ASSAY DEVELOPMENT

4.1. Immune system

All organisms are continually under attack from other organisms both externally (by predators) as well as internally (by pathogens, bacteria, virus etc.). Vertebrates have developed immune system, an elaborate protective mechanism against invading pathogens and foreign particles. The immune system has extensive ability to detect and respond to stimuli such as presence of foreign particles inside the body and it remembers what it encountered over long period of time. Immunity in vertebrates is conferred by white blood cells collectively called as lymphocytes. These cells originate from stem cells in bone marrow like all other blood cells but unlike red blood cells they leave the blood vessels and patrol the intercellular space for presence of foreign intruders or macromolecules collectively called as ‘antigens’. These cells get trained in the specialized lymphoid tissues such as thymus, lymph nodes and spleen, where they learn to differentiate between self and non-self.1

4.1.1 Antibody Production

Antibodies are serum proteins and form a part of vertebrate defense system against invading pathogens or foreign particles i.e, antigens. In 1890, it was proved that these toxins are responsible for production of special proteins in the immunized animals that were initially termed as ‘antitoxins’ now called as antibodies. It was later shown that not only toxins but also other molecules termed antigens (which included proteins, nucleic acids and polysaccharides etc.,) produced antibodies.

Antibodies are also termed as immunoglobulins (Ig’s) due to their globular protein nature and are classified into five major classes i.e., IgA, IgD, IgE, IgG and IgM. Of different types, IgG makes up for the 80% of the serum Ig’s content and thus most immunoassays are developed using IgG type antibodies.2 Studies by Rodney Porter and Gerald Edelman gave the chemical structure of the antibodies.3 As shown in figure 1, the antibody molecule consists of two identical heavy chains and two identical light chains stabilized and linked by inter and intrachain disulfide bonds.

Antibody (Ab) production is conveniently carried out in warm blooded animals, e.g. rabbits, sheep, mice or chickens.4 5 Polyclonal antibodies (pAbs) are
obtained from the serum and comprise a mixture of different Ab populations. Monoclonal antibodies (mAbs) consist of a single monospecific Ab population.\textsuperscript{6-11}

Figure 4.1: Structure of antibody.

4.1.2 Antigens

A substance that after injection into the body of a vertebrate induces a specific Ab production is called an antigen (Ag). Antigens are principally macromolecules, for instance proteins, polysaccharides or nucleic acids. Synthetic polymers can also be used as, or act as antigens. Immunogenicity is the ability to induce an immune response. Although a substance that induces a specific immune response is usually called an antigen, it is more appropriately called an immunogen. All molecules that have the property of immunogenicity also have the property of antigenicity, although the reverse is not true. Some small molecules, called haptens, are antigenic but incapable, by themselves, of inducing a specific immune response.\textsuperscript{12} In other words, they lack immunogenicity.

4.1.3 Antibody purification methods

Polyclonal antisera raised against haptens, consist of antibodies with a varying degree of specificity and affinity. Total polyclonal IgG’s have been used for development of immunoassays\textsuperscript{13,14} but affinity purification is shown to be necessary for immunodiagnostic applications, which provides antibodies of high specificity and sensitivity for the target molecule.\textsuperscript{15-17} In the formation of antibody-antigen complex,
multiple forces, such as hydrogen bonds, hydrophobic, van der Waals and coulombic interactions contribute to this interaction. Several chemicals/ reagents are effective in disrupting the above interactive forces.

Based on their physicochemical properties these disruptive reagents can be categorized broadly as the following elution conditions: extreme pH, salts, denaturants and organic solvents. Conditions of extreme pH (as low as pH 2 or as high as pH 11) weaken all forces except hydrophobic interactions. Salts such as potassium iodide etc. effect elution by changing the structure of water in and around the site of the affinity interaction. These salts primarily effect hydrophobic interactions between ligand and target molecule. Ionic solutions (such as magnesium chloride, lithium chloride etc.) disrupt the charge-charge interactions. Denaturants like urea, guanidine chloride, sodium dodecyl sulfate etc., unfold the protein structures and thus alter the stability of interaction.

On the other hand, organic solvents such as ethylene glycol, methanol, etc., are polarity-reducing agents, which are generally effective in breaking the immunocomplex formed with low molecular-weight organic molecules. However, some of these eluting reagents could cause dramatic changes in elution profiles when used to break an immuno-complex. It is, therefore, essential to select an optimal elution condition for the affinity purification of different biomolecules.

4.1.4 Antibody characterization

Overall affinity and specificity of the antibodies are the primary determinant of the performance of the assay for a given target analyte. Antibodies need to be characterized thoroughly before using them for development of immunoassays/ sensors. Functional affinity of polyclonal antibodies and the interaction forces between antigen and antibodies are prime determinant of the quality of the antibodies.

Various methods are used for determination of quality of antibodies which range from determination of functional affinity by equilibrium based ELISA method, optical methods for real-time antigen-antibody interaction analysis such as Surface Plasmon Resonance (SPR) sensors, to direct measurements of actual force of interaction between antigen and antibody using atomic force microscopy. These tools
used for determination of antibody properties have given a great leverage in fast and reliable characterization of available antibodies for development of sensitive and selective immunoassays and immunosensors.

