Chapter 3 Effect of acid, PBS, water washes and trypsinisation on NR coated tubes

3.1 Introduction

The charge of proteins and the pH of buffer are known to affect the coating of protein on solid-phase (Geerligs, 1988). pH can affect the plateau value of the adsorbed mass at hydrophilic or hydrophobic surfaces (Kondo, 1991) and exposure of proteins to a low pH environment prior to immobilisation on silica surfaces can result in an increase in activity of the immobilised proteins. Similar observations have also been reported by others (Ishikawa et al., 1980; Conradie et al., 1983). In this experiment labelled macroproteins were immobilised at different pH's to the solid phase of interest to determine the optimal pH required for protein immobilisation.

NR latex is a dispersion of latex particles, containing also very small amounts of inorganic and organic impurities (see section 1.6). To improve the sensitivity of assay using NR as a solid phase, one has to reduce the non-specific binding of the solid phase. As discussed in Chapter 2, non-rubber constituents in NR latex could be one of the causes that reduced the sensitivity of the assay. In this Chapter experiments were designed to remove the non-rubber substances from NR surface to produce a cleaner surface with lower non-specific binding. The experiments were

designed firstly to examine the effect of washing NR coated surface prior to immobilisation. 0.1M HCl, PBS and water were used as washing reagents in the experiment. The main aim was to reduce the pH of the NR solid phase and to remove the soluble non-rubber substances from the surface, including residue ammonia from the original latex which may affect the ionisation of the protein at the solid/liquid interface and thus protein adsorption. Further experiments were carried out to investigate the effect of NR latex proteins on the sensitivity of the assay by trypsinisation of the NR surface before immobilised with antibody/antigen protein.

3.2 Materials and experimental methods

3.2.1 Materials

Trypsin was purchased from Hopkin & William. Other reagents were as listed in section 2.2. Trypsin solution was prepared by dissolving 4g of trypsin in 100 ml of PBS solution (pH 7.4). The trypsin solution was then filtered through standard-grade filter paper before use.

3.2.2 Experimental methods

(a.) Binding of ^{125}I anti-HBs & ^{125}I HBsAg on NR surface at different pH

Equal volumes of labelled protein and buffer were mixed, 200 µl of the labelled mixture were then incubated WNR coated tube or PP tube overnight at 4°C. The radioactivity was then determined using a Gamma Autologic Counter before and

after washes (4 times with 1 ml of distilled water each). The buffer solution used were citrate/phosphate buffer at pH 5.4, phosphate buffer at pH 7.4 and carbonate buffer at pH 9.6. The salt concentration was calculated to give a final concentration of 0.16 mol dm^{-3} Na^{+}.

(b.) Modification on NR coated tube

1.) Acid-wash followed by water-wash

1 ml of 0.1M HCl was dispensed into each NR coated tube which was shaken gently and the content was decanted. This was repeated according to the number of times of acid wash required in the experiment. After the acid wash, the tube was washed with 1 ml of distilled water according to the number of times of water required in the experiment. After the water wash, the tube was inverted and tapped dry on a clean absorbent paper to dislodge any excess liquid. The modified NR coated tube was then immobilised with HBsAg (1.76 μg/ml) or anti-HBs (260 μg/ml) or anti-T4 (1107 μg/ml), blocked with 50% NBCS. Binding studies and assays procedure were carried out according to the protocol described in section 2.2.

2.) Phosphate buffer saline solution-wash followed by water-wash

Each tube was washed with PBS solution (pH 7.4) followed by distilled water wash as in section 3.2.2b(1). The modified NR coated tube was then immobilised with

HBsAg, anti-HBs or anti-T4, blocked with 50% NBCS and assayed as described in section 2.2.2.

3.)Trypsinisation of NR coated tube

Each NR coated tube which has been previously washed with 0.1M HCl and water (five times each) was incubated with 200 µl of trypsin solution for five hours at 37°C or 26°C respectively. The solution was discarded and the tube was washed four times with 1 ml of deionised water. The trypsinised NR surface was then immobilised with HBsAg, anti-HBs or anti-T4 was immobilised on the modified NR coated tube, the tube blocked with NBCS and assayed as described in section 2.2.

4.) Irradiation of HA latex (see appendix, pg344)

(c.) Stability studies

1.) Stability studies of NR coated tube

To study the stability of the NR coated tube, NR solid phase prepared was stored at room temperature (26°C) for 9 months. The tube was then washed five times with 1 ml 0.1M HCl and followed by five water washes before immobilisation of protein (see section 2.2.2)

2.) Stability studies of NR coated tube immobilised with anti-HBs and HBsAg

NR solid phase was prepared and stored at room temperature for 1 week. The tube was washed five times with 1 ml 0.1M HCl followed by five times 1 ml distilled water washes. Anti-HBs or HBsAg was then immobilised on the tube, the tube blocked with 50% NBCS as described in section 2.2.2. To study the stability of the

macromolecules immobilised on NR tube, the tubes were then stored at 4°C up to three months before assay. PP tubes containing immobilised macromolecules with the macromolecules were also set up as control (No washing process). Assay procedures were as carried out in section 2.2.2

3.3 Results

(a) *Binding of ^{125}I anti-HBs & ^{125}I HBsAg on WNR surface at different pH*

The percent bindings of both ^{125}I anti-HBs and HBsAg on PP and WNR surface were highest at physiological pH (pH 7.4) (Table 3.1a & 3.1b). The same trend was observed for both labelled protein. Percent binding was increased from pH 5.4 to 7.4 and decreased when pH was raised to 9.6 (Table 3.1a & 3.1b) . The optimal pH for protein immobilisation was found to be 7.4.

(b) Effect of acid, PBS and water washes

After washing of the NR coated surface with 0.1M hydrochloric acid followed by water washes, the specific (a) to non-specific (b) binding ratio (a/b) for positive and negative control sera respectively was improved slightly in HBsAg assay (using anti-HBs coated NR surface)(Table 3.3a). (This parameter was calculated as ratio of the bound radioactivity counts per minute of the positive control serum specimen to that of the negative control serum). Similarly a two-fold improvement in specific (a) to non-specific (b) binding ratio (a/b) for positive

and negative control sera respectively was observed for anti-HBs assay using HBsAg coated NR surface (Table 3.3 b). Optimal binding can be achieved by washing the coated surface 10 times with acid and water in HBsAg and anti-HBs assays (Fig. 3.1& 3.2). Decrease in specific binding was observed on both HBsAg and anti-HBs assays when the NR surface was washed more than 10 times with acid washes (Fig. 3.1 & 3.2). The non-specific binding of ^{125}I anti-HBs or ^{125}I HBsAg by NR surface immobilised with anti-HBs or HBsAg (without serum) was slightly reduced by increasing the number of acid wash followed by water wash (Fig. 3.3, Fig. 3.4). However the specific binding was improved significantly (Fig. 3.1, Fig. 3.2).

