Preferential adsorption of high density lipoprotein from blood plasma onto biomaterial surfaces

W. Breeemhaar, E. Brinkman, D.J. Ellens*, T. Beugeling and A. Bantjes

Department of Chemical Technology, Biomaterials Section, Twente University of Technology, PO Box 217, Enschede, The Netherlands

*Organon Teknika B.V., Stationsplein 1, Boxtel, The Netherlands

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In the present study a two step enzyme immuno assay (EIA) was used for the investigation of the adsorption of proteins and lipoproteins from solutions and from blood plasma onto polymer surfaces. It was found that only a small adsorption of the major blood proteins occurred from plasma. Evidence is presented that the reason for this adsorption behaviour is a preferential adsorption of high density lipoprotein (HDL).

Keywords: Biomaterial, high density lipoprotein, adsorption, protein

It has generally been assumed that the first event which takes place after contact of blood with a foreign material, is the adsorption of proteins from the blood onto the material surface. An extensive amount of work, including adsorption experiments in which single protein solutions and solutions of protein mixtures were used, has been carried out to obtain more insight into this phenomenon^{1,2}.

Protein adsorption may be followed by platelet adhesion and it was reported that this process is dependent on the composition of the adsorbed protein layer. For instance, Kim *et al.*³ showed that a more pronounced platelet adhesion occurred on surfaces precoated with fibrinogen than on surfaces precoated with albumin. However, Brash and Uniyal⁴ showed that protein adsorption data, obtained from experiments with protein solutions failed to predict the adsorption behaviour of proteins from plasma and that only a restricted adsorption of the major blood proteins occurred.

We recently demonstrated that from human plasma a small adsorption of the blood proteins, albumin, immunoglobulin G and fibrinogen, takes place, presumably due to preferential adsorption of another plasma component⁵. In the present study, in which a two step enzyme immuno assay (EIA) was used, the preferential adsorption of this plasma component was investigated in more detail.

EXPERIMENTAL

Materials

Polymers. All experiments described here were carried out with multiwell poly(vinyl chloride) (PVC) microtitre

plates (No 1–222–29, Dynatech, Virginia, USA) and polystyrene (PS) microtitre plates (Hepanostika, Organon Teknika, Turnhout, Belgium). PS latex with a particle diameter of 1.10 μm was purchased from Serva (FRG).

Proteins. The human proteins, albumin (Alb), immunoglobulin G (IgG), fibrinogen (Fb), fibronectin (FN) and citrated human plasma were purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam). Antithrombin III (AT III) was a gift of the American National Red Cross Blood Research Laboratory (Bethesda, Md., USA).

Lipoproteins. The separated fractions of very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were respectively isolated from human plasma anticoagulated with EDTA (3 mmol) by density gradient ultracentrifugation⁶.

Dilutions were made in 0.02 M phosphate buffered saline (pH 7.4).

Sera. Rabbit sera directed against Alb, IgG, Fb, AT III, FN and LDL were obtained from CLB, Amsterdam. The rabbit serum directed against HDL was purchased from Behringwerke AG (Marburg, FRG). The anti-HDL and anti-LDL are directed against the protein parts of HDL and LDL, apoprotein A I (apo-A1) and apoprotein B (apo-B) respectively.

Before use the sera were diluted a 100 times in 0.02 M phosphate buffered saline, containing 1% bovine serum albumin (PBS-BSA).

Conjugate. Purified sheep anti-rabbit immunoglobulin G (ShalGg) was covalently bound to horse radish peroxidase

(Boehringer Mannheim, FRG) by a modified thiolation method according to Carlsson, Drevin and Axen⁷.

Before use the conjugate was diluted 1 : 2000 in 0.02 M Tris buffer (pH 7.4), containing 0.02 M NaCl, 1% BSA, 1% polyethylene glycol 6000 and 25 mg.l⁻¹ ShalG.

Urea peroxide (Hepanostika, Organon Teknika) was used as the enzyme substrate and 3,3', 5,5' - tetramethylbenzidine (Fluka, Switzerland) as the leukodye⁸.

Methods

Protein adsorption studies. The wells of the PVC and PS microtitre plates were contacted with 0.075 ml of a protein or plasma solution obtained by serial dilution at 20°C for 1 h. During the protein adsorption evaporation of the solution from the wells was prevented by covering the plates.

After the contact time the wells of the plates were emptied and washed 4 times with 0.15 ml 0.01 M phosphate buffered saline (pH 7.4) containing 0.05% Tween-20^R (PBS-Tw), using a multichannel syringe. During each washing procedure the wells were contacted for 30 s with PBS-Tw³.

