Chapter 1 Introduction

1.1 Immunoassay

Immunoassays are widely used in medicine and have become an indispensable tool for modern diagnosis. The method is used routinely in many clinical laboratories. Commercial testing kits for the determination of more than 100 biochemical components are available today, and assays for new analytes (of hormones, enzymes, vitamin, drugs, virus, bacteria, residues in food and others) are being continuously developed in parallel with methodological research to further improve the precision, accuracy, sensitivity and diagnostic cost effectiveness of the assay. Immunoassays generally can be divided into unlabelled and labelled assays. Most unlabelled techniques are based on secondary immune reactions, such as precipitation and agglutination. The immune antigen-antibody complexes formed are measured by light scattering or particle counting methods. Labelled immunoassays are based on the primary immune reaction. To determine the extent of the immune reaction, the free labelled reagent has to be either separated from the bound complexes (heterogeneous assay) or the activity of the label has to be changed in such a way to make separation of bound and free reactants unnecessary (homogeneous assay).

1.2 Basic Principle of labelled immunoassays

Labelled immunoassay systems may be subdivided into two main classes consisting of (i.) *Type I ' immunometric method '*, using excess reagent as introduced by Miles and Hales (Miles & Hales,1968). (ii) *Type II ' competitive binder ligand assay '* where limited reagent (antibody or antigen) is used. In particular, immunoradiometric assay (IRMA) is a technique using antibodies/antigens immobilised onto solid phase which is allowed first to react with antibody/antigen in the serum then with radioactive labelled antibody and is known as a two-site (Addison & Hales, 1960) or sandwich technique (Salmon et al., 1969). On the other hand, a typical example of ` competitive binder ligand assay ` is radioimmunoassay (RIA) which was developed by Yalow and Berson (Yalow & Berson, 1959, 1960).

The selection of a particular type of assay for a particular analyte depends on the size of the molecules involved, availability and labelling capacity of analyte, the required sensitivity and speed of analyte determination. If the analyte is small, easy to isolate, synthesize and labelled , Type II assay using labelled antigen is the preferred method. If the analyte is of high molecular weight, difficult to purify to yield sufficient quantity for labelling and present at low concentration (require high sensitivity assay), Type I assay using labelled antibody technique will be the method of choice (Porstmann & Kiessig, 1992). Type I assays require the analyte to have at least two different epitopes clearly distinct from each other in order to permit

binding of epitopes with labelled and unlabelled antibodies without steric hindrance. It is thus not suitable for hapten quantitation (Porstmann & Kiessig, 1992).

1.3 Separation of bound and free reactants

The end-point of competitive binder ligand assay (Type II) involves determining the relative proportion of antigen that is free and antigen that is bound to the saturable reagent (immune complex). Usually the assay requires physical separation of these two components, since the bound immune complex does not precipitate spontaneously at the low concentrations employed (10^{-10} mol dm^{-3} to 10^{-12} mol dm^{-3}) and also the signal produced by the label is generally measured in the immune complex (Porstmann & Kiessig, 1992). Currently, separation technique exploits the physicochemical or immunological differences between the free and the bound moieties. Since phase separation has a strong influence on precision, sensitivity and the handling of a test, an ideal separation should meet the following requirements:

i.) it should not interfere with the primary binding reaction. Failure to meet this requirement will impair precision of the assay;

ii.) it should not be affected by plasma or serum. Failure to meet this requirement cause difficulties in standardization;

iii.) It should be simple, quick, cheap and the reagent and equipment used should be readily available.

