CHAPTER 2 LITERATURE REVIEW

2.1 *Salmonella*

Salmonella has been one of the most frequently occurring food borne pathogens affecting the microbial safety of foods [8]. *Salmonella* is a genus of bacteria with characteristic rod shaped, gram negative, non-spore forming and chenomoorganothrops. This group contains the etiologic agents of food borne salmonellosis as well as the agents that cause typhoid and paratyphoid fever. Eggs, poultry, meat, meat products, and chocolate are the most common sources of food borne salmonellosis [25].

Salmonella species cause a variety of diseases, from localized gastroenteritis to systemic illnesses. *Salmonella typhimurium* (Figure 2.1) exclusively infects humans and higher primates and causes typhoid fever, a severe systemic infection, whereas mice infected with this species get a systemic infection with pathogenesis resembling that of typhoid fever in humans [26]. The symptoms of *Salmonella* infection usually appear 12 – 72 hours after infection, and include fever, abdominal pain, diarrhoea, nausea and sometimes vomiting. The illness usually lasts 4 – 7 days.

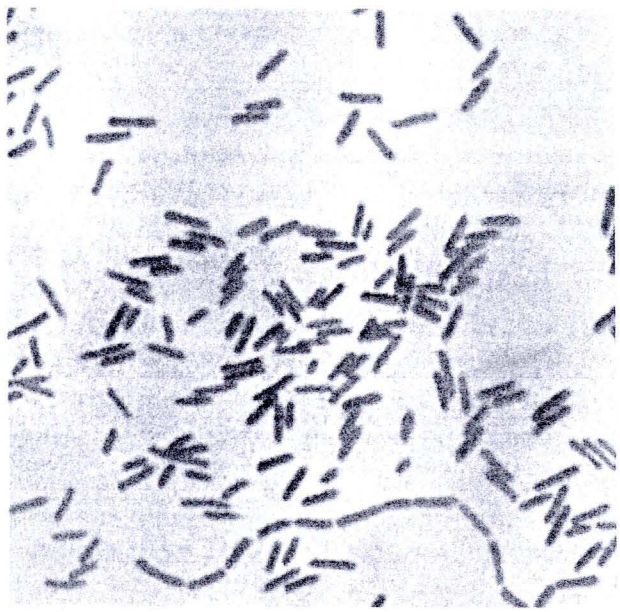

Figure 2.1 *Salmonella typhimurium* [26]

The detection of *Salmonella* in food and foodstuff is often difficult. *Salmonella* may be sub lethally injured and may be present in low numbers compared with similar, closely related organisms. Thus, they are difficult to culture insufficient numbers for easy identification. Furthermore, conventional culture and serologic identification techniques are lengthy and require costly handling and storage of food during testing [25].

The conventional methods for *Salmonella* detection involving cultural steps are labor intensive, costly and time consuming, taking 4 to 5 days for detection and confirmation. Therefore, there is an urgent need for rapid methods which would allow the industry to respond quickly to raw material and product contamination [27]. Alternative methods have been developed including enzyme linked immunoassay, immunomagnetic monoclonal antibody-based assay and DNA hybridization. Some assays still require a prolonged enrichment period whilst others suffer from low sensitivity which has limited their acceptance [28]. Among the methods used to detect pathogenic bacteria, enzyme linked immunosorbent assay (ELISA) is one of the most widely used techniques in routine sample analysis. For *Salmonella typhimurium* detection, a typical ELISA yields a sensitivity of (10 cells per 25 g) were detected in 19 h [27].

2.2 Immunoassay for *Salmonella*

During the last two decades there has been a phenomenal increase in the number and variety of immunoassay tests performed. One of the reasons for this has been the development and perfection of methods which use labeled antigens or antibodies, resulting in tests with very high levels of sensitivity and specificity [29].

Immunoassay are bioanalytical methods in which the quantitation of the analyte depends on the reaction of an antigen (analyte) and an antibody [30]. Based on the materials of protein labeling, it can be classified as radioimmunoassay, fluorescent dyes immunoassay, enzyme immunoassay, chemiluminescence immunoassay. Among these techniques, the most widely used format of immunoassay is enzyme-linked immunosorbent assay (ELISA) [31]. The advantages of immunoassay technology relative to other analytical techniques are low detection limits, high analyte selectivity, high throughput of samples, reduced sample preparation, versatility for target analytes, and cost effectiveness for large numbers of samples adaptability to field use [32].

Principally, immunoassay methods are based on a binding reaction between a fixed amount of labeled form of an analyte for a limited amount of binding sites on a highly specific anti-analyte antibody. When these immunoanalytical reagents are mixed and incubated, the analyte is bound to the antibody forming an immune complex. This complex is separated from the unbound reagent fraction by physical separation technique. Analysis is achieved by measuring the label activity (e.g. radiation, fluorescence, or enzyme) in either of the bound or free fraction. A standard curve, which represents the measured signal as a function of the concentration of the unlabelled analyte in the sample is constructed. Unknown analyte concentration is determined from this calibration curve [30].

In theory, the sensitivity and selectivity of an immunoassay are determined by the affinity of the antibody to the analyte, and hence immunogen design and antibody production are of fundamental importance to assay development. For a molecule to be immunogenic it must have a molecular mass of at least 2000 Da and possess a complex and stable tertiary structure [32].

Immunoassay is commonly used in many clinical, pharmaceutical, and scientific research laboratories. This bioanalytical technique is capable of detecting the specific antigens through the binding of the corresponding antibodies. The immunoassay is used to measure the specific reacted signal from the label attaching on either the antibody or antigen quantitatively [33]. For

example, fluorescent labels have been attached to antibodies, and these conjugates are very useful for the rapid identification of organisms responsible for infectious diseases as well as for the measurement of antibody levels especially in infectious and auto-immune diseases. Isotopes were found to be suitable, especially as labels on antigens, and radioimmunoassay (RIA) has become the method of choice for sensitive assays of both large and small molecular weight substances [29].

2.2.1 Antibody

Antibodies are host proteins found in plasma and extracellular fluids that serve as the first response and comprise one of the principal effectors of the adaptive immune system. They are produced in response to molecules and organisms, which they ultimately neutralize and/or eliminate. The ability of antibodies to bind an antigen with a high degree of affinity and specificity has led to their ubiquitous use in a variety of scientific and medical disciplines. As a reagent, there is no other material that has contributed directly or indirectly to such a vast array of scientific discoveries. Their use in diagnostic assay sand as therapeutics has had a profound impact on the improvement of health and welfare in both humans and animals [34].

The antibodies can be either polyclonal or monoclonal. However, for immunoassay development for pharmaceutical analysis purposes, monoclonal antibodies are more advantageous than polyclonal ones. This is attributed to their higher degree of affinity and specificity towards the analyte. Even that, many successful immunoassays were developed using polyclonal antibodies because it was possible to generate the antibodies with high affinity to the analyte [30].

2.2.1.1 Structure of Antibody

Antibodies are glycoproteins secreted by specialized B lymphocytes known as plasma cells. Also referred to as immunoglobulin (Ig), because they contain a common structural domain found in many proteins, antibodies are composed of four polypeptides. Two identical copies of both a heavy (~55 kD) and light (~25 kD) chain are held together by disulfide and noncovalent bonds, and the resulting molecule is often represented by a schematic Y-shaped molecule of ~150 kD (Figure 2.2) [34]. A schematic representation of an IgG antibody comprising of two heavy (green) and light (blue) chains. Carbohydrate elements are attached via the asparagine 297 amino acid residues [35].

Depending on the Ig class, up to five structural molecules may be combined to form any one antibody. In mammals, there are five classes of Ig (IgG, IgM, IgA, IgD, and IgE); and in avians, there are three classes (IgY, IgM, and IgA). In select mammals, IgG and IgA are further subdivided into subclasses, referred to as isotypes, due to polymorphisms in the conserved regions of the heavy chain. Ig class determines both the type and the temporal nature of the immune response [34].

