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COMPETITIVE ADSORPTION OF PLASMA PROTEINS AT SOLID—LIQUID INTERFACES

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SUMMARY

The competitive adsorption of human serum albumin (HSA), human immuno-γ-globulin (HIgG) and human fibrinogen (HFb) onto polystyrene (PS) at 20°C and a pH of 7.35 (phosphate-buffered saline) was studied. Protein adsorption was studied using enzyme immunoassay. The results obtained with the immunoassay were compared with those obtained using radiolabelled proteins. Recent studies revealed that the adsorption behaviour of radiolabelled proteins onto surfaces differs from that of the non-labelled proteins, which may lead to misinterpretation of adsorption data. Differences in the adsorption behaviour of the labelled proteins as compared to non-labelled proteins can possibly be explained by the formation of modified proteins during the labelling procedure as shown by ion-exchange high-performance liquid chromatography (HPLC). The competitive adsorption of HSA, HIgG and HFb onto a PS latex was studied by measuring the depletion of proteins in solution. The decrease in protein concentration in solution was determined by HPLC techniques. A strong preferential adsorption of HFb was observed with maximum adsorption values of 0.6 $\mu g/cm^2$.

INTRODUCTION

When solid materials are contacted with blood, proteins usually adsorb at the solid—liquid interface. Several investigators have tried to relate the blood compatibility of materials with the nature and the conformation of the proteins present at the interface (for reviews see refs. 1—4).

Numerous experimental techniques have been used to perform competitive protein adsorption studies at solid—liquid interfaces [5—9]. Horbett and Weathersby [5] studied the adsorption of iodine-labelled human serum albumin (HSA), human fibrinogen (HFb), human immuno-γ-globulin (HIgG), haemoglobin and prothrombin onto poly(hydroxyethyl methacrylate) (poly-HEMA) and poly(ethyl methacrylate) (poly-EMA). A mixture of proteins was present at the surface, however, HSA adsorption was not detectable. Ihlenfeld

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and Cooper [6] studied the competitive adsorption of radiolabelled proteins onto PVC and Silastic® surfaces. They concluded that fibrinogen is present on these surfaces initially, and subsequently adsorbed fibrinogen is replaced by γ-globulin and albumin. Beissinger and Leonard [7] used a multiple internal reflectance fluorescence technique to quantify multi-component adsorption of plasma proteins (HIgG and HSA) onto quartz.

Van Wagenen et al. [8] used the intrinsic fluorescence of proteins to monitor the adsorption of unlabelled proteins onto quartz surfaces. To relate total internal reflectance fluorescence (TIRF) signals to actual surface concentrations, a calibration technique with labelled proteins was developed. Gendreau et al. [9] introduced Fourier transform infrared spectroscopy (FTIR) to study the competitive adsorption of plasma proteins onto germanium. Exchange of adsorbed HSA with HFb from solution was found during the first few minutes of adsorption. Quantification of the adsorbed amount of protein is still a problem with FTIR.

A direct comparison of competitive adsorption studies is not possible. Different methods, materials and proteins are used under different adsorption conditions. Only radiolabelling of proteins, fluorescence labelling or depletion of proteins from solution can give quantitative adsorption results.

We have compared the results of the adsorption of HSA onto polystyrene latices detected by determining the depletion of protein from a solution with those obtained using radiolabelled HSA. Differences in adsorption isotherms were obtained [10—12]. A possible explanation for a different adsorption behaviour of radiolabelled HSA may be a modification of HSA during the labelling procedure. In this study, such modified albumin was detected by ion-exchange high-performance liquid chromatography (HPLC) after labelling of the protein.

The adsorption of HSA onto polystyrene microtitre plates is detected by using a two-step enzyme immunoassay. These results are also compared with those obtained using radiolabelled HSA.

Competitive adsorption data of HSA, HIgG and HFb onto polystyrene microtitre plates and polystyrene latex were obtained at 20°C and at a pH of 7.35 using phosphate-buffered saline (PBS) as a solvent. Using depletion measurements with the latex, quantitative data for the adsorption of plasma proteins at solid—liquid interfaces can be obtained.

EXPERIMENTAL

Materials

A high-performance liquid chromatograph (Waters Assoc.) was used, with a Toyo Soda Micropak TSK-Gel 3000 SW column or a 545 TSK anion-exchange column. A UV spectrophotometer (UV Cord, LKB) equipped with a microflow-through cell was used as a detector.

Polystyrene microtitre plates (110 wells) were obtained from Organon Teknika (Turnhout, Belgium). The available surface area of each well, after filling with a 75-μl protein solution, is 1.9 cm^2.

