Dye-Protein Interactions: Protein staining and Dye-IgY, Dye-Dextran-IgY Complexes for Antigen Detection

By

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"Listen to what is wise and try to understand it. Yes, beg for knowledge; plead for insight. Look for it as hard as you would for silver or hidden treasure. It is God who gives wisdom; from him comes knowledge and understanding."

Proverb 2, 2 – 4 and 6.

This work is dedicated to my parents, Anthony and Theresa Achilonu.

PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, University of KwaZulu-Natal Pietermaritzburg, from March 2002 to December 2003 under the supervision of Prof. J. P. D. Goldring.

These studies represent original work by the author and have not been submitted in any form to any university. Where use has been made of the work of others, it has been duly acknowledged in the text.

Ikechukwu Achilonu
16 January 2004.

Prof. J. P. D. Goldring
Supervisor

ABSTRACT

In order to develop a cheaper alternative to the conventional enzyme-linked immunosorbent assay system, application of dye molecules as labels in immunoassay was investigated in this study. This chromogenic dye-antibody conjugate could be used in colourimetric immunodetection diagnostic assays that could be used in a rural African setting.

The chemistry of the interaction between twenty-six dyes of anionic, cationic and ligand dye classes with IgY and other proteins were studied for protein detection and conjugation to antibodies. Out of the twenty-six dyes studied, Direct Red 81 proved to be a good protein stain on nitrocellulose and polyacrylamide gels with comparable sensitivity to Coomassie Blue R 250. Direct Red stained proteins faster (< 5 min) than Coomassie Blue R 250 in polyacrylamide gels.

Aurintricarboxylic Acid, Ethyl Red and Gallocyanine with carboxylic acid and/or hydroxyl functional groups were selected, activated with carbonyldiimidazole (CDI) to form amine reactive-imidazole intermediates and conjugated to anti-rabbit albumin IgY. Gallocyanine gave the best molar coupling ratio with IgY (76:1 dye:IgY). The dye-antibody conjugates were used to detect rabbit albumin on nitrocellulose. Aurintricarboxylic Acid-IgY and Gallocyanine-IgY detected 50 ng of rabbit albumin on nitrocellulose, which was 10 fold less sensitive than HRPO-IgY conjugate. Cross-linking of the antibodies by the dyes compromised the immunoreactivity of the Aurintricarboxylic Acid-IgY and Gallocyanine-IgY conjugates. The immunoreactivity of Ethyl Red-IgY was not compromised.

Anti-rabbit albumin IgY was conjugated to derivatized dextran as an alternative immunoassay reagent and used to detect rabbit albumin on nitrocellulose by staining the polysaccharide (dextran) in the immune complex with PAS reagent. IgY-dextran complex was able to detect 25 ng of rabbit albumin on nitrocellulose, but PAS staining resulted in high background staining of the nitrocellulose membrane.

Dextran-antibody conjugates may have better potential as immunodetecting reagent than dye-IgY conjugates, if a more sensitive and specific method of detecting the dextran in the Ag:Ab-dextran immune complex is developed.

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To the memory of my grandparents: Pa Achilonu Eke, Ma Catherine Agbaraji and Mr. Emma Agbaraji. Wherever you oaks are, I know you are proud of your Grandson. Till we meet again.

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ABBREVATIONS

3D	three-dimensional
Ab	antibody
ABTS	2,2'-azino-di-(3-ethyl)-benzthiozoloine sulfonic acid
AECM-dextran	aminoethyl-carboxymethyl-dextran
Ag	antigen
ATA	Aurintricarboxylic Acid
BLAST	basic local alignment search tool
BSA	bovine serum albumin
C.I.	colour index
ca	around or approximately
C	constant
CDI	*N,N'*-carbonyldiimidazole
CH	heavy constant region
COOH	carboxylic acid
C-terminal	Carboxyl terminal
Da	dalton
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	extinction coefficient
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding fragment
FITC	fluoresceine isothiocyanate
FW	formula weight
g	gravity

GAGs	glycosaminoglycans
GAG	glycosaminoglycan
h	hour(s)
H-chain	heavy chain
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HRPO	horseradish peroxidase
HSA	human serum albumin
I/mg	intensity per milligram
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IgY-SH	sulfhydryl-modified IgY
kDa	kilodalton(s)
L-chain	light chain
LMW	low-molecular-weight
mA	milliampere
mRNA	messenger RNA
MBS	m-maleimidobenzoyl-*N*-hydroxysuccinimide ester
MEC	molecular exclusion chromatography
Min	minute(s)
mM	millimolar
M_r	molecular weight
Na_2EDTA	ethylenediaminetetra-acetic acid disodium salt
ng	nanogram
NHS	*N*-hydroxysuccinimide ester
nm	nanometre
nM	nanomole
NPIA	nitrophenyliodoacetic acid
N-terminal	amino terminal
OH	hydroxyl

PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PEG	polyethylene glycol
pg	picogram
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PVDF	polyvinylidiene difluoride
®	registered trademark
RF	rheumatoid factor
RNA	ribonucleic acid
RT	room temperature
s	second(s)
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate- polyacrylamide gel electrophoresis
SH	sulfhydryl
TBS	Tris buffered saline
TEMED	*N,N,N',N'*-tetramethylethylenediamine
™	trademark
UV	ultraviolet
V	variable
v/v	volume by volume
v/v/v	volume by volume by volume
w/v	weight by volume

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction to dyes and dye applications

Dyes are coloured chemical substances that are used in conferring colour upon another substance in solution or dispersion. The substance to be coloured is termed the substrate to the dye. A dye and its substrate must have certain chemically or structural complementary features that enables the dye to colour or adhere to its substrate. This process by which a dye adheres to its substrate is a form of chemical reaction called dyeing. Dyeing most often depends on certain extrinsic factors such as pH of the solution, ionic strength, temperature and concentration of the solution in which the dyeing action is taking place (Clonis *et al.*, 1987). A dye molecule is made up of interdependent molecular units of chromophores and auxochromes. The chromophore is responsible for the colouristic feature of the dye molecule and it comprises a conjugated ring system that absorbs light energy. The chromophore system contains a number of π-electrons that are loosely bound, and if the aromatic or conjugated system contains enough π-electrons, the chromophore absorbs electromagnetic radiation. The light energy absorbed by the chromphore is often in the UV region of the spectrum. The number of loosely bound π-electrons remains too small to absorb the longer wavelengths of the visible spectrum. The auxochromes increase the number of loosely bound electron in the conjugated system, which shifts the absorbance towards the longer wavelength. This is because the auxochrome contains atoms such as sulfur, nitrogen and oxygen with lone pairs of electrons that add to the π-electron system and modify or intensify the spectral absorbance of the dye (Prentø, 2001).

The ability of a particular dye to bind to a particular substrate depends on the physicochemical properties of the dye and the substrate. Dye binding is of significant importance to Biochemistry and other disciplines of biomedical sciences. Dyeing, such as any other chemical reaction has an underlying physical and chemical principle, which implies that certain chemical interactions mediate the binding of dyes to their substrates. Therefore a fundamental understanding of intermolecular forces, the properties of the solvent and physical-chemical properties of the macromolecule (substrate) and the dyes is a

prerequisite to understanding the principles of dye binding (Prentø, 2001). Aromatic dyes tend to bind preferentially to the active sites of globular proteins (Glazer, 1970; Edward and Woody, 1979). This may be the result of non-specific interaction, such as general hydrophobicity, or as a result of a specific arrangement of charged groups, hydrophobic regions and hydrogen-bond donors (Glazer, 1970).

The chemical structure of dye molecules plays a significant role in binding of the dye to substrates. A greater part of the molecular architecture of a dye is made up of a conjugated heteroatomic ring structure that gives a dye a colouristic property. Attached to the heteroatomic ring structure are simple atoms or molecules, which enhance the solubility of the dye in aqueous enviroments (Prentø, 2001). Hydrogen bonding, ionic bonding, covalent bonding, hydrophobic bonding and van der Waal forces of attraction play major role in stabilising the binding of dye to biomolecules (Horobin and Bennion, 1973).

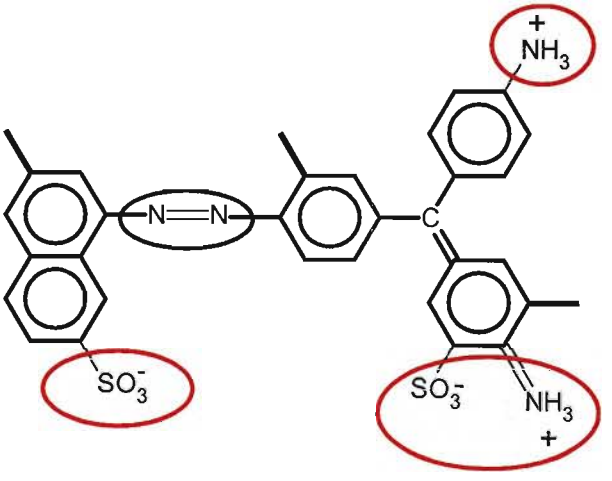


Figure 1.1 A hypothetical dye molecule
The hydrophobic parts are shown in black; the hydrophilic parts are surrounded by a red circle to indicate strong hydration and green circle to indicate weak hydration. In the triaminotriphenylmethane grouping at the right, the positive charge is actually part of the resonance system, and is delocalised (Prentø, 2001)

In addition to the coloured nature of dyes, some dyes resemble coenzymes such as folate, flavin mononucleotide and adenosine triphosphate and would be expected to follow similar principles in their reactions with macromolecules (Apps and Gleed, 1976; Thompson and Stellwagen, 1976; Clonis *et al.*, 1981; Conover and Schneider, 1981). This analogy between dyes and natural protein ligands, in addition to their low cost, has greatly influenced the use of dyes in protein and enzyme technology (Clonis *et al.*, 1985). Studies have also shown that strong binding of dyes to simple globular proteins takes place predominantly in areas overlapping the binding sites for substrates, coenzymes and prosthetic groups, in preference to other region of the protein surface. It is proposed that this phenomenon is a reflection of the special stereochemical features of sites and their hydrophobicity relative to other portions of the protein surface, and, possibly, greater flexibility in these regions of protein molecules (Glazer, 1970; Sehorn *et al.*, 2002).

Highly reactive dyes, such as the procion dyes are widely used in dye-affinity chromatography. Most procion dyes are structural analogues of the natural substrate of these enzymes. More recently, molecular modelling techniques called biomimetic modelling, have been applied in such a way that structural changes are made to some parent dyes to enhance their effectiveness in binding to enzymes by mimicking the natural ligands of these enzymes (Labrou *et al.*, 1996). Dyes bound at enzyme active sites have been subsequently used as direct spectroscopic probes of enzyme structure, function and conformational change (Edward and Woody, 1979; Federici *et al.*, 1985; Sehorn *et al.*, 2002). Cibacron Blue 3GA is an example of a dye that binds naturally to proteins containing dinucleotide folds (Thompson and Stellwagen, 1976) and it used in dye affinity chromatography (Stellwagen, 1990).

The macroscopic and microscopic characterization of biomolecular structures is due to selective colouration of these molecules by different types of dyes. Coomassie Blue is the most widely used dye in protein detection on polyacrylamide gels and in aqueous solution. This analytical application is important in biochemical analysis during protein purification (Schaffner and Weissman, 1973). Besides Coomassie Blue, other dyes and dye mixtures have been used to improve the sensitivity of protein detection on SDS-PAGE (Lin *et al.*,

1991; Choi *et al.,* 1996; Jung *et al.,* 1998). Haematoxylin, Alcian Blue 8GX and Fuchsin-based periodic acid-Schiff stains are used in the detection of Carbohydrates and glycoproteins on polyacrylamide gels during electrophoresis (Scott and Dorling, 1965; Murgatroyd and Horobin, 1969). Phospholipids and lipoproteins can be determined qualitatively and quantitatively using Victoria Blue B and Victoria Blue R (Eryomin and Poznyakov, 1989).

1.2 The basic concept of colour

An object can absorb light strongly, partially or may not absorb light at all. The part of light not absorbed by matter is reflected by the matter and transmitted to reach the retina of human eye. The reflected light wave ranges from 400 nm to 700 nm. The region of the electromagnetic spectrum that can be seen by man is known as the visible region of the spectrum and lies between 400 nm and 700 nm. The light wave initiates a photochemical reaction in the visual pigment of the eye. This reaction is then transmitted to the brain for the perception of visual colour. By and large, the perception of visual colour is, however, dependent on certain physical, physiological and even psychological processes that vary from one individual to another. Visual perception of different colours depends on the range of wavelength of the visible light that reaches the visual centre of the eye. If light in all range of the visible spectrum is transmitted by an object and reaches the eye, the brain interprets the perception as white colour. If on the other hand, no light is transmitted to the eye by an object (all region of the visible spectrum is absorbed), the brain interprets it as black colour. If however, the eye receives a constant fraction of the visible spectrum, the brain perceives it as grey colour (Zollinger, 1991).

White, black and grey are characterized by a constant absorption in the visible spectrum range. They have constant absorption of light energy over a wide range of wavelength (400 – 700 nm). These three colours are therefore called achromatic colours. Contrary to achromatic colours are chromatic colours that show maximum absorption of light energy within a narrow range of the visible spectrum. Any matter characterized by chromatic colour has a particular colour depending on the region of the visible spectrum at which the

matter absorbs light maximally. A solid that absorbs light energy maximally between 400 nm and 430 nm, and reflects the rest (430 – 700 nm) will be perceived by the brain as a yellow solid. A solid will appear orange, red, violet and blue if the solid absorbs light energy maximally at 430 – 480 nm, 480 – 550 nm, 550 – 600 nm and 600 – 700 nm respectively. A green coloured matter is characterized by two maximum absorptions at 400 – 450 nm and 580 – 700 nm (Zollinger, 1991). Absorption is sometimes likely to take place in more than one region of the visible spectrum and the intensity by which light is absorbed affects the colour perceived by the eye. Since light is made up of radiations of different wavelength and the velocity of the whole wave is constant, then the shorter the wavelength of a single component radiation, the higher the frequency of its wave. Conversely, the longer the wavelength, the lower the frequency of the wave (Gurr, 1965).

Coloured molecules such as dyes exhibit similar colour absorption to a coloured material. The conjugated ring system (chromophore) in a dye molecule responsible for the absorption of electromagnetic radiation can absorb light in the visible region of the electromagnetic spectrum due to their unsaturated nature. The presence of loose π electrons in the conjugated double bonds of the ring system causes the dye molecule to absorb in the visible region of the spectrum. The intensity of the chromophore is enhanced by auxochromes attached to the chromophore group in the dye molecule. The presence of auxochromes causes the dye molecule to shift its absorption to light energy with a longer wavelength in the visible region. This shift from shorter wavelength to longer wavelength is called *bathochromy*. The opposite of bathochromy is called *hypsochromy* (Zollinger, 1991).

According to Zollinger (1991), light absorption is an energy process that portrays frequency as a function of wave number. For the visible range, spectra are generally represented as a function of wavelength, as the perceived colour differences are then more evenly distributed. Therefore the position of the maximum of the absorption band (λ_{max}) of a chromogenic molecule, such as a dye, is given as a wavelength. This implies that light absorption can be treated quantitatively. The number of lone pair electrons in a molecular entity is related to the amount of light absorbed and the absorption intensity may be

expressed spectrophotometrically according to the Beer-Lambert law for a chromogenic sample solution according to the equation:

$$-\mathbf{Log}\ (I/I_0) = \mathbf{A} = \varepsilon d\, c.$$

Where

A = absorbance

I = intensity of light transmitted through the sample

I_o = intensity of light that passed through a sample solution

ε = absorbtion coefficient or absorptivity

d = distance of path of light through the sample

c = concentration of absorbing material in the sample (Boyer, 1993).

For technical dyes, ε has values ranging from 10^4 to more than 10^5 L mol^{-1} cm^{-1} (Zollinger, 1991).

1.3 Overview of the classification of dyes

Dyes and other colourants and pigments can be classified based on (1) area and method of applications, (2) chemical structure and (3) the type of electronic excitation occurring upon light absorption. The nature and method by which these dyes are synthesised makes it difficult to categorically classify a particular dye by these three criteria. In other words, dye classification often overlaps. Therefore, there is hardly a chemical class of dye that occurs solely in one colouristic group or class. When dyes are classified according to dyeing method, they are referred to as anionic (acidic), cationic (basic), direct, reactive, mordant and disperse dyes. When dyes are to be classified based on chemical structure, they are classified as polyenes, polymethine, di- and triarylmethine dyes and their aza [18] annulene analogues, azo dyes, anthaquinone and diphenodioxazine dyes. On the basis of the type of electronic excitation occurring on light absorption, dyes and other colourants could be classified as absorption colourants, fluorescent colourants and energy transfer colourants depending on whether the light energy absorbed is dissipated predominantly (with high quantum yields) by internal conversion, by fluorescence or by inter-system crossing (Zollinger, 1991).

Dyes used in this study will be classified based on the parent chemical structure (refer to Zollinger, 1991 for detailed description of parent chemical structures of dye and pigments) and the dyeing method. Therefore it is important to be aquatinted with some terminologies used in describing the area and method of application of dyes, namely anionic (acidic), cationic (basic), reactive, direct, mordant (ligand chelating) and disperse (colloidal) dyes.

1.3.1 Anionic (acid) dyes

These are dyes with predominantly negative charges often due to the presence of two or more sulfonate (SO_3^-) groups on the dye molecule. These negative ions increase the solubility of the dye in aqueous solution while decreasing the solubility of the dye in ethanol. Dye molecules with only one negative charge group (O^-) are not included in this class (anionic dyes) of dyes. This is because the charge-bearing electron enters the conjugated ring or double bond system. As a result, the electron is delocalised instead of being a point charge. Most anionic dyes do not form aggregates in aqueous solution due to the high number of negative charges per dye molecule. The higher the negative charge density, the lesser the tendency to aggregate in aqueous solution. However, high molecular weight anionic dyes such as Congo Red, Reactive Red 120 and Evans Blue may form aggregates due to their large sizes (Prentø, 2001)

1.3.2 Cationic (basic) dyes

Cationic dyes are characterized by the presence on a single delocalised cation that forms part of the conjugated aromatic ring chromophore group. This single positive charge is as a result of electron deficit in the molecular entity. The presence of the single positive charge on the cationic dye molecule reduces the solubility of the dye in water, while increasing its solubility in ethanol. Dyes with more than one positive charge are not included in this class of dyes. An example is Alcian Blue 8GX, which is classified as a ligand or chelate dye. Cationic dyes are capable of forming aggregates by stacking. The degree of stacking depends on the concentration of the dye in the solution. At high dye concentrations, the dye molecules readily stack to form aggregates. On the other hand, low stacking becomes evident at low dye concentration (Prentø, 2001).

1.3.3 Reactive dyes

Reactive dyes are organic coloured compounds with functionally reactive groups capable of forming covalent bonds with the substrate. These dyes contain mainly mono-or dichlorotriazinyl reactive functional groups (Figure 1.2) conjugated to the main dye molecule. These reactive functional groups are very reactive to oxygen, sulfur, or nitrogen atoms of a hydroxyl, an amine or a mercapto-group on the substrate molecule. Such covalent bonds can be formed with hydroxyl groups of cellulosic fibre, hydroxyl, mercapto and amino groups of protein fibres and amino groups of polyamides. The reaction mechanism is usually by (1) nucleophilic bimolecular substitution, (2) nucleophilic addition, (3) multiple addition and elimination reaction mechanism and (4) esterification (Stellwagen, 1990).

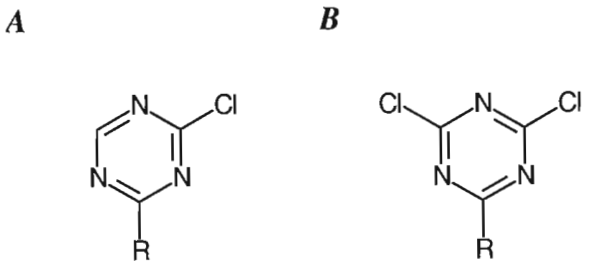


Figure 1.2 Monochlorotriazinyl (*A*) and dichlorotriazinyl (*B*)
These functional groups form the integral part of a reactive dye.

Reactive dyes are widely used in dye-affinity chromatography for the separation of proteins and enzymes that naturally bind to these dye molecules. A very good example of dyes in this class are the Procion dyes, such as Cibacron Blue 3GA (Clonis *et al.*, 1987). These dyes are covalently attached to a carbohydrate matrix by nucleophilic addition via the hydroxyl groups on the matrix and the highly reactive chlorotriazine functional group of the dye. Figure 1.3 illustrates the reaction mechanism involving a chlorotriazine-containing reactive dye and a hydroxyl-containing substrate such as a carbohydrate polymer e.g. cellulose, agarose or dextran. The nucleophilic oxygen atom on the matrix in a basic medium displaces the chlorine atoms of the chlorotriazine. This leaves the dye covalently attached on the matrix (Stellwagen, 1990).

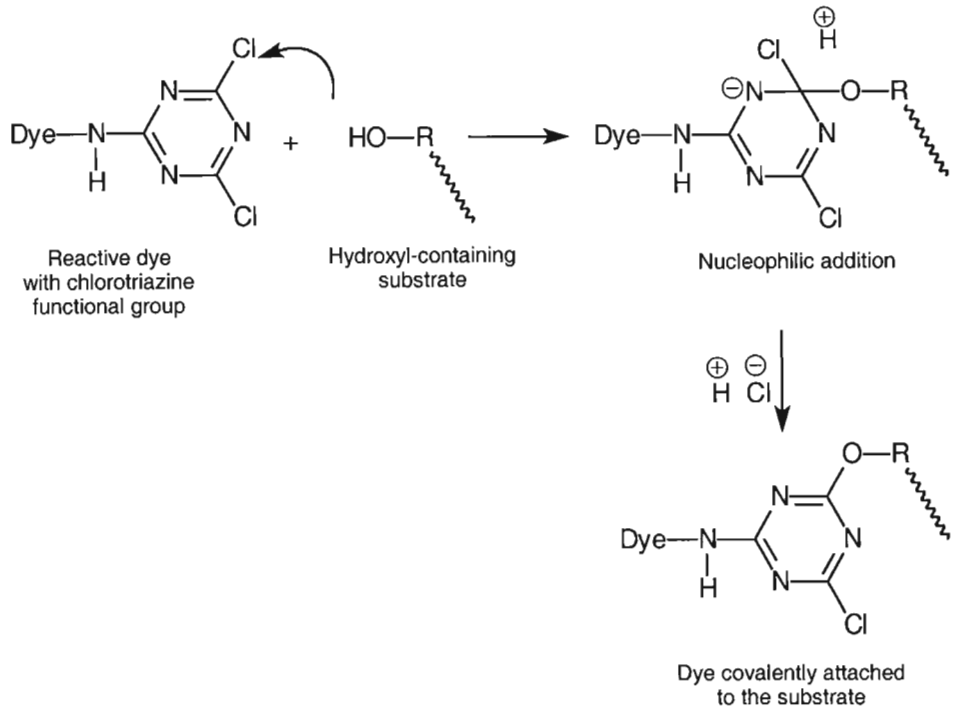


Fig 1.3 Covalent immobilisation of chlorotriazine-containing reactive dye on a hydroxyl-containing substrate by nucleophilic addition (Clonis *et al.*, 1987)

1.3.4 Direct dyes

Direct dyes (also known as substantive dyes) are chromogenic compounds, which are able to dye cellulose fibres without the aid of mordants (tannins). The name "direct" stems from the fact that reactive functional groups on these dyes form part of the general structure of the dye. Examples are Direct Red dyes (Direct Red 75 and 81), Direct Yellow dyes (Primuline) and Direct Blue Dyes (Evans Blue and Trypan Blue) (Zollinger, 1991).

1.3.5 Disperse dyes

These are dyes originally produced to dye cellulose acetate as well as synthetic fibres. They have very low solubility in water, but are highly soluble in ethanol and other organic solvents. In aqueous solution, disperse dyes readily aggregate to form micelles or dye sols due to the absence of hydrophilic groups capable of hydrogen bonding in water (Zollinger, 1991).

1.3.6 Ligand (chelating) dyes

Ligand dyes are coloured organic compounds capable of forming complexes with di- or trivalent metals. This complex formation with metals is due to the presence of orthophenol or similar groupings in the aromatic ring system. The complex between the dye and the metal is called a metal-dye complex, a metallochrome, or a dye-lake. Ligand dyes are mostly weak acids. When ligand dyes combine with divalent or trivalent ions such as Cu^{2+}, Fe^{3+} and Al^{3+}, a large positively charged metal complex is formed. This metal complex resembles cationic dyes. These complexes often interact with negatively charged biomolecules such as nucleic acids and mucopolysaccharides. A ligand dye has to have two or more ligand groups in order to form metal complexes. These ligand groups have to be hydroxyl, carboxyl or amino groups in an azo dye or other forms of dyes. The ligand occupies two or more coordination sites of the metal ion and thus can form mono-, bi-, tri-cyclic complex (chelates). Examples of ligand dyes are hematein (chelates Al^{3+}), Acid Blue 193 (chelates Cr^{3+}) and Eriochrome Black T (chelates Cr^{3+}) (Prentø, 2001).

1.4 Classification and chemistry of the dyes used in this study

A total of twenty eight dyes of various classes were used in this study. Dyes will be classified based on area and method of application and emphasis will be placed on their chemical structure, molecular weight, absorbance maximum and solubility in water. Their application will be mentioned in brief with related references. The classification of Nigrosin Water-soluble was not done due to lack of information on the structural properties of the dye. C.I. in the text is an abbreviation for colour index. Not all dyes have a colour index number. The structural formula of each dye was obtained from the Sigma web site (www.sigma-aldrich.com). Dyes names will be written as proper nouns, as they are mostly trade names. This method is in line with the naming of dyes in Colour Chemistry (Zollinger, 1991), Dyes, Indicators and Intermediates (1995, Aldrich Chemical Company Inc., international edition) and the Sigma-Aldrich web site (www.sigma-aldrich.com).

1.4.1 Acridine Orange [C.I. 46005]

Synonyms: Basic Orange 14; Molecular weight: 472.5; Molecular Formula: $C_{17}H_{19}N_3$

λ Maximum: 489 nm

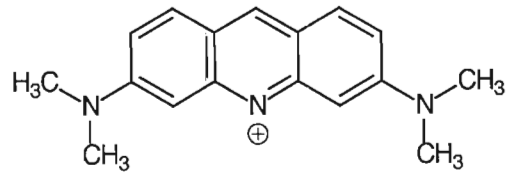


Figure 1.4 Acridine Orange

Acridine Orange (Figure 1.4), also known as Basic Orange 14, is a basic dye of the heteroatom-bridged diarylmethine class of dyes (Zollinger, 1991). It has one delocalized positive charge. Acridine Orange is not very soluble in water, but is highly soluble in ethanol. It is a fluorescent dye with maximum emission at 519 nm. Its positively charged nature makes it useful in staining negatively charged macromolecules such as nucleic acids. Acridine Orange can give rise to green nuclear fluorescence, contrasting with red emission when bound to the RNA-rich cytoplasm. The phenomenon by which a dye or a fluorochrome (such as Acridine Orange) gives rise to two or more different colours when bound by non-covalent interaction to different tissue components is called *metachromasia.* Hence the dye is used to differentiate DNA from RNA (Horobin, 1988). Acridine Orange is used as a trans-membrane pH gradient probe (Palmgren, 1991; Clerc and Barenholz, 1998) and it is also used as a competitive inhibitor of protein kinase C (Hannun and Bell, 1988).

1.4.2 Alcian Blue 8GX

Synonyms: Alcian Blue, Ingrain Blue; Molecular weight: 1298.88; λ Maximum: 615 nm.

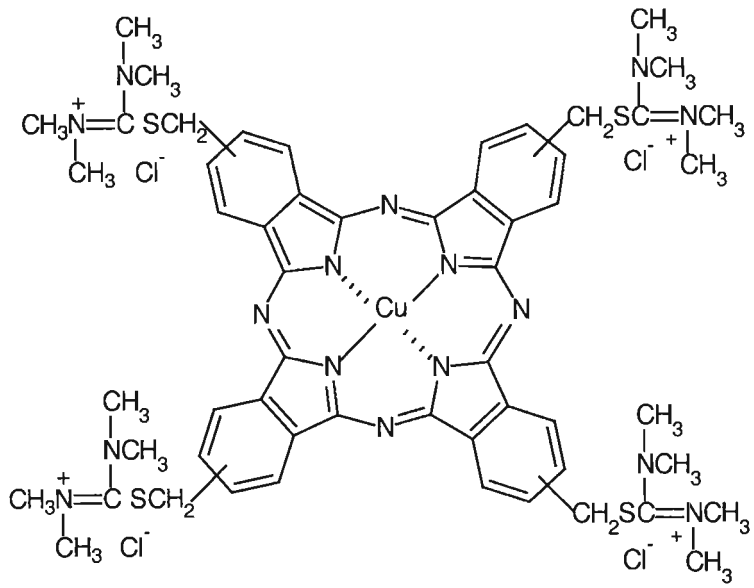


Figure 1.5 Alcian Blue 8GX

Alcian Blue 8GX (Figure 1.5) is a ligand (metallochrome) dye of the aza-annulene subclass of polymethine dyes (Zollinger, 1991). It forms metal complexes with the copper (II) ion. Alcian Blue 8GX is a dark brown powder and is deep blue when suspended in water. It is predominantly dimeric in water, with eight positive charges that gives the dye a large hydrated volume. Its dimeric nature has three effects on the dye molecule: it substantially shields the aromatic center against non-polar interactions, it impedes the penetration of the dye in to the less hydrated spaces of tissue sections and it increases charge repulsion by positively charged tissue proteins (Prentø, 2001). This makes the dye a good stain for negatively charged acidic glycosaminoglycans. Alcian Blue 8GX is used in histochemistry as a stain for detection of acidic glycosaminoglycans (Scott and Dorling, 1965; Lillie *et al.*, 1976; Marshall and Horobin, 1973).

1.4.3 Aurintricarboxylic Acid

Molecular weight: 422.35; Molecular Formula: $C_{22}H_{14}O_9$; Dye content: *ca* 85%;

λ Maximum: 552 nm

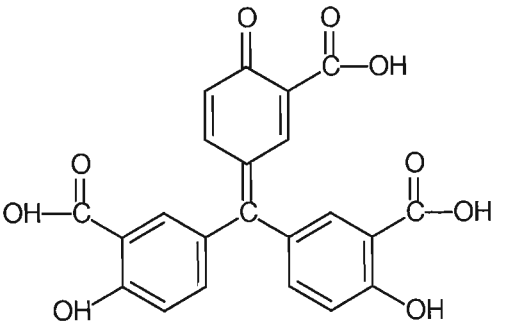


Figure 1.6 Aurintricarboxylic Acid

Aurintricarboxylic Acid (Figure 1.6) is an acidic dye of the class of triarylmethine (parental structure) dyes (Zollinger, 1991). It is a dark-red powder and gives a dark brown colouration in solution with water. Aurintricarboxylic Acid is a very attractive dye with various biochemical and physiological actions. It readily polymerizes in aqueous solution forming free radical that inhibits protein-nucleic acid interactions with the potential to inhibit ribonuclease and topoisomerase II (Gonzalez *et al.,* 1980; Nakane *et al.,* 1988; Rui *et al.,* 1998) activity. Aurintricarboxylic Acid stimulates tyrosine phosphorylation in lymphoma cells and inhibits apoptosis in many cell types (Okada and Koizumi, 1995; Okada and Koizumi, 1997; Aronica *et al.,* 1998).

1.4.4 Azure A [C.I. 52005]

Molecular weight: 291.8; Molecular Formula: $C_{14}H_{14}ClN_3S$; Dye content: *ca* 80%;
λ Maximum: 633 nm.

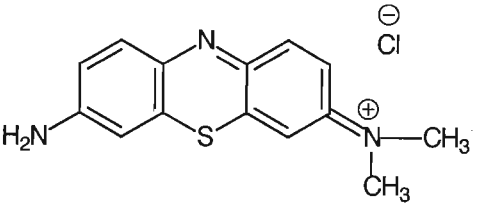


Figure 1.7 Azure A

Azure A (Figure 1.7) is a basic (due to the presence of only one positive charge) thiazine dye under the class of aza-analogues of diarylmethine dyes. It has a green appearance when dry and becomes dark violet when suspended in water. It is very soluble in water and other organic solvents. It is a certified biological stain used in the staining of tissues, cell granules and microorganisms. Azure A, in conjunction with Toluidine Blue and Methylene Blue, is used for the differentiation of metachromatic granules of the diphtheria organism (Gurr, 1960; Horobin, 1988).

1.4.5 Azure B [C.I. 52010]

Synonyms: Azure 1, Methylene Azure, *N,N,N'*-Trimethylthionin; Molecular weight: 305.84; Molecular Formula: $C_{15}H_{16}ClN_3S$; Dye content: *ca* 80%; λ Maximum: 648 nm.

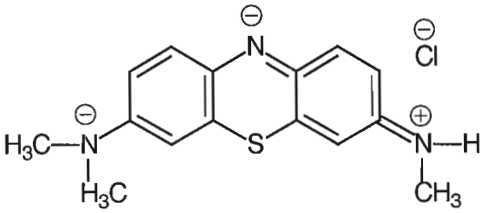


Figure 1.8 Azure B

Azure B (Figure 1.8) is a close structural analogue of Azure A. It is a basic thiazine dye in the same class with its analogue; Azure A. Azure B is green in colour when dry, but blue in solution with water. Azure B is extensively used in histology and histochemistry as tissue and macromolecular stains. It forms complexes with molecules such as RNA, DNA, and chrondroitin sulfate. It used to differentiate between cellular RNA and DNA in plant tissues, due to it ability to form complexes with different spectral characteristics (Zipfel *et al.,* 1984; Hüglin *et al.,* 1986).

1.4.6 Basic Fuchsin [C.I. 42500]

Synonyms: Basic Parafuchsin, Basic Red 9, Magenta O, Parafuchsin hydrochloride, Paramagenta hydrochloride, Pararosaniline chloride, Pararosaniline hydrochloride;

Molecular weight: 323.8; Molecular Formula: $C_{19}H_{17}N_3.HCl$; Dye content: *ca* 90%; λ Maximum: 544 nm.

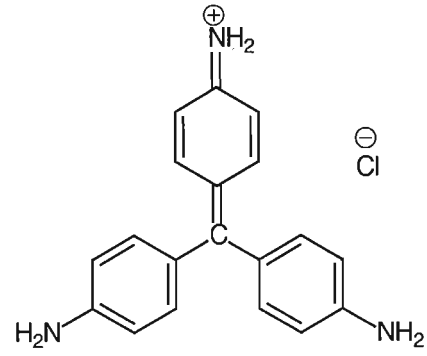


Figure 1.9 Basic Fuchsin

Basic Fuchsin (Figure 1.9) is a cationic dye of the triarylmethine class of dyes (Zollinger, 1991). It has a green to dark-green appearance in dry powder form. Basic Fuchsin not very soluble in water and ethanol but is very soluble in DMSO and DMF. Basic Fuchsin is a histological stain for bacterial cells in combination with Methylene Blue (Horobin, 1988). It is also used in the Feulgen Stain for nuclear staining (Fukuda *et al.*, 1979; Horobin, 1988).

1.4.7 Bromocresol Purple

Synonym: Bromocresol Purple; Sultone form; 5,5¢ dibromo-o-cresolsulfonaphthalein; Molecular weight: 540.2; Molecular Formula: $C_{21}H_{16}Br_2O_5S$; λ Maximum: 419 nm.

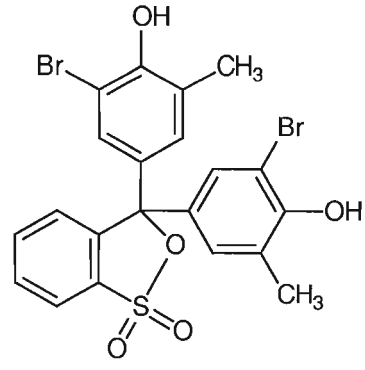


Figure 1.10 Bromocresol Purple

Bromocresol Purple (Figure 1.10) is an anionic (acidic) dye with one sulfonate group and two bromine atoms. It is a pink powder in dry form and purple in water (near neutral pH). It is sensitive to pH changes (pH range 5.2 to 6.8, yellow to purple), hence it used as a pH indicator for chemical and biochemical applications. Bromocresol Purple is widely applied in clinical chemistry for the determination of serum albumin, as the dye has an affinity for albumin (Assink *et al.,* 1984; Affonso and Lasky, 1985; McGinlay and Payne, 1988; Nakamura and Sato, 1997).

1.4.8 Cibacron Blue 3GA

Molecular weight: 774.2; Molecular Formula: $C_{29}H_{20}ClN_7O_{11}S_3$; Dye content: *ca* 60%

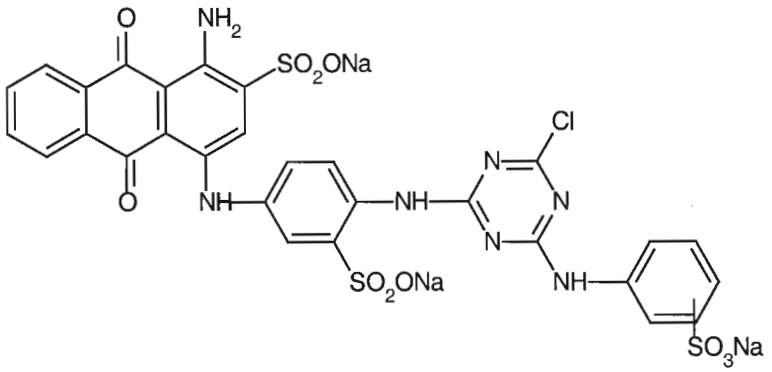


Figure 1.11 Cibacron Blue 3GA

Cibacron Blue 3GA (Figure 1.11) is a member of the triazine dyes. It has one anthaquinone molecule and three sulfonate groups that confer water solubility as well as acidity. Cibacron Blue 3GA is a dark-blue powder in dry form and forms a dark blue coloured solution with water. Cibacron Blue 3GA-immobilized agarose (Dye-immobilized agarose) is used in dye affinity chromatography (Thompson and Stellwagen, 1976; Clonis and Lowe, 1980; Stellwagen, 1990). Besides it application in chromatography, Cibacron Blue 3GA is used as a conformational probe in the study of protein structure (Apps and Gleed, 1976; Federici *et al.,* 1985).

1.4.9 Coomassie Blue R 250 [C.I. 42660]

Synonyms: Acid Blue 83, Brilliant Blue R 250; Molecular weight: 825.99; Molecular formula: $C_{45}H_{44}N_3O_7S_2$; Dye content: *ca* 90%; λ Maximum: 585 nm.

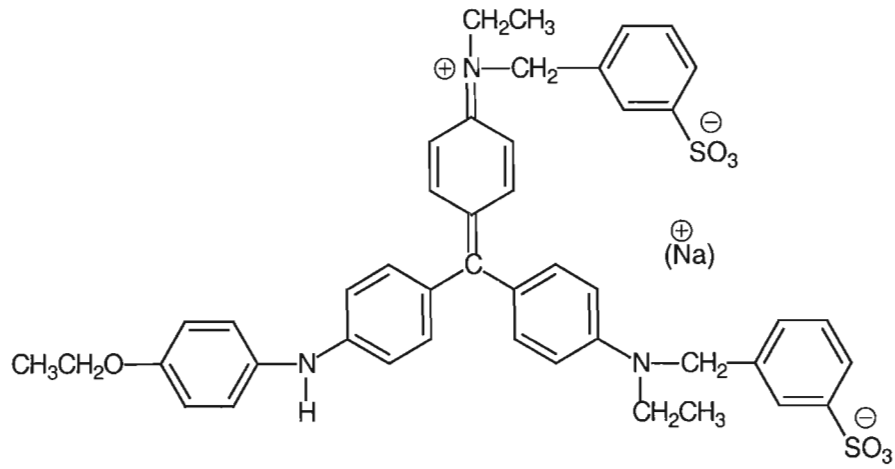


Figure 1.12 Coomassie Blue R 250

Coomassie Blue R 250 (Figure 1.12) is an acidic dye with triarylmethine parent structure (Zollinger, 1991). It has two sulfonate groups that contribute to the overall charge of the molecule. This makes it an anionic or acidic dye. The dye is a dark purple powder that turns deep blue in solution with water. It is not very soluble in water but very soluble in organic solvents such as methanol and ethanol. In methanol-acetic acid solution, Coomassie Blue R 250 forms a very good stain for detection of proteins resolved on electrophorectic gels (Fazekas De St. Groth *et al.,* 1963).

1.4.10 Direct Red 75 [C.I. 25380]

Synonyms: Benzo Fast Pink 2BL, Sirius Red BB, Chloroazol Fast Pink BK; Molecular weight: 990.8; Molecular Formula: $C_{33}H_{22}N_8Na_4O_{15}S_4$; Dye content: *ca* 30%; λ Maximum: 508 nm

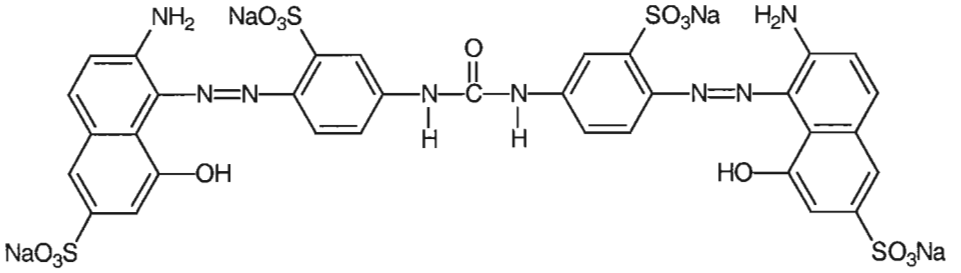


Figure 1.13 Direct Red 75

Direct Red 75 (Figure 1.13) is an acidic dye of the disazo series (Zollinger, 1991) with four sulfonate groups that confers water solubility and acidity. It is a dark-red maroon powder that turns deep red in solution with water. Direct Red 75 has anticoagulant properties (Sischy *et al.*, 1980).

1.4.11 Direct Red 81 [C.I. 28160]

Molecular weight: 675.6; Molecular Formula: $C_{29}H_{19}N_5Na_2O_8S_2$; Dye content: *ca* 50%; λ Maximum: 522 nm

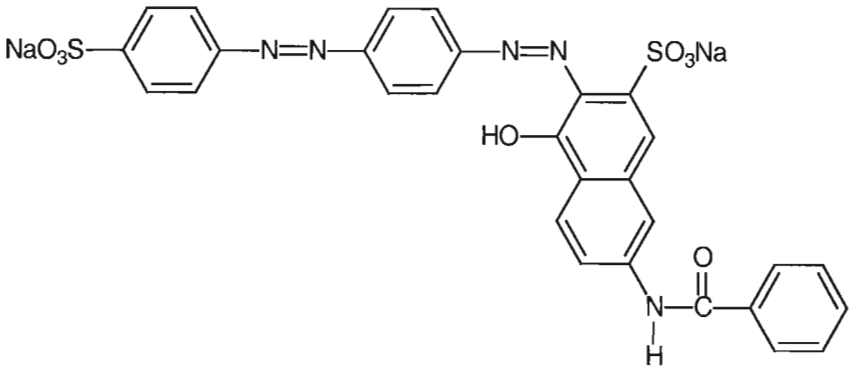


Figure 1.14 Direct Red 81

Direct Red 81 (Figure 1.14) is an acidic dye of the disazo series of direct azoic dyes (Zollinger, 1991). It has a dark brown to red brown appearance in dry form and red in solution with water. Little information could be found about this dye.

1.4.12 Eriochrome Black T

Molecular weight: 461.39; Molecular Formula: $C_{20}H_{12}N_3NaO_7S$; Dye content: *ca* 60%; λ Maximum: 503 nm.

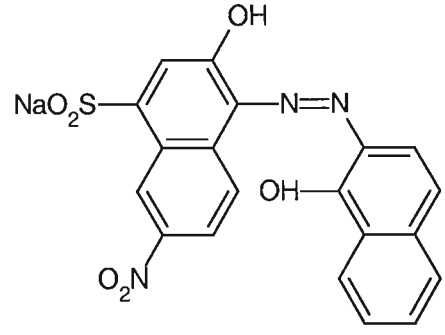


Figure 1.15 Erichrome Black T

Eriochrome Black T (Figure 1.15) is an acidic ligand dye of the mono-azo series. Eriochrome Black T supplemented with Rhodamine B can be used to detect proteins on polyacrylamide gels (Jung *et al.*, 1998). It is used as a pH indicator and it is very suitable for use as an EDTA indicator. Its ability to form chelates makes it suitable for complexiometric analysis of divalent metals. When a solution of dye chelated metal is titrated against a powerful complexing agent such as EDTA, the end point is made apparent by a brilliant colour change, which indicates that the metal has been extracted from the dye (Gurr, 1960; www.sigma-aldrich.com).

1.4.13 Ethyl Red

Synonyms: 4-(Diethylamino)azobenzene-2'-carboxylic acid; Molecular weight: 297.4; Molecular Formula: $C_{17}H_{19}N_3O_2$; Dye content: *ca* 97%; λ Maximum: 447 nm.

