison of the binding isotherms of MSA and DAPA, indicated that MSA molecules were estimated to be approximately 3.1 times more closely packed on the 15 nm nanoparticle surface than DAPA, with approximately 247 molecules compared to approximately 79 DAPA molecules bound on one nanoparticle. This finding may be compared with those of Leff et al who have shown that thiols are approximately 2.25 times more densely pack on the gold nanoparticle surface than amines (Leff et al., 1996). The slight difference in the ratio of densities may be due to the difference in structure of the model compounds. However, DAPA appears to reach saturation of binding to gold nanoparticles at a lower concentration than MSA. This would suggest that DAPA has a greater affinity for gold nanoparticles.

**Plasmon Resonance Studies**

A number of different compounds have been known to bind to nanoparticles and also interact with functional groups on other nanoparticles resulting in the nanoparticles moving closer together (Mandal et al., 2001; Selvakannan et al., 2003; Bellino et al., 2004). One such compound that behaves in this manner is the amino acid cysteine that binds to silver or gold nanoparticles through the thiol group and forms hydrogen bonds between the amine and carboxyl groups as illustrated in Figure 2.9 (Mandal et al., 2001; Li et al., 2006). This leads to particle aggregation accompanied by a plasmon frequency red-shift. The resultant colorimetric change has been used to monitor the kinetics of binding of cysteine and other molecules to gold nanoparticle surfaces (Zhang et al., 2002).

Gold nanoparticles appear red in solution because of absorption by the plasmon oscillations, which peaks at around 520 nm. When the inter-particle distance is reduced to less than 2.5
Figure 2.9: Schematic diagram of aggregation of nanoparticles by cysteine through hydrogen bonding of cysteine molecules on different nanoparticles.
times the particle diameter, as when the particles aggregate, the plasmon peak shifts to between 600 and 700 nm because of dipole-dipole interactions between adjacent particles and the colour of the solution appears blue (Rechberger et al., 2003; Su et al., 2003).

This study was designed to probe the binding of amino acids or their derivatives to gold nanoparticle surfaces by monitoring the changes in the dipole plasmon resonance band associated with particle aggregation. The compounds were chosen because of their similar structure with the only difference being one functional group; the thiol group of the cysteine molecule is replaced with an amine, hydroxyl or methyl group in 2,3-diaminopropionic acid (DAPA), serine and 2-aminobutyric acid (abu) respectively (Figure 2.1).

As noted previously, cysteine may be expected to bind to the nanoparticle surface through the thiol group, this being the only orientation that could cause a colour shift in the solution by bringing the nanoparticles together through hydrogen bonding between cysteine molecules. While DAPA could bind to the surface through either amine group, subsequent aggregation of nanoparticles would only occur if the molecule is in the same orientation as cysteine. Serine could theoretically bind to gold surfaces through the amine group, but in order to produce a colour shift through nanoparticle aggregation, it has to bind though the hydroxyl group allowing hydrogen bonds to form between the amine and carboxyl groups. Abu was not expected to bind with the gold surface in an orientation enabling it to subsequently form hydrogen bonds and therefore it was used as a negative control.
The binding of the different functional groups between pH 6.5 and 9.5 was monitored and compared. The lowest pH used in this study was 6.5, below which a high percentage of the amino acids in solution tend to be in the cationic form and thus do not form hydrogen bonds.

Figure 2.10 shows the absorption spectra of the nanoparticle solutions as seen at 30 minutes duration when incubated with 60 nmole of the different amino acids at various pH values. Interaction of the amino acid with the nanoparticles was shown by the appearance of a light absorption band between 600 and 700 nm, indicating aggregation of the nanoparticles. For nanoparticles incubated with cysteine, a greater colour-shift at higher pH values was observed. However, a greater colour shift was observed at lower pH values in nanoparticle solutions incubated with DAPA. Nanoparticles incubated with serine did not show a wavelength shift at a pH value lower than 9.5. No wavelength shift was observed in nanoparticles incubated with abu at any pH examined. The methyl group of abu was not expected to interact with gold nanoparticle surfaces in a manner similar to that found in the thiol, amine and hydroxyl groups of the other amino acids; if it did interact, hydrogen bonds would subsequently form between its amine and hydroxyl groups and a wavelength shift would have been observed. However, among the functional groups that abu possesses, the amine group is the most likely group to bind to nanoparticle surfaces. If this binding does occur, the abu molecule would be orientated in a manner that will not allow hydrogen bonds such as those observed for the other amino acids to form, resulting in no wavelength shift as predicted.
Figure 2.10: UV-visible absorbance spectra of gold nanoparticles incubated with 60 nmole of amino acid or derivatives.

Incubated for 30 minutes at pH 6.5, 7.5, 8.5 and 9.5.
As the concentration of the amino acid was increased, a proportional wavelength shift occurred. In order to be able to compare the shift, the absorbance at 700 nm was monitored to detect the new peak that was formed between 600 and 700 nm (Figure 2.11). At pH values that facilitate more interactions between the nanoparticles and the amino acids, an increased absorbance at 700 nm was observed even at lower amino acid concentrations. In the case of both cysteine and serine, there appeared to be an inverse relationship between an increase in the pH and the concentration of amino acid required to increase the absorbance. However, the increase of absorbance at 700 nm was greatest at pH 6.5 for DAPA and decreased as the pH increased. Abu showed only very small changes at pH 9.5 where a slight increase was observed. For a better comparison of the rate of adsorption of the different functional groups for gold nanoparticles, the kinetics of particle aggregation was monitored by following the absorbance at 700 nm as a function of time (Figure 2.12). For each amino acid or derivative, a maximum absorbance was reached, after which it was observed to progressively decrease. This slight decrease in absorbance is likely due to the aggregation that results in the colour change from red to blue, and then continues until very large aggregates are formed which subsequently settle on the bottom of the cuvette in time decreasing the concentration of nanoparticles in solution and hence the absorbance.

The optimum pH for functional groups to bind to gold nanoparticles is assumed to be higher than the pKₐ value of the binding group because the group is required to be unprotonated for binding to occur (Selvakannan et al., 2003). This appears to be true for cysteine, which produced a greater and faster colour shift when the pH was raised above 8.33 (the pKₐ value of the thiol group). The aggregation at high pH values may also be aided by the greater
Figure 2.11: Plot of absorbance of gold nanoparticles at 700 nm after 30 minutes as a function of concentration of amino acids or derivatives.
Figure 2.12: Plot of absorbance of gold nanoparticles at 700 nm as a function of time when incubated with 60 nmole of the corresponding amino acid or derivative.
negative charge on the carboxyl group, which leads to more hydrogen bonding (Li et al., 2006). A study using lysine (a basic amino acid) and aspartic acid (an acidic amino acid) showed that although aspartic acid was able to bind strongly at neutral pH which is above its isoelectric point, lysine showed strong binding only above its isoelectric point (Joshi et al., 2004). Using this rationale, the pH needs to be higher than 13 and 9 for serine and DAPA respectively, to bind. However, binding of serine to the nanoparticles resulted in a wavelength shift at pH 9.5 and DAPA showed fastest binding at pH 6.5 which decreased slightly as the pH was increased.

The result for DAPA binding to the nanoparticles is in agreement with Basu and colleagues (2007). They have shown that amines adsorb to gold nanoparticles at lower pH values and that the dissociation of the carboxyl group at higher pH results in a negative charge that hinders the binding of the amine group to the gold surface.

**Fourier Transform Infrared (FTIR) Spectroscopy**

FTIR spectroscopy was performed on amino acid-conjugated gold nanoparticles to verify which functional group of the amino acid binds to nanoparticles, based in the shift or disappearance of compound-specific peaks.

The spectrum of free cysteine revealed peaks at 1650 and 1392 cm\(^{-1}\), which correspond to the asymmetrical and symmetrical stretching of the carboxyl group (COO\(^{-}\)) respectively (Figure 2.13). There was also a peak at 1541 cm\(^{-1}\) and a very broad peak between 2600 and 3200 cm\(^{-1}\), which correspond to the N-H bend and N-H stretch respectively. A peak was also observed at 2554 cm\(^{-1}\) which matches with the peak for S-H stretching. However, the
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Figure 2.13: FTIR spectra of free cysteine and cysteine bound to gold nanoparticles.

Figure 2.14: FTIR spectra of free DAPA and DAPA bound to gold nanoparticles.
spectrum for cysteine associated with gold nanoparticles, showed a shift in the COO$^-$ and a narrowing of N-H peaks and the peak for S-H disappeared, as reflected by the peak at 1595 cm$^{-1}$ which corresponds to the carboxylate stretch (Mandal et al., 2001) and the narrower N-H peak between 2800 and 3200 cm$^{-1}$ respectively.

Similar to cysteine, the DAPA spectrum also had COO$^-$ peaks at 1605 and 1381 cm$^{-1}$ and N-H peaks at 1541 cm$^{-1}$ and between 2700 and 3300 cm$^{-1}$ (Figure 2.14). When conjugated to gold nanoparticles, the N-H stretch peak of DAPA shifted to between 2600 and 2900 cm$^{-1}$.

The FTIR spectrum of free serine had peaks at 1658 and 1434 cm$^{-1}$ that match with the asymmetrical and symmetrical stretching of COO$^-$, a peak at 1509 cm$^{-1}$ that corresponds to the N-H bend, a broad peak from 2800-3300 cm$^{-1}$ for the N-H stretch and a peak at 1306 cm$^{-1}$ that corresponds to the in-plane deformation of the O-H group (Figure 2.15). However, the spectrum of serine attached to gold showed a shift in the N-H stretch peak to 2700-3000 cm$^{-1}$ and the 1306 cm$^{-1}$ O-H peak was not observed.

The spectrum of free abu had COO$^-$ peaks at 1648 and 1413 cm$^{-1}$ and N-H peaks at 1520 cm$^{-1}$ and between 2450 and 3300 cm$^{-1}$. When bound to gold nanoparticles, the N-H stretch peak of abu shifted to between 2480 and 3150 cm$^{-1}$ (Figure 2.16).

The differences in the FTIR spectra of free cysteine and nanoparticle-bound cysteine include the disappearance of the S-H peak and shifts in the N-H and COO$^-$ peaks. This confirms the results of earlier studies on the binding of cysteine to gold nanoparticles (Aryal et al., 2006). The absence of the S-H peak is thought to indicate the bond that is formed between the sulphur atom and the gold. The shifts in the other peaks can be explained by the hydrogen
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Figure 2.15: FTIR spectra of free serine and serine bound to gold nanoparticles.

Figure 2.16: FTIR spectra of free abu and abu bound to gold nanoparticles.
bonds that are formed between the amine and carboxyl groups (Mandal et al., 2001). The FTIR spectra of DAPA as well as abu show a sharpening and shift of the N-H stretch peaks, indicating binding through the amine group. Serine lost the O-H peak in the FTIR spectrum when it bound to gold nanoparticles. However, a shift and sharpening of the N-H stretch peak was also observed indicating possible binding to the gold surface through the amine group as well. This would not have interfered in the plasmon resonance studies as the hydrogen bonds would only have been formed when the hydroxyl group bound, causing the wavelength shift.

The thiol group was selected as the functional group to be used for conjugating the DNA probes to gold nanoparticles, in spite of its lower affinity for gold nanoparticles compared with amine groups. This was due to the fact that they could be more densely packed on the surface of gold nanoparticles compared with amine groups. The rate of binding could be adjusted by changing the pH of the solution and could be increased by increasing the pH to above 8.5. Assuming the binding behaviour of the thiol group in the modified DNA probe is similar to that of the thiol group of cysteine, these conditions were used when conjugating the probe to gold nanoparticles.

2.3.4 Binding of Thiol-modified DNA to Gold Nanoparticles

Following the binding studies using model compounds that confirmed that thiol groups were the ideal functional group to conjugate molecules to gold nanoparticles, the two DNA probes designed in Section 2.3.1 were synthesised with a thiol group at the 5’ or 3’ end. These thiol-modified DNA probes were then used to functionalise gold nanoparticles to be used as probes in the following chapters.
Preliminary studies showed that the DNA probe was able to bind to gold nanoparticles in 10 mM phosphate buffer pH 7. Following the removal of unbound probe from the nanoparticles, an absorption peak was observed at 260 nm indicating the presence of DNA bound to the nanoparticles (Figure 2.17). The plasmon resonance peak which was originally at 518 nm had shifted to 524 nm with a slight increase in the absorption between 600 and 700 nm, which was indicative of slight aggregation. The effects of NaCl concentration and pH of the buffer on the binding of the probes to gold nanoparticles were then examined.

**Effect of Salt Concentration**

Sodium chloride was added to the DNA/gold nanoparticle solution in a concentration range from 0 to 0.5 M. The salt concentration in the solution could not be increased to the required concentration in one step as this resulted in the aggregation of nanoparticles. Therefore, the stock 5 M NaCl solution was added gradually until the required concentration was reached. This allowed a greater number of DNA molecules to bind, which stabilised the nanoparticles thus allowing subsequent additions of salt. Therefore, the increased stability of nanoparticles when NaCl was added to the solution was an indication that DNA had bound to the particles since bare nanoparticles aggregate immediately at a NaCl concentration as low as 0.05 M.

Bare gold nanoparticles in solution have an absorbance at 260 nm (Figure 2.17), thus preventing quantitative estimation of DNA bound to the nanoparticles. Instead, the amount of DNA bound to the nanoparticles was estimated using the Hoechst dye 33258 method (Stout and Becker, 1982). Once conjugation had occurred, the washing steps resulted in loss of a fraction of the nanoparticles from aggregation and therefore the supernatant solution rather than the nanoparticles was used to quantify the probe molecules that did not bind to the
Figure 2.17: Wavelength scan of bare gold nanoparticles and DNA-conjugated gold nanoparticles.

When DNA is conjugated to nanoparticles, an additional peak is observed at 260 nm.
nanoparticles. The amount of the probe bound to nanoparticles was estimated from this value.

The number of thiol-modified DNA molecules that bind to gold nanoparticles increased with rising NaCl concentrations until a saturation point was reached. At saturation, which was observed at a salt concentration of 0.35 M (Figure 2.18), each nanoparticle carried approximately 150 molecules of thiol-modified DNA. This is in agreement with a previous study that estimated a maximum of 159 DNA molecules per 15 nm particle although it was achieved at 0.1 M NaCl concentrations (Demers et al., 2000). This difference in the salt concentration required to achieve a similar coverage may be due to the extended time period during which their nanoparticles were incubated with the DNA.

By estimating the amount of DNA bound to the nanoparticles, it was observed that there is greater binding of DNA as the salt concentration was increased. This observation was also visualised by gel electrophoresis. The binding of DNA reduced the relative mobility of the nanoparticles in an agarose gel due to the increase in size of the conjugate (Figure 2.19). It was noticeable from the gel that the nanoparticles are saturated at a salt concentration of 0.35 M as the apparent size of the conjugates do not increase at higher salt concentrations.

