JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 4 (1993) 318–326

Direct immunodetection of surface adsorbed proteins

P. H. WARKENTIN, I. LUNDSTRÖM, P. TENGVALL
Department of Physics and Measurement Technology, Laboratory of Applied Physics, Linköping University S-581 83 Linköping, Sweden

A modification of standard immunoblotting techniques gives rise to a novel method of imaging surface associated proteins without removing them from their original arrangement on the surface. The original proteins and subsequent antibody probe reactions are performed directly on the surface. The substrate is adsorbed into the nitrocellulose support which, in semi-dry contact, reacts only in the regions in which the target protein is antigenically present. The sensitivity of the method is comparable to other immunological techniques and diffusion of the resulting images is negligible resulting in reproducible reblots from the same surface with high lateral resolution. The method can be applied to a wide variety of reasonably flat surfaces. It demonstrates two dimensional protein patterns as a function of varying surface characteristics as well as differing protein detectabilities in their purified form as opposed to the same molecule as a plasma protein component adsorbed onto a surface. The method promises to be a useful tool in circumstances where surface bound proteins are not completely removable from surfaces or are lost in commonly used extraction or analytical techniques.

1. Introduction

The understanding of protein behaviour, particularly in complex plasma protein mixtures, interfacing with foreign surfaces is essential to the study of biocompatibility and foreign surface–tissue interactions. The surface arrangement as well as the composition of the extracellular matrix are important aspects, but up until now highly sensitive site-specific techniques such as autoradiography and immunofluorescence have been limited to more specialized laboratories. Analytical methods such as ELISA and SDS-PAGE are indirect, focusing on contents derived from the pre-existing surface situation. The ELISA application of Merritt *et al.* [1] improves surface related protein studies with respect to quantitative analysis but provides no information regarding the arrangement on the surface. Standard immunoblotting analytical methods presently require that target proteins be transferred away from the surface and can only be applied to permeable gels. These must also be subjected to electroblotting in order for transfer to be regarded as reasonably complete and may not be suitable for materials that are labile or fail to bind nitrocellulose. SDS-PAGE and staining requires extraction and collection from the surface and results and interpretation may be biased if the surface adherent components are not extracted and isolated *in toto*.

In our laboratory, a number of studies have been undertaken to study plasma protein adsorption on surfaces by the use of ellipsometry [2–4]. While this method has provided valuable information on protein thickness and immunospecific indications with respect to the types of protein species, it is a less sensitive technique. It is also an optical technique and, subsequently, its application is restricted to reflective surfaces.

This paper outlines a simple, effective method for specific imaging of surface adherent protein patterns directly and demonstrates circumstances in which its application can provide unique benefits in extending the understanding of single protein situations as opposed to the same proteins as plasma components. It addresses the same approach utilized by Adams *et al.* [5] in 1973, where single known proteins were analysed directly on reflective surfaces based upon interference colour, stainability, and wettability. The immunospecific imaging method, presented here, generates more precise and sensitive information without regard to any of those requirements. We have applied this technique to a number of representative proteins involved in the haemostatic mechanism although the technique has a much greater range of overall applicability. Since the detection is performed directly on the surface, the composition and arrangement are minimally disrupted giving qualitative information that is more precisely representative of the surface status. Optically flat silicon surfaces were chosen in this initial test in order to obtain results comparable to previous ellipsometric work in this laboratory [3].

2. Materials and methods

2.1. Proteins

Bovine serum albumin (BSA), a product of the Sigma Chemical Company, USA, was of a quality sufficient for use in ELISA with a fatty acid composition of

0.009%. Gelatin was Type A from porcine skin, also obtained from Sigma.

Human fibrinogen obtained from KabiVitrum AB, Sweden had a stated purity of 90% coagulable protein and a composition of 41.7% protein by weight. Calculations based on this indicated 37.5% reactive fibrinogen. A more purified form of fibrinogen was obtained from Calbiochem with a coagulability of $> 95\%$ of the protein.