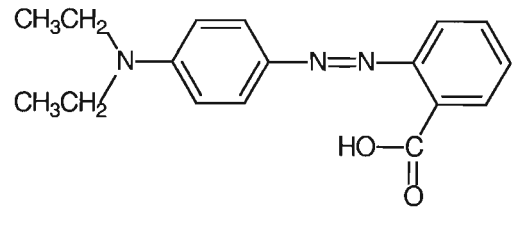


Figure 1.16 Ethyl Red

Ethyl Red (Figure 1.16) is an acidic dye of the monoazo series. It is a dark maroon powder that is insoluble in water but very soluble in solvents such as DMF and DMSO. Ethyl Red is sensitive to pH changes. It turns yellow at pH above 5.0 and maintains its red colour at pH below 5.0 (www.sigma-aldrich.com).

1.4.14 Evans Blue [C.I. 23860]

Synonym: Direct Blue 53; Molecular weight: 960.8; Molecular Formula: $C_{34}H_{24}N_6Na_4O_{14}S_4$; Dye content: *ca* 80%; λ Maximum: 611 nm.

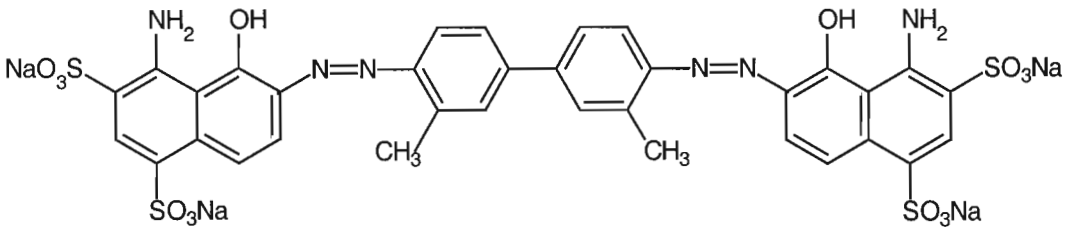


Figure 1.17 Evan Blue

Evans Blue (Figure 1.17), also known as Direct Blue 53, is a member of the direct dye series. It is an acidic dye with two azo (diazo series) groups. It has a symmetrical and planar structure (Skowronek *et al.*, 2000). Evans Blue is dark brown powder in dry form, and it turns deep blue in solution with water. Due to its size and conformational structure, Evans Blue associates readily in water to form a polymolecular dye micelle (Roterman *et al.*, 1993) that forms complexes with immunoglobulin G. Evans Blue is a strong competitive inhibitor of deoxyribonucleic acid polymerase (Nakane *et al.*, 1988) and it is used as a counter stain in immunoflourescent staining (Newkirk and Mark, 1992).

1.4.15 Gallocyanine

Molecular weight: 336.73; Molecular Formula: $C_{15}H_{16}N_2O_5Cl$; Dye content: *ca* 90%; λ Maximum: 601 nm.

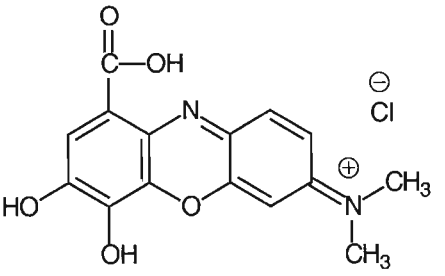


Figure 1.18 Gallocyanine

Gallocyanine (Figure 1.18) is a bluish-violet basic dye of the oxazine series that is sometimes employed in the form of iron lake as a substitute for haematoxylin. It is soluble in water but more soluble in ethanol (Gurr, 1960). It appears as a black powder, which gives a deep blue colouration in water. Gallocyanine, in combination with chromium (III) metal complex, is called Gallocyanine-chromalum. This complex is widely applied in histology/histochemistyry as a nuclear stain for DNA and RNA in tissue sections (Vejlsted, 1969; Horobin, 1988; Schulte *et al.,* 1991; Schulte *et al.,* 1992). Gallocyanine is also used in the quantification of acetylcholine (Steinhart, 1968).

1.4.16 Hematoxylin [C.I. 75290]

Synonym: Natural Black; Molecular weight: 302.3; Molecular Formula: $C_{16}H_{14}O_6$

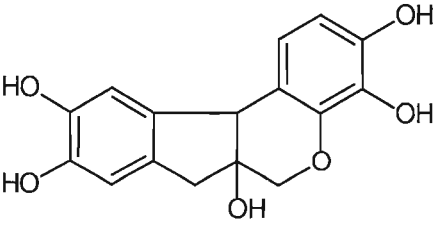


Figure 1.19 Hematoxylin

Hematoxylin (Figure 1.19) is a ligand (mordant) dye with five hydroxyl groups and an O-substituted hexane ring. It is slightly soluble in water but very soluble in ethanol. It gives a yellow colouration in ethanol. Hematoxylin forms dye lakes (dye-metal complex) with

metals such as Fe^{2+}, Fe^{3+} and Al^{3+}, to become a Hematoxylin-metal complex. These metal complexes are used to stain animal tissues and nucleic acids (Lillie and Pizzolato, 1972; Marshall and Horobin, 1973).

1.4.17 Malachite Green HCl [C.I. 42000]

Synonym: Basic Green 4; Molecular weight: 382.9; Molecular Formula: $C_{23}H_{26}N_2O.HCl$; Dye content: *ca* 80%; λ Maximum: 615 nm.

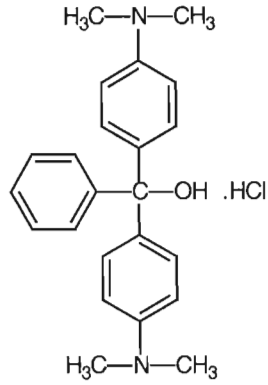


Figure 1.20 Malachite Green HCl

Malachite Green HCl (Figure 1.20) is a basic dye of the triarymethine class (Zollinger, 1991). It appears dark green in both dry powder form and when suspended in water. Malachite Green HCl is used in affinity chromatography for separation of DNA due to its ability to form complex with AT-rich region on native DNA (Weber and Cole, 1982; Fox *et al.*, 1992). It also applied in histochemistry as stain for nucleic acids. Malachite Green HCl is used, in combination with gluteraldehyde, as a fixative and preservative for revealing lipid complexes in thin sections of eukaryotic cells for electron microscopy (Kushnaryov *et al.*, 1979). Baykov *et al.* (1988) used Malachite Green HCl for a colourimetric assay for protein phosphorylase activity based on the determination of released inorganic phosphate.

1.4.18 Methyl Green, zinc chloride salt

Molecular weight: 653.25; Molecular Formula: $C_{27}H_{35}N_3BrCl.ZnCl$; Dye content: *ca* 80%; λ Maximum: 629 (423) nm.

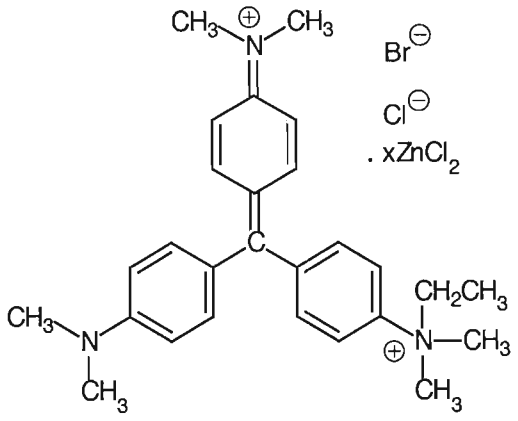


Figure 1.21 Methyl Green

Methyl Green (Figure 1.21) is a basic dye of the triarylmethine class (Zollinger, 1991). It is a dark-brown powder with a red cast. Methyl Green is very soluble in water, producing a green colouration. Methyl Green forms complexes with DNA (Krey and Hann, 1975; Kim and Nordén, 1993). This makes the dye widely used in histological tissue stains for the detection of nucleic acids (Tato *et al.*, 1991). A Methyl Green-Pryonin Y mixture is used for quantitative estimation of DNA and RNA using image cytometry (Schulte *et al.*, 1992). Methyl Green is used for colourimetric determination of DNAse activity (Sinicropi *et al.*, 1994).

1.4.19 Methyl Orange [C.I. 13025]

Synonyms: Acid Orange 52, 4-[4-(Dimethylamino) phenylazo] benzenesulfonic acid sodium salt, Orange III, Helianthin; Molecular weight: 327.3; Molecular Formula: $C_{14}H_{14}N_3NaO_3S$; λ Maximum: 507 nm.

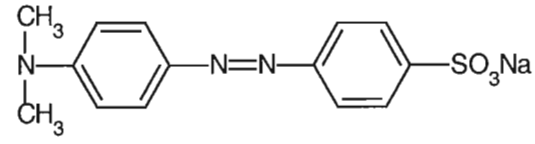


Figure 1.22 Methyl Orange

Methyl Orange (Figure 1.22) is an acidic dye with one sulfonate group that confers both solubility in water and acidity to the dye. It has a single azo group; therefore it is of the monoazo series (Zollinger, 1991). Methyl Orange has an orange to red appearance in dry form, and gives an orange colouration at neutral pH in solution. It is very sensitive to pH changes and it is thus used as a pH indicator for acid-base titrimetry (pink, at pH 3, and yellow at pH 4.4). Methyl Orange is used in the study of charge conservation in macromolecular interactions (Ford and Winzor, 1983).

1.4.20 Naphthol Blue Black [C.I. 20470]

Synonyms: Acid Black 1, Amido Black, Buffalo Black NBR, Amido Schwarz; Molecular weight: 616.5; Molecular Formula: $C_{22}H_{14}Na_2O_9S_2$; Colour Index Number: 20470; Dye content: *ca* 90%; λ Maximum: 618 nm.

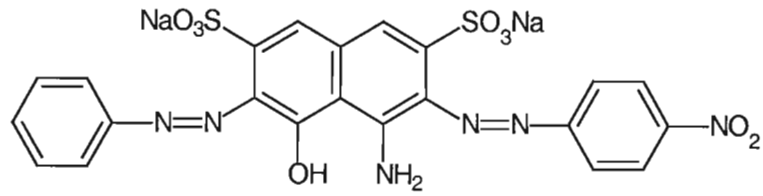


Figure 1.23 Naphthol Blue Black

Napthol Blue Black (Figure 1.23) is an acidic dye with two azo (– N=N) groups, thus making it an acidic azo dye due to the presence of sulfonate groups. It is blue in colour when suspended in water. Naphthol Blue Black can be used to stain proteins on polyacrylamide gels, nitrocellulose membranes and agarose gels. Gels are stained with 0.1% Naphthol Blue Black in 7% (v/v) acetic acid for at least 2 h and destained with 7%

(v/v) acetic acid. Detection sensitivity is 20% that of Coomassie Blue R (Towbin *et al.*, 1979; Wilson, 1983).

1.4.21 Ponceau S [C.I. 27190]

Synonyms: 3-hydroxy-4-[2-sulfo-4-(4-sulfophenylazo]-2,7-naphthalenedisulfonic acid-tetrasodium salt; Molecular weight: 760.58; Molecular Formula: $C_{22}H_{12}N_4Na_4O_{13}S_4$; Dye content: *ca* 75%; λ Maximum: 520 (352) nm.

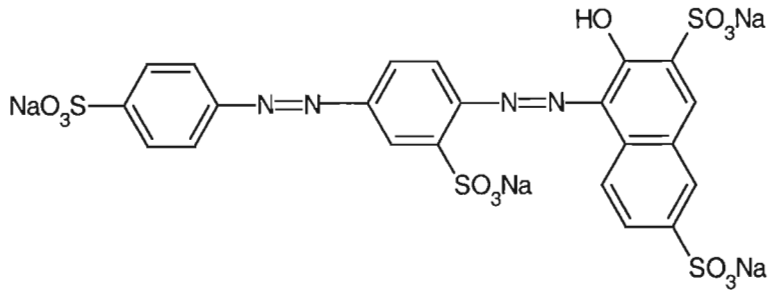


Figure 1.24 Ponceau S

Ponceau S (Figure 1.24) is an acidic dye of the bis-azo series. The dry dye appears to be brown in colour but becomes red when suspended in aqueous solution. It is highly soluble in water and other organic solvents, but less soluble in ethanol. Ponceau S is used in the reversible staining of proteins blotted on nitrocellulose and polyvinylidene difluoride membranes in electro-blotting. Ponceau S is also used for the quantitative estimation of proteins blotted on nitrocellulose and cellulose acetate membranes (Goldring and Ravaioli, 1996; Bannur *et al.*, 1999; Morçöl and Subramanian, 1999).

1.4.22 Primuline

Synonyms: Direct Yellow 59, Primuline Yellow; Molecular weight: 457.5; Molecular Formula: $C_{21}H_{14}N_3NaO_3S_3$; Dye content: *ca* 50%; λ Maximum: 229 (345) nm

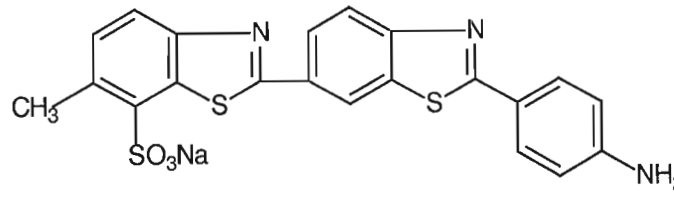


Figure 1.25 Primuline

Primuline (Figure 1.25), (also spelt Primulin) is a member of sulfur dye class with two benzothiazole ring structures. It is a dark-yellow powder or crystal that is sparingly soluble in water and ethanol, but readily soluble in DMSO and DMF. Primunline is used for the quantification of glycosphingolipids and phospholipids by thin-layer chromatography (Taki *et al.*, 1994).

1.4.23 Reactive Black 5

Synonym: Remazol Black B; Molecular weight: 991.82; Molecular Formula: $C_{26}H_{26}N_5NaO_{19}S_6$; Dye content: *ca* 75%; λ Maximum: 597 nm.

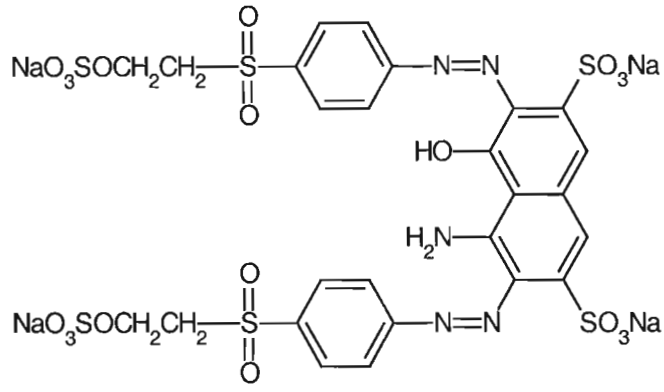


Figure 1.26 Reactive Black 5

Reactive Black 5 (Figure 1.26) is an acidic dye of the disazo series. Unlike Reactive Red 120, Reactive Black 5 does not have the trichloro~s~triazie reactive group in its structure. The acidic nature of the dye is due to the presence of four sulfonate groups, which does not only confer acidity, but also water solubility. Reactive Black appears to be navy blue in solution with water.

1.4.24 Reactive Red 120

Molecular weight: 1469; Molecular Formula: $C_{44}H_{24}Cl_2N_{14}O_{20}S_6$; Dye content: *ca* 60%

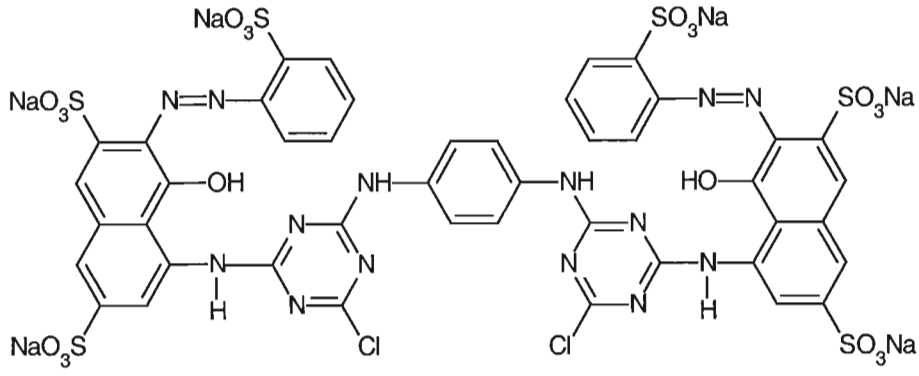


Figure 1.27 Reactive Red 120

Reactive Red 120 (Figure 1.27) is a reactive acidic dye of the bis-azo triazine dyes. It has a brown appearance when dry and deep red in solution with water where it is highly soluble. Being a reactive dye, Reactive Red 120 is capable of binding to nitrogen, oxygen and sulfur atoms on molecules with amine, hydroxyl and thio groups (Zollinger, 1991). This makes it a potential stain for proteins and carbohydrates. Reactive Red 120 and other triazine dyes such as Reactive Black 5 and Reactive Green 19 is used as an affinity ligand for glucosyltransferase in dye-affinity chromataography. The dye inhibits this class of enzymes by interacting with the sugar donor binding sites of glucosyltransferase (Kaminska *et al.*, 1999).

1.4.25 Trypan Blue [C.I. 23850]

Synonym: Direct Blue 14; Molecular weight: 960.8; Molecular Formula: $C_{34}H_{24}N_6Na_4O_{14}S_4$; Dye content: *ca* 70%; λ Maximum: 607 nm.

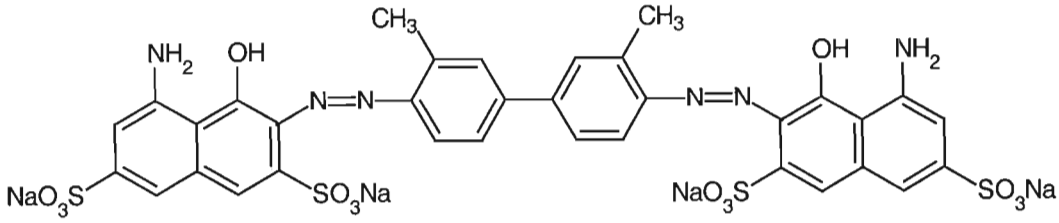


Figure 1.28 Trypan Blue

Trypan Blue (Figure 1.28) is in the class of direct azo dyes. It is a close structural analogue of Evans Blue. It is a dark brown powder and it is blue in suspension with water. Like Evans Blue, Trypan Blue associates with water molecule to form a polymoleculer micelle (Roterman *et al.*, 1993). This dye is used as a competitive inhibitor of the glutamate transporter (Roseth *et al.*, 1998). It is used as a fluorescent stain in electron microscopy (Harrisson *et al.*, 1981; Lees, 1989). It is used in the suppression of cell-mediated cytotoxicity (Scornik *et al.*, 1979) and in the treatment of *babesia* in dogs (Jack and Ward, 1980). Trypan Red, the analogue of Trypan Blue, was used as a chemotherapeutic remedy in the treatment of trypanosomiasis. It is still employed for the treatment of piroplasmosis of all species in veterinary medicine (Gurr, 1965).

1.5 Introduction to principles and mechanisms of dye binding (dyeing)

Dye binding is a chemical reaction. Like any other chemical reaction, dye binding depends on the physicochemical properties of the dye (molecular orientation, charge density, solubility in aqueous solvent and colouristic properties), macromolecules (biomolecules), solvent and the pH of the reaction (dyeing) medium. There must be complementary features between a dye and a substrate for the dye to bind to the substrate (Prentø, 2001). The term 'affinity' is used in dye binding to describe the tendency of a dye to move from a solution into the macromolecule. A dye is thus said to have affinity for a specific macromolecule if the nature of the dye complements the structural properties of the macromolecule (Horobin, 1988). Forces of attraction that are created between the dye and the substrate, when the dye comes into contact with the macromolecule, aid the process of the binding of dye to substrate. These forces, which stabilize the binding action, are covalent bonds, ionic

(coulomb) forces, hydrogen bonds, non-polar bonding, van der Waal interactions and hydrophobic interactions (Horobin, 1988; Puchtler *et al.,* 1988; Prentø, 2001).

1.5.1 Dye binding by covalent forces

Covalent bonding between a dye molecule and a macromolecule is as a result of a reaction involving reactive groups on the dye molecule and the macromolecule. A well known example is the binding of the sulfurous fuchsin of Periodic acid-Schiff reagent and aldehyde functional groups on the macromolecule. The colourless dye becomes red upon bonding with the aldehyde functional group, following elimination of sulfate anions. Reactive dyes containing triazine reactive groups are capable of forming covalent bond with oxygen, nitrogen and sulfur atoms on hydroxyl, mercapto and amine functional groups respectively on the macromolecule (Clonis *et al.,* 1987; Zollinger, 1991). Immobilisation of reactive triazine dyes on agarose for dye-affinity chromatography exploits covalent interactions between the dye and the agarose support (Stellwagen, 1990). The rate of formation of covalent bonds between the dye and the macromolecule is influenced by the amount of dye in the tissue, i.e. the affinity between the dye and the macromolecule (Horobin, 1988).

1.5.2 Dye binding by ionic (coulomb) forces

Ionic (coulomb) forces are very significant in dye binding, since macromolecules such as proteins, nucleic acids, acidic polysaccharides as well as many dyes used in the staining solution are ionic at a wide range of pH values (Horobin and Bennion, 1972; Horobin, 1988; Prentø, 2001). The binding of dyes to macromolecules by ionic interaction is mainly achieved by opposite charge attraction between the dye and the macromolecule. Anionic dye will tend to bind to cationic substrate and a cationic dye will interact with an anionic substrate. Acidic dyes are usually negatively charged at acidic pH and they tend to interact with positively charged amino acid side chains on a protein. The uptake of a basic dye by cell nuclei and ribosome-rich organelles is an example of positively charged dyes binding to negatively charged substrates. These sites contain macromolecules such as DNA and

RNA that are predominantly made up of anionic phosphates ribose sugars. Many cationic dyes will therefore bind to nucleic acid macromolecules (Lillie *et al.*, 1976; Horobin, 1988). Alician Blue 8GX is a positively charged mordant dye used in detecting acidic polysaccharides due to the presence of a high density of negative charge on the acidic polysaccharides. These negative charges readily interact with the positive charges on the dye (Scott and Dorling, 1965). Ionic groups are however not essential for dye binding (Puchtler *et al.*, 1988); rather these ionic groups increases the affinity of the dye on its substrate by guiding the dye ions towards the substrate ions and bring them within reach of short-range non-ionic forces (Prentø, 2001).

1.5.3 Dye binding by hydrogen bonding

Hydrogen bonding between dyes and the macromolecules plays a major role in dye binding to the macromolecules. Hydrogen bonding takes place when a hydrogen atom is in close contact with two strongly electronegative atoms such as oxygen and hydrogen. Hydrogen bonding certainly occurs between dye molecules with hydrogen bonding groups such as hydroxyl and amine and macromolecules with similar hydrogen bonding functional groups. The staining of fibrous proteins elastin, collagen and amyloid is mediated by hydrogen bonding (Horobin and Flemming, 1988; Prentø, 1993). The main component of hydrogen bonding is an electrostatic interaction between dipoles of the covalent bond to the hydrogen atom, in which the hydrogen atom has a partial positive (δ+) charge, and a partial negative (δ-) charge on the other electronegative atom (Creighton, 1993). However, the role of hydrogen bonding in the interaction of dye to other tissues is not very clear.

1.5.4 Dye binding by non-polar and hydrophobic interactions

Non-polar and hydrophobic interactions overlap and thus will be discussed together. Non-polar interaction between dye molecules and substrate molecules involves non-ionic groups on the dye and substrate molecules. The non-polar portion of the dye molecule is the conjugated ring system. This portion of the dye interacts with the non-polar portion on substrate molecules such as non-polar amino acid residues on proteins and non-polar

portions on lipids. Non-polar forces, as the sole interactive force in dye binding to substrate, are not very common. The use of non-polar dyes (dyes without ionic groups) to stain lipid droplets exploits non-polar interaction dye binding to the substrate. A good example is lysochrome dyes. These dyes are insoluble in water and thus they are prepared in solvent containing 70% ethanol. Due to the absence of polar groups on the dye molecules, lysochrome dyes easily leave the solvent system and diffuse into the virtually non-polar environment in the lipid molecule. The higher the concentration of ethanol in the solvent, the higher the affinity of the dye for lipid due to higher solubility of the dye in non-polar medium. Staining of non-globular proteins such as elastins is mediated by non-polar bonding (Prentø, 2001).

In hydrophobic staining, the solvent employed is usually water, which plays an important role in the staining system and participates actively in the staining mechanism (Horobin and Bennion, 1973; Horobin, 1988; Prentø, 2001). In aqueous solutions, a sheath of highly ordered clusters of water molecules will surround the hydrophobic groups in the dissolved staining solution and on the macromolecule. This results in the formation of micelles. Consequently the solvent becomes more disordered, causing an increase in entropy of the staining system. This entropy-driven interaction of the hydrophobic groups in the aqueous solution of the dye stain is what is known as hydrophobic interaction (Horobin, 1988).

1.5.5 Dye binding by van der Waal forces

All atoms and molecules attract each other, even in the absence of charged groups, as a result of mutual interactions related to induced polarization effects (the tendency of the charge distribution of a molecule to be altered by an electric field). These attractions are known as van der Waals interactions (Creighton, 1993). Van der Waals interactions are weak and close-range, varying as the sixth power of the distance between them. They arise from three types of interactions: those between two permanent dipoles; those between permanent dipoles and induced dipoles; those between two induced dipoles (known as London or dispersion forces) (Horobin, 1988). The van der Waal's interaction is generally considered to be independent of the orientation of the interacting molecules. These forces

are strongest when the dye stain and substrate molecules possess numerous dipolar and/or polarizable functional groups. Substantially polarizable and large dipoles are characteristic features of dye molecules with aromatic and conjugated ring systems (Horobin, 1988); these factors tend to become more significant as the sizes of the aromatic and conjugated ring systems increases.

1.5.6 The relationship between dye structure and dye-substrate interactions

A dye molecule is predominantly made up of a conjugated ring system that absorbs light, and charged groups called auxochromes, which enhances this light absorption or colour. The whole system forms a chromomeric unit. The structure of a dye will therefore influence the type of interaction the dye will have with its substrate. Certain classes of dyes were synthesised to colour specific types of substrates. Foremost examples are the reactive and direct dyes. These dyes were synthesised to dye cellulosic fibres. Reactive dyes were synthesised with reactive triazine groups that are capable of forming covalent bonds with hydroxyl groups on the cellulose fibres (Zollinger, 1991). These dyes were later used in dye-affinity chromatography after the discovery of the natural affinities these dye have for certain proteins (Clonis *et al.,* 1981, Clonis *et al.,* 1987; Labrou *et al.,* 1996). Therefore it would appear that a dye containing reactive triazine would interact covalently with substrates with amine, hydroxyl and sulfhydryl groups.

Dyes with charged polar groups will tend to interact with substrates containing ionic charged groups. Charged polar groups comprise phosphate, carboxyl, secondary amines, sulfonates, and divalent metal complexes and are introduced into the dye structure to increase the solubility of the dye in aqueous environment. Dyes with predominantly negatively charged polar groups such as sulfonate, will interact with positively charged substrates such as proteins at favourable pH. On the other hand, dyes with predominantly positive charges such as Alcian Blue 8GX, will interact with acidic substrates such as acidic glucosaminoglycans (Prentø, 2001). The cationic dye Methyl Green will interact with negatively charged phosphate groups of nucleic acids. Azo dyes will hydrogen-bond with substrates with hydroxyl and amine groups (Zollinger, 1991).

The hypothetical dye (Figure 1.29) illustrates the relationship between dye structure and the type of bonding interaction that the dye will form with its substrate. The hydrogen bonding groups, carbonyl, hydroxyl, amine and azo are located on one side of the dye structure circled in blue. The anionic groups such as carboxylic acid and sulfonate that will interact by opposite charge attraction with positively charged groups on the substrate are shown circled in green. The positively charged amine group that will interact with anionic groups on the substrate is circled in magenta. The triazine reactive group often found in reactive dyes (Clonis *et al.*, 1987) that is capable of forming covalent bond with substrate is circled in red. The conjugated ring system is the hydrophobic portion of the dye that will interact with the substrate by hydrophobic interaction.

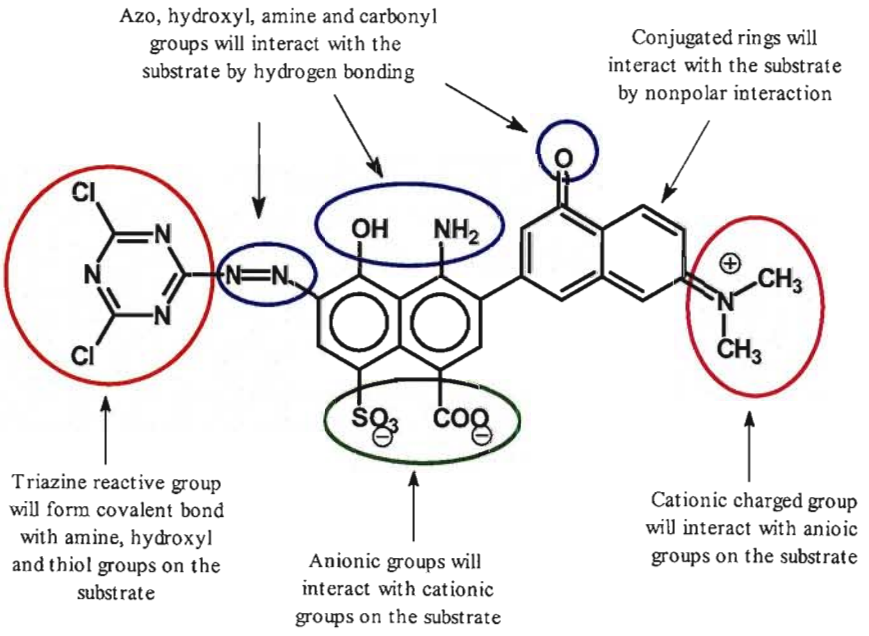


Figure 1.29 Hypothetical dye molecule with interactive functional groups

1.5.7 Physicochemical properties of macromolecules in relation to dye binding

Macromolecules are made up of repeating units of building blocks called monomers or monomeric units organised by the nature of interactions of these building blocks. The type of chemical interactions formed by these monomers influences the general three-dimensional architecture of the macromolecule. Most macromolecules are polymers with

symmetry about a rotational axis from which functional groups project. These functional groups may be polar, non-polar, charged or neutral. The nature of these functional groups determines the type of folding a macromolecule will assume and also the general mode of interaction between this macromolecule and other molecules during dye binding chemistries (Creighton, 1993; Prentø, 2001).

Understanding the structure of the macromolecules to be stained aids the understanding of the mechanisms by which dyes interact with them. The influence of the dye chemistry on the binding with its substrate has been discussed. How the 'geometry' of major biopolymers: proteins, nucleic acids, polysaccharides and lipid, influence the nature of interaction with dye stain will now be discussed. Emphasis will be laid on protein structure and staining mechanisms.

1.5.7.1 Chemistry of dye binding to proteins

In protein molecules, the orientation of the polar and non-polar amino acid residues on the protein structure determines the solubility of the protein and therefore will determine the type of dye stain and staining interaction on the protein. The proportion of non-polar amino acid residues in a globular protein is 30 – 50% of the total amino acid residues per protein molecule. The remainder are hydrophilic (soluble) amino acids bearing charged or polar functional groups. These soluble amino acids contain carboxylic or amino functional groups, which are important in protein folding. They force the protein molecule to remain in contact with water thus reducing the possibilities of folding. Charged groups in the protein tend to be surrounded by opposite charges (Wada and Nakamura, 1981; Prentø, 2001). However, occasional attraction or repulsion between charges may aid in protein folding.

Folding of proteins decreases the surface tension allowing close contact and non-polar interactions between the hydrophobic residues, which release water from the interface. The unrestrained water molecules optimise inter-water hydrogen bonding which is important in the formation of protein secondary structures (α-helices and β-sheets) (Creighton, 1993). The final folded structure of a soluble protein will tend to have the hydrophilic amino acid

residues oriented on the surface of the protein, while the hydrophobic amino acid residues will be organised inside the core of the soluble protein (Kellis *et al.*, 1988; Creighton, 1993).

Elastins and collagens do not contain complex tertiary foldings (Prentø, 2001). Collagen is the main constituent of higher animal frame works such as the bones, tendons, skin, ligaments, blood vessels and supporting membranous tissues. Non-polar and polar amino acid residues in collagens are grouped in short sequences with non-polar sequences in contact with other non-polar sequences in neighbouring chains, and residues bearing complementary charges opposite each other. The number of charged or other polar amino acid residues is low in elastin. The number of non-polar residues in elastin is 90 per 100 residues. The elastin molecule therefore lacks tertiary folding, but forms extended polypeptide chains whose peptide groups participates in inter and intrachain hydrogen bonding, which results in parts of the chain coiling into small α-helical regions. The elastin polypeptides are also connected by covalent cross-links (Eyre, 1980; Creighton, 1993; Prentø, 2001).

Hydrophobic interactions play a major role in the staining of globular proteins from an aqueous solution of dye stain. This staining solution is often prepared in a low pH medium. The low pH medium increases charge repulsion, which results to opening of the secondary structure of the globular protein with subsequent increase in mechanical tension of the protein substrate. Dye binding to exposed non-polar and other residues in globular proteins diminishes charge repulsion and allows relief of the tension. This permits at least partial re-establishment of secondary structures. Hydrogen bonding of exposed amino acid side-chains is unlikely due to steric reasons (Prentø, 2001). The conformation of globular protein system must change with each dye ion bound, and at equilibrium bound dye and protein form a 'conformative' system in which dye exchange is slow.

In collagens, most dye ions bind independently of other dye ions. The large number of proline residues on the collagen structure restricts protein folding. Collagen stains better at low pH, which ensures that carboxyl groups are uncharged at low pH. Charge repulsion at

low pH by positively charged residues increases the distance between tropocollagen units to expose non-polar residues to water (swelling) (Prentø, 1993; Prentø, 2001). The positive charged residues on the collagen can be neutralized by dye anions by virtue of ion exchange, by lodging between the side chains of the protein.

Hydrogen bonding is very much pronounced in dye-collagen interactions. This is because the peptide groups in collagen are more accessible in collagen than in globular proteins. The extent of hydrogen bonding interaction between dye molecules and collagen is evident when staining collagen with Acid Fuchsin (C.I. 42685) in a non-acidified methanolic or ethanolic solution (Salthouse, 1966; Horobin, 1988; Prentø, 1993). In such a solution, the net charge on collagen is minimal and non-polar interactions are weak. However, hydrophobic interactions contribute little in dye binding to collagen due to the semi-crystalline structure of the protein. Due to the low number of hydrophobic interactions, the total strength of dye-protein interaction in collagen is weak. This is in contrast to dye-globular protein interaction (Horobin, 1988; Puchtler *et al.,* 1988; Prentø, 1993). A dye suitable for staining collagen will have in its structure: hydrogen bonding groups, a large aromatic system and several anionic groups. Examples are the Acid Red series, Alcian Fuchsin 6B, Acid Brilliant Red 4BL, Supranol Brilliant Red 3B and Bordeaux Red (Prentø, 1993).

1.5.7.2 Chemistry of dye binding to nucleic acids

In nucleic acids, the less hydrophilic purine and pyrimidine bases are oriented inside the helical structure of the DNA. The more hydrophilic phosphate sugars on the surface of the polymer are in contact with water. Thus the DNA molecule is hydrated on the phosphate sugar residues on the surface. These phosphate sugars are predominantly negatively charged. DNA and RNA are both stained by monovalent cationic dyes. Depending on the staining conditions, the two types of nucleic acids differ in their mode of dye binding. The presence of negative charges on the structure makes it possible for cationic (positively charged) dyes to interact with DNA as well as RNA. Cationic dyes such as Alcian Blue

8GX, Azure B, Methyl Green, Toluidine Blue and Pryonin Y (Kelly *et al.*, 1969; Lillie *et al.*, 1976; Horobin, 1988; Schulte *et al.*, 1992; Prentø, 2001).

However, most nucleic acid dyes are known to interact with nucleic acids by non-polar interaction within the hydrophobic core. Acridine Orange, Pryonin Y (C.I. 45005) and Ethidium Bromide (all fluorescent dyes) are known to interact with DNA by intercalation with nitrogenous bases in the hydrophobic core of DNA molecule (Franklin and Locker, 1981). Below pH 3, intercalation of dyes in the double strand becomes virtually impossible due to the opening of the DNA double helix as a result of protonation of the nitrogenous bases (Prentø, 2001).

1.5.7.3 Chemistry of dye binding to carbohydrates

Most tissue carbohydrates are fully hydrated due to the presence of hydrogen bonding hydroxyl groups in the carbohydrate structure. Connective tissue heteropolysaccharides (glycosaminoglycans, or GAGs) and most epithelial mucins are polyanions. Apart from acetic acid ester groups (CH_3CO–), heteropolysaccharides are without non-polar groups and they are fully hydrated. This implies that hydrophobic interaction between dye molecules and polysaccharides (heteroglycans) is perhaps impossible (Scott and Dorling, 1965; Prentø, 2001).

The presence of high negative charge density on acidic GAGs and other heteroglycans permits interaction with cationic dyes, large polyvalent dyes, and metal-dye complexes. Dyes such as Toluidine Blue, Safranin O, Alcian Blue 8GX, Pryonin Y have proved very useful in staining acidic GAGs and heteroglycans. These dyes interact with polysaccharides mainly by ionic interaction (Scott and Dorling, 1965; Kelly, *et al.*, 1969; Marshall and Horobin, 1973; Horobin 1988; Prentø, 2001). Hydrogen bonding plays a major role in dye binding to non-ionic polysaccharides such as glycogen (Murgatroyd and Horobin, 1969), with the observation that dyes such as Acid Alizarin Blue, Alizarin Brilliant Blue, Alizarin Red, Haematins and Haematoxylin, carrying numerous hydrogen bonding group are good stains for non-ionic glycogen. However, hydrophobic interaction is indirectly involved in

metachromasia of heteroglycans (Kelly, *et al.*, 1969). Metachromatic binding between monovalent, planar dye and GAG is due to hydrophobic interaction between dye molecules bound on the GAG by ionic interaction.

Carbohydrates can be detected by periodic acid–Schiff (PAS) staining, which works by covalent dye binding. The mechanism of PAS staining involves periodic acid reduction of the carbohydrate diols into aldehydes. The aldehydes react with sulfurous-Fuchsin in the PAS stain to forming a magenta-coloured complex with the dye molecules (Horobin, 1988; Thornton *et al.*, 1989).

1.5.8 Effects of solvents and solvent properties on dye binding

An important and integral part of dye-substrate interactions is the solvent in which both the dye and the substrate are suspended. When a dye is solubilised in a solvent system, the dye molecules interact with the solvent such that a form of chemical bonding is established between the dye and the solvent. When the substrate to be stained is brought into the dye-solvent system, the bond between the dye and the solvent will be exchanged between the dye and the substrate. Therefore the solvent participates in the chemistry of dye binding (Prentø, 2001).

Biological fluid is mainly composed of water with dissolved electrolytes. Many biological molecules are soluble in biological fluids. They interact by hydrogen bonding and non-polar interactions. This is because of the universal nature of water as a solvent. Globular proteins interact with water by hydrophilic amino acid residues on the surface of the protein, while the non-polar hydrophobic residues are oriented inside the core of the protein. Structural proteins such as collagen fibres also have polar surfaces. The solubility of a globular protein in water generally increases at pH further away from its iso-electric point. Thus the greater the net charges on a protein, the greater the electrostatic repulsion between the molecules, which tends to keep them in solution. Nucleic acids are also soluble in intracellular fluids. The nucleotide residues are very soluble in water because of the presence of hydrogen bonding groups on the phospho-ribose sugar. The purine and

pyrimidine bases form inter-chain hydrogen bonding in DNA. This interaction results in a hydrophobic core in DNA shielded away from water. Most carbohydrates are very soluble in water. Lipids form micelles in water due to its amphiphilic nature.

The solubility of most dyes in a solvent system depends on the balance between the polar and non-polar groups on the dye molecule. The polar groups interact with water by hydrogen bonding while the non-polar groups interact with each other resulting in formation of dye micelles. Water is the solvent for most biological stains owing to its combination of a large dipole moment, its propensity to form ion-dipole, dipole-dipole and hydrogen bonding with other molecules. The dielectric constant of water is exceptionally high. However the ability of water to dissolve non-polar molecules is correspondingly low (Prentø, 2001).

1.6 Introduction to direct colourimetric immunodetection

The development of clinical chemical assays has been advanced by utilizing the specificity of antigen-antibody interactions, combined with the application of sensitive labelling techniques (Gribnau *et al.*, 1982). Coloured products, which are usually dye molecules, are employed in clinical, immunochemical and histochemical assays. One of the most common methods involves the use of antibody against an antigen of particular interest detected with an enzyme-labelled antispecies secondary antibody (Engvall and Perlmann, 1971). This enzyme then catalyses the formation of a coloured insoluble reaction product at the site, with the colour intensity being directly proportional to the assayed antigen (Mason and Sammons, 1978). This is the principle underlying enzyme-linked immunosorbent assay (ELISA).

1.6.1 FITC-labelled antibody detecting system

Covalently labelled fluorochromes and fluorescent dyes are applied in immunochemical techniques. Fluorescent dyes are attached to antibodies against single or multiple macromolecule species for use as fluorescent probes. A fluorescent dye has the ability to

absorb ultra violet (UV) radiation at one wavelength and subsequently emit the absorbed radiation at another wavelength (Hermanson, 1996). The emitted radiation is longer, with fewer quanta than the absorbed radiation. The dye Fluorescein isothiocyanate (FITC) is one of the most popular fluorescent probes. An isothicyanate derivative of fluorescein can be coupled to proteins or antibodies by nucleophilic reaction with functional targets such as amines, sulfhydryls and phenolates of tyrosine side chains (Strottmann *et al.,* 1983; Burtnick, 1984; Muramoto *et al.,* 1984; Cheng and Dovichi, 1988).

1.6.2 Antibody-labelled colloidal dye detecting system

The application of colloidal dye-sol particles as labels in immunoassay offers a cheaper alternative to enzyme labelled detecting systems (Gribnau, *et al.,* 1982; Snowden and Hommel, 1991; Nataraju *et al.,* 1993; Rabello *et al.,* 1993). This form of immunoassay, termed dye-immunoassay (DIA), makes use of colloidal textile dyes as visible detecting agents. Colloidal dyes are a class of substantially water-insoluble dyes originally introduced for dyeing cellulose acetate, and are usually applied in aqueous suspension (Gribnau *et al.,* 1982). Various colloidal disperse dyes such as Palanil Red, Samaron Blue, Terasil Brilliant Flavin and Nailene Yellow have been used to detect antigens in DIA (Gribnau *et al.,* 1982; Snowden and Hommel, 1991; Nataraju *et al.,* 1993; Rabello *et al.,* 1993; Zhu *et al.,* 2002).

By a series of preparative centrifugation steps, colloidal dyes separate into spherical or semi-spherical dye-sols of radius up to 100 nm. This offers a large surface area for antibody coating. The dye sol particles are subsequently coated with antibody molecules by means of physical adsorption (Gribnau *et al.,* 1982). By antibody-antigen interaction, 10 – 100 ng/ml of antigen in solution can be detected by antigen capture immunoassay method on solid matrix such as nitrocellulose (Snowden and Hommel, 1991) or on a micro titre plate (Gribnau *et al.,* 1982).

Colloidal dye-based immunoassays require no instrumentation for qualitative antigen detection. This makes it potentially applicable to field diagnosis. However, this technique has several shortcomings such as choice of dye, flocculation due to change in ionic

concentration, choice of labelling antibody and long-term stability of the dye-sol-antibody reagent (Snowden and Hommel, 1991).

1.6.3 Dye-encapsulated latex bead detecting system

A new dimension to immunoassays is the use of dye-encapsulated liposomes. Liposomes are spherical vesicles in which the aqueous volume is enclosed by a mono- or bilayer of membrane composed of phospholipids (Martorell *et al.,* 1999; Park and Durst, 2000). There are two different domains in liposomes, namely, the hydrophilic phase and the hydrophobic phase. The presence of these two different phases makes liposomes a multipurpose carrier for wide range of hydrophobic, hydrophilic and amphipatic agents such as dyes in particular. The hydrophilic groups on the outer surface of the liposomes offer sites for attachment of ligands such as antibodies and antigens. These features render them suitable for diagnostic applications (Park and Durst, 2000). Experimental systems have been developed for detecting pathogens in a sandwich (non-competitively) immunoassay which consist of wicking reagent containing anti-*E-coli* O157:H7 antibody-conjugated liposomes (immunoliposomes) with entrapped Sulfurhodamine B as detecting dye and a plastic–backed nitrocellulose strip with a capture zone (Park and Dusrt, 2000). The intensity of the colour in the capture zone is directly proportional to the amount of antigen.

1.6.4 Colloidal-immunogold detecting system

Colloidal gold-protein complexes are another form of a direct colourimetric immunodetection system. Antibodies are coupled to gold particles by virtue of covalent attachment of gold to free sulfhydryl residues on the protein. This coupling between the gold particle and protein form dative bonds or ionic attraction between the negatively charged gold particles and the abundant of positively charged amino acid side chains. There is also an adsorption phenomenon involving hydrophobic pockets on the protein binding to the metal surface. The visually dense nature of gold provides excellent detection qualities for techniques such as blotting, flow cytometry, and hybridisation assays (Hermanson, 1996).

Colloidal gold suspensions are made up of small particles of gold in a stable transition state. When viewed under electron microscope, gold particles appear as dense black dots. In light microscopy, gold particle are orange in colour. Colloidal gold particles act as efficient nuclei for deposition of silver, thus markedly enhancing their detection under electron microscope and providing increased sensitivity in blotting applications (Danscher and Rytter-Nörgaard, 1983; Moeremans *et al.,* 1984; Hermanson, 1996).