Detection of adsorbed proteins. Solutions (0.05 ml) of the first antibody (serum) were applied to each of the washed wells of one series. To the wells of another series 0.05 ml PBS-BSA was added to obtain negative controls. After 1 h of incubation at 20°C the wells were washed 4 times with 0.15 ml PBS-Tw. Then 0.05 ml of the conjugate solution was added to each of the wells and incubated for 1 h at 20°C. After washing 4 times with 0.15 ml PBS-Tw, 0.1 ml buffer solution containing the enzyme substrate and leukodye⁸ was added to each of the wells and incubated in the dark for 30 min. at 20°C. The enzyme induced colour reaction was then stopped by adding 0.025 ml 2 M H₂SO₄ to the substrate solution in the wells of the microtitre plate and finally the absorbance at 450 nm (A₄₅₀) was determined by using a colourimeter provided with a 80 µl flow through cell (Vitatron).

The points as shown in the figures represent average absorbance values. Each of these values is the result of at least six experiments. The error bars represent the S.D.

Semi-quantitative determination of the amount of adsorbed albumin on PS microtitre plates. PS latex depletion experiments⁹ were carried out in the following way. Alb solutions (0.543 ml) of various concentrations were added to 0.457 ml of PS latex suspensions. The surface area of PS latex varied from 200–2500 cm². After an incubation time of 1 h at 20°C the suspensions were centrifuged for 30 min. at 12000 rpm. For both the original Alb solution and the clear supernatant the Alb concentration was determined by measuring the absorbances at 280 nm. The amounts of adsorbed Alb were compared with the absorbance (450 nm) of the generated dye in the corresponding enzyme immuno assays.

RESULTS AND DISCUSSION

Adsorption isotherms of Alb, IgG and Fb which were determined for the adsorption on the materials poly(vinyl chloride) (PVC) and polystyrene (PS) are shown in Figures 1(a) and 1(b). These isotherms were obtained from serial dilution experiments in which the solutions contained one single protein.

The amount of protein adsorbed from the solution on the polymer surface is expressed as the absorbance (A₄₅₀) of the dye which is generated in the two-step enzyme immuno assay. A quantitative relationship between the absorbance and the amount of protein(s) adsorbed has not yet been determined. Preliminary investigations in which results from the two-step enzyme immuno assay were compared with those obtained from PS latex depletion experiments indicate that an absorbance of 1.0 (450 nm) corresponds to about 0.07 µg adsorbed Alb. cm⁻². The values obtained from our depletion experiments are in agreement with those reported by Norde *et al.*⁹. A more extensive study is

Figure 1 Adsorption isotherms for the adsorption of Alb, IgG and Fb obtained by serial dilution of single protein solutions. (a) on PVC; (b) on PS.

necessary in order to establish a precise relationship between the absorbance of the generated dye and the amount of protein adsorbed and to see whether or not the same relationship is found for different proteins.

The adsorption isotherms of the above mentioned proteins reach a plateau value at relatively low protein concentration; a further increase of protein concentration does not result in a further increase of the absorbance of the dye.

Adsorption measurements of Alb, IgG and Fb from protein mixtures (Alb : IgG : Fb = 10 : 5 : 1, w/w) are represented in Figures 2(a) and 2(b). The ratio (w/w) of the three proteins present in the mixtures is about the same

Figure 2 Adsorption isotherms for the adsorption of Alb, IgG and Fb obtained by serial dilution of protein mixtures. The position of the dotted line corresponds with the physiological concentration of the three proteins in the plasma. (a) on PVC; (b) on PS.

as found in human plasma. The amounts of Alb and IgG adsorbed on PVC (Figure 2a) reach a maxima at protein concentrations of approximately 10^{-2} and 10^{-1} g l⁻¹ respectively and from there decrease. Fb reaches a plateau level as found for the adsorption of this protein from single protein solutions. Evidently, a preferential adsorption of Fb from concentrated solutions of the mixed proteins Fb, Alb and IgG takes place. A preferential adsorption of Fb was also found when PS microtitre plates were used (Figure 2b). These results are in agreement with those of Kim and Lee¹⁰ who also observed a preferential adsorption of Fb from mixtures of Alb, IgG and Fb on several material surfaces.

When PVC was exposed to undiluted plasma none or only a very small adsorption of Alb, IgG and Fb took place (Figure 3a). Similar results were obtained when PS was used instead of PVC (Figure 3b). The amount of adsorbed Fb, especially, is small compared with the amount of Fb adsorbed from a solution in which the concentrations of Alb, IgG and Fb are equal to those in plasma. The results mentioned above are in agreement with those of Brash and Uniyal⁴, who showed that protein adsorption data from experiments in which protein solutions were used, failed to predict protein adsorption data from plasma and that only a small adsorption of the major blood proteins occurred.

The low adsorption of protein(s) from plasma onto polymer surfaces presumably has to be attributed to the preferential adsorption of another plasma component, probably a component which is present in a relatively high concentration. Therefore adsorption experiments were carried out in which, the adsorption of FN, AT III and lipoproteins from solutions and from plasma were studied. Moreover, the effect of the presence of these components on the Fb adsorption was investigated.

The adsorption isotherms of HDL and LDL, obtained from adsorption experiments on PVC with single lipoprotein solutions are given in Figure 4. The amounts of these lipoproteins are expressed as the concentrations of the apoproteins apo-A I and apo-B respectively.