A summary of techniques used in separating bound and free tracer is given in Table 1.1

Table 1.1: Methods of separating bound and free tracer

Principle of technique	Reagent and equipment
1. Techniques based on differential migration caused by difference in charge and molecular weight (Berson et al., 1956; Haber et al., 1965; Hunter & Greenwood, 1964; Orskov 1967)	• Paper chromatography or chromatoelectrophoresis • Electrophoresis on starch gel, cellulose acetate, or polyacryamide • Gel filtration
2. Adsorption methods (Meade & Klitgaard, 1962; Frenkel et al., 1966)	• Dextran coated charcoal • Silicates, e.g. talcum powder, Quso G-32, Fuller`earth • Anion-exchange resin e.g. amberlite and DEAE-cellulose
3. Fractionation precipitation (Heading, 1966; Thomas & Ferin 1968; Desbuquois & Aurbach, 1971; Grodsky & Forsham, 1960)	• Organic solvent eg ethanol-NaCl, ethanol, dioxan, polyethylene glycol • Salt - e.g. sodium sulphate, ammonium sulphate • Acid - e.g. trichloroacetic acid after enzyme proteolysis of free fraction
4.Immunological precipitation (double antibody technique) (Utiger et al., 1962; Morgan & Lazarow, 1963; Hales & Randle, 1963)	• Precipitation after first antibody incubation (post precipitation) • Preincubation of first and second antibodies prior to assay (pre-precipitation) • Second antibody linked to solid matrix
5. Solid-phase methods (Catt & Tregear, 1967; Donini & Domini, 1969; Miles & Hales, 1968)	• Antibody coated on disc and tubes • Antibody linked to finely divided solid phase e.g. activated sephadex, cellulose, agarose beads, magnetic particles

1.4 Solid phase immunoassay

In 1956, polystyrene latex particles were first introduced as a solid-phase adsorbent for rabbit globulin and used in agglutination tests for rheumatoid factor (Singer & Plotz, 1956). Similar latex particles have subsequently been used by Kilnman and Taylor (Kilnman & Taylor, 1969) for adsorption of protein like haptens in a solid phase antibody radioimmunoassay. However, solid-phase immunoassays became more convenient and popular when Catt and Tregear (1967) demonstrated that polypropylene or polystyrene tubes could be used as solid phase. Nowadays a variety of solid phases with either tubes, plates or spherical particles have been applied successfully to enzyme immunoassay (using enzyme label) and radioimmunoassay (using radiolabel). Stable and reproducible binding of antigens or antibodies to solid phase is a prerequisite for most of the high resolution immunoasssay of today. A variety of plastics compound of all forms and shapes are used for this purpose.

Table 1.2: Different forms of solid phase polymer commonly used for solid phase Immunoassay

Form of solid phase	Polymer Type
Tube	Polystyrene (PS), Polypropylene (PP)
Microtitre plates	PS, Covalink PS, Polyvinyl chloride (PVC)
Beads/Balls	Nylon, PS
Particles	Cellulose, Beaded Dextran, Agarose, Polyacryamide cellulose, some are magnetisable

Immobilisation of protein to solid phase can be performed either by adsorption to hydrophobic matrices or covalent coupling of protein to reactive matrices. To develope an assay to be used in the laboratory, protein adsorption on plastics surface is the most convenient method. Adsorption techniques are often easier to perform but one should be aware of the possibility of desorption. Some proteins are modified before it is immobilised to increase its binding to the substrate (Nygren & Karlsson, 1988, Jitsukawa et al., 1989; Conradie et al., 1983; O Shannessy & Quarles, 1985; O' Shannessy & Hoffman, 1987). In contrast, the chemical coupling method eliminates the desorption of bound antibodies but the operation is more difficult. Many covalent methods have been used (Wikstroem et al., 1987; Adalsteinsson et al., 1979; Loster et al., 1992, Hans et al., 1990; McConway, 1986). For adsorption, the underlying mechanism driving the binding is hydrophobic interaction (Van Oss & Singer, 1966). Both forces of protein-surface interaction and protein-protein interaction play an important role in protein adsorption. The heterogeneous distribution of the adsorbed protein indicates that cohesive forces of the protein are stronger than the surface-protein interaction (Fromherz, 1971). The adsorption of proteins to hydrophobic surface is a rapid process whereas the reverse reaction is slower. Binding is achieved by rate-limiting diffusion to the solid surface followed by rapid and irreversible adsorption (Wojeieshowskij et al., 1986) and is completed within 12-15h. The optimum conditions for adsorption of an antigen to a plastics substrate should be determined for each assay system (Portsman & Kiessig, 1992).