1.7 Chicken egg yolk immunoglobulins (IgY) as an antibody model

The antibody model used in this study was polyclonal chicken egg yolk immunoglobulin Y (IgY) raised against rabbit albumin. IgY is the functional equivalent of immunoglobulin G (IgG) in birds, reptiles and amphibia. Though many aspects of its biological functions are inadequately understood. Several studies on the genetics and functions of this molecule have increased its awareness, revealing its position as the ancestor of the distinctively mammalian antibodies, IgG and IgE (Warr *et al.*, 1995).

Chicken antibody molecules are glycoproteins called immunoglobulins. These immunoglobulins are like mammalian immunoglobulins, comprising of heavy (H) and light (L) chains bridged by disulfide bonds. The molecule is made up of a variable antigen-binding region and a constant region (Erhard and Schade, 2001). Leslie and Clem (1969) suggested that chicken serum-immunoglobulin should be called IgY. It is now known that IgY is the typical low-molecular-weight (LMW) (i.e. non-IgM) serum antibody of birds, reptiles, amphibia, and (probably) lungfish, whereas IgG occurs only in mammals. In addition, molecular cloning techniques have recently provided the evidence that not only is the IgY the evolutionary ancestor of IgG, but also IgE, another immunoglobulin unique to mammals (Leslie and Clem, 1969; Chartrand *et al.,* 1971; Leslie and Clem, 1972; Pavari *et al.,* 1988; Amemiya *et al.,* 1989; Magor *et al.,* 1992; Warr *et al.,* 1995).

1.7.1 Basic structure of IgY

IgY has two heavy (H) and two light (L) chains ($H_2 L_2$ being the configuration of all LMW immunoglobulins) and has molecular mass of about 180 kDa (Warr *et al.*, 1995). The heavy [or upsilon (υ)] chains of IgY typically posses one variable (V) and four constant (C) - region domains (Pavari *et al.*, 1988; Magor *et al.*, 1992) CH1, CH2, CH3 and CH4. The overall shape of chicken IgY revealed by electron microscopy appears as Y-shaped molecule with an angle of 90° between the antigen binding (Fab) arms. This angle represents a property of the antibody molecule itself and it is not influenced by antigen interaction on the Fab arm. The length of one Fab arm is about 10 nm on average (Noll *et al.*, 1982).

The amino acid sequences of IgY H and L chains have been published (Dahan *et al.*, 1983; Reynaud *et al.*, 1983; Pavari *et al.*, 1988; Magor *et al.*, 1992) and are available at www.expasy.org/sprot.

Based on the sequence analysis derived from ExPasy Data base (www.expasy.org/sprot) for chicken heavy chain constant region (primary accession number P01875) of *Gallus gallus* (Chicken), the IgY H chain is made up of approximately 426 amino acid (excluding *ca* 19 amino acid length for carboxyl terminal). The CH1 domain region consists of amino acids 1 – 105, CH2: 106 – 209, CH3: 210 – 316, CH4: 317 – 427 and the carboxyl terminal region: amino acid 428 – 446. The IgY H chain forms inter chain disulfide bond with the light chain at Cys^{16}, and with the other heavy chain at Cys^{289} and Cys^{445}. There are four possible points of intrachain disulfide bond (loop), one on each domain of the heavy chain. They are between Cys^{27} and Cys^{85} on CH1 domain, Cys135 and Cys^{170} on the CH2 domain, Cys^{237} and Cys^{296} on CH3 domain and Cys^{344} and Cys^{406} on CH4 domain. Potential points of N-linked glycosylation occur on asparagine at positions 45, 46, 73, 130, 198, 382 and 433. The presence of three potential glycosylation points on the CH1 region, which is closest to the Fab arm of the H chain, could mean possible occlusion of the antigen-binding site (Warr *et al.*, 1995).

The sequence of a chicken λ light chain immunoglobulin (IgY) derived from the nucleotide sequence of its mRNA (Reynaud *et al.*, 1983) is made up of a variable (V) antigen binding region and a constant (C) region. The V region consists of amino acids 1 – 103 and the C-region: 104 – 206. The chicken constant domain is 61% homologous to both human and mouse light chain constant region. Peculiar differences are observed: a deletion of one residue on both sides of Gly^{154} and addition of one residue at position 122. Both additions at Ser^{34} of the variable chain and Asn^{122} of the constant chain occur at the hairpin bends of the 3D structure of chicken IgY light chain (Poljack *et al.*, 1974; Reynaud *et al.*, 1983). Based on the Swis-Prot analysis of IgY λ-chain C-region (primary accession number P20763), the light chain has a single disulfide loop between Cys^{28} and Cys^{85}. The light chain forms an interchain disulfide linkage with the heavy chain at Cys^{103}. Table 1.2 illustrates the structural features of the IgY λ-chain C-region. Figure 1.30 illustrates the map of the IgY structure derived from sequence information published by Reynaud *et al.* (1983), Pavari *et al.* (1988) and www.expasy.org.

Table 1.1 **Structural features of chicken heavy chain constant region** (www.expasy.org/sprot)

Parameter	Amino acid	
	From	To
CH1 domain	1	105
CH2 domain	106	209
CH3 domain	210	316
CH4 domain	317	427
Carboxyl terminal	428	446
Interchain disulfide bond with light chain	16	103^{L}
Interchain disulfide bond with heavy chain	284	284^{H}
Interchain disulfide bond with heavy chain	445	445^{H}
Intrachain disulfide bond loop	27	85
Intrachain disulfide bond loop	135	190
Intrachain disulfide bond loop	237	296
Intrachain disulfide bond loop	344	40

^{L} On one light chain.

^{H} On the other heavy chain.

Table 1.2 **Structural features of chicken light chain constant region** (www.expasy.org/sprot)

Parameter	Amino acid	
	From	To
IgY λ chain C region.	Gln^{1}	Cys^{103}
Intrachain disulfide loop	Cys^{28}	Cys^{85}
Interchain disulfide linkage	Cys^{103}	Cys^{103}

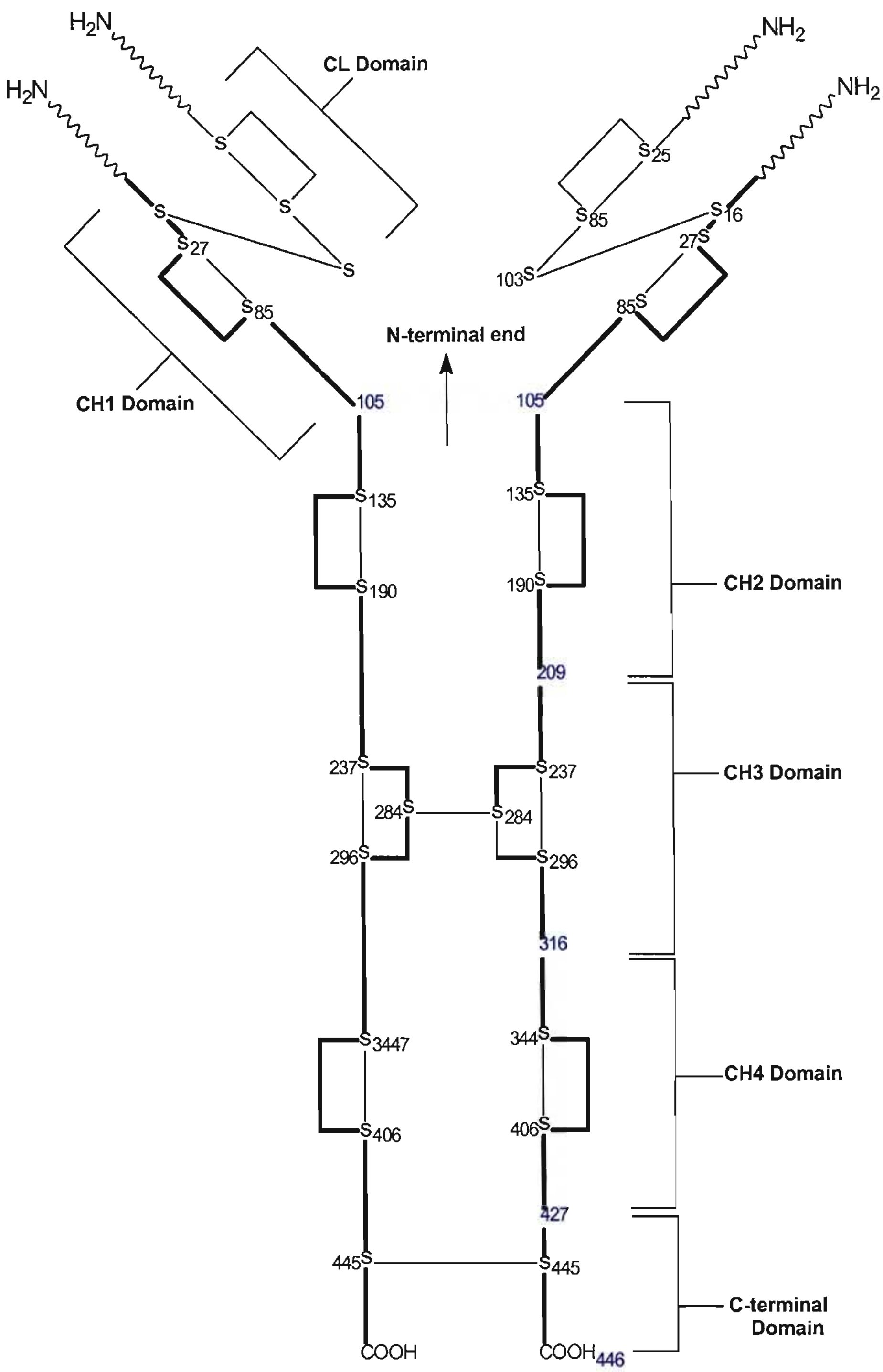


Figure 1.30 Diagrammatic illustration of chicken IgY showing its constituent chain domains Bold lines represent the heavy chain constant domain; thin lines represent light chain constant domain; the variable domains of the heavy and light chain is represented by zigzag line (Reynaud *et al.,* 1983; Pavari *et al.,* 1988; Pavari *et al.,* 1990; www.espasy.org)

1.7.2 Immunochemistry of IgY

IgY is the main serum antibody of chickens and thus plays a major role in the immune response of chickens (Larsson and Sjöquist, 1990; Janson *et al.,* 1995; Warr *et al.,* 1995; Schade *et al.,* 2001). The serum IgY in chickens is also present in egg yolk. IgY is transferred from the serum into the eggs in a manner similar to placental transportation of IgG in mammals (Larsson and Sjöquist, 1990). The active transport of IgY from the serum into the eggs results in a higher concentration in the yolk than in the serum. Thus more antibodies can be isolated per month from a laying hen compared to the serum of a rabbit. In an average egg, there is about 100 – 400 mg of yolk antibodies packaged in a convenient and sterile form (Janson *et al.,* 1995; Morrison *et al.,* 2001; Schade *et al.,* 2001). As chicken IgY can be purified from yolk, there is no need to draw blood from the animal, as for example in rabbits. The role of these maternally derived yolk antibodies is to confer immunity to the newly hatched chickens (Larsson and Sjöquist, 1990).

1.7.3 Utilizing evolutionary differences of chicken IgY

Due the evolutionary differences between IgY and mammalian IgG, IgY has several properties not found in mammalian IgG. Besides the immune functions of IgY such as opsonisation and complement fixation (Larsson and Sjöquist, 1990; Warr *et al.,* 1995) IgY, unlike mammalian immnuoglobulins does not bind to bacterial Fc receptors such as staphylococcal protein A (Langone *et al.,* 1983) or streptococcal protein G (Guss *et al.,* 1986). There is also no cross-reactivity between chicken IgY and mammalian IgG when the former is tested with rabbit anti-chicken IgY. Rheumatoid factor (RF), is an anti-immunoglobulin autoantibody that can be found in many different diseases, of which rheumatoid arthitis is the most frequent. RF is a major source of interference in many immunoassays because it reacts with IgG from different mammalian species. This cross-reactivity with mammalian IgG, such as mouse monoclonal antibodies, leads to possible erroneous results. Due to the immunological difference between IgY and IgG of mammals, IgY does not react with RF, thus can be used to avoid false positive results in immunological assays (Larsson and Sjöquist, 1988).

Evolutionary, IgY is probably related to the mammalian IgA, and it is approximately the same molecular weight and structure (Hädge and Ambrosius, 1984). However, chickens do seem to express antibody diversity differently from mammals. According to Weill and Reynaud (1996), there are three basic mechanisms behind this diversity, namely gene arrangement, gene conversion and somatic mutation. The antibody repertoire of chicken is fairly high and it is estimated to be around 10^6, only one order of magnitude higher than the antibody repertoire of the mouse. The contribution of the germ-line for the diversification of IgY light chains and heavy chains is rather limited, because their variable (V) regions are encoded by only one V_L subgroup (Reynaud *et al.,* 1987; Pavari *et al.,* 1987) and one V_H subgroup (Pavari *et al.,* 1988). The entire L-chain repertoire is derived from a single V-joining region because there is one joining gene and only one functional $V_\lambda 1$ gene, and the remaining 25 are pseudo-V_λ-genes that serve as donor sequences to diversify the rearranged $V_\lambda 1$ gene by gene conversion. Chicken IgY L-chains are further diversified by point mutations and by imprecise joining of the V and joining gene segments (Pavari *et al.,* 1990; Sapats *et al.,* 2003).

The use of chicken IgY offers numerous advantages over the conventional mammalian IgG in the design of immunodetection and immunochemical systems. A rule is that antibody response will be better if the antigen is prepared from species that is phylogenetically distant from the immunized animal (Larsson and Sjöquist, 1988). The evolutionary difference between IgY and mammalian IgG has been fully exploited in the field of immunodiagnostics. There are possibilities of the use of chicken antibodies as oral vaccines in human and animal medicine (Behn *et al.*, 2001).

1.8 Chemistries of bioconjugation and modification of antibody molecules

Protein (antibody) molecules are perhaps the most common targets for modification or conjugation techniques. As the mediator of specific biochemical activities and functions within the living organism, proteins can be used *in vitro* and *in vivo* to effect certain tasks. Having enough of a protein that can bind a particular target molecule can result in a way to detect or assay the target molecule, provided the biological function of the protein is

conserved after modification and also the protein can be measured or followed (Hermanson, 1996).

Proteins can be modified in various ways for application in biological assays by simple adsorption, or by covalent labelling of other molecules. Molecules, such as proteins (enzymes), can be covalently conjugated to other proteins as in enzyme-linked immunosorbent assay. Synthetic polymers, such as ficoll, dextran and polyethylene glycol can also be conjugated to protein molecules in the preparation of immunogens for antibody production (Inman, 1975; Brunswick *et al.,* 1988; Dintzis *et al.,* 1989; Xiao *et al.,* 1995). Small molecules such as fluorochromes and biotin could be conjugated to proteins as tags or probes in immunoassay (Strottmann *et al.,* 1983; Burtnick, 1984; Muramoto *et al.,* 1984; Cheng and Daviochi, 1988). Proteins could be linked to colloidal particles such as dyes and gold for immnuoassays (Gribnau *et al.,* 1982; Snowden and Hommel, 1991; Rabello *et al.,* 1993; Nataraju *et al.,* 1993).

Proteins are modified through the side chains of the amino acids. For carbohydrate containing proteins (glycoprotein) such as immuoglobulins, modification can take place on their carbohydrate moiety. This point of modification is usually carried out if the biological activity of the immunoglobulins is to be preserved. This is so because the carbohydrate moiety is often located on the heavy chain constant region of the immuoglobulin (Hermanson, 1996). For chicken IgY, which is the protein to be modified in this study, emphasis was placed on the lysine side chains. This implies that the molar content and the surface availability of lysine side chains may determine the extent of coupling.

Lysine has a straight four-carbon chain terminating with an ionisable amine group. This ε-amine group contributes to the overall positive charge of the protein. The ε-amine of lysine has a pK_a of 9.3 – 9.5, which differs from the pK_a of α-amines with pK_a of 7.6 – 8.0. Thus ε-amine of lysine has a slightly higher ionisation point. At pH lower than the pK_a, amine groups are generally protonated and acquire a positive charge. At a pH higher than the pK_a of ε-amine of lysine, the amine groups are unprotonated and contribute no net charge. The

ε-amine of lysine is exposed on the surface of the proteins and can be derivatized with ease (Hermanson, 1996).

The most important reactions that can occur with ε-amine groups of lysine are alkylation and acylation (Figure 1.31). In alkylation, an active alkyl group is transferred to the unprotonated amine nucleophile with loss of one hydrogen atom.

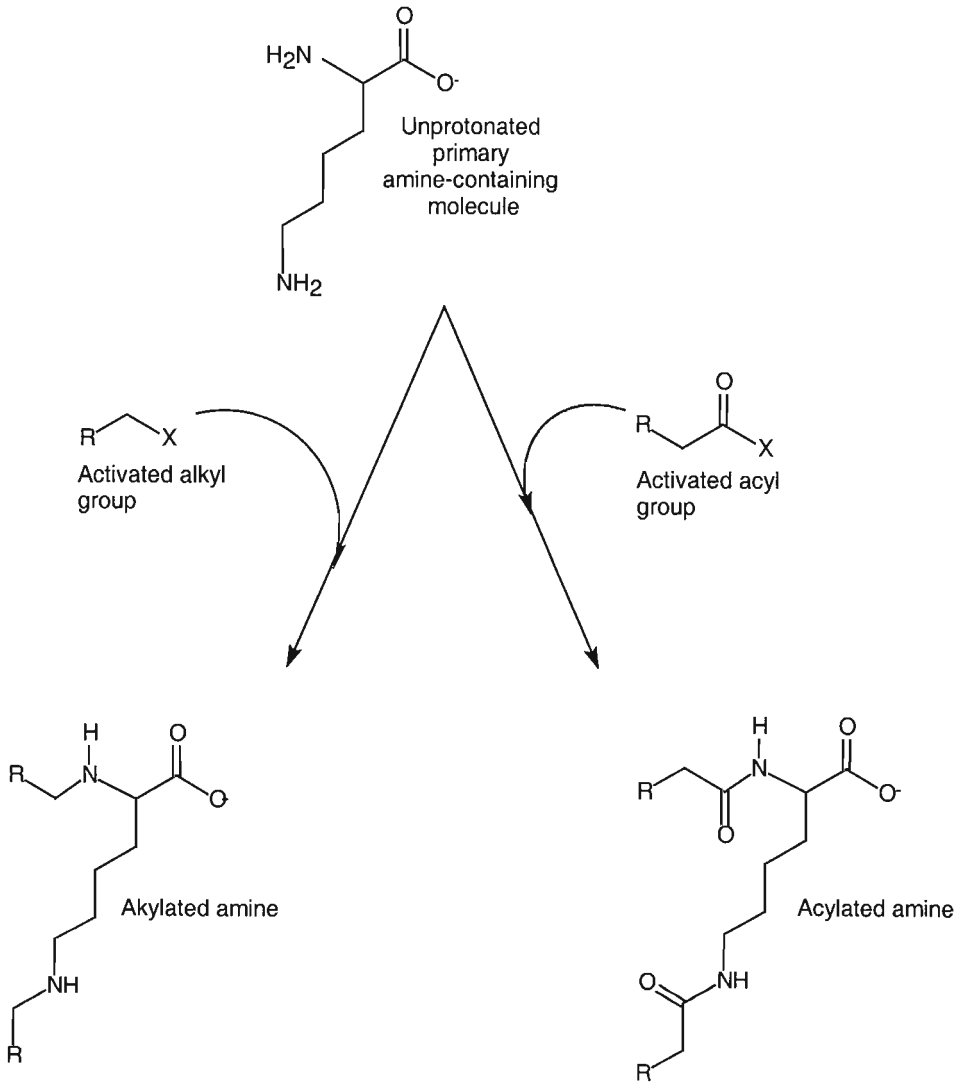


Figure 1.31 Alkyation and acylation reaction pathways for modification of protein
Proteins are often modified through ε-amine of lysine or α-amines on side chains of various α-amines containing amino acids. Unprotonated amine groups are favoured for these reactions (Hermanson, 1996).

For acylation, an active carbonyl undergoes addition with an unprotonated amine group. Thus the modification of proteins on their amine residues will generally proceed at a pH higher than the pK_a of ε-amines or α-amines. Factors such as the presence of other amino acid side chains in the vicinity of the ε-amine of lysine, salts, buffers, temperature, ionic strength and other effects of solvents medium all play a role in creating certain changes in the microenvironment that affects the ionisation of ε-amine group of lysine (Kaplan *et al.*, 1971; Schewale and Brew, 1982; Hermanson, 1996).

Modification of proteins molecules by covalent labelling with other molecules often involves the use of special molecules capable of linking the protein to be labelled with the labelling molecule. These special molecules are called cross-linkers. Examples of cross-linkers used in protein modifications are glutaraldehyde, hydrazine, carbonyldiimidazole, *p*-nitrophenyl iodoacetic acid (NPIA) and 3-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). They are often classified as zero-length, homo-bifunctional or heterobifuntional cross-linkers, depending on the chemistry of the cross-linker and the number of cross-linking funtional group on the molecule (cross-linker).

N, N'- Carbonyldiimidazole (CDI), also know as 1,1'-carbonyldiimidazole (Bethell *et al.*, 1979; Newman and Harrison, 1985; Hearn, 1987; Clough *et al.*, 1989; Chu, and Orgel, 2001), is a highly reactive carbonylating agent that is basically made up of two acyl-imidazole leaving groups. As a zero-length or one atom-length cross-linker, CDI cross-links hydroxyl or carboxylic acid containing molecule with a primary amine-containing molecule. The reaction occurs in two steps. The first step is the activation of carboxylic acid or hydroxyl-containing molecule. This step proceeds with the formation of active acyl-imidazole or imidazole carbamate intermediates depending on the activated functional group. The second step is the coupling of the activated intermediate with the primary amine-containing molecule (Bethell *et al.*, 1979; Hearn, 1987; Hermanson, 1996).

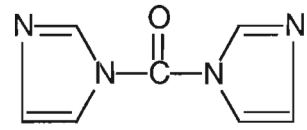


Figure 1.32 ***N, N'*- Carbonyldiimidazole,** MW 162 (Hearn, 1987)

The CDI-mediated reaction for carboxylic acid containing molecule (Figure 1.33) and hydroxyl containing molecule (Figure 1.34) occurs quite differently. With carboxylic acids containing molecule, CDI acts as a zero-length cross linker. CDI reacts with the carboxylate functional group (COOH) releasing carbondioxide and one imidazole molecule to form an active acylimidazole intermediate. The active acylimidazole then reacts with a primary amine in an amine-containing molecule to form an amide bond between the coupled molecules, releasing the remaining imidazole molecule (Hearn, 1987; Hermanson, 1996).

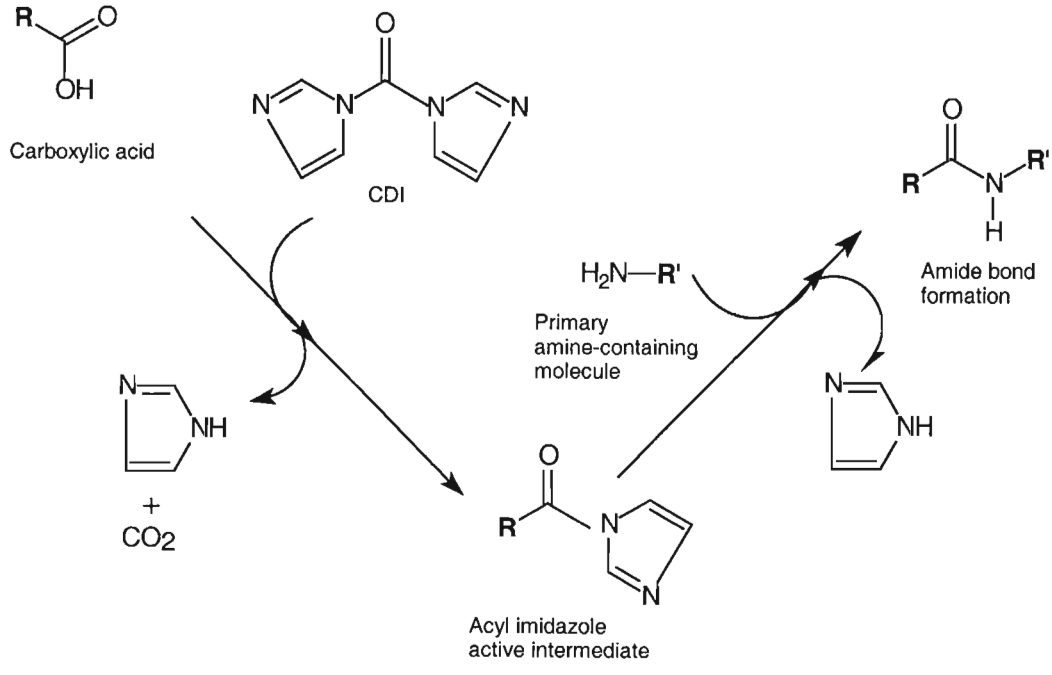


Figure 1.33 Reaction sequence of CDI activation of a carboxylate-containing molecule
Carboxylic acid–containing molecules react with CDI to form an active acylimidazole intermediate and subsequent formation of amide bond between the active acylimidazole and a primary amine-containing molecule (Hermanson, 1996).

The CDI mediated coupling of a hydroxyl-containing molecule and a primary amine-containing molecule leaves a single atom (carbonyl) between the conjugated molecules. The activation step proceeds with the release of just one imidazole molecule and the formation of an active imidazole carbamate intermediate. The coupling step results in the formation of a carbamate linkage between the conjugated molecules. The CDI molecule contributes the carbonyl group in the linkage.

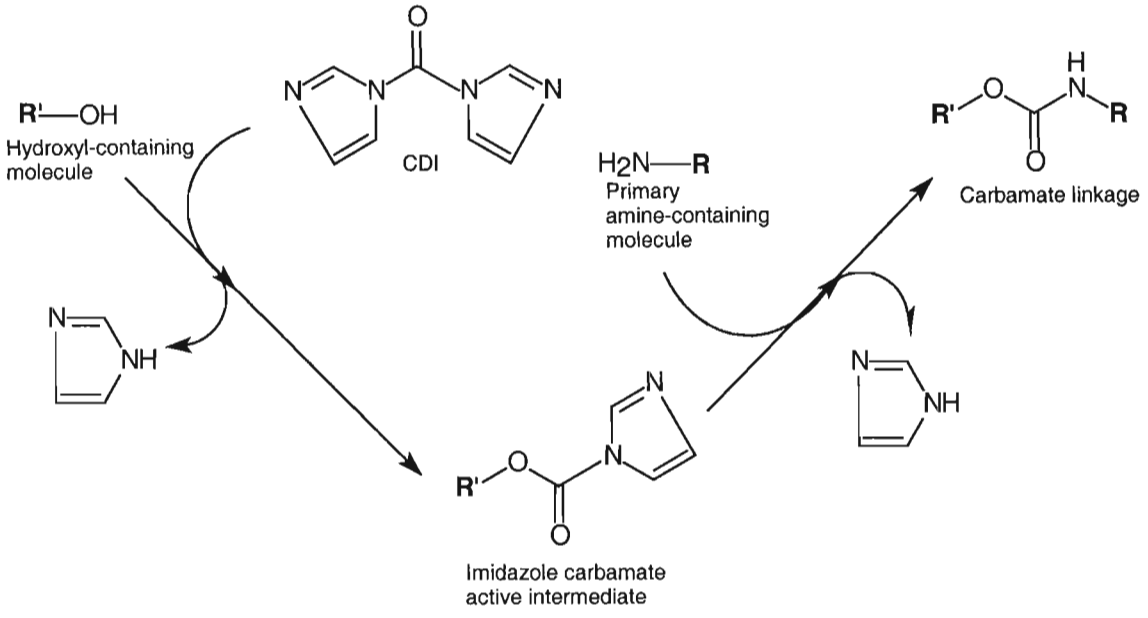


Figure 1.34 Reaction sequence of CDI activation of a hydroxyl-containing molecule
CDI reacts with a hydyoxyl-containing molecule to form an active imidazole carbamate intermediate and subsequent formation of carbamate linkage between the active imidazole carbamate and a primary amine-containing molecule (Hermanson, 1996).

CDI-mediated coupling reaction has been applied extensively to the activation of (1) hydroxyl-containing chromatographic matrices for the immobilisation of amine-containing affinity ligands (Bethell *et al.*, 1979; Hearn, 1987), (2) carboxylic acid-containing matrices for the immobilization of amine containing lysozyme as an affinity ligand (Bartling *et al.*, 1973), (3) polyethylene glycol for the modification of amine-containing macromolecules

(Beauchamp *et al.*, 1983) and (4) dextran for the modification of amine-containing mycotoxins for immunoassays (Xiao *et al.*, 1995).

Due to the sensitivity to hydrolysis of carbonylating reagents, such as CDI, the activation step is usually carried out in a non-aqueous medium. Solvents such as acetone, dioxane, DMSO, and DMF are commonly used solvents in the activation step. In the presence of water, CDI hydrolyses into carbon dioxide and two imidazole molecules. This implies that the activated species is often regenerated by slow hydrolysis of the active acylimidazole or imidazole carbamate intermediate in the presence of water (Hearn, 1987).

1.9 Aim and objectives of the study.

The specificity of antibody-antigen interactions has been greatly exploited over the past years in the development of very sensitive clinical and immunochemical assays. These assays combine the use of highly sensitive labels and detection techniques such as enzymes (in ELISA), gold particles (in immunogold assays), latex beads (in latex agglutination), fluorescent dyes (in immunofluorescent staining), colloidal gold particles (in dye-sol immunodetecting systems) and colloidal carbon particles. Of all these techniques, radioimmunoassay has proven to be a very reliable and sensitive technique. However these techniques have several pitfalls ranging from high cost of materials and production to government regulations and waste disposal problems (Gribnau *et al.*, 1982).

These problems led to the development of cheaper and more environment friendly immunoassay techniques, such as the use of colloidal dye particles (Gribnau *et al.*, 1982; Snowden and Hommel, 1991) as a replacement for expensive colloidal gold particle. It is believed that the sensitivity of immunofluorescence assay can be mimicked with more chromogenic dyes instead of fluorescent dyes. The replacement of fluorescent dye particles with coloured dye particles may be an alternative approach towards the design of cheaper immunodiagnostic systems. This will involve the covalent attachment of textile dyes on antibodies directed against a particular antigen. This antigen will be detected by the virtue of the coloured antibodies. The intensity of the colour may be proportional to the amount of antigen detected. This is the aim of this study.

- The physicochemical properties of dyes and dye binding to proteins were studied to understand the nature and class of dyes that naturally bind to proteins, which is a prerequisite to covalent attachment of dyes to IgY as a visible immunoassay agent. 26 dyes of anionic, cationic and ligand dye classes were used for this purpose. The results from the study of the physicochemical properties of dye were used in identification of dyes that were used in the covalent labelling of chicken egg yolk IgY made against rabbit albumin (antigen).

- Dyes with carboxylic acid functional groups as the only negatively charged functional groups and hydroxyl groups were used in the covalent attachment to IgY using CDI as the cross-linker. The ability of the dye-IgY conjugate to detect rabbit albumin (antigen) in a dot-immunobinding format was evaluated as possible antigen detecting reagent, using HRPO-IgY conjugate as a standard tool for comparison.

- Part of this study involved preparation of IgY-dextran conjugate as possible antigen detecting agent. IgY made against rabbit albumin was conjugated to derivatized dextran polymer. The IgY-dextran conjugate was used to detect rabbit albumin on nitrocellulose. PAS staining of the dextran was used as a means of colourimetric estimation of the antigen.

CHAPTER 2
MATERIALS AND METHODS

2.1 Introduction

This chapter describes a number of fundamental biochemical techniques used in this study. Individual methods relating to a specific area are discussed in the appropriate sections. All reagents and materials used in this study were of analytical grade except for dextran (M_r: 5000 kDa to 40 000 kDa) that was of industrial grade. The experimental methods described in this study involved (1) staining proteins bound on nitrocellulose or separated on sodium dodecyl sulfate-polyacrylamide gel with a range of dyes prepared using the standard method of preparation of Coomassie Blue R 250 stain, (2) covalent labelling of chicken IgY antibodies against rabbit albumin with appropriate dyes containing targeted functional groups and (3) covalent attachment of chicken anti-rabbit albumin IgY on high molecular weight dextran polymers.

2.2 Materials

Dextran (M_r 5000 kDa to 40 000 kDa) was purchased from Sigma, which was of industrial grade. Chemicals used for bioconjugation namely: MBS (3-maleimidobenzoic acid *N*-hydroxysuccinimide ester), 2-iminothiolane, *N,N'*-carbonyldiimidazole, chloroacetic acid, ethylenediamine dihydrochloride were all purchased from Sigma, Germany; nitrophenyl iodoacettic acid was purchased from Fluka, Switzerland. Gel filtration matrixes purchased from Sigma were Sephacryl® S-300 and Sepahdex® G-10, Bio-Gel P-6 was purchased from BioRad, U.S.A. Blue dextran from Sigma, Germany. AminoLink® was purchased from Pierce Biotech, Sweden. Tris was purchased from Roche Biochemicals, U.S.A and sodium cyanoborohydride was purchased from and Sigma, Germany. The affinity column was purchased from BioRad U.S.A. PEG 6000 and PEG 20 000 were purchased from Merck Laboratory Supplies, South Africa. Acridine orange, Alcian Blue 8GX, Aurintricarboxylic Acid, Azure B, Azure A, Bismark Brown, Bromocresol Purple, Bromophenol Blue, Cibacron Blue 3GA, Coomassie Blue R 250, Direct Red 75, Direct Red 81, Eriochrome Black T, Ethyl Red, Evans Blue, Gallocyanine, Malachite Green HCl, Methyl Green,

Methyl Orange, Naphthol Blue Black, Nigrosin Water-soluble, Pararosanilin (Basic Fuchsin), Ponseau S, Primunlin, Reactive Black 5, Reactive Green 19, Reactive Red 120 and Trypan Blue were from Aldrich Chemical Company, Germany/U.S.A. Hematoxylin was from BDH, England. Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were purchased Fluka, Switzerland. 4-Chloro-1-naphthol purchased from Sigma. Secondary antibody was rabbit-anti-chicken IgG labelled with horseradish peroxidase (IgG-HRPO) purchased from Jackson Immuno-Research Laboratories U.S.A. Horseradish peroxidase (HRPO) was purchased from Boehinger Mannheim, Germany. MINI PROTEAN™ vertical slab electrophoresis unit and Model 3000Xi computer controlled electrophoresis power supply was from BioRad, U.S.A. Low molecular weight protein calibration kit was from Amersham Biosciences, U.S.A. 10 000 molecular weight cut-off dialysis tubing was obtained from Pierce Biotechnology Tris, acrylamide and bis-acrylamide were from Roche Biochemical. *N,N,N',N'*-tetramethylethylenediamine (TEMED) and β-mercaptoethanol were purchased from Fluka, Switzerland. SDS, ammonium persulfhate were purchased from Merck. EconoPump® peristaltic pump was from BioRad. 0.45 μm nitrocellulose was purchased from Osmonics, U.S.A. Ellman's reagent (5,5'-dithio-bis[2-nitrobonzoic acid]) from Sigma, U.S.A. Imaging was carried out with CanoScan LiDE 30™ scanner (1200 × 2400 dpi with 48 bit input) from Canon, Tokyo Japan. The scanning software was ArcSoft PhotoStudio 5.0® from ArcSoft® Inc. California, U.S.A. All spectral scans were carried out with Varian Cary 50® Bio UV-Visible spectrophotometer from Varian Australia Pty Ltd, Australia. Spectral data were auto-converted to a printer-friendly Microsoft Excel® programme. Molecular structures used in the whole text were designed using ChemWindow® software programme from SoftShell® International Ltd, Connecticut, U.S.A. Nunc-Immuno™ Plates from Nalge Nunc Interantional, Denmark. ABTS (2,2'-azino-di[3-ethylbenzthiazolin-6-sulfonic acid]) from Boehinger Manniehm, Germany. Hydrogen peroxide was from BDH. VERSAmax™ ELISA plate reader and SOFTmax® PRO 3.1.2. ELISA software were from Molecular Devices Corporation, U.S.A. ScanMaker 8700® was from MICROTEX International Inc., China. Quantification of the band intensities was carried out with Soft Imaging System analySIS® software purchased from Germany.

2.3 Ethics

Laying chickens used in this study were immunized with rabbit-albumin-conjugated hapten immunogen. These chickens were kept and cared for at the University farm at Ukulinga. The use of chicken for this study were approved by the University's ethical committee, ethical clearance project number GOLD/99 and AE/GOLDRING/99/08 for 2001.

2.4 Isolation and purification of IgY from chicken egg yolks

The antibody model used in this study was polyclonal chicken egg yolk anti-rabbit albumin immunoglobulin Y (IgY). Hence the antigen was rabbit albumin. Eggs were collected from chickens immunized with synthetic peptides conjugated to rabbit albumin as a carrier protein. It is worth mentioning that these synthetic peptides were research materials from lab 39 and 40 of this department. As expected, the chickens made antibodies against rabbit albumin as well as the synthetic peptides.

IgY was isolated by the polyethylene glycol method of Polson *et al.,* (1985). Polyethylene glycol (PEG) is a linear water-soluble nonionic polymer with molecular weight varying from 200 to 20 000 depending on the extent of polymerization. It is widely used to extract proteins at room temperature without denaturation of the proteins (Polson *et al.,* 1963; Ingham, 1984; Polson *et al.,* 1985; Pohl, 1990). PEG of molecular weight 6000 was used to extract IgY from egg yolks in this study.

The solubility of proteins in an aqueous environment depends largely on the surface availability of hydrophilic amino acids of the protein. These hydrophilic amino acids form hydrogen bonding with water molecules in the hydration shell (layer of water molecules surrounding an ion or molecule in an aqueous solution) of the protein molecules. In the presence of high molecular weight soluble polymers such as dextran and PEG, the solubility of the protein in solution is altered. In most cases the solubility of the protein is decreased. These polymers (dextran and PEG) are preferentially excluded from the hydration shells around the proteins. This brings the protein solution to it solubility limit

(Polson *et al.*, 1963; Fried and Chun, 1971; Ingham, 1984; Pohl, 1990; Giorgione and Epand, 1997).

PEG with M_r 4000 to 6000 is routinely used in protein precipitation. Polymers larger than this offer no advantage, since their solutions are more viscous (Athan and Ingham, 1981). By and large, the shorter time required for precipitation of proteins by PEG and little effect on the stability of the proteins makes PEG preferable to organic solvents and ammonium sulfate extraction. In this study, PEG (M_r 6000) extraction was carried out in 100mM sodium dihydrogen phosphate buffer, pH 7.6 containing 0.1% sodium azide as preservative.

2.4.1 Reagent

Isolation Buffer [100mM $NaH_2PO_4.2H_2O$ pH 7.6, 0.1% (w/v) NaN_3]. $NaH_2PO_4.2H_2O$ (15.6 g) and 1g (NaN_3) were dissolved in 950 ml of distilled water, titrated to pH 7.6 with NaOH and made up to 1000 ml with distilled water.

2.4.2 Procedure

The method of Polson *et al.* (1985) was used. Egg yolks were separated from albumin and carefully washed under slow running tap water in order remove traces of albumin. The yolk sacs were punctured and squeezed carefully in to a measuring cylinder to determine the yolk volume. Two yolk volumes of the isolation buffer was added and stirred slowly to mix with the yolk (200 µl was kept and labelled S_1 for analysis on SDS-PAGE). 3.5% (w/v) of solid PEG was added and stirred continuously until the entire PEG dissolved. The precipitated vitellin fraction was removed by centrifuging (4420 × *g*, 30 min, RT). The supernatant fluid was filtered through a funnel packed with cotton wool. The volume of the filtrate was measured (200 µl was kept and labelled S_2 for analysis on PAGE), 8.5% (w/v) of solid PEG was added to the filtrate and stirred thoroughly. The solution was centrifuged (4420 × *g*, 10 min, RT) to pellet the precipitate. The supernatant (200 µl was kept and labelled S_3 for analysis on SDS-PAGE) was discarded and the pellet dissolved in a buffer volume equivalent to the original egg yolk volume. 200 µl of the re-dissolved pellet was

kept and labelled S_4. 12% (w/v) of solid PEG was added stirred thoroughly and centrifuged (4420 × *g*, 10 min, RT) to pellet the precipitate. The supernatant (200 µl was kept and labelled S_5 for analysis on PAGE) was discarded and the pellet dissolved in the buffer in a volume equivalent to $^1/_6$ of the original egg yolk volume (200 µl was kept and labelled S_6 for analysis on SDS-PAGE). The concentration of the IgY was determined using the equation of extinction coefficient:

$$E_{280nm}^{1mg/ml} = 1.25 \qquad \text{(Coetzer } et\ al.,\ 1985).$$

The purification profile was analyzed by applying samples collected at each step of the isolation procedure on a reducing-SDS-PAGE (Laemmli, 1970).

2.5 Affinity purification of anti-rabbit albumin IgY

Affinity chromatography is a form of liquid chromatography that exploits the principle of ligand-substrate interactions for isolation and purification of species-specific biomolecules from a mixture of other non-specific molecules. This technique offers the ultimate in specificity-separation on the basis of biological interaction. There are two major components of an affinity chromatographic system namely the matrix and the ligand. The matrix comes in different bead-like structures such as polyacrylamide, dextran and agarose polymerized beads. The matrix is insoluble and is usually commercially synthesised to acquire specific functional groups such as aldehyde, carboxylic, hydroxyl, amine, hydrazide etc functional groups. The ligand is often immobilized on the matrix (solid) support by means of several coupling chemistries. Molecules to be purified specifically and reversibly adsorb on the immobilized ligands when passed through the affinity column. The remaining components in the mixture are washed through the column. The column is washed several times with the appropriate column buffer before eluting the adsorbed molecules. A buffer of low pH or high ionic strength is often used to disrupt the affinity-based interaction between the adsorbed molecules and the ligand.

Several commercially available affinity matrixes with different coupling chemistries are widely used in protein purification. Two significant steps are involved in immobilizing ligands on the solid matrix support (Wilchek *et al.*, 1984). The first step is the activation of the matrix. This involves chemical transformation of the matrix into a more reactive form. Most commercial matrixes come in the form of activated matrixes. The second step is the ligand-coupling step. The ligand usually has functional targets such as primary amine (NH_2), sulfhydryl (SH), carboxylic (COOH) and aldehyde (R-CHO) that will react with the activated matrix. In this study, AminoLink® (Pierce) coupling gel matrix was used to purify polyclonal chicken anti-rabbit albumin IgY. Commercially lyophilized albumin was immobilized on the matrix support. AminoLink® coupling gel matrix is a 4% cross-linked beaded agarose. Diol groups on the agarose have been activated into aldehyde functional groups, which react spontaneously with primary amines found on lysine residues of the rabbit albumin (ligand) as shown in Figure 2.1. Reductive amination of the resulting Schiff base with sodium cyanoborohydride ($NaCNBH_3$) forms a stable secondary amine linkage with the ligand. The unreacted aldehyde functional groups on the gel are blocked with primary amine molecules in buffers such as, Tris-HCl (as in this experiment) and glycine. $NaCNBH_3$ is once again added to stabilize the Schiff base between the primary amine of the blocking molecule and the gel.

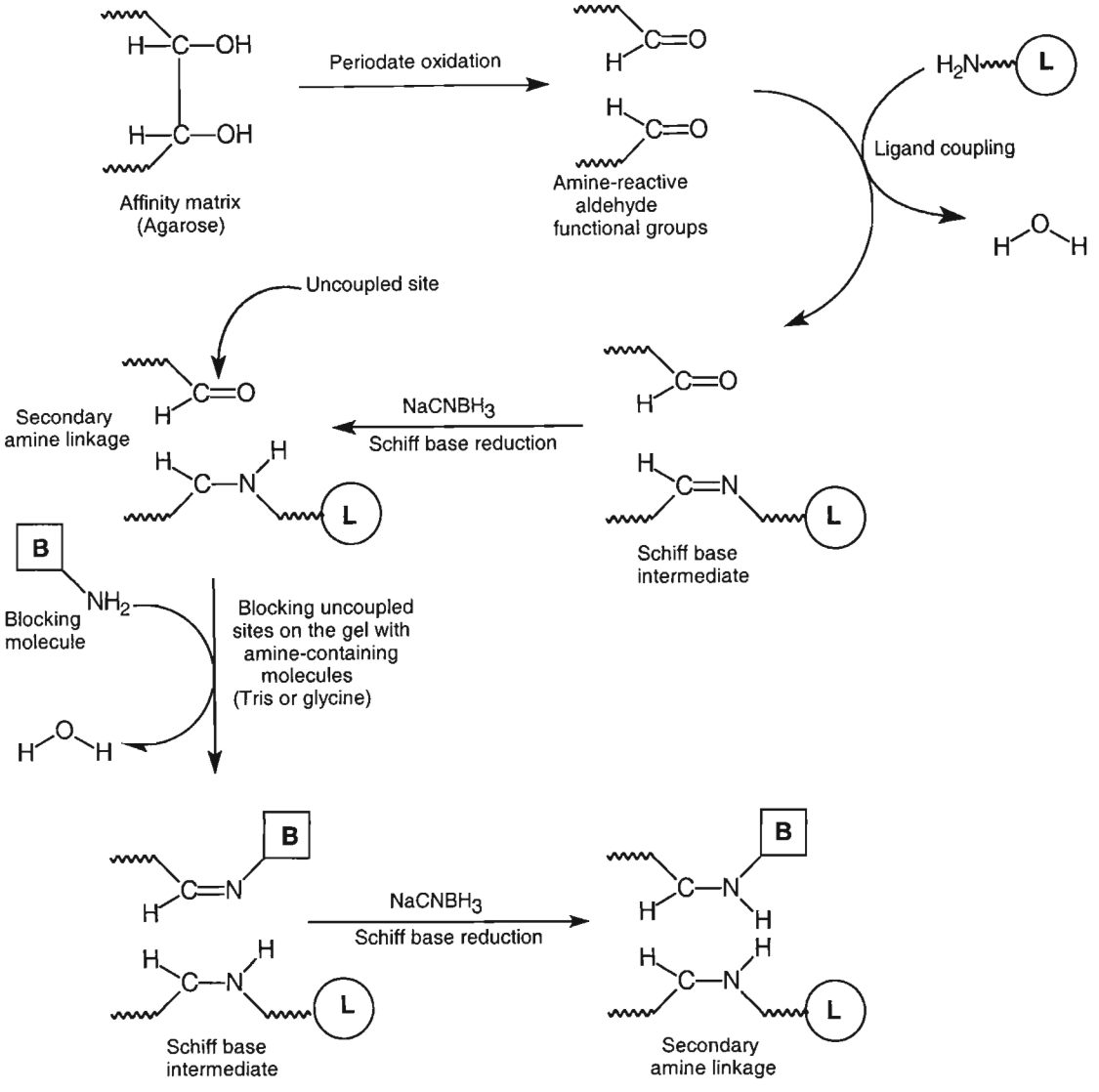


Figure 2.1 Derivatization of cross-linked agarose beads for affinity chromatography
Diol groups on the carbohydrate are oxidized to aldeyhyde with periodic acid (Hermanson, 1996). Amine-containing ligand reacts with the aldehyde, forming Schiff base intermediate that is subsequently reduced to a more stable secondary amine linkage with $NaCNBH_3$. Unreacted sites on the matrix are blocked with amine-containing molecules to prevent covalent immobilisation unwanted of molecules (Cuatrecasas, 1970; Domen *et al.*, 1990; Mohan and Lyddiatt, 1997).