It was observed that washing the NR coated tube with PBS and water improved the a/b ratio in both anti-HBs and HBsAg assays. However the a/b ratio can be improved further after ten PBS washes. The a/b ratio obtained after 15 times PBS washes, was comparable to 3 times 0.1 M HCl wash followed by 3 water washes for HBsAg and anti-HBs assays (Table 3.4a & b). PBS washes reduced the overall non-specific binding (without serum)(Fig. 3.3, Fig. 3.4). However, the non-specific binding decreased gradually with increasing number of acid washes whereas the non-specific binding showed an increase trend after an initial decrease with increasing number of PBS washes (without serum) (Fig. 3.3, 3.4). The specific binding improved as the number of PBS washes increased (Fig. 3.1&3.2).

Similarly for T4 competitive binder ligand assay, NR latex surface which had been washed with acid followed by water give higher percent binding of labelled T4

than that of unwashed NR surface (Fig. 3.5), giving two parallel displacement curves with different percentage bindings of labelled T4 in the presence of unlabelled T4 (of similar concentration).

(c) Effect of trypsinisation

The experiments showed no increase in percent binding of labelled anti-HBs and labelled HBsAg by trypsinised NR surface when compared with non-trypsinised NR surface (Table 3.5a). Trypsinisation increased the non-specific binding of labelled anti-HBs on solid phases immobilised with anti-HBs (without serum incubation) (Table 3.5b). Specific binding of labelled anti-HBs was improved after trypsinisation (Table 3.5b, Fig. 3.6). Trypsinisation at 37°C and 26 °C gave percent specific binding (a-b) of 1.37, and 1.93 respectively. Incubation of the solid phase with PBS at 37°C and 26°C after acid washes gave a percent specific binding of 1.28 and 1.42 respectively. This gave an increment of 0.33% and 0.89% of specific binding after trypsinisation at 37 °C and 26 °C respectively. However the non-specific binding of ^{125}I anti-HBs by trypsinised and non-trypsinised NR surface after preincubation with negative control serum was reduced significantly compared to the non-specific binding without serum preincubation (Fig. 3.8).

The treated NR solid phase, immobilised with HBsAg showed a different binding characteristic. The best result was obtained when the NR solid phase was treated with acid wash, water wash and incubated with 200 µl of PBS prior to immobilisation with HBsAg (Fig. 3.7). The specific binding of NR surface without

trypsinisation (but with acid and water washes and preincubation with PBS at 37°C and 26 °C were 2.99 and 3.11 respectively (Table 3.5c). NR surface trypsinised at 37°C and 26°C, washed with deionised water prior to immobilisation with HBsAg gave specific binding of 2.42 and 1.84 respectively, which was 0.57 and 1.27 less than that of non-trypsinised NR solid phase but washed with acid and water and preincubated with PBS (Table 3.5(c)). Washing the NR solid phase with acid and water only prior to immobilisation with HBsAg gave the lowest specific binding of 1.28. In the presence of serum, non-specific binding for the trypsinised and non-trypsinised NR surfaces when compared with that of the modified NR surfaces was slightly decreased without serum preincubation (Fig. 3.9).

(d) Stability studies

Experiment on dried NR coated tubes stored at 26°C showed that NR coated tube (for HBsAg assay) can be stored for up to at least six months without significant changes in specific binding (a-b) (Table 3.7). Tables 3.7(a) &(b) showed that there was a slight decrease in specific binding for anti-HBs assay after 9 months' storage. The non-specific bindings (without serum preincubation) increased with the duration of storage. Both anti-HBs or HBsAg-immobilised NR/PP tubes showed no significant change in specific and non-specific bindings for both anti-HBs and HBsAg results after 3 months of storage at 4°C (Table 3.8 a-d), except PP tube (for HBsAg assay) which showed a slight increase in non-specific binding with or without preincubation with negative serum.

Table 3.1(a) Percent binding of ^{125}I HBsAg and ^{125}I anti-HBs at different pH on NR coated surface

NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water, it was then incubated with ^{125}I labelled HBsAg or ^{125}I labelled anti-HBs overnight at 4°C. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled 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.

pH	**Percent binding of ^{125}I HBsAg	**Percent binding of ^{125}I anti-HBs
5.4	0.135±0.02	1.12±0.01
7.4	0.185±0.01	1.30±0.02
9.6	0.125±0.03	0.79±0.02

Table 3.1(b) Percent binding of ^{125}I HBs Ag and ^{125}I anti-HBs at different pH on PP surface

PP tube incubated with ^{125}I labelled HBsAg or ^{125}I labelled anti-HBs overnight at 4°C. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

pH	Percent binding of ^{125}I HBsAg	Percent binding of ^{125}I anti-HBs
5.4	0.02±0.00	0.07±0.01
7.4	0.04±0.01	0.09±0.01
9.6	0.02±0.00	0.06±0.01

Table 3.2 Percent binding of ^{125}I HBsAg and ^{125}I anti-HBs by NR surface after acid, PBS washes

(a) NR coated tube was first washed five times with 1 ml of 0.1M HCl (or 0.02M PBS) followed by five washes with 1 ml of distilled water. It was then incubated with ^{125}I labelled HBsAg or anti-HBs overnight at 4°C. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

Solid phase	Percent binding of ^{125}I HBsAg	Percent binding of ^{125}I anti-HBs
Untreated NR	0.30±0.03	2.70±0.06
NR washed 5x HCl&H_2O	0.37±0.02	2.60±0.08
NR washed 5x PBS&H_2O	0.37±0.02	2.75±0.19

(b) Percent binding of ^{125}I HBsAg and ^{125}I anti-HBs on different solid phases. 200 µl of each labelled macromolecules were incubated overnight with the solid phase at 4° C. The tube was then washed with 1 ml of distilled water according to the number of times required. (* Tube incubated overnight with 200 µl of distilled water before washing) The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

(i) On WNR coated tube (WNR = NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water.)

	Percent binding		Percentage of macromolecule remaining after washing (after 5th wash = 100%)	
No of washes	^{125}I anti-HBs	^{125}I HBsAg	^{125}I anti-HBs	^{125}I HBsAg
5	2.60±0.02	0.37±0.02	100.00	100.00
10	2.49±0.10	0.37±0.00	95.77	100.00
15	2.43±0.10	0.37±0.02	93.46	100.00
20	2.40±0.20	0.37±0.02	92.30	100.00
25*	2.25±0.02	0.34±0.02	86.60	91.89
30*	2.18±0.10	0.36±0.01	83.85	97.30

(ii) On PP tube (Without PBS and acid wash)

	Percent binding		Percentage of macromolecule remained after washing (after 5th wash = 100%)	
No of washes	^{125}I anti-HBs	^{125}I HBsAg	^{125}I anti-HBs	^{125}I HBsAg
5	0.18±0.01	0.080±0.01	100	100.00
10	0.165±0.01	0.078±0.01	91.67	97.50
15	0.158±0.02	0.072±0.01	87.78	90.00
20	0.147±0.02	0.066±0.01	81.67	82.57
25*	0.142±0.02	0.063±0.00	78.89	78.78
30*	0.142±0.02	0.050±0.01	78.89	62.57