Polystyrene latex (Lot No. 41932) was obtained from Serva and characterized as described earlier [13]. Specific surface area 15 m^2/g; poly-

styrene content 10% (w/w); density 1.0 g/l; diameter latex particles 399 nm according to scanning electron microscopy (SEM).

Human serum albumin (Batch No. A9511) was obtained from Sigma, human immuno-γ-globulin was received from the Netherlands Red Cross Blood Transfusion Service (CLB) and human fibrinogen (Batch No. 65313) was obtained from KABI (Stockholm, Sweden).

HSA, HIgG and HFb were first characterized by immunoelectrophoresis, which did not show the presence of other proteins. HSA, HFb and HIgG contain different fractions of dimer and high-molecular-weight components, which was shown by polyacrylamide gel electrophoresis (PAGE) and HPLC (using a size-exclusion column 3000 SW from Toyo Soda). HSA, HFb and HIgG were further purified by Sepharose 6B chromatography. It was not possible to completely remove the HIgG impurities. After purification, the proteins were stored at 4° C and used within seven days.

Methods

Radiolabelling of proteins. $Na^{125}I$ was purchased from Amersham (IMS 30), U.K. The labelling of proteins was carried out with the chloramine-T method [12]. After labelling, 75—85% of the radioactivity was incorporated into the protein, which was determined by thin-layer chromatography (TLC) [14]. Experiments were carried out with protein mixtures in PBS containing 3% labelled protein. The labelled proteins were characterized by HPLC using a Toyo Soda size-exclusion TSK 3000 SW column and a 545 TSK Anion-exchange column.

Adsorption of proteins onto polystyrene latex. The depletion of purified HSA solutions (PBS, pH 7.35, 20°C) in contact with a PS latex was followed by either radioactivity measurements or UV spectroscopy. The adsorption method was described earlier [13].

The competitive adsorption of HSA, HFb and HIgG from mixtures of proteins onto PS latex was studied by depletion, followed by HPLC using UV spectroscopy as a detection method. The decrease in solution concentration of each protein was determined by measuring the specific peak areas of the column liquid chromatograms.

Adsorption of proteins onto polystyrene microtitre plates. The adsorption of purified HSA onto polystyrene was followed by either radioactivity measurements or a two-step enzyme immunoassay at 20° C in PBS solution (pH 7.35).

The two-step enzyme immunoassay was developed to investigate protein adsorption onto polymeric surfaces from blood plasma or from other solutions [15, 16]. In order to detect a particular protein adsorbed on a material surface, this surface was exposed to a specific antibody that will combine with the protein. Different antibodies applied for the detection of various proteins in the first step of the assay are immunoglobulins produced by animals of the same species (rabbit). In the second step, an enzyme-labelled antibody (conjugate) was added. The antibody used for the synthesis of the conjugate has been produced by animals of other species (sheep). After the second step, an enzyme substrate (urea peroxide) and a leukodye (tetramethylbenzidine) were added. The reaction product that was formed by the action of the bound

enzyme reacts with the leukodye and a dye is generated. The absorbance (at 450 nm) of the dye was a measure of the amount of protein that had been adsorbed on the solid surface. An extensive description of this method has been given by Breemhaar et al. [16]. In Fig. 1, a schematic representation of the two-step enzyme immunoassay is shown.

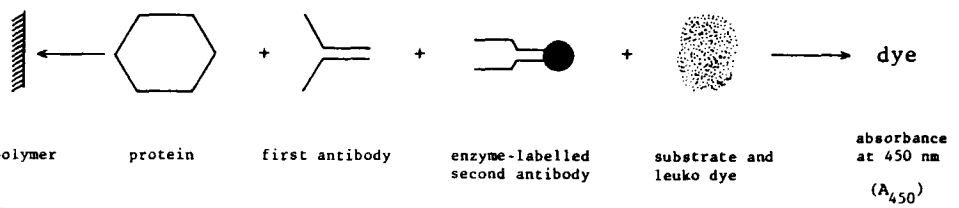


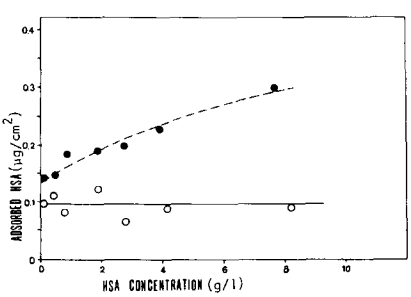
Fig. 1. Schematic representation of the two-step immunoassay.

RESULTS

The adsorption of single-labelled and non-labelled HSA, HIgG, HFb and mixtures of these proteins from PBS solutions onto polystyrene microtitre plates and polystyrene latex was measured as a function of time. Within 15 min, plateau values were observed, which were not changed after 20 h.