2.5.1 Reagents

Coupling buffer [100 mM $NaH_2PO_4.2H_2O$, 0.05% (w/v) NaN_3, pH 7.2]. $NaH_2PO_4.2H_2O$ (15.6 g) and NaN_3 (0.5 g) were dissolved in 950 ml of distilled water, stirred and adjusted to pH 7.2 with NaOH. The solution was made up to 1000 ml with distilled water.

Sodium cyanoborohydride [1 M $NaCNBH_3$, 10 mM NaOH]. 0.01 M NaOH was prepared by diluting 1 ml of 1 M NaOH (40 g/L) to 100 ml. $NaCNBH_3$ (630 mg) was dissolved in 10 ml of 10 mM NaOH.

Quenching buffer [1 M Tris.HCl, pH 7.4]. Tris (12.1 g) was dissolved in 50 ml of distilled water; adjusted to pH 7.4 with HCl and the volume finally made up to 100 ml with distilled water.

Washing buffer [1 M NaCl]. 58.4 g of NaCl was suspended in 1000 ml of distilled water and stirred until the entire salt had dissolved.

Rabbit albumin [20 mg/ml rabbit albumin]. Rabbit albumin (50 mg) was dissolved in 2.5 ml of coupling buffer. This solution was made before use.

Affinity column-washing buffer [100 mM NaH_2PO_4, 0.2% (w/v) NaN_3, pH 6.5]. NaH_2PO_4 (15.6 g) and NaN_3 (0.2 g) were dissolved in 800 ml of distilled water, stirred and adjusted to pH 6.5 with NaOH. The volume was made up to 1 000 ml.

Elution buffer [100 mM glycine, 0.2% NaN_3 (w/v), pH 2.8]. $NH_2COCH_2NH_2$ (7.5 g) and NaN_3 (0.2 g) were dissolved in 800 ml of distilled water, adjusted to pH 2.8 with HCl and the volume finally made up 1000 ml.

Eluent neutralizing buffer [1 M NaH_2PO_4, 0.2% NaN_3 (w/v), pH 8.5]. NaH_2PO_4 (15.6 g) and NaN_3 (0.02 g) were dissolved in 80 ml of distilled water, stirred, adjusted to pH 8.5 with concentrated NaOH and the volume finally made up to 100 ml.

2.5.2 Preparation of rabbit albumin affinity column using AminoLink® coupling gel

This method follows the manufacturers' procedure (Pierce Biotechnology). 5 ml of the gel slurry containing 2.5 ml of the gel was transferred into the affinity column. The gel was

washed with about 25 ml of coupling buffer and allowed to settle by standing. 2500 µl of 20 mg/ml rabbit albumin solution was added to the gel, followed by 62.5 µl of $NaCNBH_3$ solution. The affinity column was capped and mixed in an inverter (2 h, RT). After 2 h of constant mixing, the column was incubated at 4°C overnight. The following morning, 2.5 ml of quenching buffer and 125 µl of $NaCNBH_3$ solution were added to block unreacted sites on the gel. The column was mixed for 30 min and the washed with 25 ml of washing buffer.

2.5.3 Method for immunoaffinity purification of anti-rabbit albumin IgY

The affinity column was first washed with 25 ml of affinity column-washing buffer. The isolated IgY was filtered with Whatman No. 1 filter paper to prevent blocking the affinity column. The filtrate was cycled through the column overnight and the unbound antibodies were washed off the column with 25 ml of affinity column-washing buffer. With the flow rate of 200 µl/ min, the bound antibodies were eluted with the elution buffer into eppendorf tubes containing 100 µl of neutralizing buffer. The absorbance at 280 nm was read and then the elution profile was obtained by plotting absorbance at 280 nm against elution volume. Tubes expressing absorbance values > 0.2 were pooled together. 200 µl of affinity-purified IgY was kept and labelled S_7 for SDS-PAGE analysis.

2.5.4 Storage of affinity-purified polyclonal chicken anti-rabbit albumin IgY

Affinity-purified chicken IgY was stored concentrated at refrigerated temperature in a 100 mM sodium phosphate buffer, pH 7.2 containing 0.01% sodium azide as preservative.

2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a modification of ordinary native polyacrylamide gel electrophoresis that involves the incorporation of a highly polyionic detergent, SDS, in the buffer system and of the polyacrylamide gel electrophoresis. The detergent is used to prepare the protein samples to be separated on the

gel. SDS binds tightly to the proteins, giving them a 'blanket' negative charge thus transforming the protein molecules into a rod-like shape when resolving on the polyacrylamide gel. The migration of the proteins will be towards the anode in an electrical field. The extent of migration in the gel is influenced by the size, shape and composition of the protein. Large proteins will migrate slowly while small proteins will migrate faster (Weber and Osborne, 1969). Large polymeric proteins such as immunoglobulins that are linked by disulfide bonds are often reduced with reducing agents such as β-mercaptoethanol and dithiothreitol (DTT) for these proteins to resolve well on the gel. The ratio of the distance moved by the dye front and the distance of the resolved protein has an inverse relationship. The technique is useful to determine the molecular weight of unknown protein samples when compared to standard proteins of known molecular mass.

Polyacrylamide gels are prepared by free radical polymerization of acrylamide and the cross-linking agent *N,N'*-methylene-bis-acrylamide. Ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED) are used as initiators. The quantity of acrylamide/ bis-acrylamide used in the process determines the size of the pores of the gel. The higher the concentration of acrylamide the smaller the size of the gel pores and *vice versa*. Often the size of the proteins to be resolved on the gel determines the relative quantity of polyacrylamide/bis-acrylamide. Large protein will not migrate easily on highly polymerized gels due to their size; hence poor resolution of these proteins will be obtained. Therefore large proteins will require low percentage gels and small proteins will require high percentage gels (Boyer, 1993).

The discontinuous SDS-PAGE developed by Laemmli (1970) was used to study the visualization of proteins separated by SDS-PAGE using several dye stains and to evaluate the purification procedure of IgY from egg yolk. This Laemmli SDS-PAGE system comprises discontinuous two-tiered gel systems. The lower running gel that resolves the protein and an upper stacking gel with large pore size that is non-restrictive to electrophoretic mobility of resolving proteins. The buffers used in the preparation of these gels are of different ionic strength, which implies that there will be different rates of protein migration in the two gel layers.

2.6.1 Reagents

Monomer solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]. Acrylamide (73 g) and bis-acrylamide (2 g) were dissolved and made up to 250 ml with distilled water. The solution was stirred, filtered through Whatman No. 1 filter paper and stored in an amber bottle at 4°C.

Running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (45.37 g) was dissolved in 200 ml of distilled water, stirred, pH adjusted to 8.8 with HCl and the volume made up to 250 ml with distilled water. The solution was filtered with Whatman No. 1 filter paper.

Stacking gel buffer [500 mM Tris-HCl, pH 6.8]. Tris (3 g) was dissolved in 40 ml of distilled water, adjusted to pH 6.8 with HCl, and the volume was finally made up to 50 ml with distilled water. The solution was filtered with Whatman No. 1 filter paper. This solution was made up weekly.

10% (w/v) Sodium dodecylsulfate (SDS). 10 g of SDS was dissolved in 100 ml of distilled water and stirred continuously until the entire detergent had dissolved.

Initiator reagent [10% (w/v) ammonium persulfate]. 200 mg of ammonium persulfate was dissolved in 2 ml of distilled water. This reagent was prepared just before use.

Tank buffer [250 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3]. 15 g of Tris and 72 g of glycine were dissolved in about 5 L of distilled water and stirred. Before use, 5 ml of 10% SDS solution was added to 500 ml of the buffer.

Reducing sample buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20 % (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 6.8]. 2.5 ml of stacking gel buffer was mixed 4 ml of 10% SDS, 2 ml of glycerol and 1 ml of β-mercaptoethanol, mixed thoroughly and made up to 10 ml with distilled water. 35 μg of bromophenol blue was added as tracking dye.

Non-reducing sample buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20 % (v/v) glycerol, pH 6.8]. 2.5 ml of stacking gel buffer, 4 ml of 10% SDS and 2 ml of glycerol were mixed thoroughly and made up to 10 ml with distilled water. 35 μg of bromophenol blue was added as tracking dye.

Molecular weight markers (phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14 kDa). The constituent of a vial was mixed with 100 μl of the non-reducing sample buffer.

2.6.2 Preparation of polyacrylamide gel and evaluation of IgY purification on reducing Laemmli SDS-PAGE

Reducing or non-reducing SDS-PAGE was carried out as described by Laemmli (1970) using a Bio-Rad vertical slab electrophoresis apparatus. Running (separating) and stacking gels were prepared according to the recipe on Table 2.1. Prior to casting of the gel mixture, the Bio-Rad Mini PROTEAN II® vertical slab electrophoresis accessory unit was pre-cleaned and rinsed in 50% (v/v) ethanol solution. The unit was made up of a gel-casting stand with casting gaskets, two ten-well gel combs (1.5 mm thick), two 81.5 × 101.5 mm^2 and 72.5 × 101.5 mm^2 glass plates. The gel-casting unit was assembled according to the manufacturers' instructions.

Table 2.1 Recipe for two Laemmli (1970) SDS-PAGE gels for the Bio-Rad Mini PROTEAN II™ vertical slab electrophoresis apparatus

Reagent	Volume for 12.5% running gel	Volume for 7.5% running gel	Volume for stacking gel
Monomer solution	6.25 ml	3.75 ml	940 µl
Separating gel buffer	3.75 ml	3.75 ml	0.0
Stacking gel buffer	0.0	0	1.75 ml
10% SDS	150 µl	150 µl	70 µl
Distilled water	4.75 ml	7.25ml	4.20 ml
Initiator reagent	75 µl	75 µl	35 µl
TEMED	7.5 µl	7.5 µl	15 µl

Unpolymerised running gel slurry enough for two slabs were applied between the glass plates to a height of about 6.0 cm from the base and overlaid with water to prevent oxidative inhibition of polymerisation of the acrylamide. The gels were allowed 2 h polymerisation time. The water overlay was removed and then the stacking gel slurry was applied up to the brim of the 72.5 mm × 101.5 mm glass plate. The ten-well gel comb was immediately inserted in the stacking gel slurry and allowed to polymerise.

IgY purification profile was evaluated on SDS-PAGE using samples collected at each step of the isolation procedure. Briefly, 10 µl of sample of S_1, S_2, S_3 and S_5 was mixed with 100 µl of reducing or non-reducing treatment buffer. 50 µl of sample S_4 was mixed with 50 µl of the reducing or non-reducing treatment buffer. S_6 and S_7 were made up in the treatment buffers such that 10 µl of the sample loaded on the gel will contain 10 µg of protein. The samples were immersed in boiling water bath for 90 s and immediately incubated in an ice bath until they were loaded onto the gel wells. The samples of molecular weight markers were treated similarly to the IgY samples. 5 µl of each sample was loaded onto separate wells on the gel. The gel unit was immediately connected to a power pack and electrophoresis performed at 36 mA (18 mA per gel slab) until the dye front was about 0.5 cm from the bottom. The gels were immediately removed from the unit and immersed in Coomassie Blue R 250 staining solution. After 2 h staining time, the gels

were destained in methanol-acetic acid water [5:1:4 (v/v/v)] solution until background was entirely clear.

2.7 Secondary structure and sequence analysis of chicken IgY λ-chain and μ-chain regions

The sequences corresponding to the λ-chain and μ-chain regions of the chicken IgY were obtained from published literatures (Reynaud *et al.*, 1983; Pavari *et al.*, 1988). BLAST (basic local alignment search tool) /similarity search of these sequences were carried out on Expasy Search Tool (www.expasy.org/swisprot) for confirmations of the amino acid sequences. The sequences from the BLAST search were used to derive the secondary structure analysis using the Predict7® software. The data obtained from the Predict7® analysis was converted to Microsoft Excel® and the graph of the surface probability of the amino acid was plotted against the residue number. The possible position of a lysine residue on the structure was highlighted.

2.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA is an acronym for enzyme-linked immunosorbent assay. It is a form of an immunoassay that utilizes enzyme-catalysed reactions as a means of antibody or antigen detection and quantification. As the name implies, ELISA involves several parts that makes up a complete immunoassay system namely: a solid phase on which the antigen or antibody to be assayed is immobilised; antigen or antibody to be assayed; blocking molecule, which is usually protein that does not interfere with the assay; primary antibody, which interacts with the antigen or antibody immobilized on the solid phase. Depending on the ELISA format the primary antibody may be linked the enzyme in direct ELISA system. In an indirect ELISA system a secondary antibody that interacts with the primary antibody is linked to the enzyme. Peroxidase, glucose oxidase and alkaline phosphatase are widely used in ELISA. These enzymes catalyse the conversion of colourless substrates or non-fluorescent molecules into coloured or fluorescent molecules that are quantified. These

substrates form part of the ELISA system. The buffer used in ELISA is commonly a high pH (9.6) sodium carbonate-bicarbonate buffer or neutral phosphate buffered saline (PBS).

There are three major systems (arrangement and uses of reagent in an ELISA test) in ELISA, which are direct, indirect and sandwich systems. Direct and indirect systems, as described above, involve the use of labelled primary and secondary antibody respectively, and the antigen is directly immobilised on the solid phase. In sandwich ELISA system, the antibody directed against the antigen to be assayed is immobilized on the solid phase before introduction of the antigen. Sandwich ELISA may be direct or indirect depending on whether the primary or secondary antibody is labelled with the detecting enzyme. ELISA technique may be designed to be a competitive or non-competitive method (Crowther, 2001).

Competitive ELISA technique utilizes antigen-enzyme or antibody-enzyme conjugate as competing molecule in addition to a standard or sample antigen/antibody to be assayed. The concentration of the enzyme-modified chromogenic/fluorescent product is usually inversely proportional to the concentration of the standard or test antigen/antibody. This method of ELISA has been useful in the assay of a wide variety of antigens including IgG, human chrorionic gonadotrophin, ATP, and carcino-embyonic antigen (Hammstrom *et al.,* 1975). Non-competitive ELISA technique is also called immunoenzymometris assay. In this type of technique, the antigen is reacted with excess of antibody. The degree of antigen-antibody reaction is measured in a step involving labelled primary or secondary antibody. Non-competitive ELISA requires lesser assay time and it is very sensitive compared to the competitive technique. However, the precision and reproducibility of non-competitive ELISA may be compromised if not well controlled (Hammstrom *et al.,* 1975; Nakamura *et al.,* 1986).

In this study, ELISA was used to demonstrate the immunoreactivity of IgY used in the study against rabbit albumin (antigen) in an indirect/non-competitive technique and to evaluate the immunoreactivity of dye-IgY conjugates towards rabbit albumin in a competitive ELISA technique.

2.8.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 1000 ml of distilled water.

0.5% (w/v) ovalbumin-PBS. Ovalbumin (0.5 g) was dissolved in 100 ml of PBS.

0.15 M citrate-phosphate buffer at pH 5.0. A solution of citric acid monohydrate (21.0 g/l) was titrated with a solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer]. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissoloved in citrate-phosphate buffer at pH 5.0 (15 ml).

2.8.2 Method

A 96-well microtitre plate was coated with 150 µl of 1 µg/ml of rabbit albumin in PBS over night at 4°C. The following day, the plates were washed with PBS and blocked with 0.5% ovalbumin-PBS (30 min, RT). After blocking, the plate was washed three times with PBS. 150 µl of 100.0 – 1.5625 µg/ml of anti-rabbit albumin IgY (affinity-purified), non-affinity-purified immune IgY, non-immune IgY and 0.1 M ovalbumin-PBS were added to the plates in triplicate wells. The plates were incubated (2 h, 37°C) and washed three times with PBS. 150 µl of 1:20 000 dilution of rabbit-anti-chicken-HRPO was added to each well and incubated (1 h, 37°C). After incubation, the plates were washed three times with PBS and 150 µl of substrate (ABTS) was added to each well. The colour was allowed to develop for 30 min before the absorbance reading was obtained at 405 nm. The results were converted to Microsoft Excel® and the absorbance versus the concentration was plotted.

2.9 Detection of proteins blotted on nitrocellulose membrane using range of dyes

Dyes are often used to detect and quantify protein immobilized on a solid support such as nitrocellulose, polyvinylidene diflouride (PVDF), and acetate membranes. Nitrocellulose membrane is the most popular protein-immobilizing matrix used in electro-blotting of proteins and peptides separated on by PAGE and in dot-blotting immunoassays. (Towbin *et al.*, 1979; 1984; Li *et al.*, 1988). Solid phase assays that use nitrocellulose as protein immobilisation matrix, combined with dye staining techniques, have been successfully used in quantitative estimation of proteins in nanogram range. In this case, the intensity of the protein-dye complex spot is proportional to the amount of protein in the complex when scanned on a densitometer (Nakamura *et al.*, 1985, Li *et al.*, 1988, Li *et al.*, 1989; Thornton *et al.*, 1989; Morçöl and Subramanian, 1999,). Proteins stained on nitrocellulose using a range of organic dyes, such as Amido Black (Naphthol Blue Black), Ponceaus S, Coomassie Blue and Aurodye, can also be quantified by eluting the dye-protein complexes into solvents such as dimethyl sulfoxide (DMSO) and determining the absorbance of the resulting solution spectrophotometrically (Parek *et al.*, 1985; Goldring and Ravaioli, 1996).

2.9.1 Preparation of dye staining reagents

Dye stains were prepared in methanol-acetic acid solution at pH approximately 2.3 [50% (v/v) methanol and 10% (v/v) acetic acid], methanol-NaOH solution at pH approximately 13.0 [50% v/v methanol, 10% (v/v) NaOH] and 0.1 M sodium phosphate buffer at pH 7.2. All dye solutions were of equal molar concentration of 0.75 μmol/ml. The dye was dissolved in the buffer and stirred for 1 h at room temperature. The solution was filtered with a filter paper and stored in a bottle covered with aluminium foil prior to usage. Not all dyes were soluble in the solution in which the dye was suspended. Eriochrome Black T and Hematoxylin were suspended in 1 ml of methanol before suspending in 100 mM sodium phosphate buffer at pH 7.2. Primuline was dissolved in 1 ml of DMSO before suspending in any of the solutions.

2.9.2 Method

Nitrocellulose membrane sheets were cut to 5 cm × 1.5 cm. Gloves were worn while handling the nitrocellulose membrane sheets to prevent contamination of the membrane by keratin. 0.5 µl dot of 0.05 – 10.0 mg/ml IgY in 0.1 M phosphate buffer at pH 7.2 were blotted on nitrocellulose membrane (0.5 µl of 0.1 M phosphate buffer was blotted as a negative control), allowed to dry (1 h, RT) and stained in various dye stains for approximately 5 min before destaining in the solution without the dye (destaining solution) until the background was reduced. The protein stains were observed by eye.

2.10 Detection of proteins resolved on SDS-polyacrylamide gels using range of dyes

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a very reliable and widely used technique for the separation, identification and characterization of proteins and protein mixtures (Jung *et al.,* 1998). Many commercially available organic dyes can be employed for the detection of proteins separated on sodium dodecylsulfate-polyacrylamide gels, though they may not have equal sensitivity. Some of these organic dyes are Coomassie Blue R 250 (Fazekas De St. Groth *et al.*, 1963; Meyer and Lambert, 1965), EosinY (Lin *et al.*, 1991), Eriochrome Black T, Rhodamine B (Jung *et al.*, 1998), Bismark Browm R-Coomassie Blue R 250 mixture for higher sensitivity (Choi *et al.*, 1996), Amido Black (Chrambach *et al.*, 1967) and PAS-staining of glycoproteins on sodium dodecylsulfate-polyacrylamide gel (Zaccharius *et al.*, 1969).

A silver staining technique was developed to improve sensitivity limits on protein staining (Merrill *et al.*, 1979; Wray *et al.*, 1980). However, the sensitivity of silver staining can be comparable to Eosin Y (Lin *et al.*, 1991).

The focus of this section is the dye-based detection of proteins separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein-binding abilities and sensitivities of various listed dyes (section 2.2) at acidic pH will be studied in a bid to discover new dyes that might have equal or greater sensitivity than Coomassie Blue R 250.

In this section, dyes that stained proteins on nitrocellulose membrane in methanol-acetic acid solution were used in the first phase study under this section. The dyes were Naphthol Blue Black, Eriochrome Black T, Reactive Green 19, Reactive Red 120, Aurintricarboxylic Acid, Reactive Black 5, Cibacron Blue 3GA, Nigrosin Water-soluble, Trypan Blue, Evans Blue, Methyl Orange, Direct Red 75, Direct Red 81, Primuline, Hematoxylin, Bromocresol Purple and Ponseau S. 7.5 μmol of dye in 50 ml of methanol-acetic acid solution. All dye stains were prepared in methanol-acetic acid solution as in section 2.6.2. The study was divided into four phases of experiments.

2.10.1 Methods for general screening of dyes

10 μg of chicken IgY was loaded into 10-well 12% reducing-Laemmli SDS-polyacrylamide gel and resolved electrophoretically. When the dye front reached approximately 0.5 cm from the base, electrophoresis was terminated and the gel was cut into strips of a single lane containing the resolved protein. The gel strips were stained overnight at room temperature in the dye solutions and destained in methanol-acetic acid solution.

2.10.2 Sensitivity study using dyes that worked well in section 2.10.1 in comparison with Coomassie Blue R 250

Dyes that gave a clear background after destaining in methanol-acetic acid solution were employed in this section. Dyes used were Reactive Black 5, Direct Red 81 and Cibacron Blue 3GA. The objective of this second phase study was to determine the sensitivity of three dyes that gave a clear background in the first phase, in comparison with Coomassie Blue R 250 stain. 10 μl containing 10.0, 5.0, 2.5, 1.25, 0.625 and 0.3125 μg of chicken IgY were loaded into 12% reducing-Laemmli SDS-polyacrylamide gel. The control sample had no protein but 10 μl of buffer in which the protein was dissolved. The samples were treated according to standard reducing-Laemmli-SDS-PAGE procedure (section 2.6.2). When the dye front reached approximately 0.5 cm from the base of the gel, electrophoresis was terminated and gels carefully removed and immersed in the staining solution. Staining was carried out at room temperature overnight and gels were destained the following morning

with methanol-acetic acid solution. After the background cleared, the gels were soaked in distilled water to swell before imaging. Imaging was carried out with Canon CanoScan LiDE 30 scanner. The gels were placed in between two transparent sheets, scanned and the image captured with ArcSoft PhotoStudio 5.0® software. Images were saved as JPG files and copied directly, without any alteration on the scanned images, into Microsoft Word® programme.

2.10.3 Comparing staining ability of Direct Red 81 with Coomassie Blue R 250

This section involves staining variety of proteins resolved on SDS-polyacrylamide gel with Direct Red 81 and comparing it with Coomassie Blue R 250 stain. Equimolar amounts (7.5 μmol/ml in methanol-acetic acid solution) of Direct Red 81 and Coomassie Blue R 250 were used. Commercial low molecular weight calibration kit for SDS-PAGE (Amersham-Biosciences) was the model for protein variety. 10 μl of the sample containing phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin was loaded into four wells (for duplicate results) of 12% reducing Laemmli SDS-polyacrylamide gel and electrophoresis performed according to the standard Laemmli-SDS-PAGE procedure. When the dye front reached *ca* 0.5 cm from the base of the gel, electrophoresis was terminated and the gels stained in either Direct Red 81 stain or Coomassie Blue R 250 stain overnight at RT. The gels were destained with methanol-acetic acid solution until the background became very clear.

2.10.4 Comparing the staining time of Direct Red 81 to Coomassie Blue R 250

In this section, the time it took Direct Red 81 to visibly 7.5 μg of IgY resolved on 12% SDS-polyacryamide gel was compared to Coomassie Blue R 250. 10 μl of the sample containing 7.5 μg of IgY was loaded into gel and resolved using the standard Laemmli procedure. When the dye front reached *ca* 0.5 cm from the base of the gel, electrophoresis was terminated. The gels were removed from the unit and carefully cut into strips of single lane. The gel strips were stained in either Direct Red 81 staining reagent or Coomassie Blue R 250 staining reagent (five strips per stain). A strip was removed from the stain after 5, 10,

15, 20, 30, 40, 50 and 60 min and then destained in 100 ml of methanol-acetic acid solution until the background became clear.

2.11 Covalent labeling of affinity-purified anti-rabbit albumin IgY with dyes

The use of antibody molecules for immunoassays, targeting, or detection techniques encompasses a broad variety of applications affecting nearly every field of life sciences. The availability of relatively inexpensive polyclonal and monoclonal antibodies with exact specificity has made possible the design of reagent systems that can interact with high affinity with targets or antigens that are difficult to visualize ordinarily. The specificity of purified immunoglobulins provides a powerful tool for constructing immunological techniques. Using bioconjuagation techniques, antibodies can be modified by covalent labeling of molecules (called probes) for direct and/or indirect immunodetection. These labelled antibodies provide the means by which antigens can be detected by colourimetric or fluorimetric means (Hermanson, 1996).

In this section, affinity-purified polyclonal chicken egg yolk immunoglobulins (IgY) against rabbit albumin will be covalently labelled with dyes bearing hydroxyl and/or carboxylic functional groups. These dyes are Aurintricarboxylic Acid, Ethyl Red, Gallocyanine and Malachite Green HCl (Figure 2.2). These conjugate were used as direct colourimetric immunoassay tool for detecting rabbit albumin on a nitrocellulose membrane. The dye provides the colour for detection of the antigen.

The covalent conjugation of dyes to IgY was mediated by CDI. These dyes were activated in either dimethylformamide (DMF) or dimethylsulfoxide (DMSO) because CDI is unstable in an aqueous environment (Bethel *et al.,* 1979; Hearn, 1987; Xiao *et al.,* 1995). Several biochemical techniques were employed to study the activation and conjugation strategies. Secondary structure sequence analysis of chicken IgY was carried out with the Predict7® software in order to establish the surface availability of lysine residue in the structure of IgY.

The reaction mechanism involved in the conjugation process was a two-step reaction. The first step is the activation of dye with CDI and the second step being the coupling of activated dye to IgY. Figure 2.3 illustrates the possible reaction mechanism. CDI reacts with COOH and/or OH groups on the dye molecule to form acyl imidazole (if COOH was activated) and/or imidazole carbamate (if OH was activated) intermediates. The acyl imidazole and imidazole carbamate will proceed to react with ε-amine residues on the IgY molecule.

The reacting molar ratio of the dye to CDI used in the activation was not uniform for all dyes used in the experiment. This was because the number of functional targets per molecule of dye is not equal for all dyes. Therefore, the reacting molar ratio was estimated based on the number of CDI-target functional groups (COOH and OH) on one dye molecule. The activation was designed so that there will be two CDI molecules per functional target on each dye molecule. The coupling step was carried out at alkaline pH. This pH is suitable for CDI-mediated conjugation because the rate of hydrolysis of activated acyl imidazole or imidazole carbamate is retarded at alkaline pH (Roberts *et al.*, 1987; Hermanson, 1996).

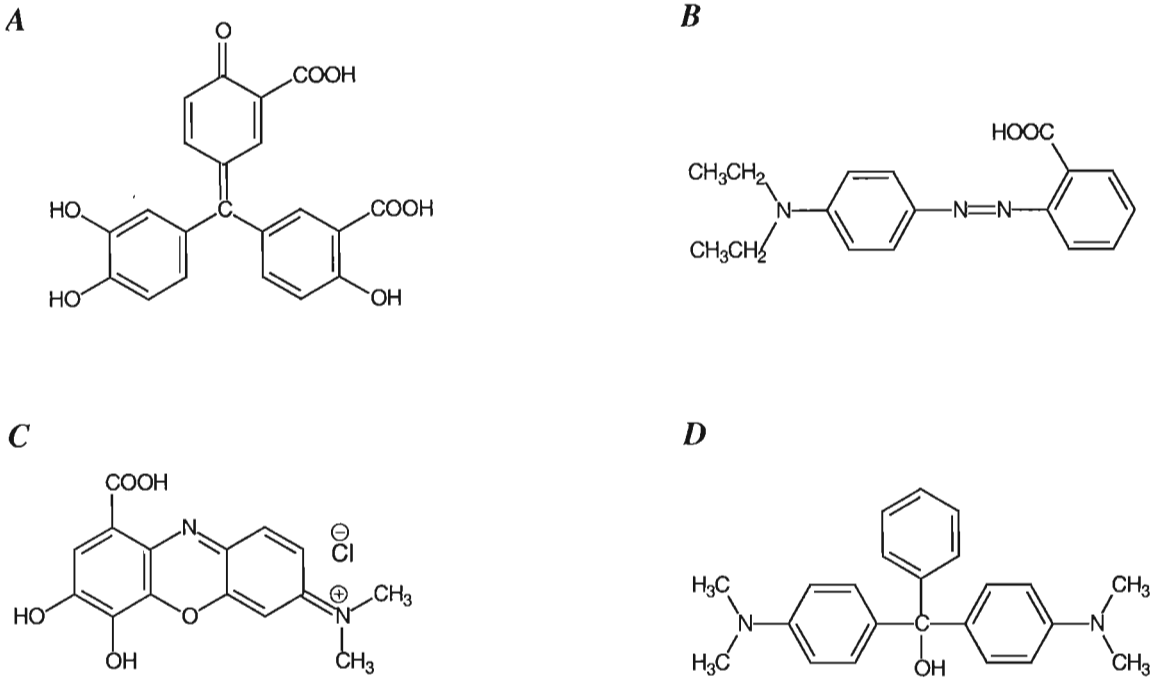


Figure 2.2 Molecular structure of dyes used in covalent conjugation to IgY
A: Aurintricarboxylic Acid, *B*: Ethyl Red, *C*: Gallocyanine and *D*: Malachite Green HCl. (www.sigma-aldrich.com)

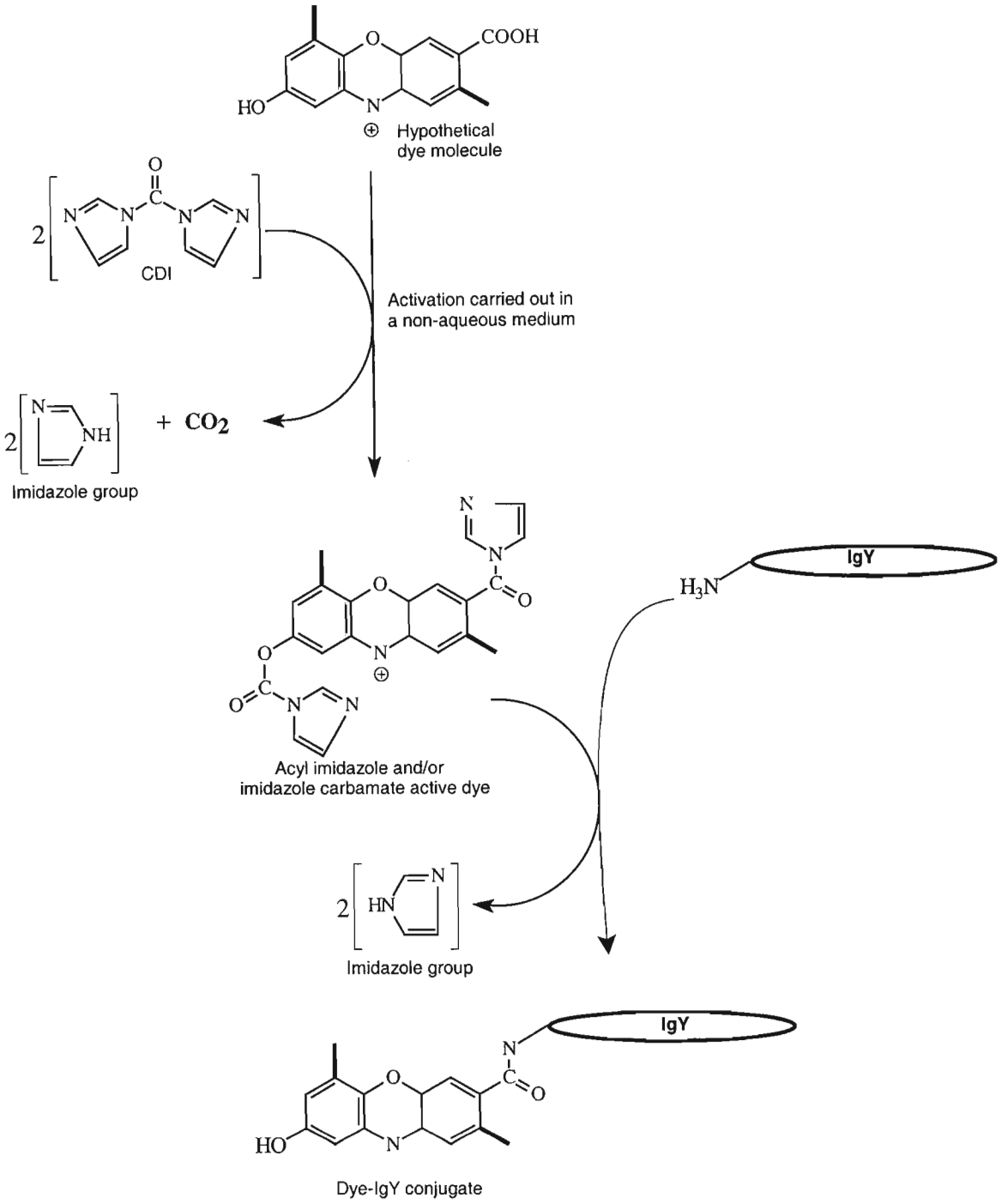


Figure 2.3 Schematic illustration of reaction mechanisms for activation and coupling of a hypothetical dye with hydroxyl and carboxylic acid functional groups on chicken IgY using CDI

2.11.1 Reagents

Sodium carbonate-bicarbonate buffer [100 mM Na_2CO_3, 100 mM $NaHCO_3$, pH 9.6]. Na_2CO_3 (8.4 g) was dissolved in 1 L of distilled water. $NaHCO_3$ (42.4 g) was dissolved in 4 L of distilled water. Na_2CO_3 solution was adjusted to pH 9.6 with approximately 3 L of $NaHCO_3$ solution.

Dialysis solution [200 mM NaCl]. NaCl (11.7 g) was dissolved in 1000 ml of distilled water.

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 1000 ml of distilled water.

2.11.2 Method for activation of dyes with CDI

A modification of Roberts *et al.* (1987) and Xiao *et al.* (1995) was used. Non-aqueous activation of dyes was carried out in two media, DMF and DMSO so as to determine the best solvent for the activation process. Due to insolubility of Aurintricarboxylic Acid in acetone, acetone was not used as a medium for activation. 100 mg of each dye was suspended in 2.5 ml of either DMF or DMSO with equivalent amount of CDI that will give the estimated reacting molar ratios of dye to CDI. Table 2.2 shows the recipe for activation of various dyes used in this experiment. The number of functional target for CDI is on the second column. The amount of CDI (mg) used per 100 mg of each dye is given in the fifth column. The last column shows the molar ratio between the dye and CDI. The control dyes were not activated (had no CDI). The mixture was stirred overnight at room temperature in the dark.

Table 2.2 Recipe for activation of various dyes used in this experiment

Dye	Number of CDI functional targets per dye molecule	Formula mass	Mass of dye used (mg)	Mass of CDI used (mg)	Reacting molar ratio dye : CDI
Aurintricarboxylic Acid	5	422.35	100.0	384.0	1 : 10
Ethyl Red	1	297.36	100.0	109.0	1 : 2
Gallocyanine	3	336.73	100.0	290.0	1 : 6
Malachite Green HCl	1	346.48	100.0	93.5	1 : 2

2.11.3 Spectral analysis of activated dyes

In order to show that the dye was covalently modified in either DMF or DMSO, spectra of activated dyes were compared to the non-activated dyes. All samples were prepared immediately before scanning. 1 µl of activated dye solution was dissolved in 4 ml of 0.1 M sodium carbonate-bicarbonate buffer at pH 9.6. 1 ml of this solution was scanned between 350 nm and 750 nm. The non-activated dye control was prepared in the same way as the activated dye sample. For the blank, 1 µl of either DMSO or DMF was dissolved in 4 ml of 0.1 M sodium carbonate-bicarbonate buffer at pH 9.6.

2.11.4 Coupling of activated dyes to IgY

Prior to coupling reaction, affinity-purified IgY was dialyzed overnight in 1000 ml of 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6. The dialyzed solution was concentrated with PEG 20 000. 100 µl of activated dye or the non-activated dye control was mixed drop wise with 2.5 ml of 5 mg/ml IgY in 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6 in glass test tubes. The tubes were sealed with parafilm, wrapped with aluminum foil and mixed gently by inverting the tubes several times. The tubes were incubated (48 h, 4°C).

2.11.5 Purification of dye-IgY conjugates

Dialysis tubing (15 cm) was neatly cut with a clean pair of scissors. Gloves were worn while handling dialysis tubing to prevent contamination of the dialysis bag with keratin.

The dialysis tubing was pre-treated according to the manufacturers' instructions. The conjugate solution was carefully transferred into the dialysis tubing and the tubes sealed using special plastic clips to prevent leakage of the sample into the dialysis solution. The sample was dialyzed for 48 h against 4 changes of 0.1M sodium carbonate-bicarbonate buffer at pH 9.6 and one change of PBS containing 0.01% (w/v) NaN_3 as preservative for 24 h. The dialysis procedure lasted for approximately 72 h. After dialysis, the conjugate solution was centrifuged (1000 × *g*, 10 min, RT) to sediment aggregated dye particles and precipitated protein. The conjugate solution was stored in clean glass vials away from direct light before use.

2.11.6 Spectral analysis of dye-IgY conjugates

100 µl of dye-antibody conjugate solution was made up to 1000 µl with PBS. This solution was scanned between 250 nm and 750 nm using quartz cuvettes. PBS was used as the blank solution.

2.11.7 Estimation of molar coupling ratio of dye to IgY

Aurintricarboxylic Acid, Ethyl Red and Gallocyanine were each dissolved in DMSO to a concentration of 500 µg/ml to make stock dye solutions. From the stock solutions, 0.1 – 50 µg/ml of each dye was prepared in PBS in triplicate solution. The absorbance of each set of concentration was determined against a reagent blank (100 µl of DMSO in 900 µl of PBS). The standard calibration graph of the absorbance against the concentration was obtained. The calibration graph was used to estimate the dye concentration in each conjugate after purification by dialysis. The amount of dye/IgY ratio was deduced.

2.11.8 Analysis of dye-IgY conjugates by SDS-PAGE

The electrophoretic pattern of the conjugates was carried out on a 10% SDS-polyacrylamide gel in both reducing and non-reducing denaturing conditions. The test samples were IgY alone, dye-IgY with activation in DMF, dye-IgY without activation in

DMF, dye-IgY with activation in DMSO, dye-IgY without activation in DMSO, activated dye alone in DMF and non-activated dye alone in DMSO. 10 µl of activated dye in DMF or non-activated dye in DMSO was diluted to 200 µl with distilled water. 100 µl of this diluted dye samples was mixed with 50 µl of reducing or non-reducing treatment buffers. 100 µl of dye-IgY conjugate samples in DMF and DMSO were treated with 50 µl of reducing or non-reducing treatment buffers. Gels were imaged before and after staining.

2.12 Detecting rabbit albumin on nitrocellulose

The use of the dye-IgY conjugates in detection of antigen (rabbit albumin) was carried out on a nitrocellulose membrane. Only conjugates prepared from dyes activated with CDI in DMF were used. Several dilutions (500 – 5.0 ng) of rabbit albumin in PBS were blotted on a nitrocellulose membrane. Non-specific sites were blocked with 0.5% (w/v) ovalbumin-PBS (30 min, RT) and then the nitrocellulose membranes were incubated in three dilutions (300, 200 and 100 µg/ml) of each dye-IgY conjugates in PBS (2 h, 37°C). The membranes were washed with PBS and coloured spots were observed. Similar range of rabbit albumin concentrations was also used in the control experiment with anti-rabbit albumin IgY-HRPO. Ovalbumin was used as the negative control in both experiments.

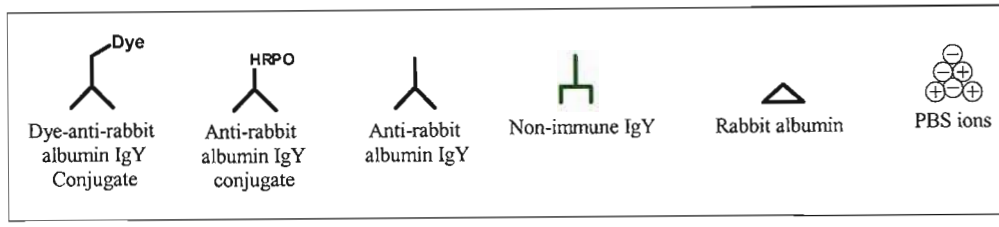
2.13 Evaluation of immunoreactivity of dye-antibody conjugates using ELISA

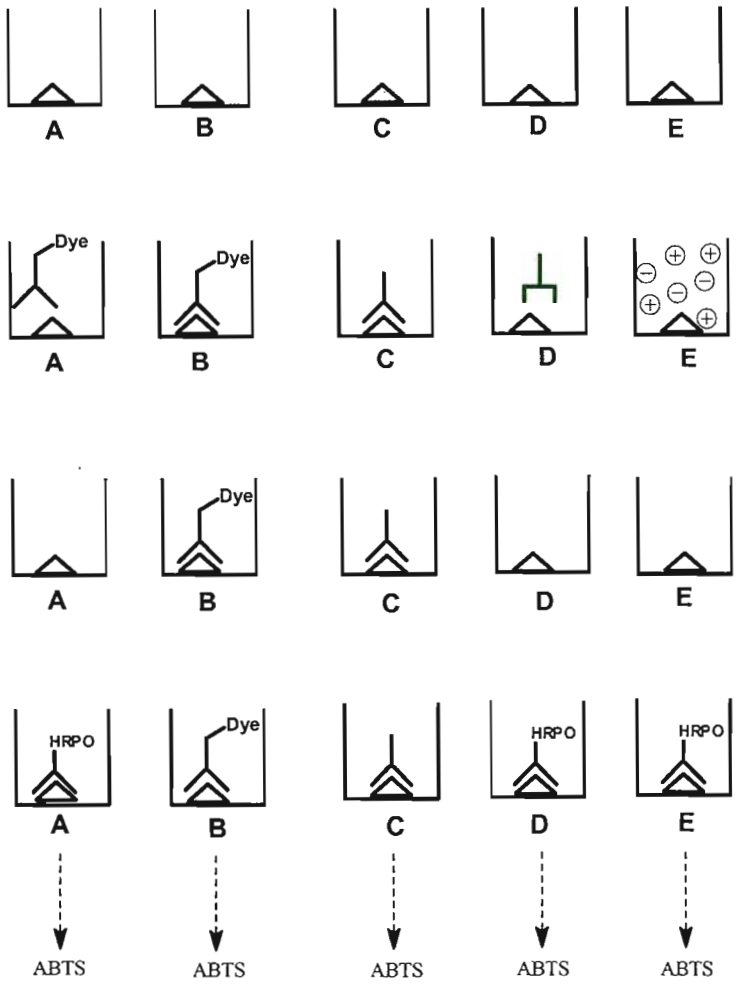
A reagent used in section 2.8.1 was used in this method. 96-well ELISA plates were coated with 150 µl of 1 µg/ml of rabbit albumin in PBS and incubated overnight at 4°C. The plates were washed three times with PBS and blocked with 0.5% ovalbumin-PBS for 1 h at 37 °C. After blocking the plates were washed three 3x with PBS. 150 µl of 100.0 – 1.5625 µg/ml dye-antibody conjugates, anti-rabbit albumin IgY (affinity-purified), non-immune IgY and 0.1 M PBS were added to the plates in triplicate wells as shown in Table 2.3 below.

