Chapter 4 Effect of concentration of adsorbate

4.1 Introduction

Sensitivity and reproducibility (Tijssen, 1985; Aldao and Vides, 1990) are two of the most important properties of immunoassay which depends on, among other factors, the concentration of the protein adsorbed onto the solid phase (Pesce et al., 1981; Makela and Peterfy, 1983) and the non-specific binding of the protein components in the biological specimen (Bjerckle et al., 1986; Gripenberg and Kuruki, 1986; Shields and Turner 1986). The appropriate concentration of protein used for immobilisation is critical for the optimisation of the assay (Makela & Peterfy, 1983; Steward & Lew, 1985). Excessive coating of the solid phase with antibody may produce weak protein-protein interaction and thus reduce antigen binding (Pesce et al., 1981) . Thus optimum assay range of antigen (for antibody assay) or antibody (for antigen assay) concentration must be determined to ensure optimal sensitivity. An acceptable method is required to determine these range of concentration. The effect of concentration on an assay performance has been extensively studied using polystyrene and polyvinyl chloride as solid phase (Cantatero et al., 1980; Vogt et al., 1987; Mc Ginlay and Bardsley,1989; Zollinger et al., 1976). The present work is to determine the effect of varying the concentrations of both anti-HBs and HBsAg in the coating solution for HBsAg and anti-HBs assays using WNR as a solid phase.

4.2 Materials and experimental methods

4.2.1 Materials (See 2.2)

4.2.2 Experimental methods

Coating reagent was diluted in 0.02 M PBS, pH 7.4, to the predetermined concentrations to give 260, 130, 65, 32.5 μg/ml for anti-HBs and 1.76, 0.88, 0.44, 0.22 μg/ml for HBsAg. It was then coated on NR tube which had been prewashed five times with 1 ml of 0.1M HCl and five times with 1 ml of deionised water. Immobilisation, blocking and assay procedures were carried out as described in section 2.2.2.

4.2.3 Calculation

(a) Concentration of ^{125}I anti-HBs and HBsAg used

Concentration of ^{125}I anti-HBs and HBsAg = 20 μCi/μg or 1 μCi/ml

1μCi = 1/20μg

1 ml = 1μCi

This gives the concentration of 5×10^{-2} μg/ml

$\therefore$ 200 μl = (1/5) x (1/20 μg)

= 1/100 μg

Concentration of ^{125}I anti-HBs and HBsAg before dilution

= (1/20) μg/ml

= 0.05 μg/ml

(b) *Surface concentration of bound ^{125}I anti-HBs and HBsAg on PP surface at saturation level*

(1) Surface concentration of bound ^{125}I anti-HBs

= mass of ^{125}I anti-HBs / area immobilised

Radius of PP tube = 6 mm

200 µl of solution in PP tube filled up to the semisphere of the tube

Area immobilised $= 2\pi (6)^2$ mm^2

$= 226.19$ mm^2

Percent binding of ^{125}I anti-HBs on PP surface at saturation level = 0.14% (from Table 4.2)

Mass of ^{125}I anti-HBs on PP surface = (0.05/1000) x 200 x (0.14/100) µg

$= 1.40 \times 10^{-5}$ µg

Surface concentration of ^{125}I anti-HBs on PP surface at saturation level

$= 1.40 \times 10^{-5}$ µg / 226.19 mm^2

$= 6.19 \times 10^{-2}$ µg m^{-2}

(2) Surface concentration of bound ^{125}I HBsAg

= mass of ^{125}I HBsAg / area immobilised

Percent binding of ^{125}I anti-HBs on PP surface at saturation level =0.08% (from Table 4.2)

Mass of bound ^{125}I HBsAg = (0.05/1000) x 200 x (0.08/100) μg

= 8.00×10^{-6} μg

Surface concentration of ^{125}I HBsAg on PP surface at saturation level

= 8×10^{-6} μg / 226.19 mm^2

= 3.54×10^{-2} μg m^{-2}

4.3 Results

The percent binding of labelled anti-HBs and HBsAg at different concentrations onto the solid phase are shown in Table 4.1, Table 4.2, Fig. 4.1a & 4.1b. The amount of adsorbed protein by WNR surface was greater than that on PP surface. On both WNR and PP surfaces, saturation was not reached even at concentration up to $5x10^{-2}$ µg/ml of ^{125}I anti-HBs or HBsAg as shown by Fig. 4.1 a &b.

