Chapter 5 Effect of precoating on NR and PP tubes

5.1 Introduction

Polymers with a low degree of adsorptivity have found application in many fields, such as various separation techniques, diagnostic methods, and even biosensors. After being transformed into biomolecule-covered polymers, they possess unique surface characteristics. For many applications, (e.g. in affinity chromatography, immunoassay and enzyme linked immunosorbent assay techniques), surfaces with a low degree of adsorptivity have been transformed into other types of surfaces by chemical modifications which introduce reactive groups to which proteins can be covalently attached. However, antibodies covalently immobilised on solid phase are also functionally inactivated in the process and could result in the increase of non-specific bindings. The biomolecule-covered polymers obtained have adsorption characteristics which are very different from those of the original polymers. Implants with good biocompatibility will become covered by biomolecules after a short time in contact with biological fluids. Depending on the polymer involved, the first layer of biomolecules adsorbed onto the surface will undergo various degree of conformational changes (Norde et al., 1986). As a result of these changes, new structures in the immobilised biomolecule will become exposed and the original surface will acquire new characteristics.

How does this newly deposited layer adsorb additional biomolecules upon further contact with fresh biological fluid? Does the surface change continuously upon each new contact with biological fluids? What type of adsorption characteristics does this biomolecule-covered surface have compared to the original naked polymer surface? The aim of the study described in this chapter is to look into how precoating agent affects the binding of anti-HBs, HBsAg and anti-T4 and whether the non-specific bindings could be reduced in this process.

5.2 Materials and experimental methods

5.2.1 Materials (refer 2.2)

Reagent / Abbreviation	Source and/or formulation
Newborn Fetal calf serum (NBCS)	Paesel + Lorei GMBH & Co. Serile filtered, Art No 36-101-00003
Gelatin powder	B.D.H Chemicals
Albumin Bovine Powder (BSA)	Sigma Chemical Company, No A-45031
Neonatal serum (NEO)	In- house preparation (see text)

Preparation of solution

I) Bovine serum albumin (BSA)

0.25g of BSA was dissolved in 50 ml PBS (0.02M, pH 7.4) solution at 26°C to give BSA solutions of 0.5% (w/v).

2.)Gelatin

0.25g of gelatin powder was dissolved in 50 ml of warm (37°C) PBS (0.02M, pH 7.4) to give solutions of 0.5% (w/v).

3.) Neonatal sera (NEO)

NEO were pooled from new born cord sera which were anti-HBs and HBsAg negative (assayed by using IMK-441 system CIAE HBsAb SPRIA and IMK-413 system CIAE HBsAg SPRIA modified kit procedure (refer 2.2)). Anti-HBs and HBsAg coated polystyrene beads were gift from Dr. He of CIEA, Beijing. The procedure of preparation of HBsAg and anti-HBs coated polystyrene beads were as outlined in section. 2.2

4.) Newborn Calf serum (NBCS)

NBCS was diluted to 50%(v/v) in PBS 0.02M pH 7.4 at 26°C.

5.2.2 Determination of precoating concentration

Total protein concentration of 50% NBCS and NEO were determined using the DuPont Dimension system AR (DuPoint, USA). The concentration of 50% NBCS and NEO were 19.6 g dm^{-3} and 52.8 g dm^{-3} respectively.

5.2.3 Experimental methods

(a) Assay procedure

NR coated tubes were washed five times with 1 ml 0.1M HCl followed by five times distilled water washes before incubated with 200 µl of various preblocking solutions (BSA, Gelatin, NBCS, NEO) at 4°C overnight. Tubes were then washed four times with 1 ml PBS, 0.02M, pH 7.4 solution. After the last wash, the tubes were inverted and tapped on a clean absorbent paper to drain of all liquid. Immobilisation of anti-HBs or HBsAg, blocking and assay procedures were carried out as described in section 2.2.2 (a). Precoating on PP tube was similarly carried out, and immobilised with anti-HBs and HBsAg. Triplicates were carried out for each experiments. For T4 assay, gelatin and NBCS were chosen as precoating agent. The precoating process was as described above.

(b) Preparation of samples for SEM and AFM observation (as described in section 2.2.2)

5.3 Results

Table 5.1 and 5.2 (Fig. 5.1 a & b and Fig. 5.2 a & b) show adsorption of ^{125}I anti-HBs on different types of precoated modified surfaces. Solid surface was precoated with blocking solution before being immobilised with anti-HBs reagent and then further blocked with 50% NBCS. HBsAg serum was allowed to react with

the prepared solid phase for two hours followed by two hours incubation with ^{125}I anti-HBs at 45°C. Precoating the solid phase with gelatin and NEO enhanced the specific binding of anti-HBsAg on NR surface immobilised with anti-HBs (in HBsAg assay) by 74.07 and 57.41% respectively (Table 5.7) as compared with control. However for WNR surface precoated with BSA and NBCS, specific binding was reduced by 46.30% and 59.26% respectively (Table 5.7). On PP surface , the specific bindings were reduced by 74.78%, 73.37%, 51.42% and 87.60% after precoating with 0.5% gelatin, 0.5% BSA, NEO and NBCS respectively (Table 5.7). Non-specific binding of ^{125}I anti-HBs (without serum) decreased on both WNR and PP surfaces when compared with the control (Fig. 5.1a & 5.2a). NEO serum , 50% NBCS, 0.5% BSA, 0.5% gelatin and PBS were effective precoating agents in reducing non-specific bindings in the absence of serum on WNR. Further blocking of the WNR precoated solid phase with negative serum before adding ^{125}I anti-HBs showed an additional decrease in non-specific binding. On WNR surface, with gelatin as precoating agent, the non-specific binding of ^{125}I anti-HBs was high without serum preincubation but it was comparatively low after preincubation with negative control serum. The reverse was seen when 0.5% gelatin or 0.5% BSA, or 50% NEO or 50% NBCS was precoated on PP surface (Fig. 5.1a & 5.2a). Precoating of WNR surface with 0.5% gelatin, and NEO gave a lower non-specific binding (preincubated in HBsAg negative serum) and higher specific binding when compared with precoating using 0.5% BSA and NBCS. Thus precoating the WNR surface with 0.5% gelatin or NEO could enhance the sensitivity of the HBsAg assay, whereas with PP surface, precoating reduced the specific binding capacity and

increased the non-specific binding (with HBsAg negative serum preincubation) (Table 5.1 & 5.2) and thus reduced the sensitivity of the assay.

Fig. 5.3 & 5.4 (Table 5.3 & 5.4) show the adsorption of ^{125}I HBsAg on different types of precoat modified surfaces. Solid surface was precoated with blocking reagents before immobilisation of HBsAg reagents and blocked with 50% NBCS. Anti-HBs serum was then added and the tubes were incubated for two hours followed by water washes and two hours incubation with ^{125}I HBsAg. The bound radioactivities after water washes were counted. The specific binding capacity of ^{125}I HBsAg by both PP or WNR surface was reduced after precoating with different reagent (Fig. 5.3b & 5.4b). For WNR surface precoated with gelatin, BSA, NEO and NBCS, specific bindings of ^{125}I HBsAg binding were reduced by 65.50%, 89.92%, 98.45% and 89.92 % respectively (Table 5.7). Similarly for precoated PP surface, the specific binding was reduced by 78.90%, 65.33%, 75.88% and 77.89% respectively. However non-specific binding of ^{125}I HBsAg (without serum) by WNR surface precoated with the reagent was not reduced significantly (Table 5.3) when compared with control tube (WNR). Immobilisation of the WNR with HBsAg, or PP surface, followed by preincubation with negative control serum, caused a slight decrease in non-specific binding for both WNR and PP surfaces. (Fig. 5.3a & Fig. 5.4a). Thus, precoating reduced the sensitivity of anti-HBsAg assay. On unprecoated control WNR tube immobilised with HBsAg, the specific/non-specific binding ratio

for ^{125}I HBsAg was 7.00 (Table 5.3), however this ratio decreased to 3.28, 1.63, 1.10 and 1.74 respectively after precoating the surface with gelatin, BSA, NEO and NBCS respectively. On PP tube (Table 5.4) precoating with different reagents reduced slightly the non-specific binding of ^{125}I HBsAg in the presence of negative control sera and in the absence of serum significant reduction was observed for BSA and NBCS (Fig. 5.4a). For BSA, NEO, NBCS precoated PP surface, the non-specific binding of precoated tube immobilised with HBsAg or anti-HBs in negative serum and without serum were comparable except those with BSA and NBCS precoating which show a significant decrease without serum preincubation (Table 5.3 & 5.4).

Fig. 5.5 a to e, Fig. 5.6 a to g (Table 5.8) show that WNR surfaces precoated with gelatin and immobilised with anti-T4 exhibited an increase in displacement of labelled T4. No specific trend in binding of ^{125}I T4 was observed except that at anti-T4 dilution of 1/10 where binding increased with increasing number of precoating. At this dilution, precoating 3 times with gelatin increase the slope of the dose response curve over the concentration range of 0 to 24 μg/dL of T4. WNR surface precoated with gelatin show a greater percent binding of ^{125}I T4 at zero dose of T4 as compared with WNR surface precoated with NBCS, and immobilised with anti-T4 at all dilutions. WNR surfaces precoated three times with 0.5% gelatin and immobilised with anti-T4 at dilutions of 1/10 and 1/25 showed that the displacement of ^{125}I T4 occurred throughout the standard curve which comparable to that of PP tube (no precoating, see Fig. 5.6 c and g & Table 5.8).

Table 5.1 : Binding of ^{125}I anti HBs by precoated WNR surface immobilised with anti-HBs (in HBsAg assay)

WNR coated tube precoated with different reagents before immobilised with anti-HBs, blocked with 50% NBCS and preincubated with positive HBsAg control serum or negative HBsAg control serum (or without serum incubation). ^{125}I anti-HBs was then added and incubated. The tube was washed and the bound radioactivities were counted. WNR = NR coated tube washed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of distilled water. Control = WNR tube without precoating.

*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.