Table 2.3 **ELISA plate layout for evaluation of immunoreactivity of dye-antibody conjugates**

	µg/ml of IgY			
	dye-IgY	IgY	Non-immune-IgY	PBS
Well	1 – 3	4 – 6	7 – 9	10 – 12
A	0	0	0	100 mM
B	1.5625	1.5625	1.5625	100 mM
C	3.125	3.125	3.125	100 mM
D	6.25	6.25	6.25	100 mM
E	12.5	12.5	12.5	100 mM
F	25	25	25	100 mM
G	50	50	50	100 mM
H	100	100	100	100 mM

The plates were incubated (2 h, 37°C), washed three times with PBS. 150 µl of 480 pg/ml of HRPO-labelled to anti-rabbit albumin IgY was added to each well and incubated (1 h, 37°C). After incubation, the plates were washed three times with PBS and 150 µl of substrate was added to each well. The colour was allowed to develop for 30 min before the absorbance reading was obtained at 405 nm. The results were converted to Microsoft Excel® and the absorbance versus the concentration was plotted. The scheme illustrating this procedure is shown in Figure 2.4.





Step 1
The well is incubated with 1μg/ml of rabbit albumin overnight at 4°C

Step 2
Following blocking with 0.5% ovalbumin-PBS and washing with PBS, the plate was incubated with dye-IgY, immune IgY, non-immune IgY in two fold serial dilution and PBS (2 h, 37°C)

Step 3
Unbound primary antibodies were washed off the well with PBS.

Step 4
The well is incubated with IgY-HRPO (1 h, 37°C)

Step 5
The plate was washed with PBS and incubated with ABTS (substrate) and the absorbance read at 405 nm using an ELISA plate reader.

Figure 2.4 **A schematic representation of the evaluation of the immunoreactivity of dye-IgY conjugates by ELISA**

2.14 Preparation of horseradish peroxidase-labelled IgY

Peroxidases are enzymes that catalyse the oxidation of various substrates. These enzymes are found in multiple isoenzyme forms and have optimal activity at pH 7.6. Cyanides, sulfides and azides inhibit peroxidases. High sensitivity, flexibility and availability of variety of substrates make horseradish peroxidase the enzyme of choice for many applications. Horseradish peroxidase (HRPO) is a 40 000 Da enzyme that interact with hydrogen peroxide (H_2O_2) and special electron donors to yield detectable products. HRPO has a high turnover rate yielding abundant reaction product molecules at very short time interval (www.piercenet.com).

HRPO-IgY conjugate was prepared and used as a standard for comparing the dye-IgY conjugate immunoassay reagent prepared in section 2.11. A modification to the method of Duncan *et al.* (1983) was used to prepare the HRPO-IgY conjugates. In this method, sulfhydryl groups were introduced to the IgY molecules while the enzyme will be modified with sulfhydryl-reactive maleimide ester molecule. The modified enzyme was then conjugated to the sulfhydryl-modified IgY. Figure 2.5 illustrates the chemistry of the two-step conjugation procedure. Introducing terminal sulfhydryl groups on the molecule using 2-iminothiolane (Traut' reagent) modifies the IgY (Traut et al., 1973). The enzyme was modified with MBS (3-maleimidobenzoyl-*N*-hydroxysuccinimide ester). MBS reacts with primary amine residues on the enzyme molecule, therefore conferring upon the enzyme, a sulfhydryl reactive molecule. The 2-iminothiolane reacts with primary amine residues on the antibody molecule in a ring-opening mechanism. The MBS-activated enzyme molecule then reacts with the terminal sulfhydryl residue on the modified IgY to form a stable covalent linkage between the IgY and the HRPO.

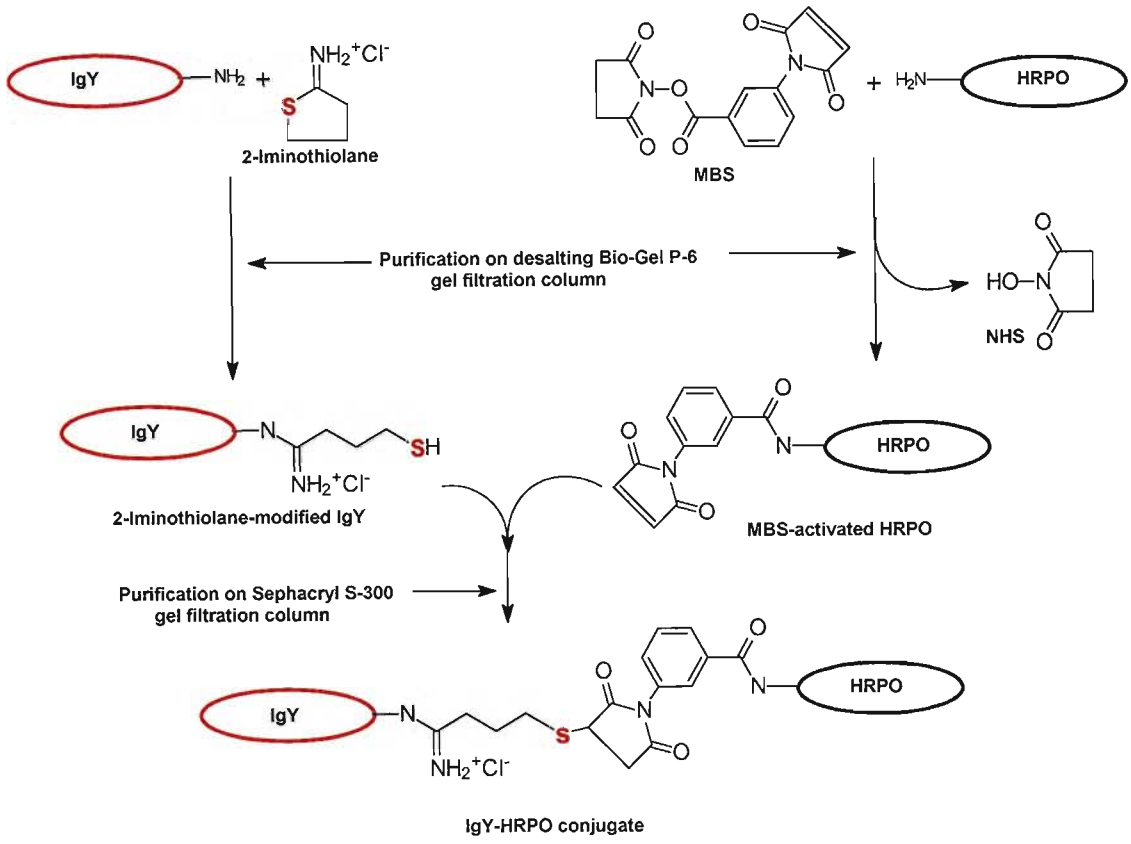


Figure 2.5 **Schematic illustration of conjugation of MBS-activated horseradish peroxidase to 2-iminothiolane modified IgY**

Molecular exclusion chromatography (MEC) was employed in the purification processes. This is a form of liquid chromatography in which molecules are separated by virtue of differences in molecular sizes and/or shape. Molecular exclusion chromatography is also known as molecular sieve, gel permeation, gel filtration or size exclusion chromatography. The separation is achieved by percolating the sample through a bed of porous and non-ionic gel matrix. These matrices are made up of beaded synthetic polymers such as agarose, dextran and acrylamide. The gel matrix represents the stationary phase of the chromatographic system and the solvent system in which the sample is dissolved is the mobile phase. Prior to separation by gel filtration, the column is equilibrated with the mobile phase. Sample containing a mixture of molecules with different molecular weight to be separated by gel filtration is passed through the column packed with the appropriate gel

matrix, depending on the molecular range of the mixture of molecules. Molecules of low mass will penetrate the matrix (depending on the size of the pore and the molecule), thus having restricted migration. On the other hand, molecules with higher molecular mass are excluded from permeating the gel matrix because of their size. Molecules with larger molecular sizes elute the column more rapidly compared to molecules that permeat the beads of the gel matrix (Andrews, 1965; Reiland, 1971).

The excess MBS was purified from the MBS-modified HRPO using Bio-Gel P-6 desalting column. Similarly, excess 2-iminothiolane was purified from the sulfhydrylated IgY using Bio-Gel P-6 column. This was preferred to dialysis because of possible loss of activity of the sulfhydryl groups by metal-catalyzed oxidation. EDTA was added to the column buffer to chelate divalent cations that might catalyze oxidation of sulfhydryl groups. Unconjugated IgY and HRPO was removed from the conjugate by molecular exclusion chromatography over pre-calibrated Sephacryl® S-300 column.

2.14.1 Reagents

Molecular exclusion chromatography (MEC) buffer [100 mM $NaH_2PO_4.2H_2O$, 150 mM NaCl, 1 mM Na_2EDTA, pH 7.5]. $NaH_2PO_4.2H_2O$ (15.6 g), NaCl (8.77 g) and Na_2EDTA (0.372 g) were dissolved in 950 ml of distilled water, adjusted to pH 7.5 with NaOH and made up to 1000 ml with distilled water.

Sulfhydryl-modification buffer [100 mM $NaH_2PO_4.2H_2O$, 150 mM NaCl, 20 mM Na_2EDTA, pH 7.5]. $NaH_2PO_4.2H_2O$ (15.6 g), NaCl (8.77 g) Na_2EDTA (7.40 g) were dissolved in 950 ml of distilled water, adjusted to pH 7.5 with NaOH and made up to 1000 ml with distilled water.

Ellman's reagent buffer [100 mM Tris, 1 mM Na_2EDTA, 0.1% (w/v) SDS, pH 8.0]. Tris (0.605 g), Na_2EDTA (0.186 g) SDS (50 mg) were dissolved in 45 ml of distilled water, adjusted to pH 8.0 with HCl and the volume was made up to 50 ml with distilled water.

Ellman's Reagent [0.4% (w/v) 5,5'-dithio-bis(2-nitrobenzoic acid), 100 mM Tris-HCl, 1 mM Na_2EDTA, 0.1% (w/v) SDS, pH 8.0]. 10 mg of 5,5'-dithio-bis(2-nitrobenzoic acid) was dissolved in 2.5 ml of Ellman's reagent buffer.

Sulfhydryl-modification reagent [10 mg/ml 2-iminothiolane]. 5 mg of 2-iminothiolane was dissolved in 500 µl of distilled water.

MBS reagent [100 mg/ml]. 10 mg of MBS was dissolved in 100 µl of DMF in an eppendorf tube wrapped with aluminum foil.

Column calibration sample solution. Blue dextran (10 mg) was dissolved in 20 ml of MEC buffer containing 10 mg of chicken IgY.

Tris-HCl buffered saline (TBS) [20 mM Tris-HCl, 500 mM NaCl, pH 7.5]. Tris (2.4 g), NaCl (29.2 g) were dissolved in 950 ml of distilled water, adjusted to pH 7.5 with HCl and the volume made up to 1000 ml with distilled water.

Blocking Solution [0.5% low fat milk in TBS]. Low fat milk (2.5 g) was suspended in 50 ml of TBS and stirred for 30 min at room temperature.

Substrate (4-chloro-1-naphthol) solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2]. 4-chloro-1-naphthol (0.03g) was dissolved in methanol (10 ml). Two ml of this solution was diluted to 10 ml with TBS, with addition of 30% hydrogen peroxide (4 µl).

2.14.2 Preparation of Bio-Gel P-6 desalting size exclusion chromatography column

From calculations made to obtain a packed bed volume of 20 ml, 3.1 g of dry gel was weighed into a beaker containing 40 ml gel filtration buffer. The gel was mixed gently with a glass rod before incubating (4 h, 37°C) to allow the gel swell (hydration). After hydration, half the volume of the supernatant was decanted. The gel suspension was transferred into a

vacuum flask containing 40ml of degassed buffer and degassed using a vacuum pump with occasional swirling of the flask. After the gel materials had settled, approximately 90% of the supernatant was removed by suction in order to remove fine particles. This step was repeated four times. The slurry of degassed gel was transferred onto the column (containing 6 ml of buffer with its exit closed) with a pre-cleaned long funnel in a single, smooth movement to prevent trapping of air bubble in the column. The column exit was opened to pack the gel properly to a height of 11 cm. The column was equilibrated with the gel filtration buffer 2 h before use.

2.14.3 Preparation of Sephacryl® S-300 size exclusion chromatography column

Sephacryl® S-300 (molecular exclusion: 10 kDa to 1500 kDa) was supplied in hydrated form from Sigma. The hydrated gel slurry was poured into the column (90.0×3.2 cm^2) and allowed to pack to a height of 82.5 cm. The packed bed volume was 438.2 ml. The column was set to run at a volumetric flow rate of 13.2 ml/h, equivalent to a linear flow rate of 1.6 cm/h. The column was stored and used at 4°C.

2.14.4 Calibration of Sephacryl® S-300 size exclusion chromatography column

Prior to calibration of Sephacryl® S-300 column, the column was equilibrated in MEC buffer for 24 h. After equilibration of the column, 20 ml of column calibration sample solution was loaded onto the column. Elution was set at the flow rate of 13.2 ml/h (section 2.14.3). 80 fractions of 5 ml per fraction were collected with an automated fraction collector synchronized with the column flow rate. The total elution volume was 400 ml. The absorbance each fraction was read at 280 nm, and then the absorbance was plotted against the elution volume to obtain the column calibration curve.

2.14.5 Activation of horseradish peroxidase with MBS

50 µl of MBS reagent was added to 2 ml of 10 mg/ml HRPO in 100 mM $NaH_2PO_4.2H_2O$, 150 mM NaCl, 10 mM Na_2EDTA at pH 7.5 buffer solution. The mixture was allowed to

react for (2 h, RT) in the dark (tube was wrapped with aluminum foil because MBS is light sensitive). By inverting the tube occasionally during the reaction interval, the reaction mixture was mixed to ensure favourable reaction. After the completion of the reaction, the activated enzyme was purified from excess reagents and reaction products by gel filtration over Bio-Gel P-6 desalting 11 cm × 1.6 cm column (linear flow rate of 8.45 cm/h) at 4°C. The brown coloured fractions were pooled and concentrated with PEG 20 000.

2.14.6 Modification of anti-rabbit albumin IgY with 2-iminothiolane

Prior to sulfhydryl modification, the affinity-purified IgY sample to be modified was re-precipitated with PEG 6000 and re-suspended in sulfhydryl-modification buffer in order to remove sodium azide and glycine present in the IgY sample. Sodium azide inhibits HRPO while glycine may interfere with the sulfhydryl-modification process. The sample was dialysed overnight against 1000 ml of sulfhydryl-modification buffer. 100 µl of sulfhydryl-modification reagent was (10 mg/ml of 2-iminothiolane solution) added to 2 ml of 13.7 mg/ml affinity-purified anti-rabbit IgY. The mixture was allowed to react (1 h, RT) in the dark with occasional swirling of the tube at 10 min interval to mix the reactants properly. Sulfhydryl-modified IgY was purified from excess reagent through the Bio-Gel P-6 desalting 11 cm × 1.6 cm column (linear flow rate of 8.45 cm/h) at 4°C. Fractions of 0.5 ml were collected and the presence of sulfhydryl groups in the fraction was tested by mixing 10 µl of each fraction with 10 µl of Ellman's reagent. The presence of sulfhydryl group was confirmed by yellow colour. The first set of fractions producing a yellow colour with the Ellman's reagent were pooled together and concentrated with PEG 20 000.

2.14.7 Coupling of MBS-activated HRPO with 2-iminothiolane-modified IgY

MBS-activated HRPO solution (5 ml) was divided into 1 ml aliquot in 5 pre-cleaned test tubes. One ml of pooled sulfhydry-modified IgY was added to each test tube containing MBS-HRPO. The tubes were coved with parafilm, wrapped with aluminium foil and incubated (15 h, 4°C) and then pooled into a single test tube.

2.14.8 Purification of IgY-HPO conjugates

The IgY-HRPO conjugates were purified from uncoupled IgY and HRPO through the Sephacryl® S-300 column. The flow rate was similar to calibration flow rate (section 2.14.3). 80 fractions of 5 ml per fraction were collected with an automated fraction collector synchronized with the column flow rate. The total elution volume was 400 ml. The absorbance of each fraction was read at 280 nm and 403 nm. The absorbance was plotted against the elution volume to obtain the elution profile. The fractions containing IgY-HRPO conjugates were pooled together and concentrated to 13 ml, which was divided into 1 ml aliquots. To the 1 ml aliquots of IgY-HRPO conjugate solution, 10 µl of 1% (w/v) thimerosal (10 mg/ml in MEC buffer) was added as preservative. The solutions were stored at 4°C.

2.14.9 Standard calibration curve of HRPO and estimation of coupling yield

Concentrations of 13.25 µg/ml to 1 mg/ml HRPO were prepared in triplicates in 0.1 M $NaH_2PO_4.2H_2O$, 0.15 M NaCl, and 0.001 M Na_2EDTA at pH 7.5. The absorbances of the standard solutions were read at 403 nm against phosphate buffer blank. The graph of mean absorbance against concentration was plotted using Microsoft Excel® software package. Samples from HRPO-IgY purification showing high absorbance at 403 nm were pooled together and concentrated to 13.5 ml using PEG 20 000. The absorbance of this solution (in quadruplicate) was read at 403 nm. Using the standard curve equation, the amount of HRPO in the conjugate was estimated and the molar coupling ratio was deduced.

2.14.10 Characterising and optimising HRPO-IgY conjugates using Dot-ELISA

Rabbit albumin (5 – 100 ng) prepared in TBS were blotted on nitrocellulose membrane in triplicates and allowed to dry at room temperature. 1 µl of TBS and 100 ng of BSA were blotted on the same membrane as the rabbit albumin dots as negative controls. Non-specific sites on the membrane were blocked with 5% (w/v) non-fat milk in TBS at room temperature for 30 min. After blocking, the membranes were washed 3 × 5 min with TBS and incubated in several dilutions on the HRPO-IgY conjugate (1/50 000; 1/10 000;

1/5000; 1/2500; 1/1000 and 1/500) in TBS (2 h, 37°C). After incubation, the membranes were washed 3 × 5 min with TBS before incubating in substrate solution for 15 – 30 min. A positive result was indicated by dark coloured dots on the nitrocellulose membrane.

2.15 Preparation of anti-rabbit albumin IgY-dextran conjugate

Dextran is a linear (with few branches) bacterial polysaccharide of glucose subunit joined mainly by α 1 – 6 glycosidic bonds and with few α 1 – 2, α 1 – 3 and α 1 – 4 glycosidic bonds. It has varying molecular weights, ranging from 10 – 40 000 kDa depending on the source of the polymer. It is very soluble in water due to its high content of hydroxyl group (Dintzis *et al.,* 1989). When cross-polymerized, dextran becomes insoluble in aqueous solutions. Cross-polymerized dextran is used as chromatographic matrix support known as Sephadex®.

Protein and toxin haptens have been covalently attached to dextran as immunogenic agents (Inman, 1975; Brunswick *et. al.,* 1989; Dintzis *et al.,* 1989; Xiao *et al.,* 1995). Several cross-linking agents and coupling chemistries were used to attach proteins and toxins to dextran by virtue of the seemingly unreactive hydroxyl groups on the polymer. The use of dextran-antibody conjugates as an immunoassay tool for antigen detection is not very common.

In this study, affinity-purified polyclonal chicken anti-rabbit albumin immunoglobin Y (IgY) as antibody, was covalently attached to industrial grade dextran. This conjugate was used to detect rabbit albumin (antigen) spotted directly on a nitrocellulose membrane. The carbohydrate polymer was detected by periodic acid-Schiff (PAS) staining. It is believed that the amount of antigen detected might be proportional to the colour intensity of the coloured dextran.

Dextran was derivatized to sulfhydryl reactive iodoacetyl-dextran in a three-step reaction involving chloroacetic acid, ethylenediamine and *p*-nitrophenyl iodoacetic acid (NPIA). The first step is carboxymethylation of dextran. Chloroacetic acid reacts with the hydroxyl

groups on the dextran, converting it to carboxymethyl-dextran. The carboxymethyl-dextran is derivatized to have functional amine groups in the second step by reaction with ethylenediamine in the presence of *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC), thus converting carboxylmethyl-dextran to aminoethylcarboxymethyl-dextran (AECM-dextran). In the third step, AECM-dextran is converted to a sulfhydryl-reactive derivative, by reaction with *p*-nitrophenyl iodoacetic acid. The intermediate, iodoacetyl dextran will react with IgY modified to have terminal sulfhydryl residues (SH-IgY) by 2-iminothiolane. The final conjugate was made up of the antibody-dextran conjugate separated by at 13-atom spacer linker arm (Figure 2.7).

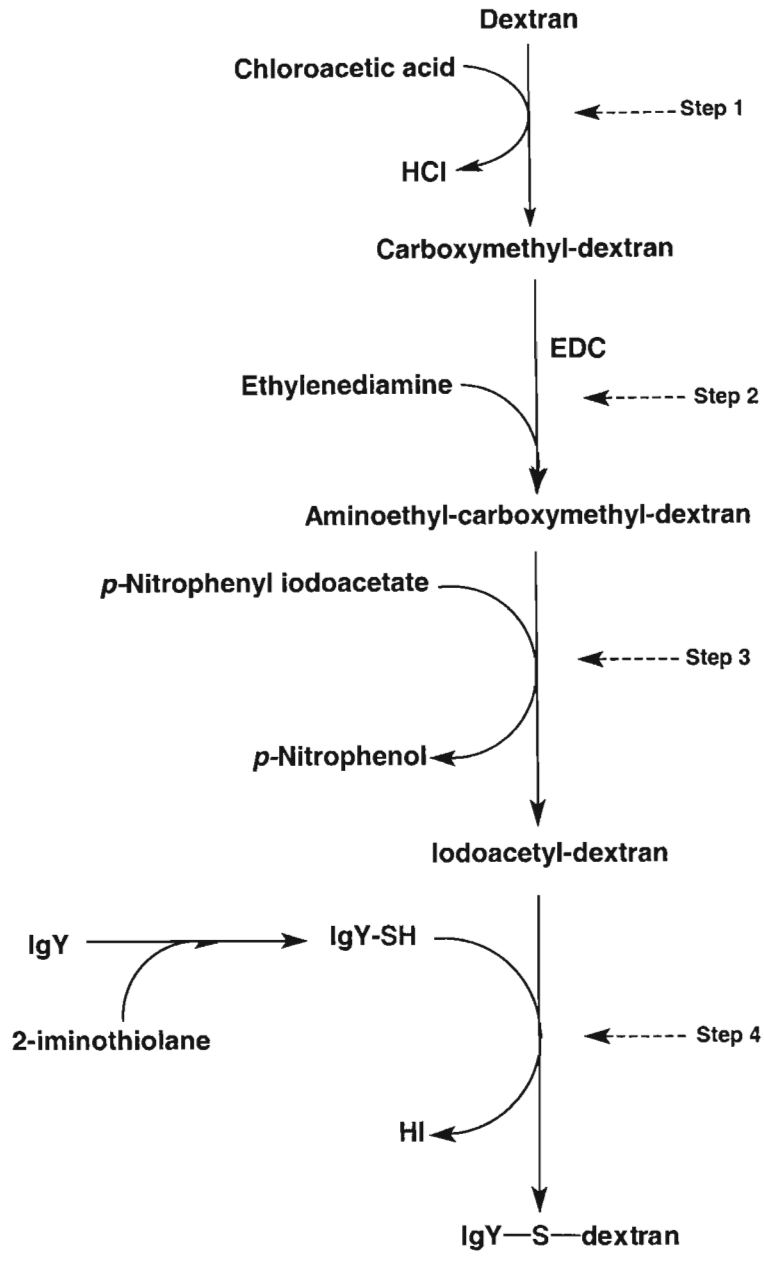


Figure 2.6 Schematic illustration of coupling steps used to covalently couple IgY to dextran

Step 1 is carboxy-methylation of dextran to carboxymethyl-dextran; step 2 is amine-modification of carboxymethyl-dextran to AECM-dextran in the presence of EDC; step 3 is alkylation of AECM-dextran to iodoacetyl-dextran and step 4 is the final coupling of SH-IgY to SH-reactive iodoacetyl-dextran.

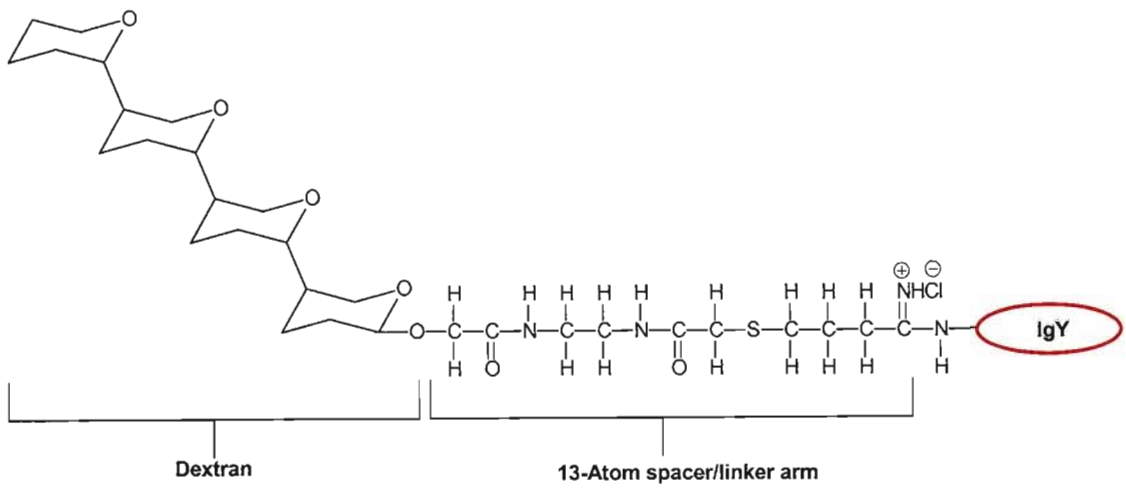


Figure 2.7 Schematic representation of the final antibody-dextran conjugate.

2.15.1 Reagents

Carboxylating reagent [1 M chloroacetic acid, 3 M NaOH]. Chloroacetic acid (9.45 g) and NaOH (12 g) were dissolved in 100 ml of distilled water.

Neutralizing solution [6 *N* HCl]. 58.9 ml of 32% HCL was made up to 100 ml with distilled water. The acid was added to water.

Alkylating buffer (borate buffered saline) [50 mM sodium borate decahydrate, 10 mM Na_2EDTA, 100 mM NaCl, pH 8.3]. Sodium borate decahydrate (19.0 g), Na_2EDTA (3.72 g) and NaCl (5.8 g) were dissolved in 950 ml of distilled water, adjusted to pH 8.3 with HCl and volume made up to 1000 ml with distilled water.

Alkylating reagent [10% (w/v) *p*-nitrophenyl iodoacetic acid]. 100 mg of *p*- nitrophenyl iodoacetic acid (NPIA) was dissolved in 1 ml of DMF in an eppendorf tube. The eppendorf tube was immediately wrapped with aluminium foil, as NPIA is sensitive to light.

Periodic acid solution [0.1% (v/v) periodic acid, 7% (v/v) acetic acid]. 200 µl of 50% periodic acid and 7 ml of acetic acid were made up to 100 ml with distilled water.

Periodic acid-Schiff (PAS) reagent for spectrophotometric assay of dextran. 1 g of Basic Fuchsin HCl (C.I. 42500) was dissolved in 100 ml of boiling distilled water. The solution was stirred for 5 min and cooled to 50°C and 20 ml of 1 M HCl was added. 1 g of activated charcoal was added and stirred for 5 min. After repeated addition of activated charcoal and shaking, the mixture was filtered. This stock solution was stored in an amber bottle for 6 weeks. Before use, 0.1 g of sodium metabisulfite was added to 6 ml of the stock solution and this mixture was incubated at 37°C until it became colourless or pale yellow. The mixture was discarded on turning pink (Kirkeby *et al.*, 1992).

Periodic acid-Schiff (PAS) reagent for staing dextran on nitrocellulose. 0.5 g of Basic Fuchsin HCl (C.I. 42500) was dissolved in 100 ml of boiling distilled water. The solution was stirred for 5 min and cooled to 50°C. 2.5 ml of 2 *N* HCl and 1.7 g of of sodium metabisulfite were added and stirred for overnight at 4°C. The following morning, 1.0 g of activated charcoal was added. After repeated addition of activated charcoal and shaking, the mixture was filtered with No. 1 Whattman filter paper. This stock solution was stored in an amber bottle for 6 weeks. The mixture was discarded on turning pink (Doerner and White, 1990).

Bradford reagent [0.01% (w/v) Coomassie Blue G (C.I. 42655), 10% (v/v) phosphoric acid, 4.7% (v/v) ethanol]. 50 ml of 85% phosphoric acid was added to 23.5 ml of 95% ethanol. This solution was made up to 500 ml with distilled water. 50 mg of Coomassie Blue G was dissolved in the solution, stirred (30 min, RT) and filtered into an amber bottle. The solution was kept at room temperature for 6 month and visual checks for precipitation was made prior to use (Bradford, 1976).

Standard protein solutions. Chicken IgY (1.0 mg/ml) was serially diluted in 0.1 M sodium phosphate buffer to give the concentrations ranging from 50 – 250 µg/ml. The protein solutions were diluted to1 in 10 before calibration.

2.15.2 Preparation of iodoacetyl derivative of dextran

200 mg of dextran was dissolved in 10 ml of freshly prepared carboxylating reagent and stirred (3h, 45 min, RT). 40 mg of dry $NaH_2PO_4.10\ H_2O$ was added to stop the reaction and the solution was neutralized with 2.5 ml of 6 *N* HCl. 4ml of the solution containing approximately 64 mg of dextran was quickly transferred into dialysis tubing (molecular weight cut off: 10 000 Da) and dialyzed against 200 mM NaCl for 15 h with several changes of the dialysis solution (end of step 1, Figure 2.6). After dialysis, the volume of the solution was determined, 20% (w/v) ethylenediamine dihydrochloride and 1.5% (w/v) EDC were added to the solution and then stirred (4 h, RT). The solution was dialysed against four changes of 1000 ml of 0.2 M NaCl for 15 h and finally against 1000 ml of alkylation buffer (end of step 2, Figure 2.6). 600 µl of alkylation reagent was added to the dextran solution and stirred (4h, RT) in the dark. The resulting solution was dialysed against several changes of distilled water until the dialysed dextran solution regained its original milky colour. The yellow colour was *p*-nitrophenol, a reaction by-product of the alkylation of dextran (end of step 3, Figure 2.6).

2.15.3 Covalent conjugation of iodoacetyl-dextran with SH-IgY

The dextran solution was further dialysed overnight (15 h) against 1000 ml of sulfhydryl-modification buffer and concentrated to 4 ml with PEG 20 000. 4 ml of the concentrated dextran was divided equally into 4 clean test tubes and 1.5 ml of 2.8 mg/ml of sulfhydryl-modified IgY (section 2.14.6) was added to each ml of dextran solution. The solution was allowed to react (150 min, RT) in the dark, while mixing the solution every 10 min interval (end of step 4, Figure 2.6).

2.15.4 Purification of IgY-dextran conjugate

After coupling, the solutions were pooled together and the final volume was made up to 25 ml with MEC buffer in order to decrease the viscosity of the solution caused by the high molecular weight dextran (*ca* 0.25%). High viscosity could lead to hydrostatic pressure in the MEC column. The conjugate was purified over Sephacryl® S-300 molecular exclusion

chromatography column (sections 2.14.8), pre-equilibrated for 48 h with MEC buffer. The column was set to run at a volumetric flow rate of 13.2 ml/h, equivalent to a linear flow rate of 1.6 cm/h. 5 ml fractions were collected. To obtain the elution profile, the absorbance at 280 nm of each fraction (80 fractions) was plotted against the column volume.

2.15.5 Standard calibration curve for spectrophotometric PAS assay of dextran

The method of Kirkbey *et al.* (1992) was used for spectrophotometric calibration of standard dextran (M_r 5000 kDa to 40 000 kDa) curve. The stock standard solution of dextran was 1 mg/ml in MEC buffer. The stock solution was diluted to series of concentration ranging from 20.0 µg/ml to 120 µg/ml with MEC buffer. The blank was the buffer alone. 500 µl of each concentration (5 samples per concentration) was oxidized with 2 ml of periodic acid solution (2 h, 37°C). Then 200 µl of PAS reagent was added, the mixture was vortexed and the colour allowed to develop for 30 min. After colour development, the absorbance of each concentration was read at 555 nm against the blank. The plot of absorbance against concentration was obtained to give the standard calibration curve for dextran.

2.15.6 Standard calibration curve for protein using Bradford dye binding assay

50 µl of IgY solutions containing 1 to 5 µg serial concentrations of IgY were mixed with 950 µl of Bradford reagent in eppendorf tubes by inverting the tubes slowly. The colour was allowed to develop for 2 min. The absorbance of each tube was read at 595 nm against reagent blank containing 50 µl of the dissolution buffer (100 mM phosphate buffer at pH 7.5). The graph of the absorbance against concentration was plotted, the equation and the regression square was also obtained using Microsoft Excel® package.

2.15.7 Determination of coupling yield

The quantity of IgY in fractions 155 ml to 210 ml corresponding to the first peak on the elution profile of IgY-dextran conjugate was determined using Bradford protein assay in triplicates. The fractions were pooled together before determining the total protein concentration using Bradford protein assay.

2.15.8 Immunodetection of rabbit albumin on nitrocellulose using IgY-dextran conjugate by PAS staining

10 – 200 ng of rabbit albumin was blotted on a nitrocellulose membrane in triplicate dots. 200 ng of BSA and HAS, and 1 µl of PBS were also blotted on the same membrane as negative controls. Non-specific sites on the membrane were blocked with 0.5% low fat milk (30 min, RT). After blocking, the membrane was washed three times with PBS. The membrane was incubated in the IgY-dextran reagent (200 µg/ml of dextran) (2h, 37°C). The membrane was washed three times with PBS and the incubated in 0.1% periodic acid solution (1h, RT) in order to oxidize the vicinal diols of dextran to aldehyde. Following oxidation, the membrane was washed in distilled water and incubated in PAS reagent (1h, RT), washed and the coloured spots were observed.

2.15.9 Evaluation of the immunoreactivity of IgY-dextran conjugate

10 – 100 ng of rabbit albumin was blotted on a nitrocellulose membrane in triplicate dots. 100 ng of ovalbumin and 1 µl of PBS were also blotted on the same membrane as negative controls. Non-specific sites on the membrane were blocked with 0.5% low fat milk (30 min, RT). After blocking, the membrane was washed three times with PBS. The membrane was incubated in the IgY-dextran reagent (200 µg/ml of dextran) or anti-rabbit 150 µg/ml of anti-rabbit albumin IgY (2h, 37°C). The membrane was washed three times with PBS and the incubated rabbit-anti-chicken-HRPO (1 h, RT). Following incubation in the secondary antibody, the membrane was washed three times with PBS and incubated with ABTS (substrate) and coloured spots were observed.

2.15.10 Storage of IgY-dextran conjugate

The IgY-dextran conjugate was stored at 4°C in closed glass vials for several weeks. Visual checks of the solution were made before use.

CHAPTER 3
STAINING PROTEINS ON NITROCELLULOSE AND IN POLYACRYLAMIDE GELS

3.1 Introduction

Proteins can be effectively quantified on nitrocellulose membrane as a solid support with sensitivities comparable to assays in solutions (Nakamura *et al.*, 1985; Li *et al.*, 1989; Goldring and Ravaioli, 1996; Bannur *et al.*, 1999; Morçöl and Subramanian, 1999). Dye binding to proteins resolved on polyacrylamide gels has been investigated since 1963, when Fazekas de St Groth *et al.* (1963) introduced the Coomassie dye for protein detection on polyacrylamide gels. This dye was originally designed as an acid wool stain for the textile industry to commemorate the 1896 British occupation of the Ashanti capital, Kumasi or "Coomassie", now in Ghana (Merril, 1987). Besides Coomassie Blue R 250, Amido Black (Wilson, 1983), Eosin Y (Lin *et al.*, 1991) and Erichrome Black T-Rhodamin complex (Jung *et al.*, 1998) have been established as protein stains on polyacrylamide gels.

In this study, 26 dye stains were used to stain different concentrations of proteins on nitrocellulose and proteins resolved in polyacrylamide gels. Classes of dyes and dye binding conditions were compared.

3.2 Staining proteins blotted on nitrocellulose membrane with 26 dye stain preparations

26 dye stain preparations were used to stain a series of IgY concentration blotted onto nitrocellulose membranes. The sensitivity of each dye used in the experiment was evaluated. 26 dyes stains were prepared in acidic buffer (methanol-acetic acid solution) at pH 2.3, 100 mM phosphate buffer at pH 7.2, and alkaline medium (methanol-sodium hydroxide solution [5:1:4 (v/v/v)] at pH 13.0). Aurintricarboxylic Acid, Basic Fuchsin, Eriochrome Black T and Hematoxylin were insoluble in 100 mM phosphate buffer at pH 7.2 and were not evaluated at this pH. Between 10 ng and 5.0 μg of IgY prepared in 0.1 M phosphate buffer at pH 7.2 were blotted on nitrocellulose membrane. 0.5 μl of phosphate

buffer was spotted on nitrocellulose as a negative control. The spots were allowed to dry at room temperature for 1 h and stained in the dye stains in buffer at pH 2.3, 7.0 and 13.0 for 5 min before destaining in the same buffer without the dye (destaining solution).

Table 3.1 shows the results obtained with dye prepared in acidic buffer (methanol–acetic acid solution) at pH 2.3. Acridine Orange, Alcian Blue 8GX, Azure A, Azure B, Bismark Brown R, Gallocyanine, Malachite Green HCl and Methyl Green produced a visible protein stain at all of the range of protein concentrations used on nitrocellulose at pH 2.3. Bromocresol Purple, Hematoxylin and Primuline stained 250 ng of protein with weak intensity. Aurintricarboxylic Acid, Cibacron Blue 3GA, Coomassie Blue R 250, Evans Blue, Naphthol Blue Black, Nigrosin Water-soluble, Ponceau S, Reactive Black 5, Reactive Red 120 and Trypan Blue, all stained 50 ng of protein darkly. Direct Red 75, Direct Red 81 and Primunlin stained 10 ng of protein making them the most sensitive of the dyes tested at pH 2.3. None of the dyes stained the negative control on the nitrocellulose membrane.

Table 3.2 presents the results obtained when dye prepared in 100 mM sodium phosphate buffer at pH 7.2 was used to stain a range of IgY concentration on nitrocellulose. Acridine orange, Alcian Blue 8GX, Azure A, Azure B, Bismark Brown R, Bromocresol Purple, Malachite Green HCl HCl, Methyl Green and Methyl Orange did not stain any proteins over the range of protein concentrations used at pH 7.2. Cibacron Blue 3GA, Coomassie Blue R 250, Evans Blue, Naphthol Blue Black, Nigrosin Water-soluble, Ponceau S, Reactive Black 5, Reactive Red 120 and Trypan Blue, all stained 50 ng of protein. Direct Red 75, Direct Red 81 and Primuline detected 10 ng of protein making them the most sensitive of the range of dyes tested at pH 7.2. None of the dyes stained the negative control on the nitrocellulose membrane.

None of the dye preparations at pH 13.0 stained proteins on nitrocellulose.

Table 3.1 Proteins detected by 26 dyes in acidic (methanol-acetic acid solution) buffer at pH 2.3

IgY in 0.1 M phosphate buffer at pH 7.2 was blotted (in duplicate) on nitrocellulose membrane, allowed to dry at RT for 1 h and stained with each of the dye stains for 5 min and destained. The protein spots were visualized and recorded as + + for a dark staining, + – for a light staining and – – for no staining

Dye	protein concentration							
	0 ng	10 ng	50 ng	100 ng	250 ng	500 ng	1.0 µg	5.0 µg
Acridine orange	– –	– –	– –	– –	– –	– –	– –	– –
Alcian Blue 8GX	– –	– –	– –	– –	– –	– –	– –	– –
Aurintricarboxylic Acid	– –	+ –	+ –	+ –	+ –	+ +	+ +	+ +
Azure A	– –	– –	– –	– –	– –	– –	– –	– –
Azure B	– –	– –	– –	– –	– –	– –	– –	– –
Basic Fuchsin	– –	– –	– –	– –	– –	– –	– –	– –
Bismark Brown R	– –	– –	– –	– –	– –	– –	– –	– –
Bromocresol Purple	– –	– –	– –	– –	+ –	+ –	+ –	+ –
Cibacron Blue 3GA	– –	+ –	+ +	+ +	+ +	+ +	+ +	+ +
Coomassie Blue R 250	– –	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Direct Red 75	– –	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Direct Red 81	– –	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Eriochrome Black T	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +
Evans Blue	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +
Gallocyanine	– –	– –	– –	– –	– –	– –	– –	– –
Hematoxylin	– –	– –	– –	– –	+ –	+ –	+ –	+ –
Malachite Green HCl	– –	– –	– –	– –	– –	– –	– –	– –
Methyl Green	– –	– –	– –	– –	– –	– –	– –	– –
Methyl Orange	– –	– –	– –	– –	– –	– –	– –	+ –
Naphthol Blue Black	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +
Nigrosin Water-soluble	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +
Ponseau S	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +
Primuline	– –	– –	– –	– –	+ –	+ –	+ –	+ –
Reactive Black 5	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +
Reactive Red 120	– –	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Trypan Blue	– –	+ –	+ –	+ +	+ +	+ +	+ +	+ +

Table 3.2 Proteins detected by range of dyes in 0.1 M phosphate buffer at pH 7.2
IgY in 0.1 M phosphate buffer at pH 7.2 was blotted (in duplicate) on nitrocellulose membrane, allowed to dry at RT for 1 h and stained with each of the dye stains for 5 min and destained. The protein spots were visualized and recorded as + + for a dark staining, + − for a light staining and − − for no staining

Dye	Amount of Protein detected							
	0 ng	10 ng	50 ng	100 ng	250 ng	500 ng	1.0 µg	5.0 µg
Acridine orange	− −	− −	− −	− −	− −	− −	− −	− −
Alcian Blue 8GX 8GX	− −	− −	− −	− −	− −	− −	− −	− −
Aurintricarboxylic Acid	NS*	NS	NS	NS	NS	NS	NS	NS
Azure A	− −	− −	− −	− −	− −	− −	− −	− −
Azure B	− −	− −	− −	− −	− −	− −	− −	− −
Basic Fuchsin	NS	NS	NS	NS	NS	NS	NS	NS
Bismark brown R	− −	− −	− −	− −	− −	− −	− −	− −
Bromocresol Purple	− −	− −	− −	− −	− −	− −	− −	− −
Cibacron Blue 3GA	− −	+ −	+ +	+ +	+ +	+ +	+ +	+ +
Coomassie Blue R 250	− −	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Direct Red 75	− −	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Direct Red 81	− −	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Eriochrome Black T	NS	NS	NS	NS	NS	NS	NS	NS
Evans Blue	− −	+ −	+ −	+ +	+ +	+ +	+ +	+ +
Gallocyanine	− −	− −	− −	− −	− −	− −	− −	− −
Hematoxylin	NS	NS	NS	NS	NS	NS	NS	NS
Malachite Green HCl	− −	− −	− −	− −	− −	− −	− −	− −
Methyl Green	− −	− −	− −	− −	− −	− −	− −	− −
Methyl Orange	− −	− −	− −	− −	− −	− −	− −	+ −
Naphthol Blue Black	− −	+ −	+ −	+ +	+ +	+ +	+ +	+ +
Nigrosin Water-soluble	− −	+ −	+ −	+ +	+ +	+ +	+ +	+ +
Ponseau S	− −	+ −	+ −	+ +	+ +	+ +	+ +	+ +
Primuline	− −	+ −	+ +	+ +	+ +	+ +	+ +	+ +
Reactive Black 5	− −	+ −	+ −	+ +	+ +	+ +	+ +	+ +
Reactive Red 120	− −	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Trypan Blue	− −	+ −	+ −	+ +	+ +	+ +	+ +	+ +

*NS is not soluble due to the insolubility of the dye in the solvent used for dye stain preparation.

3.3 Staining proteins resolved on SDS-polyacrylamide gels with the 16 dyes that stained proteins on nitrocellulose membranes

16 dyes that stained proteins on nitrocellulose in section 3.2 were screened for their ability to stain proteins in a gel with a minimal background stain. About 10 μg of IgY was resolved in each lane of a 12% SDS-polyacrylamide gel. The gel was cut into strips representing each lane and stained overnight at room temperature in the dye stain at pH 2.3. After staining, the gels were destained and protein bands on the gels were observed.

Table 3.3 illustrates the result obtained in this screening experiment. Most dyes used for this study were acidic dyes. Exceptions were Hematoxylin, which is a ligand dye and Nigrosin Water-soluble that could not be classified.

Direct Red 75, Eriochrome Black T, Evans Blue and Trypan Blue all stained proteins strongly on the polyacrylamide gel. But after destaining the gel, all left a high background stain. As a result, they were assessed to be poor protein stains on SDS-polyacrylamide gels. Aurintricarboxylic Acid, Ponceau S and Reactive Red 120, stained proteins weakly on the gel, and were therefore disregarded from further studies. Bromocresol Purple, Hematoxylin, Methyl Orange and Nigrosin Water-soluble did not stain the protein on the gel. Cibacron Blue 3GA, Direct Red 81, Naphthol Blue Black and Reactive Black 5 detected 10 μg of the protein on the gel without background staining. Primuline stained proteins weakly on the gel and the staining was readily observed when viewed under the UV light where protein stains appeared as dark bands in fluorescent background.