For WNR or PP tubes coated with different concentrations of anti-HBs and blocked with NBCS, after incubation in HBsAg positive sera for two hours, it was found that specific binding of ^{125}I anti-HBs increased with an increase in the concentration of anti-HBs (Table 4.3, 4.4, Fig. 4.2 & 4.3). However, increase in concentration does not affect the non-specific binding significantly on PP surface and only a slight increase in WNR tube (Table 4.3 & 4.4).

Similarly for WNR tube coated with different concentrations of HBsAg and blocked with NBCS, after incubation in anti-HBs positive serum for two hours, it was found that specific binding of ^{125}I HBsAg increased with an increase in concentration of HBsAg (Fig 4.4, Table 4.5). On PP tube, specific binding of ^{125}I HBsAg increased as concentration of HBsAg (in coating solution) increased initially but peaked at concentration about 0.88 µg/ml and it then decreased above the concentration of 1.00 µg/ml (Fig. 4.5 & Table 4.6). Increasing the concentration of HBsAg did not affect the non-specific binding (with or without serum) significantly on both the surfaces (Table 4.5 & 4.6).

Table 4.1 Percent binding of ^{125}I HBsAg and ^{125}I anti-HBs by WNR coated tube without prior immobilisation with HBsAg or anti-HBs and in the absence of serum incubation
WNR coated tube was incubated in 200 µl labelled protein at different concentrations for overnight at 4 °C. The tube was washed and the bound radioactivities were counted. WNR = NR coated tube prewashed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of deionised water.
**The numbers indicated in all the tables are the mean of three determinations together with their standard errors. This applies to all the Tables in the Chapter.

Concentration x 10^{-3} (µg/ml) of ^{125}I HBsAg or ^{125}I anti-HBs	Percent binding of ^{125}I HBsAg **	*Surface concentration of ^{125}I HBsAg (µg/m^2)	Percent binding of ^{125}I anti-HBs **	*Surface concentration of ^{125}I anti-HBs (µg/m^2)
5.00	0.07±0.01	0.39 x 10^{-2}	0.37±0.03	0.016
6.25	0.08±0.02	0.44 x 10^{-2}	0.37±0.03	0.02
7.14	0.15±0.00	0.95 x 10^{-2}	0.77±0.04	0.05
10.00	0.18±0.01	1.60 x 10^{-2}	1.02±0.01	0.09
16.70	0.22±0.02	3.24 x 10^{-2}	1.22±0.10	0.18
25.00	0.26±0.02	5.75 x 10^{-2}	2.23±0.00	0.50
50.00	0.37±0.04	16.36 x 10^{-2}	2.60±0.08	1.15

* As calculated in section 4.2.3

Table 4.2 Percent binding of ^{125}I HBsAg and ^{125}I anti-HBs by uncoated PP tube without prior immobilisation with HBsAg or anti-HBs and in the absence of serum incubation
PP tube incubated in 200 µl labelled protein overnight at 4 °C. The tube was washed and the bound radioactivity were counted.

Concentration x 10^{-3} (µg/ml) of ^{125}I HBsAg or ^{125}I anti-HBs	Percent binding of ^{125}I HBsAg	*Surface concentration of ^{125}I HBsAg (µg/m^2)	Percent binding of ^{125}I anti-HBs	*Surface concentration of ^{125}I anti-HBs (µg/m^2)
5.00	0.03±0.00	0.13 x 10^{-2}	0.05±0.00	0.22x 10^{-2}
6.25	0.02±0.00	0.11 x 10^{-2}	0.03±0..00	0.17 x 10^{-2}
7.14	0.04±0.01	0.13 x 10^{-2}	0.06±0.01	0.38 x 10^{-2}
10.00	0.04±0.01	0.35x 10^{-2}	0.07±0.00	0.62 x 10^{-2}
16.70	0.07±0.00	1.03 x 10^{-2}	0.10±0.01	1.47 x 10^{-2}
25.00	0.08±0.00	1.77 x 10^{-2}	0.13±0.01	2.88 x 10^{-2}
50.00	0.08±0.01	3.54 x 10^{-2}	0.14±0.01	6.19 x 10^{-2}

* As calculated in section 4.2.3

Table 4.3: Effect of immobilising the solid phase with different concentrations of anti-HBs on the binding of HBsAg and ^{125}I anti HBs by WNR surface (in HBsAg assay)

WNR coated tube immobilised with different concentrations of anti-HBs, blocked with 50% NBCS and preincubated with HBsAg positive control serum or HBsAg negative control serum, along with tube without serum preincubation. The solid phase was then allowed to react with ^{125}I anti-HBs. The tube was washed and the bound radioactivities were counted . WNR = NR coated tube prewashed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of deionised water.