Bare gold nanoparticles were not analysed on the gel because they tended to aggregate due to the high ionic strength of the TBE buffer used in the electrophoresis. In contrast, DNA-conjugated gold nanoparticles have greater stability and do not aggregate in the TBE buffer.

The increase in the binding of the thiol-modified probe to gold nanoparticles with increasing salt concentrations is likely due to prevention of non-specific binding of the DNA bases to the gold surface. Although the thiol group would be expected to have a greater affinity than
Figure 2.18: Effect of NaCl concentration on the binding of thiol-modified DNA to gold nanoparticles.

Figure 2.19: Agarose gel loaded with DNA-conjugated gold nanoparticles. The NaCl concentration is increased from 0.05 M to 0.5 M in 0.05 M increments from Lane 1 to Lane 10.
the DNA bases for the gold surface, it can be hindered by the non-specific binding of the bases. This would lead to the probes lying flat on the nanoparticle surface thus preventing attachment by other probe molecules in solution. This difficulty can thus be avoided by increasing the ionic strength of the solution, which may be expected to cause the bases to desorb and allow the remaining thiol groups to bind.

**Effect of pH**

Although there have been many studies on the binding of thiol-modified DNA to gold nanoparticles, none have examined the effect of pH which was examined in this study. This was performed by estimating the amount of DNA bound to the nanoparticles at different pH values in the range of 5 to 10. The data presented in Figure 2.20 shows that as the pH of the solution was increased, the binding of DNA to gold nanoparticles increased. It was expected to increase further if the pH was increased. The number of DNA molecules per nanoparticles was lower that those obtained by varying NaCl concentrations and may be due to the shorter incubation time used in this study. This indicated that the DNA molecules have not reached equilibrium within about 35 hours which was the incubation time.

Increasing the pH of the solution increased the binding of DNA to the gold nanoparticles, possibly due to deprotonation of the thiol group as described in Section 2.3.3. Since an alkaline pH was not suitable for hybridisation studies, the pH was lowered to pH 7 following completion of the conjugation prior to use of the conjugated nanoparticles in the detection studies.
Figure 2.20: Effect of pH of the solution on the binding of thiol-modified DNA to gold nanoparticles.
2.4 Summary

The sequence of the \textit{porA} gene, which is only expressed by \textit{N. meningitidis}, was examined and a target designed to a conserved region in the gene. The \textit{porA4} sequence was selected as it could be used to specifically detect \textit{N. meningitidis} DNA. The BLAST search revealed a high level of conservation in the target region among meningococci strains. The Southern blot analysis confirmed that the target sequence is present in the genome of the \textit{N. meningitidis} MC58 strain. Therefore the methods used for detection of the chosen target sequence in the following chapters used this sequence to detect the presence of \textit{N. meningitidis} DNA.

The ideal functional group to use in the conjugation of DNA to gold nanoparticles was established by comparing the binding characteristics of thiol, amine and hydroxyl groups. When comparing the binding of thiol and amine groups to gold nanoparticles it was found that although amine groups had a greater affinity (lower $K_d$) than thiol groups, they did not pack as densely on the nanoparticle surface. The model studies using amino acids and derivatives to compare the binding of thiol, amine and hydroxyl groups gave an estimation of the kinetics of adsorption as well as the optimum pH for binding. At lower pH values, amines bound at a greater rate than thiol or hydroxyl groups and the rate decreased as the pH was increased. However, in the case of thiol and hydroxyl groups, a direct relationship between pH and rate of binding was observed. Since thiol groups had greater coverage on gold nanoparticles compared with amine groups, it was selected as the ideal functional group whose rate of binding could be increased by increasing the pH.
The two DNA sequences designed as probes were synthesised with a thiol group on either the 5’ or 3’ end and the effect of salt concentration and pH on their binding to gold nanoparticles was examined. As expected, by increasing the salt concentration, more DNA bound to the nanoparticles until a maximum of about 150 DNA molecules per nanoparticle was observed. Although it may not be necessary to saturate nanoparticles with the probes, it is important to be able to adjust the packing density to give the optimum coverage for hybridisation of the target DNA. As observed for thiol groups in the model binding studies, the amount of DNA binding to nanoparticles could be increased by increasing the pH.

In Chapter 3, the probes porA4-A and porA4-B were conjugated to gold nanoparticles using conditions determined in this chapter. These conjugates were then used for the detection of target DNA using a plasmon resonance shift.
Chapter 3

Detection of DNA Using a Plasmon Resonance Shift
3.1 Introduction

The plasmon resonance shift caused by the aggregation of DNA-functionalised gold nanoparticles in the presence of complementary DNA and its use for the detection of specific DNA sequences was conceptualised by Mirkin and colleagues (Elghanian et al., 1997). There is considerable interest in this method for the detection of target DNA sequences as the colour-shift can be observed with the naked eye without the need for instrumentation or alternatively, using visible spectroscopy for greater sensitivity (Jin et al., 2003). Hence this method may possibly be employed in a point of care setting for the detection of pathogenic DNA to facilitate the immediate treatment of the infection.

This method employs a set of DNA probes that are attached to gold nanoparticles. The nucleotide sequences of the probes are complementary to different sections of the target DNA, but not complementary to each other. Therefore the probes do not hybridise with each other, but they hybridise to the target when it is introduced to the mixture, which brings the attached nanoparticles closer together. This controlled aggregation allows the dipole plasmon resonances of the gold nanoparticles attached to the probes to couple. The resultant change in the colour of the solution from red towards blue can be observed with the eye or UV-visible spectroscopy may be used to monitor the dipole plasmon resonance absorbance peak shift from 520 to between 600 and 700 nm.

Previous studies have shown that the melting properties of nanoparticle aggregates linked by DNA as in the scheme described above, are influenced by various factors including probe coverage, salt concentration and inter-particle distance (Jin et al., 2003). When the probe surface density was increased, the melting temperature ($T_m$) of the DNA-linked nanoparticle
aggregates increased, signifying increased stability of the aggregates due to multiple DNA links between nanoparticles (Jin et al., 2003). However other studies have shown that a high surface coverage of probe can decrease hybridisation efficiency of target DNA due to the steric hindrance presented by the nanoparticle-bound probe to the hybridising target (Demers et al., 2000; Peterson et al., 2001). Therefore, it is of importance to find the optimum probe coverage in order to maximise hybridisation efficiency. The optimum coverage would ideally be sufficiently high to stabilise gold nanoparticles in high salt concentrations and at the same time sufficiently low as not to cause steric hindrances to hybridisation. At salt concentrations less than 0.05 M, nanoparticle-bound probes do not hybridise at all, indicating that the presence of salt is necessary in facilitating the hybridisation of nanoparticle-bound probe with the target DNA. While keeping target concentration constant, as the salt concentration of the solution was increased $T_m$ was also found to increase (Jin et al., 2003).

Two different schemes have been used previously that result in nanoparticles coming closer together. The first employs a pair of probes that are each attached to nanoparticles through their 5’ or 3’ ends. When these probes hybridise to the target DNA, they will be oriented in a head-to-tail arrangement (Elghanian et al., 1997). The second arrangement involves one probe that is attached to nanoparticles through the 5’ end, with the other probe attached through the 3’ end. When these probes hybridise to the target DNA, they will be oriented in a tail-to-tail arrangement (Storhoff et al., 1998). These arrangements may be used to study the effect of inter-particle distance on the target’s efficiency in producing a plasmon resonance shift.
In this chapter, the effect of probe density on the surface of gold nanoparticles as well as the effect of the salt concentration of the solution on the hybridisation efficiency was examined. The information from the effect of probe density on hybridisation was used when determining the best conditions for conjugation of the probes to gold nanoparticles, so that the probe coverage was optimised. After the DNA-nanoparticle conjugates were prepared, target DNA was added to the nanoparticle solutions with a range of NaCl concentrations to study the effect of salt concentration on the target-mediated aggregation of nanoparticles.

The two possible nanoparticle arrangements for their aggregation, the head-to-tail and tail-to-tail arrangements, were explored in this context to examine the influence of distance between the nanoparticles on their aggregation in the presence of target DNA. Once the method was established, meningococcal DNA was used to determine if this method can be used for the detection of pathogenic DNA.

### 3.2 Methods

The DNA probes and targets used in this study are listed in Table 3.1. The probes porA4-A and porA4-B contain a thiol group at the 5’ and 3’ end respectively. They are each complementary to the two sections of the target DNA porA4 and were used in the tail-to-tail arrangement. The probe porA4-B is also complementary to three sections of the target DNA B3-comp and was used in the head-to-tail arrangement. The lyophilised probes porA4-A and porA4-B were dissolved in molecular biology grade H₂O (Sigma, USA) to give a final concentration of 1 mM. Prior to conjugation to gold nanoparticles, the thiol-modified probes were reduced using tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) as described in Chapter 2.
Table 3.1: Oligonucleotide list.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’- 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>porA4-A</td>
<td>SH-CATCGTGTCCTGG</td>
</tr>
<tr>
<td>porA4-B</td>
<td>CGCTTGCTGAA-SH</td>
</tr>
<tr>
<td>porA4</td>
<td>TTCAGCCAAGCGCCAGACACGATG</td>
</tr>
<tr>
<td>B3-comp</td>
<td>TTCAGCCAAGCGTTCCAGCCAAGCGTTCCAGCCAAGCG</td>
</tr>
</tbody>
</table>
3.2.1 Hybridisation Studies

As described in Chapter 2, the DNA-nanoparticle conjugates were prepared by incubating 3 nmole of the reduced probe porA4-A per mL of gold nanoparticle solution in 10 mM phosphate buffer pH 9 for 16 hours at room temperature. The NaCl concentration was then increased in 0.05 M increments using a 5 M NaCl solution. The solutions were left for a period of at least 1 hour between each addition until the appropriate NaCl concentrations of 0 to 0.4 M was reached. The nanoparticles were separated from the unbound probe by centrifugation at 10,000 × g for 15 minutes. A 100 µL sample of the supernatant was used to quantify the probe remaining in solution using the Hoechst dye 33258 assay (Stout and Becker, 1982). This assay was used to estimate the coverage of the probe on the nanoparticle surface.

As the NaCl concentration of the solution was increased during conjugation of DNA probes to nanoparticles probe coverage increased (Chapter 2). The different NaCl concentrations used in this study resulted in a probe coverage ranging from 50 to 100%, where 100% was the maximum density of the thiol-modified probe on a 15 nm gold nanoparticle as determined previously (Section 2.3.4). The maximum density was estimated to be approximately 150 DNA molecules per nanoparticle.

The nanoparticle pellet was washed twice in 10 mM phosphate buffer pH 7 with the corresponding NaCl concentration. Since there was a loss of nanoparticle during the wash steps due to reduced stability, the final concentration of DNA-conjugated nanoparticle was estimated by spectrophotometric analysis using the plasmon resonance absorption peak at 518 nm.
One nmole of target DNA (porA4) was added to each nanoparticle solution. Hybridisation of nanoparticle-bound probe to the target DNA was allowed to occur for 1 hour at room temperature, followed by the separation of the unhybridised target DNA from the nanoparticles by centrifugation at 10,000 ×g for 15 minutes. One hundred microlitres of the supernatant fluid was taken to quantify the amount of target DNA remaining in solution and thus estimate the degree of hybridisation of the target DNA to gold nanoparticle-bound probe.

3.2.2 Preparation of Probe Conjugated Gold Nanoparticles

The DNA-gold nanoparticle conjugates were prepared by incubating 3 nmole of the reduced probes porA4-A or porA4-B per millilitre of gold nanoparticle solution in 10 mM phosphate buffer pH 9 for 16 hours at room temperature. The NaCl concentration was then increased in 0.05 M increments using a 5 M NaCl solution until a final concentration of 0.1 M was reached. The nanoparticles were then separated from the unbound probe by centrifugation at 10,000 ×g for 15 minutes. The nanoparticle pellet was washed twice in 10 mM phosphate buffer pH 7 containing 0.1 M NaCl before being resuspended in 10 mM phosphate buffer pH 7 containing 0 to 0.7 M NaCl.

3.2.3 Polymerase Chain Reaction (PCR)

Meningococcal genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer’s protocol. Briefly, the bacteria was mixed with nuclei lysis solution and incubated at 80°C for 5 minutes. The cell lysate was further incubated at 37°C in the presence of ribonuclease (RNase) for 30 minutes. Protein was
precipitated and the supernatant fluid was removed to a clean microfuge tube followed by the addition of isopropanol to precipitate the DNA. The genomic DNA was then washed with 70% (v/v) ethanol and air-dried before it was dissolved in 10 mM Tris, 1 mM EDTA pH 8.0 (TE buffer) at 65°C for 1 hour. The meningococcal genomic DNA was stored at -20°C and used as the template in a polymerase chain reaction (PCR).

A PCR was performed to amplify an 897 base pair (bp) section of the *porA* gene of *N. meningitidis* containing the selected *porA*4 target sequence. A pair of primers was designed using Primer Designer 5, Version 5.11 (Scientific & Educational Software, USA) and was synthesised by Sigma-Genosys (Australia) (Table 3.2). A PCR Master kit (Roche, Germany) was used according to the manufacturer’s instructions. Approximately 100 ng of genomic DNA were used as template DNA. The reaction mix is listed in Table 3.3. The reactions were prepared in a final volume of 50 µL. The PCR was done in a Thermo thermal cycler (Px2) (Thermo, USA) using the reaction conditions described in Table 3.4 for 40 amplification cycles. The amplified PCR product was separated by electrophoresis on a 1% (w/v) agarose gel using TAE electrophoresis buffer and visualised by staining with ethidium bromide. The size of the PCR product was estimated by comparison to fragments of lambda (λ) DNA (Promega, USA) digested with the restriction enzyme *PstI* (Promega, USA), that was separated on the gel together with the PCR product (see Appendix A for band sizes of the marker).

The PCR product was purified using Wizard PCR preps DNA purification kit (Promega, USA) following the manufacturer’s protocol. Briefly, PCR product was mixed with direct
Table 3.2: Primers for amplification of *porA* gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’-3’</th>
<th>T$_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>porA-For</td>
<td>GAATTTGTGGCGCAACC</td>
<td>60</td>
</tr>
<tr>
<td>porA-Rev</td>
<td>CAGCTTGAGCAAGACGTATC</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 3.3: PCR reaction mix.

<table>
<thead>
<tr>
<th>PCR reagent</th>
<th>Volume added</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milli-Q H$_2$O</td>
<td>17 µL</td>
<td>N/A</td>
</tr>
<tr>
<td>PCR master mix</td>
<td>25 µL</td>
<td>1×</td>
</tr>
<tr>
<td>Forward primer (5 µM)</td>
<td>3 µL</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Reverse primer (5 µM)</td>
<td>3 µL</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>2 µL</td>
<td>~2 ng/µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 µL</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Reaction conditions for PCR.