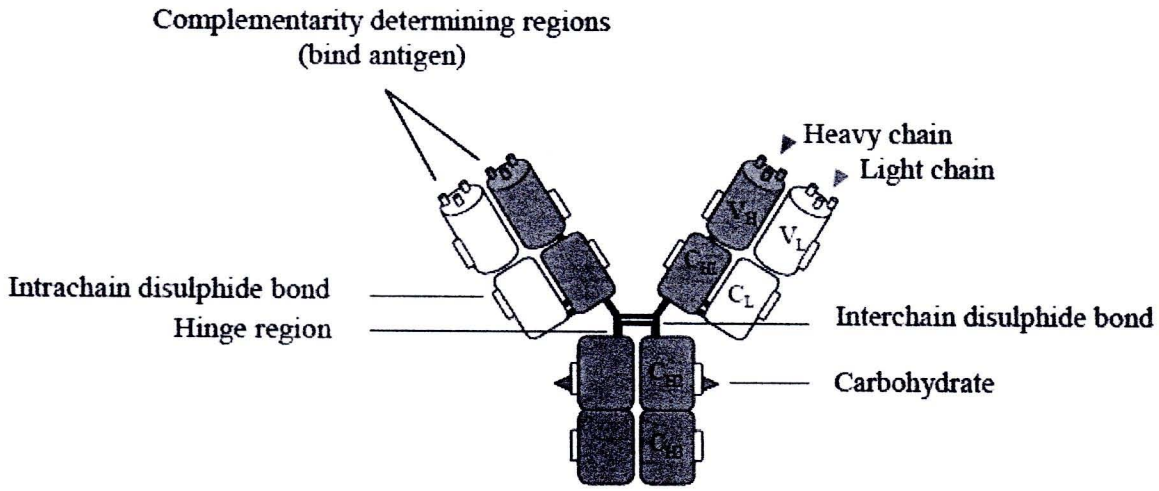

Figure 2.2 A schematic representation of antibody [35]

2.2.1.2 Production and Purification of Antibody

Polyclonal, monoclonal and recombinant antibodies have frequently been selected for a wide variety of applications, including immunodiagnostics and biomarker detection. Their production involves the exploitation of the immune system of murine, leporine, ovine or avian hosts (Figure 2.3) [35].

A. Polyclonal Antibodies

Polyclonal antibodies (pAb) are typically raised in rabbits, goats or sheep, and their popularity is illustrated by the fact that they are frequently selected in immunosensor based assays for pathogen detection. It should be stressed that the inherent nature of pAbs means that a selection of different epitopes may often be recognized on a single cell. In cases where this is undesirable, such as in the case where high specificity is a requirement, monoclonal or recombinant antibodies may be more applicable.

B. Monoclonal Antibodies

Monoclonal antibodies are generated through the use of hybridoma technology and murine hosts are commonly selected for immunization. The bone marrow, primary lymph nodes and, most commonly, the spleen are selected as a source of antibody-producing B cells which are harvested and fused to immortal myeloma cells. The resulting hybrid cells (referred to as hybridomas) subsequently secrete full-length antibodies that are directed towards a single epitope. Suitable candidates, identified by ELISA based analysis, are then 'cloned out' to ensure that a single cell, producing antibody specific for an individual epitope, is present and the antibody generated can be used for assay development.

C. Recombinant Antibodies

Recombinant antibodies, generated through the use of phage display technology and the biopanning of antibody repertoires (libraries) against a target of interest, have been selected for

the detection of a range of structurally diverse antigens, including proteins, haptens and carbohydrate moieties.

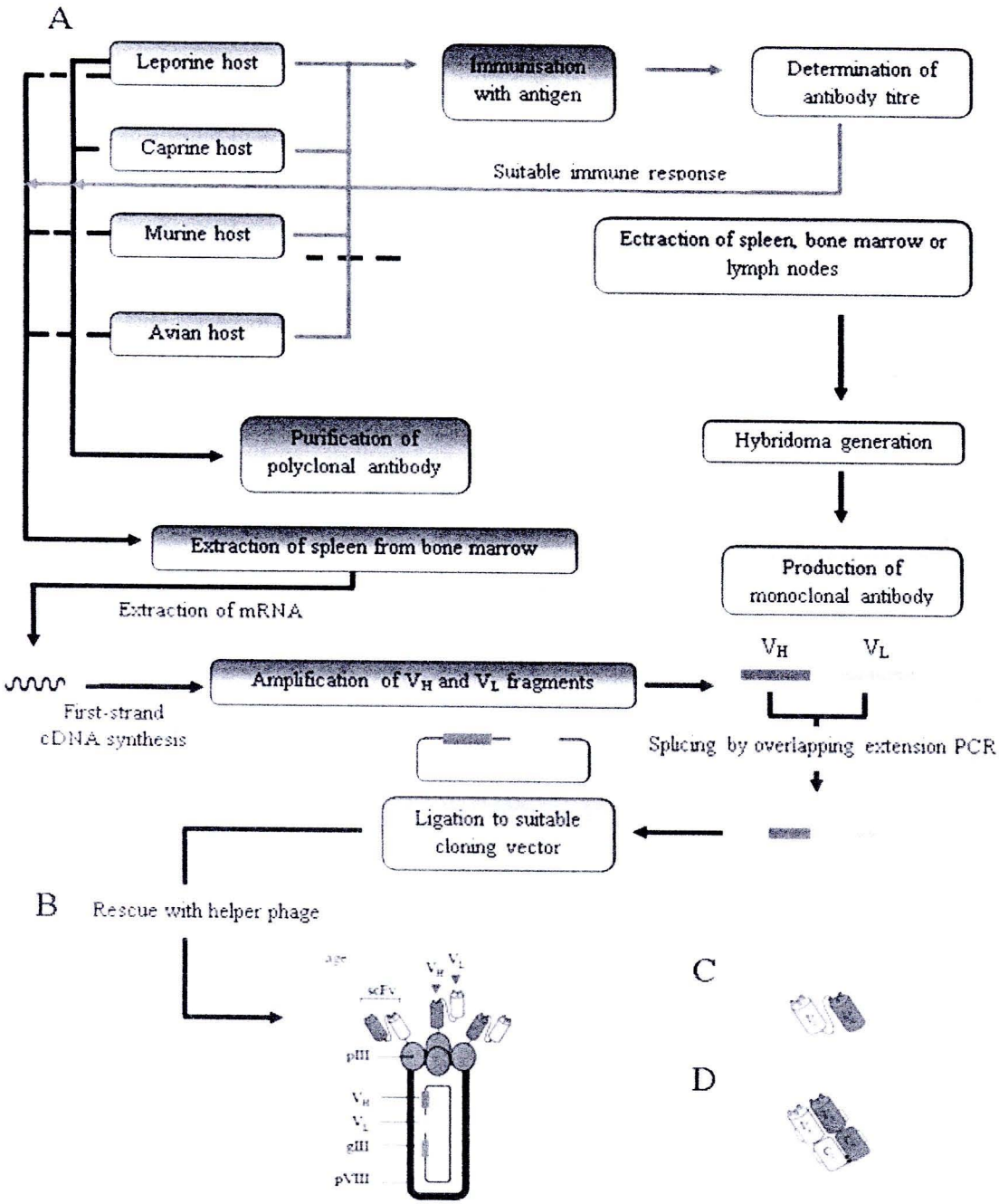

Figure 2.3 An overview of monoclonal, polyclonal and recombinant antibody production [A]. Immunizations related stages are represented by a red line, with those involving antibody productions shown in black. A filamentous phage displaying scFv antibody fragments [B] and two recombinant antibody fragments, the scFv [C] and Fab [D], are also illustrated [35].

2.2.2 Antigen

A molecule that binds to an antibody is called an antigen (Ag). An antigen is a molecule that can be recognized by the immune system (immunogenicity) and bound specifically to an antibody (reactogenicity). Molecules with both immunogenicity and reactogenicity are called as complete antigens; while molecules that only possess reactogenicity are defined as incomplete antigens or haptens. The antigen used in immunoassay is protein, hapten conjugates, lipopolysaccharide that can stimulate immune response by inducing the secretion of antibody [36].