4.1.5 Diabetes diagnosis

As medical diagnostics is increasingly relying on molecular markers and highly specific therapies targeted at disease specific receptors, novel methods are emerging for the detection and quantification of low abundant bio-molecules. Diagnosis requires selectivity and quantification. “Selectivity” refers to how well an assay can detect particular molecules in a complex mixture without interference from other molecules. Biomarker validation and measurement on the other hand depends on accurate and reproducible protein quantitation. The emergence of novel nanotechnologies not only increased sensitivity but also enabled biologists to achieve high specificity by enriching very low abundant proteins from complex mixtures. Recent introduction of nanomaterials with promising new properties have the potential to revolutionise the diagnostics industry and provide solutions to problems “traditional” proteomics has been unable to solve. The recognition of a particular biomarker depends highly on one’s ability to detect and/or observe it. Nanomaterials now enable to monitor concentration changes that were unattainable in the past. Moreover, with these new tools one can go beyond observing and improving detection for known biomarkers to discovering novel ones.

Over the past several decades, diabetes mellitus has become a major health problem worldwide, reaching epidemic proportions in many developing countries as well as in minority groups in the developed world.25,26 Worldwide projections suggest that more than 220 million people will have diabetes by the year 2011.27 By 2025, India might become the diabetic capital of the world. Making the situation more complex, most of persons suffering from diabetes will also be affected with kidney failure, if they do not take corrective measures. Diabetes mellitus is associated with increased cardiovascular and overall mortality. In fact, diabetic patients diagnosed before 70 years of age, have only 70% of the life expectancy of non-diabetic people.28,29
Diabetes is accompanied by long-term micro- and macrovascular complications, the primary causes of morbidity and mortality in these patients. Diabetic nephropathy, as the single most common cause of end-stage renal disease, accounts for more than one-third of all cases. The core of the issue is glucose (glycemic) control. Amongst the various markers of glycemic control, glycated hemoglobin (GHb) has now been established as the most reliable, though many other proteins are also glycated in the diabetic and non-diabetic states.

The mainstay of monitoring diabetes control is measurement of the blood glucose concentration and the extent to which certain circulating proteins have become nonenzymatically glycated. The major proteins for which it is has found useful to measure the amount of nonenzymatic glycation are hemoglobin and albumin. Glucose monitoring in blood samples, in general, is a well accepted and widespread method for diabetic condition monitoring. However, it is not a fool-proof method since the level of glucose depends on the metabolism of the body. This level keeps on changing with various activities such as eating, walking etc. Recently, there has been an inclination towards monitoring albumin, glycated haemoglobin and insulin instead of glucose. This is so because these markers do not change with the metabolic activities. Haemoglobin concentrations remain relatively constant in most individuals. Infact, the HbA1c (Glycated haemoglobin) has become the gold standard for the therapeutic management of diabetes mellitus in research and in the clinical setting.

The failure to make insulin or insufficiency of insulin is termed as diabetes mellitus. Insulin is a natural hormone which controls the level of the sugar glucose in the blood. Insulin allows cells to use glucose for energy. Cells cannot utilize glucose without insulin. Excess glucose builds up in the bloodstream, increasing the risk of diabetes. Glucose is the body's primary source of fuel. Insulin enables the body cells to take glucose from the bloodstream. The cells might use glucose for production of energy if required, or it is sent to the liver to preserve it, in the form of glycogen.

Glycated albumin is formed in the body from a reaction between glucose and albumin which is the principal protein in serum. The primary factor which influences the amount of albumin that becomes glycated is the concentration of glucose in the blood. Thus, when blood sugar is elevated in diabetic people, whose diabetes is not
well controlled, high levels of glycated albumin are present. Because the reaction is slow and continuous, the absolute amount of glycated albumin in a person's blood reflects the average blood glucose concentration to which albumin has been exposed during its life in the circulation. This period is about 2 weeks. A single glycated albumin determination, therefore, provides a window for monitoring overall diabetic control during the preceding 10-20 days.

Glycated hemoglobin (GHb), also commonly referred to as glycated haemoglobin, glycohaemoglobin, HbA1c, HbA1, or A1C, is a term used to describe a series of stable minor haemoglobin components formed slowly and nonenzymatically from haemoglobin and glucose. The rate of formation of GHb is directly proportional to the ambient glucose concentration. Because erythrocytes are freely permeable to glucose, the level of GHb in a blood sample provides a glycaemia history of the previous 120 days, the average erythrocyte lifespan. GHb testing first became available to the routine clinical laboratory in the late 1970s. Since then, use of the test for both research and patient care has increased steadily. Routine use of GHb testing in patients with diabetes is recommended, first to document the degree of glycemic control at initial assessment, then as part of continuing care.\(^ {30} \) GHb is used both as an index of mean glycaemia and as a measure of risk for the development of diabetes complications. The test is also being used increasingly by quality assurance programs to assess the quality of diabetes care.\(^ {31,32} \)

In normal human erythrocytes, HbA comprises about 90% of the total haemoglobin. As early as 1955, investigators noted that adult human haemoglobin was heterogeneous.\(^ {33,34} \) In 1958, Allen et al\(^ {35} \) reported that with cation-exchange chromatography, human haemoglobin could be separated into at least three minor components that had more negative charges than HbA. These minor components were named HbA1a, HbA1b, and HbA1c, in order of their elution from the column. The significance of this finding in relation to diabetes was not appreciated until Rahbar et al.\(^ {36,37} \) using gel electrophoresis, reported an elevation of these minor haemoglobin fractions in patients with diabetes. By the mid-1970s, it became clear that HbA1c resulted from a modification of HbA by glucose and that there was a relationship between HbA1c and fasting plasma glucose and mean glucose levels over the preceding weeks.\(^ {38-43} \) By the early 1980s, GHb testing became widely available.
4.1.5.1 Chemistry and terminology