<table>
<thead>
<tr>
<th>Program</th>
<th>Temperature</th>
<th>Time</th>
<th>cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>2 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>54°C</td>
<td>45 seconds</td>
<td>25-40</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 seconds</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>3 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>hold</td>
<td>1</td>
</tr>
</tbody>
</table>
purification buffer and 1 mL of resin. The mix was then passed through a minicolumn, washed in isopropanol and the purified PCR product was eluted using Milli-Q H$_2$O. The purified PCR product was used as a target in the plasmon resonance shift assay.

A PCR was also done to determine the sensitivity of this PCR using the primers porA-For and porA-Rev when 25 amplification cycles were used. It was carried out with the template DNA used in the PCR reaction mix varied between $6 \times 10^7$ and $1.2 \times 10^2$ copies of genomic DNA. The intensity of the PCR products was compared with the $PstI$ digested fragments of $\lambda$-DNA to estimate the quantity of the amplified DNA.

### 3.2.4 Plasmon Resonance Shift Assays

Two schemes were used to produce a colour-shift in nanoparticle solutions in the presence of target DNA. The first scheme used only probe porA4-B, against the target B3-comp (Table 3.1), which consists of three repeat sequences complementary to the probe porA4-B. This arrangement allowed the nanoparticles to be arranged in a head-to-tail arrangement as shown in Figure 3.1a, separated by 12 DNA bp. The second scheme used both probes porA4-A and porA4-B. In this case, the target, porA4 (Table 3.1), which is derived from a meningococcal sequence (Chapter 2), is comprised of two halves, each complementary to one of the probes. This arrangement allowed for a tail-to-tail arrangement as shown in Figure 3.1b, with the length of 24 bp separating the nanoparticles. When genomic DNA and the PCR product were used as the target, the second scheme employing both probes porA4-A and porA4-B was used.
Figure 3.1: Schemes used for producing colour shift as a result of hybridisation of probes to target DNA.
When using B3-comp as the target sequence, 1 to 5 nmole of B3-comp were added to 500 µL of porA4-B conjugated gold nanoparticle solution with NaCl concentrations of 0 to 0.5 M. The optical properties of the solutions were monitored as a function of time and concentration of target DNA, using a Varian Cary 500 spectrophotometer (Varia, Australia). The absorbance was recorded at 1 nm intervals in the range of 400-800 nm. The spectrum of each solution was monitored for 1 hour with wavelength scans recorded every 15 minutes.

When using porA4 as the target DNA, 250 µL of both porA4-A and porA4-B conjugated nanoparticles were combined for each study. The NaCl concentrations used in this study were varied from 0.1 to 0.7 M. Quantities of 1 to 5 nmole of target DNA were added and the spectrum of each solution monitored for 1 hour with wavelength scans recorded every 15 minutes.

Using an absorbance measurement at 260 nm, the concentration of meningococcal genomic DNA used was 2.21 mg/mL, equivalent to approximately 1.5 pmole/mL based on the molecular weight of *N. meningitidis* genome being approximately $1.45 \times 10^9$. Prior to use, the genomic DNA was denatured by heating at 95°C for 5 minutes, then immediately placed on ice. The detection nanoparticle solution contained 250 µL each of porA4-A and porA4-B conjugated nanoparticles with NaCl concentrations od 0.5 and 0.7 M. Volumes of 10 to 50 µL of genomic DNA solution and the spectrum of each solution monitored for 1 hour with wavelength scans recorded every 15 minutes.

When the *porA* gene PCR product was used as the target DNA, 250 µL each of porA4-A and porA4-B conjugated nanoparticles were combined for the detection solution containing 0.7 M NaCl. The PCR product was denatured heating at 95°C for 5 minutes, then immediately
placed on ice. Amounts of PCR product used in this study were varied from 0.5 to 2.5 nmole. The spectrum of each solution monitored for 1 hour with wavelength scans taken every 15 minutes.

### 3.3 Results and Discussion

#### 3.3.1 Hybridisation Studies

The effect of both the coverage of the probe on the nanoparticle and the NaCl concentration of the solution upon the hybridisation efficiency of target DNA to the nanoparticle-bound probes were examined. As detailed in Chapter 2, the coverage of the probe on the nanoparticle surface could be adjusted by changing the NaCl concentration of the solution during conjugation and increasing the pH to 9 resulted in a decrease in the time taken for the probe to bind to the gold nanoparticles. In this study, the coverage of the probe on the nanoparticle surface was adjusted between approximately 70 and 138 probe molecules per nanoparticle by changing the NaCl concentration between 0 and 0.4 M.

Although citrate acts as a good stabilising agent for gold nanoparticles in the absence of NaCl, it is not a good stabilising agent at salt concentration as low as 0.05 M. When NaCl was added to bare nanoparticles, immediate aggregation of the nanoparticles was observed due to the charge screening effects of salt that allowed nanoparticles to come closer together. When the nanoparticles are functionalised with DNA, they are further stabilised electrostatically by the negatively charged DNA molecules (see Section 1.1.1.). However, as the ionic strength of the nanoparticle solution was increased, the DNA layer was insufficient for keeping the nanoparticles stable. Therefore during conjugation of the probe, the solutions
of nanoparticles with higher NaCl concentrations were not as stable as the solutions with lower ionic strength. Due to this lack of stability, there was some loss of nanoparticles during the washing steps. This loss was accounted for by calculating the final nanoparticle concentrations of the solutions using the molar absorptivity coefficient of the nanoparticles as determined in Chapter 2 \( (3.222 \times 10^8 \text{ M}^{-1}\text{cm}^{-1}) \). The hybridisation efficiency was then adjusted for the nanoparticle concentration finally obtained.

Once the probe-nanoparticle conjugates were prepared, the hybridisation efficiency or the percentage of nanoparticle-bound probe involved in hybridisation to target DNA was estimated as a function of coverage as well as NaCl concentration in the solution. As seen in Figure 3.2, for nanoparticles with probe coverage ranging from 50 to 100% maximum coverage, an increase in the concentration of NaCl in the solution increased the hybridisation efficiency between the target and functionalised nanoparticles. This increase could be due to the charge-screening effect of NaCl. The salt is able to screen the negative charges between DNA molecules and minimise electrostatic repulsion, allowing the target DNA to approach the nanoparticle-bound probes and hybridisation to occur (Demers et al., 2000).

As probe coverage on the surface of the nanoparticle was decreased, the hybridisation efficiency increased, with maximal hybridisation efficiency observed between 50 and 62% probe coverage (Figure 3.3). This decrease in the hybridisation efficiency as the coverage was increased may be due to the steric hindrance presented by the tight packing of the probe molecules that left little space for the target DNA to hybridise (Demers et al., 2000; Peterson et al., 2001).
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Figure 3.2: Effect of NaCl concentration on DNA hybridisation efficiency on the surface of gold nanoparticles.
Figure 3.3: Effect of probe coverage on the DNA hybridisation efficiency on the surface of gold nanoparticles.
The optimum coverage of probe on the nanoparticle would be sufficiently low to maximise the probes available for hybridisation, increasing the interaction of the nanoparticles resulting in a maximum change in the plasmon resonance. At the same time, the coverage needs to be sufficiently high to make the nanoparticle stable at high salt concentrations. The high coverage is necessary to prevent non-specific aggregation due to NaCl concentration and not due to presence of target DNA. During conjugation, the NaCl concentration needed to be raised to at least 0.1 M to give a probe coverage sufficient to stabilise the nanoparticles in the buffers containing up to 0.7 M NaCl used during hybridisation. Therefore, nanoparticles with a coverage of 62% were prepared rather than with a coverage of 50% due to the stability that this coverage conferred to the nanoparticles at a higher NaCl concentration of 0.7 M.

3.3.2 PCR of porA Gene

Two primers porA-For and porA-Rev were designed to amplify a 897 bp section of the porA gene containing the target sequence porA4. The PCR was first performed using excess genomic (template) DNA containing approximately $6 \times 10^7$ copies and 40 amplification cycles to obtain sufficient PCR product to use as the target in the plasmon resonance shift assays. When electrophoresed on an agarose gel, the resultant PCR product appears as a single band with a size of approximately 900 bp (Figure 3.4). This PCR product was purified before it was used as a target in the plasmon resonance shift assay. The concentration of the PCR product was estimated to be 120 µg/mL by absorbance at 260 nm.

A second PCR was performed using amounts of template DNA varying from $6 \times 10^7$ and $1.2 \times 10^2$ copies to determine the sensitivity of this PCR. All samples that contained $1 \times 10^4$
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Figure 3.4: Amplification of *porA* gene from meningococcal genomic DNA.

Lane 1: λ-DNA digested with *Pst*I, Lane 2: Amplified PCR product with 897 bp size.

Figure 3.5: Sensitivity of PCR.

Lane 1: λ-DNA digested with *Pst*I, Lane 2: $6 \times 10^7$, Lane 3: $3 \times 10^7$, Lane 4: $1.2 \times 10^7$, Lane 5: $1 \times 10^6$, Lane 6: $2.4 \times 10^5$, Lane 7: $8 \times 10^4$, Lane 8: $1 \times 10^4$, Lane 9: $1 \times 10^3$, Lane 10: $1 \times 10^2$ copies of template DNA used for PCR.
copies template DNA copies or more produced amplified DNA with a concentration greater than 10 ng/µL, while the sample containing $1 \times 10^3$ copies resulted in a solution containing about 1 ng/µL (Figure 3.5). Therefore this PCR required a starting template DNA copy number of at least $10^3$ to amplify the target gene and is similar to required template DNA copy numbers described in the literature (Saunders et al., 1993). The amount of the PCR product formed when the copy number is low (minimum of $10^3$) may be increased by increasing the number of amplification cycles but it is important to ensure that there is sufficient primer present and that it is not a limiting factor.

### 3.3.3 Plasmon Resonance Shift Assays

**Inter-particle Distance of 12 Base Pairs**

The probe porA4-B, which is 12 bases in length, was directionally conjugated to gold nanoparticles via a thiol group. These conjugates were used together with the target B3-comp to allow the hybridisation of the conjugate to the target in a head-to-tail arrangement. The hybridisation resulted in controlled aggregation of the attached nanoparticles with an inter-particle distance of 12 bp.

The probe-nanoparticle conjugates using porA4-B as the probe were prepared in a 10 mM phosphate buffer and the NaCl concentration was increased to 0.1 M. The conjugates were washed and resuspended in phosphate buffer containing a range of NaCl concentrations up to 0.5 M.

The presence of at least 0.3 M NaCl was necessary for hybridisation of the probes to the target DNA as indicated by a lack of plasmon resonance shift in the absence of NaCl and at
an NaCl concentration of 0.1 M when up to 5 nmole of target DNA was added to the solution (Figure 3.6a and b, also see Appendix B). At NaCl concentrations of 0.3 and 0.5 M, a shift in the plasmon resonance peak was observed within 1 hour when target DNA was added (Figure 3.6c and d). The shift was indicated by an increase in the absorbance between the wavelengths 600 and 700 nm. The peak that formed between 600 and 700 nm was due to the dipole plasmon resonance peak of nanoparticle aggregates (Basu et al., 2007). The peak at about 520 nm, which was due to a dipole plasmon resonance absorbance peak of the individual nanoparticles, was observed to red-shift slightly. In nanoparticle aggregate solutions, this peak now represents the quadrupole plasmon resonance peak (Basu et al., 2007). As described previously in Section 3.1, the requirement of salt for hybridisation to occur may be due to the stability of the nanoparticles. The bound DNA probes stabilise the nanoparticles, preventing their aggregation. This stabilisation also inhibits hybridisation of the probes to target DNA. In the presence of salt, the negative charge of the DNA is screened, allowing target DNA to approach and hybridise to the probes. This trend is also observed for free DNA molecules in solution, which are able to hybridise more easily in high salt concentrations as indicated by their higher $T_m$ (Owczarzy et al., 1997; Tan and Chen, 2006). In this setting, where the probes are attached to gold nanoparticles, the charge-screening effect of salt also allows the nanoparticles to come closer together, assisting in the aggregation that leads to a plasmon resonance shift (Jin et al., 2003).
Figure 3.6: Plasmon resonance shift when B3-comp was used as target DNA.
(a) 5 nmole of target DNA used with no NaCl present (b) 5 nmole of target DNA used with 0.1 M NaCl present (c) 5 nmole of target DNA used with 0.3 M NaCl present and (d) 5 nmole of target DNA used with 0.5 M NaCl present.
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When the NaCl concentration was 0.5 M, no plasmon resonance shift was observed in the nanoparticle solution within 1 hour when up to 2 nmole of target was added (Figure 3.7a to c). However, an increase in the absorbance between 600 and 700 nm was observed when at least 3 nmole of target was present (Figure 3.7d). As the amount of target was increased further to 4 and 5 nmole, a more rapid red-shift in the plasmon resonance peak was observed (within 30 to 45 minutes) (Figure 3.8a and b).

To better understand the kinetics of target DNA linking the nanoparticles to form aggregates, the formation of the dipole plasmon resonance peak of the aggregates was monitored as a function of time. The absorbance at 600 nm was observed to increase within 30 minutes when at least 3 nmole of target is present (Figure 3.9). It has been suggested that the shift in the colour (or plasmon resonance peak wavelength) of the nanoparticle solution is due to an increase in the aggregate size or the number of particles that the aggregates are comprised of (Storhoff et al., 2000). Therefore the greater shifts in plasmon resonance at higher target concentrations indicate that larger nanoparticle aggregates were formed due to greater linking between the nanoparticles in solution.

Inter-particle Distance of 24 Base Pairs

The probes porA4-A and porA4-B, each 12 bases in length, were conjugated to gold nanoparticles. These conjugates were used together with the target porA4 to allow the conjugates to hybridise to the target in a tail-to-tail arrangement. This arrangement caused controlled aggregation of the attached nanoparticles with an inter-particle distance of 24 bp. The conjugates were prepared in 10 mM phosphate buffer pH 9 and the NaCl concentration was increased to 0.1 M and then washed and resuspended in 10 mM phosphate buffer pH 7.
Figure 3.7: Plasmon resonance shift at 0.5 M NaCl concentrations when B3-comp was used as target DNA.

(a) no target present (b to d) represent 1 to 3 nmole of target DNA present.
Figure 3.8: Plasmon resonance shift at 0.5 M NaCl concentrations when B3-comp was used as target DNA.

(a) 4 nmole and (b) 5 nmole of target DNA present.
Figure 3.9: Increase in nanoparticle absorbance at 600 nm when B3-comp was used as target DNA as a function of time.

The NaCl concentration in the nanoparticle solution was 0.5 M.
containing 0.1 M NaCl. They were then combined in a 1:1 ratio in solutions containing up to 0.7 M NaCl. The target DNA was added to the combined solutions.

At NaCl concentrations between 0 and 0.3 M, no shift was observed in the plasmon resonance of the nanoparticle solution within 1 hour when up to 5 nmole of target DNA was added (data shown in Appendix B). This was similar to what was observed in the previous section where the presence of at least 0.5 M NaCl was a requirement for hybridisation when B3-comp was used as the target DNA.