The human IgG used was a commercial gammaglobulin fraction from KabiVitrum AB, Sweden of a quality suitable for human injection. Human albumin was obtained commercially as a lyophilized powder from AB Kabi. The source of purified high molecular weight kininogen (HMWK) was Calbiochem USA.

Citrated human plasma and human serum were obtained from apparently healthy donors and diluted 1/10 in phosphate buffered saline (PBS). HMWK deficient plasma was obtained from Sigma as lyophilized powder which was reconstituted immediately before use with deionized water.

2.2. Antisera

Rabbit anti-human IgG, rabbit anti-human albumin, rabbit anti-human fibrinogen, and alkaline phosphatase conjugated swine anti-rabbit IgG were all polyclonal antibody fractions from Dakopatts, Denmark.

Rabbit anti-human HMWK was a polyclonal antibody fraction from Calbiochem while goat anti-human HMWK was an antisera from Nordic, The Netherlands. Alkaline phosphatase conjugated rabbit anti-goat IgG antibodies came from Sigma.

2.3. Reagents and buffers

Nitro blue tetrazolium (NBT) (2,2′-di-p-nitrophenyl-5,5′-diphenyl-3-3′-(3,3′-dimethoxy-4,4′-diphenylene)-ditetrazolium chloride), 5-bromo-4-chloro-3-indolyl phosphate substrate (BCIP) and p-nitrophenyl phosphate were obtained from Sigma. Nitrocellulose filter paper, (NC), with a 0.2 μm pore size was obtained as sheets from Pharmacia. NaCl, KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, KCl, $NaHCO_3$, $MgCl_2$, and NaOH were all of reagent grade.

Tris buffered saline (TBS) was prepared as 20 mM Tris(hydroxymethyl)-aminomethane, 0.5 M NaCl, adjusted to pH 7.5 with HCl. Phosphate buffered saline, (PBS) was prepared as 8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KCl, and 0.2 g NaN_3. This was brought to 1 l, pH 7.4 without adjusting.

2.4. ELISAs

ELISAs were performed on 96 well Nunc Maxisorp plates. The standard ELISA assay buffer and ELISA wash buffers were used in some assays while other assays involving surface adsorbed proteins, as opposed to surface coated proteins, employed the same buffer in the absence of Tween 20. ELISA Assay buffer consisted of PBS, pH 7.4, with 0.05% Tween 20 and 0.5% BSA (0.5 g). ELISA wash buffer was PBS, pH 7.4, with 0.5% Tween 20 (0.5 ml). Diethanolamine buffer was prepared as 10% diethanolamine containing 0.2 g NaN_3 and 100 mg $MgCl_2:6H_2O$, pH to 9.8 with HCl, brought to a volume of 1 l and used to prepare 1 mg ml^{-1} of p-nitrophenylphosphate substrate solutions.

2.5. Solid surfaces

Silicon wafers were used in hydrophilic and hydrophobic flat surface experiments. Commercially produced P-type (100) boron doped wafers with a diameter of 76.2 ± 0.5 mm were obtained from Okmetic and cut to required sizes. Silicon gradient surfaces, 10 mm × 37 mm, were also cut from the same silicon wafers. These were treated as described below to obtain the desired surface characteristics.

2.6. Silicon surface treatments

Silicon surfaces were cleaned and made hydrophilic by heating to 80 °C in a 1:1:5 (volume) solution of $NH_3:H_2O_2:H_2O$ (deionized). They were washed three times in deionized water heated to 80 °C in a 1:1:6 solution (volume) of $HCl:H_2O_2:H_2O$ (deionized) and finally washed three times in deionized water and stored until use in acidified H_2O.