Table 3.3 Staining proteins in a gel with 16 dyes that stained proteins on nitrocellulose membrane

10 μg of IgY was resolved in several lanes on a 12% reducing SDS-polyacrylamide gel. Each lane of the gel was stained overnight in a solution of each one of the 16 dyes and destained. + + indicates strong staining/high background, + – weak staining and – – no staining/ no background.

Dye	Classification	Detection	Background
Aurintricarboxylic Acid	Acidic	+ –	+ +
Bromocresol Purple	Acidic	– –	+ +
Cibacron Blue 3GA	Acidic-triazine	+ +	+ +
Direct Red 75	Acidic-direct	+ +	– –
Direct Red 81	Acidic-direct	+ +	+ +
Eriochrome Black T	Acidic	+ +	– –
Evans Blue	Acidic-direct	+ +	– –
Hematoxylin	Ligand dye	– –	+ +
Methyl Orange	Acidic	– –	+ +
Naphthol Blue Black	Acidic	+ +	+ +
Nigrosin Water-soluble[1]		– –	– –
Primuline	Acidic-Direct	+ –	+ +
Ponseau S	Acidic	+ –	– –
Reactive Black 5	Acidic	+ +	+ +
Reactive Red 120	Acidic-triazine	+ –	– –
Trypan Blue	Acidic-Direct	+ +	– –

[1] Details on the structure and chemistry of Nigrosin Water-soluble was not available.

3.4 Staining proteins resolved on a SDS-polyacrylamide gel with Cibacron Blue 3GA, Direct Red 81 and Reactive Black 5

Direct Red 81, Reactive Black 5 and Cibacron Blue 3GA that stained proteins in a gel and had a clear background (section 3.3) were compared with the standard protein stain, Coomassie Blue R 250. Naphthol Blue Black (Amido Black or Amido Schwartz) is established as a dye for protein detection on polyacrylamide gels (Towbin *et al.*, 1979; Wilson, 1983) and thus was left out of the study. The amount of protein detected (sensitivity) of each dye was established with 312.5 ng to 10 µg of IgY resolved on SDS-polyacrylamide gel and stained overnight at RT. The following day, the gels were destained and evaluated.

The result (Figure 3.1***A***) shows that the three dye stains produced a protein stain that was darker with increasing protein concentration and was compared to Coomassie Blue R 250. Reactive Black 5 and Cibacron Blue 3GA were less sensitive than Coomassie Blue R 250 and detected1.25 µg of IgY. The sensitivity of Direct Red 81 was comparable to Coomassie Blue R 250, detecting 312.5 ng of IgY. The intensity of each stained protein band was quantified by measuring the pixels for 'true colour' images of each band using soft imaging system analysis (analySIS®). The intensity of each band was derived from the difference between the intensity of the gel background (without protein) and the intensity of each protein band. The mean of the difference was plotted against the concentration of the protein (Figure 3.1***B***). The trend is the same as that seen in Figure 3.1***A***, as the concentration of the protein increased, the band intensity increased. The intensity/ng protein (I/ng) of Cibacron Blue 3GA staining increased from 0.004 – 0.0884. The I/ng protein of Reactive Black 5 staining increased from 0.009 – 0.106. The I/ng protein of Direct Red 81 staining increased from 0.034 – 0.139. The I/ng protein of Coomassie Blue R 250 staining increased from 0.022 – 0.125 I/ng. The intensity profile of Direct Red 81 was comparable to Coomassie Blue R 250. A linear relationship between dye staining and protein concentration was also demonstrated.

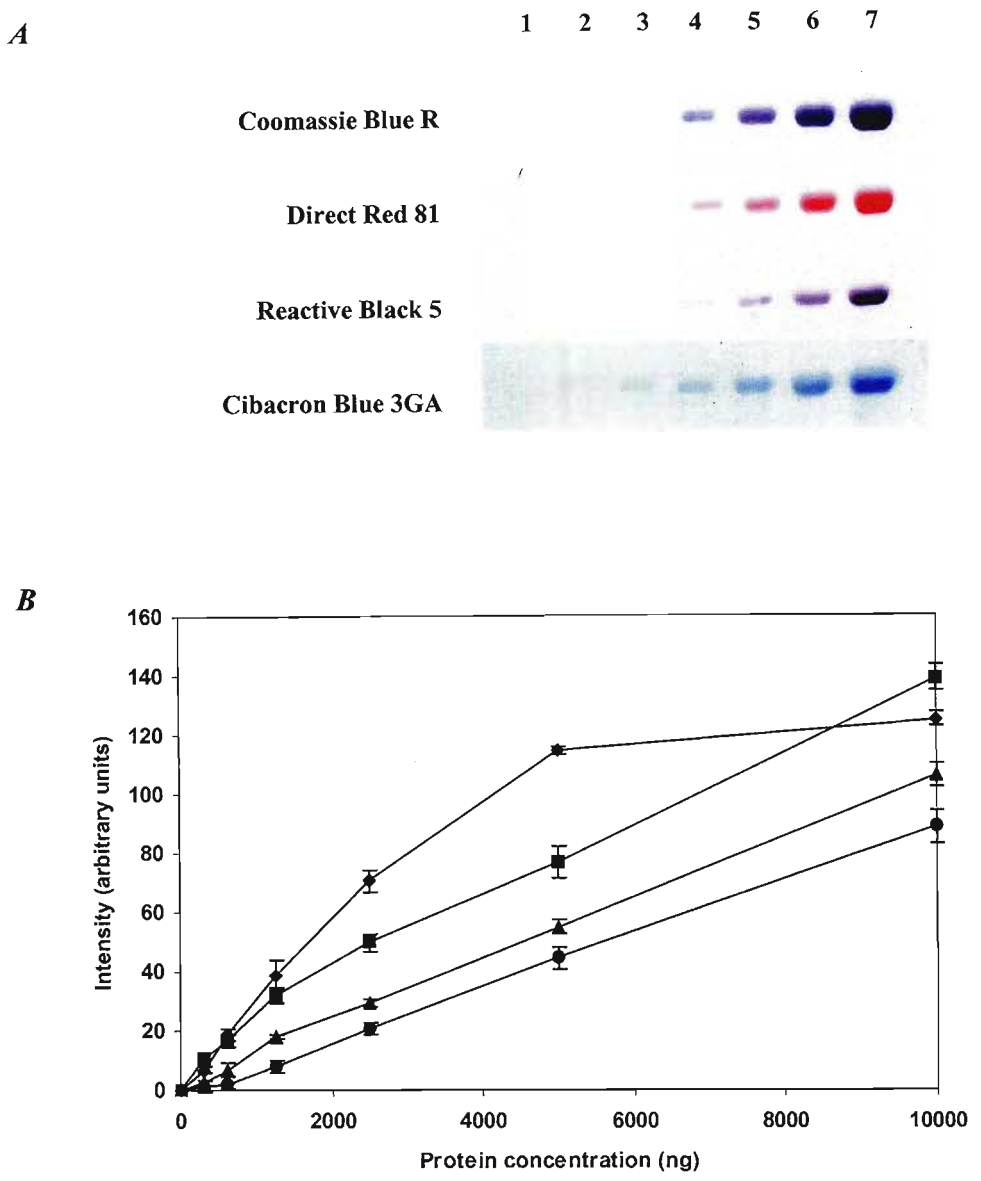


Figure 3.1 **Comparing the sensitivity of Direct Red 81, Reactive Black 5 and Cibacron Blue 3GA with Coomassie Blue R in a polyacrylamide gel**

A: *lane 1*, no protein (control); *lane 2*, 312.5 ng; *lane 3*, 625 ng; *lane 4*, 1.25 μg; *lane 5*, 2.5 μg; *lane 6*, 5.0 μg and *lane 7*, 10.0 μg of IgY were resolved on 12% reducing Laemmli SDS-polyacrylamide gel, stained with Coomassie Blue R 250, Direct Red 81, Reactive Black 5 or Cibacron Blue 3GA. ***B***: Intensity of staining of (◆) Coomassie Blue R 250; (■) Direct Red 81; (▲) Reactive Black 5 and (●) Cibacron Blue 3GA.

3.5 Staining six molecular weight marker proteins with Direct Red 81 in comparison with Coomassie Blue R 250.

Direct Red 81 staining was further compared to Coomassie Blue R 250. Molecular weight marker proteins; phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin and lysozyme, were resolved on a 12% SDS-polyacrylamide gel. After electrophoresis, gel strips containing a single lane of the resolved proteins were stained in either Direct Red 81 or Coomassie Blue R 250 and destained under identical conditions until a clear background was obtained.

The six proteins were stained by both dyes with similar staining intensity (Figure 3.2***A***). The intensities of staining of each protein appeared to be identical. Quantitatively, the staining intensity (Figure 3.2***B***) showed that Direct Red 81 stained five of the six proteins better than Coomassie Blue R 250 dye. The staining intensity of trypsin inhibitor appeared to be comparable for both dyes.

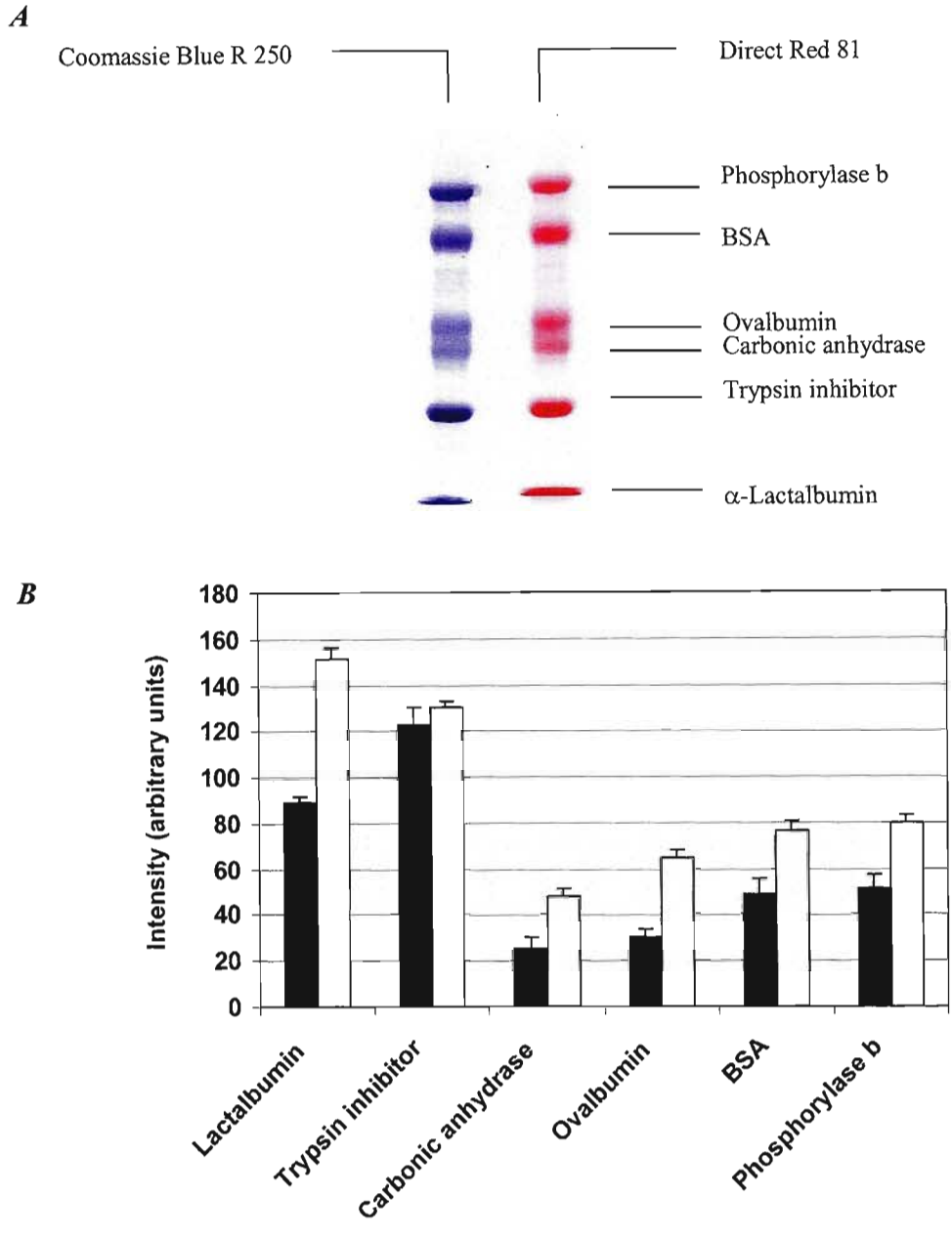


Figure 3.2 Staining six proteins with Direct Red 81 in comparison with Coomassie Blue R 250

A: Phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin were resolved on a 12% SDS-polyacrylamide gel, stained in either Direct Red 81 or Coomassie Blue R 250. *B*: The quantitative evaluation of the staining intensity of Coomassie Blue R 250 (■) and Direct Red 81 (□).

3.6 Rate of staining of Direct Red 81 compared to Coomassie Blue R 250 in a polyacrylamide gel

The rate at which Direct Red 81 stains proteins resolved in polyacrylamide gel was compared to Coomassie Blue R 250. 7.5 μg of IgY was resolved in 12% reducing SDS-polyacrylamide gel and incubated with either dye for 5 – 60 min, destained and a picture taken (Figure 3.3***A***). The intensity of each band was evaluated and plotted against the staining time (Figure 3.3***B***).

Direct Red 81 produced a stain of protein within 5 min and increased marginally over a further 55 min (Figure 3.3). The intensity of the Direct Red 81 stain was *ca* 12 times higher than that of Coomassie Blue R 250 in the first 5 min. The staining intensity of Direct Red 81 was higher than Coomassie Blue R 250 throughout the staining period.

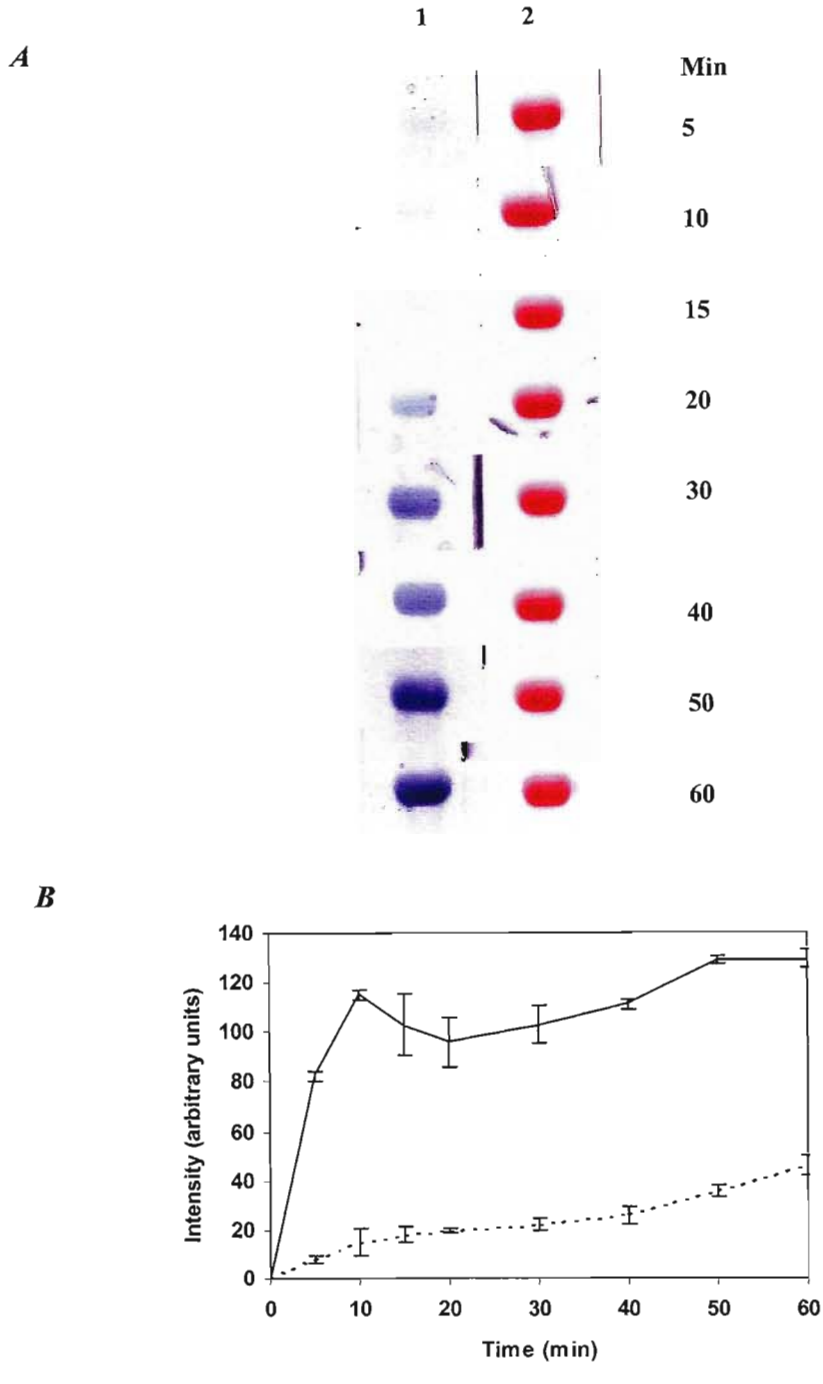


Figure 3.3 Rate of staining of Direct Red 81 compared to Coomassie Blue R 250 in a polyacrylamide gel

A: 7.5 µg of IgY was resolved in 12% SDS-polyacrylamide gel, stained between 5 – 60 min in either Coomassie Blue R 250 (*lane 1*) or Direct Red 81 (*lane 2*) and destained. ***B***: The quantitative evaluation of the staining intensity for (-------) Coomassie Blue R 250 and (——) Direct Red 81.

3.7 Discussion

In this work, the ability of several dyes to stain proteins blotted on nitrocellulose or separated by SDS-PAGE was evaluated. Dye stains were prepared in various solutions with different pH values in order to understand the suitable conditions required for protein staining. Three questions were to be answered in this section, which were: what class of dye really binds well to proteins? What pH favours the binding of dyes to proteins? How will the study help in the selection of dyes that will be subsequently used for the preparation of dye-antibody conjugates for visible immunodetection? The dyes used were of acidic, mordant (ligand dyes), and basic dye classes. All dye stains were prepared to have equal molar concentrations. Dye solubility varied from one solvent to another depending on the physicochemical properties of the dye. However, most dyes were soluble in methanol-acetic acid.

Table 3.1 showed the sensitivity of 26 dyes used in staining proteins in methanol-acetic acid solution. All the dyes used were soluble in methanol-acetic acid solution. Most dyes that stained protein on nitrocellulose in Table 3.1 are acidic dyes, except Hematoxylin that was a ligand dye. The acidic dyes had one or more sulfonate groups attached to the dye molecule. Aurintricarboxylic Acid has three carboxylic groups instead of sulfonate. The presence of acidic groups on the dye molecule makes it possible for the dye to interact with positively charged amino acid side chains on a protein molecule by ionic interaction (Prentø, 2001). At acidic pH, these sulfonate groups will be negatively charged (Puchtler *et al.,* 1988; Prentø, 2001). Reactive Red 120 with six sulfonate groups has equal sensitivity with Ponceau S with three sulfonate groups or Direct Red 81 and Coomassie Blue R 250 with only two sulfonate groups. In addition, the presence of reactive triazine functional groups (Clonis *et al.,* 1987) on reactive dyes such as Reactive Red 120 and Cibacron Blue 3GA did not show any visible enhancement of dye binding to protein, though the possibility of enhancement of dye-protein interaction by triazine functional groups may not be ruled out (Clonis *et al.,* 1980; Clonis *et al.,* 1981; Clonis *et al.,* 1985). From the results obtained, it appears that the number of sulfonate groups per dye molecule or negative charge density is unrelated to the extent of dye binding and hence the sensitivity of the dye as evaluated by visible colour. The findings by Puchtler *et al,* (1988), showed that increased

number of sulfonate groups on a dye molecule does not necessarily increase the binding of dyes to proteins, but rather decreases the substantivity of the dye for its substrate, hence reducing dye binding. Hematoxylin, though a ligand dye, may have interacted with the protein by hydrogen bonding due to the high number of hydroxyl groups on the dye molecule (Prontø, 2001). Dyes that did not stain proteins on nitrocellulose were either basic dyes or ligand dyes. These dyes may not stain proteins at acidic pH due to the positively charged nature of the dye.

Table 3.2 illustrated the sensitivity of the same dyes in staining proteins in 100 mM phosphate buffer at pH 7.2. Aurintricarboxylic Acid, Basic Fuchsin, Eriochrome Black T and Hematoxylin were not soluble in this buffer and as a result they were not evaluated. Dyes that stained proteins in methanol-acetic acid solution and were soluble in phosphate buffer also stained proteins in this buffer with comparably equal sensitivities. This perhaps shows that at neutral pH, acidic dyes still interact with proteins by ionic interaction. However some acidic dyes such as Bromocresol Purple and Methyl Orange that stained proteins weakly at acidic pH did not stain proteins in phosphate buffer. Basic (cationic) dyes remained unable to stain proteins at pH 7.2. The binding of dyes at neutral pH has been shown to be possible by Nakamura *et al* (1985). They showed that acidic dyes would bind to soluble proteins such as human albumin, BSA, and human γ-globulin with comparable sensitivity and linearity at a pH range of 3.7 – 8.5.

None of the dyes stained proteins at alkaline pH. This however contradicted the results obtained by Horobin and Bennion (1973) for Basic Fuchsin and Acridine Orange with stained tissue sections at pH 4, 7 and 9. Their findings suggested that basic dyes could stain cytoplasm-rich tissue section at alkaline pH. A different staining mechanism different to the Horobin and Bennion (1973) method was used in this work.

The results of the study confirmed that acidic dyes were better protein stains at low pH for soluble proteins compared to basic dyes. None of the 7 basic dyes used in this study bound to protein on nitrocellulose at acidic, neutral and alkaline pH, while 16 acidic dyes used all bound to proteins on nitrocellulose in acidic pH.

To further characterize the staining of proteins by acidic dyes, proteins separated on reducing SDS-polyacrylamide gels were stained with all the acidic dyes (except Coomassie Blue R 250) that stained proteins on nitrocellulose membranes. Table 3.4 shows the results of staining 10 μg of IgY resolved on SDS-polyacrylamide gels with 16 dye-stain preparations in methanol-acetic acid solution at pH 2.3. The suitability of a dye in staining proteins on a gel was judged by the ability of the dye to stain at least 10 μg of the protein and the ability of the dye to diffuse out of the gel when immersed in methanol-acetic acid solution. The results obtained showed that there was no relationship between dye size and staining of the gel since these dyes had different molecular weight ranging from 461.39 – 1469.0. Reactive Red 120 has two triazine functional groups that may have influenced the inability of the dye to diffuse out of the gel because triazines react covalently with amine groups (Clonis *et al.,* 1987; Zollinger, 1991). With polyacrylamide containing a high quantity of amine groups, there is the possibility of triazine-containing dyes strongly binding to polyacrylamides. However, this is unlikely as Cibacron Blue 3GA, which is also a triazine-containing dye, stained proteins in the gel but did not stain the gel. Other dyes that were judged as suitable protein stains on gels were Direct Red 81, Naphthol Blue Black and Reactive Black 5. With exception of Naphthol Blue Black that is a known protein detection dye in SDS-PAGE, Direct Red 81, Reactive Black 5 and Cibacron Blue 3GA are not well known as possible protein stains on gels. Cibacron Blue 3GA however has been employed in spectroscopic evaluation of protein structures (Thompson and Stellwagen, 1976). The results showed that Cibacron Blue 3GA, Direct Red 81 and Reactive Black 5 were good candidates for further studies.

In Figure 3.1, the sensitivities of Cibacron Blue 3GA, Direct Red 81 and Reactive Black 5 were compared to standard Coomasie Blue R 250 at pH 2.3. If the extent of dye binding to protein is dependent on the number of charges, then Reactive Black 5 may be expected to detect proteins with higher sensitivity compared to Coomassie Blue R 250. That was not however the case as Direct Red 81 proved to be very comparable to Coomassie Blue R 250. Direct Red 81 detected 31.25 ng of IgY in 12% polyacrylamide gel. The molecular mass of the dyes did not correlate to the penetration and detection of proteins in the gel. Smaller dyes may be assumed to penetrate the gel more rapidly and stain proteins in the gel than

larger dyes. The molecular mass of Cibacron Blue 3GA, Direct Red 81 and Reactive Black 5 is 774.4, 675.6 and 927.9 respectively.

The ability of Direct Red 81 to stain a variety of proteins on polyacrylamide gel was compared to Coomassie Blue R 250 (Figure 3.2). To be comparable to Coomassie Blue R 250, Direct Red 81 should have equal or more staining sensitivity on a variety of proteins. Direct Red 81 stained six standard proteins with a comparable sensitivity to Coomassie Blue R 250 after 16 h of incubation in the respective dye stains. The rate of staining of protein (Figure 3.3) in a polyacrylamide gel by Direct Red 81 compared to Coomassie Blue R 250 showed that Direct Red 81 stained proteins more rapidly compared to Coomassie Blue R 250 after only 5 min. The short staining time of Direct Red 81 may be related to the relatively smaller structure of the dye as regards to penetration of the gel matrix by the dye.

The use of Direct Red 81 as a substitute for Coomassie Blue R 250 has been shown in this work as a possibility. Direct Red 81 is a textile dye with carcinogenic potential (Przybojewska *et al.,* 1988). With Direct Red 81 (50% dye content) being 6 times cheaper (Aldrich 2003 – 2004 chemical catalogue) than Coomassie Blue R 250 (90% dye content) and a faster acting stain, Direct Red 81 may be a more convenient dye in protein detection than Coomassie Blue R 250. The most accepted mechanism by which Coomassie Blue R 250 interacts with proteins in a gel is by ionic interaction with basic amino acid residues such as arginine, lysine and histidine (Righetti and Chillemi, 1978; Tal *et al.,* 1978). Wilson (1983) also proposed dye-dye interaction with dyes ionically or hydrophobically bound on protein structures. The mechanisms by which Direct Red 81 binds to proteins needs to be investigated further and compared to Coomassie Blue R 250 and other protein binding dyes. It is likely due to the commonality of dye structure that this will be the case.

CHAPTER 4

PREPARATION OF DYE-ANTIBODY CONJUGATES FOR IMMUNODETECTION

4.1 Introduction

Dyes were covalently conjugated to anti-rabbit albumin IgY isolated from chickens immunized with rabbit albumin with conjugated haptens. The dye coupling strategy required the use of linker molecules that can effectively cross-link the dye with the antibody without compromising the biological activity of the antibody and retaining the colour of the dye. *N, N'*-carbonyldiimidazole (CDI) was used as the linker. This molecule is a zero-length cross-linker that is able to link a hydroxyl and/or carboxylic acid functional group with an amine functional group. Primary and secondary structural analysis of chicken IgY was carried out using Internet databases and Predict7® software (Cármenes *et al.*, 1987). This was intended to establish the number of lysine residues in an IgY because the ε-amine residues of lysine was targeted for covalent dye conjugation and to understand how many of these lysine residues are located on the surface of the antibody. The dyes used in the study all contained hydroxyl and/or carboxylic acid functional groups. Dye-antibody conjugates were evaluated by ELISA and compared to the same antibody coupled to horseradish peroxidase.

4.2 Isolation and purification of chicken egg yolk IgY

Chicken IgY was isolated from the egg yolk using PEG 6000 precipitation as described by Polson *et al.* (1985) and the profile of the purification procedure evaluated with reducing SDS-PAGE. The purification profile (Figure 4.1) shows that the number of protein decreased from the starting material (lane 2) to the final product (lane 7). Lane 2 had multiple protein bands ranging from sizes greater than 97 kDa to 20 kDa. Many yolk proteins were precipitated with the lipid fraction after 3.5% PEG precipitation, leaving only two prominent protein bands of M_r 66 kDa and 40.4 kDa (lane 3). The number of protein bands in lane 4 increased after increasing the PEG concentration to 8.5% and two major protein bands were evident. There was a single 66 kDa protein band in lane 5. This 66 kDa appeared to correspond to the same band in lanes 2, 3, 5, 7 and 8. Besides the 66 kDa

protein band in lanes 7 and 8, there was a 25 kDa protein band. The 66 kDa and 25 kDa protein bands in lanes 7 and 8 indicates the heavy chain and the light chain of IgY respectively (Goldring and Coetzer, 2003; Schade *et al.*, 2001).

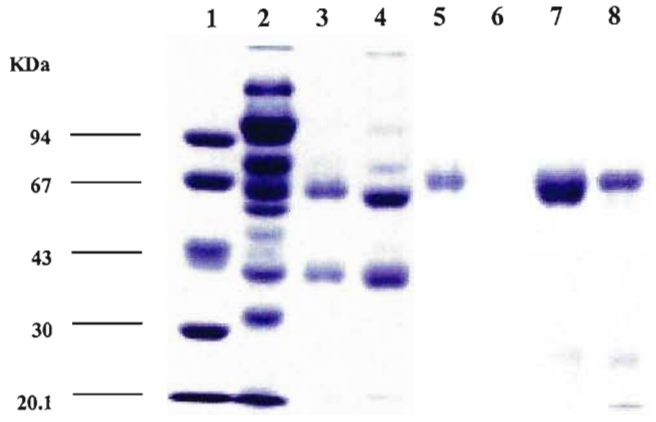


Figure 4.1 SDS-PAGE purification profile of IgY
Samples were taken from each purification step and ran on a 12% reducing SDS-polyacrylamide gel. *Lane* 1: molecular weight markers (phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase 30 kDa; trypsin inhibitor 20.1 kDa), *lane 2*: yolk in buffer; *lane 3*: supernatant after 3.5% PEG precipitation; *lane 4*: supernatant after first 12% PEG precipitation; *lane 5*: pellet after first 12 % PEG precipitation; *lane 6*: supernatant after second 12% PEG precipitation; *lane 7*: final pellet after second 12% precipitation; *lane 8*: affinity-purified IgY.

4.3 Immunoaffinity purification of anti-rabbit albumin IgY

Immunoaffinity purification was used to selectively separate species-specific anti-rabbit albumin IgY from a pool of chicken antibodies isolated from the egg yolk. 20 – 25 mg/ml of antibody was isolated from egg yolk. Approximately 6% of this amount was isolated as species-specific antibody after affinity purification. Figure 4.2 is representative of several elution profiles obtained from immunoaffinity purification of anti rabbit albumin IgY. There was no significant change in absorbance of the fractions at 280 nm from fraction 1 to fraction 7. The absorbance increased at fraction 8 reaching maximum absorbance at fraction 10 to 11 and decreased at fraction 15. Fractions 8 to 13 were pooled together for conjugation studies.

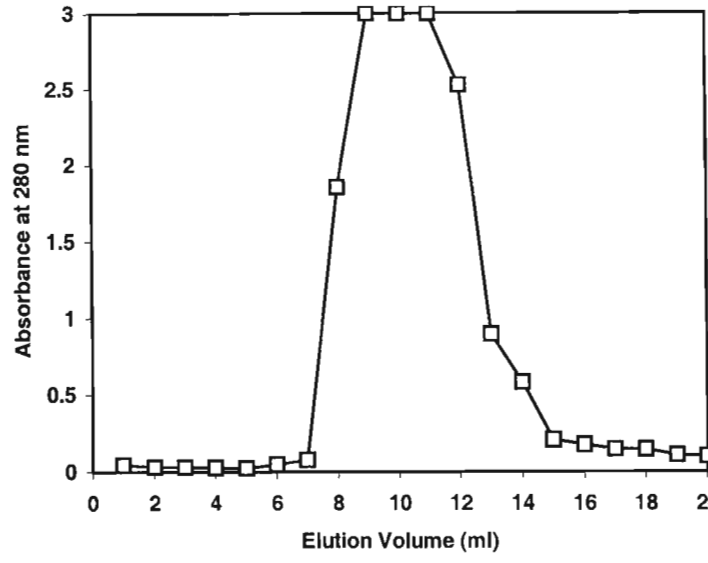


Figure 4.2 Elution profile of anti-rabbit albumin IgY
Rabbit albumin was coupled to an affinity matrix. Anti-rabbit albumin IgY was eluted from the affinity matrix with glycine-HCl at pH 2.8.

4.4 Evaluation of affinity-purified anti-rabbit albumin IgY using ELISA

ELISA was used as a quantitative and qualitative means to examine the extent of affinity purification and immunoreactivity of the immune-IgY towards rabbit albumin. The results are illustrated in Figure 4.3. Non-immune antibody served as the control. IgY that did not bind to the affinity matrix did contain some anti-rabbit albumin IgY and indicated that not all the antibody against rabbit albumin was removed during the affinity purification step. There was however, significant decrease in the titre of anti-rabbit albumin IgY compared to the original sample. The affinity-purified IgY had higher absorbance values in ELISA than with the original IgY sample and the IgY sample that did not bind to the affinity matrix.

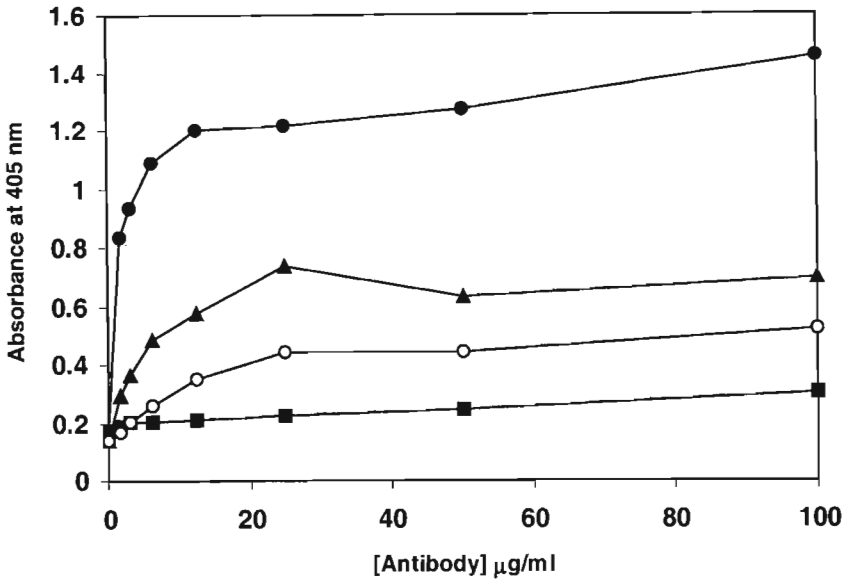


Figure 4.3 ELISA evaluation of affinity-purified IgY
A 96-well micro-titre plate was coated with rabbit albumin (1 µg/ml) and incubated with serial dilutions of affinity-purified IgY (•); original IgY sample (▲); unbound IgY (o); non-immune IgY (■). This was followed with incubation with rabbit-anti-IgY-HRPO secondary antibody and ABTS-H_2O_2 substrate.

4.5 Analysis of the number and the surface probability of lysine residues in chicken IgY

The sequence of chicken egg yolk IgY was initially derived from published literature (Reynaud *et al.*, 1983; Pavari *et al.*, 1988; Pavari *et al.*, 1990) and a BLAST search was carried out on the Expasy web site (www.expasy.org) to obtain the updated IgY sequence. The primary and secondary structural analysis was further carried out using on Expasy web site. The Predict7® software (Cármenes *et al.*, 1987) was used to obtain the surface probability of lysine residues as well as the hydrophilicity of IgY (data not shown). The results of the analysis (Figure 4.4*A*) revealed that out of the 103 amino acids that make up the light chain constant region of IgY, five are lysine (Lys^4, Lys^{15}, Lys^{23}, Lys^{96} and Lys^{99}) residues. Figure 4.4*B* illustrates that most of the lysine residues appeared to be located on the surface of the IgY light chain constant region. Most of the lysine residues appeared to be located on the hydrophilic regions of the light chain.

A

```
        10         20         30         40         50         60
         |          |          |          |          |          |
QPKVAPTITL FPPSKEELNE ATKATLVCLI NDFYPSPVTV DWVIDGSTRS GETTAPQRQS

        70         80         90        100
         |          |          |          |
NSQYMASSYL SLSASDWSSH ETYTCRVTHN GTSITKTLKR SEC
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B

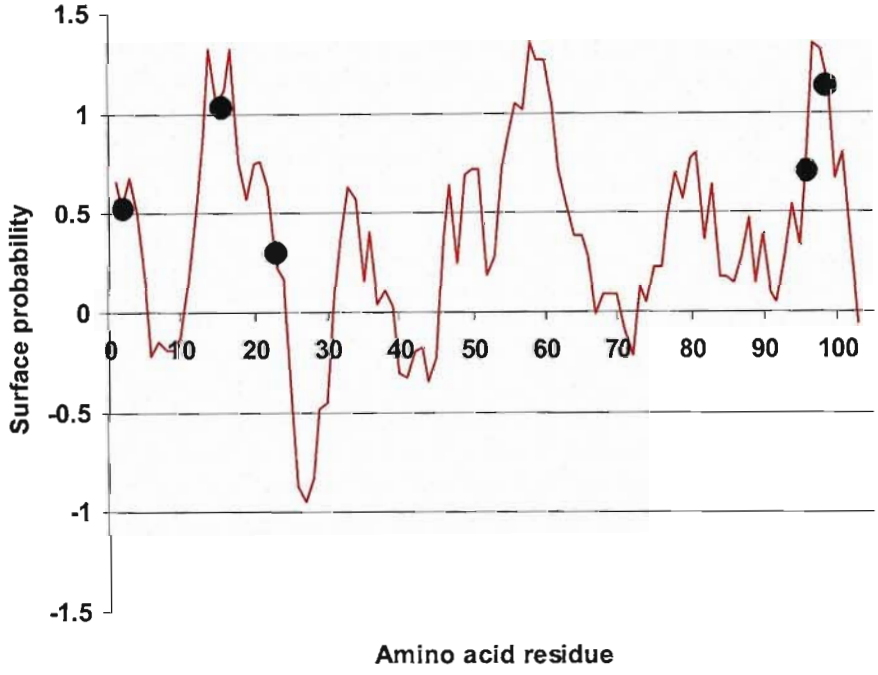

Figure 4.4 Primary and secondary structure analysis of IgY light chain
A: Amino acid sequence of IgY light chain constant region derived from www.expasy.org, (Primary accession number P20763). *B*: The secondary structure analysis of the surface availability of the various amino acids constituting the light chain constant region of IgY. The black dots on the figure represent the position of lysine residues (Cármenes *et al.*, 1989).

Figure 4.5*A* represents the primary sequence of the heavy chain constant region of chicken IgY derived from www.expasy.org (Primary accession number P20763). There are 446 amino residues making up the heavy chain constant region of the IgY, of which 16 are lysine residues. The number of lysine residues is different for each region of the heavy chain constant region. CH1 region contains three lysine residues (Lys^{57}, Lys^{78} and Lys^{80}); CH2 contains two lysine residues (Lys^{150} and Lys^{201}); CH3 region contains nine lysine residues (Lys^{231}, Lys^{254}, Lys^{256}, Lys^{259}, Lys^{266}, Lys^{297}, Lys^{310}, Lys^{313} and Lys^{315}); CH4 region and the C-terminal domain have one lysine residue at position 420 and 428 respectively.

Figure 4.5*B* illustrates the surface probability of the lysine residues on the heavy chain constant region of the IgY. 10 of these lysine residues have surface probability > 0.5, while the remainder have surface availability < 0.5. Just like IgY light chain, most of the lysine residues are located on the hydrophilic regions of IgY heavy chain.

This means that there are up to 42 lysine residues on the light and heavy chain constant regions of IgY that may be available for coupling reactions.

A

```
          10          20          30          40          50          60
          |           |           |           |           |           |
ASASPSPPRL FPLVLCSPSD SVYTVGCAAF DFQPSSIAFT WFDSNNSSVS GMDVIPKVIS

          70          80          90         100         110         120
          |           |           |           |           |           |
GPPYRAVSRI QMNQSEGKEK QPFRCRAAHP RGNVEVSVMN PGPIPTPNGI PLFVTMHPPS

         130         140         150         160         170         180
          |           |           |           |           |           |
REDFEGPFRN ASILCQTRGR RRPTEVTWYK NGSPVAAAAT TATTVGPEVV AESRISVTES

         190         200         210         220         230         240
          |           |           |           |           |           |
EWDTGATFSC VVEGEMRNTS KRMECGLEPV VQQDIAIRVI TPSFVDIFIS KSATLTCRVS

         250         260         270         280         290         300
          |           |           |           |           |           |
NMVNADGLEV SWWKEKGGKL ETALGKRVLQ SNGLYTVDGV ATVCASEWDG GDGYVCKVNH

         310         320         330         340         350         360
          |           |           |           |           |           |
PDLLFPMEEK MRKTKASNAR PPSVYVFPPP TEQLNGNQRL SVTCMAQGFN PPHLFVRWMR

         370         380         390         400         410         420
          |           |           |           |           |           |
NGEPLPQSQS VTSAPMAENP ENESYVAYSV LGVGAEEWGA GNVYTCLVGH EALPLQLAQK

         430         440
          |           |
SVDRASGKAS AVNVSLVLAD SAAACY
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B

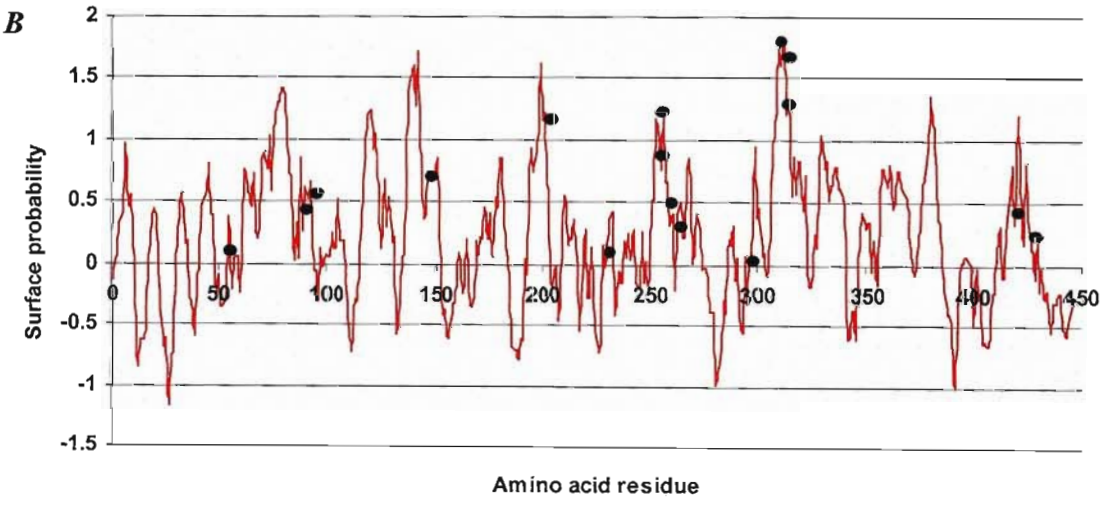

Figure 4.5 Primary and secondary structure analysis of IgY heavy chain

A: Amino acid sequence of IgY heavy chain constant region derived from www.expasy.org (Primary accession number P20763). *B*: The secondary structure analysis of the surface availability of the various amino acids constituting the heavy chain constant region of IgY. The black dots on the figure represent the position of lysine residues (Cármenes *et al.*, 1989).

4.6 Preparation and evaluation of immunoreactivity of HRPO-IgY conjugates

Affinity-purified anti-rabbit albumin IgY was labelled with horseradish peroxidase (HRPO). The activated HRPO-MBS and SH-IgY were coupled and purified by gel filtration over Sephacryl® S-300 gel filtration column calibrated with blue dextran and IgY (Figure 4.3). Blue dextran eluted at 175 ml (V_o), while IgY eluted at 240 ml. IgY-HRPO conjugates eluted at 175 ml (peak *a*) and 225 ml (peak *b*), and the unconjugated HRPO eluted the column at 285 ml (peak *c*). The presence of HRPO in each elution volume was determined by absorption at 403 nm. Elution samples corresponding to peaks *a* and *b* in Figure 4.3 were pooled and concentrated with PEG 20 000. The molar ratio of IgY:HRPO in the conjugate was determined to be 1:1. The conjugates were stored at 4°C in 0.01% thimerosal.

Table 4.1 illustrates results obtained from immunoreactivity and optimisation of the IgY-HRPO conjugate using dot-ELISA format. The test was carried out in triplicates. 50 – 100 ng of rabbit albumin was detected by IgY-HRPO conjugate at all dilutions used. 1/500 – 1/2500 dilution of IgY-HRPO conjugate detected 5 ng of the antigen. However, 1/500 and 1/1000 dilution cross-reacted with BSA (negative control). 1/2500 dilution appeared to be optimal as it detected 5 ng of the antigen and did not detect the negative control.