When the NaCl concentration was 0.5 M, no shift in the plasmon resonance was observed within 1 hour when 1 and 2 nmole of target was present. However, when the amount of target was increased to 3 nmole a slight increase in the absorbance between 600 and 700 nm was observed (Figure 3.10). A shift in the plasmon resonance was observed when 4 and 5 nmole of target was present (Figure 3.11). When the absorbance at 600 nm was monitored as a function of time, in the presence of 5 nmole of target the absorbance was observed to increase within 30 minutes, 4 nmole of the target cause an increase within 45 minutes and 3 nmole of the target DNA within 60 minutes (Figure 3.12).

This change in the plasmon resonance was comparable to that seen for the B3-comp target for the same NaCl concentration. However, the kinetics were slightly slower for target amounts of 4 nmole or lower, with 4 nmole of porA4 requiring at least 45 minutes, compared with 30 minutes required by the same amount of B3-comp, to produce a shift in the plasmon resonance peak. This may be due to the shorter distance (equivalent of 12 DNA bp) between the nanoparticles with B3-comp when compared with the bigger spacing (equivalent of 24 bp) when porA4 was used as the target. This finding is similar to the findings of Storhoff.
Figure 3.10: Plasmon resonance shift when porA4 was used as target DNA with a NaCl concentration of 0.5 M.

(a) no target present (b to d) represent 1 to 3 nmole of target DNA present.
Figure 3.11: Plasmon resonance shift at 0.5 M NaCl concentrations when porA4 was used as target DNA.

(a) 4 nmole and (b) 5 nmole of target DNA present.
Figure 3.12: Increase in nanoparticle absorbance at 600 nm as a function of time when porA4 was used as target DNA with a NaCl concentration of 0.5 M.
and colleagues (2000), who showed that there was a greater shift in the plasmon resonance peak when a shorter linker was used. This was attributed to be due to the larger nanoparticle aggregates that were formed with the shorter linkers rather than due to closer packing (Storhoff et al., 2000).

When the NaCl concentration of the nanoparticle solution was increased to 0.7 M, an increase in the absorbance between 600 and 700 nm was observed within 1 hour when at least 2 nmole of target DNA was present (Figure 3.13). The plasmon resonance shift was more pronounced and occurred at a faster rate when 4 and 5 nmole of target was present (Figure 3.14). When the absorbance at 600 nm was monitored as a function of time, it was observed to increase within 15 minutes when 5 nmole of target was present, within 30 minutes with 3 to 4 nmole of the target and within 45 minutes when 2 nmole of the target DNA was present (Figure 3.15). Therefore, as may be expected, the plasmon resonance shift was greater at higher NaCl concentration because the hybridisation efficiency was increased.

**Meningococcal Genomic DNA as Target**

The same two sets of probes (porA4-A and porA4-B) that were used in the previous section with porA4 as the target, was also used with meningococcal genomic DNA as the target. This may be expected to give a tail-to-tail arrangement of the nanoparticle probes as before. Since porA4 was derived from the genomic sequence of *N. meningitidis* and was shown to bind to the expected restriction fragment of the meningococcal genomic DNA (Chapter 2), these probes may be expected to recognise the target in this system. However, no plasmon resonance shift was observed in the nanoparticle probe solution when up to 0.11 mg of meningococcal genomic DNA was added (data shown in Appendix B).
Figure 3.13: Plasmon resonance shift when porA4 was used as target DNA with a NaCl concentration of 0.7 M.

(a) no target present (b to d) represent 1 to 3 nmole of target DNA present.
Figure 3.14: Plasmon resonance shift at 0.7 M NaCl concentrations when porA4 was used as target DNA.

(a) 4 nmole and (b) 5 nmole of target DNA present.
Figure 3.15: Increase in nanoparticle absorbance at 600 nm as a function of time when porA4 was used as target DNA with a NaCl concentration of 0.7 M.
The plasmon resonance shift in nanoparticles brought about by the controlled aggregation of the nanoparticles by the target DNA may be used as a detection method for DNA as shown previously when synthetic target sequences (B3-comp and porA4) were used. Therefore it may be expected to be able to detect DNA derived from biological samples. However, meningococcal DNA was not able to produce a colour shift although the NaCl concentration was similar to that used in the system with a 24 bp inter-particle distance. This lack of plasmon resonance shift may be due to the low quantities of genomic DNA used. The maximum amount of genomic DNA used was 0.75 pmoles compared with 2 nmole of porA4 which was the minimum amount that was required to produce a shift. This was then confirmed by using PCR to amplify the copies of the gene and then the shift in plasmon resonance of the nanoparticles was used for its detection.

Since this amount of genomic DNA was equivalent to about 0.75 pmoles of the genome and represented DNA extracted from $4.5 \times 10^{11}$ bacterial cells, this system is unlikely to be useful for direct detection of meningococcal DNA within a clinical sample which may be expected to contain much less DNA.

**porA gene PCR Product as a Target**

The 897 bp PCR product that was obtained from the amplification of the porA gene of *N. meningitidis* was used as target DNA. The probes porA4-A and porA4-B were used in this system as for genomic DNA. No significant plasmon resonance shift was observed in the nanoparticle probe solution within 1 hour when up to 1.5 nmole of the porA PCR product was present (Figure 3.16). However, when the amount of PCR product was increased to 2 and 2.5 nmole, the absorbance is seen to increase between 600 and 700 nm (Figure 3.17).
Chapter 3: Detection of DNA Using a Plasmon Resonance Shift

Figure 3.16: Plasmon resonance shift when \textit{porA} PCR product was used as target DNA with a NaCl concentration of 0.7 M.

(a) no target present (b to d) represent 0.5 to 1.5 nmole of target DNA present.
Figure 3.17: Plasmon resonance shift at 0.7 M NaCl concentrations when porA PCR product was used as target DNA.

(a) 2 nmole and (b) 2.5 nmole of target DNA present.
The corresponding colour shift of the nanoparticle solutions from red to blue observed by eye is shown in Figure 3.18. As indicated by the wavelength scans (Figure 3.16 and Figure 3.17), the colour shift from red to blue was evident when a minimum of 2 nmole of PCR product was present (Figure 3.18).

By amplifying the number of copies of the target DNA, it could be detected by a plasmon resonance shift in nanoparticle solutions. Although this detection method cannot be used directly on clinical samples due to lack of sensitivity, it may be used in conjunction with PCR. This amplification process required about 1.5 hours when 25 amplification cycles were used and requires a longer time if the starting DNA concentration is low.

### 3.4 Summary

The aim of this study was to evaluate plasmon resonance shift of gold nanoparticles as a detection method for target DNA sequences and optimise the conditions that influence the plasmon resonance or colour shift.

The effect of the NaCl concentration of the solution and the coverage of the probe on the nanoparticle on the hybridisation efficiency of target DNA to the nanoparticle-bound probes was examined. As the concentration of NaCl in the solution was increased, the hybridisation efficiency between the target and functionalised nanoparticles also increased. The coverage of the probe on the nanoparticle surface was adjusted between approximately 70 and 138 probes per nanoparticle (50 to 100% maximum coverage) by changing the NaCl concentration from 0 and 0.4 M during conjugation. As probe coverage on the surface of the nanoparticle was decreased, the hybridisation efficiency increased, with maximal
Figure 3.18: Colour shift in the nanoparticle solution after 1 hour when *porA* PCR product was added.

(a) no target present (b to f) represent 0.5, 1.0, 1.5, 2.0 and 2.5 nmole of target DNA present.
hybridisation efficiency observed between 50 and 62% probe coverage. These findings agree with those of Peterson et al. (2001) as well as Jin et al. (2003).

Systems that allowed for controlled aggregation of nanoparticles when target DNA was present were examined for the effect of the presence of NaCl. In such systems, more than one probe may be expected to hybridise to the target sequence, bringing the attached nanoparticles closer together and aggregating them. It was observed that at least 0.5 M NaCl was required for hybridisation of the probes and target DNA to occur and subsequently produce a plasmon resonance shift in the nanoparticle solution that can be monitored. By increasing the NaCl concentration to 0.7 M, an even greater plasmon resonance shift could be produced. This finding is similar to those reported by Jin and colleagues (2003).

The influences of the distance between the nanoparticles when linked with the target DNA was examined by designing two model systems containing 12 and 24 bp inter-particle distances. Comparing both systems with a NaCl concentration of 0.5 M, showed that the kinetics of the 12 bp system was faster possibly due to the larger size of aggregates formed when the inter-particle distance was shorter (Storhoff et al., 2000).

The optimised method using a probe coverage of 62% and a NaCl concentration of 0.7 M was used to determine if this technique may be used for the detection of meningococcal DNA. One of the limitations of this technique was its low sensitivity (Rosi and Mirkin, 2005). It was first tested on genomic DNA, which was not able to be detected perhaps due to low concentrations used.
In order to enhance the sensitivity of the technique, PCR was used to amplify the *porA* gene prior to addition of the nanoparticle probes. The PCR produced a 897 bp fragment that contained the target sequence. The resultant PCR product, when used in the nanoparticle system containing probes against it, was able to produce a plasmon resonance shift that can also be observed by eye. However the assay required a minimum of 2 nmole of the target to produce a shift that could be detected within 1 hour. The PCR required a starting genomic DNA copy number of at least $10^3$ to be able to amplify the target gene within approximately 1.5 hours and is similar to what has been reported in the literature (Saunders *et al.*, 1993).

Another limitation of this technique is the time required for a plasmon resonance change to be observed. The more target there is available, the earlier it can be detected. However, samples with lower target amounts of less than 2 nmole take more than an hour to produce a change that may be observed. This may be due to the fact that this technique relies on Brownian motion to bring the nanoparticle probes and target close together to hybridise and form aggregates that are sufficiently large to shift the plasmon resonance of the solution (Ray *et al.*, 2005).

The PCR may be performed directly on bacterial cells by boiling the bacterial suspension as done by in several studies (Speers and Jelfs, 1997; Richardson *et al.*, 2003; de Filippis *et al.*, 2005) rather than using extracted genomic DNA as used in this study. This would decrease the time taken to amplify the gene by eliminating the DNA purification process that required approximately 1.5 hours. Another strategy that may be used to shorten the time require for detection of the PCR product is using an inter-particle distance of 12 bp. As seen previously, this scheme resulted in a faster shift in the plasmon resonance peak. However, for this
scheme to be used on meningococcal DNA, a thiol group needs to be incorporated at the 3’ end of the probe porA4-A, so that it aligns itself in a head-to-tail manner with respect to porA4-B when they both hybridise to the target DNA.

The method that uses the target DNA-directed shift in the plasmon resonance peak of gold nanoparticles has been shown to be able to detect meningococcal-specific DNA sequences. Although it needs to be used in conjunction with PCR to enhance its sensitivity, it is a convenient technique for the detection of specific DNA sequences. Unless real-time PCR is used, a PCR assay requires the PCR product to be electrophoresed and visualised on an agarose gel. The technique described in this study may be used instead of gel electrophoresis to confirm the presence of the PCR product.
Chapter 4

Detection of DNA using

Fluorescence
4.1 Introduction

4.1.1 Interaction Between Fluorophores and Gold Nanoparticles

Gold nanoparticles are highly polarisable and therefore capable of producing enhanced optical fields near their surface when irradiated with light (Cannone et al., 2006). These enhanced fields have an effect on molecules such as fluorescent dyes that are able to interact with the plasmon resonance of nanoparticles when located near their surface.

Fluorophores are able to reversibly bind to the surface of gold nanoparticles (Maxwell et al., 2002), and when in close proximity to the gold nanoparticle surface, their fluorescence is quenched. The emitted fluorescence intensity of a fluorophore depends on the manner in which it releases energy, which is generally a combination of both radiative (photon emission) and non-radiative (loss of energy to the environment) energy transfer (Anger et al., 2006). The quenching of the fluorophore’s fluorescence occurs through surface energy transfer (SET) from the fluorophore to the nanoparticle by non-radiative means, which decreases the energy lost through radiation (through photons that can be detected). Therefore an increase in the rate of non-radiative energy transfer is observed as well as a decrease in the rate of radiative energy transfer (Dulkeith et al., 2005). These changes in the non-radiative rate and radiative rate lead to a lower quantum yield, which is the ratio of the photons emitted to the photons absorbed. The relationship between quantum yield and the non-radiative and radiative rates has been described as follows.

\[ Q = \frac{\Gamma}{\Gamma + k_{nr}} \]  

(1)
where Q is the quantum yield, Γ is the radiative rate and \( k_{nr} \) is the non-radiative rate (Lakowicz, 1999). Therefore the quantum yield decreases with an increase in the non-radiative rate.

The lifetime of the fluorophore, or the average time during which it remains in the excited state is also affected by the changes in the non-radiative and radiative rates. The relationship between the fluorophore lifetime (\( \tau \)) and the non-radiative and radiative rates has been described as follows.

\[
\tau = \frac{1}{\Gamma + k_{nr}}
\]  

(2)

where \( \tau \) is the lifetime of the fluorophore, Γ is the radiative rate and \( k_{nr} \) is the non-radiative rate (Lakowicz, 1999). From equations (1) and (2), it can be seen that the quantum yield is directly proportional to the lifetime of a fluorophore.

Two types of fluorescent measurements may be determined, steady state and time-resolved measurements which examine the quantum yield and lifetime of the fluorophore respectively. Steady-state measurements are the most commonly used and measure fluorescence intensity at constant excitation. A decrease in quantum yield can be detected by the decrease in fluorescence emission intensity.

Alternatively, time-resolved measurements use a pulse of light to excite the fluorophore and measure the subsequent intensity decay. These measurements are typically of a nanosecond timescale and can be used to determine the lifetime of the fluorophore. More information may be derived from the shape of the decay curve, compared with steady-state
measurements. Samples that contain more than one conformation of the fluorophore exhibit decay curves that have more than a single exponential decay curve. The decay curve may be separated into its component parts so that the lifetimes of each conformation may be calculated (Lakowicz, 1999).

### 4.1.2 Binding of DNA to Gold Nanoparticles

Single stranded (ss) DNA molecules are highly flexible with a persistence length of 8 to 13 Å compared to double stranded (ds) DNA, which is more rigid and has a persistence length of about 450 Å (equivalent to approximately 132 bp) (Tinland et al., 1997). Due to the low persistence length and the availability of its bases ss-DNA can adsorb to the surface of gold nanoparticles. Although it possesses a negatively charged phosphate backbone that may be expected to be repelled by the citrate layer on the nanoparticle surface (Section 1.1.1.), its conformationally flexible backbone unwinds to expose the bases, allowing such bases to interact with the nanoparticle surface through nitrogen or oxygen atoms depending on the base (Jang, 2002; Storhoff et al., 2002; Sandström et al., 2003). However, in ds-DNA these bases are involved in base-pairing through hydrogen bonds, forming a double helical structure. The bases are therefore buried within the structure of the double helix and protected by the sugar-phosphate backbones, making them unavailable to interact with gold nanoparticles.