Adsorption isotherm of purified HSA onto polystyrene latex

This is determined by either radioactivity measurements or by UV spectroscopy. Using both detection methods, a significant difference in the isotherms is obtained (Fig. 2). The albumin is characterized before and after labelling by immunoelectrophoresis, PAGE and HPLC using a size-exclusion column (Toyo



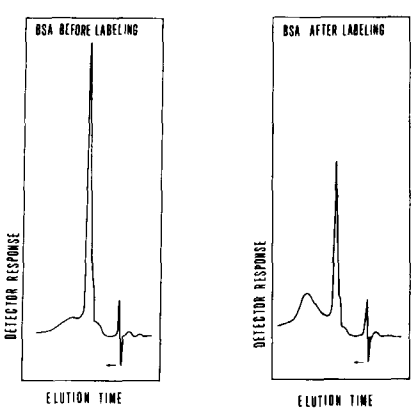


Fig. 2. Adsorption isotherms of [^{125}I]HSA onto polystyrene latex after 1 h of adsorption using UV spectroscopy (○) or radioactivity measurements (●). All protein concentrations are equilibrium concentrations.

Fig. 3. HPLC of albumin (concentration 1 g/l) before and after labelling with iodine. The column used is a 545 TSK anion-exchange column; elution buffer water—0.11 *M* ammonium acetate; pH 5.5; injection volume 50 μl; flow-rate 0.5 ml/min; UV detection at 280 nm.

Soda Micropak TSK, Gel 3000 SW). It is shown that no differences in immunoactivity or molecular weight are obtained by labelling the albumin with iodine using the chloramine-T method.

Characterization of labelled albumin using a TSK ion-exchange column shows a decrease in the unmodified peak in the chromatogram and the formation of a new component with a higher affinity to the column matrix (Fig. 3). In table I, ion-exchange HPLC analyses of BSA solutions in the presence of different labelling components are presented in relation to the formation of a new component in the solution. It is shown that the presence of chloramine T is responsible for the modification of BSA.

TABLE I

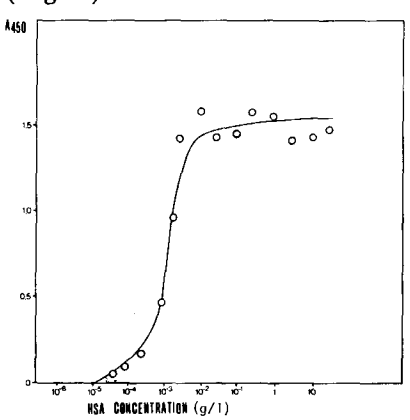
HPLC ANALYSIS OF BSA IN THE PRESENCE OF DIFFERENT LABELLING COMPONENTS

+: Present in solution; —: not present in solution.

BSA	Iodine	Chloramine-T	Sodium bisulphate	Formation of a new component
+	+	+	+	Yes
+	—	+	+	Yes
+	+	—	—	No
+	+	—	+	No

Adsorption isotherms of HSA onto polystyrene microtitre plates

The adsorption of HSA from HSA solutions onto PS surfaces obtained by serial dilution adsorption experiments of pure HSA solution, are given in Fig. 4. The absorbance ($A_{450\ nm}$) of the dye that is generated in the two-step enzyme immunoassay is representative of the amount of protein adsorbed from solution on the polymer surface. The adsorption isotherm can be compared with that obtained from radioactivity measurements under similar conditions (Fig. 5).



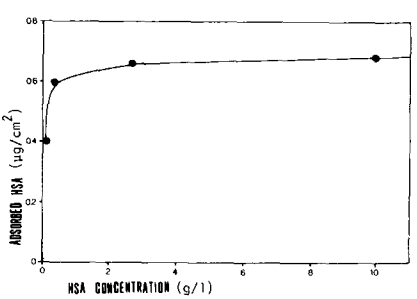


Fig. 4. Adsorption isotherm of HSA onto polystyrene determined by the two-step enzyme immunoassay. The adsorption time was 1 h. Temperature 20° C; pH 7.35 in PBS.

Fig. 5. Adsorption isotherm of [^{125}I]HSA onto polystyrene. The adsorption conditions are the same as in Fig. 4. All protein concentrations are equilibrium concentrations.

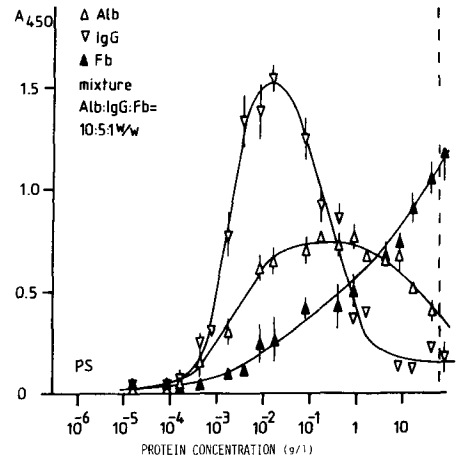


Fig. 6. Adsorption isotherms for the adsorption of HSA, HIgG and HFb onto polystyrene microtitre plates obtained by serial dilution of protein mixtures. The position of the dotted line corresponds with the physiological concentration of the three proteins in plasma.