The ability of reducing sugars to react with the amino groups of proteins is now widely recognized, as is the natural occurrence of many non-enzymatically glycated proteins. The initial step in the reaction is the condensation of a free primary amine on haemoglobin with the carbonyl of the glucose, resulting in the formation of a Schiff base (scheme 1). This Schiff base is not stable and may either dissociate or undergo an Amadori rearrangement to form a stable ketoamine. There is now considerable evidence for an Amadori-type rearrangement for the adduct of glucose with the NH$_2$-terminal valine (amino acid) of the β chain (HbA1c) as well as the NH$_2$-terminal valine of the α chain and for amino groups of certain lysine residues on α and β chains. The rate of formation of GHb is directly proportional to the ambient glucose concentration. Because haemoglobin circulates in each erythrocyte for about 120 days, there is some opportunity in this cell for Maillard reactions and the extent of these changes appears to correlate with GHb values.

Scheme 1: The Amadori rearrangement as the initial step of Maillard reaction

GHb is a general term used to describe haemoglobin that has been modified by addition of glucose through a nonenzymatic process. HbA1c is one of several GHbs that reflect glycemic status; it is the form of GHb that has been studied most extensively, probably because it was the first haemoglobin species observed to be increased in individuals with diabetes. Because circulating erythrocytes are incapable
of initiating protein synthesis after the reticulocyte stage (3 days in the circulation), HbA1c and the other forms of GHb are produced only as posttranslational modifications of haemoglobin. The post synthetic modifications of haemoglobin to form GHbs are essentially irreversible, and rates of synthesis reflect the minute-to-minute glucose environment in which the erythrocyte circulates. The term HbA1 is used to describe all the fast-eluting haemoglobins as quantified with cation-exchange chromatographic methods (or fast-migrating ones, as quantified with electrophoretic methods) and include HbA1a, HbA1b, and HbA1c. Glycated haemoglobin reflects blood glucose concentrations averaged over the previous 6-8 weeks. If patients are assessed more frequently than this, there is a possibility that assays more sensitive to rapid blood glucose fluctuations may provide significant additional information.

4.1.6 Nanoparticle based Immunoassays

4.1.6.1 Principles of Immunoassays

Immunoassays are biochemical tests that utilize the reaction of monoclonal or polyclonal antibodies to their antigens to test for particular disorders. They have been used for everything from the diagnosis of the flu to heart attacks to HIV. Traditional approaches for immunoassays are the enzyme linked immunosorbent assay (ELISA), the ELISA sandwich, latex agglutination (irreversible flocculation), and sol particle agglutination. ELISA and ELISA sandwich assays are enzyme-linked assays that utilize fixed and immobilized primary and secondary antibodies and an enzyme-substrate reaction. These immunoassays often require extensive preparation, rinses, time, and sophisticated instrumentation. Latex agglutination tests have been used since 1956 to detect a wide range of analytes and have been applied to the detection of over 100 infectious diseases. In a latex agglutination, an antibody coats and sensitizes latex particles. When a sample with specific antigen is mixed with sensitized latex, visible agglutination occurs. However, the colloidal stability of the latex particles often depends on environmental factors such as pH and stabilizing surfactants. The sol particle agglutination immunoassay is a test that capitalizes on the agglutination and absorbance shift of antibody-coated gold sol granules. These assays, however, only use samples of dilute, buffered serums since whole blood turbidity and components often interfere with detection. The main drawbacks to these existing
solutions are the required preparation of samples, sophisticated instrumentation and technical proficiency. The cost and technical proficiency needed to conduct and interpret these immunoassays are also issues that reduce their marketability. Thus, there is a need for an inexpensive immunoassay that ensures particle stability, reduces the sample preparation steps and lowers the technical proficiency required without sacrificing the accuracy afforded by current immunoassays.

Most recently, a rapid optical immunoassay for whole blood was conducted.\(^5^3\) The immunoassay using gold nanoshells utilizes the underlying mechanisms for the latex agglutination and sol particle agglutination immunoassays, but provides the particle stability and optical detection upon aggregation in whole blood that these assays lack. This immunoassay minimizes nonspecific binding and maximizes rate, sensitivity, and specificity, yielding results within 10-30 minutes and requiring less technical proficiency. Colloidal stability is guaranteed as reproducibility was ensured after one week of nanoshell storage, however, longer times have not yet been tested. Two drawbacks of this method were reduced sensitivity in whole blood compared with saline due to nonspecific binding and nanoshell-antibody degradation and nanoshell storage.