Dubertret and colleagues (2001) have applied the difference in conformation of ss-DNA and ds-DNA to a scheme to detect target DNA. They used gold nanoparticles as quenchers in a molecular beacon system that contained a ss probe labelled with a fluorophore and directionally attached to a gold nanoparticle. Hybridisation of the probe to target DNA
changed the conformation of the probe and moved the fluorophore away from the nanoparticle surface. This increase in distance between the fluorophore and the nanoparticle allowed the fluorescence to increase.

The difference electrostatic properties of ss and ds-DNA have been recently exploited to develop a detection system for target DNA (Li and Rothberg, 2004a). This system used a ss probe that can bind to and stabilise gold nanoparticles when the salt concentration of the solution is increased. However in the presence of target DNA, the probe hybridises to the target instead of stabilising the nanoparticles. This causes the nanoparticles in the solution to aggregate when the salt concentration is increased. The change in colour may be detected by eye and acts as an indicator of the presence of target DNA. The electrostatic properties of DNA have also been used with fluorophore-labelled DNA probes (Li and Rothberg, 2004b; Ray et al., 2005). In these schemes, the ss probe binds to gold nanoparticles in the absence of target DNA. However when the target DNA was present, the probe hybridised to it and did not bind to the nanoparticles, allowing the fluorescence of the label to be detected.

### 4.1.3 Overview of Chapter

In this chapter, the differences in the flexibility of ss and ds-DNA and the manner in which they interact with gold nanoparticles were exploited to detect hybridization of the target DNA to fluorophore-labelled probes. Two different probes were used, one with a thiol group incorporated at the 3’ end (porA4-B) to permit directional binding to gold nanoparticles and the other oligonucleotide with no modification (porA4-C) and which relied on interactions between the constituent bases and the gold nanoparticle surface. When the thiol-modified probe porA4-B was used, it was tethered to gold nanoparticles through their thiol group. The
flexible probe interacted with the gold surface and allowed the fluorophore attached to its 5’ end to become quenched due to its proximity to the surface. However when the probe hybridised to target DNA a rigid double helix was formed, creating a distance between the fluorophore and the nanoparticle surface that allowed emission of fluorescence that could be detected. When the oligonucleotide porA4-C was used, it adsorbed to the gold nanoparticles in solution because it was single stranded, consequently allowing the fluorescence of the label to be quenched. However, when the target DNA was present, the probe hybridised to it, hence preventing itself from adsorbing to the nanoparticles. This led to an assay that could detect the hybridisation of the probe to the target by measurement of the fluorescence of the labels.

Time-resolved fluorescence measurements were used to understand the interaction of the probes with gold nanoparticles under different conditions, such as varying probe concentration and the amount of target DNA present. The time required for changes in the fluorophore lifetime was also determined. Once the optimum conditions were determined, the use of steady-state fluorescence measurements that require a simple spectrofluorometer was examined as a method for the detection of target DNA.

4.2 Methods

4.2.1 Labelling Probes

Amine-modification of 5’ Phosphate Groups

One micromole of the DNA probe porA4-B or porA4-C (Table 4.1) was combined with 750 µL of reaction buffer and 500 µL of ethylenediamine solution. This was added to 625 µmole
### Table 4.1: Oligonucleotide sequences used in these studies.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>porA4</td>
<td>TTCAGCCAAGCGCCAGACACGATG</td>
</tr>
<tr>
<td>porA4-B</td>
<td>CGCTTGCTGAA-SH</td>
</tr>
<tr>
<td>porA4-C</td>
<td>CATCGGTCTGGCGCTTGGCTGAA</td>
</tr>
</tbody>
</table>
of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Pierce, USA) before 200 µL of 0.1 M imidazole pH 6 was added. The mixture was mixed and incubated overnight at 37°C. The modified probe was purified by extracting it three times with an equal volume of chloroform/isoamyl alcohol (24:1). The DNA was then precipitated using 145 µL of 3 M NaCl and 3.625 mL of cold absolute ethanol. The mixture was incubated at -20°C for 30 minutes before the DNA was separated as a pellet by centrifugation at 16,000 ×g for 30 minutes. The DNA pellet was then washed with 70% (v/v) ethanol before being dried under vacuum.

**Labelling Probes with Carboxyfluorescein Succinimidyl Ester (FAM)**

Two hundred micrograms of 5-(and-6)-carboxyfluorescein succinimidyl ester (FAM) (Invitrogen, USA) dissolved in DMSO to a concentration of 14 µg/µL was used per 100 µg of purified probe. Seven microliters of Milli-Q water and 75 µL of labelling buffer were added per 100 µg of probe. The amine-modified probe pellet was dissolved in Milli-Q water to a concentration of 25 µg/mL and then added to the labelling mixture and incubated shaking at room temperature overnight. The FAM-labelled probe was precipitated using 3 M NaCl and cold absolute ethanol as described previously. It was separated as a pellet by centrifugation at 16,000 ×g for 30 minutes and the probe pellet was washed with 70% (v/v) ethanol before it was air dried. The probe was dissolved in molecular biology grade water (Sigma, USA) to a final concentration of 1 mM. The probes were stored at -20°C, wrapped in aluminium foil.
4.2.2 Attachment of FAM-labelled porA4-B to Gold Nanoparticles

Gold nanoparticles were conjugated with the FAM-labelled thiol-modified probe (porA4-B) by incubating 0.5 nmole of the TCEP-reduced probe with 1 mL of gold nanoparticle solution in 10 mM phosphate buffer pH 9 for 16 hours at room temperature in the absence of light. The NaCl concentration was then increased to 0.05 M and after being left for at least 1 hour, increased to 0.1 M. The nanoparticles were then separated from the unbound probe by centrifugation at 10,000 ×g for 15 minutes. The nanoparticle pellet was washed twice in 10 mM phosphate buffer pH 7 containing 0.1 M NaCl before resuspension in the same buffer.

4.2.3 Time-resolved Fluorescence Studies

Fluorescence decay curves of gold nanoparticle-associated probes were obtained using a time-correlated single-photon counting (TCSPC) system, FluoroHub (HORIBA Jobin Yvon IBH, UK). A pulsed laser diode (NanoLED-460, wavelength 460 nm) with a pulse rate of 1 MHz was used as the excitation source and the emitted fluorescence was recorded at 520 nm. The DataStation (v2.3) software (HORIBA Jobin Yvon, IBH, UK) was used to acquire the raw data. The time calibration was 55.6 ps per channel. The peak was preset at 10,000 counts. A quartz cuvette with a 1 cm path length and a nominal volume of 3 mL was used to hold the samples. All gold nanoparticle solutions were diluted ten-fold so that the absorbance of the solution at 460 nm was less than or equal to 0.1, to avoid inner filter effects. Other samples were diluted if the rate of the time-to-amplitude converter (TAC) was above 2%.
Once the data was acquired, the Decay Analysis Software, DAS6 (v6.1) (HORIBA Jobin Yvon) was used to recover one or two exponential decay components from the raw decay data. This software uses a reconvolution procedure to estimate the emission profiles from the raw data and calculate the fluorescence lifetime information as well as relative amplitudes of the components by fitting the raw decay data to the pre-established models. The data was fit to a single exponential function model when the sample contained only gold nanoparticles or the FAM-labelled probe whereas a two exponential function model was used when the sample contained a combination of gold nanoparticles and the probe.

4.2.4 Steady State Fluorescence Studies

Fluorescence spectra and intensities were measured with a Hitachi F-2000 spectrofluorometer (Hitachi, Japan) with excitation at 495 nm and emission at 520 nm. A quartz cuvette with a path length of 1 cm was used for the samples. The standard curves were derived from solutions containing 0 to 4 nmole/mL of FAM-labelled probes in 10 mM phosphate buffer at pH 7 and 9 respectively due to the sensitivity of the fluorescence intensity of FAM to pH of the solution (Zhao et al., 1989). The amount of FAM-labelled probe bound to gold nanoparticles was estimated by quantifying the amount remaining in solution when the nanoparticles were centrifuged. In the assays for the detection of target DNA, samples that included gold nanoparticles were diluted three-fold to minimise light scattering by the nanoparticles.
4.3 Results and Discussion

4.3.1 Thiol-modified porA4-B as Probe

The probe porA4-B had a thiol group incorporated at its 3’ end and was labelled with carboxyfluorescein succinimidyl ester (FAM) at its 5’ end. This probe was chosen rather than porA4-A which has a thiol modification at its 5’ end, because the 5’ end of porA4-B could be amine-modified and labelled with FAM, leaving the 3’ thiol group free to be used for conjugation to gold nanoparticles. When the probe was conjugated to nanoparticles, the FAM group was expected to reversibly adsorb to the nanoparticle surface and therefore emitting no fluorescence. The following time-resolved and steady state fluorescence studies were carried out to examine the changes in the fluorescence under different conditions and assess the effectiveness of this probe for the detection of target DNA.

Time-resolved Fluorescence Studies

Colloidal silica was used to obtain a prompt trace, which is the response of the instrument at $\tau=0$ and was used to compare the spectra obtained from the samples so that their fluorescence decays may be analysed. The profile of the time-resolved decay curve of gold nanoparticles in solution was similar to the prompt profile, which implied that although the nanoparticles do not have fluorescence, they are able to scatter the incident pulse of light (Figure 4.1).
Figure 4.1: Time-resolved decay curve of gold nanoparticle solution.
The time-resolved fluorescence decay curve of the thiol-modified probe (porA4-B) in 10 mM phosphate buffer pH 7 had a 1-exponential function curve as shown in Figure 4.2a. However when the probe was conjugated to gold nanoparticles, the decay curve was seen to comprise of two components; the light scattering of the gold nanoparticles and the fluorescence of the probe (Figure 4.2b). When this curve was corrected by subtracting the component with the short lifetime, which was assumed to represent the scattering of the light by gold nanoparticles, the decay curve of the probe bound to gold nanoparticles remains (Figure 4.2c). All decay curves that contained gold nanoparticles as well as FAM-labelled probes were corrected for the nanoparticle component by this subtraction. The background caused by gold nanoparticles was removed to leave only the decay curve of the nanoparticle-bound probe.

Time-resolved fluorescence measurements were made to examine the interaction of the FAM-labelled probe with nanoparticles under different conditions. One of the conditions examined was the effect of probe concentration used during its conjugation to nanoparticles.

During conjugation of the thiol-modified probe (porA4-B) to gold nanoparticles, a final concentration of 0 to 5 nmole/mL of probe was used. The nanoparticles were then washed to remove any unbound probe remaining in solution. Time-resolved measurements of the nanoparticle-bound probes showed an upward trend in the fluorophore lifetime from 3.886 ns to 4.390 ns as the probe concentration was increased during conjugation (Table 4.2).

As the concentration was increased, the slight increase in lifetime may be due to the fact that a larger amount of the probe bound to the surface of the nanoparticle. The greater coverage
Figure 4.2: Time-resolved decay curve of thiol-modified probe (porA4-B).

(a) in 10 mM phosphate buffer; (b) conjugated to gold nanoparticles; (c) corrected decay curve of nanoparticle-bound probe by subtraction of nanoparticle curve
Table 4.2: Lifetime and relative amplitude of nanoparticle-bound thiol-modified probe (porA4-B) at different probe concentrations.

<table>
<thead>
<tr>
<th>porA4-B concentration (nmole/mL)</th>
<th>Lifetime (ns)</th>
<th>Relative Amplitude (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.063</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>3.886</td>
<td>1.15</td>
</tr>
<tr>
<td>2</td>
<td>3.971</td>
<td>2.32</td>
</tr>
<tr>
<td>3</td>
<td>4.056</td>
<td>4.33</td>
</tr>
<tr>
<td>4</td>
<td>4.001</td>
<td>3.20</td>
</tr>
<tr>
<td>5</td>
<td>4.390</td>
<td>6.84</td>
</tr>
</tbody>
</table>
on the nanoparticle surface limited the space each strand of probe had for attachment, forcing it to be in a more linear conformation. This conformation would extend the fluorophore away from the influence of the plasmon resonance of the gold nanoparticle surface (Dulkeith et al., 2005).

The length of the porA4-B probe is 12 bases which is calculated to be approximately 4.08 nm when in the B-form conformation (rise per nucleotide is 0.34 nm). Therefore when the probe, which is tethered to a nanoparticle, is stretched to its maximum, the distance of the fluorophore from the nanoparticle surface will be only 4.08 nm. This is likely to result in only a small difference in lifetimes between a flat conformation and a stretched conformation since the quenching efficiency would be expected to decrease only about 0.1 (from 1 to about 0.9) (Figure 4.3). Therefore during conjugation, although the increase in concentration of the probe may have been sufficiently high for stretching the fluorophore away from the nanoparticle surface, only a small increase in the fluorescence lifetime from 3.886 to 4.390 ns for 1 to 5 nmole/mL of probe concentration used was observed.

Once the probe was conjugated to gold nanoparticles at different concentrations (1 to 5 nmole/mL), 5 nmole of the synthetic target DNA (porA4), which is complementary to porA4-B, was allowed to hybridise to nanoparticle-bound probe. An increase in the lifetime of the probe was observed for all probe concentrations (Figure 4.4). However, there was no correlation between change in lifetime and probe concentration. When the target DNA hybridised to the probe, the resulting changes in conformation led to movement of the fluorophore away from the nanoparticle surface resulting in an increased lifetime. Therefore as may be expected, an increase in lifetime of the fluorophore was observed when the target
Figure 4.3: Theoretical curve of quantum efficiency of the fluorophore FAM as a function of distance from the nanoparticle surface (Jennings et al., 2006).

Maximum length of probe (porA4-B) is indicated on graph (4.08 nm).
Figure 4.4: Time-resolved spectra of gold nanoparticle conjugated probe porA4-B.

(a-e) 1-5 nmole of probe used during conjugation to nanoparticles. An increase the fluorophore lifetime was observed when the probe was hybridised to the target DNA.
hybridised (Dubertret et al., 2001).

For detection of target DNA it is necessary that the FAM-labelled probe is completely quenched so that when it hybridises to target DNA, the movement of the fluorophore away from the nanoparticle surface may be detected by a detection of fluorescence emission. A final concentration not exceeding 1 nmole/mL of the probe was used during conjugation to ensure that the distance between the fluorophore and the nanoparticle surface was close to 0 nm. This was done to ensure maximum sensitivity.

The hybridisation of the probe bound to the nanoparticle to the target DNA (porA4) was monitored as a function of time over a period of 1 hour after porA4 was added by observing the change in lifetime of the probe. The results in Table 4.3 show that there was an increase in lifetime of the probe from 3.720 ns to 4.399 ns within 1 hour. There is also an increase in the percentage (relative amplitude) of the component with the longer lifetime, which is assumed to be the percentage of probe hybridised to target DNA.