	Percent binding				
Concentration (μg/ml)	With HBsAg (Positive serum) (a)	With HBsAg (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
32.50	2.58±0.12	2.04±0.08	2.89±0.18	0.54	1.26
65.00	2.74±0.24	2.12±0.14	2.89±0.01	0.62	1.29
130.00	2.88±0.16	2.22±0.07	2.95±0.16	0.66	1.30
260.00	3.44±0.01	2.25±0.02	2.94±0.05	1.19	1.53

Table 4.4: Effect of immobilising the solid phase with different concentrations of anti-HBs on the binding of HBsAg and ^{125}I anti-HBs by PP surface (in HBsAg assay)

PP tube immobilised with different concentrations of anti-HBs, blocked with 50% NBCS and preincubated with HBsAg positive control serum or HBsAg negative control serum, along with tube without serum preincubation. The solid phase was then allowed to react with ^{125}I anti-HBs. The tube was washed and the bound radioactivities were counted.

	Percent binding				
Concentration (μg/ml)	With HBsAg (Positive serum)(a)	With HBsAg (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
32.50	2.13±0.39	0.16±0.01	0.24±0.01	1.97	13.31
65.00	2.42±0.40	0.23±0.02	0.22±0.03	2.19	10.52
130.00	2.68±0.20	0.22±0.03	0.23±0.03	2.46	12.18
260.00	2.95±0.39	0.19±0.02	0.20±0.03	2.76	15.53

4.5 Effect of immobilising the solid phase with different concentrations of HBsAg on inding of anti-HBs and ^{125}I HBsAg by WNR surface (in anti-HBs assay)

coated tube immobilised with different concentrations of HBsAg, blocked with 50% and preincubated with anti-HBs positive control serum or anti-HBs negative control serum, with tube without serum preincubation. The solid phase was then allowed to react with ^{125}I g. The tube was washed and the bound radioactivities were counted. WNR = NR coated tube shed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of deionised

	Percent binding				
centration ug/ml)	With anti-HBs (Positive serum)(a)	With anti-HBs (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
0.22	0.77±0.10	0.35±0.03	0.40±0.03	0.42	2.20
0.44	1.13±0.09	0.31±0.02	0.41±0.03	0.82	3.65
0.88	2.08±0.37	0.34±0.04	0.42±0.01	1.74	6.12
1.76	2.41±0.12	0.34±0.02	0.43±0.01	2.07	7.09

4.6 Effect of immobilising the solid phase with different concentrations of HBsAg on iding of anti-HBs and ^{125}I HBsAg by PP surface (in anti-HBs assay)

ed tube immobilised with different concentrations of HBsAg, blocked with 50% NBCS incubated with anti-HBs positive control serum or anti-HBs negative control serum, along ube without serum preincubation. The solid phase was then allowed to react with ^{125}I . The tube was washed and the bound radioactivities were counted.

	Percent binding				
ntration g/ml)	With anti-HBs (Positive serum)(a)	With anti-HBs (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
22	6.18±0.53	0.11±0.10	0.06±0.01	6.07	56.18
44	7.29±0.51	0.11±0.11	0.06±0.00	7.18	66.27
88	7.66±0.33	0.12±0.12	0.10±0.01	7.54	63.83
76	5.35±0.06	0.09±0.09	0.09±0.02	5.26	59.44

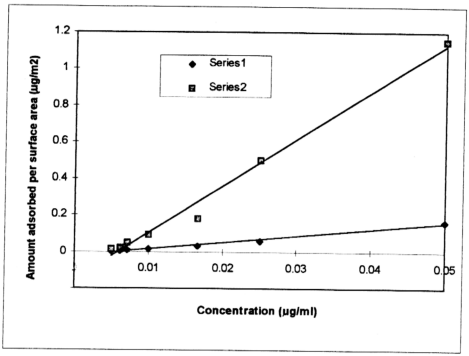


Fig. 4.1a Adsorption isotherm of ^{125}I HBs Ag and ^{125}I anti-HBs by WNR coated tube without prior immobilisation with HBsAg or anti-HBs and in the absence of serum incubation

WNR coated incubated in 200 µl labelled protein overnight at 4 °C. The tube was washed and the bound radioactivities were counted. WNR = NR coated tube prewashed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of deionised water.