	*Percent binding				
Precoat reagent	With HBsAg (Positive serum)(a)	With HBs Ag (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
0.5% gelatin	2.92±0.16	1.98±0.11	3.07±0.72	0.94	1.47
0.5% BSA	2.43±0.17	2.14±0.13	3.03±0.06	0.29	1.13
50% NEO	2.81±0.21	1.96±0.08	2.77±0.05	0.85	1.43
50% NBCS	2.30±0.18	2.08±0.01	2.89±0.08	0.22	1.11
PBS(0.02M, pH 7.4)	2.91±0.23	2.19±0.16	3.01±0.03	0.72	1.33
Control (WNR)	2.87±0.14	2.33±0.06	3.21±0.03	0.54	1.23

Table 5.2 Binding of ^{125}I anti HBs by precoated PP surface immobilised with anti-HBs (in HBsAg assay)

PP tube precoated with different reagents before immobilised with anti-HBs , blocked with 50% NBCS and preincubated with positive HBsAg control serum or negative HBsAg control serum (or without serum incubation). ^{125}I anti-HBs was then added and incubated. The tube was washed and the bound radioactivities were counted. Control = PP tube no precoating

	Percent binding				
Precoat reagent	With HBsAg (Positive serum)(a)	With HBsAg (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
0.5% gelatin	1.42±0.19	0.18±0.02	0.12±0.01	1.24	7.89
0.5% BSA	1.44±0.58	0.13±0.03	0.10±0.00	1.31	11.07
50% NEO	2.53±0.20	0.14±0.01	0.11±0.00	2.39	18.07
50% NBCS	0.75±0.27	0.14±0.01	0.13±0.00	0.61	5.35
Control (PP)	5.07±0.36	0.15±0.01	0.15±0.00	4.92	33.80

Table 5.3 Binding of ^{125}I HBsAg by precoated WNR surface immobilised with HBsAg (in anti-HBs assay)

WNR coated tube precoated with blockers before immobilised with HBsAg, blocked with 50% NBCS and preincubated with positive anti-HBsAg control serum or negative anti-HBsAg control serum (or without serum incubation). ^{125}I HBsAg was then added and incubated. The tube was washed and the bound radioactivities were counted. WNR = NR coated tube washed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of distilled water. Control = WNR tube without precoating

	Percent binding				
Precoat reagent	With Anti-HBs (Positive serum)(a)	With Anti-HBs (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
0.5% gelatin	1.28±0.11	0.39±0.03	0.36±0.02	0.89	3.28
0.5% BSA	0.67±0.16	0.41±0.04	0.38±0.03	0.26	1.63
50% NEO	0.45±0.02	0.41±0.03	0.37±0.03	0.04	1.10
50% NBCS	0.61±0.03	0.35±0.02	0.40±0.00	0.26	1.74
PBS(0.02M, pH 7.4)	3.25±0.72	0.44±0.05	0.39±0.03	2.81	7.39
Control (WNR)	3.01±0.15	0.43±0.00	0.37±0.03	2.58	7.00

Table 5.4 Binding of ^{125}I HBsAg by precoated PP surface immobilised with HBsAg (in anti-HBs assay)

PP tube precoated with different reagents before immobilised with HBsAg, blocked with 50% NBCS and preincubated with positive anti-HBs control serum or negative anti-HBs control serum (or without serum incubation). ^{125}I HBsAg was then added and incubated again. The tube was washed and the bound radioactivities were counted. Control = PP tube no precoating

	Percent binding				
Precoat reagent	With Anti-HBs (Positive serum)(a)	With Anti-HBs (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
0.5% gelatin	0.58±0.07	0.16±0.03	0.14±0.00	0.42	3.63
0.5% BSA	0.86±0.10	0.17±0.01	0.06±0.00	0.69	5.06
50% NEO	0.62±0.06	0.14±0.02	0.12±0.02	0.48	4.43
50% NBCS	0.62±0.06	0.18±0.00	0.06±0.00	0.44	3.44
Control (PP)	2.18±0.21	0.19±0.01	0.13±0.01	1.99	11.47

Table 5.5 Non specific percent binding of ^{125}I HBsAg and ^{125}I anti-HBs by WNR coated tube after precoating without immobilisation with HBsAg or anti-HBs and in the absence of serum incubation

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

Precoat reagent	Percent binding of ^{125}I HBsAg	Percent binding of ^{125}I anti-HBs	% increase /decrease of ^{125}I HBsAg in binding after precoating	% increase /decrease of ^{125}I anti- HBs in binding after precoating
0.5% gelatin	0.33±0.02	2.62±0.19	-17.50	+0.77
0.5% BSA	0.33±0.01	2.51±0.55	-17.50	-3.46
50% NEO	0.23±0.03	1.61±0.08	-42.50	-38.00
50% NBCS	0.25±0.02	1.69±0.06	-37.50	-35.00
PBS	0.34±0.02	2.44±0.19	-	-
Control (WNR)	0.40±0.05	2.60±0.06	-	-

note :

+ Increase in percentage of percent binding (as compared with control tube -WNR)

- Decrease in percentage of percent binding (as compared with control tube - WNR)

Table 5.6 Non specific percent binding of ^{125}I HBsAg and ^{125}I anti-HBs by precoated PP tube but without immobilisation with HBsAg or anti-HBs and in the absence of serum incubation

PP tube precoated with different blocker then incubated in 200 µl labelled protein overnight at 4°C. The tube was washed and the bound radioactivities were counted. Control = PP tube without precoating

Precoat reagent	Percent binding of ^{125}I HBsAg	Percent binding of ^{125}I anti-HBs	% increase /decrease of ^{125}I HBsAg in binding after precoating	% increase /decrease of ^{125}I anti- HBs in binding after precoating
0.5% gelatin	0.086±0.003	0.196±0.003	+7.50	+38.03
0.5% BSA	0.060±0.002	0.129±0.002	-25.00	-9.15
NEO	0.085±0.008	0.130±0.002	+6.25	-8.45
NBCS	0.065±0.002	0.108±0.013	-18.75	-23.58
Control (PP)	0.008±0.002	0.142±0.002	-	-

note :

+ Increase in percentage of percent binding (as compared with control tube - PP)

- Decrease in percentage of percent binding (as compared with control tube - PP)

Table 5.7 Effect of precoating on % change of specific binding on ^{125}I anti HBs (in HBsAg assay) & ^{125}I HBsAg (in anti-HBs assay) by the solid phase immobilised with anti-HBs and HBsAg respectively

	^{125}I anti-HBs (%)		^{125}I HBsAg (%)	
Precoat reagent	PP	WNR	PP	WNR
0.5% gelatin	-74.78	+74.07	-78.90	-65.50
0.5% BSA	-73.37	-46.30	-65.33	-89.92
50% NEO	-51.42	+57.41	-75.88	-98.45
50% NBCS	-87.60	-59.26	-77.89	-89.92
PBS	-	+33.30	-	-9.34

note :

+ Increase in percentage of specific binding (as compared with control tube - WNR or PP tube without precoating)

- Decrease in percentage of specific binding (as compared with control tube - WNR or PP tube without precoating)

WNR = NR coated tube washed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of distilled water

Table 5.8 (a) Effect of immobilising the PP solid phase with anti-T4 of different dilution on displacement of labelled T4

PP (no precoating) tubes immobilised with anti-T4 at various dilutions. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of standard T4 were incubated at room temperature with side-to-side rotation at 190 rpm for 60 min. The tube was washed and the bound radioactivities were counted.

	Concentration of T4 standard solution (µg/dL)			
	PBS	0	6	24
Dilution of anti-T4	(The bound radioactivities were expressed as percentage of total counts)			
1/10	26.30±0.28	15.30±2.03	5.15±0.88	3.03±0.52
1/25	20.45±0.98	14.01±1.68	4.45±1.16	2.55±0.38
1/50	17.78±1.09	12.11±0.30	2.80±0.19	2.42±0.50
1/100	14.17±2.14	9.32±0.50	3.15±0.99	2.40±0.21
1/200	13.81±1.12	9.14±0.52	2.77±0.27	2.01±0.34

Table 5.8 (b) Effect of precoating on displacement of labelled T4

WNR tubes precoated with various reagents as outlined below and immobilised with anti-T4 with different dilution of anti-T4. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of standard T4 were incubated at room temperature with side-to-side rotation at 190 rpm for 60 min. WNR = NR coated tube washed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of distilled water (X= number of precoating). The tube was washed and the bound radioactivities were counted. a. T4=1/10 b. T4=1/25 c. T4=1/50 d. T4=1/100 e. T4=1/200 Control = WNR without precoating

	Concentration of T4 standard solution (µg/dL)			
	PBS	0	6	24
Dilution of anti-T4	(The bound radioactivities were expressed as percentage of total counts)			
(a) 1/10				
control	7.08±0.18	4.64±0.12	2.90±0.11	1.85±0.13
gelatin, 1x	18.27±5.45	9.06±1.59	3.53±0.18	2.48±0.49
gelatin, 2x	19.09±1.22	9.68±1.75	3.88±0.60	2.86±0.31
gelatin, 3x	20.10±2.32	15.44±0.59	7.45±0.42	3.85±0.39
NBCS, 1x	15.80±4.77	4.85±0.18	2.71±0.38	1.88±0.16
NBCS, 2x	15.78±2.38	5.08±1.12	2.51±0.48	1.93±0.33
NBCS, 3x	11.05±1.18	5.60±1.02	2.36±0.36	2.09±0.31
(b) 1/25				
control	9.66±0.47	6.65±0.11	2.62±0.11	2.32±0.10
gelatin, 1x	17.18±2.58	9.06±0.94	3.04±0.53	2.03±0.10
gelatin, 2x	15.80±1.80	7.37±1.27	3.55±0.48	3.15±0.25
gelatin, 3x	14.71±0.33	13.89±1.82	3.39±0.30	3.15±0.30
NBCS, 1x	12.59±1.16	4.87±0.65	1.58±0.15	1.33±0.25
NBCS, 2x	13.96±0.83	4.60±0.22	2.31±0.66	1.67±0.12
NBCS, 3x	10.70±0.47	5.03±0.78	2.36±0.32	1.54±0.15
(c) 1/50				
control		4.78±0.94	2.38±0.35	2.23±0.23
gelatin, 1x	15.66±3.39	5.81±0.69	2.73±0.5	2.04±0.27
gelatin, 2x	14.24±1.14	7.17±0.35	3.32±0.49	1.88±0.28
gelatin, 3x	12.13±1.10	7.14±0.47	2.92±0.36	1.70±0.31
NBCS, 1x	10.92±0.38	4.24±0.58	2.10±0.21	1.76±0.19
NBCS, 2x	10.24±1.16	4.04±0.10	2.13±0.73	1.56±0.34
NBCS, 3x	8.80±0.52	3.54±0.04	1.95±0.27	1.67±0.04
(d) 1/100				
control		3.86±0.34	1.89±0.24	1.44±0.30
gelatin, 1x	10.04±1.22	4.09±0.46	2.07±0.22	1.67±0.18
gelatin, 2x	10.87±0.76	4.73±1.07	2.70±0.36	2.04±0.13
gelatin, 3x	8.72±0.87	5.47±0.00	1.82±0.27	2.01±0.46
NBCS, 1x	9.96±0.52	2.47±0.75	1.75±0.16	1.61±0.25
NBCS, 2x	7.60±0.82	2.00±0.33	1.65±0.36	1.71±0.35
NBCS, 3x	6.66±0.40	2.82±0.30	1.84±0.39	1.85±0.11