Aggregation of metallic nanoparticles, induced by specific biomolecular interactions, is a practical tool for the development of simple colorimetric assays for the detection of DNA hybridization,\(^5^4-6^6\) and has potential applications in immunoassays\(^5^7\) and controlled-assembly of nanoparticles.\(^5^8\) Gold nanoparticles are frequently used in a variety of applications due to their exceptional optical and electronic properties.\(^5^9-6^2\) Their amenability for the attachment of biomolecules and ligands makes them a very attractive tool, especially for biosensing applications. Facile immobilization of biomolecules and ligands onto gold surfaces can be performed by means of thiol chemistry, which provides well-defined monolayers.\(^6^3\) Aggregation of ligand-modified gold nanoparticles induced by specific biomolecular interactions can be characterized by optical absorption spectroscopy,\(^5^7\) light scattering\(^5^8,6^4a\) small-angle X-ray scattering,\(^6^4b\) and transmission electron microscopy.\(^5^8,6^4b\) Despite the promising progress in the development of aggregation-based immunoassays and controlled assembly of nanoparticles, certain issues such as
nonspecific interactions of proteins with the nanoparticle surface and reversibility of the nanoparticle aggregation process are yet to be addressed completely.\textsuperscript{57, 58, 64}

Accurate analysis and highly sensitive detection of biomarker molecules are essential for early detection, treatment and management of diabetes. A typical heterogeneous immunoassay can usually take hours to days to complete. It involves antibody immobilization, multiple steps of incubation and washing cycles, followed by signal amplification and reading. From the initial antibody immobilization to the final assay results, the entire immunoassay can usually take hours to days to complete. A traditional immunoassay is time-consuming and labour-intensive. To circumvent these problems, the development of single-step and washing-free homogeneous immunoassays is of tremendous interest and value to the scientific community.\textsuperscript{65}

Use of nanoparticles in aggregation-based immunoassays offers several advantages; suspensions of nanoparticles do not appreciably scatter visible light, and reduces the background signal in turbidimetric assays which lowers the detection limit. In addition, nanoparticles form more stable suspensions and are, therefore, less susceptible to nonspecific aggregation.\textsuperscript{66} Gold nanoparticles have been previously used in aggregation-based immunoassays in conjunction with micrometer-sized latex particles to develop a home pregnancy test.\textsuperscript{67} In this assay, the colorless micro- and nanoparticles are derivatized using antibodies to human chorionic gonadotropin, α-hCG, a hormone released by pregnant women. When mixed with a urine sample containing this hormone, the micro- and nanoparticles are coagglutinated to form noticeably pink aggregates.

Gold nanoparticles attract great scientific and technological interest because of their physical and chemical characteristics. Because of their high electron density, colloidal gold particles are commonly used as tracers in electron microscopic studies of cellular biological samples.\textsuperscript{68-73} Gold nanoparticles are red in color because of their surface-plasmon oscillation peak at 520 nm.\textsuperscript{74} The aggregation of gold nanoparticles leads to the formation of a new absorption band at longer wavelengths as a result of electric dipole-dipole interaction and coupling between the plasmons of neighbouring particles in the formed aggregates. Nanoparticle aggregates with interparticle distances substantially greater than the average particle diameter appear red, but as the interparticle distance in these aggregates decreases to less than approximately the
average particle diameter, the color of the aggregates turns blue. Mirkin et al recently developed a colorimetric DNA hybridization assay using the spectral properties of gold nanoparticles. In their experiments, gold nanoparticles averaging 13 nm in diameter were coated with single-stranded DNA oligonucleotides. The gold nanoparticles agglutinated in the presence of target oligonucleotides with a matching sequence as indicated by a color change of the sample from red to purple. They were able to detect femto levels of matching oligonucleotides using this technique. Otsuka et al used gold nanoparticles modified with R-lactosyl-6-mercaptopoly(ethylene glycol) to induce selective aggregation of the particles in the presence of lectin. The intensity of the plasmon resonance absorption band at 520 nm was used to monitor the aggregation of the particles. This led to a long assay time of about 8 hours, since the intensity of the plasmon band decreases only when large aggregates are formed. In a similar study, Hupp et al used functionalized gold nanoparticles to sense spectroscopically silent heavy metal ions via an ion-chelation-induced aggregation process.

The flocculation parameter was measured based on the Mie’s scattering theory. The semiquantitative measurement of flocculation parameter of aggregated colloidal nanoparticles was defined as the integral of the UV absorption spectra between 600 to 800 nm. According to the Mie’s scattering theory, metallic nanoparticles with a radius much smaller than the wavelength of light absorb strongly at certain wavelength due to resonance excitation of surface plasmon which is around 520 nm for colloidal gold nanoparticles. When the colloidal particles aggregate and the distance between aggregating spheres becomes small compared to their radius, the additional resonance will occur towards longer wavelength which results in red shifting and broadening of the absorption spectra. Since the absorbance of colloidal solution increases at longer wavelength upon particle aggregation, the flocculation parameter increases with the extent of particle aggregation.