Table 3.3(a) : Effect of acid and water washes on ^{125}I anti HBs bindings (HBsAg assay)
WNR coated tube immobilised with anti-HBs and blocked with 50% NBCS and preincubated with HBsAg positive control serum or HBsAg negative control serum or without serum preincubation before ^{125} I anti-HBs was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
No. of acid and water washes	With HBsAg Positive serum (a)	With HBsAg Negative serum (b)	Without serum	a-b	a/b
3x acid & 3x water	3.09±0.17	2.04±0.19	2.67±0.07	1.05	1.51
10x acid & 10x water	3.10±0.26	2.06±0.14	2.62±0.07	1.04	1.50
15x acid & 15x water	3.03±0.16	2.00±0.11	2.56±0.17	1.03	1.51
Original NR tube	2.66±0.03	2.05±0.07	2.73±0.13	0.61	1.30

Table 3.3(b) Effect of acid and water washes on ^{125}I HBsAg bindings (anti-HBs assay)
WNR coated tube immobilised with HBsAg and blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti-HBs negative control serum or without serum preincubation before ^{125}I HBsAg was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
No. of acid and water washes	With anti-HBs Positive serum (a)	With anti-HBs Negative serum (b)	Without serum	a-b	a/b
3x acid & 3x water	1.29±0.40	0.23±0.01	0.28±0.01	1.06	5.6
10x acid & 10x water	1.42±0.19	0.24±0.01	0.26±0.01	1.18	5.91
15x acid & 15x water	1.10±0.09	0.21±0.01	0.26±0.01	0.89	5.24
Original unwashed NR tube	0.61±0.15	0.21±0.03	0.27±0.07	0.40	2.90

Table 3. 4 (a) Effect of PBS and water washes on ^{125}I anti-HBs bindings (HBsAg assay)
WNR coated tube immobilised with anti-HBs and blocked with 50% NBCS and preincubated with HBsAg positive control serum or HBsAg negative control serum or without serum preincubation before ^{125}I anti-HBs was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
No. of PBS and water washes	With HBsAg Positive serum(a)	With HBsAg Negative serum (b)	Without serum	a-b	a/b
3x PBS & 3x water	2.92±0.13	2.07±0.03	2.57±0.20	0.85	1.41
10x PBS & 10x water	3.17±0.24	2.01±0.03	2.64±0.11	1.16	1.57
15 x PBS & 15x water	3.24±0.31	2.13±0.22	2.58±0.10	1.11	1.52
Original unwashed NR tube	2.66±0.03	2.05±0.07	2.73±0.13	0.61	1.3

Table 3.4 (b) Effect of PBS and water washes on ^{125}I HBs Ag bindings (anti-HBs assay)
WNR coated tube immobilised with HBsAg and blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti-HBs negative control serum or without serum preincubation before ^{125}I HBsAg was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
No. of acid and water washes	With anti-HBs Positive serum (a)	With anti-HBs Negative serum (b)	Without serum	a-b	a/b
3x PBS & 3x water	0.84±0.07	0.22±0.00	0.26±0.02	0.62	3.82
10x PBS & 10x water	1.07±0.23	0.23±0.02	0.27±0.04	0.84	4.65
15 PBS & 15x water	1.21±0.22	0.22±0.04	0.28±0.03	0.99	5.50
Original unwashed NR tube	0.61±0.15	0.21±0.03	0.29±0.02	0.40	2.90

Table 3.5: Effect of trypsinisation of NR surface

Table 3.5(a) : Percent binding of ^{125}I HBsAg and ^{125}I anti-HBs by NR surface after various treatment

200 µl of each labelled macromolecules were incubated overnight with the solid phase at 4°C. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

Types of NR surface	Percent binding	Percent binding
	^{125}I HBsAg	^{125}I anti-HBs
(a)	0.37±0.05	2.57±0.06
(b)	0.40±0.04	2.60±0.08
(c)	0.33±0.03	2.48±0.06
(d)	0.34±0.02	2.44±0.10

(a) Original unwashed NR surface
(b) NR surface washed five times with 1 ml of 0.1 M HCl and followed by five washes with 1 ml of distilled H_2O
(c) NR surface washed five times with 1 ml of 0.1 M HCl and followed by five washes with 1 ml of distilled H_2O, then trypsinised for five hours at 37°C
(d) NR surface washed five times with 1 ml of 0.1 M HCl and followed by five washes with 1 ml of distilled H_2O, and incubated in 0.02 M PBS overnight at 4° C.

Table 3.5(b) : Percent binding of ^{125}I anti-HBs by NR coated tubes (HBsAg assay)

Tubes coated with NR were treated (see explanation below) before immobilised with anti-HBs. The tubes were 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 bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
modification on NR tube	With HBsAg positive serum (a)	With HBsAg Negative serum (b)	Without serum	a-b	a/b
(1)	3.63±0.10	2.26±0.11	3.01±0.11	1.37	1.60
(2)	4.17±0.11	2.24±0.20	3.03±0.11	1.93	1.86
(3)	3.37±0.20	2.09±0.20	2.62±0.20	1.28	1.61
(4)	3.70±0.21	2.28±0.20	2.93±0.20	1.42	1.62
(5)	3.10±0.11	2.06±0.20	2.62±0.20	1.04	1.50

1. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at 37° C for 5 hours.(WT37)
2. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at room temperature (26 C).(WT26)
3. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02 M PBS at 37° C for 5 hours.(PBS37)
4. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02M PBS at room temperature (26° C) for five hours.(PBS 26)
5. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water .(W)

Table 3.5(c): Percent binding of ^{125}I HBsAg on NR tube (anti-HBs assay)
Tube coated with NR was treated before immobilised with HBsAg. The tubes were then blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti-HBs negative control serum. Along with tube without serum preincubation, the treated solid phase were allowed to react with ^{125}I HBsAg. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
modification on NR tube	With anti-HBs Positive serum (a)	With anti-HBs Negative serum (b)	Without serum	a-b	a/b
(1)	2.64±0.20	0.22±0.01	0.25±0.01	2.42	12.00
(2)	2.06±0.21	0.22±0.04	0.28±0.02	1.84	9.36
(3)	3.21±0.11	0.22±0.01	0.27±0.01	2.99	14.59
(4)	3.33±0.10	0.22±0.03	0.26±0.02	3.11	15.14
(5)	1.49±0.20	0.21±0.01	0.27±0.02	1.28	7.09

1. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at 37° C for 5 hours.(WT37)
2. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at room temperature (26 C).(WT26)
3. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02 M PBS at 37° C for 5 hours.(PBS37)
4. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02M PBS at room temperature (26° C) for five hours.(PBS 26)
5. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water .(W)

Table 3.6 Percent binding of T4 on unwashed NR and WNR coated surface
Unwashed NR and WNR coated tube immobilised with anti T4 (1/25 dilution) (1.10 mg/ml). It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of standard T4 were incubated at room temperature while rotating at 190 rpm for 60 min. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. WNR = NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water before precoating.