Series 1 - ^{125}I HBsAg

Series 2 - ^{125}I anti-HBs

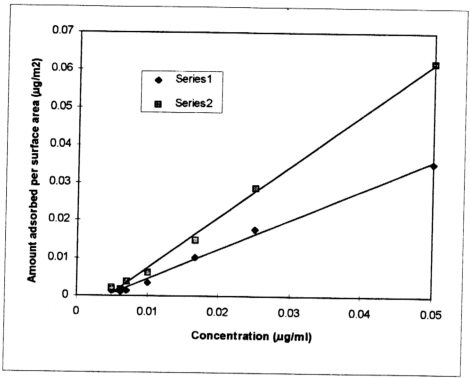


Fig. 4.1b Adsorption isotherm of ^{125}I HBsAg and ^{125}I anti-HBs by PP tube without prior immobilisation with HBsAg or anti-HBs and in the absence of serum incubation

PP tube incubated in 200 µl labelled protein overnight at 4 °C. The tube was washed and the bound radioactivities were counted. Series 1 - ^{125}I HBsAg Series 2 - ^{125}I anti-HBs

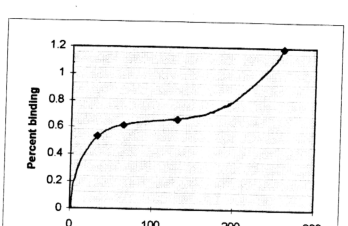


Fig. 4.2 Effect of different concentrations of anti-HBs in coating solution on specific binding of ^{125}I anti-HBs by WNR tube in the presence of HBsAg positive and negative serum (in HBsAg assay)

WNR coated tube immobilised with different concentrations of anti-HBs, blocked with 50% NBCS then preincubated with HBsAg positive control serum or HBsAg negative control serum before ^{125}I anti-HBs was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in the presence of positive control serum - percent binding in the presence of negative control serum . WNR = NR coated tube prewashed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of deionised water.

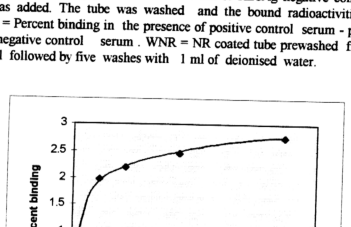


Fig. 4.3 Effect of different concentrations of anti-HBs in coating solution on specific binding of ^{125}I anti-HBs by PP tube in the presence of HBsAg positive and negative serum (in HBsAg assay)

PP tube immobilised with different concentrations of anti-HBs, blocked with 50% NBCS then preincubated with HBsAg negative control serum or without serum preincubation before ^{125}I anti-HBs was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in positive control serum - percent binding in negative control negative serum .

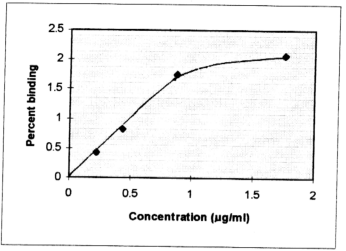


Fig. 4.4 Effect of different concentrations of HBsAg on specific binding of ^{125}I HBsAg by WNR tube in the presence of anti-HBs positive and negative serum (in anti-HBs assay)
WNR coated tube immobilised with different concentrations of HBsAg, blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti-HBs negative control serum before ^{125}I HBsAg was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in the presence of positive control serum - percent binding in the presence of negative control serum . WNR = NR coated tube prewashed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of deionised water.

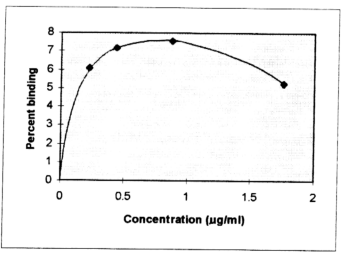


Fig. 4.5 Effect of different concentrations of HBsAg on specific binding of ^{125}I HBsAg by PP tube in the presence of anti-HBs positive or negative serum (in anti-HBs assay).
PP tube immobilised with different concentrations of HBsAg, blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti-HBs negative control serum before ^{125}I HBsAg was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in the presence of positive control serum - percent binding in the presence of negative control serum .