It has been revealed that the optical properties of gold nanoparticles are dominated by the surface plasma resonance (SPR), which is associated with the collective oscillation of particles’ free electrons in the conduction band and is indeed a small particle effect, nanometre in scale, since it is absent in individual atom as well
as in bulk. The SPR band(s) depend(s) strongly on the size, shape, composition, and dielectric environment. In addition to these studies on individual gold nanoparticles, optical properties of gold nanoparticle pairs,\textsuperscript{85,86} aggregates,\textsuperscript{87-91} and molecularly bridged nanoparticle arrays\textsuperscript{92-96} have also been investigated. For aggregated gold nanoparticles, the individual nanoparticle size, the arrangement of the nanoparticles in the aggregates, and the number of nanoparticles in the aggregates all affected the longitudinal resonance mode energy significantly.\textsuperscript{86} For the antibody labelled gold nanoparticle assemblies, the aggregation size controlled the optical properties of the assemblies.\textsuperscript{93a} With an increased aggregation size, there was a red shift of as well as broadening of the SPR band. The gold nanoparticle aggregates showed a substantial decrease of the SPR band at 520 nm, along with the development of a broad new band at 620 nm.\textsuperscript{90} The peak around 520 nm represents the SPR absorption for isolated GNPs, which is ascribed to the collective oscillation of particles’ free electrons in the conduction band. The intensity of this band increases as the size of the individual nanoparticles increases, accompanied by a slight red-shift. The observed features are consistent with the literature reports.\textsuperscript{97-101} Upon addition of NaCl aqueous solution, the color of the gold colloids gradually changes from red to blue, indicating the occurrence of aggregation. Electrolyte-induced aggregation of GNPs with various surface protecting groups has been observed by several groups using sodium salts (NaCl or Na\textsubscript{2}CO\textsubscript{3}).\textsuperscript{104,105,107,108} Such an aggregation is ascribed to the screening of the electrostatic repulsion between the surface-charged nanoparticles by electrolytes. As a result of aggregation, the interparticle distance becomes shorter, and electromagnetic coupling between the surface plasma modes of adjacent nanoparticles is feasible.

Depending on the size of individual nanoparticles, the relative distance of nanoparticles, the particle arrangement within the aggregates, and the size of aggregates, the interaction can be dipolar or multipolar, resulting in SPR band broadening, red-shifting, and even splitting into two distinct bands: a visible band corresponds to the transverse mode, and a near-IR band corresponds to the longitudinal mode. This phenomena has been clearly demonstrated in the literature.\textsuperscript{102-113} For gold colloids the intensity of the 520 nm band decreases, suggesting the decrease in the numbers of isolated GNPs. In contrast, a broad band appears between 600 and 800 nm and it red-shifts at increased NaCl concentration.\textsuperscript{102-105} In such a case,
the transverse and longitudinal polaritons produce nonequivalent resonances, with a broadening and red-shifting of the longitudinal resonance, while the transverse resonance remains almost unchanged. This has been clearly demonstrated by Nurmikko et al.\textsuperscript{102} for gold nanoparticle pairs. Aggregation further turns to flocculation and reduces the UV-vis absorption\textsuperscript{106,107} which manifested by the studies carried out during the course of the present investigation.

The surface plasmon absorption band, and consequently the color of a metal nanoparticle solution, is dependent on a number of parameters, viz., the size and shape of the particle, the type of metal, the dielectric properties of the medium, and the distance between particles.\textsuperscript{114,115} Gold nanoparticles, with an interparticle distance greater than the average particle diameter, appear red as a consequence of the surface plasmon absorption band centered at 520 nm. As the interparticle distance decreases to less than the diameter of the particles, coupling interactions result in a broadening and a shift to longer wavelengths of the surface plasmon absorption band that results in a solution of aggregated gold nanoparticles appearing blue.

Keeping this in mind, the current work aims at developing a sensitive immunoassay for diabetic markers glycated haemoglobin (GHb), glycated albumin (GAb) and insulin using glutamic acid reduced gold nanoprobes. Antibodies against GHb and GAb were generated in the lab and antibodies against insulin were purchased from BARC, Mumbai.

4.2 Experimental details

4.2.1 Immunization and sera collection in rabbits

Young four to six weeks old New Zealand white rabbit was immunized subcutaneously (beneath the skin) with 250 \( \mu \text{g} \) of immunizing antigen (glycated albumin and glycated haemoglobin) emulsified with Freund’s Complete Adjuvant (FCA) (\textbf{Adjuvants} (from Latin \textit{adjuvare}, to help) are substances that, when mixed with an antigen and injected with it, enhance the immunogenicity of that antigen). Further boosters were given at an interval of 21 days using 1:1 emulsion of antigens in Freund’s Incomplete Adjuvant (FIA). Boosters were given at the interval of 21 days and the blood was collected from the ear vein of the rabbit. Blood was collected from the rabbit after 4\textsuperscript{th} day at alternate days after the booster dose was given. The blood was allowed to clot for one hour at room temperature. The clot thus formed was
punctured and serum was allowed to separate overnight at 4°C. Serum was collected after centrifugation for 30 minutes at 10000 revolutions per minute (rpm) and mixed. The antibody titers (concentration where OD₄₅₀nm =1) in the pooled serum were determined using ELISA assay.

Note: A preparation known as Freund’s incomplete adjuvant contains antigen in aqueous solution, mineral oil and an emulsifying agent such as mannide monooleate, which disperses the oil into small droplets surrounding the antigen; the antigen is then released very slowly from the site of injection. This preparation is based on Freund’s complete adjuvant, the first deliberately formulated highly effective adjuvant, developed by Jules Freund many years ago and containing heat-killed Mycobacteria as an additional ingredient. Muramyl dipeptide, a component of the mycobacterial cell wall, activates macrophages, making Freund’s complete adjuvant far more potent than the incomplete form. Activated macrophages are more phagocytic (capable of ingesting foreign particles) than unactivated macrophages (macrophages are white blood cells within tissues).