S. typhimurium detection can be occurred due to the presence of lipopolisacharide (LPS) as an integral component in outer membrane of their cell walls. There are three general components of LPS such as the O-specific side chain (O-antigen), the core oligosaccharide and lipid A [37]. O-antigen composed of repeating units of five to eight monosaccharides (galactose, rhamnose, and mannose in *S. typhimurium*). The O-antigen has several biological activities such as, serving as receptors for bacteriophage, modulating the activation of the alternative complement pathway, and inhibiting the attachment of the membrane attack complex to the bacterial outer membrane. The core oligosaccharide supported the structure of bacteria membrane, has a function as the anchor to connect the molecules within the membrane. Both of the outer and inner core oligosaccharide carries epitopes for antibodies. Lipid A has the toxic effect of gram negative bacteria. When specific receptor bound with lipid A, it will be recognized by immune cells as a pathogen. Lipid A also able to induce non- specific resistance to bacterial and viral infection. The structure of gram negative bacterial membrane shown in Figure 2.4 [38].

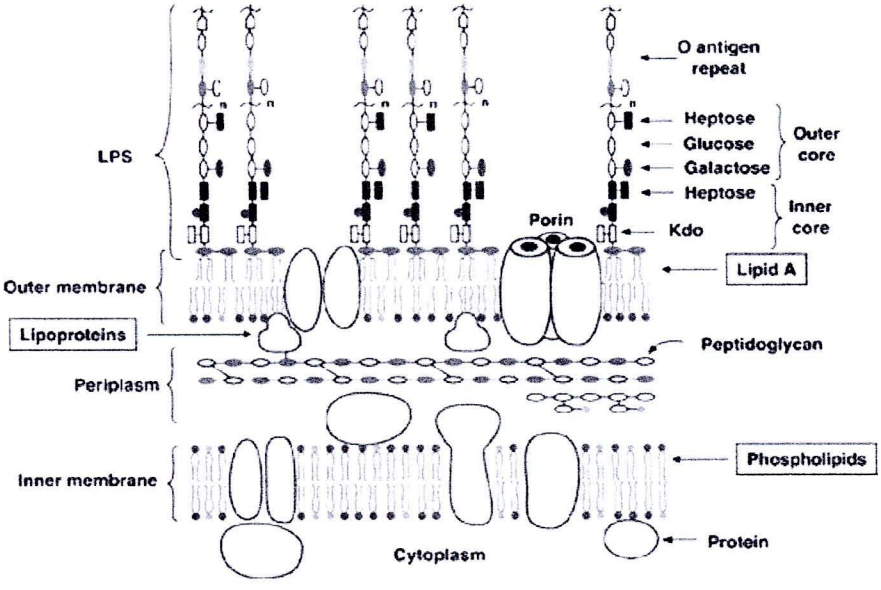

Figure 2.4 The composition of a gram-negative bacteria membrane. The inner or cytoplasmic membrane surrounds the bacterial cell. The periplasm, which contains peptidoglycan, is surrounded by the outer membrane. Lipopolysaccharide (LPS) is embedded in the outer leaflet of the outer membrane and is composed of three distinct components; lipid A, oligosaccharide core, and O-antigen [38].

2.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

Among the methods used to detect pathogenic bacteria, enzyme linked ELISA is one of the most widely used techniques in routine sample analysis. ELISA is a biological technique which is used in the detection and measurement of the antibody and antigen in the body. ELISA has been widely used for routine analysis due to its ease of use, ability to handle a large number of samples and automation [16]. ELISA has been used with enthusiasm by workers on infectious diseases, where the need is often for measurement of antibody [29].

Typical ELISA assays are comprised of a number of steps; namely blocking, washing, incubation of primary and secondary antibodies and substrate development. These can take several hours to complete and, understandably, this may be problematic in instances where rapid detection is a requisite [35].

2.2.3.1 Types of ELISA

A. Competitive ELISA

Competitive ELISA is involved in the quantification of antigens (Figure 2.5). Enzyme labeled antigen is mixed with the test sample containing antigen, which competes for a limited amount of antibody. The reacted (bound) antigen is then separated from the free material, and its enzyme activity is estimated by addition of substrate. For competitive ELISA, the higher the sample antigen concentration, the weaker the eventual signal. The major advantage of a competitive ELISA is the ability to use crude or impure samples and still selectively bind any antigen that may be present.

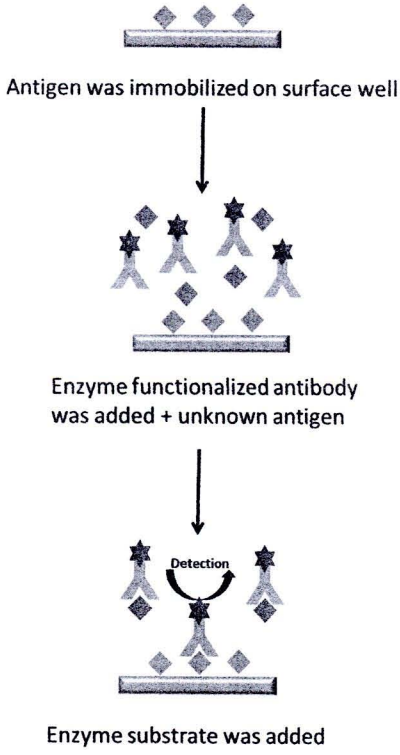

Figure 2.5 Competitive ELISA for assay of antigen [29]

B. Sandwich ELISA

In this process, the detection of antigen takes place between two plates of antibodies that is why it is called as sandwich ELISA (Figure 2.6). In this modification a solid phase is coated with specific antibody. This is then reacted with the test sample containing antigen, then enzyme labeled specific antibody is added, followed by the enzyme substrate. The antigen in the test sample is captured and immobilized on to the sensitized solid phase where it can itself then fix the enzyme labeled antibody. Sandwich ELISA is the most common type of immunoassay used for the detection of proteins. Unlike the competitive immunoassays described in Figure 2.6, the absorbance in the sandwich immunoassay is directly proportional to the concentration of the analyte in the sample solution [32].

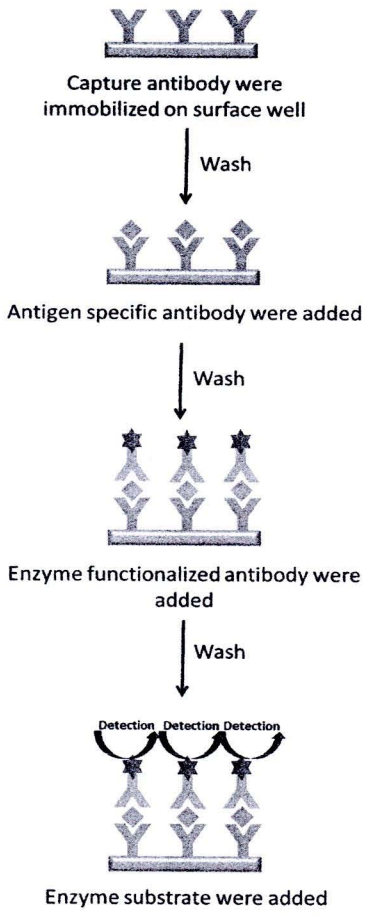

Figure 2.6 Sandwich ELISA [29]

In the Figure 2.7 shown a system where the second antibody used in the double antibody sandwich method is from a different species, and this is then reacted with an anti-immunoglobulin enzyme conjugate. The advantage of this is that it avoids the labeling of the specific antibody, which may be in short supply and of low potency. This same method can be used to assay antibody (in step 3) where only an impure antigen is available, the specific reactive antigens are selected by the antibody immobilized on the solid phase.