4.4 Discussion

The experiments showed the binding characteristics of ^{125}I HBsAg and ^{125}I anti-HBs by the solid-phase (PP & WNR) respectively. Figure 4.1a & b illustrate the relationship between the surface concentration of labelled macromolecules versus the concentration of the reacting labelled macromolecules (^{125}I anti HBs and ^{125}I HBsAg) in the medium. The results showed that immobilisation of the labelled ligand onto the solid phase increased with increasing concentration of the labelled ligand used as coating solution. The percent binding of ^{125}I anti-HBs and ^{125}I HBsAg with concentration of 0.05 μg/ml by PP surface reached a value of 0.08 and 0.14, but for NR surface, the percent binding of both ^{125}I anti-HBs and ^{125}I HBsAg with concentration of 0.05 μg/ml were 2.60% and 0.37%. Calculated surface concentration of bound ^{125}I anti-HBs and ^{125}I HBsAg were approximately 6.2×10^{-2} μg m^{-2} and 3.5×10^{-2} μg m^{-2} respectively on PP surface when immobilised with coating solution of ^{125}I anti-HBs and ^{125}I HBsAg at concentration of 50×10^{-3} μg/ml. Table 4.7 shows the plateau values of IgG adsorbed on different surfaces determined using different analytical methods from the literature. In general adsorption plateau of IgG ranges from about 2 to 60 mg m^{-2}. The very low adsorption obtained in this study was possibly due to the low concentration of labelled ligands used in the experiments (0.05 μg/ml) (200 to 6000 times lower). Monolayer adsorption was unlikely to be achieved after overnight

incubation of labelled proteins at 0.05 μg/ml. Table 4.8 showed some examples on adsorption values plateau and concentrations of different proteins used for immobilisation.

Table 4.7 Plateau values of adsorption of IgG on different surfaces.
[Different analytical methods were used (Lowry, UV 280 nm, radiolabelled protein, BCA-bicinchoninic, etc.). More experimental details can be found in the references]

Solid phase – Immunoglobulin	Adsorption plateau $\Gamma_{pl,\,max}$ (mg m^{-2})	Conditions	Reference
Latex (-PS) - BIgG	5.5-15, two plateaus	pH 7.4, 5 mM	Fair & Jamieson, 1980
Latex (-PVT) - RIgG	7.5	pH 7.8, 2 mM KNO_3	Bagchi & Birnbaum, 1981
Latex (+ and -PS) - Mab	6-7	pH5-6, 5 mM NaH_2PO_4	Elgersma et al., 1991
Latex (-PS) - BIgG	5.5	pH 7.4, 150 mM NaCl	Bale et al., 1989
Latex (-PS/PAA) - BIgG	8.0	pH 7.4, 150 mM NaCl	Bale et al., 1989
Latex (-PS/PMAA) - BIgG	4.0	pH 7.4, 150 mM NaCl	Bale et al., 1989
Latex (-PS/PHEA) - BIgG	1.5	pH 7.4, 150 mM	Bale et al., 1989
Latex (-PS) - (Mabs) and RIgG	5-6	pH 7, 2 mM phosphate	Serra et al., 1992
Latex (-PS) - BIgG -P(S/HEMA) - BIgG -P(S/MAA) - BIgG -P(MAA/HEMA-BIgG)- Silica - BIgG	7.0 6.8 10.0 3.0 6.0	pH 7, 10 mM pH 8.5, 10 mM pH 7, 10 mM pH 7, 10 mM pH 8, 10 mM	Kondo et al., 1991
- PS - BIgG	5.8	pH 7.4, 150 mM	Bale et al., 1988
-PS- HIgG PEO (11%)-PS 3 - HIgG PEO (40%)-PS 2 - HIgG PEO (60%)-PS 1 - HIgG	2.7 2.25 2.0 1.75	pH 7.4, 150 mM PBS	Grainger et al., 1988
Polyesterterepthalate (PET) - HIgG Colloidal hematite - IgG	2.62±0.36 18.5	PBS	Tang et al., 1993
Colloidal chromium hydroxide - IgG	10.6	pH 7, 10 mM $NaNO_3$	Johnson & Matijevic, 1992
-PS - RIgG	7-8	pH 6, 2 mM	Martin et al., 1992
-PS - RIgG	6.5	pH 6, 2 mM	Galisteo et al., 1994