	Percent binding	
Standard (µg/µl)	WNR	Original NR
0	9.75±0.14	8.14±0.44
3	7.25±0.53	6.00±0.17
6	4.02±0.13	3.24±0.13
12	2.68±0.06	2.36±0.10
24	2.52±0.06	2.02±0.01

Table 3.7(a) Effect of storage of NR coated tube (at 26°C) on ^{125}I anti-HBs specific binding (HBsAg assay)

Dried NR coated tube stored at room temperature (26°C) for a specific duration, then washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water. Washed NR coated tube then immobilised with anti-HBs and blocked with 50% NBCS and preincubated with HBsAg positive control serum or HBsAg negative control serum or without serum preincubation before ^{125}I anti-HBs was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
Months of storage	With HBsAg Positive serum(a)	With HBsAg Negative serum (b)	Without serum	a-b	a/b
9	3.50±0.02	2.45±0.20	3.10±0.13	1.05	1.43
6	3.11±0.01	2.16±0.10	2.88±0.12	0.95	1.44
0.5	3.10±0.02	2.06±0.11	2.62±0.13	1.04	1.50

Table 3.7(b) Effect of storage of NR coated tube (at 26°C) on ^{125}I HBsAg specific binding (anti-HBs assay)

Dried NR coated tube stored at room temperature (26°C), then washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water. Washed NR coated tube was then immobilised with HBsAg and blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti-HBs negative control serum or without serum preincubation before ^{125}I HBsAg was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
Months of storage	With anti-HBs Positive serum (a)	With anti-HBs Negative serum (b)	Without serum	a-b	a/b
9	1.21±0.11	0.24±0.03	0.30±0.05	0.97	5.04
6	1.46±0.12	0.22±0.04	0.29±0.02	1.24	6.64
0.5	1.42±0.10	0.24±0.05	0.26±0.01	1.18	5.92

Table 3.7(c) Effect of storage of NR coated tube(at (at 26°C) on ^{125}I anti-HBs and ^{125}I HBsAg binding

Dried NR coated tube stored at room temperature (26°C), then washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water. Washed NR coated tube was then incubated with ^{125}I labelled HBsAg or anti-HBs overnight at 4°C. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. (WNR = NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water)

Months of storage	Percent binding of ^{125}I HBsAg	Percent binding of ^{125}I anti-HBs
9	0.30±0.01	2.70±0.13
6	0.31±0.02	2.93±0.15
0.5	0.32±0.03	2.83±0.20

Table 3.8 (a)Stability of WNR coated surface-immobilised anti-HBs tube stored at 4°C (HBsAg assay)

WNR coated tube immobilised with anti-HBs and blocked with 50% NBCS. The tube was stored at 4° C before assay carried out. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. (WNR = NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water)

Storage times	Percent binding			Specific binding %	
	With HBsAg Positive serum(a)	With HBsAg Negative serum (b)	Without serum	a-b	a/b
3 months	3.23±0.10	2.17±0.20	2.67±0.30	1.06	1.498
2 months	3.24±0.11	2.14±0.22	2.14±0.22	1.10	1.51
1 day	3.10±0.20	2.06±0.10	2.62±0.10	1.04	1.50

Table 3.8(b) Stability of WNR surface-immobilised HBsAg tube stored at 4° C (anti-HBs assay)

WNR coated tube immobilised with HBsAg and blocked with 50% NBCS . The tube was stored at 4 °C before assay was carried out. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. (WNR = NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water)

Storage times	Percent binding			Specific binding %	
	With anti--HBs Positive serum(a)	With anti-HBs Negative serum (b)	Without serum	a-b	a/b
3 months	1.38±0.02	0.25±0.01	0.25±0.01	1.13	5.52
2 months	1.38±0.10	0.23±0.04	0.24±0.02	1.15	6.0
1 day	1.42±0.20	0.24±0.02	0.26±0.02	1.18	5.91

Table 3.8 (c)Stability of PP surface-immobilised anti-HBs tube stored at 4° C (HBsAg assay)

PP coated tube immobilised with anti-HBs and blocked with 50% NBCS. The tube was stored at 4°C before assay was carried out. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

Storage times	Percent binding			Specific binding %	
	With HBsAg Positive serum(a)	With HBsAg Negative serum (b)	Without serum	a-b	a/b
3 months	2.99±0.10	0.125±0.01	0.27±0.01	2.86	23.92
2 months	2.90±0.20	0.15±0.02	0.19±0.01	2.75	19.33
1 day	3.00±0.03	0.08±0.02	0.19±0.01	2.92	37.50

Table 3.8(d) Stability of PP surface-immobilised HBsAg tube stored at 4° C (anti-HBs assay) PP tube immobilised with HBsAg and blocked with 50% NBCS. The tube was stored at 4° C before assay was carried out. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water.

	Percent binding			Specific binding %	
Storage times	With anti--HBs Positive serum(a)	With anti-HBs Negative serum (b)	Without serum	a-b	a/b
3 months	3.95±0.01	0.07±0.01	0.07±0.01	3.88	56.43
2 months	4.36±0.02	0.07±0.01	0.06±0.01	4.29	62.28
1 day	4.33±0.01	0.07±0.01	0.07±0.01	4.2	61.86

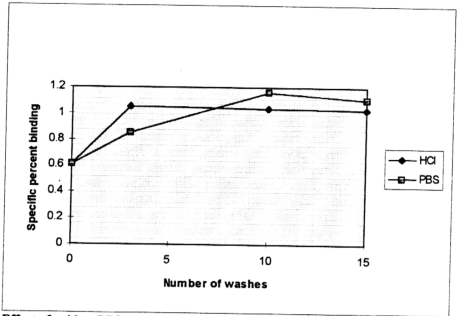

Fig. 3.1 Effect of acid or PBS washes on specific binding of ^{125}I anti-HBs (in HBsAg assay) NR coated tube washed with acid or PBS followed by distilled water, immobilised with anti-HBs and blocked with 50% NBCS, washed and then incubated in HBsAg positive serum or HBsAg negative serum. ^{125} I anti-HBs was then added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. Specific binding = Percent binding in positive serum - percent binding in negative serum.

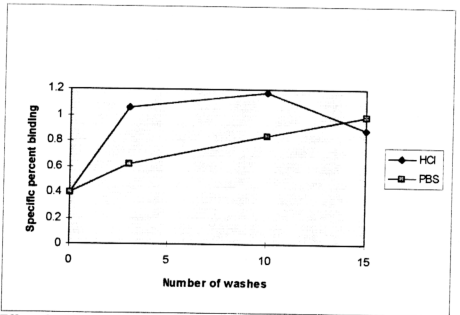

Fig. 3.2 Effect of acid and PBS washes on specific binding of ^{125}I HBsAg (in anti-HBs assay) NR coated tube washed with acid/PBS followed by distilled water immobilised with HBsAg blocked with 50% NBCS, washed and then incubated in anti HBs control positive and anti-HBs control negative serum. ^{125} I HBsAg was then added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. Specific binding = Percent binding in positive serum - percent binding in negative serum.