Dilution of anti-T4/ Precoat surface	PBS	0	6	24
Dilution of anti-T4	(The bound radioactivities were expressed as percentage of total counts)			
(e) 1/200				
control		3.03±0.21	1.62±0.33	1.47±0.20
gelatin, 1x	8.35±0.33	3.53±0.21	2.04±0.32	1.88±0.31
gelatin, 2x	9.06±1.19	3.46±0.59	2.45±0.42	1.94±0.18
gelatin, 3x	5.96±0.44	3.48±0.26	1.73±0.09	1.21±0.15
NBCS, 1x	7.46±1.06	2.98±0.62	1.80±0.13	1.76±0.15
NBCS, 2x	5.47±0.32	2.00±0.29	1.62±0.14	1.38±0.14
NBCS, 3x	6.18±0.83	2.50±0.44	2.35±0.13	2.31±0.27

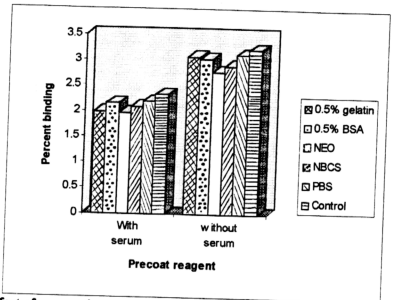


Fig. 5.1a : Effect of precoating on non-specific binding of ^{125}I anti-HBs (in HBsAg assay) by WNR coated tube

WNR precoated with reagent before immobilised with anti-HBs and blocked with 50% NBCS. The surface was washed and incubated with negative control HBsAg serum (or without serum incubation) . ^{125}I anti-HBs was then added and incubated. The tube was washed and the bound radio activities were counted. WNR = NR coated tube washed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of distilled water. Control = WNR tube without precoating. NSB (serum) = non specific binding with negative control serum. NSB (without serum) = non specific binding without serum preincubation.

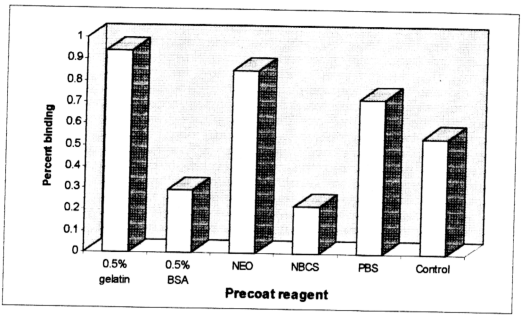


Fig. 5.1 b Effect of precoating on specific binding of ^{125}I anti-HBs by WNR coated tube (in HBsAg assay)

WNR coated tube precoated with reagent before immobilised with anti-HBs and blocked with 50% NBCS. The surface was washed and incubated in positive control HBsAg serum or negative control HBsAg serum. ^{125}I anti-HBs was then added and incubated again. The tube was washed and the bound radioactivities were counted. Percent of specific binding = Percent binding in positive control serum - percent binding in negative control serum. WNR = NR coated tube washed five times with 1 ml 0.1M HCl followed by five times washes with 1 ml of distilled water. Control - WNR tube without precoating.

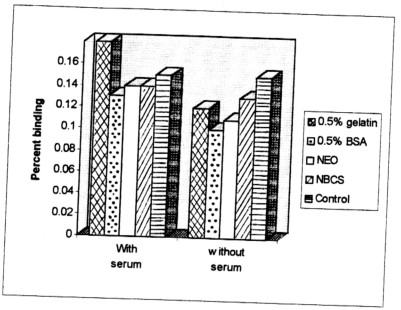


Fig. 5.2a : Effect of precoating on non-specific binding of ^{125}I anti-HBs (in HBsAg assay) by PP tube

PP tube precoated with reagent before immobilised with anti-HBs and blocked with 50% NBCS. The surface was washed and incubated with negative control HBsAg serum (or without serum incubation) . ^{125}I anti-HBs was then added and incubated. The tube was washed and the bound radioactivities were counted. Control = PP tube without precoating. NSB (serum) = non specific binding with negative control serum. NSB (without serum) = non specific binding without serum preincubation.

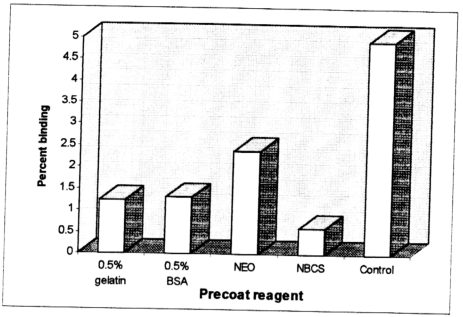


Fig. 5.2b Effect of precoating on specific binding of ^{125}I anti-HBs by PP tube (in HBsAg assay) PP tube precoated with reagent before immobilised with anti-HBs and blocked with 50% NBCS. The surface was washed and incubated in positive control HBsAg serum or negative control HBsAg serum. ^{125}I anti-HBs was then added and incubated again. The tube was washed and the bound radioactivities were counted. Percent of specific binding = Percent binding in positive control serum - percent binding in negative control serum. Control = PP tube without precoating

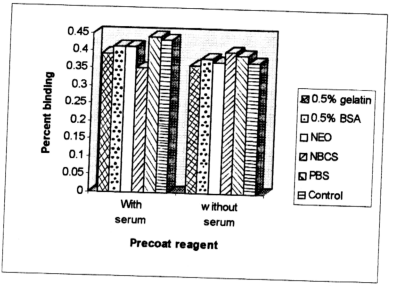


Fig. 5.3a : Effect of precoating on non-specific binding of ^{125}I HBsAg (in anti-HBs assay) by precoated WNR tube immobilised with HBsAg

WNR coated tube precoated with reagent before immobilised with HBsAg and blocked with 50% NBCS. Tube was then incubated with negative anti-HBsAg serum (or without serum incubation). ^{125}I HBsAg was added and incubated. The tube was washed and the bound radioactivities were counted. Control = WNR tube without precoating. WNR = NR coated tube washed five times with 1 ml 0.1M HCl followed by five times 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.

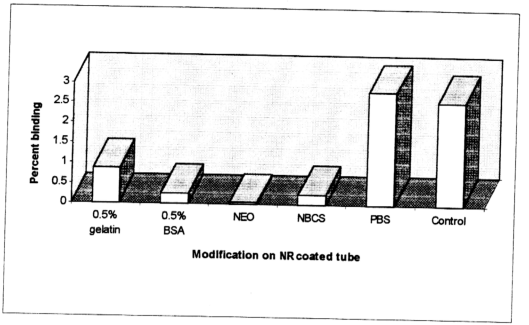


Fig. 5.3b Effect of precoating on specific binding of ^{125}I HBsAg (in anti-HBs assay) by precoated WNR tube

WNR coated tube precoated with reagent before immobilised with HBsAg and blocked with 50% NBCS. The surface was washed and incubated in positive control anti-HBs serum or negative control anti-HBs serum (or without serum incubation) .^{125}I HBsAg was then added and incubated again. The tube was washed and the bound radio activities were counted. Percent of specific binding = Percent binding in positive control serum - percent binding in negative control serum. Control = WNR tube without precoating. WNR = NR coated tube washed five times with 1 ml 0.1M HCl followed by five times washes with 1 ml of distilled water.

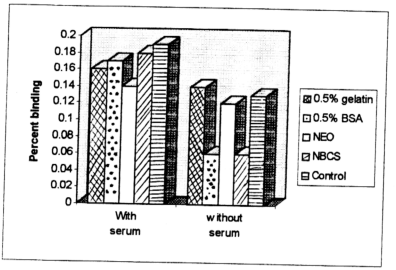


Fig. 5.4a : Effect of precoating on non-specific binding of ^{125}I HBsAg (in anti-HBs assay) by precoated PP tube immobilised with HBsAg

PP tube precoated with reagent before immobilised with HBsAg and blocked with 50% NBCS. The tube was then incubated with negative anti-HBsAg serum (or without serum incubation). ^{125}I HBsAg was added and incubated. The tube was washed and the bound radioactivities were counted. Control = PP tube without precoating

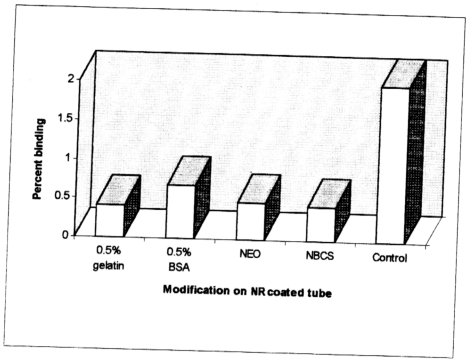


Fig. 5.4b Effect of precoating on specific binding of ^{125}I HBsAg (in anti-HBs assay) by precoated PP tube

PP tube precoated with blocker before immobilised with HBsAg and blocked with 50% NBCS. The surface was washed and then incubated in positive control anti-HBs serum or negative control anti-HBs serum. ^{125}I HBsAg was then added and incubated again. The tube was washed and the bound radioactivities were counted. Percent of specific binding = Percent binding in positive control serum - percent binding in negative control serum. Control = PP tube without precoating

Fig. 5.5a-e Percent binding of ^{125}I anti-T4 on WNR tube precoated surface (number of precoating X is as shown) immobilised with anti-T4 (at various dilution) and blocked with 50% NBCS

Precoated WNR tube was immobilised with anti-T4 at different dilution. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of PBS or standard T4 [concentration : zero µg/dl; 6 µg/dl; 24µg/dl] were incubated at room temperature with side-to-side rotation at 190 rpm for 60 min.The tubes were then washed and bound radioactivities were counted. WNR = NR coated tube washed five times with 1 ml 0.1M HCl and five times 1 ml of distilled water before precoating. a. T4=1/10 b. T4=1/25 c. T4=1/50 d. T4=1/100 e. T4 =1/200 X = number of precoating