Solid phase - Immunoglobulin	Adsorption plateau $\Gamma_{pl,\,max}$ (mg m^{-2})	Conditions	Reference
-PS - RIgG	5.8	pH 6, 2 mM	Galisteo et al, 1994
Polymer films Polystyrene- IgG Polyethylene - IgG Polydimethysiloxane - IgG Teflon FEP -IgG	 7 10 18 0	pH 7.4	Brash & Lyman, 1969
Glass beads - HIgG	0.2-0.3	Tris buffer, pH 7.35	Cornelius et al., 1992
PEO/PPO block-copolymers - RIgG	1.5-3.25	Temperature 20-50°C	Tiberg et al., 1992
Poly(n - alkylmethacrylates) - RIgG	0.0545-5.45	PBS	van Damme et al., 1991
Polymer surface Polyelectrolyte complexes - IgG Acrylic copolymers - IgG Polymers monolayer - IgG Crystalline polymers - IgG Polyacetals - IgG	 17-60 6-12 0.51-0.96 0.66-0.99 0.38 - 0.54	pH 7.2, 150mM NaCl	Lebedeva et al., 1991
PS - coated silicon wafers - Mab	2.75	pH 5.5, 5mM phosphate	Elgersma et al.,1992
Nylon balls - IgG Soda-lime glass - IgG Fused silica glass - IgG	14.7± 0.6 1.6± 0.3 2.4 ±0.3	pH 9.5, 100 mM	Plant et al, 1991
Silica derivative - IgG	From (0.11±0.04) to (0.73±0.04)	pH 7.5, tris buffer 50 mM	Oscarsson, 1994
Polymer films - HIgG	0.2-5.3	PBS, pH 7.4	Rabinow et al, 1994
Polymer biomaterials: PS- BIgG PHEMA -BIgG	 5.8±0.3 from 0.3 to 6.0	pH 7.4, 10 mM	Tashiro et al, 1990
PS latex - radiolabelled IgG	5.4±0.2	Phosphate pH 7.4	Ball et al, 1994
PS latices - MAbs	0.2-1.8	Phosphate pH 7.4	Lichtenbelt, 1993
PS - latex - HIgG: radioactivity UV spectroscopy	 7.4 (non-plateau) 4.0±0.1	PBS, pH 7.35	Lensen, 1984

PS - Polystyrene
PVT - Polyvinyl toluene
PAA Polyacrylic acid
PMAA - Polymehtacrylic acid
PHEA - Poly(2-hydroxyethyl acrylate)
PHEMA - Poly(2-hydroxyethylmeth acrylate)
Mab - monoclonal antibody

PEO - Polyethylene oxide
PET - Polyestertereplhalate
PPO - Polypropylene oxide
IgG - Immunoglobulin
BIgG -Bovine IgG
RIgG - rabbit IgG
HIgG - Human IgG

Table 4.8 Plateau values of adsorption of IgG on different surfaces in immobilisation studies

Substrate	IgG	Concentration of solution (μg/ml)	Adsorption plateau mg/m^2	Conditions	Reference
PS*	RgG	10	6.50	pH 6, 2 mmol dm^{-3}, Phosphate buffer	Galisteo et al., 1994
PS^-	IgG 1B	300	4.00	pH 5, 5 mmol dm^{-3}, Acetic buffer	Buijs. J et al., 1995
PS^+	IgG 1B	350	2.00	pH 6, 5 mmol dm^{-3}, Phosphate buffer	Buijs. J et al., 1995
Silica	IgG	300	0.44	pH 7.5, 0.05 mmol dm^{-3} Tris buffer	Oscarsson, 1994

PS* : Polystyrene plates

PS^- : negatively charged PS

PS^+ : positively charged PS

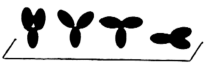
IgG 1B : mouse-anti hCG (human Chorionic Gonadotropin) from isotype IgG -1