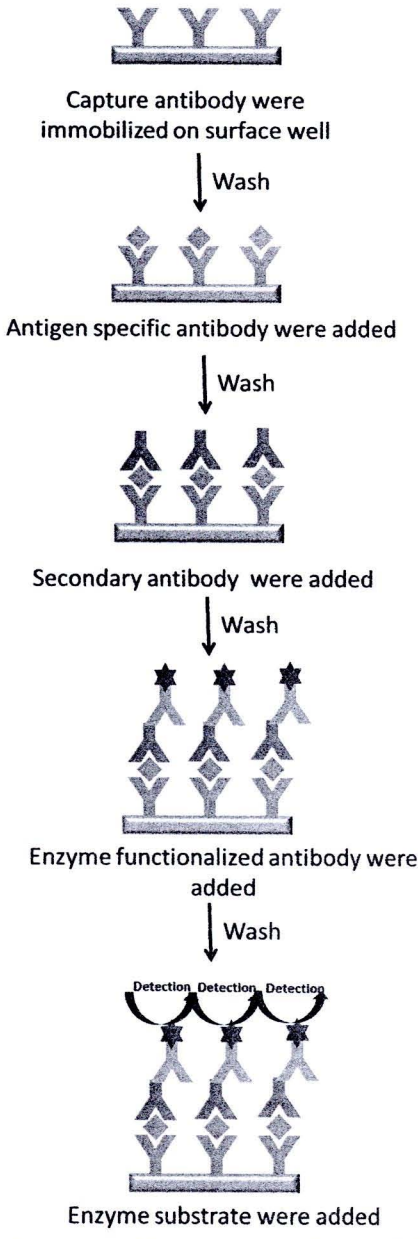

Figure 2.7 Double antibody in sandwich ELISA [29]

C. Indirect ELISA

In the indirect method the antigen is immobilized by passive adsorption on to the solid phase (Figure 2.8). Test sera are then incubated with the solid phase and any antibody in the test sera becomes attached to the antigen on the solid phase. After washing to remove unreacted serum components an antiglobulin enzyme conjugate is added and incubated. This will become attached to any antibody already fixed to the antigen. Washing again removes unreacted material and finally the enzyme substrate is added. Its color change will be a measure of the amount of the conjugate fixed, which is itself proportional to the antibody level in the test sample.

A major disadvantage of the indirect ELISA is that the method of antigen immobilization is non-specific; when serum is used as the source of test antigen, all proteins in the sample may stick to the microtiter plate well, so small concentrations of analyte in serum must compete with other serum proteins when binding to the well surface.

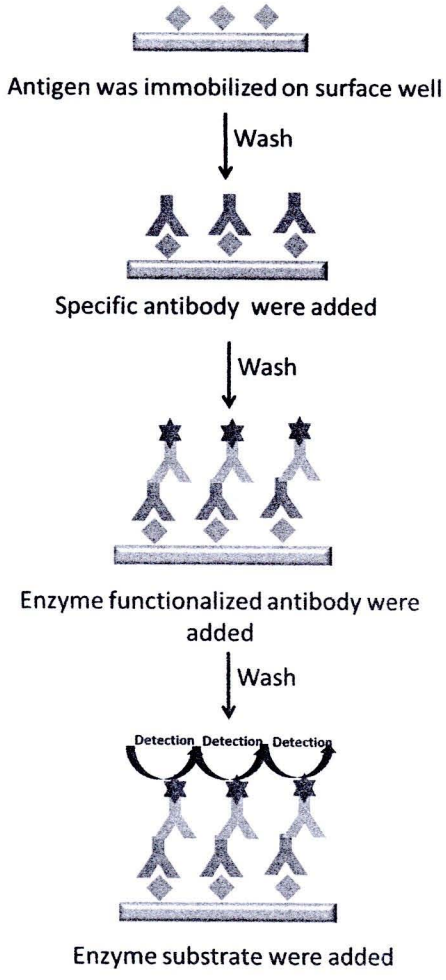

Figure 2.8 Indirect ELISA [29]

In Figure 2.9 another assay for antigen is illustrated. Plates are coated with the specific antigen and these are then incubated with a mixture of reference antibody and the test sample. If there is no antigen in the test sample the reference antibody becomes fixed to the antigen sensitized surface. If there is antigen in the test solution this combines with the reference antibody, which cannot then react with the sensitized solid phase. The amount of antibody attached is then indicated by an enzyme labeled antiglobulin conjugate and enzyme substrate. The amount of inhibition of substrate degradation in the test sample (as compared with the reference system) is proportional to the amount of antigen in the test system.

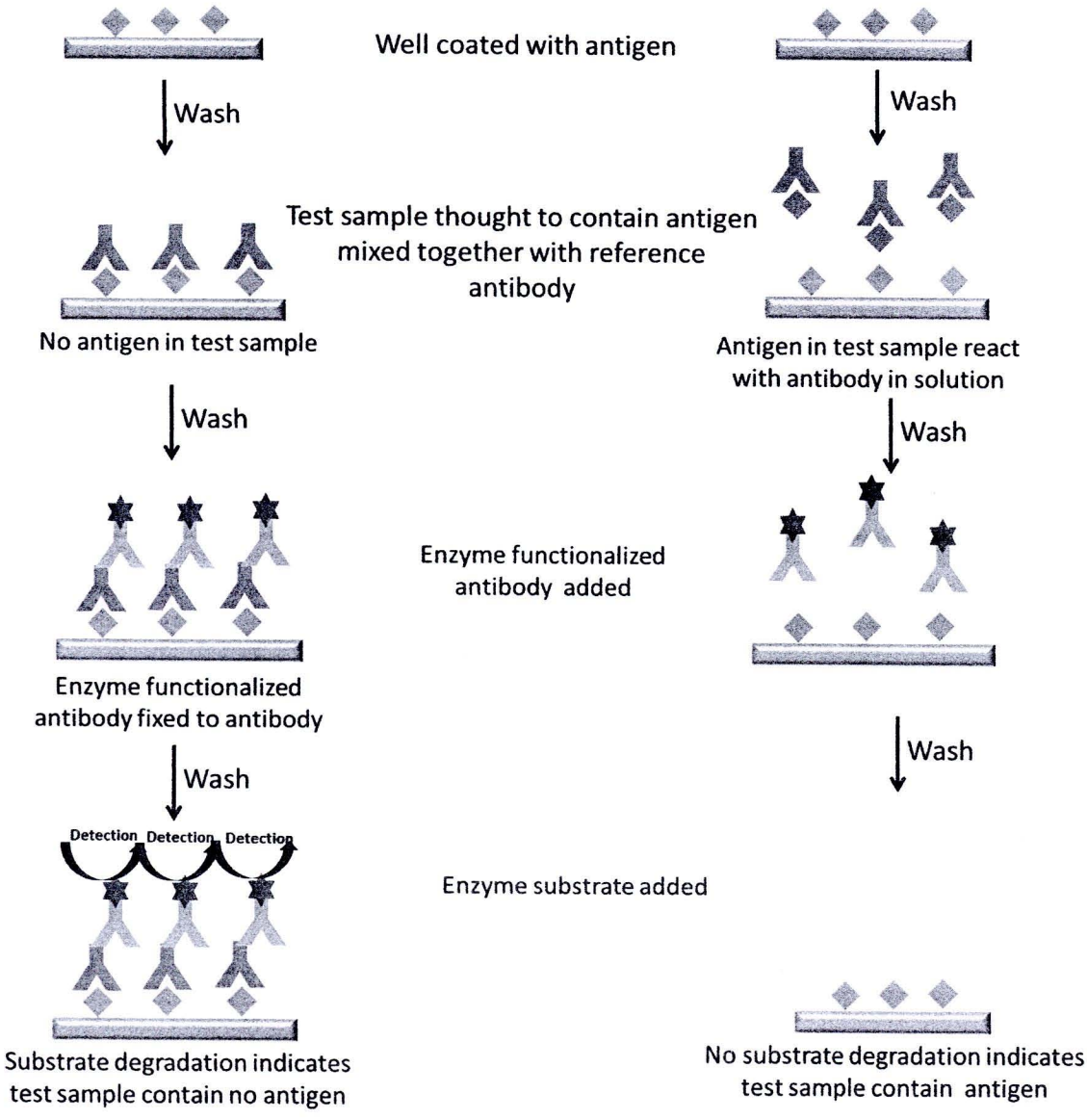

Figure 2.9 Competitive antigen modification of the indirect ELISA for assay of antigen [29].