It is assumed that the single stranded probe adsorbed to the nanoparticle surface, quenching the attached fluorophore. However, when the probe hybridised to the target, the more rigid double helix moved the fluorophore away from the nanoparticle surface, while still being attached to the nanoparticle through the thiol group of the probe. The progress of hybridisation was observed by the increase in relative amplitude of the long-life component which is assumed to be due to the probe that has hybridised to the target DNA. The rate of hybridisation of the target to the nanoparticle-bound probe to the target DNA appeared to be high until 15 minutes, during which time 5% of the probe hybridised. After 15 minutes, the rate of hybridisation slowed until 8.15% of the probe hybridised by 1 hour of incubation with
Table 4.3: Lifetime and relative amplitude of nanoparticle-bound probe (porA4-B) after target DNA (porA4) was added

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Lifetime (ns)</th>
<th>Relative Amplitude (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.720</td>
<td>0.99</td>
</tr>
<tr>
<td>15</td>
<td>3.842</td>
<td>5.99</td>
</tr>
<tr>
<td>30</td>
<td>4.433</td>
<td>6.03</td>
</tr>
<tr>
<td>60</td>
<td>4.399</td>
<td>8.15</td>
</tr>
</tbody>
</table>
the target DNA. It is assumed that the hybridisation would continue increasing if monitored for longer than 1 hour. This may be due to the fact that the bases of the probe interact with the nanoparticle surface, therefore the target may have difficulty in gaining access to the bases in order to hybridise, requiring at least an hour for all the target DNA strands to hybridise to the nanoparticle bound probe strands.

The surface energy transfer (SET) from the fluorophore to the nanoparticle is inversely proportional to separation distance (d) (Jennings et al., 2006). The length of the hybridised probe/target ds-DNA used here is calculated to be 4.08 nm. However, as shown previously in Figure 4.3, at this distance of separation the fluorophore is still able to transfer energy to the nanoparticle and is partially (90%) quenched (Jennings et al., 2006). Therefore, for a probe with a thiol-modification to be useful in detecting target DNA by hybridisation the probe is required to be longer than 12 bases (the length of the probe porA4-B). Thus for example, a probe that consisted of 44 bases (having a calculated length of 15 nm) would be have negligible SET (5%) when hybridised and may prove to be a better probe compared with porA4-B for the detection of target DNA.

**Detection Using Steady-state Fluorescence**

Steady-state intensity is the average fluorescence during the decay of a fluorophore (Lakowicz, 1999). Therefore, although the properties of different components in the system such as the fluorophore at and away from the nanoparticle surface cannot be separated from this information, basic measurements of fluorescence intensity, which indicate the average quenching of the fluorophore or lack thereof, may be carried out. While time-resolved measurements have helped in understanding the interaction of the probes with nanoparticles,
simple steady-state measurements can be used for a detection assay of hybridisation of the probe to the target by changes in the fluorescence intensity.

The thiol-modified probe (porA4-B) was conjugated to gold nanoparticles using 1 nmole of the probe for every mL of gold nanoparticle solution. The amount bound was estimated by measuring the fluorescence intensity of the supernatant when the nanoparticles were separated from the unbound probe molecules by centrifugation. Of the 1 nmole used, only 0.43 nmole of porA4-B bound to nanoparticles while the remaining unbound fraction was removed by two washing stages.

The synthetic target DNA (porA4) was added to the nanoparticle-conjugated probe and allowed to hybridise at room temperature for 1 hour. The amount of target DNA used varied from 0 to 0.8 nmole to examine the effect of different probe: target ratios, including excess target. An increase in the fluorescence intensity of the solution was observed when the target amount was increased from 0.1 to 0.8 nmole (Figure 4.5).

Since only 0.43 nmole of the probe bound to 1 mL of gold nanoparticles, the maximum amount of target DNA that could be detected is expected to be less than 0.43 nmole. This was confirmed by observation, with the fluorescence intensity increasing as increasing amounts of the target DNA porA4 was added up to 0.4 nmole. This suggests that although this method can be used for the detection of target DNA, it cannot be used to quantify it above a limit of 0.4 nmole for the quantity of nanoparticle solution used in this study.

When \textit{N. meningitidis} genomic DNA was used as the target, a small, but significant change (95% confidence interval) in the fluorescence was observed when it was denatured and
Figure 4.5: Thiol modified probe (porA4-B) conjugated to gold nanoparticles and used for detection of synthetic target (porA4).
allowed to hybridise to probe bound to nanoparticles (Figure 4.6). While the increase in fluorescence intensity was significant, the relatively small change in fluorescence likely reflects the picomole quantities of genomic DNA used. This shows the technique is able to detect extremely small amounts of bacterial genomic DNA. However, as patient samples tested may contain much lower bacterial DNA yields, the sensitivity of such a scheme is likely to require a form of DNA amplification prior to hybridisation.

4.3.2 Oligonucleotide porA4-C as Probe

The probe porA4-C was labelled with carboxyfluorescein succinimidyld ester (FAM) at its 5’ end. When the probe was added to gold nanoparticles, it may be expected to bind to the nanoparticles through its constituent bases and the FAM group expected to reversibly adsorb to the nanoparticle surface and therefore emitting no fluorescence. However if the probe was allowed to hybridise to the target DNA before its addition to the nanoparticles, it was not expected to bind to nanoparticles therefore the FAM group would emit fluorescence. The advantage that this scheme had over the previous that used a thiol-modified probe was that the hybridisation of the probe to the target occurred in a separate buffer, which did not contain gold nanoparticles and therefore were not influenced by them. This meant that gold nanoparticles were not required to be taken into account when considering hybridisation conditions. Also the ss probe may be expected to bind to nanoparticles almost immediately after addition to the nanoparticle solution, eliminating time period before which the fluorescence could be read. The following time-resolved and steady state fluorescence studies were carried out to examine the changes in the fluorescence under different conditions and assess the effectiveness of the probe porA4-C for the detection of target DNA.
Figure 4.6: Thiol modified probe (porA4-B) conjugated to gold nanoparticles and used for detection of *N. meningitidis* genomic DNA.
Time-resolved Fluorescence Studies

Varying Probe Concentration

The effect of varying the ratio of the oligonucleotide probe (porA4-C) to the nanoparticle solution on its fluorescence lifetime was examined to determine the optimal concentration to be used for the detection of target DNA. Since porA4-C possesses no thiol group to attach itself to gold nanoparticles, it interacts with the surface through its bases.

To determine the optimal amount of probe for detection of target, amounts of probe varying from 0.5 to 3 nmoles were added to 1 mL of gold nanoparticle in 10 mM phosphate buffer pH 7. After 10 minutes, by which time the probe was expected to have bound to nanoparticles (Li and Rothberg, 2004b), the time-resolved spectra showed an increase in the average lifetime as the probe concentration was increased. When these decay curves were fitted to 2-exponential decay curves, a change in the relative amplitude of the longer-life component, which represents the free probe, was observed (Table 4.4). The lifetime of the free probe remained almost the same. However, the relative amplitude increases from 0.79% to 12.37% as the amount of probe added to the solution was increased from 0.5 to 3 nmoles.

The optimal concentration was assumed to be when a majority of the probe binds to the nanoparticle, so that there is no fluorescence detected in the solution in the absence of target DNA. However the amount of probe present was required to be sufficiently high for its fluorescence to be detected when it was not adsorbed to the nanoparticles that is, when target DNA was present. Therefore, 0.5 nmole/mL, where only 0.79% of the probe was detected free in solution was chosen as the probe concentration used for further studies.
Table 4.4: Effect of concentration of oligonucleotide probe (porA4-C) on the relative amplitude of free probe.

<table>
<thead>
<tr>
<th>porA4-C concentration (nmole/mL)</th>
<th>Lifetime (ns)</th>
<th>Relative Amplitude (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>3.822</td>
<td>0.79</td>
</tr>
<tr>
<td>1.0</td>
<td>3.760</td>
<td>2.16</td>
</tr>
<tr>
<td>1.5</td>
<td>3.838</td>
<td>2.29</td>
</tr>
<tr>
<td>2.0</td>
<td>3.790</td>
<td>3.88</td>
</tr>
<tr>
<td>2.5</td>
<td>3.915</td>
<td>6.19</td>
</tr>
<tr>
<td>3.0</td>
<td>3.950</td>
<td>12.37</td>
</tr>
</tbody>
</table>
Concentrations below 0.5 nmole/mL of the probe were not considered due to lack of sensitivity in that range.

*Time Required for Probe to Bind to Nanoparticles*

The amount of time required by the probe and hybridised probe/target to adsorb onto the nanoparticle surface was determined in this study by time-resolved fluorescence measurements taken over a period of 1 hours. A hybridisation solution containing 0.5 nmole of the probe porA4-C and 1 nmole of the target DNA (porA4) in 10 mM phosphate buffer pH 7 containing 0.3 M NaCl was denatured at 95°C for 5 minutes and annealed at 50°C for 1 minute. The hybridisation solution was then added to 1 mL of gold nanoparticle in 10 mM phosphate buffer pH 7. Time-resolved measurements were taken at 1, 10 and 60 minutes. As seen in Figure 4.7, there is no change in lifetime of the probe or probe/target, indicating that the probe adsorbed to the nanoparticles in less than one minute.

Since the oligonucleotide probe hybridised to the target DNA in the absence of gold nanoparticles, there was no interference to hybridisation by the nanoparticle as observed for the thiol-modified probe (porA4-B) that was bound to nanoparticles. When the hybridisation solution was added to the nanoparticle solution, the binding of the probe to the nanoparticles occurred within 1 minute whereas, no binding to nanoparticles was observed for hybridised probe/target due to the probes bases being involved in base-pairing. As a detection method, this scheme is ideal as there is virtually no prolonged time between addition of the sample and subsequent hybridisation and detection.
Figure 4.7: Effect of time following addition of unhybridised (ss) probe (porA4-C) and hybridised (ds) probe/target DNA.

(a) 1 minute (b) 10 minutes (c) 60 minutes after addition of probe or probe/target.
**Varying Target Concentration**

Different target: probe ratios were used to examine if this scheme using porA4-C as the probe with gold nanoparticles could be used for the detection of target DNA. This studied the effect of the presence of target DNA on the fluorescence of the probe when the solution was added to gold nanoparticles. Amounts of target DNA (porA4) ranging from 0 to 0.75 n mole were hybridised to 0.5 n mole of the oligonucleotide probe before being added to the gold nanoparticle solution. Although there was no significant change in the fluorescence lifetime of free probe, the relative amplitude increased from 0.79% to 32.26% as the amount of target DNA was increased from 0 to 0.75 n moles (Table 4.5). While the relative amplitude of free probe increased with increasing amounts of target, a plateau was observed when the amount of target reached 0.5 n mole. When the target amount was increased further, there was no significant change in the relative amplitude indicating that all of the probe was hybridised to the target when the amount was about 0.5 n mole and no ss probe was present to bind to the nanoparticles. There was no significant increase in the lifetime of the hybridised probe/target as the target amount was increased. This could be due to the fact that if the probe was hybridised to the target, it has no interaction with nanoparticles and the observed lifetime was the maximum for the probe.

**Detection Using Steady-state Fluorescence**

Varying amounts of the oligonucleotide probe (porA4-C) from 0 to 2 n mole were added to 1 mL of gold nanoparticle solution in 10 mM phosphate buffer pH 7. After allowing the probe to bind for 1 hour to reach equilibrium, the amount bound was estimated by measurement of the fluorescence intensity of the supernatant when the nanoparticles were separated from the
Table 4.5: Lifetime and relative amplitude of oligonucleotide probe (porA4-C) hybridised to target DNA (porA4) in gold nanoparticle solution.

<table>
<thead>
<tr>
<th>Amount of target porA4 (nmole)</th>
<th>Lifetime (ns)</th>
<th>Relative Amplitude (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.822</td>
<td>0.79</td>
</tr>
<tr>
<td>0.25</td>
<td>3.999</td>
<td>21.77</td>
</tr>
<tr>
<td>0.50</td>
<td>3.988</td>
<td>31.68</td>
</tr>
<tr>
<td>0.75</td>
<td>4.001</td>
<td>32.26</td>
</tr>
</tbody>
</table>
unbound probe molecules by centrifugation. At all probe concentrations used (0.17 to 2 nmole/mL), between 84.4 and 77.0% respectively of the probe bound to nanoparticles. A probe concentration of 0.5 nmole/mL was used in the assays for detection of target DNA since a maximum percentage (82.7%) of the probe bound to nanoparticles associated with minimal fluorescence in the solution (8.6% of fluorescence intensity of free probe). Also the optimum concentration of probe as determine by time-resolved fluorescence measurements was 0.5 nmole/mL.

The probe (0.5 nmole) and varying amounts of target were denatured at 95°C for 5 minutes and annealed at 50°C for 1 minute in 10 mM phosphate buffer pH 7 containing 0.3 M NaCl. The amount of the target DNA porA4 used varied from 0 to 0.8 nmole. When the hybridisation solution was added to a gold nanoparticle solution in 10 mM phosphate buffer, an increase in the fluorescence intensity of the solution was observed when the target amount was increased from 0.1 to 0.4 nmole, after which the intensity appears to remain constant (Figure 4.8). The greatest fluorescence intensity of the nanoparticle-bound probe in the presence of target DNA was 72.4% of the intensity when the probe was free in solution.

When porA4-C was hybridised to the target DNA porA4, there was an increase in fluorescence intensity observed when compared with the probe in absence of the target. As observed for the thiol-modified probe porA4-B, the maximum detection limit is approximately 0.4 nmole of target. It can be assumed that since only 0.5 nmole of the probe is used per millilitre of gold nanoparticle solution, of which only about 82.7% binds to nanoparticles within 1 hour, this is the maximum detection limit.
Figure 4.8: Oligonucleotide probe (porA4-C) with gold nanoparticles used for detection of synthetic target porA4.
However, the increase in fluorescence at highest target concentration was 72.4% of the intensity when the probe was free in solution compared with only 22.8% for the thiol-modified probe porA4-B indicating that this scheme is more sensitive compared with that using a thiol-modified probe. This is most likely due to the fact that hybridisation of the oligonucleotide probe to the target DNA prevents it from having any interaction with the nanoparticles. Conversely, in the case of the thiol-modified probe, although hybridisation to the target DNA causes the fluorophore to be less under the influence of the nanoparticle, it still is quenched to a great degree due to the short length of the probe.

The amount of *N. meningitidis* genomic DNA used for detection in this study was varied from 0 to 1 pmole of the complete meningococcal genome. No fluorescence intensity increase was observed when the amount of target genomic DNA was lower than 0.25 pmole which appears to be the detection limit (Figure 4.9). At the highest target amount used, the fluorescence intensity was only 16.9% of the intensity when the probe was free in solution.