The coating process of antigen and antibody is simple. The coating solution should be as pure as possible when left in contact with the solid phase. The solid phase is then washed in assay buffer and reincubated with an excess of protein to ensure that any remaining protein binding sites on the plastics support are occupied , and after further washing in assay buffer the coated solid surface is ready to be used. Among the variables which are optimised are the protein concentration, the pH of the coating solution, the incubation temperature and the length of incubation time. Other factors like agitation and enhancement of reaction temperature accelerate the rate of diffusion (King et al., 1990; Ijsselmuiden et al., 1989).

1.5 Objective of the project

The main objective of the present study is to develop an applicable solid phase system based on natural rubber film and to study its possible uses in competitive and two-site immunoassay. The solid phase format is chosen because the technique has proven superior compared to other methods due to the following advantages:

1.) Universal applicability of such system independent of the analyte' s nature.

2.) Near complete separation of the immune complexes formed is possible. It provides easy and convenient way to separate bound from free fraction which leads to high accuracy and precision.

3.) Possess characteristics which favour automation at a low cost.

Most of the solid matrices are based on simple adsorption of antigen or antibodies to plastic surfaces. In such cases it is difficult to prevent non-specific adsorption of imunoreagents or the desorption of antigen or antibody from the solid immunosorbents. To diminish these problems, covalent binding of antigen or antibodies have been studied in relation to the preparation of solid immunosorbents but satisfactory solutions have, to date, yet to be found. Besides, in solid phase immunoassay, it is desirable to use the materials that can bind effectively large quantity of proteins so that the anti-sera can be used directly without prepurification to isolate the IgG fraction and thus the sensitivity of the assay can be improved. Natural rubber latex, a cheap material readily available locally is selected because it has a high affinity for proteins and could possibly enhance the sensitivity of the assay. Deproteinised latex film has also been used as a solid phase for this system, however the results indicate that NR latex give better assay results. Therefore NR latex has been chosen for this study. The following aspects of the work are investigated.

i.) Comparison of adsorption behavior of anti-Hepatitis B surface antigen (anti-HBsAg), Hepatitis B surface Antigen (HBsAg), Sheep anti-thyroxine (Anti-T4) on natural rubber (NR) latex coated tubes and polypropylene (PP) tubes.

ii.) Assays of anti-HBs, HBsAg and Thyroxine using NR latex coated tubes and PP tubes (as reference)

iii.) Modification of NR latex coated tube to enhance its sensitivity and reduce its non specific binding

iv.) Studies on the effect of blocker in reducing non specific binding for both the solid phases

v.) Studies on the kinetics of protein binding on both solid surfaces

vi.)Electron and scanning probe microscopy studies of the macromolecules immobilised on the different surfaces.

1.6 Natural rubber latex :Production, Properties and Composition

1.6.1 Introduction

Natural rubber latex has been commercially available since 1930 and subsequently has been thoroughly exploited for the manufacture of a great variety of products.

1.6.2 The production of natural rubber latex concentrates

Natural rubber latex from the *Hevea brasiliensis* tree has a rubber content of 25 - 40% by weight. This material is not utilized in its original form due to its high water content and susceptibility to bacteria attack. Field latex is collected from the trees and preliminary ammoniation to about 0.05-0.2% of NH_3 is carried out to protect it from bacterial attack prior to delivery to the factory. The latex is then concentrated by centrifugation to yield a concentrate (containing 60% or more rubber content) and a skim latex (3-6% rubber content). The concentrate is ammoniated with ammonia and treated with preservative. Three types of latex

concentrate are commercially available : namely doubled centrifuged latex, creamed latex and evaporated latex. The latex concentrate used in this project is double centrifuged high ammonia latex which is preserved with ammonia.