2.3 Carbon Nanotube

Carbon nanotubes (CNTs) are carbon-based nanomaterials, exhibiting outstanding physico-chemical and mechanical properties (high tensile strengths, ultra-light weight, thermal and chemical stability, metallic and semi-conductive electronic properties) [39]. The shapes of CNTs are hexagonal networks of carbon atoms of approximately 1 nm diameter and 1 to 100 microns of length. They can essentially be thought of as a layer of graphite rolled-up into a cylinder [40]. An ideal CNTs can be described as a rolled up graphene sheet consisting solely of carbon atoms arranged in hexagonal ring structure with aromatic bonds [41].

Depending on the arrangement of their graphene cylinders, there are two types of nanotubes: single-walled nanotubes (SWNTs), which are hollow tubes of carbon capped at either end with a hemi-fullerene, and multi-walled nanotubes (MWNTs) consisting of concentric layers of graphene sheets rolled up, where smaller diameter tubes are encased in larger diameter tubes [42]. The MWNTs can reach diameters of up to 100 nm, on other hand SWNTs have been observed with diameters ranging from 0.4 to 3 nm [43]. MWNTs have many layers (approximately 50), while SWNTs have only one single layer of graphene cylinders, as shown in Figure 2.10 [40]. SWNTS is a strikingly inert material in the form of bundles due to Van Der Waals attractive interactions. MWNTs are always electrically conductive and found to have an electrical conductivity of approximately 1.85 x 10^3 S/cm. The different types of SWNTs are presented in Figure 2.11 [15].

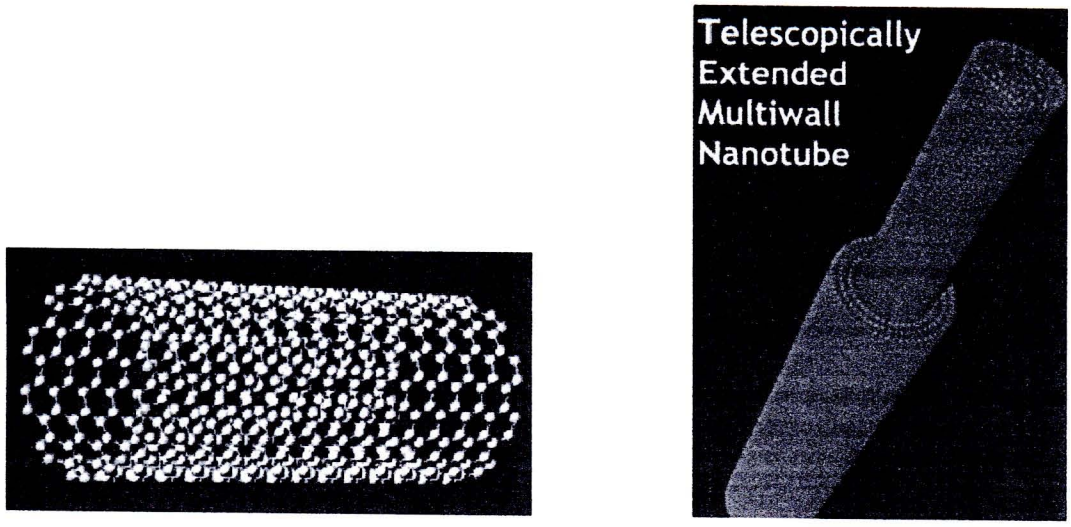

Figure 2.10 Structures of SWNTs (a) and MWNTs (b) [40].

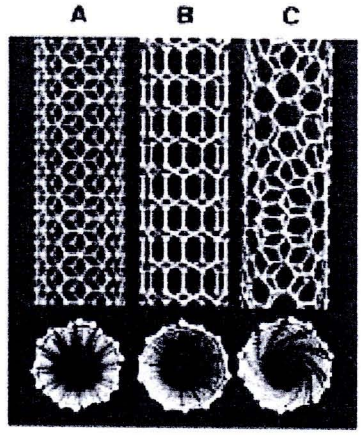

Figure 2.11 Different types of SWNTs. A: Armchair; B: Zigzag and C: Chiral [15].

The single- and multi-wall nanotubes are interesting nanoscale materials for the following four reasons [44]:

1. Single- and multi-wall nanotubes have very good elastic mechanical properties because the two dimensional (2D) arrangement of carbon atoms in a graphene sheet allows large out of plane distortions, while the strength of carbon-carbon in-plane bonds keeps the graphene sheet exceptionally strong against any in-plane distortion or fracture. These structural and material characteristics of nanotubes point towards their possible use in making next generation of extremely light weight, but highly elastic, and very strong composite materials.

2. A single-wall nanotube can be either conducting or semi conducting, depending on its chiral vector (n, m), where n and m are two integers. The rule is that when the difference n-m is a multiple of three, a conducting nanotube is obtained. If the difference is not a multiple of three, a Semi conducting nanotube is obtained. In addition, it is also possible to connect nanotubes with different chiralities creating nanotube hetero-junctions, which can form a variety of nanoscale molecular electronic device components.

3. Nanotubes, by structure, are high aspect-ratio objects with good electronic and mechanical properties. Consequently, the applications of nanotubes in field emission displays or scanning probe microscopic tips for metrological purposes have started to materialize even in the commercial sector.

4. Since nanotubes are hollow, tubular, caged molecules, they have been proposed as lightweight large surface area packing material for gas storage and hydrocarbon fuel storage devices, and gas or liquid filtration devices, as well as nanoscale containers for molecular drug delivery and casting structures for making nanowires and nano capsulates.

2.3.1 Synthesis of Carbon Nanotubes

Various techniques have been developed to produce carbon nanotubes (CNTs) and well known techniques are arc discharge, laser ablation and chemical vapour deposition (CVD). Most of these processes take place in vacuum or with inert gases mixed with precursor flow. CVD growth of CNTs can take place in vacuum or at atmospheric pressure. Large quantities of nanotubes can be synthesized by these methods; however, advances in catalysis, and continuous growth processes are making CNTs more commercially viable [15].

A. Carbon arc-discharge technique

Two carbon electrodes are used in the carbon arc-discharge technique to generate an arc by DC current. The electrodes are kept in a vacuum chamber and an inert gas is supplied to the chamber. The purpose of the inert gas is to increase the speed of carbon deposition. Initially, the two electrodes are kept independent. Once the pressure is stabilized, the power supply is turned on (about 20 V) and the positive electrode is then gradually brought closer to the negative electrode to strike the electric arc. On arcing, the electrodes become red hot and a plasma forms. Once the arc stabilizes, the rods are kept about a millimeter apart while the CNT deposits on the negative

electrode. The power supply is cut-off and the machine is left for cooling once a specific length is reached [40].

The two most important parameters to be taken care of in this method are: (i) the control of arcing current and (ii) the optimal selection of inert gas pressure in the chamber.

B. Laser ablation technique

In the laser-ablation technique for producing CNTs, Intense laser pulses are utilized to ablate a carbon target. The pulsed laser-ablation of graphite in the presence of an inert gas and catalyst forms CNTs. In general, some of the major parameters that determine the amount of CNTs produced are: the amount and type of catalysts, laser power and wavelength, temperature, pressure, type of inert gas present, and the fluid dynamics near the carbon target [40].

C. Chemical vapor deposition (CVD) technique

In the CVD technique, CNTs are synthesized by imparting energy to hydrocarbons. The imparted energy breaks the molecule into reactive radical species in the temperature range of 550–750 ^{0}C. These reactive species then diffuse down to the substrate, which is heated and coated in a catalyst (usually a first row transition metal such as Ni, Fe, or Co) where it remains bonded. There are several parameters that affect the synthesis of CNTs by CVD technique. The key parameters are the nature of hydrocarbons, catalysts, and the growth temperature [40].