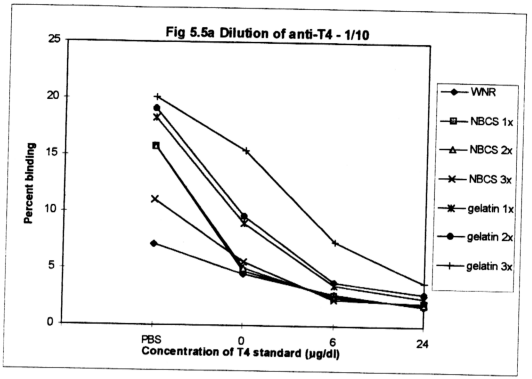


Fig 5.5a Dilution of anti-T4 - 1/10
Percent binding
25
20
15
10
5
0
PBS
0
6
24
Concentration of T4 standard (µg/dl)
WNR
NBCS 1x
NBCS 2x
NBCS 3x
gelatin 1x
gelatin 2x
gelatin 3x

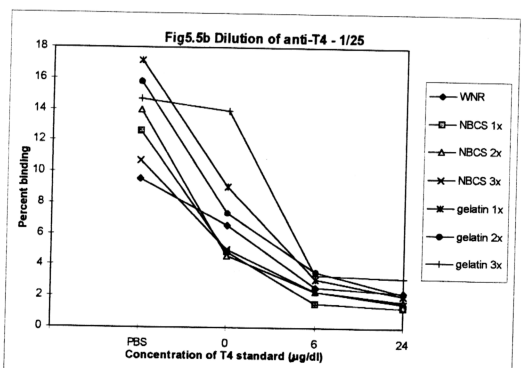


Fig5.5b Dilution of anti-T4 - 1/25
Percent binding
18
16
14
12
10
8
6
4
2
0
PBS
0
6
24
Concentration of T4 standard (µg/dl)
WNR
NBCS 1x
NBCS 2x
NBCS 3x
gelatin 1x
gelatin 2x
gelatin 3x

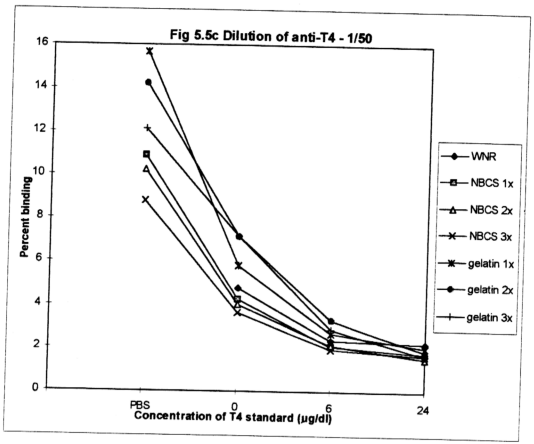


Fig 5.5c Dilution of anti-T4 - 1/50
Percent binding
16
14
12
10
8
6
4
2
0
PBS
0
6
24
Concentration of T4 standard (µg/dl)
WNR
NBCS 1x
NBCS 2x
NBCS 3x
gelatin 1x
gelatin 2x
gelatin 3x

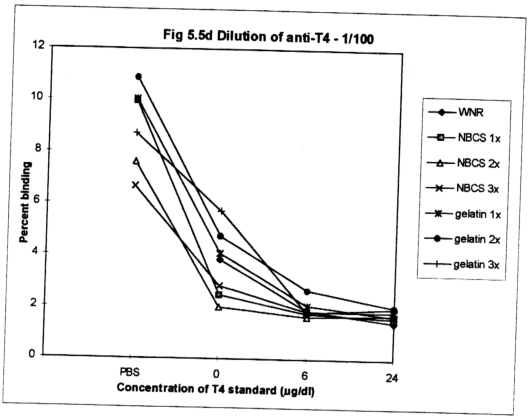


Fig 5.5d Dilution of anti-T4 - 1/100
Percent binding
12
10
8
6
4
2
0
PBS
0
6
24
Concentration of T4 standard (µg/dl)
WNR
NBCS 1x
NBCS 2x
NBCS 3x
gelatin 1x
gelatin 2x
gelatin 3x

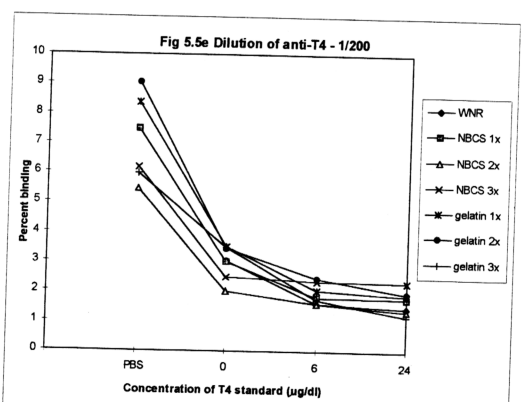


Fig 5.5e Dilution of anti-T4 - 1/200
Percent binding
10
9
8
7
6
5
4
3
2
1
0
PBS
0
6
24
Concentration of T4 standard (µg/dl)
WNR
NBCS 1x
NBCS 2x
NBCS 3x
gelatin 1x
gelatin 2x
gelatin 3x

Fig. 5.5 f Percent binding of ^{125}I anti-T4 on PP tube without precoating and WNR precoated three times with 0.5% gelatin

Unprecoated PP tube and PP tube precoated three times with 0.5% gelatin were immobilised with anti T4 at 1/25 dilution. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of standard T4 were incubated at room temperature with side-to-side rotation at 190 rpm for 60 min. The tubes were washed and the bound radioactivities were counted. WNR = NR coated tube washed five times with 1 ml 0.1M HCl and five times 1 ml of distilled water before precoating.

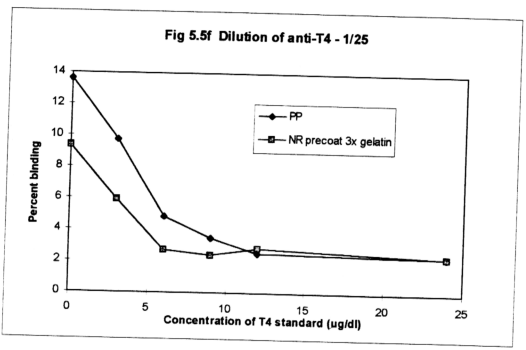
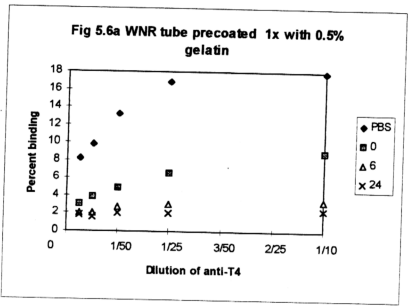
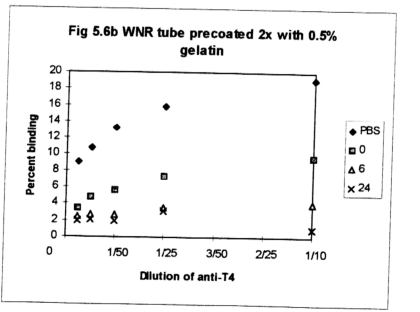


Fig. 5.6 a-f Percent binding of ^{125}I anti-T4 on WNR tube precoated surface (number of precoating X is as shown) immobilised with anti-T4 (at various dilution) and blocked with 50% NBCS

Precoated WNR tube was immobilised with anti-T4 at different dilution. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of PBS (♦) or standard T4 [concentration : zero µg/dl (□); 6 µg/dl (Δ); 24µg/dl (×)] were incubated at room temperature with side-to-side rotation at 190 rpm for 60 min.The tubes were then washed and bound radioactivities were counted. WNR = NR coated tube washed five times with 1 ml 0.1M HCl and five times 1 ml of distilled water before precoating. a. T4=1/10 b. T4=1/25 c. T4=1/50 d. T4=1/100 e. T4 =1/200 X = number of precoating





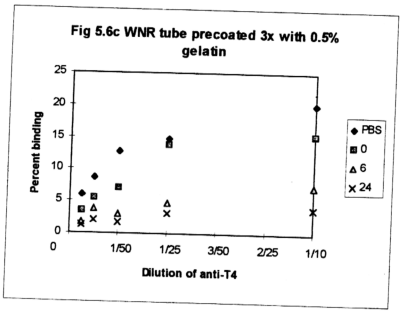


Fig 5.6c WNR tube precoated 3x with 0.5% gelatin
Percent binding
25
20
15
10
5
0
0
1/50
1/25
3/50
2/25
1/10
Dilution of anti-T4
PBS
0
6
24

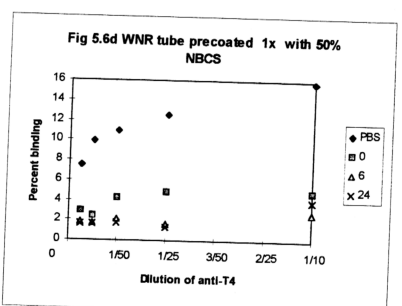


Fig 5.6d WNR tube precoated 1x with 50% NBCS
Percent binding
16
14
12
10
8
6
4
2
0
0
1/50
1/25
3/50
2/25
1/10
Dilution of anti-T4
PBS
0
6
24

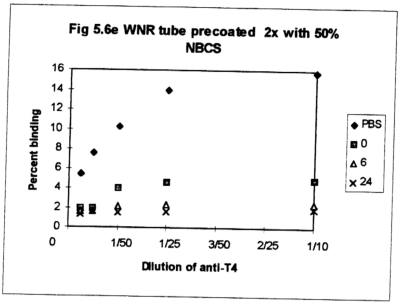


Fig 5.6e WNR tube precoated 2x with 50% NBCS
Percent binding
16
14
12
10
8
6
4
2
0
0
1/50
1/25
3/50
2/25
1/10
Dilution of anti-T4
PBS
0
6
24

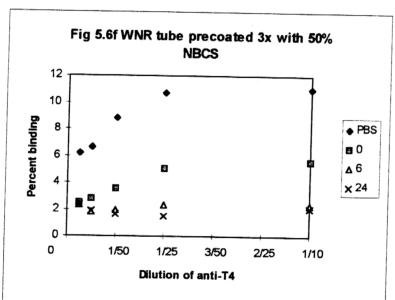


Fig 5.6f WNR tube precoated 3x with 50% NBCS
Percent binding
12
10
8
6
4
2
0
0
1/50
1/25
3/50
2/25
1/10
Dilution of anti-T4
PBS
0
6
24

Fig. 5.6g Percent binding of ^{125}I anti-T4 on PP (without precoating) surface immobilised with anti-T4 (at various dilution) and blocked with 50% NBCS

PP tubes immobilised with different concentration of anti T4. It was then blocked with 50% NBCS. 100 µl of ^{125}I T4 and 10 µl of standard T4 were incubated at room temperature with side-to-side rotation at 190 rpm for 60 min. The tube was washed and the bound radioactivities were counted.