4.2.2 Purification of antibodies using affinity chromatography

4.2.2.1 Purification of whole IgG using protein-A

The purification of IgG was done in two stages. The first stage included precipitation of IgG fraction and removal of unwanted serum proteins by saturated ammonium sulphate precipitation. In the second step, protein-A affinity purification was carried out to purify total IgG content from the serum.

**Saturated ammonium sulfate (SAS) precipitation**

The IgG fraction of serum proteins from the antisera was precipitated using saturated ammonium sulfate (761 g/L, pH adjusted to 7.0 by triethylamine) precipitation method. In brief, the antiserum was centrifuged at 10000 rpm for 30 minutes to remove any cellular debris. An equal volume of SAS was slowly added to the serum supernatant while stirring, to achieve a final concentration of 50% ammonium sulfate and kept the mixture at 4°C overnight with constant stirring. Solution was centrifuged at 10000 rpm for 30 minutes and the precipitates were resuspended in minimum amount of PBS after removing the supernatant. The protein solution was then dialysed against PBS, overnight at 4°C with frequent changes of
dialysis buffer. The dialyzed protein solution was centrifuged at 10000 rpm for 30 minutes and the protein concentration was determined by taking optical density (OD) at 280 nm.

**Figure 4.3:** Procedure of separation of immunoglobulin from blood of animal.

**Protein-A affinity purification**

The protein solution was passed through the Protein-A column twice, flow through was collected and the column was washed till the OD$_{280}$ of the washing buffer (PBS, pH=7.4) fell down to a minimum of 0.03 or lower. The elution was done using minimum amount of elution buffer i.e., glycine-HCl (50 mM, pH=2.5). The buffer coming out of the column was monitored for pH change and the fractions were collected as soon as the pH started dropping. One mL fractions were collected, till the absorption at 280 nm fell down to base line. The fractions collected were neutralized immediately with 50 μL of 0.1 M tris buffer and kept at 4°C. The fractions with maximum protein were pooled and dialyzed against PBS containing 0.01% sodium azide, overnight at 4°C with frequent changes of the elution buffer. The concentration of the purified antibodies was determined using absorption at 280 nm.
4.2.3 Enzyme Linked Immunosorbent Assay (ELISA)
The following protocol was used for ELISA (scheme 2):

- Coating antigen was coated on 96 well Elisa plate (figure 4.4) (Nunc, USA) at a concentration of 5 μg/mL in carbonate buffer (50 mM, pH=9.6) by adding 100 μL per well and incubating overnight at 4°C.

- After washing twice with phosphate buffer saline (PBS), the non-specific binding sites were blocked with 10% defatted skim milk in PBS using 200 μL/well and incubating for 1 hour at 37°C.

- Plates were washed thrice with PBS containing 0.05% Tween-20 (PBST) and finally with PBS. 100 μL/well of primary antibodies (antiserum) solution prepared in PBS containing 0.01% defatted skim milk was added at various dilutions as required and incubated for 2 hrs at 37°C.

- The plates were thoroughly washed thrice with PBST and finally with PBS. 100 μL/well of Horse Radish Peroxidase (HRP) conjugated goat anti rabbit-IgG was used as secondary antibody at a dilution of 1:10000.

- Plates were incubated for one hour at 37°C followed by a thorough washing (as in earlier step) to remove any nonspecifically bound secondary antibody.

- Color was developed using 3,3′,5,5′-tetramethylbenzidine (TMB)/H₂O₂ as substrate (100 μL/well).

- The development of blue color was stopped after 15 minutes using 50 μL/well 1N H₂SO₄ solution. The blue color is converted to bright yellow color which was monitored at 450 nm using a micro plate reader (Biotech, USA).
**Scheme 2**: General ELISA protocol.

**Anti glycated albumin antibody (GAb):**

To generate antibodies against GAb, 250 μg of human glycated albumin (Sigma, USA) were used as the antigen. Immunization, sera collection, antibody purification and characterization was carried out as mentioned earlier. For ELISA, glycated albumin was used as the coating antigen. Three boosters were given to the rabbit and the serum showed good reactivity and specificity towards the antigen as evident from figure 4.5.

The amount of the antibody increased after every booster. A significantly high reactivity of the antibody was found against the specific antigen while cross reactivity with other antigens were relatively low. The slight cross reactivity with Human Serum Albumin (HSA) was due to the fact that the antigen used was of human origin.
**Figure 4.5**: Dilution curve for sera collected from rabbits immunized with Glycated Albumin (a) 1st booster (b) 2nd booster (c) 3rd booster (d) Dilution curve of antisera for glycated albumin with various boosters. BSA, OVA, HSA and GAb stand for bovine serum albumin, ovalbumin, human serum albumin and glycated albumin respectively.

**Figure 4.6**: Binding of anti glycated albumin antibody with glycated albumin (GAb), human serum albumin (HSA), bovine serum albumin (BSA) and ovalbumin (OVA).
4.2.4 Antibody generation against Glycated Haemoglobin, HbA1c (GHb)

To generate antibodies against GHb, 250 µg of glycated (Sigma, USA) was used as the antigen. Immunization, sera collection, antibody purification and characterization were carried out as mentioned earlier. For ELISA, specific antigen, glycated haemoglobin was used as the coating antigen alongwith non specific antigens (BSA, OVA and HSA). Three boosters were given to the rabbit and the serum showed good reactivity and specificity towards the antigen as evident from figure 4.7.