A comparison among these three CNT synthesis techniques indicates that arc-discharge and laser-ablation methods produce high yields (>70%) of SWNTs, and the cost of producing CNTs by arc-discharge method is cheaper. However, the main disadvantages with these processes are: (i) tangled CNTs are synthesized that make the purification and applications of CNTs difficult; and (ii) these processes rely on evaporation of carbon atoms at temperatures >3000 ^{O}C. In addition to materials scale-up, CVD technique offers controlled synthesis of aligned and ordered CNTs. Although the microstructures of the CNT tips synthesized by CVD technique have well-formed caps compared to other techniques, they often have interrupted graphite layers. In applications such as scanning probe microscopy, tips are very important. Although CVD process appears technologically easier, the required quality of tips can be made by arc-discharge method. The purity of CNTs can be evaluated qualitatively using Raman spectroscopy; while near infrared spectroscopy can be used for the quantitative assessment. The most common impurities are carbonaceous materials, whereas metals are the other types of impurities generally observed. The impurities can be purified by oxidation in the carbon arc-discharge technique as the carbonaceous impurities have high oxidation rates. However, when 95% of the starting materials are oxidized, only 10 to 20% of the remaining material comprises pure nanotubes. For purification by oxidation, generally two approaches are followed: (i) gas phase and (ii) liquid phase purification [40].

2.3.2 Properties of Carbon Nanotubes

Unlike other nanomaterials, CNTs have a large surface area to volume ratio which makes them an ideal candidate for immobilization applications. Additionally, the ability of CNTs to be easily introduced new functional groups such as carboxyl and amine has extended another dimension to their uses by being able to integrate with biological elements. On the other hand, these biomolecules can be coupled with CNTs directly with no further modification required. In this respect, the use of CNTs as a carrier to incorporate biomolecules such as enzymes and antibodies to use in bioanalytical applications could help promoting signal and therefore detection sensitivity [16].

The characteristic dimensions of CNTs are strictly related to their unique properties, for example, the mechanical strength (i.e. buckling force) is a function of length and diameter [41], while the small size and the high surface define the chemical reactivity of CNTs and induce changes in permeability or conductivity of biological membranes [39].

Mechanically, CNTs are currently the strongest known fibers because the carbon–carbon bond observed in graphite is one of the strongest in nature. CNTs show good load transfer characteristics with metal matrix composites. Efficient load transfer between a matrix and CNTs plays a key role in the mechanical properties of composites and can lead to the development of many super strong nanocomposites. The specific heat and thermal conductivity of CNTs are dominated by phonons as the electronic contribution is negligible due to low density of free charge carriers [45].

The electronic properties of CNTs are such that they may be metallic or semiconducting depending on their diameter and the arrangement of graphitic rings in the walls. Besides, they show exceptionally good thermal and mechanical properties. The unique properties of carbon nanotubes have led to their use in areas as diverse as sensors, actuators, field-emitting flat panel displays, energy, and gas storages [45].

The dielectric responses of the CNTs are found to be highly anisotropic. Owing to their nearly one dimensional electronic structure, the electronic transport in metallic SWNTs and MWNTs occurs ballistically (without scattering) over long lengths. This enables nanotubes to carry high currents with negligible heating. It was observed through experiments by Wei et al [46] that MWNTs can carry high current densities up to 109 −1010 A/cm2 and can conduct current without any measurable change in their resistance or morphology for extended periods of time at temperature up to 250 ^{O}C.

The electrical and electronic properties of nanotubes are affected by distortions like bending and twisting. Pentagon-heptagon pair is introduced in CNTs by bending, which results in metal–metal and semi conductor metal nanoscale junctions that can be used for nanoswitches. The effect of bending becomes important when bending angles are more than. At this stage, kinks appear in the structure of the tube, resulting in the reduction in conductivity of CNTs. However, the presence of a metal nanowire inside the nanotube greatly suppresses the tube-buckling instability. In this case, increased tube diameter leads to an increase in the bending strength [40].

Recent studies demonstrated that CNT can enhance the electrochemical reactivity of important biomolecules, and can promote the electron-transfer reactions of proteins (including those where the redox center is embedded deep within the glycoprotein shell) [47]. In addition to enhanced electrochemical reactivity, CNT-modified electrodes have been shown useful to accumulate important biomolecules (e.g., nucleic acids) and to alleviate surface fouling effects (such as those involved in the NADH oxidation process).The remarkable sensitivity of CNT conductivity to the surface adsorbates permits the use of CNT as highly sensitive nanoscale sensors. These properties make CNT extremely attractive for a wide range of electrochemical biosensors ranging from amperometric enzyme electrodes to DNA hybridization biosensors [48].

2.3.3 Immunoassay Applications of Carbon Nanotubes

The application of CNTs in next-generation of sensors has the potential of revolutionizing the sensor industry due to their inherent properties such as small size, high strength, high electrical and thermal conductivity, and high specific surface area [39]. CNTs have specific physico-chemical and electrical properties that are useful for telecommunications, medicine, materials, manufacturing processes and the environmental and energy sectors [49].

Application of CNTs was developed by Villamizar et al. for *Salmonella infantis* detection based on transduction power of field effect transistor carbon nanotubes (CNTFETs) combined with the recognition capacity of the antigen – antibody interaction. Anti-*Salmonella* antibodies were adsorbed onto the single wall carbon nanotubes. SWCNTs acts as the conductor channel. It can detect at least 100 CFU/mL of *S. infantis* in just 1 h [50]. Villamizar et al. also developed biosensor based on field effect transistor (FET) for *Candida albicans* detection. A single wall carbon nanotube (SWCNTs) was used as aconductor channel. Monoclonal anti-*Candida* antibodies were adsorbed onto the SWCNT to provide specific binding sites for fungal antigens. FET devices were exposed to increasing concentrations of *C. albicans* and were able to detect atleast 50 CFU/mL in only 1 h. The sensor response remained stable for more than 10 days [51]. Chunglok et al. employed SWCNTs as labeling to enhance the detection sensitivity of enzyme linked immunosorbent assay (ELISA) for *S. enterica* serovar Typhimurium. Antibody and horseradish peroxidase (HRP) were co - immobilized on surface SWCNTs to improve the signal. Limit of detection (LOD) was found to be as low as 10^3 and 10^4 CFU/mL for direct and sandwich ELISA [16].

2.3.4 Functionalization of Carbon Nanotubes

Integrate CNTs with biomolecule is required to apply the CNTs in biosensor. Selectively immobilized biological molecules can be performed by modification and functionalization of surface CNTs. Basically, functionalization is a process that creates defects or oxides on the ends and sidewalls of the CNTs. Methods that has been widely used to immobilize of biological species to nanotubes are covalent bonding and noncovalent bonding or physical adsorption [52].

Covalent bonding can be applied to immobilize biomolecules with the use of proper coupling agents such as 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (NHS) and *N,N'*dicyclohexyl carbodiimide (DCC) for activated

amidation. Functional group on the CNTs requires to covalently bond to molecules. The best choice functional group is carboxylic acid group because it can undergo a variety of reactions and is easily formed on carbon nanotubes via oxidizing treatments, for example sonication in sulfuric and nitric acid, refluxing in nitric acid, ozonolysis, and air oxidization. As shown in Figure 2.12, CNTs were acid-oxidized to form carboxylic acid groups on the surface of the carbon nanotubes. Then, EDAC activate carboxylic acid groups, forming a highly reactive *O*-acylisourea active intermediate. In the presence of *N*-hydroxysuccinimide (NHS), a more stable active ester (succinimidyl intermediate) can beformed. The active ester was formed to allow the cross-linked of protein onto CNTs [53].