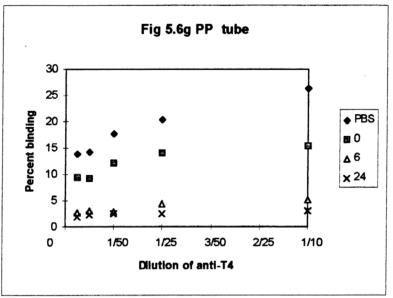
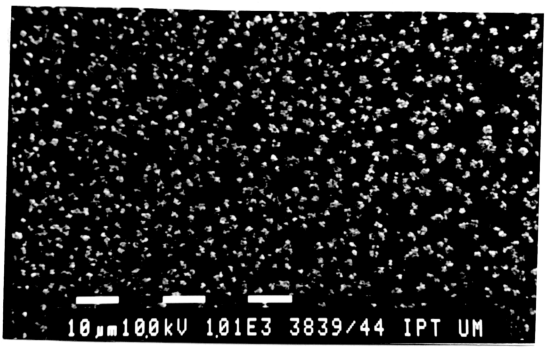


Fig. 5.7 (a) Scanning electron micrograph showing WNR tube immobilised with 0.5% gelatin (w/v). Magnification (i) $1x10^3$ (ii) $1x10^4$
200 µl of 0.5% gelatin w/v was incubated overnight at 4°C in WNR tube (NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water), the supernatant was then discarded and washed four times with 1 ml of distilled water.

a(i)



a(ii)

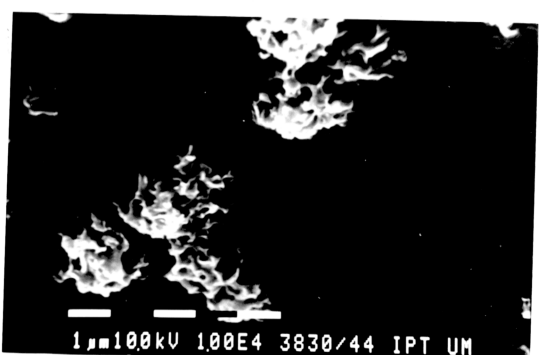
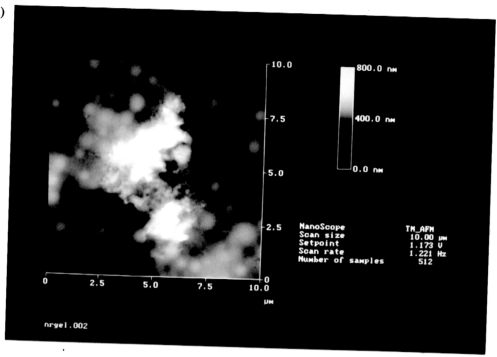


Fig. 5.7(b) Atomic force microscope images of gelatin immobilised on WNR tube.(i) top view surface (10x10 μm) (ii) three-dimension surface plot (10x10 μm)
200 μl of 0.5% gelatin w/v was incubated overnight at 4°C in WNR tube (NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water), the supernatant was then discarded and washed four times with 1 ml of distilled water.

b(i)



b(ii)

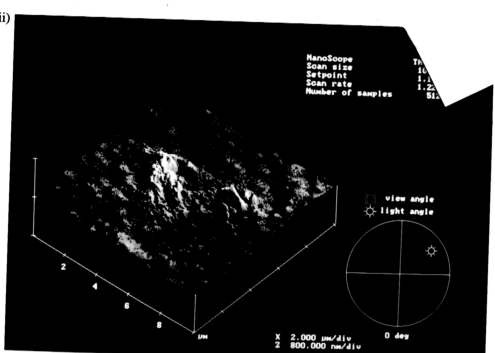
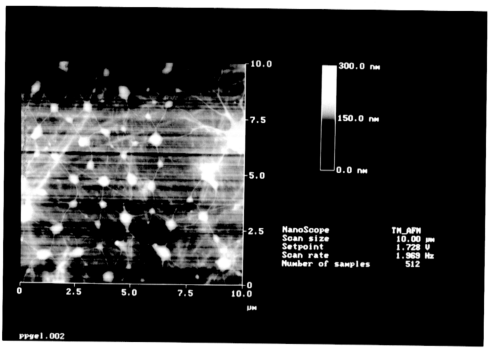


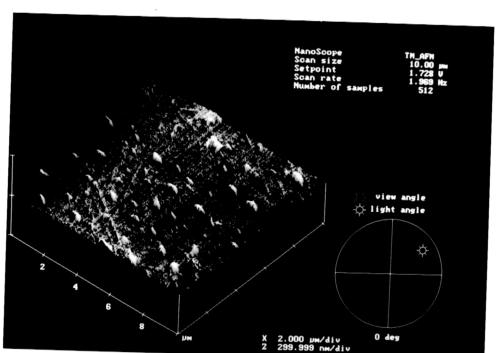
Fig. 5.8 Atomic force microscope images of gelatin immobilised on PP tube.(i) top view surface (10x10 μm) (ii) three-dimension surface plot (10x10 μm) (iii) top view surface (10x10 μm) (iv) three-dimension surface plot (10x10 μm)

200 μl of 0.5% gelatin w/v was incubated overnight at 4°C in PP tube, the supernatant was then discarded and washed four times with 1 ml of distilled water.

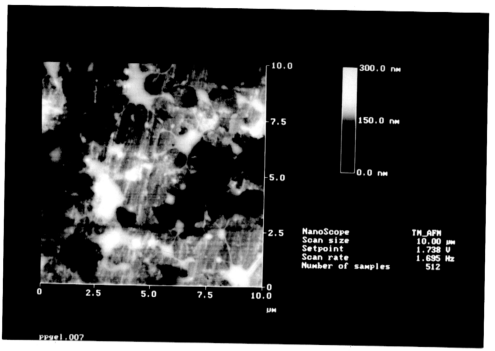
(i)



(ii)



(iii)


10.0
7.5
5.0
2.5
0
0
2.5
5.0
7.5
10.0
μm
300.0 nm
150.0 nm
0.0 nm
NanoScope
Scan size
Setpoint
Scan rate
Number of samples
TM_AFM
10.00 μm
1.738 V
1.695 Hz
512
PPpel.007

(iv)

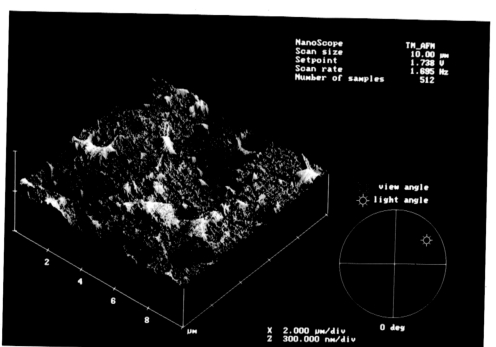
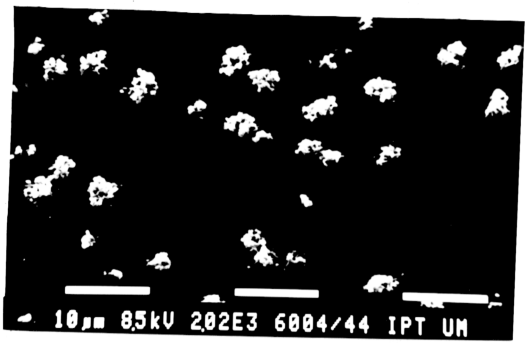

NanoScope
Scan size
Setpoint
Scan rate
Number of samples
TM_AFM
10.00 μm
1.728 V
1.695 Hz
512
view angle
light angle
2
4
6
8
μm
X 2.000 μm/div
Z 300.000 nm/div
0 deg

Fig. 5.9 (a) Scanning electron micrograph showing WNR tube immobilised with 0.5% BSA (w/v). Magnification (i) $2x10^3$ (ii) $1x10^4$

200 µl of 0.5% BSA w/v was incubated overnight at 4°C in WNR tube (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), the supernatant was then discarded and washed four times with 1 ml of distilled water.

a(i)



a(ii)

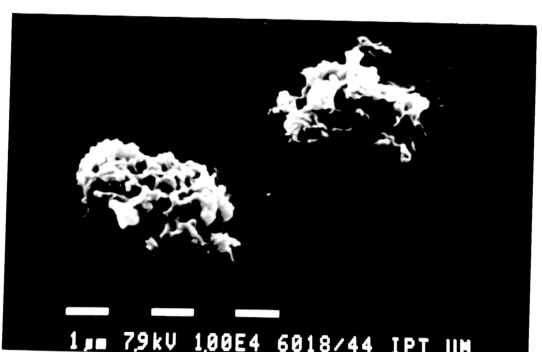
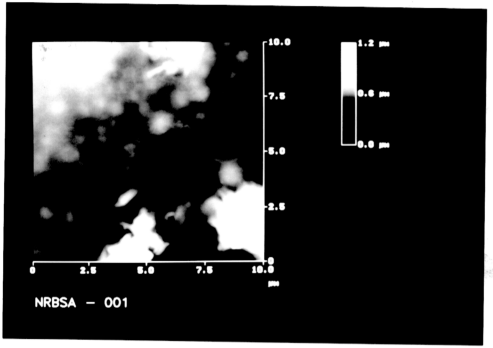


Fig. 5.9 (b) Atomic force microscope images of BSA immobilised on WNR tube.(i) top view surface (10x10μm) (ii) three-dimension surface plot (10x10 μm)
200 μl of 0.5% BSA w/v was incubated overnight at 4°C in WNR tube (NR coated tube was first washed five times with 1 ml of 0.1M HCl followed by five washes with 1 ml of distilled water), the supernatant was then discarded and washed four times with 1 ml of distilled water.

b(i)



b(ii)