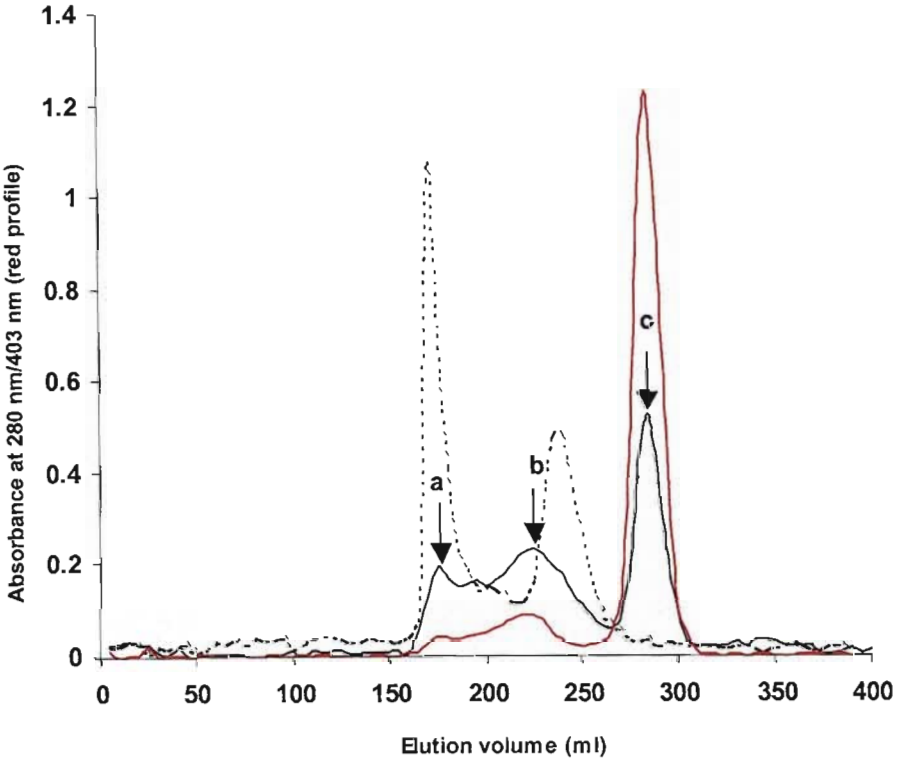

Figure 4.6 Elution profile of HRPO-IgY conjugates from Sephacryl® S-300 size exclusion column

IgY-HRPO conjugate prepared from MBS-activated HRPO and sulfhydryl-modified IgY was purified by size exclusion chromatography using Sephacryl® S-300 column (82.5 × 3.2 cm^2). (——): represents elution profile of HRPO-IgY monitored at 280 nm, (——) represents elution profile of HRPO-IgY monitored at 403 nm and (-------) represents calibration profile. The flow rate was 1.6 cm/h.

Table 4.1 Immunoreactivity and optimisation of HRPO-IgY conjugate on nitrocellulose

The number of positive (+) signs indicates the colour intensity of the spots (+++ for high intensity; ++: for medium and +: for weak intensity). A negative sign (–) represents absence of coloured spot. The test was carried out in triplicates.

HRPO-IgY dilutions	Rabbit albumin (ng)				TBS (µl)	BSA (ng)
	100	50	10	5	1	100
1/500	+++	+++	+++	+++	–	++
1/1000	+++	+++	+++	+++	–	+
1/2500	+++	+++	++	+	–	–
1/5000	+++	+++	++	–	–	–
1/10000	+++	++	–	–	–	–
1/50000	++	+	–	–	–	–

4.7 CDI-activation of dyes and spectral analysis of activated dyes

Dyes were activated in either DMF or DMSO in order to establish which solvent is suitable for activating a particular dye with CDI. The ratio to dye to CDI was estimated based on the number of functional targets of CDI (hydroxyl and carboxylic acid functional groups) on each dye molecule. Since the process of dye activation with CDI is a chemical coupling process, the colouristic properties of the dyes was altered due to modification of the dye auxochromes. An alternate control involved reacting Basic Fuchsin and Azure A that lack hydroxyl and carboxylic acid groups with CDI in DMF or DMSO. To monitor the activation of each dye, the spectral patterns of the activated dyes were compared with the spectral pattern of non-activated dye.

Figure 4.7 illustrates the spectral patterns of dyes activated in DMF. The spectrum of activated Aurintricarboxylic Acid (Figure 4.7*A*) exhibited a peak absorbance at 560 nm while the non-activated dye did not show a peak absorbance when scanned from 250 – 750 nm. The spectrum of activated Ethyl Red (Figure 4.7*B*) showed a peak absorbance at 500 nm compared to a peak of 450 nm for the non-activated dye, a spectral change referred to as "bathochromy". The activated Gallocyanine (Figure 4.7*C*) exhibited a single peak absorbance at 565 nm while the non-activated dye showed two peaks at 515 nm and 630 nm. The spectrum of activated Malachite Green HCl (Figure 4.7*D*) exhibited a lower absorbance at 615 nm, at the same wavelength as the non-activated dye control that showed a higher peak absorbance. However, the non-activated Malachite Green HCl also exhibited a second peak at 420 nm.

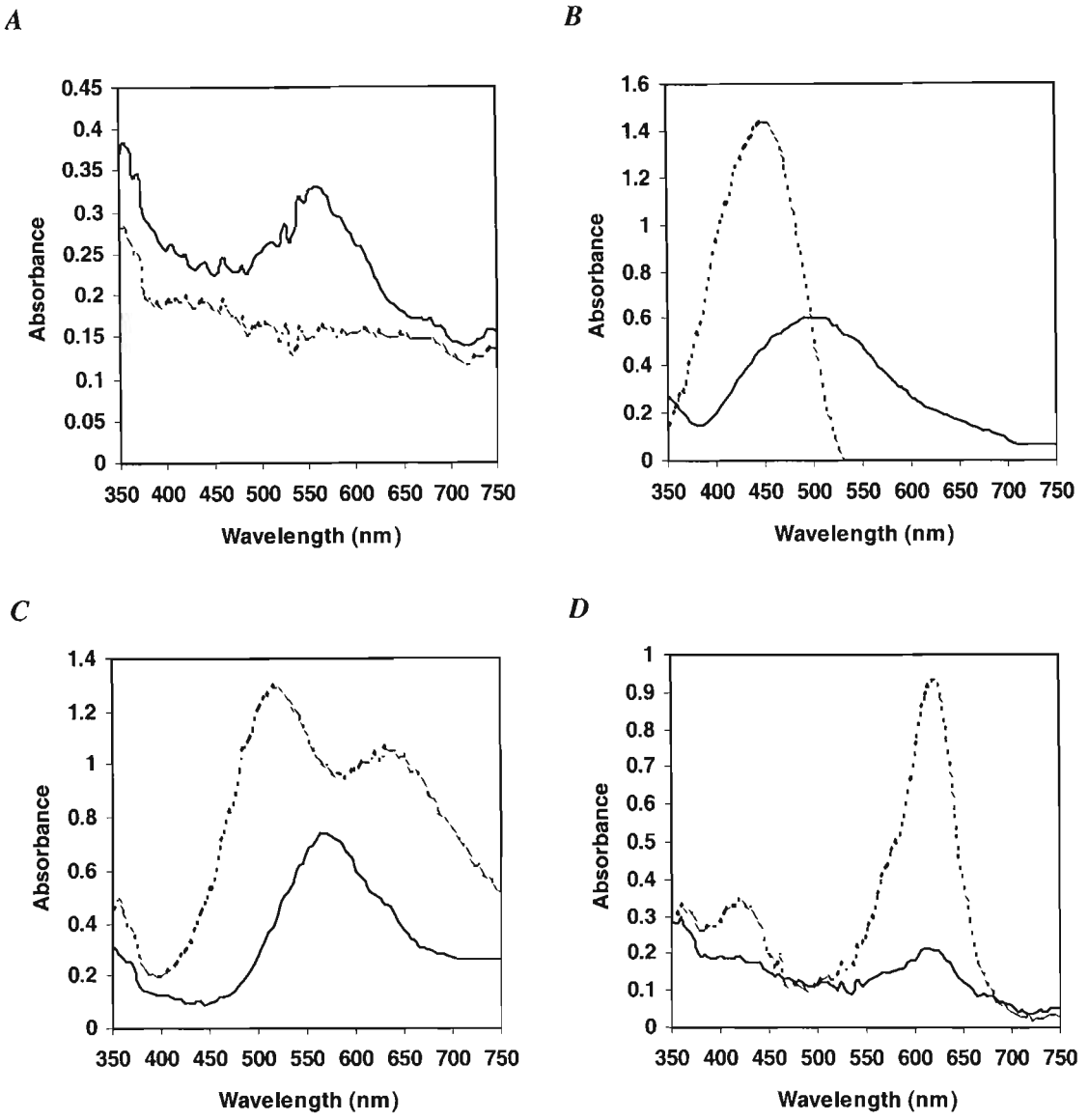

Figure 4.7 Spectral patterns of dyes activated with CDI in DMF
(*A*) Aurintricarboxylic Acid, (*B*) Ethyl Red, (*C*) Gallocyanine and (*D*) Malachite Green HCl were activated or not activated with CDI in DMF. 1 µl of each dye solution (activated/non-activated) was suspended in 4 ml of bicarbonate buffer at pH 9.6 and scanned between 350 nm and 750 nm using Cary 50 ™ Bio-UV visible spectrophotometer. The profile of activated dyes is represented by (——) and the profile of: non-activated dye control is represented by (-------).

The spectral patterns of activated/non-activated dyes in DMF shown in Figure 4.7 are similar to the spectral patterns of the same dyes (activated/non-activated) in DMSO. Therefore the figures illustrating the spectral pattern of activation/non-activation in DMSO were not shown.

Figure 4.8 illustrates the spectral patterns of Basic Fuchsin in (*A*) DMF and (*B*) DMSO and Azure A in (*C*) DMF and (*D*) DMSO. There was no significant difference in absorption spectrum of both dyes in the different solvents as in the presence or absence of CDI. The absorbance maximum of Azure A and Basic Fuchsin were at 625 nm and 540 nm respectively in both solvents. The absorbance of the dye reacted with CDI was marginally higher than the absorbance of the dye without CDI. The decrease in absorbance at 625 and 540 nm was due to alteration in dye concentration during the experiment.

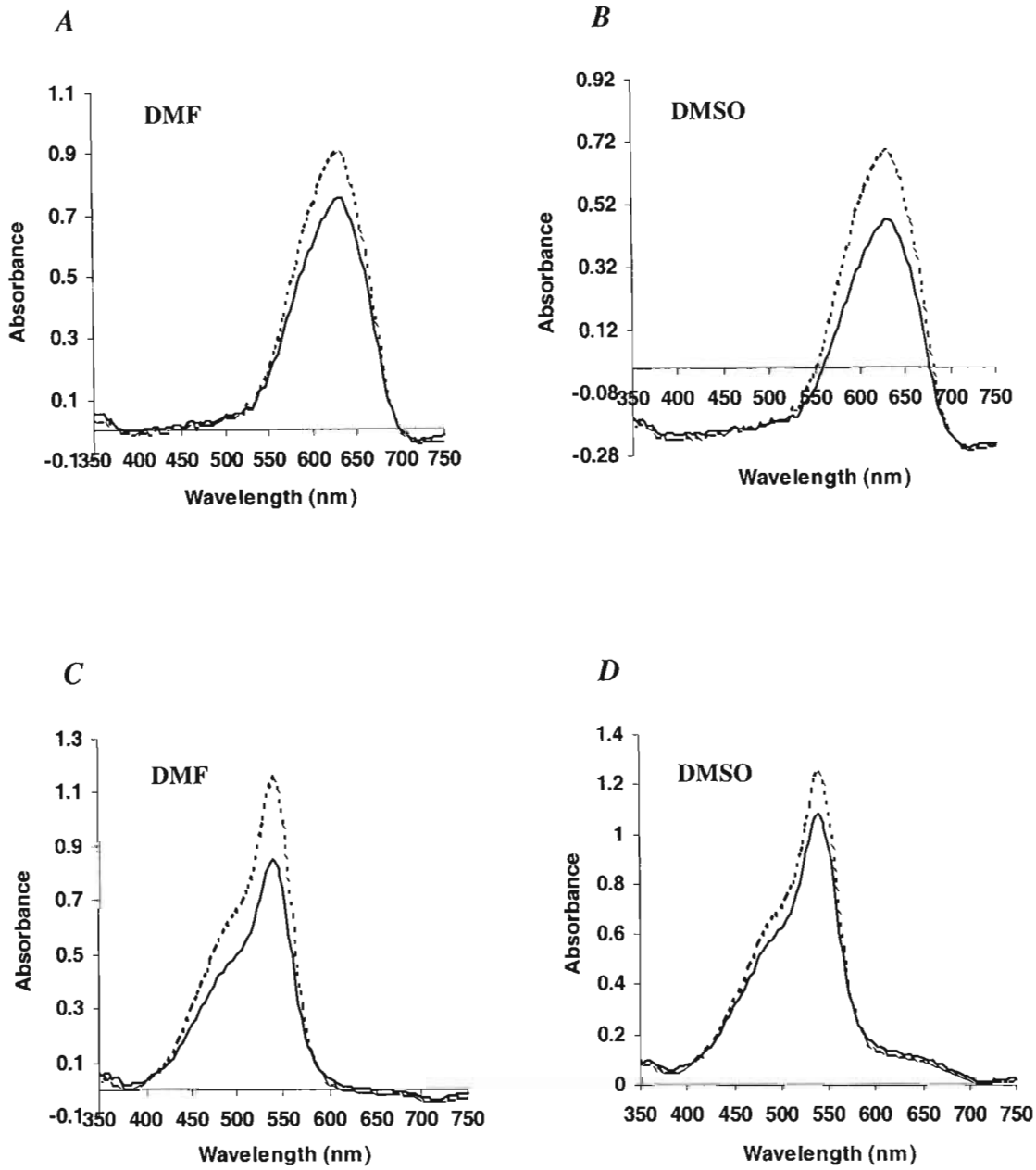

Figure 4.8 The spectral patterns of dyes lacking CDI-functional targets
Azure A (*A* and *B*) and Basic Fuchsin (*C* and *D*) were suspended with or without CDI in DMF or DMSO. 1 µl of each dye solution was suspended in 4 ml of bicarbonate buffer at pH 9.6 and scanned between 350 nm and 750 nm using Cary 50™ Bio-UV visible spectrophotometer. The profile of dye with CDI is represented by (——) and the profile of dye without CDI is represented by (-------).

4.8 Purification and characterization of dye-IgY conjugates

Colourimetric verification of the covalent conjugation of dyes to IgY and dialysis was assessed by the difference in spectra of dye-IgY conjugates prepared from CDI-activated dyes and non-activated IgY-dye (control). SDS-PAGE was also used to evaluate the size of the IgY-dye conjugates. CDI-activated-Malachite Green HCl lost its green colour and precipitated the protein; therefore Malachite Green HCl was not studied further.

Figure 4.9 illustrates the spectra of IgY-dye conjugates prepared from dyes activated in DMF compared to non-activated dye-IgY. The spectrum of IgY alone (Figure 4.9*A* – *C*) exhibited characteristic absorbance at 280 nm. The spectra of IgY-activated dye conjugate and IgY-non-activated dye solution all absorbed at 280 nm. The spectra of IgY-activated-Aurintricarboxylic Acid conjugate and IgY-non-activated-Aurintricarboxylic Acid (Figure 4.9*A*) exhibited no defined peak in the visible region of the spectrum. The IgY-activated-Ethyl Red conjugate spectrum had a single peak at 455 nm (Figure 4.9*B*), while the IgY-non-activated Ethyl Red control had a similar profile to IgY alone, without an absorbance peak in the visible region of the spectrum. The spectrum from IgY-activated-Gallocyanine conjugate (Figure 4.9*C*) exhibited a broad peak ranging from 550 nm to 630 nm, while IgY-non-activated-Gallocyanine had a similar profile to IgY alone, without an absorbance peak in the visible region of the spectrum.

The spectral patterns of dye-IgY conjugates prepared from dyes activated/non-activated in DMSO were not shown due to the similarity of the spectra to that shown in Figure 4.9.

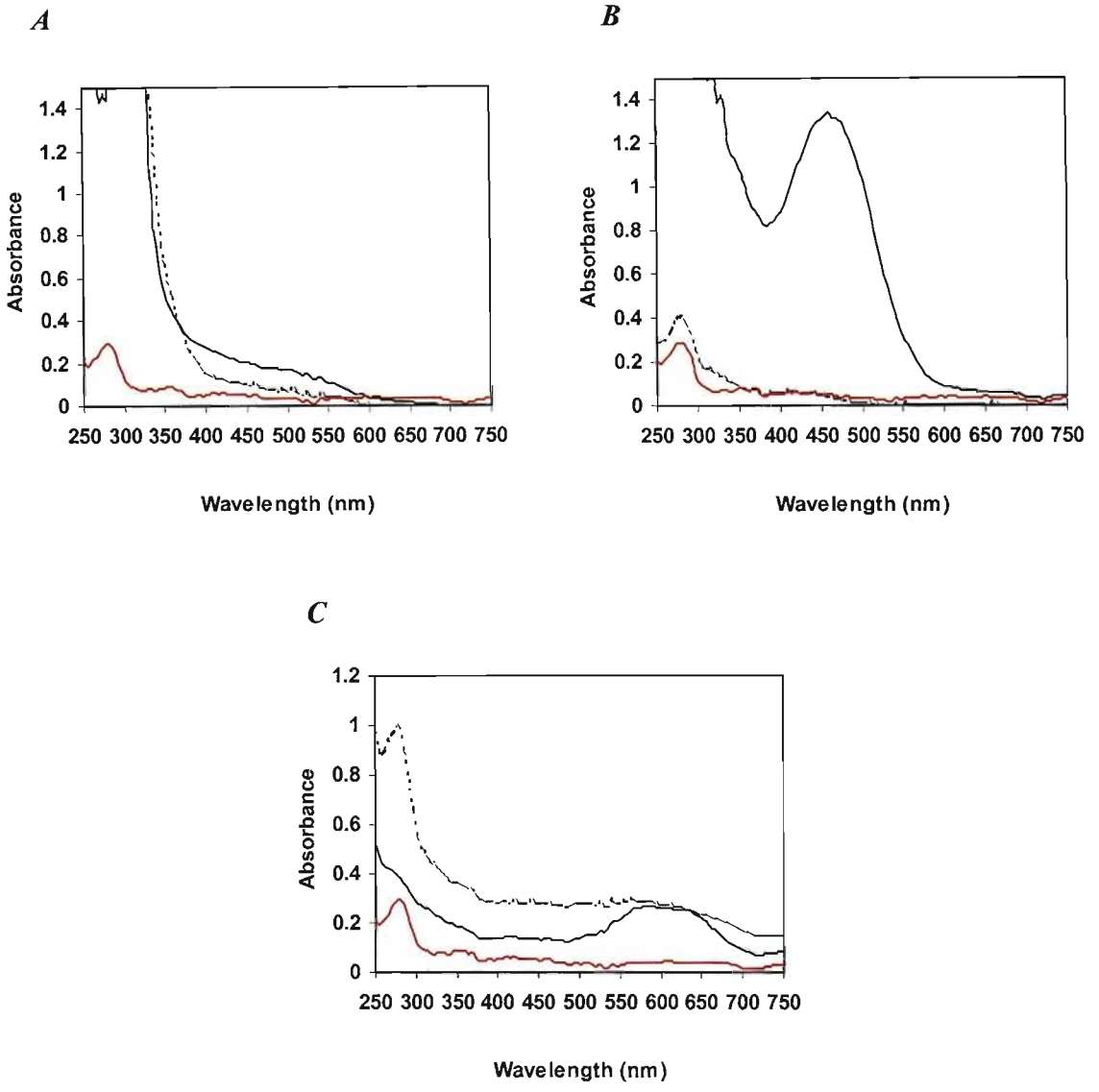

Figure 4.9 Analysis of the spectral patterns of dye-IgY conjugates
(*A*): Aurintricarboxylic Acid; (*B*): Ethyl Red and (*C*): Gallocyanine. A spectral pattern of IgY- CDI-activated dyes conjugates (——); IgY-non-activated dyes solution (-------) and IgY alone (——).

SDS-PAGE evaluation of dye-IgY conjugates is shown in Figures 4.10 – 4.12. In the non-stained gels (Figures 4.10*A* and *C*), a red coloured protein band is evident on lane 3 representing Aurintricarboxylic Acid-IgY conjugate prepared from the dye activated in DMF. The feint band (lane 5) represents the conjugate prepared from the dye activated in DMSO. There were no other bands in other lanes of the gels. After staining (Figures 4.10*B* and *D*), the band in lane 2 is IgY at *ca* 97 kDa (Figure 4.10*B*) under non-reducing conditions. When reduced the IgY has an approximate M_r of 67 kDa representing the heavy chain (Figure 4.10*D*), the light chain was not visible on the gel due to the low percentage of the gel. The band in lane 3 (Figure 4.10 *B* and *D*) at $M_r > 97$ kDa shows that the conjugation reaction produced dye-IgY conjugate that was not effectively reduced. In lanes 4, 5 and 6 (Figures 4.10 *B* and *D*), there was a major band corresponding to the IgY in lane 2. However, there were a range of high molecular weight protein bands in lane 5 (Figure 4.10*D*) suggesting the presence of different conjugation products of Aurintricarboxylic Acid-IgY conjugate prepared from the dye activated in DMSO.

Figure 4.8 illustrates the characteristics of Ethyl Red-IgY conjugates. Two red bands were evident (lane 3) indicating Ethyl Red-IgY conjugates prepared from dyes activated in DMF (Figure 4.11*A*), though not very distinguishable in the picture. Three red bands were visible (lane 3) from Ethyl Red-IgY conjugates prepared from the dye activated in DMF (Figure 4.11*C*). The position of the band (lane 3) moved down the gel upon reduction. No other bands were visible. After staining (Figures 4.11*B* and *D*), the IgY (lane 2) is > 97 kDa (Figure 4.11*B*). In Figure 4.11*D*, two bands of 67 kDa and 25 kDa (lane 2) corresponding to the heavy and light chains of IgY were visible upon reduction in lanes 2, 3, 4, 5 and 6. The bands in lane 3 (Figures 4.11*B* and *D*) correspond to the bands in the same lane of the pre-stained gels (Figures 4.11*A* and *B*). The intensity of the Coomassie stain is lower in lane 3 than in bands in the other lanes. There were no bands on lanes 7 and 8 corresponding to unconjugated dye controls.

Figure 4.12 illustrates the evaluation of Gallocyanine-IgY conjugates by SDS-PAGE on a 12% gel. There are 4 visible bands at the top of lanes 3, 4, 5 and 6 (Figures 4.12*A* and *C*). However, the bands on Figure 4.12*C* were not clear on the picture due to the resolution of

the scanner. The colour of the bands in lane 3 and 5 is blue, corresponding to Gallocyanine-IgY conjugates prepared from Gallocyanine activated in DMF and DMSO respectively. The black-coloured bands on lanes 4 and 6 are Gallocyanine-IgY conjugates prepared from non-activated dyes (control) in DMF and DMSO respectively. No other bands were evident on the pre-stained gels. Staining revealed proteins (Figures 4.12*B* and *D*) that were not visible in the pre-stained gels. Molecular weight markers are in lane 1. Lane 2 is IgY alone, which is > 97 kDa (Figure 4.12*B*), and 67 and 25 kDa (Figure 4.12*D*) corresponding to the heavy and light chains. There was a prominent band at the top of lanes 3, 4, 5 and 6 that is > 97 kDa (Figure 4.12*B*). These bands correspond to the IgY band in lane 2. There was a single protein band > 97 kDa that is prominent in lane 3 and minor in lanes 4, 5 and 6 of Figure 4.9*D*. Two protein bands of 67 and 25 kDa corresponding to the heavy and light chain are in lanes 4, 5 and 6 (Figure 4.12*D*). These protein bands match the bands on lane 2 of Figure 4.912. Bands were not noticeable in lane 7 and 8.

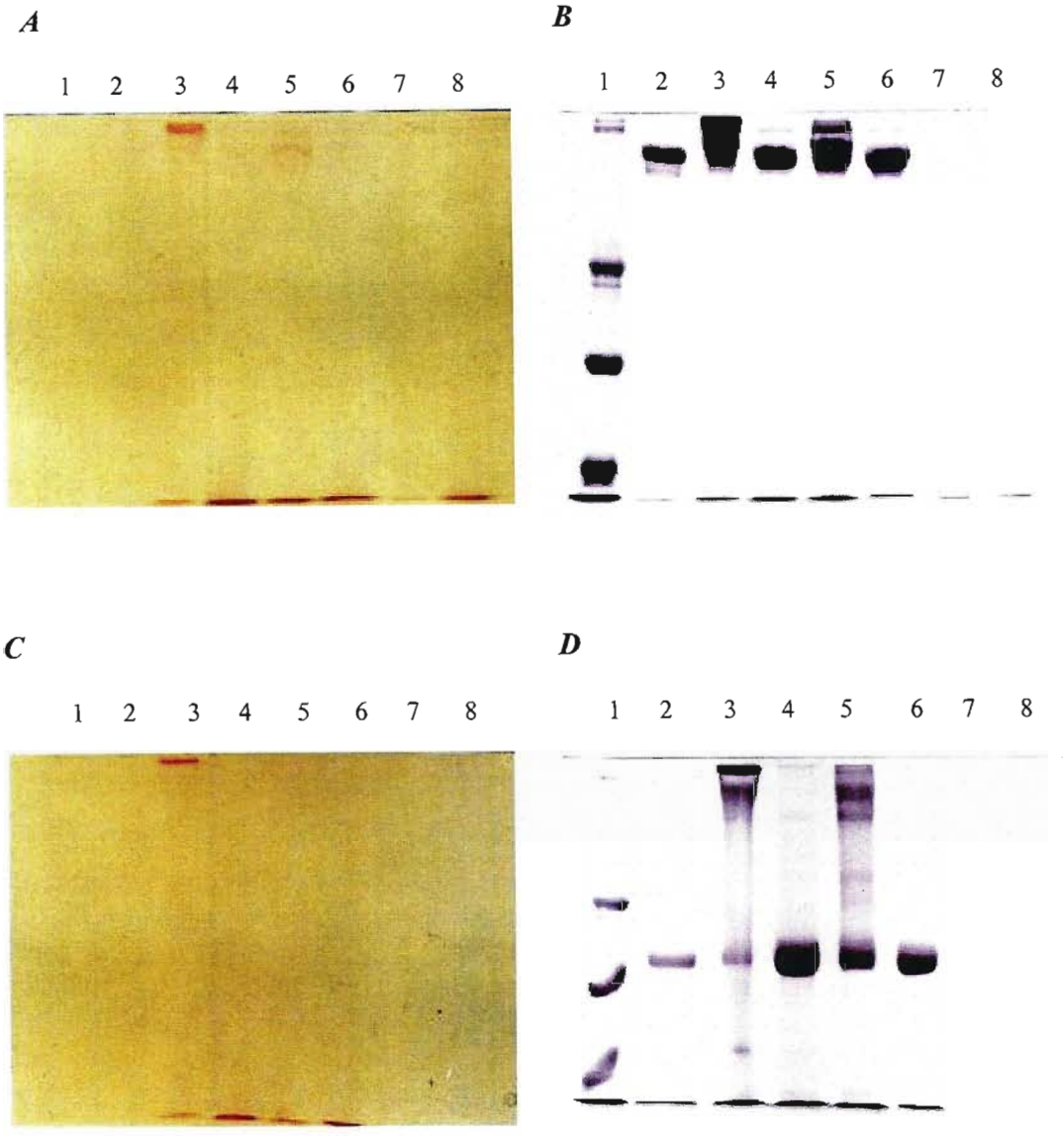

Figure 4.10 SDS-PAGE evaluation of Aurintricarboxylic Acid-IgY conjugates
IgY-ATA-IgY conjugate prepared from dyes activated or non-activated in either DMF or DMSO was run on 7.5% non-reducing and reducing SDS-polyacrylamide gel. *Lane 1*: molecular weight markers (97, 65, and 45 kDa); *lane 2*: 10 μg unconjugated IgY: *lane 3*: 15 μg ATA-IgY conjugate from dye activated in DMF; *lane 4*: 15 μg ATA-IgY conjugate from dye non-activated in DMF; *lane 5*: 15 μg ATA-IgY conjugate from dye activated in DMSO; *lane 6*: 15 μg ATA-IgY conjugate from dye non-activated in DMSO; *lane 7*: ATA activated in DMF; *lane 8*: ATA activated in DMSO.

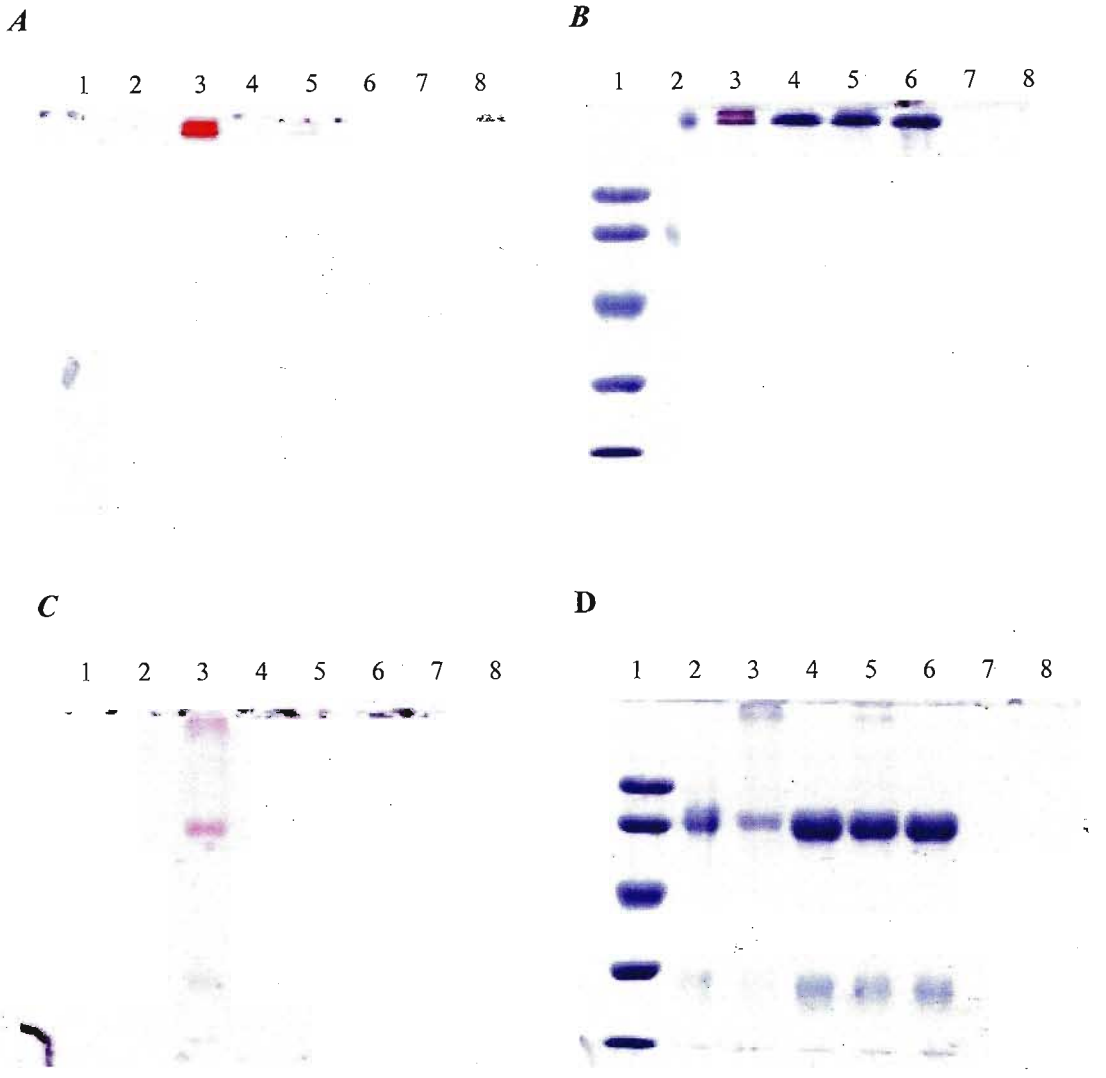

Figure 4.11 SDS-PAGE evaluation of Ethyl Red-IgY conjugates
Ethyl Red-IgY conjugate prepared from dyes activated or non-activated in either DMF or DMSO was run on 12% non-reducing and reducing SDS-polyacrylamide gel. *Lane 1*: molecular weight markers (97, 65, 45, 30 and 20 kDa); *lane 2*: 10 µg unconjugated IgY: *lane 3*: 15 µg Ethyl Red-IgY conjugate from dye activated in DMF; *lane 4*: 15 µg Ethyl Red-IgY conjugate from dye non-activated in DMF; *lane 5*: 15 µg Ethyl Red-IgY conjugate from dye activated in DMSO; *lane 6*: 15 µg Ethyl Red-IgY conjugate from dye non-activated in DMSO; *lane 7*: Ethyl Red activated in DMF; *lane 8*: Ethyl Red activated in DMSO.

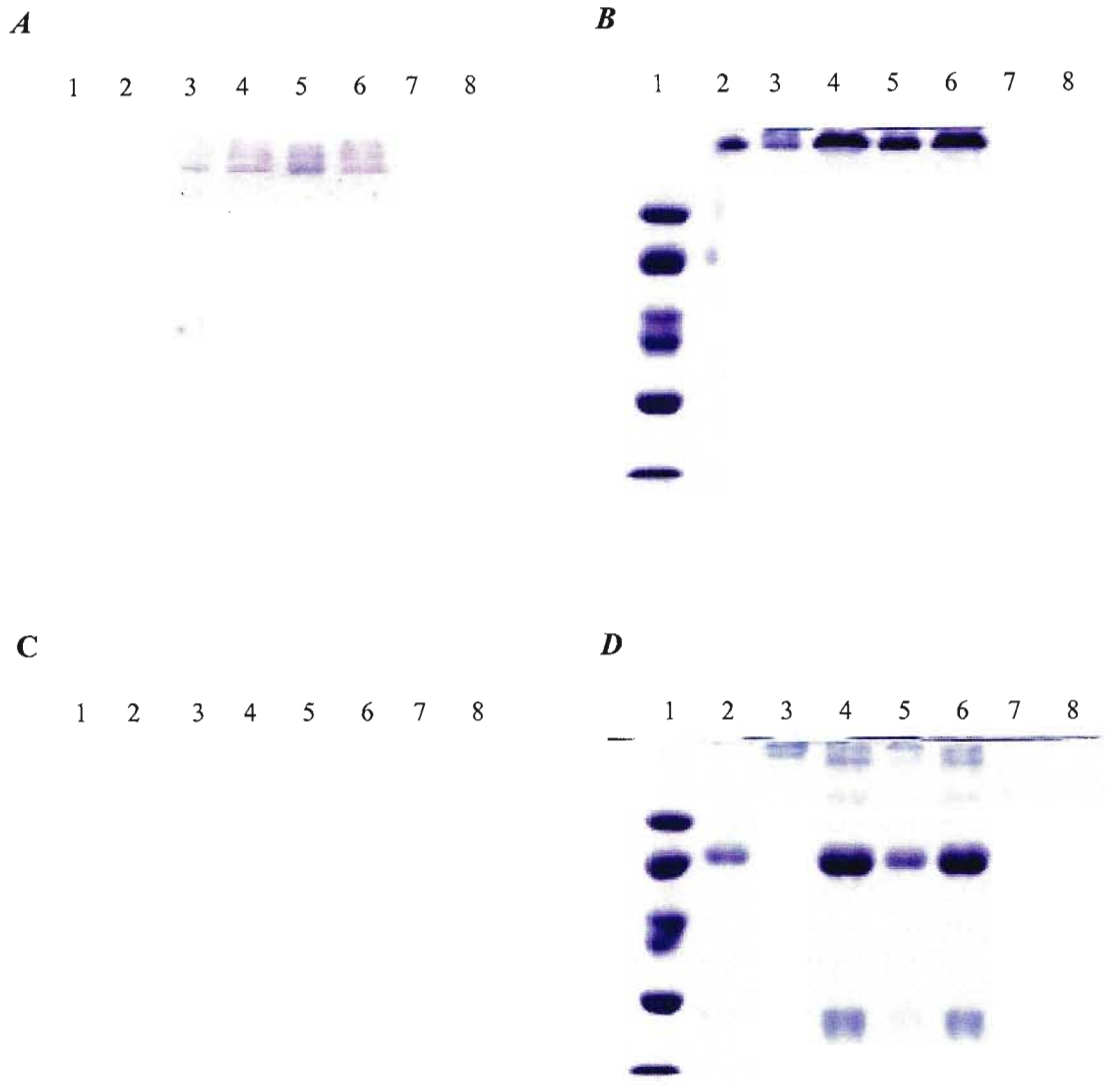

Figure 4.12 SDS-PAGE evaluation of Gallocyanine-IgY conjugates
Gallocyanine-IgY conjugate prepared from dyes activated or non-activated in either DMF or DMSO was run on 12% non-reducing and reducing SDS-polyacrylamide gel. *Lane 1*: molecular weight markers (97, 65, 45, 30 and 20 kDa); *lane 2*: 10 μg unconjugated IgY: *lane 3*: 15 μg Gallocyanine-IgY conjugate from dye activated in DMF; *lane 4*: 15 μg Gallocyanine-IgY conjugate from dye non-activated in DMF; *lane 5*: 15 μg Gallocyanine-IgY conjugate from dye activated in DMSO; *lane 6*: 15 μg Gallocyanine-IgY conjugate from dye non-activated in DMSO; *lane 7*: Gallocyanine activated in DMF; *lane 8*: Gallocyanine activated in DMSO.

4.9 Estimation of the molar coupling ratio of dye to IgY

The molar coupling ratios of the dye-IgY conjugates were estimated using standard calibration curves of each dye. Table 4.2 represents the molar coupling ratio of dye-IgY conjugates prepared from dyes activated in DMF. Gallocyanine-IgY appeared to be most efficient with a conjugation reaction ratio of 76 dye molecules per IgY molecule. The coupling ratio of Ethyl Red-IgY was the least efficient conjugation reaction, producing two dye molecules per IgY molecule and Aurintricarboxylic Acid-IgY was in between at a ratio of 34:1 dye to antibody.

Table 4.2 Molar coupling ratios of dyes to IgY

dye-IgY Conjugate	No. of CDI-functional targets per dye molecule	Initial molar ratio Dye:IgY	Final molar ratio Dye:IgY
Aurintricarboxylic Acid-IgY	5	350:1	34:1
Ethyl Red-IgY	1	489:1	2:1
Gallocyanine-IgY	3	440:1	76:1

4.10 Detection of rabbit albumin on nitrocellulose with dye-anti-rabbit albumin IgY conjugates

The use of the dye-IgY conjugates to detect antigen (rabbit albumin) was carried out on a nitrocellulose membrane. Only conjugates prepared from dyes activated with CDI in DMF were used. The test was carried out in triplicates. Aurintricarboxylic Acid-IgY conjugate detected 50 ng of rabbit albumin at dye-IgY concentration of 200 – 300 μg/ml (Table 4.3*A* and *B*) Aurintricarboxylic Acid-IgY conjugate did not detect antigen at any of the concentration of the antigen used. 100 – 300 μg/ml of Gallocyanine-IgY conjugate detected 50 ng of rabbit albumin (Table 4.3). In comparison, IgY-HRPO conjugate detected 5 ng of the antigen. The most sensitive dye-IgY conjugate was Gallocyanine-IgY, but this was 10-fold less sensitive than IgY-HRPO conjugate.

Table 4.3 Result obtained from the immunodetection of rabbit albumin with dye-IgY conjugates.

The number of positive (+) signs indicates the colour intensity of the spots (+++ for high intensity and +: for weak intensity). A negative sign (–) represents absence of coloured spot.

A

Conjugate (300 μg/ml of dye-IgY)	Rabbit albumin (ng)							Ovalbumin (500 ng)
	500	100	50	25	10	5	0	
ATA*-IgY	+	+	+	–	–	–	–	–
Ethyl Red-IgY	–	–	–	–	–	–	–	–
Gallocyanine-IgY	+	+	+	–	–	–	–	–
IgY-HRPO[c]	+++	+++	+++	+++	+++	+++	–	–

B

Conjugate (200 μg/ml of dye-IgY)	Rabbit albumin (ng)							Ovalbumin (500 ng)
	500	100	50	25	10	5	0	
ATA*-IgY	+	+	+	–	–	–	–	–
Ethyl Red-IgY	–	–	–	–	–	–	–	–
Gallocyanine-IgY	+	+	+	–	–	–	–	–
IgY-HRPO[c]	+++	+++	+++	+++	+++	+++	–	–

C

Conjugate (100 μg/ml of dye-IgY)	Rabbit albumin (ng)							Ovalbumin (500 ng)
	500	100	50	25	10	5	0	
ATA*-IgY	+	–	–	–	–	–	–	–
Ethyl Red-IgY	–	–	–	–	–	–	–	–
Gallocyanine-IgY	+	+	+	–	–	–	–	–
IgY-HRPO[c]	+++	+++	+++	+++	+++	+++	–	–

*Aurintricarboxylic Acid

[c]230 pg/ml of the enzyme in the IgY-HRPO conjugate

4.11 Evaluation of the immunoreactivity of dye-antibody conjugates using competitive ELISA

The immunoreactivity of the Aurintricarboxylic Acid-IgY, Ethyl Red-IgY and Gallocyanine-IgY conjugates was evaluated with a competitive ELISA (Figure 4.13). There was a gradual decrease in absorbance with an increase in competing immune-IgY concentration up to 25 μg/ml, and then the decease became marginal. The profile showed that increasing immune-IgY concentration competed with the secondary antibody-HRPO and this demonstrates good binding of the antibody to the antigen. Non-immune-IgY showed bound poorly to the antigen.

The competitive ELISA for the Aurintricarboxylic Acid-IgY conjugate (Figure 4.13*A*) has a similar profile to the non-immune IgY and this showed poor binding of the IgY-Aurintricarboxylic Acid conjugates (primary antibody) to the antigen (like the non-immune-IgY). The competitive ELISA Ethyl Red-IgY conjugate (Figure 4.13*B*) showed comparable profile to the immune-IgY. Gallocyanine-IgY conjugate (Figure 4.13*C*) was the same as Aurintricarboxylic Acid-IgY conjugate and showed no significant decrease in absorbance value with increasing concentration of the primary antibody. This result suggested that the dyes (Aurintricarboxylic Acid and Gallocyanine) that coupled well to the IgY produced conjugates that bound poorly to the antigen, whereas Ethyl Red that did not couple well to IgY resulted in a conjugate that bound effectively to the antigen.

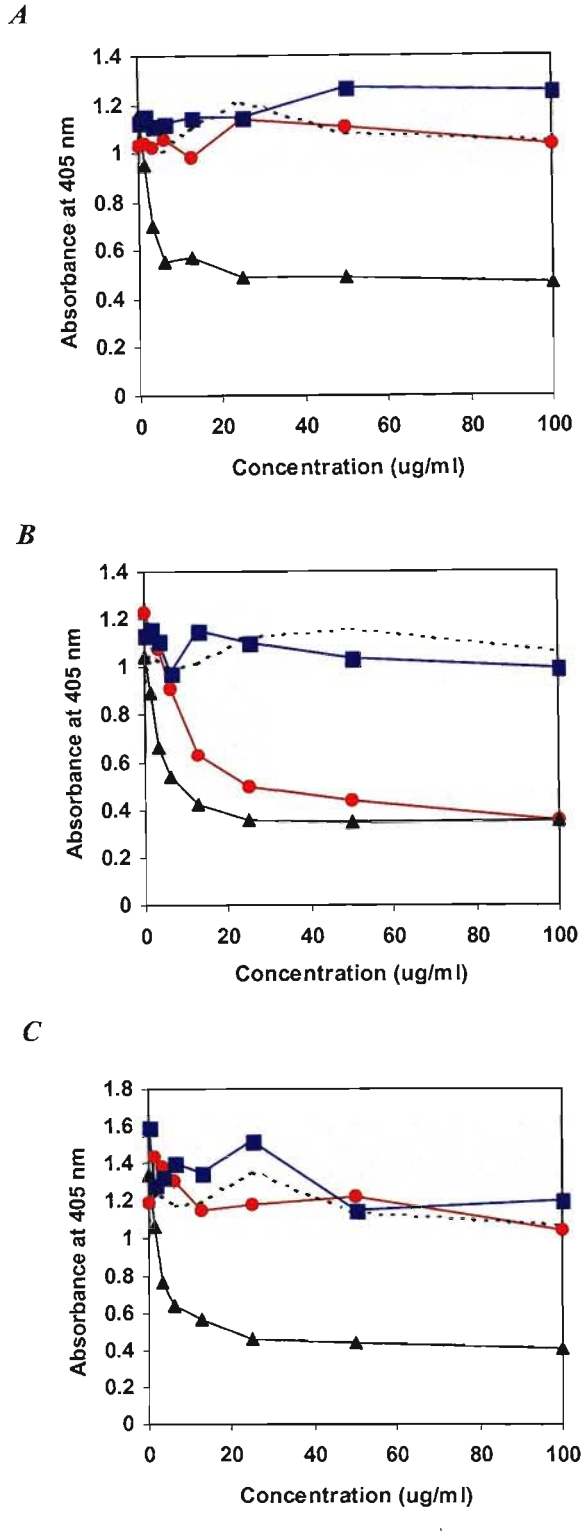

Figure 4.13 Competitive ELISA results for the evaluation of dye-IgY conjugates
A: Aurintricarboxylic Acid-IgY (●); *B*: Ethyl Red-IgY (●); *C*: Gallocyanine-IgY (●) were compared with (▲) affinity-purified anti-rabbit albumin IgY; (■) non-immune-IgY and (-----) PBS. Anti-rabbit albumin IgY-HRPO used as the competing secondary antibody.