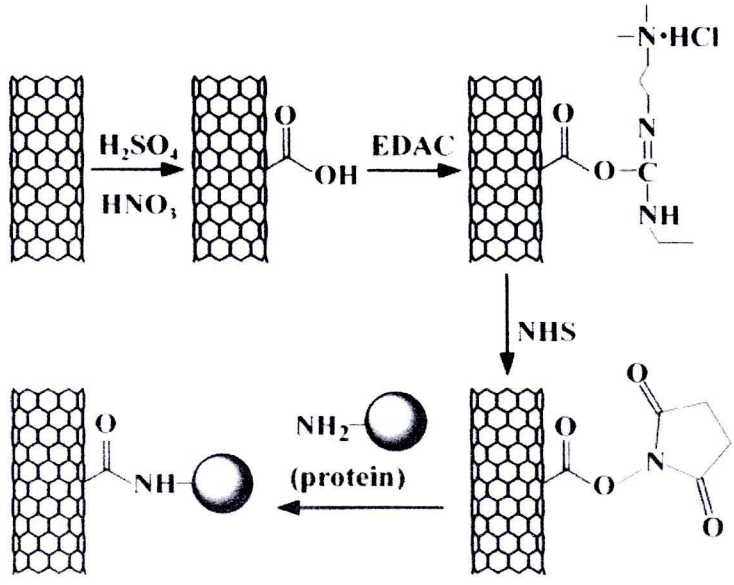

Figure 2.12 Schematic view of the attachment of proteins to carbon nanotubes [53].

Besides covalent bonding, the other method for functionalized CNTs is non-covalent bonding, coating or wrapping CNTs with polymers such as poly (vinyl pyrrolidone), polypyrrole, poly ethylene glycol),and poly (styrene sulfonate) makes the CNTs water soluble and biocompatible so can be easily functionalized for protein conjugation [52]. Chen et al. reported a simple approach to noncovalent functionalization of the sidewall single wall carbon nanotubes (SWCNTs). The noncovalent functionalization involves a bifunctional molecule, 1-pyrenebutanoic acid, succinimidyl ester, irreversibly adsorbed onto the inherently hydrophobic surfaces of SWNTs in an organic solvent dimethylformamide (DMF) or methanol. The Pyrenyl group interacts strongly with sidewall of SWCNTs via π-stacking. This leads to the functionalization of SWNTs with succinimidyl ester groups that are highly reactive to nucleophilic substitution by primary and secondary amines that exist in abundance on the surface of most proteins [54]. The mechanism of protein immobilization on nanotubes, then, involves the nucleophilic substitution of *N*-hydroxy succinimide by an amine group on the protein, resulting in the formation of an amide bond. This technique enables the immobilization of a wide range of biomolecules on the sidewalls of SWNTs with high specificity and efficiency [54].

2.4 Gold Nanoparticles (AuNPs)

Recently, gold nanoparticles (AuNPs) have been remarkably used in various imaging and biosensing systems. Owing to their excellent versatility, biocompatibility and special properties, AuNPs have showed their significant roles as ligand anchors or transducing agents, they possess great promise in cellular and biomolecular identification, medical diagnostics, and environmental monitoring [55].

AuNPs have received greatest interests because they have several kinds of intriguing properties. AuNPs, with the diameter of 1-100 nm, have high surface to volume ratio and high surface energy to provide a stable immobilization of a large amount of biomolecules retaining their bioactivity. Moreover, AuNPs have an ability to permit fast and direct electron transfer between a wide range of electroactive species and electrode materials. In addition, the light-scattering properties and extremely large enhancement ability of the local electromagnetic field enables AuNPs to be used as signal amplification tags in diverse biosensors [56].

Catalytic activity of AuNPs would be a typical example which attracts extensive attention for a rapid and sensitive detection. AuNPs can catalyze for both ionic gold or ionic silver, causing depositions of gold or silver atoms on the surface of the AuNPs. The gold or silver depositing on the AuNPs then serves as a biological transducer, transforming the phenomena of nanoscale biorecognition events to a colorimetric signal that could be discriminated by an unaided eye or inexpensive equipments. This strategy has been performed in both liquid and solid phases by many others [57].

AuNPs have attracted a great interest in fabrication of various biosensor systems for analysis of cellular and biomolecular recognitions. In conjunction with vast conjugation chemistry available, the materials are easily coupled with biomolecules such as nucleic acids, antigens or antibodies in order to achieve their many potential applications as ligand carriers or transducing platforms for preparation, detection and quantification purposes. Furthermore, the nanoparticles possess easily tuned and unique optical/ physical/chemical characteristics, and high surface areas, making them ideal candidates to this end. By coupling with biomolecules (e.g. DNA, aptamer, protein, enzyme, etc.), AuNPs have been implemented to detect and quantify the biological interactions as optical labels, electrochemical markers, surface plasmonic amplifiers, catalytic probes, or FRET probes [57].

2.4.1 Synthesis of Gold Nanoparticles (AuNPs)

AuNPs can be prepared by both chemical and physical methods. Normally, gold derivatives (e.g. chloroauric acid) are reduced and controlled to grow particles with nanometer scale in chemical methods. The chemical synthesis methodologies include redox synthetic method, electrochemical method, photochemical method, seed-growth method, and template synthesis, micro-emulsion template synthesis, and microwave synthesis, etc [58].

The Turkevich–Frens method is the most representative and popularly used procedure to synthesize the AuNPs with sizes between 10 and 60 nm in diameter by adjusting the ratio of reducing/stabilizing agents (the trisodium citrate) and gold (III) derivatives (the hydrogen/sodium

tetrachloroaurate (III)) in boiling water. This method is very often used even now because the loose shell of citrates on the particle surfaces is easily replaced by other desired ligands (e.g. thiolated DNA) with valuable function [59, 60].

Most hydrophobic AuNPs with diameters in the range of 1 to ca. 8 nm are prepared by the Brust–Schiffrin method. In this case, the gold (III) derivatives are reduced by sodium borohydride ($NaBH_4$) in an organic solvent in the presence of thiol capping ligands using either a two-phase liquid/liquid system or a suitable single-phase solvent. Typically, sodium tetrachloroaurate (III) is transferred to toluene using tetraoctylammonium bromide (TOAB) as the phase-transfer reagent and reduced by $NaBH_4$ in the presence of dodecanethiol (DDT). Smaller average core sizes were obtained with larger thiol/gold mole ratios, and fast reductant addition and cooled solutions produced smaller and more monodisperse particles [61].

The preparation of water-soluble or hydrophobic AuNPs with size less than 10 nm usually can be achieved by polymer ligands–protection method, which is based on using polymers (e.g. polyethylene glycol, thiol- or thioether-functionalized polymer) as ligand, $NaBH_4$ as reducing agent. The solubility of AuNPs is dependent on the polymer stabilizer [61].

2.4.2 Immunoassay Applications of Gold Nanoparticles

AuNPs have been widely used in biological and pharmaceutical fields because AuNPs have unique optical properties (i.e. surface plasma resonance (SPR) absorption and resonance light scattering (RLS), a variety of surface coatings and great biocompatibility (generally, unmodified AuNPs is nontoxic, and the biological toxicity of the functional AuNPs is dependent on their ligands) [58].

AuNPs have also been employed for many other applications such as immunoassay, protein assay, time of flight secondary ion mass spectrometry, capillary electrophoresis, and detection of cancer cells [62]. Furthermore, AuNPs can also be applied to enhance the signal of surface plasmon resonance (SPR) biosensor. The SPR signal enhancement of sandwich immunoassay can be achieved by labeling the secondary antibodies using gold nanoparticles [63].

Chuang et al. reported, protein activity assay was established based on the surface resonance property of AuNPs. AuNPs with size around 13 nm were modified with gelatin as proteinase substrate and subsequently modified with 6-mercaptohexan-1-ol (MCH). The AuNPs will lose shelter and MCH increase the attractive force between the modified AuNPs after proteinase (tryosin or gelatinase) digestion, resulting aggregation of AuNPs. The limit of detection of this method was as low as 20 ng/mL in gelatinase detection [64]. Ambrosi et al. have developed an ELISA immunoassay. Gold nanoparticles were used as carriers of signaling antibody in order to achieve an amplification of the optical signal for the analysis of breast cancer biomarker. In the range between 0 and 60 U/mL, the assay adopting AuNPs as an enhancer resulted in higher sensitivity and shorter assay time when compared to classical ELISA procedures [65]. An improved scanometric immunoassay based on dual enlargement of AuNPs on a solid support was successfully carried out by Cao et al. to detect *Campylobacter jejuni*. The combining Au enlargement and Ag enhancement the detection signal could be greatly enhanced, resulting in

more distinct images that could be quantified by a simple flatbed scanner. This method have limit of detection as low as 10^3 CFU/mL [57].