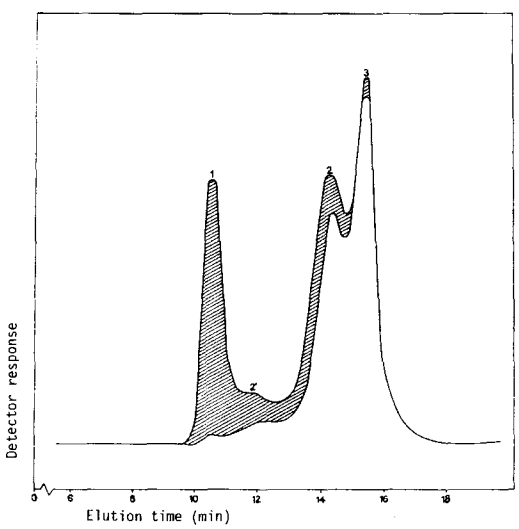


Fig. 7. HPLC profiles of a mixture of HFb (0.45 g/l, peak 1), HIgG (0.76 g/l, peaks 2 and 2') and purified HSA (2.0 g/l, peak 3) in PBS, pH 7.35, 20°C; elution rate 0.5 ml/min, elution buffer PBS. Shaded and unshaded area before adsorption, unshaded area after 4 h of adsorption time onto PS latex.

Competitive adsorption of HSA, HIgG and HFb onto polystyrene

Adsorption measurements of HSA, HIgG and HFb from protein mixtures onto polystyrene microtitre plates are depicted in Fig. 6. The amounts of HSA and HIgG adsorbed onto PS reach a maximum at protein concentrations of approx. 10^{-2} and 10^{-1} g/l, respectively. A preferential adsorption of HFb is observed from concentrated solutions of the mixed proteins (10 g/l).

The change in the HPLC profile of a mixture of HSA (2.0 g/l), HFb (0.45 g/l) and HIgG (0.76 g/l) after addition of PS latex is presented in Fig. 7. In these experiments, the PS latex surface area was 1430 cm^2 and the total buffer

volume was 1.5 ml. A strong preferential adsorption of HFb is observed. Almost all HFb originally present in the solution is adsorbed.

After adsorption, the surface concentration of HFb and HIgG are, respectively, 0.5 and 0.2 $\mu g/cm^2$; almost no HSA is adsorbed under these circumstances.

DISCUSSION

Quantitative adsorption results can be obtained by radiolabelled proteins, but the presence of an extrinsic label may alter the physical properties and subsequently the adsorption behaviour of the protein [10, 11]. This is illustrated in Fig. 3. The column liquid chromatograms show that by labelling of proteins using the chloramine-T method a part of the protein in the solution is modified in such a way that a change in ionogenic interactions exists by labelling. This indicates a difference in affinity for the surface compared with the unmodified protein. After labelling, a new component is introduced. Interpretation of the adsorption data using these labelled proteins is very complicated and leads in most cases to unreliable results. In Table I it is shown that the iodination method is responsible for the modification of a part of the protein in solution and not the incubated iodine label in the protein. The differences in the adsorption isotherms obtained via radioactivity measurements from those obtained with UV measurements can be explained in this way (Fig. 2). Radiolabelled proteins are often used to quantify the results obtained by other adsorption methods [8]. In this study, the plateau level ($A_{450\ nm} = 1.5$) of the HSA isotherm obtained by the two-step enzyme immunoassay corresponds with a surface concentration of 0.6 $\mu g/cm^2$ (Figs. 4 and 5).

The plateau level of the isotherm in Fig. 5 determined by the two-step enzyme immunoassay is already observed at protein concentrations in solution as low as 0.01 g/l. In Fig. 5, the plateau level is reached at a HSA concentration of 2 g/l. This difference can be explained by steric hindrance of the larger globulins (antibodies), necessary to detect the adsorption of smaller HSA molecules. A maximum absorption at 450 nm does not always represent a maximum surface concentration of HSA.

The two-step enzyme immunoassay is a selective and qualitative technique to determine protein adsorption on biomaterial surfaces, when these surfaces are in contact with plasma or mixtures of proteins; however, the only reliable technique for quantitative measurement of the competitive adsorption of plasma proteins onto solid—liquid surfaces is by measuring solution depletion from mixtures of proteins by UV in combination with HPLC, as shown in Fig. 7. However, applicability to materials with a low surface area is often minimal.

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