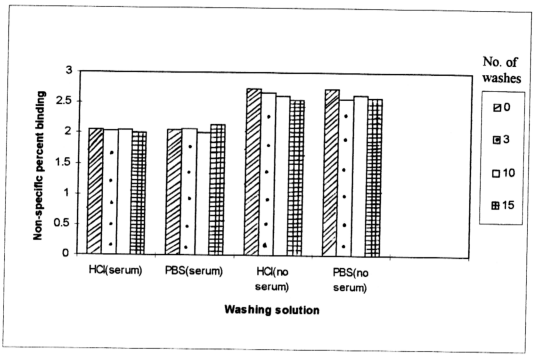

Fig. 3.3 : Effect of acid, PBS and water washes on non-specific binding of ^{125}I anti-HBs (in HBsAg assay)

NR coated tube washed with acid or PBS followed by distilled water before immobilised with anti-HBs and blocked with 50% NBCS. The tube was then preincubated with HBsAg negative control serum or without serum preincubation before ^{125}I anti-HBs was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. NSB (serum) - non specific binding with negative control serum; NSB (no serum) - non - specific binding without serum preincubation.

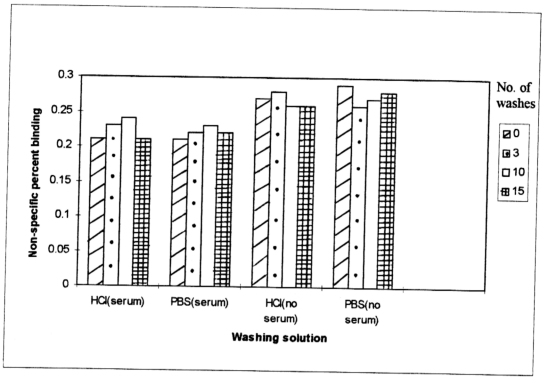

Fig. 3.4 : Effect of PBS , acid and water washed on non-specific binding of ^{125} I HBsAg (in anti-HBs assay)

NR coated tube washed with acid or PBS followed by distilled water before immobilised with HBsAg and blocked with 50% NBCS .The tube was then preincubated with anti-HBs negative control serum or without serum preincubation before ^{125} I HBsAg was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. NSB (serum) - non specific binding with negative control serum; NSB (no serum) - non specific binding without serum preincubation.

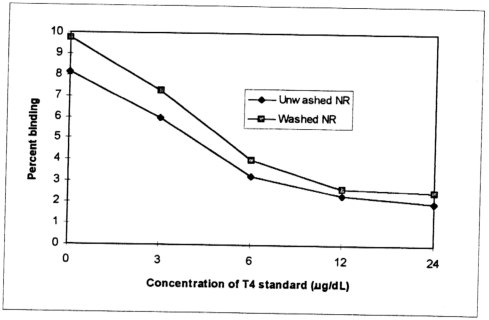

Fig. 3.5 Comparison of ^{125}I T4 percent binding on unwashed and WNR coated tube immobilised with anti-T4

Unwashed NR and WNR were immobilised with anti T4 at dilution of 1/25. Total protein concentration = 1.1 mg/ml. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of standard T4 were incubated at room temperature while rotating at 190 rpm for 60 min. The tube was washed and the bound radioativities were counted. (WNR = NR coated tube was first washed five times with 0.1M 1 ml of HCl and followed by five washes with 1 ml of distilled water)

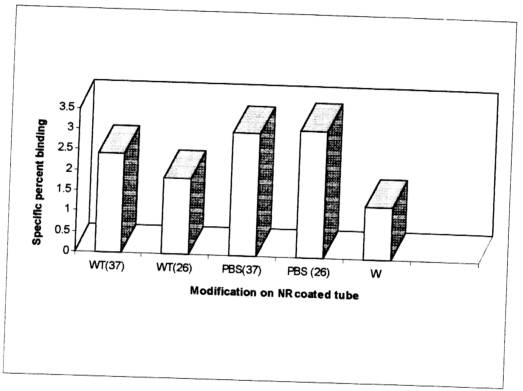

Fig. 3.7 Effect of trypsinisation on ^{125}I HBsAg specific binding (in anti-HBs assay)
Tube coated with NR was modified before immobilised with anti-HBs. Tube blocked with 50% NBCS and preincubated with anti-HBs positive control serum or anti- HBs negative control serum or without serum preincubation before ^{125} I HBsAg was added. The bound radioactivities were counted in an Abbott Gamma Counter after four washes with 1 ml of distilled water. Specific binding = percent binding in positive control serum - percent binding in negative control serum

1. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at 37° C for 5 hours.(WT37)
2. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at room temperature (26 C).(WT26)
3. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02 M PBS at 37° C for 5 hours.(PBS37)
4. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02M PBS at room temperature (26° C) for five hours.(PBS 26)
5. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water .(W)

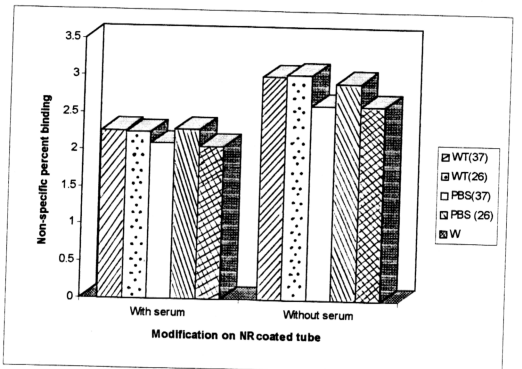

Fig. 3.8: Effect of typsinisation on ^{125}I anti-HBs non-specific binding (in HBsAg assay)
Tube coated with NR was modified before immobilised with anti-HBs. The tube was first blocked with 50% NBCS and preincubated with HBsAg negative control serum or without serum preincubation before ^{125}I anti-HBs was added. The bound radioactivities were counted with Abbott Gamma Counter after four washes with 1 ml of distilled water. NSB (serum) - non specific binding with negative control serum; NSB (without serum) - non specific binding without serum preincubation.

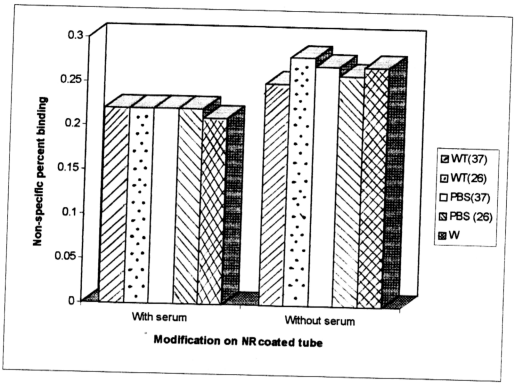

Fig. 3.9: Effect of trypsinisation ^{125}I HBsAg non-specific binding (in anti-HBs assay)
Tube coated with NR was modified before immobilised with HBsAg. Tube first blocked with 50% NBCS and preincubated with anti-HBs negative control serum or without serum preincubation before ^{125}I HBsAg was added. The bound radioactivities were counted with Abbot Gamma Counter after four washes with 1 ml of distilled water. NSB (serum) - non specific binding with negative control serum; NSB (without serum) - non specific binding without serum preincubation.

1. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at 37° C for 5 hour.(WT37)
2. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by trypsinisation at room temperature (26 C).(WT26)
3. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02 M PBS at 37° C for 5 hour.(PBS37)
4. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water followed by incubation in 0.02M PBS at room temperature (26° C) for five hours.(PBS 26)
5. Tube coated with NR washed five times with 1 ml of 0.1M HCl & five times with 1 ml of distilled water .(W)

3.4 Discussion

3.4.1 Effect of pH of labelled macromolecules on the percent binding of labelled macromolecules, ^{125}I anti-HBs and ^{125}I HBsAg by WNR coated and PP surfaces

Protein adsorption at a charged surface involves overlap of the electrical double layers at the solvated solid surface and the solvated protein surface. This overlap will result in electrostatic attraction if the protein macro-ion and the sorbent have opposite charges or in repulsion if the charges are of the same sign. Though global electrostatic forces undoubtedly affect adsorption, they do not dominate it. It is noticed that maximum adsorption of protein was found in the region of the isoelectric point of the protein (IEP). At low or high pH, the adsorption is small. This is because the increase lateral electrostatic repulsion between charged proteins on the surface prevents the formation of close-packed monolayer. Such observation was noted for anti-IgG with IEP 5.5-7.5 (Elgerma et al., 1991), the percent binding was highest in this region but decreased at pH 9.6. The anti-HBs showed poor immobilisation at pH 9.6 possibly because of the acidic residues. These acidic groups are dissociated at pH 9.6, resulting in strong negative charges and thus the repulsive force increased and the percent binding decreased. For HBsAg, the IEP is 3.5-5.0. Thus high pH would reduce the percent binding as explained above. The higher percent binding observed at pH 7.4, the physiological pH, was not clear and could be due to other factors not identified in this study. In view of the high percent

binding of labelled macroproteins at pH 7.4, protein immobilised on the solid-phase was performed at this pH (Table 3.2 a & b).

3.4.2 Effect of washing and trypsinisation

From the above results, it is obvious that treatment of NR latex surface with acid significantly improved its surface activity. Acid treatment will reduce residue ammonia content of the NR surface. Extreme pH can significantly affect the binding property of immobilised macromolecule (pH of ammonia latex is about 10.5). Both anti-HBs and HBsAg coating reagents were prepared in PBS pH = 7.4 which may not be sufficiently effective in buffering the residue ammonia at the sorbent/solution interface. Scanning electron micrographs and AFM pictures of acid washed NR surface are shown in Fig. 3.10 (a) & 3.10 (b). Some possible explanations for the increased binding capacity of the immobilised macromolecules after treating the NR surface with acid and PBS are : (i) an increase in NR solid phase adsorption of macromolecule at pH nearer to neutrality (ii) increase in anti-HBs or HBsAg affinity and (iii) removal of non-rubber constituents from NR surface. Each of these is discussed.

(i) Before washing, at high pH 10.5, dissociation of the acidic group of coated protein resulted in strong negative charges which contributed to repulsive force, and reduction in binding of the protein molecule onto the negatively charge solid phase. The negative charge of the NR solid phase arose partly from protein molecules of the NR latex (with isoelectric point 3.8) which were negatively charged at pH 10.5.

After ammonia was removed from the NR coating by washing with acid or PBS, the pH at the sorbent/solution interface would decrease resulting in a reduction of the negative charge of the NR surface and the immobilised macromolecules (anti-HBs or HBsAg). A reduction in repulsive force between protein-protein (anti-HBs and binder) and protein-sorbent will contribute to improve binding of protein (antibody/antigen) to NR surface. Geerding et al. (Geerding et al., 1988) had shown that variation in pH affected strongly the adsorption of peptides onto solid phase. Stringent pH conditions were needed to coat some peptides on solid phases while others could be coated over a broad pH range. Neutral peptides could be coated with buffers covering a relatively wide pH range in contrast to peptides which contained many acidic residues with high negative charge at alkaline pH. The resulting strong repulsive forces would reduce its binding to solid phase which was negatively charged at pH 9.6. Thus protein (antibody/antigen) with acidic residues would coat more effectively at lower pH (pH 4.6-7.6) rather than pH 9.6. By changing the pH of coating solution from 1.5 to 9.6, Conradie et al (Conradie et al., 1983) showed that solid phase adsorption of polyclonal antibodies of anti-HBs and ferritin was higher if the proteins were pretreated at lower pH . It was found that the greatest effect occurred between pH 1.5 and 4 with an optimal pH at 2.5, which agreed with the findings of Ishikawa et al. (1980). This is due to the exposure of hydrophobic regions of the protein to hydrophobic region of the solid phase subsequent to denaturation of the Fc region of the IgG molecule at pH 2.5. Treatment at pH 2.5 is known to transiently expose a trypsin sensitive-site in IgG

which cleaved the molecule between the $C_{\gamma 2}$ and $C_{\gamma 3}$ domains (Ellerson et al., 1976). However washing the NR surface with acid or PBS did not increase significantly the binding of ^{125}I anti-HBs (monoclonal antibody) and ^{125}I HBsAg by the WNR surface. Binding of ^{125} I HBsAg and ^{125} I anti-HBs on WNR surface was increased only slightly as compared to unwashed surface (Table 3.5 a). This showed that the increased binding capacity was unlikely to be due to an increase in the quantity of protein adsorbed on WNR surface.

(ii) Lowering the pH of the WNR surface resulted in a change of the charges of the amino acids through the Nernst' layer of immobilised protein. This may affect the antigen-antibody interaction. Such alterations in the charge of specific amino acids may change the binding affinity of the immobilised antigen-antibody (Laver et al., 1981; Bartholomew et al., 1982). For example, the binding of monoclonal antibodies to chlamydial antigen was independent of pH between 4 and 9. However binding was reduced at extreme pH conditions (Marta-Terttu., 1984). Dissociation of antibody-antigen complexes at extreme pH conditions, has also been shown for polyclonal antibodies (Singer, 1957; Hardie and Van Erp., 1977). Protein (antibody/antigen) at the sorbent/ liquid interface region with high pH, prior to its physical adsorption may lose their active binding sites. The loss of active-sites at extreme pH depends on the properties of the coated protein. It was shown by van Erp et al. (1992), that acidic treatment of monoclonal antibodies directed against IgG prior to its physical adsorption at pH 2 gave a positive contribution to the total number of active binding site per square area (Ag binding capacity). In

contrast , Lin et al., (1989) found that the active sites of anti-HSA (Human serum albumin) IgG were considerably reduced if immobilised at low pH. Alteration of pH may also cause changes not only in ion pairs but also in hydrogen bonds, dipole interactions and hydrophobic forces between structural features. Minor chemical changes in the structure of antigen or antibody molecules could also significantly affect antigen-antibody interaction, e.g. in influenza virus haemagglutinin test. It has been shown that a change of proline in position 143 causes a 100-fold decrease in agglutination titre (Laver et al., 1971). With influenza A virus, a single amino acid substitution (serine to leucine) causes a change of about five orders of magnitude in binding affinity by a monoclonal antibody (Laver et al., 1979). Changes in structural integrity of the bound macromolecule resulting in improved orientation of the molecules and thus enhanced antibody-antigen interaction.