4.12 Discussion

Chicken egg yolk antibodies were chosen as the antibody model in this study. Chickens were immunized with a peptide hapten conjugated to a rabbit albumin carrier. The choice of IgY over mammalian antibodies is because of the ease with which egg yolk antibodies can be isolated from the chicken egg yolk and it is not necessary to bleed the animal. Chicken egg yolks have higher antibody titres than in chicken serum due to the active transport of IgY from the serum into the egg yolk (Larsson and Sjöquist, 1990). Up to 1g/month of antibodies can be produced by a laying hen, a higher yield than that obtained from antibodies isolated from rabbit blood (Larsson and Sjöquist, 1990). Chicken egg yolk IgY can retain biological activity for a several years when stored at 4°C in the presence of anti-bacterial agents such as sodium azide (Goldring and Coetzer, 2003). The long shelf life of chicken antibodies offers advantages over mammalian serum antibodies.

The isolation of anti-rabbit albumin IgY was carried out with PEG 6000 precipitation and rabbit albumin-immobilized affinity matrix was used to affinity-purify the specific antibodies. Figure 4.1 shows the purification steps and the purity of the purified IgY used in this study. PEG 6000 is a very good water-extracting agent. It is non-ionic and so it is useful in the isolation of proteins as the biological activity of the protein can be retained (Ingham, 1984). The final product of the purification of IgY was a 67 kDa and a 27 kDa fragments representing the heavy and the light chains of IgY (Figure 4.1) and identical to that reported by Goldring and Coetzer (2003).

Due to the coupling strategy that was employed in the covalent attachment of dyes to antibodies, dyes with carboxyl and hydroxyl groups (COOH and OH) were chosen. CDI is a zero-length cross-linker that converts hydroxyl and carboxylic derivatives into imidazole intermediates that will subsequently react with primary amine-containing molecules (Hearn, 1987; Bethel 1987; Hermanson, 1996). The activation of dyes was carried out in a non-aqueous medium (DMSO and DMF) due to the degenerative effect water has on CDI, even at very low concentrations of water (Hearn, 1987). The activation step in the coupling reactions changed the colour of the dyes (Figure 4.7) due to the modification of the auxochrome groups on the dye molecule. The difference in the spectra of the activated dye

when compared to the non-activated dye and dyes lacking CDI-functional targets (Figure 4.8) confirmed that CDI activated Aurintricarboxylic Acid, Ethyl Red and Gallocyanine. The alteration in colour (Figure 4.7) resulted in a bathocromic, a hypschromic or a hyperchromic shift in absorption spectra of the activated dyes depending on the dye chemistry or solvent used. The principle of ELISA and immunohistochemistry is based on the modification of the colour of dyes, as a result of the modification of dye auxochromes. Unlike in CDI-mediated activation of dyes, enzymes mediate the modification of the dye auxochromes (Bunea and Zarnescu, 2001) by oxidation (horseradish peroxidase and glucoseoxidase) or dephosphorylaltion (alkaline phosphatase) of the substrates.

Activated dyes were conjugated to IgY in sodium carbonate-bicarbonate buffer at pH 9.6 as the rate of degeneration of activated intermediate at alkaline pH > 9 is reduced. Spectral patterns of activated and non-activated- dye-IgY conjugates were compared to each other. This was to establish if the activation of the dye with CDI was necessary for the covalent conjugation of the imidazole-active-dye to IgY. The result showed that the activation of the dyes with CDI did enhance the covalent coupling of the dyes to the antibody molecules (Figure 4.9). Ethyl Red-IgY conjugate prepared from Ethyl Red activated in DMF showed a defined absorbance peak at 450 nm (Figure 4.9*B*) and the dye-antibody conjugate appeared pale yellow. Interestingly, uncoupling of the imidazole functional group from the activated dye changed the λ_{max} of the dye from 500 nm (for the activated dye) to 450 nm. When the activation was conducted in DMSO, the specific shift in λ_{max} of the dye did not occur and may be due to the effect of the solvent on the CDI-activation chemistry of Ethyl Red. Gallocyanine-IgY conjugate showed a defined absorbance peak at 575 nm for Gallocyanine activated in both DMF (Figure 4.8*C*) and DMSO (data not shown). The high colour intensity of Gallocyanine suggested that the dye could be useful for the purpose of designing visible dye-antibody conjugate as an immunodiagnostic tool.

To confirm the covalent attachment of dyes to the antibodies and to evaluate possible alterations in electrophoretic mobility of dye-antibody conjugates, SDS-PAGE was used (Roberts *et al.*, 1987). Figures 4.10, 4.11 and 4.12 showed that dyes were successfully conjugated to the antibodies as indicated by the presence of high molecular weight coloured

bands on the on the unstained gels. Gallocyanine appeared to bind to IgY without activation (Figure 4.12 *A* and *C*). However, Gallocyanine did not bind to IgY at the coupling pH (pH 9.0) on nitrocellulose (data not shown).

Coomassie Blue R 250 stained all the proteins on the gels. Aurintricarboxylic Acid produced dye-IgY conjugates with undefined multiple molecular weights. This was evident as the multiple banding patterns revealed after Coomassie Blue R 250 staining (Figure 4.10*B* and *D*). Aurintricarboxylic Acid has five CDI-functional targets per dye molecule compared to Gallocyanine and Ethyl Red with three and one CDI-functional targets respectively. It is suggested that a dye with five functional targets is more likely to form large complexes with the antibody compared to a dye with three functional targets. In addition, the orientation of the CDI-functional targets on the dye molecule may have influenced the formation of the large molecular weight complexes. The functional targets on Aurintricarboxylic Acid seem to be more accessible to CDI due to their orientation compared to Gallocyanine with the CDI-functional targets. Ethyl-red has only one CDI-functional target; hence the possibility of cross-linking of IgY by activated Ethyl Red is unlikely and large molecular weight complexes were not observed in the gels (Figure 4.11).

These conjugates were used to detect 10 – 500 ng of rabbit albumin on a nitrocellulose membrane. Aurintricarboxylic Acid-IgY and Gallocyanine-IgY conjugates detected 50 ng of rabbit albumin and the resulting coloured spots were not very distinct. The Ethyl Red-IgY conjugate did not produce a visible signal. The sensitivity of the dye-IgY conjugates was 10 – 50 fold lower than the conventional HRPO-antibody labelled detection system. This is probably due to the amplification step in the enzyme-mediated detecting system.

The number of dye molecules coupled to the antibodies and alterations of the conformational architecture of the IgY molecule due to the covalent attachment of dyes may be responsible for the relatively low detection of antigen by the dye-IgY conjugates. Using the primary amino acid sequence of the heavy chain (Magor *et al.,* 1992, www.expasy.org) and light chain (Reynaud *et al.,* 1983; www.expasy.org) about 40 lysine residues appeared to be available on the constant heavy and light region of IgY for primary

amine-targeted covalent immobilisation of dyes or other molecules. The Predict7® programme (Cármenes *et al.*, 1989) was used to predict the surface probability of these lysine residues (Figures 4.4 and 4.5) for covalent attachment of dyes. A surface probability plot suggested that many lysine residues reside on the surface of the molecule.

A competitive ELISA was designed to investigate if dyes bound on the antigen-binding site on the antibody, thereby interfering with antibody-antigen interaction. Aurintricarboxylic Acid and Gallocyanine coupled well to the antibody (Table 4.2), but this coupling appears to block the antibody from binding to the antigen in a competitive ELISA (Figure 4.13***A*** and ***C***). However, Ethyl Red, which coupled less effectively to the antibody, did not block the binding of the antibody to the antigen in a competitive ELISA (Figure 4.13***B***). Ethyl Red-IgY conjugate bound as well to the antigen as immune-IgY. Moreover, Ethyl Red has only one functional group that will be activated by CDI for attachment of the IgY molecule. The competitive ELISA results seemed to be quite promising. Roberts *et al.* (1987) showed that cross-linking did not alter the immunoreactivity of IgG when porphyrin-IgY conjugates were prepared. In the covalent attachment of dyes to IgY, it appeared that the chemistry of the dye or the coupling chemistry or the occlusion of Fab sites on the antibody might have affected the immunoreactivity of the antibody; hence the detection limit of the dye-antibody conjugates. The possible alteration of the tertiary conformation of antibodies often results in a profound inhibitory effect on the biological activities of protein molecules such as antibodies (O'Shannessy, 1990). Biotin and most fluorescent dyes have been derivatized to have a single functional group for attachment on protein molecules (Wilchek and Bayer, 1988; Hermanson, 1996; Haugland and You, 1998). FITC has been shown to label antibodies at ratio of 37 dye molecules per antibody molecule. Being mono-functional, FITC does not cross-link proteins. Their use in this work for comparative purposes would have been very useful.

The three dyes used in this study were not originally designed for covalent labeling of proteins and other antibodies (sections 1.4.3, 1.4.13 and 1.4.15). The covalent attachment of dyes on antibody molecules may have to mimic the biotinylation and labeling of antibodies with flourophores (Wilchek and Bayer, 1988; Hermanson, 1996). Biotin and fluorescent

dyes used for protein labeling are derivatized to acquire functional groups that can easily react with protein molecules. This will not require complex coupling chemistries that are often difficult to control. Staining the antibodies with acidic dyes for use as an immunoassay tool (Gribnau *et al.,* 1982) is not a workable alternative to using covalently conjugated dyes. Unlike protein staining, covalent conjugation does not often lead to destabilization of the structure of the protein (Prentø, 2001) and it is more site-directed.

The design of dyes or derivatives of certain dyes with amine and hydroxyl functional reactive groups might be a future prospect on molecular dye-antibody conjugates as immunodetecting tools. The extent of labeling will determine the efficacy of the dye-antibody conjugates as a tool for immunodetection. From a theoretical point of view, IgY is a bivalent antibody that binds to two antigens at its antigenic sites on the Fab fragments. 10 ng of rabbit albumin will theoretically be bound to 1.25×10^{-13} M of IgY. Assuming that up to 40 moles of dye can be attached to an antibody molecule based on the available lysine residues on the IgY molecule, only 5.0×10^{-12} M of the dye will be present in the IgY-rabbit albumin complex. For Gallocyanine (Fw = 336.73) 1.68 ng of the dye will be on the IgY-rabbit albumin complex, while for Reactive Red 120 (Fw = 1469) about 7.3 ng of the dye will be the IgY-rabbit albumin complex. The least amount of Gallocyanine that can be seen on a nitrocellulose is about 25 ng. However, this amount may vary from one dye to another, depending on the colour intensity and the molar mass of the dye.

A more effective way of increasing the number of dyes covalently bound to antibody is, perhaps, to target both the ε-amine residues and hydroxyl residues on the antibody molecule. This can be achieved by designing dyes with either N-hydroxy-succinimide (NHS) or hydrazide functional derivatives. These functional derivatives are cross-linkers. Designing coloured dyes with cross-linker derivatives would solve the problems encountered in cross-linking chemistries and labelling would be easier to control. These dyes must be monofunctional so as to avoid the complications of cross-linking the antibody molecules and must lack sulfonate functional groups, which tends to bind to proteins without the need of cross-linkers. An example is illustrated in Figure 4.17. The NHS-Gallocyanine would form covalent bonding with ε-amine residues, while Gallocyanine

hydrazide would form a stable hydrazone linkage with aldehyde groups on the oxidized oligosaccharide residues on the antibody.

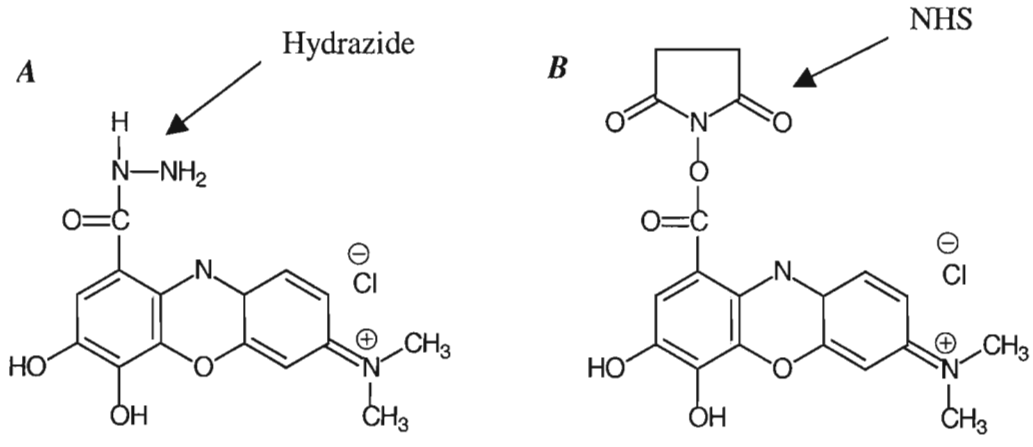

Figure 4.14 (*A*) hydrzide and (*B*) NHS derivative of Gallocyanine dye

CHAPTER 5
COVALENT CONJUGATION OF IgY ON DEXTRAN FOR IMMUNOASSAY

5.1 Introduction

In this study, anti-rabbit albumin IgY was isolated from chickens immunized with rabbit albumin conjugated haptens. IgY was conjugated to soluble dextran with a molecular weight range of 5000 – 40 000 kDa. The conjugation strategy required the modification of the hydroxyl groups on the dextran to acquire terminal amine functional groups. These terminal amines were reacted with IgY modified by 2-iminothiolane to SH-IgY. The conjugate was purified by molecular sieve chromatography. The IgY-dextran conjugate was tested at various concentrations to detect rabbit albumin blotted on a nitrocellulose membrane using PAS staining and compared to HRPO-IgY.

5.2 Preparation of IgY-dextran conjugate

Dextran polymer was modified to acquire terminal amine residues via a three-step derivatization reaction. Dextran was converted to carboxy methyl-dextran using chloroacetic acid. The carboxymethyl-dextran was subsequently converted to aminoethyl-carboxymethyl-dextran (AECM-dextran) using ethylenediamine di-HCl mediated by *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide. The AECM-dextran was finally converted to SH-reactive iodoacetyl-dextran using *p*-nitrophenyl iodoacetic acid. The SH-reactive idoacetyl dextran was coupled to IgY modified to have a terminal SH groups at the ε-amine residues of the antibody molecule. The preparation of IgY-dextran conjugate took approximately 4 days due to the extensive dialysis at each step of dextran modification. The conjugate was purified by size exclusion chromatography with a Sephacryl® S-300 matrix.

Figure 5.1 shows the elution profile of IgY-dextran conjugates. The first peak on the calibration profile is blue-dextran (M_r 2000 kDa) eluting after 170 ml (V_o) and the second peak is IgY (M_r 180 000 Da), eluting after 235 ml. IgY-dextran eluted with two major

peaks. The first peak of the IgY-dextran elution profile coincided with the void volume at 170 ml and the second peak was at 250 ml.

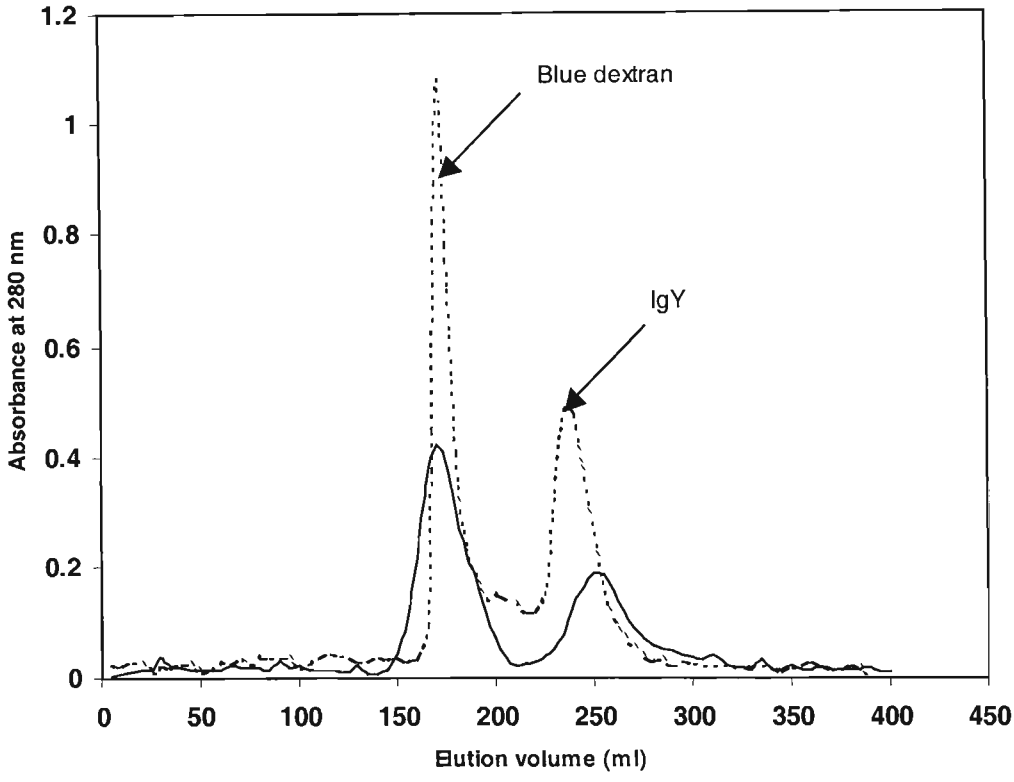

Figure 5.1 Elution profile of IgY-dextran conjugate from a Sephacryl® S-300 column
IgY-dextran was separated from unconjugated IgY by size exclusion chromatography on a Sephacryl S-300 column. (-------) represents the calibration profile obtained with a blue dextran-IgY mixture. (——) represents the elution profile of the IgY-dextran conjugate. The column dimension was 82.5 × 3.2 cm^2 and the linear flow rate was 1.6 cm/h.

5.3 Determination of carbohydrate/protein ratio

The protein/carbohydrate ratio was determined in order to establish the possible presence of IgY coupled to dextran in the Sephacryl® S-300 elution profile and to calculate the molar coupling ratio of the IgY-dextran conjugate. Fractions 155 – 300 corresponding to the two peaks on the IgY-dextran (Figure 5.1) elution profile were used. By using a standard dextran and a standard protein calibration curve obtained by PAS (Kirkbey *et al.*, 1992) and Bradford assays respectively, the concentration of dextran and protein in each fraction was obtained (Figure 5.2).

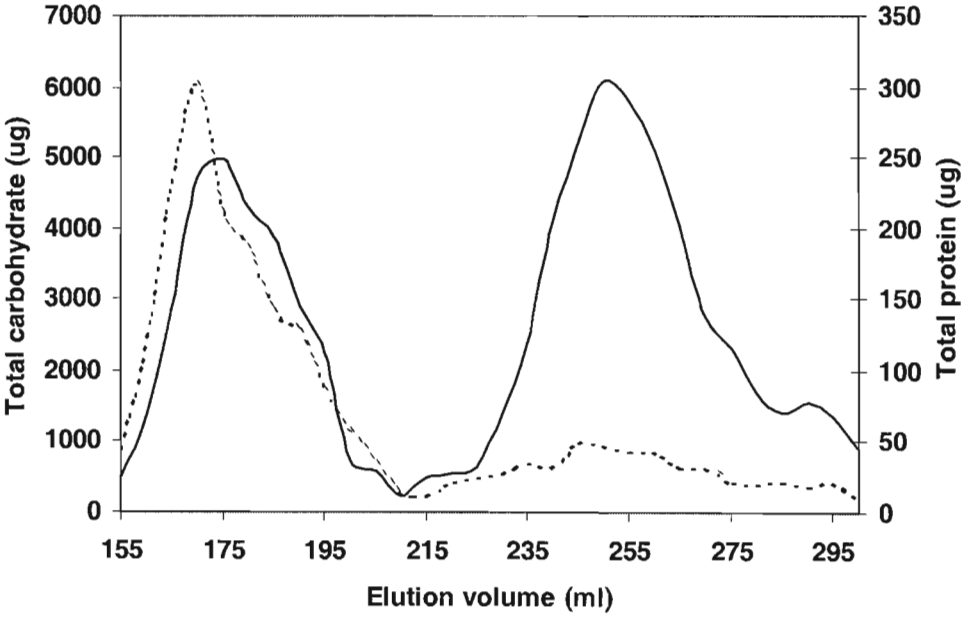

Figure 5.2 Profile of carbohydrate and protein content in fraction 155 – 300
The concentration of carbohydrate and protein in fractions 115 – 300 corresponding to the two peaks of IgY-dextran elution profile (Figure 5.1) were estimated using PAS (for carbohydrate) and Bradford (protein). (---------) represents total carbohydrate concentration and (———) represents total protein concentration in each fraction.

The highest carbohydrate concentration was in fraction 170 with a total carbohydrate concentration of 6.05 mg (1.21 mg/ml). The total protein content increased from fraction 155 ml to reach a peak of 249.1 μg (50 μg /ml) in fraction 175. There was a second protein peak of 50.0 μg in fraction 250. This result indicates that IgY coupled to dextran eluted in fraction 155 – 215. The molar coupling ratio was obtained using the mean protein concentration/mean carbohydrate concentration of fractions 155 – 215. The molar coupling ratio was estimated to be 6 moles of IgY per mole of dextran polymer (average M_r of dextran *ca* 22 500 kDa).

5.4 Detection of rabbit albumin with IgY-dextran conjugate by PAS staining

Rabbit albumin (antigen) was detected by PAS staining of dextran in the antigen-IgY-dextran immune complex on a nitrocellulose membrane (Table 5.1). The picture was not shown due to poor resolution when scanned. 25 ng of the antigen was indirectly detected by staining dextran in the IgY-dextran complex. PAS reagent stained BSA and human serum albumin as they contain carbohydrate. The background staining of the nitrocellulose by the PAS reagent was very high. The IgY-HRPO detected 10 ng of the antigen and human serum albumin and BSA producing a feint signal. This was probably due to the high concentration of these reagents.

Table 5.1 Detection of rabbit albumin with IgY-dextran conjugate
0 – 200 ng of rabbit albumin was blotted on nitrocellulose in triplicate and incubated in IgY dextran conjugate or IgY-HRPO. The dextran was stained with PAS reagent and IgY-HRPO was evaluated with ABTS substrate. (+++) dark stained dot; (++) denotes a medium stained dot; (+) denotes a weakly stained dot and (–) denotes no staining.

Reagent	Rabbit albumin (ng)					HSA[a]	BSA
	200	50	25	10	0	(200 ng)	(200 ng)
IgY-dextran	+++	+++	+++	–	–	++	++
IgY-HRPO	+++	+++	+++	+++	–	+	+

[a] Human serum albumin.

5.5 Evaluation of the immunoreactivity of the IgY-dextran conjugate using dot-ELISA

The immunoreactivity of IgY-dextran conjugate was evaluated by dot-ELISA on a nitrocellulose support to establish if the covalent binding of the IgY to dextran alters the immunogenic property of the antibodies. Immune-IgY and non-immune IgY were used as a positive and a negative control respectively. IgY was detected by a rabbit-anti-IgY-HRPO conjgate. The results (Table 5.2) showed that 10 ng of antigen was detected by IgY-dextran conjugate and the IgY (alone) control in a dot-ELISA format. The conjugate did not recognize 100 ng of ovalbumin (control) and the non-immune IgY control did not detect the antigen. The result illustrated that the IgY-dextran conjugate recognized the antigen and this was not compromised by dextran binding to IgY. Dextran did not interfere with recognition of IgY by a secondary antibody.

Table 5.2 Evaluation of the immunoreactivity of IgY-dextran conjugate by dot-ELISA on nitrocellulose

0 – 100 ng of rabbit albumin was blotted on nitrocellulose in triplicate and incubated in IgY-dextran conjugate, immune IgY or non-immune IgY. Rabbit-anti-IgY-HRPO and ABTS substrates were used for the detection step. (+++) dark stained dot; (++) denotes a medium stained dot; (+) denotes a weakly stained dot and (–) denotes no staining.

Reagent	Rabbit Albumin (ng)					Ovalbumin
	100	50	25	10	0	(100 ng)
IgY-dextran	+++	+++	+++	+++	–	–
Immune IgY	+++	+++	+++	+++	–	–
Non-immune IgY	–	–	–	–	–	–

5.6 Discussion

In an effort to develop a dye-based colourimetric assay system, anti-rabbit albumin IgY was cross-linked to high molecular (M_r: 5000 – 40 000 kDa) weight dextran to form an IgY-dextran conjugate. The conjugate was used to detect rabbit albumin (antigen) on nitrocellulose by PAS staining. Thornton *et al.* (1989) reported that up to 50 ng of mucin (a mucus glycoprotein with up to 90% carbohydrate content) could be detected on nitrocellulose using PAS staining. Several reports have indicated the use of protein-dextran conjugates as immunogen in antibody production. Haptenated dextran polymers have been shown to elicit strong B-cell response (Brunswick *et al.,* 1988; Dintzis *et al.,* 1989; Xiao *et al.,* 1995).

In this study, dextran was derivatized to iodoacetyl-dextran via a series of chemical reactions aimed at introducing an iodo-acetyl group on the hydroxyl functional groups of the dextran. The derivatization of dextran was initiated by two fairly common chemical reactions: (carboxylmethyl-modification and amine-modification) that resulted in an aminoethyl-carboxymethyl-derivative of dextran (AECM-dextran). The initial step (carboxy-methyl-modification) was carried out at alkaline pH. The termination of this reaction required neutralization of the reaction medium with NaH_2PO_4. The second step involved amide formation between the carboxylic acid and the diamine, and was carried out in excess diamine in the presence of *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (Inman, 1975; Brunswick *et al.,* 1988; Kim *et al.,* 2003) in order to minimize cross-linking through amide formation at both ends of any given diamine molecule. A shorter version of diamine (ethylenediamine) was preferred to larger α, ω homologues due to the electrostatic interactions between the two amino groups (Inman, 1975). The AECM-dextran was modified to acquire iodoacetyl groups that provided an anchor for attachment of sulfhydryl-derivatized antibodies. In this reaction, the production of yellow colour was an index of favourable iodoacetyl-derivatization of AECM-dextran. The yellow colour was from *p*-nitrophenol, a breakdown product of the *p*-nitrophenyl iodoacetate (Hiratsuka, 1987; Hermanson, 1996). Dialysis was carried out extensively to ensure efficient removal of unwanted products of conjugation reactions.

Anti-rabbit albumin IgY modified to acquire sulfhydryl groups by modification with 2-iminothiolane was conjugated to the iodoacetyl-derivatized dextran by formation of a thiol-ester bond. EDTA prevents thiol-dependent metal-catalyzed oxidation of sulfhydyls to form disulfide linkages (Park and Floyd, 1994; Maier *et al.,* 1996; Netto *et al.,* 1996). EDTA was therefore added to chelate divalent metal cations that might initiate oxidation of SH groups. The ester and amide linkages established in these reactions are stable to chemical and enzymatic breakdown (Inman, 1975). Amine-containing Good's buffers such as Tris-HCl, PIPES, HEPES, and Glycine were avoided in the conjugation reaction so as not to compete with the ε-amine of IgY.

An obvious and less complex method to couple IgY to the dextran would have been via activation of the dextran with cyanogen bromide and subsequent coupling of the activated dextran to IgY via the terminal ε-amine group of lysine residues on the antibody. However, the resulting linkages (isourea, N-substituted imidocarbonate and N-substituted carbamate types) are not very stable (Porath, 1976). Cuatrecasas and Parikh (1972) have reported the spontaneous breakdown of bonds established between cyanogen bromide-activated agarose and amino-functional ligands with a chemical half-life of 40 days.

The series of coupling reactions used in this study to create a covalent linkage between the antibody and the dextran polymer offers advantages, such as reduction in steric hindrance of the antibody and unrestricted movement of the immobilized antibody due to the 13-atom spacer arm between the antibody and the dextran (Cuatrecasas and Anfinsen, 1971; Hannache and Boulanger, 1977; Smith *et al.,* 1991; Deguchi *et al.,* 1999; Weimer *et al.,* 2000; Hernandez *et al.,* 2001). Sephacryl® S-300 size exclusion chromatography column was used to purify the conjugate in order to eliminate any unconjugated IgY in the reaction mixture that may interfere with the assay. To avoid high hydrostatic pressure in the column due to the high viscosity of dextran, the solution to be purified was diluted prior to purification.

It appeared that not all the IgY was conjugated to the dextran (Figure 5.2). Brunswick *et al.* (1988) reported a possible high molar coupling ratio of 17 – 33 antibody molecule per

molecule of 2000 kDa of dextran. The coupling yield of 6:1 protein per dextran molecule may be as a result of the degree of derivatization of the dextran and/or thiol-modification of IgY. The concentration of dextran may be overestimated as a mixture of different sizes of dextran was used due to the high cost of using a more refined reagent.

The strategy was to indirectly detect the antigen on nitrocellulose by the colourimetric detection of dextran in the immune complex using PAS staining. PAS staining involves oxidation of adjacent vicinal diol in the polysaccharide to aldehyde and the subsequent covalent binding of sulfourous-Fuchsin to the aldehyde to form a red complex (Horobin, 1988; Kirkeby *et. al.,* 1992). This implies that the presence of a hydroxyl is involved for PAS staining (Doerner and White, 1990). As the hydroxyl group was the point of derivatization of the dextran, the number of hydroxyl groups could have been reduced due to the modification of the dextran molecule. This might have reduced the sensitivity of the assay. The conjugate detected 10 ng of the antigen on a dot-ELISA format. The high background staining by the PAS reagent made observation of coloured dots on the nitrocellulose quite difficult. The backround was not improved with a number of alterations in the protocol (data not shown). The immunoreactivity (Table 5.2) of the antibody was conserved after the coupling chemistry as the IgY-dextran conjugate detected the rabbit albumin with comparative sensitivity to the immune-IgY. A more sensitive method other than the PAS staining would be required for IgY-dxetarn detection to be comparable with HRPO-coupled antibody.

The pitfalls to this approach are possible interferences by glycoproteins; high background staining by PAS reagent and the time required for the assay to be completed. These pitfalls could be avoided if low carbohydrate content antibodies such as monoclonal antibodies are used as the antibody for coupling. Basic Fuchsin is a hydrophobic dye (Gurr, 1960; www.sigma-aldrich.com) and was not easily destained from nitrocellulose with aqueous and non-aqueous solutions. Nitrocellulose is an ester of cellulose formed from esterification of the hydroxyl groups of cellulose. Therefore, the staining of nitrocellulose by PAS reagent is not due to covalent bond formation as a result of oxidation of the nitrocellulose because of the absence of vicinal diols in nitrocellulose. PAS stained nylon and PVDF

membranes similarly to nitrocellulose. Other methods of staining carbohydrates carried out in this study could not stain dextran on nitrocellulose (data not shown).

To avoid the problems encountered with PAS staining it may be possible to use hyrdrazide-derivatized hydrophilic dyes (with high colour intensity). Oxidation of dextran yields aldehydes at the position of the vicinal diols. These aldehydes could react with the hyrdrazide-derivatized hydrophilic dye to form a covalent linkage (hydrazone) with the dextran-IgY:Ag complex (Figure 5.3). Non-covalently bound dyes could be washed off with a buffer. This approach may lead to a novel staining method for polysaccharides. One of the major advantages of the hydrazine-functional group is that it has a significantly lower p*K* than primary amines (O'Shannessy and Wilchek, 1990; Inman and Dintzis, 1969) and will therefore react with an aldehyde under conditions where primary amines will be protonated and unreactive. The reaction between an aldehyde and a hydrazine is specific and acid-catalyzed. It reaches maximal rate in aqueous solutions at pH 4 (O'Shannessy and Wilchek, 1990). Hydrazine reacts more readily with aldehydes than do primary amines (as in PAS staining) because hydrazine is a stronger nucloephile. A hydrazone (product of condensation between a hydrazine and an aldehyde) is much more stable than Schiff base and does not require stabilization by reduction. The uses of hydrazide-activated enzyme, avidin or streptavidin have been applied in the detection of glycoproteins in solution or cell surfaces and have been used to form conjugates with glycoproteins (O'Shannessy *et al.*, 1987; Bayer and Wilchek, 1990; O'Shannessy and Wilchek, 1990; Bayer *et al.*, 1990).

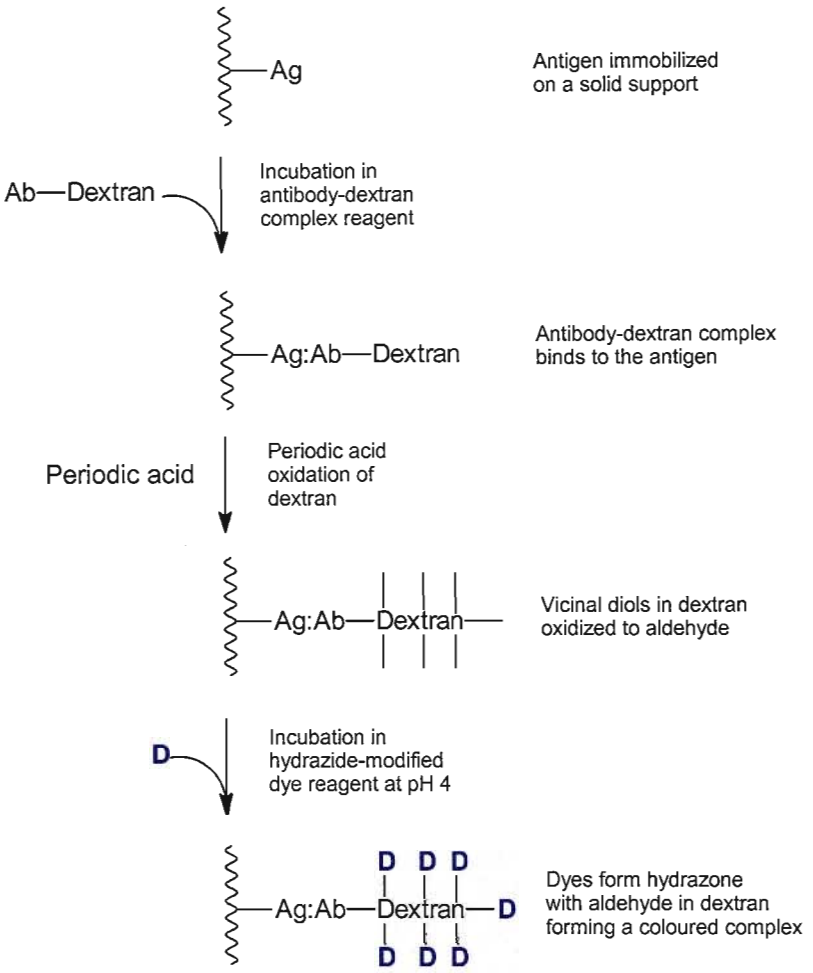

Figure 5.3 **A schematic illustration of a hydrazide-based immunoassay system**
This assay system will utilize antibody-dextran complex specifically stained with a hydrazine-functional derivative of a coloured hydrophilic dye.

CHAPTER 6
GENERAL DISCUSSION

Immunoassay techniques are important aspects of applied natural and medical sciences. These techniques often exploit the chemistry of the specificity of interaction between an antigen and an antibody to quantify or confirm the presence of an antigen or antibody in a biological sample. These techniques are employed daily in hospitals, medical research laboratories, food industries, water purification plants, and vertinary research centers. Over the years, numerous immunodetection (immunoassay) techniques have been developed to meet the growing need for diagnosic methods. One of the most common colourimetric immunodetection techniques is an enzyme-linked immunosorbent assay (Engvall and Perlmann, 1971). This technique involves the use of enzymes that catalyses the modification of colourless substrate into coloured product. The presence of colour, in most cases, confirms the presence of the assayed parameter. In quantitative format, the concentration of the coloured product may be directly or inversely proportional to the assayed parameter.

Other immunoassay techniques involve the use of fluorescent-labelled antibody, gold-labelled antibody, colloidal dye-labelled antibody and liposome-labelled antibody. However, the signal measured in these immunoassay techniques (including ELISA) is an outcome of the collective sum of the reporter labels present in the assay site, including false results from non-specific interactions (Sheldon *et al.,* 2000). Recently, there has been the evolution of more technically advanced immunoassay techniques such as the semi-conductor quantum-dot nanocrystals (QDOT^{TM}) (http://www.qdots.com/live/index.asp) and colloidal silver plasmon-resonant particles (PRPs) (Sheldon *et al.,* 2000). These new immunodetection techniques exploit the chemistry of antibody-antigen and avidin-biotin interactions as well as the physical properties of metals such as Zn, Cd, Se and Ag in the presence of an electromagnetic spectrum. Qdot nanocrystals and PRPs have unique properties that organic fluorescent dyes do not have. The quantum-dot nanocrystals and the PRPs are used in immunofluorescence microscopy, microplate-based analysis, flow cytometry and molecular techniques (Sheldon *et al.,* 2000; Schultz *et al.,* 2000; Dahan *et*

al., 2003; Hanaki *et al.,* 2003; Lingerfelt *et al.,* 2003). The major drawback to these assays is the cost of their production, application, and the technicalities involved in using them.

The applications of visible coloured dyes as antibody labels in the development of a simple diagnostic immunoassay tool has not been widely investigated other than the use of fluorescent dyes as labels in immunofluorescent assays. This study was aimed at the application of visible coloured textile dyes as antibody labels in developing an immunoassay reagent. An alternative strategy involved the covalent immobilisation of coloured dye (Basic Fuchsin) on Dextran coupled to antibody was also investigated. In order to develop these assay systems, the chemistry of the dyes and mechanisms by which dyes interact with proteins was evaluated.

26 dyes of anionic, cationic and ligand dye classes were employed for this work. Only acidic (anionic) dyes stained IgY on nitrocellulose at acidic and neutral pH, but did not stain proteins at alkaline pH (Tables 3.1 and 3.2). Out of the 16 dyes that stained IgY on nitrocellulose, Cibacron Blue, Direct Red 81, and Reactive Black 5 stained proteins in polyacrylamide gels (Figure 3.1) with minimal background staining of the gels. Direct Red 81 stained proteins faster than Coomassie Blue R 250 and with comparable sensitivity (Figures 3.2 and 3.3). The presence of negatively charged functional groups on dyes is needed in order to stain proteins. Anionic dyes used for protein staining in polyacrylamide gels and tissue sections, and in protein quantification interact with positively charged amino acid residues such as lysine and arginine (Fazekas De St. Groth *et al.,* 1963; Chrambach *et al.,* 1979; Wada and Nakamura, 1981; Horobin, 1988; Whaheed *et al.,* 2000; Prentø, 2001).

In this study, Direct Red 81 has proved to be a good alternative to Coomassie Blue R 250 as protein in gel electrophoresis, based on its rate of protein staining (Figure 3.3), staining intensity and the low cost. There is little literature available about this dye. The applications of Direct Red 81 as a nuclear, lipid or carbohydrate stain in histochemistry and histology could be investigated. The chemistry of the mechanism of Direct Red-protein interactions could be investigated. If the interaction between Direct Red 81 and protein could be

reversed, the dye may be applied in dye-affinity chromatographic technique as protein ligands.

Aurintricarboxylic Acid, Ethyl Red and Gallocyanine were chosen as labels in covalent conjugation to IgY because they have CDI-functional targets (COOH and OH). Aurintricarboxylic Acid has numerous applications in Physiology and Biochemistry. It inhibits protein-nucleic acid interaction (Okada and Koizumi, 1995) and stimulates tyrosine phosphorylation in lymphoma cell (Gonzalez *et al.,* 1998). Aurintricarboxylic Acid also protects against cell death caused by lipopolysaccharide in macrophages through decreasing inducible nitric-oxide synthase induction. This protection of macrophages (by Aurintricarboxylic Acid) from lipopolysacharide-mediated cell death is primarily the result of the inhibition of nitric oxide production (Tsi *et al.,* 2002). Gallocyanine is widely used in histology as a nuclear stain in a complex called Gallocyanine-chromalum (Horobin, 1988; Schulte, *et al.,* 1991; Schulte *et al.,* 1992). Ethyl Red is an acid-base indicator (www.sigma-aldrich.com).

Aurintricarboxylic Acid stained IgY on nitrocellulose at acidic pH (methanol-acetic acid solution) (Table 3.1) but not at pH 9.6 (Na_2CO_3-$NaHCO_3$ buffer) or when chemically modified with CDI (data not shown). These dyes were modified by CDI in DMSO and DMF into active imidazole derivatives, resulting in alteration of the colour of the dyes. This was monitored by the absorbance spectrum of the activated dye, using non-activated dyes and dyes lacking CDI-functional targets as controls. The activated dyes were conjugated on IgY at pH 9 and the conjugates were purified by dialysis. SDS-PAGE was used to confirm the covalent attachment of the dyes on IgY. The molar coupling ratio of dye to antibody varied from one dye to another. Gallocyanine gave the best coupling ratio followed by Aurintricarboxylic Acid and Ethyl Red.

The conjugates were used to detect rabbit albumin (antigen) on nitrocellulose, using HRPO-IgY as control immunoassay reagent. Aurintricarboxylic Acid and Gallocyanine detected 50 ng of rabbit albumin, which was 10-folds less sensitive than HRPO-IgY conjugate. Ethyl Red-IgY conjugate produced a feint colour. The low sensitivity of the dye-

IgY conjugates compared to HRPO-IgY lead to the evaluation of their immunoreactivity using competitive ELISA system. The competitive ELISA result showed that the chemistry of the dyes compromised the activity of the antibodies. The presence of more than one CDI-functional target per dye molecule in Aurintricarboxylic Acid and Gallocyanine lead to cross-linking of the antibodies and poor immunoreactivity of the conjugates. The Ethyl Red-IgY conjugate exhibited good immunoreactivity results, which might be as a result of either CDI-mono-functionality or low molar coupling ratio of the dye.

Two questions that may be posed by the results obtained from the covalent conjugation of dye to IgY are the chemistry of the dyes that were used in this study and coupling chemistry used. Aurintricarboxylic Acid and Gallocyanine, with multifunctional CDI target groups, gave a better coupling efficiency but exhibited low immunoreactivity; while Ethyl Red gave very low coupling yield but exhibited good immunoreactivity. Could the low coupling yield of Ethyl Red have resulted from mono-functionality for CDI and as result, the biological properties of IgY were not compromised? These questions may be answered if a dye with reactive groups such as hydrazide is designed for the covalent coupling to antibodies for the purpose of immunodetection. Using dyes designed to have reactive functional groups such as thiocyanate and hydrazide will eliminate the complications encountered in the coupling chemistry. A useful control in this study will be the use of fluorescent dye-labelled antibodies, example FITC-IgY (Hommel *et al.,* 1996; Staak, 1996; Cipolla *et al.,* 2001).

In the alternative assay system, IgY was covalently attached to derivatized dextran polymer. Derivatized-dextran has various biological, immunological and medical applications. As a biomaterial, cross-linked dextran is used in chromatography as a matrix support and as hydrogels (Chiu *et al.,* 2002). Sulfonate and aldehyde-modified dextran matrices are used in chromatography as affinity biosensors. The aldehyde groups on the matrix forms covalent bonds with biomaterials while the sulfonate groups interacts with positively charged biomaterials by electrostatic interaction (Chegel *et al.,* 2002). Dextran-coated activated charcoal is used in the removal of highly reactive membrane-solubilising reagent in a protein mixture prior to immunoassay and it is very useful to a variety of other

techniques that are often affected by the presence of detergents, such as in protein assays (Malhas *et al.,* 2002). Sulfated dextran has been reported to have various biological properties. Sulfated dextran boosts CD36-dependent adhesion of *Plasmodium falciparum* erythrocytes to human microvascular endothelial cells (McCormick *et al.,* 2000), protects antinuclear antibodies against inactivation by acylating agents during protein modification (Samokhin *et al.,* 2001) and enhances cell-type dependent protein transduction in synthetic and naturally occurring protein transduction domains (Mai *et al.,* 2002). Protein-dextran conjugates elicit immunological response when used as immunogens in antibody production. The level of immune response is often dependent on the size of the dextran used in the preparation of the immunogen (Brunswick *et al.,* 1989; Dintzis *et al.,* 1989; Xiao *et al.,* 1995; Ediriwickrema *et al.,* 2000; Kobayashi *et al.,* 2001). Dextran also has wide applications in immunoassays. Antibody and a horseradish peroxidase-labelled dextran backbone allows for multiple detection of a broad spectrum of antigens in frozen section in immunohistochemistry (Kämmerer *et al.,* 2001)

For this study, dextran was derivatized by series of cross-linking reactions that yielded a 13-atom spacer arm between the antibody and the polymer. The presence of covalently immobilized IgY was confirmed by a Bradford protein assay. The molar coupling ratio was estimated to be six IgY molecules per dextran polymer. PAS staining of the dextran in the immune complex (Ag:Ab-dextran) detected 25 ng of rabbit albumin on nitrocellulose. However, enzyme-linked immunostaining of the immune complex revealed that the IgY-dextran could detect 10 ng of the antigen on nitrocellulose. A better approach is a hydrazide-based immunoassay system that could be used to detect aldehydes as described in chapter 5 (Figure 5.3) and thus reduce non-specific staining.

The information derived from this study could provide various routes for dye-protein interactions. The discovery of Direct Red 81 as a potentially excellent protein stain was fortuitous. The prospect of Direct Red 81 in enzyme and protein technology needs to be investigated further. The labelling chemistry used in this study was effective enough to covalently label dye on IgY. However, the coupling may be improved with appropriately designed dyes. Covalently attaching antibodies to synthetic carbohydrate polymers such as

dextran was shown to have a potential application as an immunoassay tool. The dextran polymer did not compromise the biological properties of the antibody. The conclusion derived from this study suggested that antibody-dextran conjugate, as a colourimetric immunoassay tool, appears to have a better potential as immunoassay tool than dye-antibody conjugate. If a more effective means of detecting the dextran polymer could be used, antibody-dextran conjugate could be a cheaper alternative in immunoassay techniques.

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