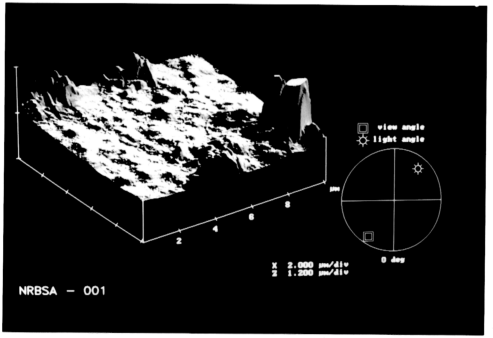
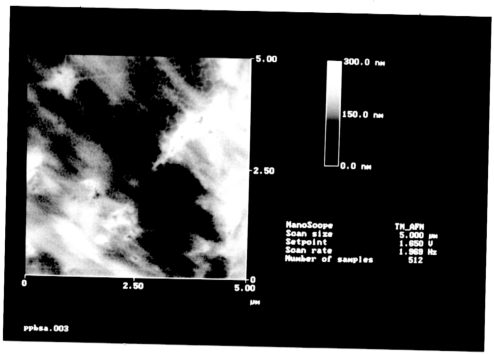


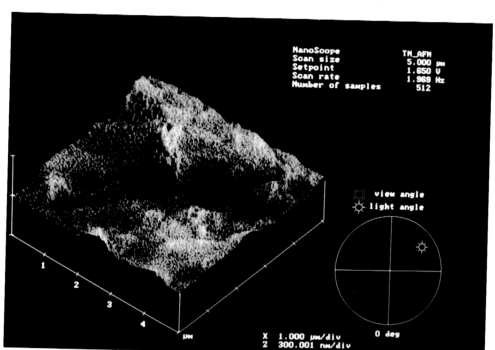
Fig. 5.10 Atomic force microscope images of BSA immobilised on PP tube.(i) top view surface (5x5 μm) (ii) three-dimension surface plot (5x5 μm).(iii) top view surface (2x2 μm) (iv) three-dimension surface plot (2x2 μm)

200 μl of 0.5% BSA w/v was incubated overnight at 4°C in PP tube, the supernatant was then discarded and washed four times with 1 ml of distilled water.

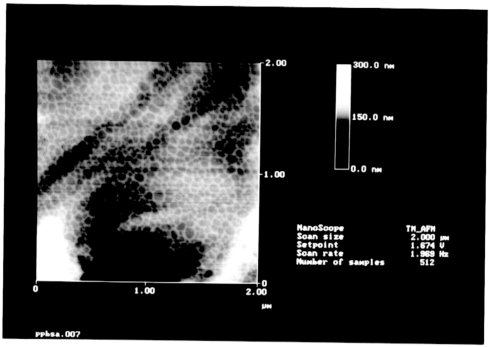
(i)



(ii)



(iii)


2.00
1.00
0
0
1.00
2.00
µm
300.0 nm
150.0 nm
0.0 nm
NanoScope
Scan size
Setpoint
Scan rate
Number of samples
TM_AFM
2.000 µm
1.674 V
1.969 Hz
512
PPhsa.007

(iv)

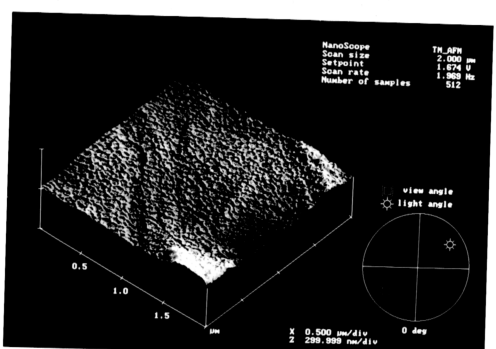

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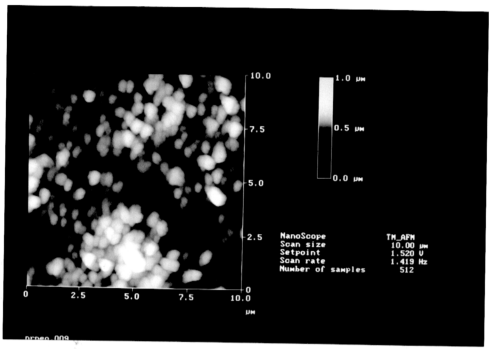
Fig. 5.11 (a) Scanning electron micrograph showing the various features of WNR tube immobilised with NEO. Magnification (i) $2x10^3$ (ii) $1x10^4$

200 µl of 50% NEO was incubated overnight at 4°C in WNR tube (NR coated tube was first washed five times with 1 ml 0.1M HCl followed by five washes with 1 ml of distilled water), the supernatant was then discarded and washed four times with 1 ml of distilled water.

a(i)



a(ii)

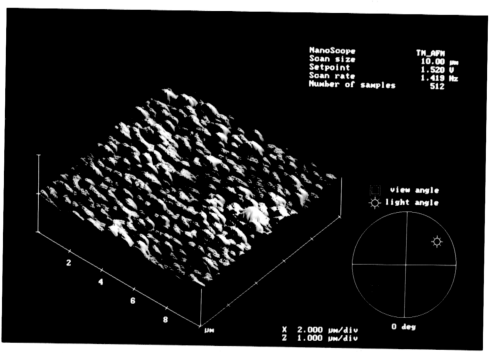
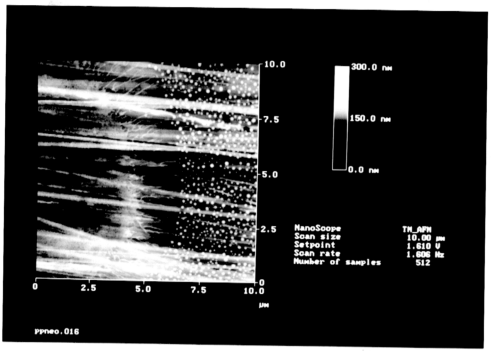


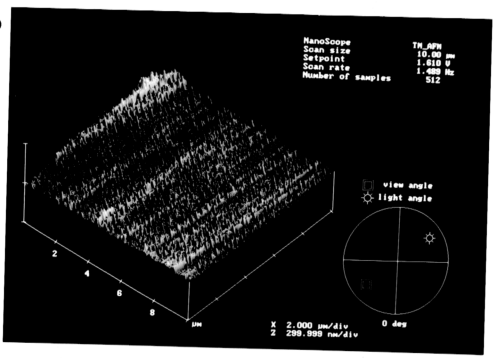
Fig. 5.11(b) Atomic force microscope images of NEO immobilised on WNR tube. (i) top view surface (10x10 μm) (ii) three-dimension surface plot (10x10 μm) iii) top view surface (2 x 2 μm) (iv) three-dimension surface plot (2 x 2 μm)

200 μl of 50% NEO was incubated overnight at 4°C in WNR tube (NR coated tube was first washed five times with 1 ml 0.1M HCl followed by five washes with 1 ml of distilled water), the supernatant was then discarded and washed four times with 1 ml of distilled water.

b(i)



b(ii)



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b(iv)

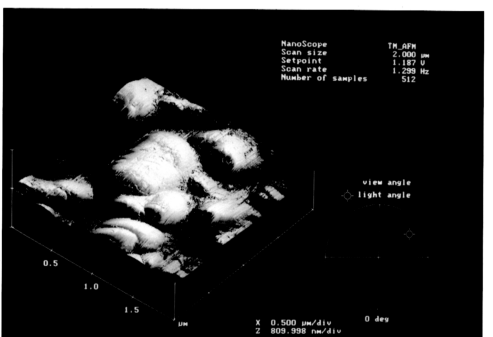
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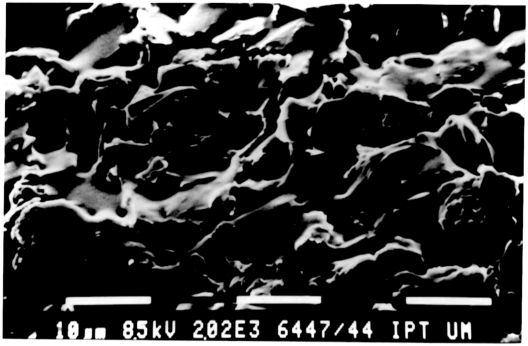
Fig. 5.12 (a) Scanning electron micrograph showing PP tube immobilised with 50% NEO. Magnification (i) $2x10^3$ (ii) $1x10^4$
200 µl of 50% NEO was incubated overnight at 4°C in PP tube, the supernatant was then discarded and washed four times with 1 ml of distilled water.

a(i)



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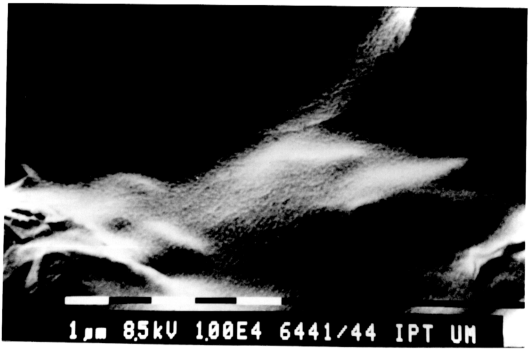
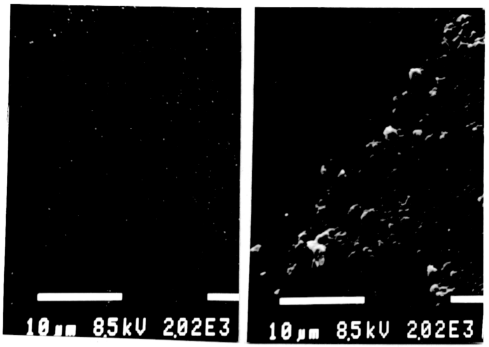


Fig. 5.12(b) Atomic force microscope images of 50% NEO immobilised on PP tube.(i) top view surface (10x10 μm) (ii) three-dimension surface plot (10x10 μm) (iii) top view surface (2x2 μm) (iv) three-dimension surface plot (2x2 μm)

200 μl of 50% NEO was incubated overnight at 4°C in PP tube, the supernatant was then discarded and washed four times with 1 ml of distilled water.

b(i)