![Figure 4.7: Dilution curve for sera collected from rabbits immunized with glycated haemoglobin (a) 1st booster (b) 2nd booster (c) 3rd booster (d) Dilution curve of antisera for glycated haemoglobin with various boosters.](image)

A high titre (taken as concentration where OD_{450nm} = 1) was obtained which increased with successive boosters. As evident from figure 4.8, the amount of antibody increased with increasing boosters. A significantly high reactivity of the antibody was found against the specific antigen while cross reactivity with other
antigens were relatively low. The observed reactivity with human serum albumin (HSA) was due to the fact that the glycated albumin used was of human origin. The antibody was obtained in high amounts as illustrated by figure 4.8.

Figure 4.8: Binding of anti glycated haemoglobin antibody with with bovine serum albumin (BSA), ovalbumin (OVA), human serum albumin (HSA), glycated haemoglobin (GHb). A significantly high reactivity of the antibody was found against the specific antigen while cross reactivity with other antigens were relatively low.

4.3 Assay Development and Validation

It was aimed to develop a simple, easy-to-use and inexpensive assay for specific biomarker detection based on gold nanoprobes. The assay is based on a non-cross-linking hybridisation method, where aggregation of the gold-nanoprobes is induced by an increasing salt concentration – the presence of antigen prevents aggregation and the solution remains red; absence of antigen does not prevent gold-nanoprobe aggregation resulting in a change of colour from red to blue. This method has been successfully applied to detect eukaryotic gene expression without retrotranscription or PCR amplification\textsuperscript{116} and is a fast and straightforward assay for Mycobacterium tuberculosis DNA detection in clinical samples\textsuperscript{117}.

The method is based on the colour change of a gold nanoprobe solution, upon increasing salt concentration, in presence or absence of antigen (figure 4.9). As mentioned earlier, following an increase in salt concentration, negative samples show extensive gold-nanoprobe aggregation, noticeable by the blue colour of the respective solutions. Conversely, the positive sample containing the antigen does not show that
Mechanism of electrolyte mediated aggregation of nanoprobes in presence or absence of antigen. The presence of antigen prevents aggregation of functionalised GNP and hence the solution remains red while absence of antigen leads to aggregation resulting in a visible change of colour from red to purple blue.

Effect and the solution retains the initial red color. Binding of antigen to the gold-nanoprobe stabilises the gold-nanoprobe in solution, thus avoiding aggregation. Colour change upon aggregation of gold-nanoprobes is corroborated by visible spectra, wherein an intense plasmon resonance band appears at 600–650 nm, with a concomitant decrease of the intensity of the original plasmon resonance at 520 nm. The intensity of the plasmon resonance at 620 nm is a convenient way to measure gold-nanoprobe aggregation. After binding and upon salt addition, a slight colour change from red to blue was observed.

4.3.1 Experimental

4.3.1.1 Synthesis of GNP-Ab bioprobe

For the synthesis of GNP-Ab bioprobe, flocculation assay was first carried out to confirm the minimum amount of antibody required to stabilize the GNP. This was done for all the three antibodies viz. GHb, GAb and insulin. The homogeneous immunoassay was then conducted in solution using the antibody-conjugated nanoparticles. For preparing Ab-GNP conjugates, antibodies prepared in phosphate
buffer were added separately into 1 mL colloidal gold solution under mild stirring conditions. The pH of the colloidal gold solution was maintained at 7.4 by addition of dilute 0.1 M K₂CO₃ before adding the antibody concentration derived from CFC measurements. The mixture was incubated overnight at 4°C and centrifuged at 12,000 rpm for 30 minutes. The pellet obtained was further washed twice with 10 mM tris buffer (pH 8.0) under centrifugation at 12,000 rpm for 30 minutes to remove traces of unconjugated antibody. The amino acid capped GNPs (20, 40 and 70 nm) were used to bind to the antibody to make stable gold-antibody conjugate. The adsorption is established through electrostatic interaction between the surface-terminated anionic groups (–COO⁻) on the GNPs and the positively charged amine groups (–NH₃⁺) on the lysine residues of the antibody and ionic/hydrogen bonding between –NH₃⁺ and COO⁻ functional groups.

### 4.3.1.2 Nanoprobe based immunoassay

The homogeneous immunoassay was conducted in solution using the antibody-conjugated nanoparticles (50 μL) into each well of micro titre strip. Serial dilutions of the antigen were prepared in carbonate buffer and 50 μL of sample was added to each well, followed by the addition of 50 μL of NaCl (5 M) after 5 min. The absorbance at 520 nm and 620 nm were measured using an ELISA plate reader. All experiments were performed at room temperature.

In this colorimetric detection method, GNPs aggregate when interparticle distance becomes substantially smaller than the average particle diameter. As a result, the color of the aggregates turns purple blue. Using this simple detection approach, the present studies demonstrate a non-cross-linking hybridisation method, where aggregation of the nanoprobes is induced by an increasing salt concentration.