1.6.3 Composition

a.) Dispersed phase

The latex particles in NR latex is cis-1:4 polyisoprene with a molecular weight of about one million. The molecular weight can vary if gel (an insoluble rubber), is present the content of which will increase with storage of the latex. However, this does not produce any discernible change in the film-forming properties of the latex.

b.) Non-rubber substances in the serum

NR latex is a dispersion of latex particles in an aqueous phase (serum) containing small amount of various inorganic and organic substances comprising of amines, amino acids, carbohydrates, proteins and nuclei acids, neucleotides and lipid (Robert, 1988). In addition to water and rubber hydrocarbon, these substances are either dissolved in the serum or associated with the rubber particles. The composition of acid coagulated dry NR is given (Table 1.3) (Nair, 87). The ionic contents in latex concentrate have been analysed and it contains ammonium, potassium, sodium, magnesium, copper , iron and calcium ions.

Table 1.3 Composition of acid coagulated NR

Component	Percentage (wt.%)
Rubber Hydrocarbon	93.7
Neutral lipids	2.4
Glycolipids and Phospholipids	1.0
Proteins	2.2
Carbohydrates	0.4
Inorganic constituents	0.2
Others	0.1

c.) Non rubber substances adsorbed on latex particle surface

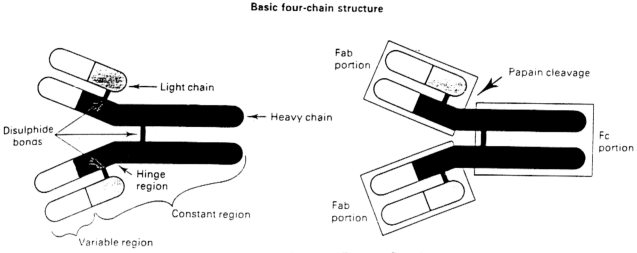
The rubber latex particles are surrounded by an adsorbed layer of protein and lipid. When ammonia is added to the latex, the phopholipids are hydrolysed to long chain fatty acid soaps which then become adsorbed onto the latex particle surface. The hydrolysis of phopholipids and proteins continues in the latex concentrate.

1.7 The structure of immunoglobins (Ig) (Turner, 1977)

The basic Ig structure is that of four polypeptide chains, two identical heavy (molecular weight 50000-77000) and two identical light (MW 22000) chains, linked by disulphide bonds (Fig 1.1). Papain, a protein-digesting enzyme, splits the antibody molecules into three large pieces. Two of the pieces are identical and contain antibody binding sites, which have been termed the Fab fragments. They are composed of the entire light chain and about half of the heavy chain . The Fab portions contain what is known as the variable regions of the molecule . It is this variable region that provides high specific binding for a particular antibody to

antigen. The third fragment (composing mainly of the heavy chain) plays no part in the combination with antigen but has many other important functions. They are specific receptors for a number of other molecules such as complement and secretory component and determinants bound by special cell receptor. When the molecule is chemically split, this fragment can be obtained in a crystalline form. Ig can be divided into five classes (IgA, IgD, IgE, IgG, IgM) according to characteristics of the heavy chain and their specific effector functions. Two kinds of IgG used in this study are polyclonal IgG, which is separated as the immunological fraction from the serum of sensitised animals, and monoclonal IgG, artificially reproduced from one T-cell type in ascites fluid. The first kind is less specific as it is a mixture of a multitude of slightly different IgG molecules, but is cheaper and recognizes different antigen epitopes. Monoclonal IgG, nevertheless, is a very homogeneous antibody, and suitable for basic investigation of specific epitopes of the macromolecules . Monoclonal IgG used in this study was labelled using ^{125}I which has a half life of 60 days.

Fig 1.1 Schematic representation of the four-chain structure of Immunoglobulins



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