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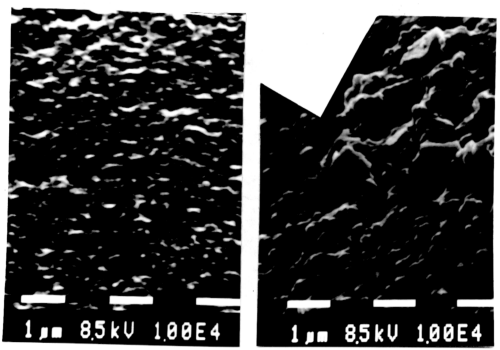
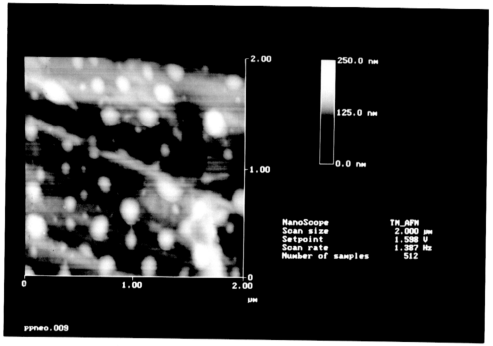


b(iii)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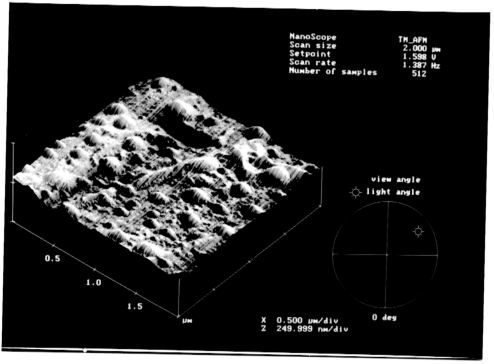
ppneo.009

b(iv)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5.4 Discussion

5.4.1 General discussion

Adsorption of protein at interface may be considered as a three-step process (MacRitchie & Alexander, 1963) :

Step1. Diffusion of solute molecules from bulk solution to the subsurface region

Step2. Adsorption of molecules from subsurfacesto sorbent/water interface. Fairly rapid and reversible uptake of proteins occurs.

Step3. Conformational rearrangement of adsorbed protein molecules. Each molecule on the surface undergoes a structural transition as a function of time that occurs in the direction of optimising protein/surface interaction. As the protein increases its interaction with the surface (and their entropy), desorption becomes less likely.

The properties of BSA and gelatin are given below.

i.)Physical and Chemical Properties of BSA (Peters,1980; Hamilton et al.,1991)

Table 5.9 Properties of BSA

Isoelectric point	pH = 4.7
Secondary structure	(3-D)*
Dimension	2.7 x 2.7 x 11.6 nm
Molecular weight	67 kDa

*This is the number of α-helices & β-sheet strands. The (3-D) notation for BSA denotes that it has three probable structural domains (Andrade et al, 1990). Andrade proposed a 'tennis ball' model, in which the three domains line up like three tennis balls within a cylinder. The N-terminus is contained in domain I and the C-terminus

in domain III. The domains differ in electrostatic charge, pH characteristics and overall stability. At pH 7, the net charge on BSA is -18. However at pH 7 each of the three domains has a distinct electrostatic charge: domain I is -10, domain II is -8, and domain III is 0.

ii.) Physical and chemical properties of gelatin

Gelatin is an amphoteric polyelectrolyte that is obtained from denatured (alkali or acid treated and heated) collagen by extraction. Gelatin, which is chemically modified collagen, contains fibrous protein (However, much of the fibrous structure is lost during modification). The fibrous proteins are the main constituents in bone and connective tissue. They are large, often sparingly soluble in water, very elongated and rather rigid and inflexible. Fibrous proteins are often built up of subunits bound closely together. Hence these proteins have neither a typical globular structure nor a random structure. The gelatin sample investigated by Kawanishi et al.(1990) was polydisperse with an average molecular weight of 300 kDa and an isoelectric point of 5. The interfacial behavior of partly denatured collagen, gelatin, can to a large extent be understood by considering the behaviour of polyelectrolytes at interfaces. It is likely that the amphoteric properties of gelatin provide the means for making the interaction between these molecules and the surfaces favourable for a wide range of surface compositions than for a synthetic homopolyelectrolyte.

5.4.2 Immobilisation of proteins on solid surfaces

Scanning electron micrographs and AFM pictures of immobilisation of precoating agent on both WNR and PP surfaces are shown in Fig (5.7-5.12). Both 0.5% BSA and 0.5% gelatin form isolated aggregates and clusters on WNR surface and large portions of the surface remained uncoated and bare (Fig 5.7 a & b, 5.9 a & b). About 23.36% and 5.44% of the area are occupied by gelatin and BSA respectively (The surface area of the different shapes of immobilised protein molecules was calculated manually using SEM micrographs with 2 x 10^3 magnification). Both NBCS and NEO formed a layer covering almost completely the WNR surface (Fig 2.19 & 5.11a). Fig 5.11b shows spherical granule shape protein of even size on NEO-coated WNR surface. On PP surface, BSA (Fig 5.10) adsorbed as dense reticulated network of aggregates while gelatin (Fig 5.8 a & b) aggregates formed larger non-uniform clusters. NBCS (Fig 2.19) and NEO (Fig 5.12 a & b) form thin homogeneous layer with strands and spherical particles on the PP surface. Some factors affecting the pattern of immobilisation are as follows:

(i) Type of sorbent

All proteins appeared as clusters on WNR surface. However on PP surface they formed homogeneous thin layer covering the solid surface (NEO and NBCS) or in small spherical / reticulated dendrite (gelatin and BSA). As discussed in Chapters 2 & 3, the different patterns of adsorption may be due to the difference in hydrophobicity of the surface or their surface structure. Bale et al.(1989) studied the influence of copolymer composition of the substrate on immunoglobulin adsorption.

The amount of saturated adsorption of IgG on various polymer surfaces (PS, PS/PMAA, PS/PAA, PS/PHEA) was different. These results suggest that small changes in the polystyrene composition of the polystyrene copolymer drastically influenced both initial adsorption of proteins and their interactions with proteins at the surface.

(ii) Concentration and molecular weight

On PP surface at lower protein concentrations of BSA and gelatin (0.5% (w/v)), separated protein aggregates (with 2-dimensional net work structure) were observed. Their apparent size increased with molecular weight of the protein. Gelatin with higher molecular weight (300 kDa) formed larger individual aggregates. However BSA which has the same concentration (0.5%w/v) but much lower molecular weight (67 kDa) showed a much denser surface coverage with smaller aggregates (Fig 5.8 & 5.10). With increase in protein concentration of non-homogeneous composition, NBCS and NEO, the networks merged, forming near continuous film of proteins covering the surface almost uniformly (Fig 2.19 & Fig 5.12).

(iii) Size of proteins

Proteins of different sizes can fill up the gaps between the adsorbed ones. NBCS and NEO containing molecules of various sizes are bound more homogeneously on WNR surface.

(iv) Types of proteins

BSA, a *soft protein with extremely hydrophilic surface (Augenstein & Ray 1957), may unfold during adsorption. It is known to adsorb onto hydrophobic solids (Andrade & Hlady1987), and the relatively soft BSA molecule may partially unfolds upon adsorption at the hydrophobic PP surface (Fig 5.7).

*Hard and soft protein
Protein structural hardness - Globular proteins with a high degree of conformational stability and a low degree of flexibility are considered to be rigid or 'hard' (MacRitchie, 1991 & Maksymiw, 1991) and tend to resist large, irreversible changes in conformation upon adsorption at interfaces. Conversely, 'soft' globular proteins are relatively flexible and can arrange their tertiary structures to facilitate adsorption at interfaces. Hard proteins are generally identified by their relatively great resistance to thermal, chemical and shear-induced denaturation.

5.4.3 Effect of precoating

There are a number of possible explanations for the diminished adsorption capacity of the immobilised binder (anti-HBs and HBsAg) after the surface was precoated with the various reagents. After the surface was precoated, there are two possible interactions between anti-HBs and HBsAg bindings with the precoated surface: a.) Interaction with the solid phase -PP or WNR surface or b.) interaction with the precoated protein layer:

(i) Reduction in the surface area

After precoating, bare surface of solid substrate for immobilisation of anti-HBs / HBsAg are reduced. Therefore the diminished binding capacity may be due to insufficient exposed substrate surface. The presence of various sizes of protein molecules in NBCS and NEO serum, resulted in more effective binding to both PP and WNR surfaces, thus bare surfaces left were relatively less compared to surface precoated with biomolecules of uniform size such as 0.5% gelatin and 0.5% BSA. On WNR surface, precoating with NEO and NBCS (without immobilisation of anti-HBs, HBsAg) which occupied larger surface area, reduced the percent binding of ^{125}I HBsAg by 42.5% and 37.5% and of ^{125}I anti-HBs by of 38.0% and 35.0% respectively, whereas precoating with 0.5% BSA and 0.5% gelatin reduced the percent binding of ^{125}I HBsAg by only 17.5%. Precoating with 0.5% BSA reduced the binding of ^{125}I anti-HBs by 3.46% (Table 5.5). However precoating with 0.5% gelatin did not result in any significant change of the percent binding of ^{125}I anti-HBs. A decrease in bare surface area reduced specific and non-specific bindings. Despite this, WNR precoated surface immobilised with HBsAg (Table 5.3) did not seem to reduce non-specific binding of ^{125}I HBsAg (with or without negative serum). On WNR surface, immobilisation of HBsAg without precoating but instead blocked with 50% NBCS was sufficient to reduce NSB (with or without HBsAg serum). One of the reasons could be HBsAg is bigger than anti-HBs and it is more difficult for it to get through the gaps between the protein molecules and immobilised on the WNR surface itself.