The saturation levels of various sorbents were influenced by spatial distribution, lateral interaction, conformation and orientation of the proteins at the sorbent surface. Crystallographic studies of immunoglobulin have revealed its molecular flexibility. This kind of flexibility is expected to facilitate the formation of antibody-antigen complexes. The Fab and Fc fragments are relatively compact, however the IgG molecule as a whole is not compact. [Its scattering curves are anomalous and the radii of gyration of the whole molecule are larger than expected for overall close packing of regions (Sarma & Siverton, 1971))]. This segmental flexibility could be explained by the variation in dimensions of the immunoglobulin G molecule. The distance between binding sites of an elongated antibody in crystalline state is 12 nm which can expand to reach 25 nm in aqueous phase (end-to-end distance in solution) (Silverton et al., 1977; Sarma et al., 1971). This segmental flexibility could be the

reason for poor agreement among the IgG adsorption data obtained by different authors. Also the area per molecule depends on the configuration of the IgG at the solid-liquid interface. The projected area in an end-on configuration is 20 nm^2, whereas that for side-on is 103 nm^2 (Brash & Lyman, 1969). A monolayer of side-on immunoglobulin G was reported to correspond to an adsorbed amount of about 3 mg m^{-2}, while a monolayer of end-on immunoglobulin G corresponded to approximately 15 mg m^{-2} (Merrill et al., 1986). For the three different monoclonal antibodies (IgG 6.5, IgG-7.8 and IgG-9.0) of 156 kDa and diffusivity of 3.84x 10^{-7} cm^2 s^{-1}, Young et al. (1988) found that a monolayer of these antibodies was equivalent to 6.3-7.9 mg m^{-2} for the acid monoclonal antibody, and 4.0-4.4 mg m^{-2} for the other basic antibodies. These values were obtained by adsorption of Mab (monoclonal antibodies) on polyether urethane urea, polyvinyl chloride, silicone rubber, and polyethylene. These authors gave IgG's dimensions as 23.5 nm x 4.4 nm (Doolittle et al., 1978). Buijs. et al. (1995) obtained the dimensions of proteins from the structure of crystallised Fc fragments (Deisenhofer, 1981) and F(ab) fragments (Marquart et al., 1980) to be 7.0 x 6.3 x 3.1 nm^3 and 8.2 x 5.0 x 3.8 nm^3 respectively. Based on these dimensions, the amount adsorbed were calculated for some orientations of the IgG shown in Fig. 4.6.

From SEM micrographs, the average area occupied by each cluster of polyclonal anti-HBs and HBsAg at 260 and 1.76 μg/ml after overnight incubation was 3.85 μm^2 and 13.19 μm^2 on WNR surface (The surface area of the different shapes of immobilised protein molecules was calculated manually using SEM

Fig. 4.6 Schematic drawing and calculated adsorbed amount of some orientations of the adsorbed IgG . A, B and C show that the proteins are adsorbed end-on, whereas orientation D is for side-on. The adsorbed amounts were computed from the dimension of fragments and assuming closed-packed monolayers (Buijs et al., 1995)



Orientation	A	B	C	D
Γ (mgm^{-2})	5.5	3.7	2.6	2.0

micrographs at 2 x 10^3 magnification), implying 13.79% & 31.35% of the surface were occupied. Thus we presumed that labelled macromolecules with concentration of 0.05 μg/ml was not the saturated concentration required for optimal adsorption. Therefore by increasing the concentration of labelled protein, percent binding could be increased.

As the concentration of anti-HBs coated on the PP tube increased, specific binding of ^{125}I anti-HBs increased in the beginning and then it reached a plateau value (Fig. 4.2, Fig. 4.3). However a further increase in adsorption after the first plateau value was observed on WNR surface. From SEM micrographs, adsorption of anti-HBs on WNR surface at concentration of 260 μg/ml occupied a surface area of 13.79%. The specific binding of ^{125}I anti-HBs increased from 0.54 to 1.19 % on WNR surface and from 1.97 to 2.76% on PP surface (Table 4.3 & 4.4). Specific binding was enhanced as antigen-antibody binding increased because of increased concentration of immobilised binders. Anti-HBs concentration of 260 μg/ml was required to give optimal binding on WNR surface (Table 4.3) with the corresponding increase in non-specific binding in the presence of negative serum (Table 4.3). A concentration of 260 μg/ml anti-HBs gave the highest specific binding in the concentration range of anti-HBs tested in this experiment. Further increase in the anti-HBs concentration used may give rise to further increase in NSB (in the presence of negative control serum) and also will contribute to additional cost to the production of immunoassay procedure using the NR solid phase. Based on these consideration we decided to further study immobilisation process using

anti-serum at a concentration of 260 μg/ml to optimise the assay specificity and sensitivity, without further increase in the concentration of the anti-HBs in the coating solution. Further immobilisation of anti-HBs on PP surface showed that the specific binding of ^{125}I anti-HBs in the presence of HBsAg positive sera reached an optimal level when coating solution of 260 μg/ml anti-HBs was used. For the WNR surface, the highest specific binding of ^{125}I anti-HBs was not reached even at 260μg/ml anti-HBs (Fig 4.3 & Fig 4.4). However a concentration of 260μg/ml of anti-HBs or HBsAg was used for coating the solid phases (PP & WNR) in all the experiments so that a comparison between PP and WNR solid phases can be made.