(iii) The largest component of the non-rubbers of NR are lipids (3.4%) and these comprise of neutral lipids, phospholipids and glycoplipids. Natural rubber latex also contains proteins. High speed centrifugation separates the latex into three fractions in which proteins are distributed. These three fractions are the rubber latex particles, the serum (C-serum) and the bottom fraction (the B-serum). The C-serum and the bottom serum (B-serum) are rich in soluble proteins. In fresh field latex, 27% of the proteins are associated with the rubber particles, 48% in C serum and 25% in B-serum (Tata., 1980). The proteins associated with the latex particle surface are not soluble in their native state, but could be solubilised to some extent by the ammonia added to the latex. These proteins are largely anionic proteins with IEP (Isoelectric

point) between pH 3.5 to 6 and molecular weights of 14 and 24 kD. The B and C serum proteins consist of both anionic and soluble cationic proteins with IEP ranging from pH 3.5 to 9.5. The majority are anionic proteins with molecular weights between 14.0 x 10^3 to 66.0 x10^3 (Hasma, 92). Hevein is one of the major protein component (5 x 10^3; IEP 4.7) and has been isolated and characterised (Archer., 60). The rubber particles of the fresh latex are stabilised by adsorbed proteins and phospholipids (Ho, 1989 & 1996). Hydrolysis of the phospholipids produced long chain fatty acid soaps during the production of HA latex. Thus latex particle of HA latex concentrate is covered mainly with adsorbed long chain fatty soaps which contributed about 86% towards the charges on the surface. The remaining negative charges come from the carboxyl groups of adsorbed proteins.

Upon drying this layer of NR latex coated on a surface , the milky colloidal dispersion was transformed into transparent continuous latex film. The following mechanism has been proposed for latex film formation (Vanderhoff, 1970). Initially, the latex particles move about freely, with characteristic Brownian motion. As the water evaporates, their motion becomes more restricted, and eventually the water-air interfacial tension forces them together into 2a packed array, with their double layers hindering their mutual approach. As the water evaporates further, the water-air interface necks or contracts between the packed spheres in the outer layer, exerting a force to cause coalescence. This coalescence is prevented by the electrostatic forces of repulsion. However, as the force exerted exceeds the critical

value needed to overcome this barrier, the particles move closer together and form polymer-polymer contacts. Once this occurs, the forces arising from the polymer-water interfacial tension also act to coalesce. This brings the polymer-water interfacial tension into play, to reinforce and complement the water-air interfacial tension. If the combined forces are sufficient to deform the polymer spheres, they form a continuous film. As coalescence proceeds, incompatible substances like rubber protein and inorganic materials are exuded or squeezed to the surface. On storage of the NR latex film for two weeks, it becomes more homogeneous as gradual coalescence and exudations continue to occur. Proteins in the NR latex are exuded to the surface of the film when dried. AFM pictures (Fig 2.9 b) show unwashed NR surface was rather flat with lots of tiny particles (exudates) accumulated on the surface. After washing, the flat surface became rough with protruding latex particles as revealed by AFM (Fig. 3.10 b). By washing the NR surface with acid, water and buffer, the soluble NR proteins at the surface and other organic and inorganic substances were removed. Thus washing produced a cleaner surface. Other investigations (Shamsul et al., 1993), using optical microscopy examination of the cross section of gloves also showed a distinct region of soluble proteins stained at the inner surface. The staining was much reduced in sample where the protein had been earlier removed by leaching with water (Shamsul et al., 1993). The protruding particles on the NR surface were individual polyisoprene latex particles. Latex film dried at room temperature has been shown experimentally to give film with relatively low extractable proteins (Shamsul et al., 1993)

compared to latex film dried at higher temperature. More extractable NR proteins can be leached out from latex films if the latex film has been heated. Water-soluble proteins in wet natural rubber latex films co-migrated with water during evaporation to the surface when the film was dried at 100°C (Yeang et al., 1995). This shows that some of the NR proteins are not transferred to the surface and remain within the interior of NR film dried at room temperature. As the NR film coated on PP tube was thick, it will take a longer time for the NR proteins to migrate to the surface. Thus by trypsinisation and PBS incubation NR surface for a long period of time (five hours or overnight), the soluble NR proteins which were 'locked ' within could migrate to the surface and be leached out. Besides, longer period of PBS incubation could remove hydrophilic materials from the NR film surface yielding a cleaner WNR film (compare Fig. 3.10 c & 2.9 b). Removal of NR proteins will reduce protein interference on antigen-antibody binding and thus enhanced the sensitivity of the assay. Trypsinisation can remove both soluble and insoluble NR protein, therefore it would enhance specific binding to a higher level. However this phenomenon was only observed in anti-HBs immobilised tube but not in HBsAg immobilised tube. From the SEM micrographs (Fig. 3.11), it can be seen that after trypsinisation of the NR surface, some trypsins are immobilised on the surface. These individual trypsin particles on NR surface after washing with water could interfere with the assay result.

Several methods have been suggested to effectively reduce water extractable protein from NR latex film.

i.) Wet gel leaching, i.e. washing of the wet gel (gelled deposit on solid surface prior to drying) (Ng et al., 1994).

ii.) Dry film-leaching. Latex film is heated up before leaching (Yeang et al., 1995). One major effect of heating is to transfer soluble NR proteins to the surface where they are easily extracted. Water-soluble NR proteins in wet natural rubber latex films co-migrate with water during evaporation to the surface when film is dried at 100°C and moisture allowed to evaporate from the air/rubber interface. The effect of evaporation on NR protein migration is significant only when the evaporative temperature is sufficiently high (100°C).

iii.)Recentrifugation (Ng et al., 1994). The centrifugation of the fresh latex removes the bottom fraction of non-rubber constituents including soluble NR proteins. The most effective method of reducing extractable protein contents in NR latex is by re-centrifuging latex in combination with a protocol of wet gel leaching and dry-film leaching.

3.4.3 Formation of aggregates on NR surfaces

When the hydrophilic NR proteins and non-protein substances are removed , the hydrophilicity of the NR surface is reduced. The solid surface tension of synthetic polyisoprene latex film was found to be 24.1 - 29.6 mJ m^{-2} (Table 2.4) which is rather similar to the solid surface tension of PP (25.7 mJ m^{-2}) (Table 2.4). Thus we may expect immobilised antigen- antibody proteins to unfold on WNR surface. However, AFM and SEM micrographs showed that all proteins

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(antibodies and binders) formed aggregates on the NR latex film surface. Therefore aggregates of proteins (antibody or antigen) on WNR surface was probably not solely due to the surface hydrophilicity as explained in Chapter Two. Since all coatings are washed with acid and water before being immobilised with antigen / antibody and blocker, the effect of these non-rubber proteins which result in aggregations (antibody and antigen) is perhaps of a more specific nature.