(ii) Both WNR and PP surfaces are hydrophobic. The hydrophobic portion of the blocker orients toward the hydrophobic surface, and, as a consequence, the hydrophilic surface of the protein would be in contact with the surrounding solution. If the outer part of the blocker is hydrophilic the originally hydrophobic surface of the solid phase surface is transformed by protein immobilisation to become hydrophilic and will be in contact with the surrounding solution. A hydrophilic surface to which anti-HBs or HBsAg become adsorbed would now possess new adsorption characteristics as the polarity of the surface is reversed and it may not favour the adsorption of the macromolecules. Thus this could weaken the binding of anti-HBs or HBsAg. However, different behavior was seen on gelatin. When the WNR surface was precoated with gelatin, the specific binding was enhanced and this could be explained as follows. Precoating with 0.5% gelatin on WNR surface (in HBsAg assay) did not cover the whole surface, which allowed the anti-HBs added to be adsorbed onto the uncoated area of the WNR surface. Further anti-HBs or HBsAg could bind to the gelatin coating on WNR surface. After the blocking process, the bare WNR surface area was reduced compared prior to precoating surface. On adding the labelled protein, less of it would be bound to the WNR bare surface, thus reducing the non-specific binding. At the same time, competition by immobilised binder protein and bare surface to adsorb labelled protein specifically and non-specifically respectively takes place. Since non specific adsorption of protein decreased owing to reduced bare WNR surface area, the specific binding would increase accordingly. On PP surface the non-specific percent binding of labelled macromolecuIs (in the presence of HBsAg negative serum) increased after

precoating the surface with 0.5% gelatin and immobilised with binder protein. However the increase in non-specific binding of ^{125}I labelled anti-HBs (in the presence of HBsAg negative serum) by PP surface precoated with gelatin and immobilised with anti-HBs was probably due to adsorption onto precoated gelatin. ^{125}I anti-HBs appears to be able to compete more effectively with other serum proteins in HBsAg negative serum for binding on to the precoated gelatin. Precoating of gelatin on PP surface also reduced the available bare PP surface for immobilisation by anti-HBs and hence the specific binding and the sensitivity of the assay.

Precoating of gelatin give higher percent binding of labelled T4 than that in its absence and this could be explained as follows: Precoating with gelatin reduced the surface area for the immobilisation of anti-T4 on WNR surface. After precoating, anti-T4 were either immobilised onto bare WNR surface or gelatin surface. The binding of labelled T4 to WNR surface gave rise to non-specific binding, while the binding to anti-T4 protein was the specific binding. Precoating reduced non specific binding as a result of reduction of bare WNR surface area. It also reduced the available effective anti-T4 binding sites by restricting the amount of anti-T4 which could be immobilised on to the precoated WNR surface. Competition of labelled hapten ^{125}I T4 and unlabelled hapten (T4) to adsorb specifically to immobilised proteins (anti-T4) was enhanced. When in the presence of precoating serum and anti-T4, the binding of labelled T4 to immobilised anti-T4 was greater as compared

to zero standard serum (without T4). This is due to non-specific competition between proteins in zero standard serum and labelled T4 for binding to immobilised anti-T4, giving rise to a larger differential change of percent binding.

(iii) Both NBCS and NEO sera, contained proteins of various sizes. Small proteins which were initially adsorbed might not desorb from the surface. Preadsorption of these proteins effectively blocked further adsorption of other proteins. It has been found that, in some cases, high molecular weight proteins like fibrinogen, fibronectin and high molecular kininogen are preferentially adsorbed relative to low molecular weight proteins such as human serum albumin (HSA) (Malmsten & Lassen, 1995). However, since the latter was present in a higher concentration, e.g. in blood, and was smaller than the former, initial adsorption kinetics would favour HSA adsorption. When a surface was in contact with NEO or NBCS, small molecules present would dominate the adsorption initially, due to their small dimensions and high concentration which favoured rapid diffusion (the limited transport of this protein) to the interface, followed by large molecules. Thus the surface was fully occupied by precoated small molecular weight proteins of various sizes (Fig. 5.11 a & 2.19 a & 2.19 b). These small molecules may not be displaced by the added anti-HBs or HBsAg and could reduce the percent binding of anti-HBs or HBsAg (Table 5.5 & 5.6). This behaviour was also observed in the adsorption of human serum albumin (HSA) which was displaced with difficulty by other proteins, such as fibrinogen, fibronectin and high molecular weight kininogen (Andrade,1985 & Norde,1986)

5.4.4 Effect of precoating on specific binding of anti-HBs and HBsAg - a comparison between PP and WNR surfaces

In general, precoating of the PP surface reduced the specific binding of ^{125}I anti-HBs (in HBsAg assay) to a greater extent than that of WNR surface (Table 5.7). These may be due to

(i) Greater conformation change occurred on protein (used in precoating) adsorbed on to the PP surface compared to that on WNR surface. This resulted in greater area covered and reduced the binding of immobilised antibody (or binder) more efficiently (Fig. 5.7-5.12 & 2.18 & 2.19 a & b). Both *hard and *soft proteins (see page 243), examined in the literature, adsorbed onto hydrophobic surfaces irrespective of the electrostatic charges of the surfaces. The argument is that the decrease in Gibbs energy by dehydration of the hydrophobic contact surface ($G = -15.7$ mJ m^{-2} at 3 °C) (Norde & Lyklema,1991) together with the entropy gain by unfolding of the protein (release of 50 amino acids from α-helix or β-sheet into a random coil corresponds to about $G = -70$ kT (2.64×10^{-19} J)(Norde & Lyklema,1991) can easily overcome any repulsive interaction. Soft proteins such as human plasma albumin (Kochwa et al., 1977; Uzgiris et al. 1976; Soderquistet al., 1980) and immunoglobulin (Hlady & Andrade, 1988) were found to unfold partially upon adsorption. It is generally accepted that the interaction between a protein and a surface increased with increasing hydrophobicity of either the surface or the protein (Lyklema,1984; Andrade, 1986, Iwamoto et al., 1985). It is expected that as the protein-surface interaction increased, the conformational changes of the adsorbed protein occurred to a bigger extent. Therefore they covered greater area and resulted

in lower percent binding of immobilised antibodies or binder. A number of examples have been shown in Chapter 2 to illustrate this fact. Other examples are :

(A). On hydrophobic surfaces, albumin has been found to be displaced by, for example, fibrinogen and IgG to a smaller extent (Andrade,1985 & Norde,1986). The marked difference between hydrophilic and hydrophobic surface is reflected in the different state of adsorption of albumin on these surfaces. Total internal reflectance flourescence spectroscopy (TIRF) was used to analyse the conformational changes of BSA upon adsorption onto silica surfaces. It was found that albumin underwent conformational changes on adsorption on both hydrophobic and hydrophilic surfaces (Hlady & Andrade, 1988; Soderquist & Walton, 1980). However conformational changes were greater on hydrophobic surface.

(B.) The extent of conformational changes of fibrinogen adsorbed on germanium, poly(hydroxyethyl methacrylate) [poly(HEMA)], Biomer, and polystyrene surfaces was studied using Fourier transform infrared spectroscopy (FTIR) coupled with attenuated total reflectance (ATR) (Donghao et al., 1991). Studies on adsorption kinetics of fibrinogen from 1 mg/ml solution showed that the amount of fibrinogen adsorbed increased as the surface became more hydrophobic. The spectra of fibrinogen in the bulk solution and of fibrinogen tightly adsorbed on germanium, poly(HEMA), Biomer(segmented polyether polyurethane), and polystyrene surfaces were treated using Fourier self deconvolution and the synthetic single-peak fitting

techniques to resolve the overlapped peaks in the amide I and II regions (Donghao et al, 1991). It was found that some α-helical structures were changed into the unordered structures and the content of β-turns increased upon adsorption. A weighted-peak shift method was used to examine the extent of the protein conformational changes upon adsorption on hydrophobic and hydrophilic surfaces (Donghao et al., 1991). The result of decreased α-helical content upon protein adsorption was consistent with the work involving other proteins (Lenk et al. 1989; Dev & Rha, 1984). It was found that the adsorption of albumin on polyurethane or soft contact lens surfaces reduced the α-helical content in the protein (Castillo et al., 1984; Pitt et al., 1987). This result indicated that adsorbed fibrinogen underwent larger degree of conformational changes as the surface hydrophobicity was increased.

(ii) The greater conformational changes on PP surface resulted in a lower degree of desorption of protein from its surface as compared to WNR surface. Therefore the antibody/antigen added cannot displace the immobilised proteins. For example, the interfacial exchange process between human serum albumin (HSA) and fibrinogen on different surfaces was investigated with an in situ ellipsometry and total internal reflection fluorescence (TIRF) spectroscopy (Malmsten & Lassen,1995). It was ellipsometrically found that the total amount adsorbed on silica made hydrophilic by preadsorption with HSA was similar to that obtained without HSA preadsorption. From TIRF, it was concluded that preadsorbed HSA was displaced, although not

completely on the addition of fibrinogen. On the other hand, preadsorbed HSA effectively blocked further adsorption of fibrinogen and IgG on hydrophobic surfaces such as methylated silica (Malmsten & Lassen, 1995). A similar conclusion was reached with hexametyldisiloxane (HMDSO) treated surface (Malmsten & Lassen, 1994). The two hydrophobic surfaces behaved in a similar way. Several investigators have found that on hydrophilic surfaces like glass, fibrinogen displaced HSA to a large extent (Andrade,1985 & Norde,1986). Depending on the nature of the conformational changes on adsorption [which are likely to be different on silica (hydrophilic) and methylated silica (hydrophobic)], this may result in different degrees of irreversibility. From the results on different types of protein coating on both WNR and PP surfaces, it could be said that all proteins (NEO, NBCS, BSA and gelatin) underwent greater conformational changes on PP as compared to WNR surface (Fig. 5.7-5.12 & 2.18-2.19). Thus additional adsorption of anti-HBs or HBsAg appeared to be one of coadsorption rather than one of classical competition adsorption on PP surface. Thus less of the anti-HBs and HBsAg can be immobilised on the PP surface compared to WNR. It is possible that the displacement of the preadsorbed protein was a statistical process, that is proteins underwent large conformational changes on adsorption, and their desorption will be exceedingly low on PP surface. The difference in displacement behaviour between hydrophilic and hydrophobic surfaces could then be due to different degree and mode of conformational changes of protein during adsorption on these surfaces.

5.5 Conclusion

Precoating reduced the non-specific binding but its effect on specific binding was dependent on the hydrophobicity of the solid phase. This was due to the low adsorption of the immobilised protein (antibody or binder) after the precoating step because the bare surface area of the substrate for adsorption was reduced. Further, the characteristics of the solid phase were changed in the process. NEO and NBCS, both at higher protein concentration and contained mixture of various size proteins, were able to adsorb onto hydrophobic solid surface more efficiently. Therefore it prevented anti-HBs or HBsAg from adsorbing on to the precoated surface. As a consequence, specific binding was reduced to a greater extent. However relatively hydrophilic precoated WNR surface with 0.5% of gelatin was able to enhance the sensitivity of HBsAg and T4 assay. In the hydrophobic PP tube, the greater conformational changes of immobilised proteins resulted in greater surface area being covered and stronger binding . Thus they were not easily displaced by addition of binder protein such as anti-HBs or HBsAg in the second immobilisation process. Therefore precoating on PP surface reduced the sensitivity of the assay to a greater extent as compared to WNR surface.

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