Fig. 4.4 & 4.5 show that specific binding of ^{125}I HBsAg on both PP and NR surfaces immobilised with HBsAg and reacted with anti-HBs positive sera increased as the concentration of antigen in the coating solution increased. The specific percent binding of ^{125}I HBsAg increase from 0.42 to 2.07% on WNR surface and from 6.07 to 7.54 percent on PP surface (Table 4.5 & 4.6). However, on PP surface the percent binding reached a maximum level (when coating solution of HBsAg at 0.44-0.88 μg/ml was used) and then decreased gradually, whereas the WNR surface showed a steady increase in specific binding until a plateau values was reached at 1.76 μg/ml HBsAg. Thus a concentration of 1.76 μg/ml HBsAg was selected for anti-HBs assay (in the presence of anti-HBsAg positive and negative control sera) because at this concentration the assay system gave the highest percentage binding of ^{125}I anti-HBs [in the presence of anti-HBs positive sera]. Although at this concentration the PP surface immobilised with HBsAg gave a

slightly lower binding of ^{125}I anti-HBs, it was decided to use this concentration because for ease of comparison between the PP and WNR surfaces, and also for the optimum use of assay material system based on WNR surface.

An equilibrium concentration of 0.88 μg/ml HBsAg was needed to give optimal specific binding of ^{125}I HBsAg by anti-HBs on HBsAg coated PP surface. The following deductions were made about the binding sites of HBsAg on the polypropylene tubes.

SEM miocrograghs (Fig. 2.10 a & 2.10 b) show that the PP surface was fully covered by HBsAg. The micrographs showed that the thin layer could be due to immobilisation of multilayer of proteins. In addition, nucleation of a large number of islands of protein superimposing on thin layer coverage of HBsAg was observed. The nucleation of high density island on thin layer coverage of HBsAg could be due to the self assembly arrangement of HBsAg molecules on the solid phase. The thin layer of adsorbed HBsAg could be side-on adsorption while nucleation of these islands could probability due to end-on plus side-on adsorption.

In summary the possible mechanism of adsorption of HBsAg on PP surface consisted of a layer of (a) HBsAg immobilised on the solid surface in side-on configuration, (b) a second layer of HBsAg adsorbed on top of the adsorbed layers of HBsAg via protein-protein association, (c) HBsAg on the solid surface in an end-on manner. The apparent protein-protein association beyond the saturation level and end-on protein adsorption can lead to a loss of antibody-antigen interaction

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during the various steps in the assay resulting in the dissociation of the bound protein (for evidence of dissociation see Chapter 7). When anti-HBs control serum was added, anti-HBs macromolecules formed complex either with HBsAg immobilised on PP surface or with HBsAg which was loosely bound (which could be dissociated) on the PP surface. Competition between the binding of anti-HBs to the coated HBsAg or loosely bound HBsAg in the solution could occur. This would reduce the specific binding of ^{125}I HBsAg. Arrangement of (b) and (c)(vide supra) could contribute to the microscopic island as observed (AFM or SEM) on PP surface immobilised with HBsAg (Fig. 2.10 a & 2.10 b).

4.5 Conclusion

The optimum concentration of HBsAg for adsorption on PP tube was 0.88 μg/ml, however higher concentration of 1.76 μg/ml was required to saturate the WNR surface. In the case of PP surface immobilised with anti-HBs, the optimal concentration was about 260 μg/ml, but higher anti-HBs concentration would be required to increase further the surface binding of ^{125}I anti-HBs by WNR surface immobilised with anti-HBs with a concomitant increase in the non-specific binding. For the various reasons outlined above (see page 186-187) concentration of 260 μg/ml was used for anti-HBs and 1.76 μg/ml for HBsAg in the subsequent experiments.

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