Another possible reason for the aggregations of antibody and antigen on NR surface could be the result of other functional groups on the film surface. In fact the chemical structure of the NR molecule may not be that simple. It has been shown recently that-non-rubber proteins may be linked to the NR molecules (Eng et al., 1994). Long-chain fatty acid ester groups were found to remain in *Hevea* rubber to the extent of 5-8 mmol/kg even after deproteinisation of latex and reprecipitation or acetone extraction of the rubber. The quantity of ester groups was 1.5-2.5 per chain for fractionated samples which was comparable to that of trans isoprene units at the initiating terminal of the polymer chain (Eng et al., 1994). The ester groups were removed from rubber by transesterification. Isolated methyl esters were found to compose to mainly of saturated $C_{16\text{-}18}$ fatty acids. Besides, natural rubber is also known to contain small amounts of abnormal functional groups such as aldehyde, epoxide, and lactone. These may contribute to the formation of protein aggregates on WNR surface. However, the detailed structure and role of these abnormal groups are still not fully elucidated (Eng et al., 1994).

Fig. 3.10 (a) Scanning electron micrograph showing WNR surface. Magnification (i) $1x10^4$ WNR = NR coated tube was washed five times with 0.1 M 1 ml of HCl and followed by five washes with 1 ml of distilled water.

a(i)

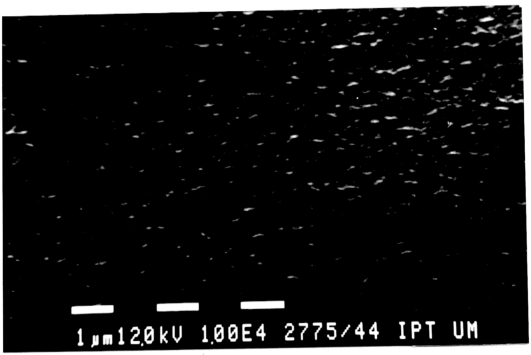
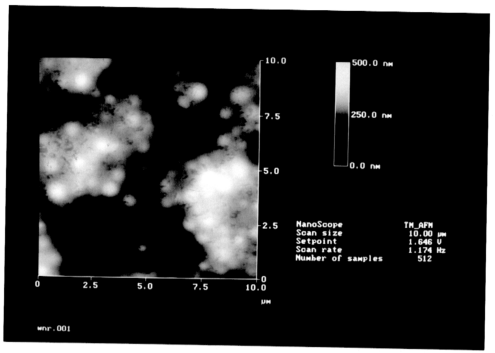


Fig. 3.10 (b) AFM images showing WNR surface (i) 10x10 μm top view surface (ii) 10x10 μm three dimension surface plot
WNR = NR coated tube was washed five times with 0.1 M 1 ml of HCl and followed by five washes with 1 ml of distilled water.

b(i)



b(ii)

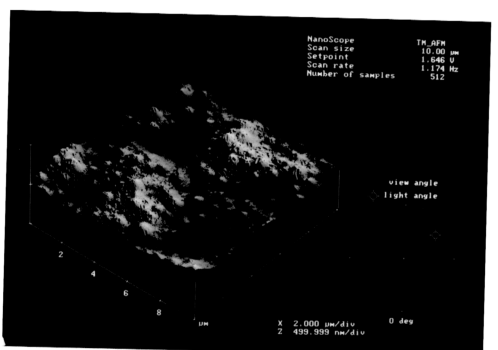
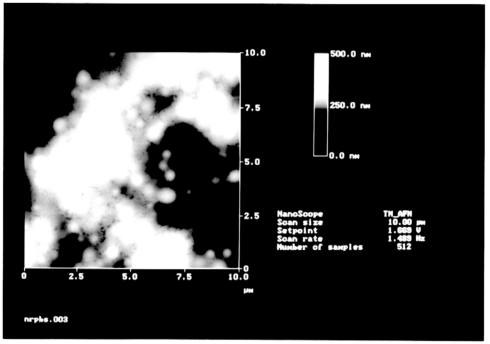


Fig. 3.10 (c) AFM images showing acid, PBS and water washes of NR coated surface (i) 10x10 µm top view surface (ii) 10x10 µm three dimension surface plot

NR coated tube washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water, the tube was then incubated overnight in PBS at 26°C followed by 4 times of 1 ml distilled water washes

c(i)



c(ii)

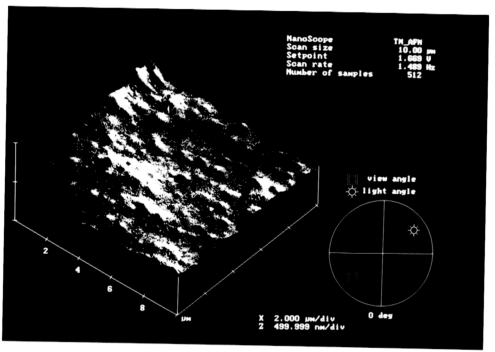
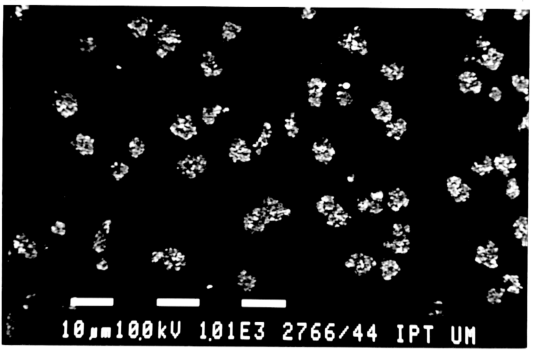


Fig. 3.11 Scanning electron micrograph showing washed and trypsinised NR surface. Magnification (i) $1.01x10^3$ (ii) $5.00x10^3$
Tube coated with NR was washed five times with 1 ml of 0.1M HCl and five times with 1 ml of distilled water followed by typsinisation at 37°C for five hours. The tube was then washed five times with 1 ml of distilled water

(i)



(ii)

3.5 Conclusion

In conclusion, we found that treatment of NR latex film surface with acid, PBS, water and trypsinisation improved its surface sensitivity. The above treatments did not increase percent of antibody or antigen adsorbed on the latex film surface. The treatments were able to remove non-rubber materials on NR film surface. This probably includes proteins which are exuded to the surface of the NR film when dried. Longer incubation time of trypsin and PBS was able to produce cleaner surface with higher sensitivity because the NR proteins embedded in the interior of the NR film could migrate to the surface and be easily leached out from the film surface. Upon trypsinisation, trypsin immobilised on NR surface gave rise to non-specific interference. Moreover, the removal of ammonia lowered the pH of the NR film surface resulting in the reduction of the charges of amino acid through the Nernst's layer of immobilised antigen-antibody proteins. Such alterations in the charge of specific amino acids may change the binding affinity of the immobilised antigen-antibody complex. In addition, changes in of the pH may change hydrogen bonds, dipole interactions and hydrophobic forces between the structural features. Changes in structural integrity of the bound macromolecules resulted in improved orientation of the molecules and thus enhanced antibody-antigen interaction. Washing the NR surface improved the specific binding capacity of immobilised anti-HBs and HBsAg but was ineffective in preventing the formation of aggregates of

immobilised protein on WNR surface and could be due to other surface functional groups which were not easily removed by washing. To avoid introduction of additional variable, the following washing procedure was selected for subsequent experiments :- NR surface is washed five times with 1 ml of 0.1M HCl followed by five times with 1 ml of distilled water before immobilisation with anti-HBs or HBsAg.

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