Addition of antigen to the GNP-Ab conjugate prevented aggregation and the solution remained red while its absence led to aggregation on salt addition. Figure 4.10 shows the change in the UV-Vis. extinction spectra of 40 nm GNP bioprobes after the addition of NaCl. GNP-immunocomplexes with non specific antibody, (c2) or antigen (ovalbumin, c3) showed significant aggregation. However, GNP-Ab conjugates with specific target molecule (HbA1c) were able to resist aggregation as evident from clear surface plasmon resonance (SPR) bands.
The change in the absorbance ratio profile of the different sized GNPs labeled with anti-HbA1c antibody with increasing amounts of antigen followed by the addition of 5 M NaCl is shown in figure 4.11. Similar observations were also seen for anti glycated albumin antibody as well as anti insulin antibody (figure 4.12 and 4.13 respectively).

The aggregation ratio (620/520) of antibody functionalised nanoprobes gradually decreased with increase in concentration of antigen. The GNP aggregates showed a substantial decrease of the SPR band at 520 nm, along with the development of a broad new band at 620 nm. It was attributed that 40 nm GNPs functionalised with specific antibody showed better linearity with high sensitivity as compared to the others sizes of GNPs for all the three antigens. On the other hand, 70 nm GNPs could not show consistent results, possibly because of poly-dispersed nature and self-aggregation of these particles. This may be due to the reason that in case of 70 nm particles, the interparticle distance is less than the particle diameter which increases the possibility of aggregation.

The changes in size and surface charge of the gold-nanoprobes induced by adding salt and increasing concentrations of antigens were further characterized by using dynamic and electrophoretic light scattering techniques. The zeta potential measurements of complexed nanoprobes were carried out to confirm the aggregation kinetics with increase in antigen concentrations. Zeta (ζ) potential is the electrostatic potential that exists at the shear plane of a particle, which is related both to the surface charge and the local environment of the particle.
**Assay Development**

**Figure 4.12:** Change in the absorption ratio (620/520 nm) with increasing amounts of antigen (GA) added to the different sized GNP-Ab complexes. (a) 20 nm GNPs (b) 40 nm GNPs (c) 70 nm GNPs. There was a decrease in the aggregation ratio with increase in the concentration of GA.

**Figure 4.11:** Change in the absorption ratio (620/520 nm) with increasing amounts of antigen (HbA1c) added to the different sized GNP-Ab complexes. (a) 20 nm GNPs (b) 40 nm GNPs (c) 70 nm GNPs. There was a decrease in the aggregation ratio with increase in the concentration of HbA1c.
Assay Development

Zeta potential measurements provide an important criterion for the stability of a colloid system.\textsuperscript{120} The concept of zeta potential has been used to study cell biological activation, cell agglutination and cell adhesion which are related to cell surface charge properties.\textsuperscript{121} In particular, Altankov et al\textsuperscript{122} stated that the zeta potential might be a critical parameter for cellular interaction. Although zeta potential measurements have been utilized in probing the interaction between cells and biomolecules, it still remains an untapped method for studying the interaction between nanoparticles and immunocomplexes. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system.

In this study, it was observed that the decrease in the zeta potential of the aggregates perfectly correlated with the increase in the concentration of the HbA1c (figure 4.14). This observation is also supported by the dynamic light scattering (DLS) data (figure 4.14). The observed decrease in the magnitude of the zeta potential can be attributed to the screening of the negative charges, which otherwise contribute

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**Figure 4.13:** Change in the absorption ratio (620/520 nm) with increasing amounts of antigen (insulin) added to the different sized GNP-Ab complexes. (a) 20 nm GNPs (b) 40 nm GNPs (c) 70 nm GNPs. There was a decrease in the aggregation ratio with increase in the concentration of insulin.
Assay Development

Figure 4.14: Zeta potential (ζ) profile (a, c, e) and size distribution profile (b, d, f) of the GNPs aggregates with increasing concentrations of the antigen for different sizes of GNPs. It is attributed that in the electrolyte-induced aggregation of GNPs, the interparticle distance becomes shorter and consequently electromagnetic coupling between the surface plasma modes of adjacent nanoparticles is feasible.\textsuperscript{123}
Depending on the size of individual nanoparticles or the size of aggregates, the interaction can be dipolar or multipolar, resulting in surface plasmon resonance band broadening, red-shifting, and even splitting into two distinct bands (a visible band corresponding to the transverse mode and a near-IR band corresponding to the longitudinal mode). For gold colloids, the intensity of the 520 nm band decreases, suggesting the decrease of the numbers of isolated GNPs. In contrast, a broad band appears between 600 and 800 nm. The appearance of this near-IR band normally indicates that the geometry of nanoparticles in the aggregates changes, causing a “shunting” of dipole-dipole interaction and an initiation of quadrupole interactions due to conductive contact.

This assay proved to be a promising one-step homogeneous immunoassay using gold nanoprobes. The work describes an easy and inexpensive method of detection of diabetic markers in samples. Results can be assessed without the need of further signal enhancement or complicated technological solutions, which are common with other nanoparticle based methodologies. The method presented here proved to be selective, sensitive and inexpensive, as the overall cost for analysis is very low. The assay does not involve any washing cycle. Moreover, extremely small amounts of samples are needed for the assay (in this study, about 50 µL sample solution was used for each assay). The proposed colorimetric method is very easy to perform, taking less than 15 minutes, rendering it suitable for use at point-of-care diagnosis.
REFERENCES