The sensitivity of this detection scheme that used porA4-C as the probe is seen by its ability to detect *N. meningitidis* genomic DNA. However, as discussed in Section 4.3.1, the ability to detect the amount of genomic DNA at the order of magnitude of 0.25 pmole may not be sufficient to support the diagnosis of meningococcal disease as it represents DNA extracted from approximately $1.5 \times 10^{11}$ bacterial cells, which is far greater than the number expected to be isolated from clinical samples. Therefore, it is likely that this method for the detection of meningococcal DNA for the identification of the pathogen in clinical samples will require a form of DNA amplification prior to detection.
Figure 4.9: porA4-C-FAM conjugated to gold nanoparticles used as probe while *N. meningitidis* genomic DNA used as target.
4.4 Summary

In this chapter, two different fluorophore-labelled probes were assessed for their ability to detect a specific DNA sequence at a level of less than 1 pmole sensitivity. The two different probes used were (a) a 12 base thiol-modified probe porA4-B and (b) a 24 base oligonucleotide with no modification (porA4-C). The thiol-modified probe was conjugated to the gold nanoparticle prior to being used in the detection assay, whereas the oligonucleotide probe could be added to the nanoparticles directly before detection.

Time resolved fluorescence measurements were used to optimise conditions such as nanoparticle and probe concentration, amount of target DNA present and time required for hybridisation of the probe to target DNA.

The use of steady-state fluorescence measurements was examined as a method for the detection of target DNA. Both probes could detect the presence of synthetic target DNA. However, the change in fluorescence in the presence of the target DNA was greater for the oligonucleotide probe compared with the thiol-modified probe indicating that the probe is more sensitive when not attached to the nanoparticle.

It would be of interest to explore the effect of the length of the thiol modified probe on its sensitivity in detecting target DNA. As discussed in Section 4.3.1, a longer probe with a minimum length of 44 bases may be expected to be more sensitive due to the greater distance that the ds probe/target can provide between the fluorophore and the nanoparticle surface. Therefore the hybridisation of the target DNA to the nanoparticle-bound probe would cause a
greater increase in the fluorescence detected in the solution due to the fluorophore being moved away from the influence of the nanoparticle.

Although the use of synthetic target DNA demonstrated the technique operated in a controlled system, it was necessary to show that it worked in a similar manner with biological samples. When meningococcal genomic DNA was used as the target, probes porA4-B and porA4-C were able to detect meningococcal DNA with a detection limit of 0.125 pmol and 0.25 pmol, respectively.

However, there is a need for greater sensitivity for the detection of meningococcal DNA in diagnosing the disease. The concentration of meningococcal cells in blood can be less than 1 cfu/mL; while in cerebrospinal fluid the concentration can vary from 0 to $10^7$ cfu/mL (Olsen and Fredlund, 2001). Any method that is used in the diagnosis of this disease would require sensitivity in this range. Therefore, in order for these fluorescence detection methods using gold nanoparticles to be clinically applicable, the samples need to undergo a form of DNA amplification such as polymerase chain reaction (PCR) to enhance the sensitivity prior to detection. However, while the method is rapid, the addition of the DNA amplification step will add a time of at least 1 hour to the whole detection process.
Chapter 5

General Discussion and

Future Directions
Chapter 5: General Discussion and Future Directions

*N. meningitidis* infection results in invasive diseases such as meningitis and sepsis which progress rapidly and can result in death within 24 hours of the first symptoms. The mortality reaches 100% if the disease is untreated and remains 7-10% even if treated (Goering *et al.*, 2008). Rapid diagnosis and identification of *N. meningitidis* in clinical samples is essential to exclude other pathogens and for the administration of appropriate and effective treatment. Current methods for detection of *N. meningitidis* which include direct microscopy, culture, PCR and serology for detection of antigens in CSF, blood and other clinical samples have problems with sensitivity or the time required for results (Zollinger and Boslego, 2002; Gray *et al.*, 2006; Nadel and Kroll, 2007).

The primary aim of this project was to utilise the unusual size-dependent properties of gold nanoparticles to develop methods for the detection of a *N. meningitidis*-specific DNA sequence. These methods were then optimised and evaluated as diagnostic tests for the rapid detection of the pathogen.

In this study, a meningococcal-specific DNA sequence porA4 was selected from the *porA* gene that is only expressed in *N. meningitidis* (Unemo *et al.*, 2005). The probe sequences porA4-A and porA4-B were designed to be complementary to sections of the porA4 target sequence. These DNA probes used needed to be directionally conjugated to gold nanoparticles through the incorporation of a functional group that would preferentially bind to the nanoparticle surface. Binding studies using model compounds were carried out to determine the ideal functional group and optimal conditions required to conjugate the DNA probes to nanoparticles. The binding isotherm studies showed that amine groups had a higher affinity for gold nanoparticles compared with thiol groups although they are less
densely spaced on the nanoparticle surface. Leff and colleagues (1996) have also reported that amines tend to less packing on the surface of nanoparticles compared with thiols. The pH of the solution was found to have no influence on the affinity of the thiol and amine groups for the gold nanoparticle surface. However, the kinetics of binding was influenced by the pH. It had been assumed that the functional group, whether thiol or amine, needed to be unprotonated to allow binding to gold surfaces (Selvakannan et al., 2003). This is achieved by increasing the pH of the solution to above the pKₐ of the functional group. In this study, as may be expected, the kinetics of the binding of thiol groups was increased when the pH was above 8.33, the pKₐ of thiol groups. This phenomenon was also observed for hydroxyl group which has not been previously shown to bind to gold nanoparticles. However, the amine groups demonstrated more rapid binding at lower pH values well below 9.5 at which they are protonated. A more recent study by Basu and colleagues (2007) reported a similar increase in binding of the α-amine group of glutathione at lower pH values. Since thiol groups had greater coverage on gold nanoparticles compared with amine groups, it was selected as the functional group for conjugation of the probes used in this study where the rate of binding could be increased by increasing the pH of the solution.

The two DNA sequences designed as probes were synthesised with a thiol group on either the 5’ or 3’ end, conjugated to gold nanoparticles and used for the detection of target meningococcal DNA. However prior to their use for detection, the effects of salt concentration and pH on their binding to gold nanoparticles were examined. The influence of probe density on the nanoparticle surface and the effect of salt concentration on the hybridisation of target DNA were also determined. Similar to results from previous studies (Demers et al., 2000; Hurst et al., 2006), increasing the salt concentration led to increased
DNA binding to the nanoparticles until a maximum of about 150 DNA molecules per nanoparticle was observed. This coverage is in good agreement with a coverage of 159 DNA molecules of similar size per 15 nm gold nanoparticle reported by Demers and colleagues (2000). The effect of the pH of the solution on the binding of thiol-modified DNA to gold nanoparticles has not been previously examined. This study observed that similar to the thiol groups in the model binding studies, the rate at which of thiol-modified DNA bound to nanoparticles could be increased by increasing the pH. Once the probes were conjugated to gold nanoparticles, as the concentration of NaCl in the solution was increased, the hybridisation efficiency between the target and functionalised nanoparticles also increased. However, as probe coverage on the surface of the nanoparticle was increased, the hybridisation efficiency decreased, with maximal hybridisation efficiency observed between 50 and 62% probe coverage. These findings are in agreement with previous reports (Peterson et al., 2001; Jin et al., 2003).

The plasmon resonance shift of gold nanoparticles caused by target DNA directed aggregation was evaluated as a potential detection method for target DNA sequences and the optimised conditions for hybridisation of DNA to nanoparticle-bound probes were used to achieve the maximal plasmon resonance colour shift. An inter-particle distance of 24 bp rather than 12 bp was used although the rate of the plasmon resonance shift was shown to be greater in the system with a 12 bp inter-particle distance. Storhoff and colleagues (2000) used DNA linkers ranging in length from 24 to 72 bp to aggregate gold nanoparticles. They showed that the linker length kinetically controlled the formation of the aggregated and consequently the shift in the plasmon resonance peak, with 24 bp linkers producing the fastest shift of the plasmon resonance peak wavelength. The 12 bp inter-particle system was
not used in this study because a porA4-A probe with a 3’ end thiol modification was needed. If these 3’ thiol-modified probes were used, it would be envisaged that less time would be required for detection of DNA through the plasmon resonance shift assay.

Alternatively, a greater shift in the plasmon resonance peak may occur with the use of larger gold nanoparticles. Studies on the surface plasmon resonance of gold nanoparticles have shown the when the distance between the nanoparticles is less than 1.5 times their diameter, a shift in the plasmon resonance peak is observed. The smaller the distance between the particles is relative to the diameter, the greater the shift in the plasmon resonance peak (Rechberger et al., 2003; Su et al., 2003). Therefore the use of larger nanoparticles for example 50 nm instead of 15 nm, is likely to produce a greater plasmon resonance peak shift for the same inter-particle distance used.

It has been previously reported that a major limitation of this plasmon resonance shift detection method is low sensitivity (Rosi and Mirkin, 2005). In this work, 0.75 pmol of genomic DNA could not be detected using this assay however, the PCR product of the porA gene was detected with a minimum of 2 n mole of the target required to produce a shift that could be detected within 1 hour. The PCR required a starting genomic DNA copy number of at least $10^3$ to amplify the target gene and is similar to that which has been reported in the literature (Saunders et al., 1993). One approach to overcome the low sensitivity of the colour-shift method would be to undertake a detailed sequence analysis of the N. meningitidis genome to identify species-specific sequences that are repeated multiple times within the genome. These sequences could be targeted to increase the sensitivity of the technique. An alternative is to use the mRNA of a chosen gene as the target. Another potential target is
rRNA, which contains species-specific sequences. The multiple copies of target RNA in a single cell will increase the sensitivity of the assay. However the lack of stability of RNA may require the test to be completed without delay or used in the presence of an RNAse inhibitor.

Another limitation of the colour-shift method was the time required for a plasmon resonance change to be observed. Samples with lower target amounts of less than 2 nmole take more than an hour to produce a change that may be observed. This may be due to the fact that this technique relies on Brownian motion to bring the nanoparticle probes and target close together to hybridise to form aggregates that are sufficiently large to shift the plasmon resonance peak of the system (Ray et al., 2005).

A range of probes may be used in an array that target different genes or sequences. The target genes may be from the same organism, in which case this scheme could enhance the sensitivity of the assay. Another potential application of this arrangement is in the area of pharmacogenomics where different genes in the same organism may be probed. The presence of a gene may influence the treatment used, for example the penA gene in *N. meningitidis* that confers resistance to penicillin (Bowler et al., 1994; Rosenstein et al., 2000), thus providing for a more specific and effective chemotherapeutic regime. This scheme may also be used to screen for several organisms at the same time by targeting species-specific sequences.

The colour-shift method for the detection of target DNA may be further explored to study the effects of point mutations in the target sequence on hybridisation to the probe and plasmon resonance shift. These studies need to be performed in combination with T$_m$ studies in order
to distinguish between perfect matches and mismatches. Previous studies have shown that differences in the melting temperatures of the mismatched target and the probe compared with probe bound to complementary target enable the identification of point mutations within sequences (Elghanian et al., 1997; Storhoff et al., 1998). This may be useful in the detection of *N. meningitidis* strains that have single polymorphisms within the target sequence by lowering hybridisation temperatures to accommodate single mismatches. This strategy may also be used for the diagnosis of a large range of genetic disorders that are caused by single mutations in humans.

The stability of the double helix formed by the hybridisation of the probe to the target DNA could be increased by using peptide nucleic acid (PNA) probes instead of DNA. PNA’s are nucleic acid analogues in which the sugar-phosphate backbone of DNA is replaced by a neutral pseudo-peptide backbone that consists of N-(2-aminoethyl)glycine units to which the nucleotide bases are attached (Egholm et al., 1993; Nielsen, 2001). These molecules are completely synthetic and therefore are not recognised by either nucleases or proteases, making them highly stable. Moreover, due to their neutral backbone, they bind to complementary DNA sequences with higher affinity than the equivalent DNA molecule (Nielsen, 1999). The PNA molecules of the porA4-A and porA4-B probes would be predicted to have melting temperatures of 64 and 69°C respectively at a salt concentration of 1 µM, which is much higher than the melting temperatures of 44 and 48°C for the equivalent DNA probe that were used in this study. In addition, PNA molecules are also more sensitive to a mismatch in the complementary DNA sequence that is, a single mismatch destabilises the double helix and the melting temperature decreases sharply. Their stability, higher melting temperatures and greater sensitivity to mismatches make PNA’s better probes.
(Chakrabarti and Klibanov, 2003) and could be investigated in combination with gold nanoparticles for further optimisation of the plasmon resonance shift DNA detection method.

The second detection method evaluated in this study involved the use of DNA probes labelled with fluorophores permitting the monitoring of the hybridisation of target DNA to the probes by associated changes in fluorescence. Although time-resolved fluorescence measurements provided an understanding of the mechanisms of interaction of the probes with gold nanoparticles to optimise the detection method, steady-state fluorescence was the method assessed for the detection of meningococcal DNA in practice. There is potential application of time-resolved fluorescence as a diagnostic test, although it requires expensive instrumentation. The data obtained in this study show that the hybridisation of the target to the probe may be monitored and also quantified by separation of the decay curve into its components using fluorescence decay analysis software.

Two different fluorophore-labelled probes were assessed for their ability to detect a specific DNA sequence at a sensitivity level of less than 1 pmole. The two different probes used were (a) a 12 base thiol-modified probe that was conjugated to gold nanoparticles and (b) a 24 base oligonucleotide with no modification that was added to the nanoparticle solution after hybridisation to target DNA. Both probes could detect the presence of synthetic target DNA. However, the change in fluorescence in the presence of the target DNA was less for the thiol-modified probe compared with the unmodified probe. This indicated that the probe was more sensitive when not attached to the nanoparticle.

It would be of interest to explore the effect of the length of the thiol modified probe on its sensitivity in detecting target DNA because this length limited the probe’s ability to move the
fluorophore away from the quenching influence of the gold nanoparticle surface. The quenching efficiency of the gold nanoparticle has an inverse relationship to $d^4$, where $d$ is the distance between the nanoparticle surface and the fluorophore (Jennings et al., 2006). The length of the probe used in this study (4.08 nm) was insufficient to move the fluorophore completely away from the influence of the nanoparticle as calculated by the quenching efficiency of gold nanoparticles for FAM, the fluorophore used in this study. Therefore, the use of a longer probe may increase the sensitivity of this detection method.

The fluorescence method may be used with a set of probes, each labelled with a different fluorophore that have emission spectra that do not overlap. These probes may then be used for the simultaneous detection of different DNA sequences, either different genes in the same organism or sequences specific to different pathogens, by monitoring the associated changes in fluorescence.

When the fluorescence method of detection was used on meningococcal genomic DNA, both probes were able to detect meningococcal DNA in the order of 0.1 pmol. The sensitivity of this method was at least $10^4$ fold greater compared with the plasmon resonance shift method which required a minimum of 2 nmole of target DNA. Another advantage of this method over the colour shift assay was the shorter time required for detection, which was in the order of minutes, when the unmodified probe was used. However, this method requires specialised instrumentation for the detection of fluorescence while the colour shift may be detected by the naked eye.