The above surfaces were rendered hydrophobic by first washing and drying in an oven or under nitrogen. They were then incubated for 20–30 min in a xylene solution containing 1% dichlorodimethylsilane (DDS) by volume. These surfaces were then washed sequentially in ethanol, xylene, and ethanol and stored in ethanol until use.

Gradient surfaces were produced using silicon wafers using the method of Elwing *et al.* [4]. Silicon wafers were cut into sizes of 10 mm × 37 mm and made hydrophilic as indicated above. These were placed vertically in a container filled with xylene and a 0.05% solution of DDS in trichloroethylene which was carefully layered below the xylene phase and permitted the DDS to diffuse into the upper phase for 90 min. The contents were then drained from the bottom and the surfaces washed sequentially with ethanol, trichloroethylene, and ethanol and finally dried under nitrogen stream prior to use.

2.7. Determination of immunospecificity

Initial experiments were performed on the purified human proteins; albumin, IgG, fibrinogen, and HMWK as well as human plasma. All proteins were brought to 1/10 physiological concentrations in PBS as reported in the review of Andrade and Hlady [6] or as indicated by Müller-Esterl [7] with respect to human HMWK. Specifically, albumin was used at 4.0 mg ml^{-1}, IgG at 1.25 mg ml^{-1}, and fibrinogen at 0.25 mg ml^{-1}. HMWK was diluted to final working concentration of 0.0074 mg ml^{-1}. Plasma was diluted 1:9 in PBS for comparisons with the single protein solutions.

Hydrophilic and hydrophobic surfaces 1.0 cm × 1.0 cm were incubated four per protein or plasma

solution with a volume of 1 ml cm^{-2} for 1 h. These were then washed three times in TBS and blocked in a 1% solution of BSA in TBS (except for albumin treated surfaces and samples of each of the hydrophilic and hydrophobic plasma treated surfaces which were blocked with 0.1% gelatin) for 1 h. After washing once more, one surface of each was placed in the following antisera diluted in TBS and 0.1% of the appropriate blocking agent overnight at room temperature: rabbit anti-human albumin, 1:1000; rabbit anti-human IgG, 1:1000; rabbit anti-human fibrinogen, 1:2500; and rabbit anti-human HMWK, 1:100. Surfaces were washed three times in TBS and incubated for 2 h at 37 °C in swine anti-rabbit IgG in TBS containing 1% BSA in all assays except those for albumin, in which case, 0.1% gelatin was used.

After the second antibody treatment, surfaces were washed three times in TBS and rinsed in deionized water. Surface thickness was read after every incubation step by ellipsometry and surfaces were then subjected to the NBT/BCIP direct imaging technique.

2.8. Comparison of TBS versus PBS in detection of HMWK

In order to determine if the choice of buffer was affecting the detection using the imaging technique an experiment to compare images obtained from discrete hydrophobic and hydrophilic surfaces with respect to the two buffers was performed. Triplicate 1 cm × 1 cm surfaces were prepared. These were incubated with 1 ml solutions of the 1/10 normal plasma or purified HMWK in PBS for 1 h at room temperature. These were then washed in either PBS or TBS and then incubated for 1 h in the same buffers supplemented with 1% BSA. Surfaces which were not protein treated were also blocked with BSA as a negative control. Surfaces were washed once more with the appropriate buffer and incubated overnight at room temperature with 1:100 rabbit anti-HMWK in either PBS or TBS with 0.1% BSA. They were again washed with the appropriate buffer and incubated with alkaline phosphatase conjugated-swine anti-rabbit IgG in either PBS or TBS containing 1% BSA for 2 h at room temperature. After washing with deionized water and drying under nitrogen, the surfaces were read by ellipsometry to establish surface protein thicknesses and then imaged using the NBT/BCIP direct imaging method.