2.5 Nanocomposite of Carbon Nanotubes and Gold Nanoparticles

CNTs and metal nanocomposites are of great interest due to combination of the unique properties of these two kinds of materials to provide new applications. The most interest metal to composite on CNTs is AuNPs. AuNPs have several benefit properties such as easily synthesized and functionalized and easy to link to many molecules or groups. There are two common method to composited AuNPs with CNTs, indirect and direct deposition [66].

Indirect composite method reported by Jiang et al. [67] AuNPs was attached on CNTs surface using cationic polyethylene amine or anionic citric acid as the dispersant. CNTs surface would be changed to yield basic or acidic, and AuNPs successfully coated CNTs surface with AuNPs that has size about 10 nm . The other method is direct method, which means that AuNPs are coated on the sidewall of CNTs without any link of other molecules or groups. Zhang et al. mixed MWCNTs with sodium citrate and using ultrasonication, shells of sodium citrate were formed on the carbon nanotube. When $HAuCl_4$ was added to the reaction system, Au^{3+} was directly reduced at the surface of the MWCNTs and gold nanoparticles were assembled along the MWCNTs [66].

Nanocomposites of CNTs and AuNPs have been employed for many applications in nanoelectric, biosensor, etc. Immunosensor for carcino embryonic antigen (CEA) detection was fabricated using chitosan–carbonnanotubes–gold nanoparticles (CS–CNTs–GNPs) nanocomposite film by electro deposition method. The porous three-dimensional CS–CNTs–GNPs nanocomposite film, which offered a large specific surface area for immobilization of antibodies, exhibited improved conductivity, high stability and good biocompatibility. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used for characterization of the immunosensor. This method have detection limit as low as 0.04 ng/mL under the optimal condition [68]. Huang and co-worker [69] reported, electrochemical immunosensor for carcino embryonic antigen (CEA) detection based on AuNPs/MWCNTs–chitosans (Chits) composite film. AuNPs was in situ synthesize at the composite MWCNTs-Chits in acetic acid solution. The mixture was dripped on the glassy carbon electrode (GCE) and then CEA antibody (anti-CEA) was immobilized on the resulted modified electrode to construct the immunosensor. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used for characterization of the immunosensor. This method has limit of detection around 0.01 ng/mL. This electrochemical immunoassay combines the specificity of the immunological reaction with the sensitivity of the AuNPs and MWCNTs amplified electrochemical detection.

2.6 Magnetic Bead

Magnetic nanoparticles as special biomolecule immobilizing carrier offer a promising alternative to conventional methodology. Magnetic nanoparticles have been used in immunoassays, enzyme, DNA, protein immobilization, and DNA purification [70]. Magnetic microbeads used in biomedical applications present usually a core/shell structure. Such microbeads have an inorganic core e.g. iron oxide, surrounded by an outer layer of shell wall that consists of long-chain organic

ligands or inorganic/organic polymers. The attachment of bioactive ligands to the surface of the outer shell is the key to bioapplication of magnetic microbeads [71].

Magnetic nanoparticles and microparticles offer an additional advantage: having embedded magnetic entities, they can be magnetically manipulated using permanent magnets or electromagnets, independently of normal microfluidic or biological processes This extra degree of freedom is the basis of a still improved exposure of the functionalized bead surface to the surrounding liquid and of higher sample preconcentration efficiencies, due to the increased relative motion of the bead with respect to the fluid [72].

Magnetic microbeads are now used as magnetic markers for bioassays. Typically, magnetic micro beads are iron oxide based particles that may be encapsulated in a polymeric layer, the total particle having a diameter in the range of one to several micrometers. The use of magnetic markers has been shown to offer advantages such as, low interference, little or no background signal, no transducer fouling and no sample treatment [71].

Several immunomagnetic biosensors have been developed for label-free detection of *E. coli*. For example, Mujika et al. reported a magneto resistive immunosensor for the analysis of *E. coli* O157:H7 in food and clinical samples. This biosensor was capable of detecting and quantifying small magnetic field variations caused by the presence of super paramagnetic beads bound to the antigens previously immobilized on the sensor surface via an antibody–antigen reaction [73]. Maalouf and co-workers described an immunomagnetic biosensor for *E. coli* comprising streptavidin-functionalized paramagnetic nanobeads attracted to a gold-electrode surface via a magnetic field. Biotinylated-anti *E. coli* antibodies then interacted with the nanobeads. Detection was in this case by impedimetric measurements yielding a working range of $10 - 10^3$ CFU/mL [74].

2.7 Scanometric Detection

Scanometric detection is one of the most interesting approaches for detecting DNA. This method was develop by Mirkin and co-workers [12]. They developed quantitative signal amplification method based on nanoparticle promoted reduction of silver and used a conventional flatbad scanner as a reader. First, capture oligonucleotides were attached on surface glass slide. AuNPs modified with oligonucleotides were used to indicate the presence of DNA target. Nanoparticle probes and DNA target were co hybridized to the capture nucleotides on glass slide surface. To facilitate visualization silver ion was reduced by hydroquinone to silver metal at the surface of AuNPs. This process increased the scanned intensity and therefore help amplifying the signal. Scanometric detection method not only enables very low surface coverages of nanoparticle probes to be visualized by a simple flatbed scanner or by the naked eye but also permits the quantification of target hybridization on the basis of the imaged grayscale of the darkened area. This method has a limit of detection as low as 50 fM [12]. Scanometric array detection is illustrated in Figure 2.13.

In this work, AuNPs and MWCNTs nanocomposites were used to immobilize antibody by covalent linkage to form a label. This label was then employed to detect *Salmonella serovar* enterica Typimurium based on a scanometric method.

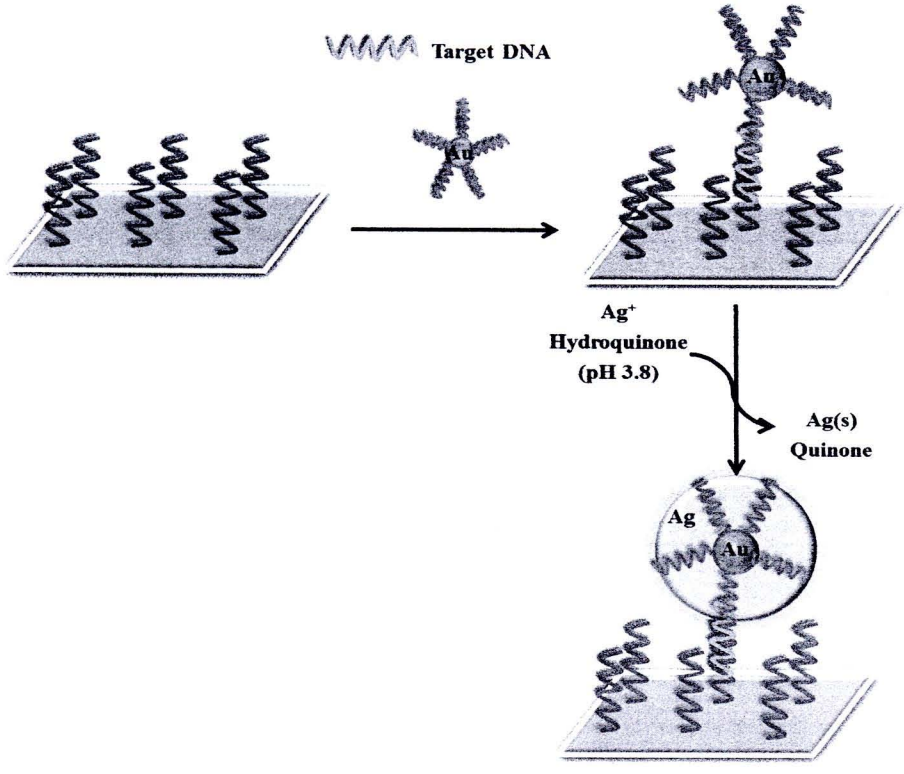

Figure 2.13 Scanometric DNA assay [12].