Resonance light scattering is another property of gold nanoparticles that has potential to be used in the detection of DNA sequences. The resonance light scattering increases as the
nanoparticles aggregate and may be measured using a fluorometer (Yguerabide and Yguerabide, 2001; Du et al., 2006; Li et al., 2006). Unlike the fluorescence method used in this study, resonance light scattering does not require the probes to be labelled. Similar to the plasmon resonance shift, this method uses the target DNA-directed aggregation of nanoparticles for the monitoring of hybridisation and has a sensitivity in the picomolar range (Du et al., 2006).

The concentration of meningococcal cells in blood can be less than 1 cfu/mL; while in cerebrospinal fluid the concentration can vary from 0 to $10^7$ cfu/mL (Olcen and Fredlund, 2001). Therefore, any method that is used in the diagnosis of this disease would require sensitivity in this range. The detection methods analysed in this study have only been attempted on either synthetic DNA or DNA obtained from bacterial cultures and does not reflect results that may be obtained from clinical samples containing meningococci. In order for either of the methods evaluated in this study to be used in a clinical setting, the samples need to undergo a form of DNA amplification such as PCR to enhance the sensitivity prior to detection. However, certain body fluids found in the clinical samples such as CSF, blood and urine contain substances known to inhibit the PCR enzyme Taq polymerase (Panaccio and Lew, 1991). Therefore it may be necessary to separate the bacteria from the sample by centrifugation if possible, as in the case of CSF or urine, or extract bacterial DNA from blood samples (Newcombe et al., 1996; Taha and Fox, 2007) prior to performing PCR. This would mean that if these DNA detection methods were further optimised for clinical samples and used in combination with PCR, a result could be obtained within three hours, which is necessary when working with a highly pathogenic organism such as *N. meningitidis*. 
The results obtained for the methods analysed in this study are promising to warrant further investigation for these detection methods using gold nanoparticles. These methods have been shown to be rapid, requiring just one hour for the colour-shift method and less than 10 minutes for the fluorescence method using unmodified probes. The probes used in this study were highly specific, hybridising to only the target sequence within the entire genome. Moreover, other studies using the colour-shift method have shown that point mutations within the target DNA sequence may be identified by controlling the temperature during hybridisation (Elghanian et al., 1997; Storhoff et al., 1998). The colour-shift method was able to detect a minimum of 2 nmole of target DNA within an hour while the fluorescence method had an even greater sensitivity, detecting 0.1 pmole of target DNA. These two methods provide flexibility in the use of gold nanoparticles for the detection of DNA. The fluorescence method is ideal in a setting where sensitivity is an advantage and speed is required. However, when large copy numbers of target DNA are known to be present, the colour-shift method is a simple test not requiring instrumentation and can also be used to detect point mutations. The properties of these tests suggest that gold nanoparticles have great potential to be used in a wide range of applications including the detection of a various bacteria and other pathogens, as well as in genetic screening through identification of either the presence or absence of genes and the detection of point mutations.
References


pulsed-field gel electrophoresis versus phenotypic methods. Journal of Medical Microbiology 47: 993-998.


References


References


**References**


References


References


Appendix A

Materials

Milli-Q water (18.2 MΩ cm⁻¹) was produced by purification with a Millipore Milli-Q water system. Unless otherwise stated, all solutions were made using Milli-Q water. All salts and reagents are analytical grade unless otherwise stated.

The bacterial strain used in this thesis is *Neisseria meningitidis* (MC58). The growth media used to culture the bacteria was Mueller-Hinton broth (Oxoid, UK).

The DNA sequences used are listed in Table A1. The porA4 sequence is the synthetic target DNA derived from a meningococcal sequence, while the sequence B3-comp is an artificial target. They were both obtained from Sigma-Genosys (Australia). The probes porA4-A and porA4-B have a thiol modification at the 5’ and 3’ end respectively. They were obtained from Operon Biotechnologies (Cologne, Germany). The lyophilised probes and targets were dissolved in molecular biology grade H₂O (Sigma, USA) to give a final concentration of 1 mM and stored at -20°C.

Reagents for DNA Extraction

Chloroform/Isoamyl alcohol (CI): 96% (v/v) chloroform (Mallinckrodt, USA), 4% (v/v) isoamyl alcohol (SAFC).

CTAB/NaCl solution: 4.1 g NaCl, 10 g CTAB in 100 mL H₂O

Ethanol (Merck, Germany)

Isopropanol: Propan-2-ol (Mallinckrodt, USA)
Lysis buffer: 9.4 mL TE buffer, 0.1 mL of 0.5 M EDTA, 50 µL proteinase and 0.5 mL of 10% (w/v) SDS (BioRad, USA)

5 M NaCl solution

Phenol/chloroform/isoamyl alcohol (PCI): 50% (v/v) phenol (Sigma, USA), 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol (SAFC).

Proteinase K: 20 mg/mL (Invitrogen, USA) in sterile Milli-Q H₂O

TE buffer: 10 mM Tris (Sigma, USA), 1 mM EDTA (J.T. Baker, USA) pH 8.0

Reagents for Southern Blot Hybridisation

Agarose: x% (w/v) Ultrapure Agarose 1000 (Invitrogen, USA) in 1×TAE buffer, solubilised by heating in a microwave oven.

Anti-digoxigenin (DIG) Fab fragment-alkaline phosphatase (Roche Diagnostics, Germany)

Anti-DIG solution: Anti-DIG-alkaline phosphatase (1:5,000) in blocking solution.

Blocking reagent: supplied by Roche Diagnostics, Germany

Blocking reagent stock solution (10%): 10% (w/v) Blocking reagent (Roche Diagnostics, Germany) in maleic acid buffer. The blocking reagent was heated at 65°C with constant stirring for 1h or until completely dissolved. The solution was autoclaved and stored at 4°C.

Blocking solution: 1% Blocking reagent diluted in maleic acid buffer

Depurination solution: 0.2 M HCl

Denaturation solution: 0.5 M NaOH, 1.5 M NaCl

DNA loading buffer (11×): 10% (w/v) ficoll®-400 (BDH), 50% (v/v) glycerol (BDH), 0.5% (w/v) Orange G (Sigma-Aldrich, USA), 1% (w/v) SDS, 10 mM EDTA, 50 mM Tris pH 8.0.

DNA marker: 1 kb DNA ladder (Promega, USA) see Figure A1 for bands.
Ethidium bromide (Boehringer Mannheim, Germany)

Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl. Adjusted to pH 7.5 with NaOH. Autoclaved.

Maleic acid wash buffer: 100 mM maleic acid, 150 mM NaCl, 0.3% (v/v) Tween® 20 pH 7.5.

Neutralization solution: 0.5 M Tris-HCl pH 7.5 and 3 M NaCl

Restriction enzymes: BamHI (10 U/µL) (Promega, USA) and EcoRI (12 U/µL) (Promega, USA)

Roche DIG-labelling kit for Southern blot included 10× dNTP labelling mixture, 10× hexanucleotide mixture, Klenow enzyme (100 U/mL) and Southern blot labelled control DNA (5 µg/mL DIG-labelled pBR328 linearized by EcoRI)

SSC (20×): 3 M NaCl, 300 mM trisodium citrate (BDH) pH 7. Autoclaved.

SSC (2×) wash buffer: 2× SSC, 0.1% SDS

SSC (0.5×) wash buffer: 0.5× SSC, 0.1% SDS

Standard hybridisation solution: 5× SSC, 0.1% N-lauroylsarcosine (Sigma-Aldrich, USA), 0.02% SDS, 1% Blocking reagent (from 10% stock solution) diluted in Milli-Q H₂O.

Tris-Acetate-EDTA (TAE) buffer (×50): 2 M Tris, 1 M acetic acid (BDH), 0.1 M EDTA

Western Blue Stabilised Substrate for alkaline phosphatase (Promega, USA)

Gold Nanoparticle Synthesis

Hydrogen tetrachloroaurate (HAuCl₄·3H₂O) (Sigma-Aldrich, USA)

Trisodium citrate (BDH)
Appendices

Reagents for Model Binding Studies

Binding Isotherm Studies

4 M Acetate buffer: 4 M sodium acetate pH 5.5
2,3-diaminopropionic acid monohydrochloride (DAPA) (Sigma, USA)
5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB) solution: 10 mM DTNB (Calbiochem, USA) in
0.1 M phosphate buffer pH 8.0
Mercaptosuccinic acid (MSA) (≥ 99%) (Fluka, Switzerland)
Ninhydrin reagent: 0.8 g of ninhydrin (Aldrich), 0.12 g of hydridantin (Sigma, USA) in 30
mL of dimethyl sulfoxide (Sigma, USA) and 10 mL of 4 M acetate buffer
Phosphate buffer: Prepared by combining 0.2 M NaH$_2$PO$_4$ and 0.2 M Na$_2$HPO$_4$ with ratios
according to the Henderson Hasselbach equation to give the desired pH at a final
concentration of 0.1 M. Phosphate buffers of lower strength were prepared by diluting the
0.1 M buffer

Amino Acids and their Derivatives

DL-2-aminobutyric acid (abu) (Aldrich, USA)
L-Cysteine (Sigma, USA)
2,3-diaminopropionic acid monohydrochloride (DAPA) (Sigma, USA)
DL-Serine (Fluka, Switzerland)
Appendices

**DNA quantification**

Hoechst dye 33258 solution: $4.5 \times 10^{-7}$ M Hoechst 33258 (Sigma-Aldrich, USA) in 25 mM phosphate buffer pH 7

5 M NaCl solution

Tris-Borate-EDTA (TBE) buffer ($\times10$): 89 mM Tris, 2.5 mM EDTA, 89 mM borate (BDH)

Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma, USA)

**PCR**

Wizard Genomic DNA Purification Kit (Promega, USA)

PCR Master Kit (Roche, Germany)

Agarose: x% (w/v) Ultrapure Agarose 1000 (Invitrogen, USA) in 1×TAE buffer, solubilised by heating in a microwave oven.

DNA loading buffer ($11\times$): 10% (w/v) ficoll®-400 (BDH), 50% (v/v) glycerol (BDH), 0.5% (w/v) Orange G (Sigma-Aldrich, USA), 1% (w/v) SDS, 10 mM EDTA, 50 mM Tris pH 8.0.

DNA Marker: Lambda DNA digested with *Pst*I: 20 µg λ-DNA (Promega, USA) was incubated with 10 U of *Pst*I (Promega, USA) in 1× Buffer H (Promega, USA) at 37°C overnight. Twenty microlitres of 11× DNA loading buffer was then added to stop the digest, and Milli-Q H$_2$O was added to make the total volume 200 µL. The marker was stored at 4°C, with 10 µL used on a DNA agarose gel. A diagram of *Pst*I digested λ-DNA is shown in Figure A2.

Wizard PCR Preps DNA Purification Kit (Promega, USA)

Primers used for the amplification of the *porA* gene were obtained from Sigma-Genosys (Australia) and are listed in Table A2.
Appendices

**FAM-labelling**

5-(and-6)-carboxyfluorescein succinimidyl ester (FAM) (Invitrogen, USA)

Chloroform/Isoamyl alcohol (CI): 96% (v/v) chloroform (Mallinckrodt, USA), 4% (v/v) isoamyl alcohol (SAFC).

Dimethylsulphoxide (DMSO) (Sigma, USA)

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) (Pierce, USA)

Ethylenediamine solution: 0.25 M ethylenediamine in 0.1 M imidazole pH 6

Labelling buffer: 0.1 M Sodium tetraborate pH 8.5, made fresh

Phenol/chloroform isoamyl alcohol (PCI): 50% (v/v) phenol (Sigma, USA), 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol.

Reaction buffer: 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA pH 7.2
### Table A1: Oligonucleotide sequences used in this study

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<tr>
<th>Name</th>
<th>Sequence 5’- 3’</th>
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<tr>
<td>porA4-A</td>
<td>$SH$-CATCGTGTCTGG</td>
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<tr>
<td>porA4-B</td>
<td>CGCTTTGCGCTGAA-$SH$</td>
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<td>porA4-C</td>
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<tr>
<td>porA4</td>
<td>TTCAGCCAAGCGCCAGACACGATG</td>
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<tr>
<td>B3-comp</td>
<td>TTCAGCCAAGCGTTCAAGCCAGACGCTCAGCCAGCAGC</td>
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### Table A2: Primers used for the amplification of the porA gene

<table>
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<th>Name</th>
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<tr>
<td>porA-For</td>
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<td>porA-Rev</td>
<td>CAGCTTGGAGCAAGACGTATC</td>
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</table>
Figure A1: 1 kbp DNA Ladder (Promega, USA)
**Figure A2: PstI digested λ-DNA separated by agarose gel electrophoresis**

Diagram obtained from Fermentas Life Sciences website

([http://www.fermentas.com/catalog/electrophoresis/convlambdamarkers.htm#24](http://www.fermentas.com/catalog/electrophoresis/convlambdamarkers.htm#24))

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0.5μg/lane, 8cm length gel, 1X TAE, 7V/cm, 1h
Appendix B

Gold Nanoparticle Plasmon Resonance

Absorption spectra of DNA-gold nanoparticle conjugates in the presence of target DNA (B3-comp, porA4 and meningococcal genomic DNA) at salt concentrations ranging from 0 to 0.7 M.
Figure B1: Plasmon resonance shift when 3B-comp was used as target DNA with a NaCl concentration of 0 M.

(a) No target DNA present  (b-f) represent 1 to 5 nmole of target DNA.
Figure B2: Plasmon resonance shift when B3-comp was used as target DNA with a NaCl concentration of 0.1 M.

(a) No target DNA present (b-f) represent 1 to 5 nmole of target DNA.
Figure B3: Plasmon resonance shift when B3-comp was used as target DNA with a NaCl concentration of 0.3 M.

(a) No target DNA present (b-f) represent 1 to 5 nmole of target DNA.
Figure B4: Plasmon resonance shift when porA4 was used as target DNA with a NaCl concentration of 0.1 M.

(a) No target DNA present (b-f) represent 1 to 5 nmole of target DNA
Figure B5: Increase in nanoparticle absorbance at 600 nm as a function of time when porA4 was used as target DNA with a NaCl concentration of 0.1 M.
Figure B6: Plasmon resonance shift when porA4 was used as target DNA with a NaCl concentration of 0.3 M.

(a) No target DNA present (b-f) represent 1 to 5 nmole of target DNA
Figure B7: Increase in nanoparticle absorbance at 600 nm as a function of time when porA4 was used as target DNA with a NaCl concentration of 0.3 M.
Figure B8: Plasmon resonance shift when meningococcal genomic DNA was used as target with a NaCl concentration of 0.5 M.

(a) No target DNA present (b-f) represent 0.22 to 0.11 mg of target DNA
Figure B9: Plasmon resonance shift when meningococcal genomic DNA was used as target with a NaCl concentration of 0.7 M.

(a) No target DNA present (b-f) represent 0.22 to 0.11 mg of target DNA