Adsorption isotherms of these lipoproteins from plasma (Figure 5) are different compared with those mentioned above. LDL (apo-B) shows an overall low adsorption level; a maximum could not be detected. At increasing plasma concentration the HDL adsorption on PVC increases and reaches a plateau value of about 80% of the one obtained for the experiments in which solutions of the single lipoprotein were used (Figures 4 and 5). Evidently, the adsorption of HDL is not much influenced by the presence of large amount of proteins in plasma. As is also shown in Figure 5 the amount of Fb on the PVC surface reaches a maximum at low plasma concentration and thereafter decreases.

The adsorption isotherms of Fb obtained by serial dilution of a mixture of Fb and VLDL show that the adsorption of Fb is not influenced by the presence of VLDL (Figures 6 and 1a). When similar experiments were carried out in which mixtures of Fb and HDL (Fb : HDL = 3.3 : 1, w/w) or Fb and LDL (Fb : LDL = 4.2 : 1, w/w) were used, the adsorption of Fb from concentrated solutions was found to be less compared with the adsorption from the pure Fb solutions (Figure 6). However, this decrease in Fb adsorption is much more pronounced when HDL is present in the mixture. This adsorption behaviour of Fb corresponds well to the one found from experiments in which plasma was used (Figure 4).

Figure 3 Adsorption isotherms for the adsorption of Alb, IgG and Fb obtained by serial dilution of citrated plasma. (a) on PVC; (b) on PS.

The effect of the presence of HDL on the adsorption of Fb onto polymer surfaces is also shown in Figure 7. In the experiments concerned solutions with a fixed concentration of Fb ($2.5 \text{ g} \cdot \text{l}^{-1}$) and an increasing concentration of HDL were contacted with PS. At low HDL concentrations ($< 10^{-5} \text{ g} \cdot \text{l}^{-1}$) the adsorption of Fb on PS is about the same as found from pure Fb solution, but at relatively high concentrations of HDL the adsorption of Fb is severely reduced.

As reported previously⁵ competition experiments as described above were also carried out with solutions of Alb which contained a fixed concentration ($0.2 \text{ g} \cdot \text{l}^{-1}$) FN or AT III and with solutions of Fb which contained a

fixed concentration of Alb ($25 \text{ g} \cdot \text{l}^{-1}$). The adsorption behaviour of Alb was not found to be influenced by the presence of FN or AT III. Moreover, it was found that minimal amounts of FN and AT III adsorbed from plasma (and mixed protein solutions) onto polymer surfaces. Also the adsorption behaviour of Fb was not influenced by the presence of Alb when the protein concentrations were about the same as found in plasma.

Figure 8 shows the result of experiments in which the Fb adsorption from solutions of Fb was measured after PS surfaces were precoated with solutions containing

Figure 4 Adsorption isotherms for the adsorption of HDL and LDL on PVC obtained by serial dilution of single lipoprotein solutions.

Figure 5 Adsorption isotherms for the adsorption of Fb, HDL and LDL on PVC obtained by serial dilution of citrated plasma.

different concentrations of HDL or LDL. PS surfaces precoated with LDL seem to be hardly altered with respect to the adsorption behaviour of Fb. Even when PS is precoated with a relatively concentrated solution of LDL (apo-B $\sim 1 \text{ g} \cdot \text{l}^{-1}$) the amount of Fb adsorbed from a solution of Fb is not very much reduced compared with the amount of Fb adsorbed onto a pure PS surface. When the PS surface is precoated with HDL a decrease of the Fb adsorption is observed, even if the HDL coating solution is very diluted.

Figure 6 Adsorption isotherms for the adsorption of Fb on PS in the presence of VLDL, LDL and HDL, respectively.

Figure 7 Adsorption of Fb on PS from mixtures with a fixed concentration of Fb ($2.5 \text{ g} \cdot \text{l}^{-1}$) and increasing concentrations of HDL.

Figure 8 Adsorption of Fb from a Fb solution ($2.5 \text{ g} \cdot \text{l}^{-1}$) on PS, precoated with LDL and HDL, respectively.

The present experimental results strongly suggest that a preferential adsorption of HDL from plasma is the cause of severely reduced adsorption of blood proteins onto polymer surfaces compared with the adsorption of these proteins from protein solutions.

As reported in the literature^{11, 12} the amount of HDL in the blood plasma of haemodialysis patients is decreased compared to normal subjects. It is conceivable that the contact of the circulating blood with large surface area of a dialyser membrane results in a relatively large adsorption of HDL. Therefore, a frequent use of artificial kidneys may be responsible for the reduced HDL levels reported. As a low HDL level has been related to the occurrence of cardiovascular disease¹¹ it would be of interest to investigate the effect of precoating biomaterials, such as used in extracorporeal blood treatment devices, with HDL in order to counteract the negative effects mentioned above.

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