2.9. Anti-HMWK versus fibrinogen

Experiments were performed in order to establish if the anti-HMWK was reacting with some component of fibrinogen. ELISA plates were coated with 0.01 mg ml^{-1} of either purified fibrinogen or purified HMWK in coating buffer overnight and then washed 3 × 300 µl/well with PBS containing 0.5% Tween 20 and blocked with 1% BSA in PBS containing 0.5% Tween 20 for 1 h. 1:1 titrations of goat anti-human HMWK were performed in triplicate beginning with a 1:100 dilution of the antisera in 0.1% BSA, 0.5% Tween 20 in PBS, 100 µl/well. Plates were incubated overnight at room temperature and washed 3 × 300 µl/well. Wells were then incubated for 2 h at 37 °C, 100 µl/well of 1:2500 rabbit anti-goat IgG conjugated to alkaline phosphatase in 1% BSA, 0.5% Tween 20 in PBS, 100 µl/well. The wells were once again washed in the wash buffer followed by a wash in PBS only and incubated in 100 µl/well of a 1 mg ml^{-1} o-nitrophenyl phosphate in carbonate buffer, pH 9.6, substrate solution for 45 min, stopped with 50 µl of 3 M NaOH and read at 405 nm.

2.10. Direct imaging method

An overview of the method used is given in Fig. 1. Nitrocellulose (NC) sheets were obtained from Pharmacia and were cut to appropriate sizes to serve as support for the Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate. For 1 cm^2 flat surfaces, 1.5 cm × 1.5 cm papers were used to overlay the entire surface. Silicon gradient surfaces required NC papers of 1.5 cm × 4.5 cm. In the case of small well ELISA plates (inside diameter 6.2 mm), paper punch disks of NC (6.0 mm diameter) were produced.

Alkaline phosphatase detection was a modification of basic methods used in immunoblotting procedures [8]. In this instance however, since all protein and antibody reactions occurred on the surface to be analysed, the nitrocellulose filter papers were incubated in solutions of NBT and BCIP prior to coming into contact with the analytical surface as follows:

Nitro blue tetrazolium (Sigma N 6876) was dissolved in 140 µl dimethylformamide (DMF) and 60 µl deionized H_2O in a glass test tube. In a separate solution 3 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was dissolved in 200 µl DMF (Sigma B-85039) in a glass test tube. Both solutions were mixed with 20 ml of Carbonate buffer, pH 9.8 (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$ adjusted with NaOH) and nitrocellulose papers were added in a ratio of 1.25 ml of substrate solution per cm^2 of nitrocellulose and allowed to sit for 10 min prior to application.

Application is 'semi-dry' such that papers are glossy, but not dripping. Papers are placed in direct firm contact with the surface being analysed for 20 min and covered to avoid drying. The nitrocellulose sheets are then removed and rinsed thoroughly with deionized water to stop the reaction.

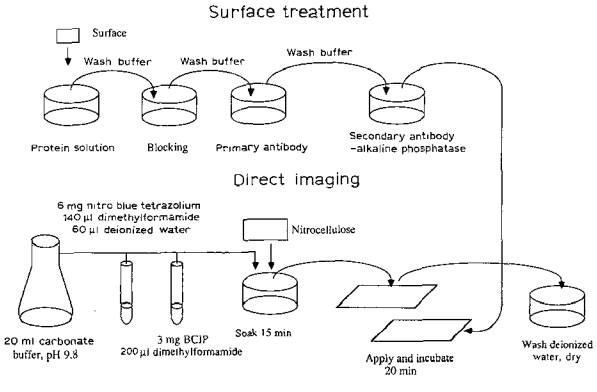

Figure 1 Schematic outline of the direct imaging technique.

2.11. Adsorption of NBT by nitrocellulose

In order to establish the degree of adsorption of the substrate an experiment was undertaken in which an initial concentration of 30 mg ml^{-1} NBT, 15 mg ml^{-1} BCIP was prepared in carbonate buffer, pH 9.8. This was then serially diluted out 1:1 in the same buffer. Since the NBT component is chromogenic, adsorption was determined as OD 405 before and after incubation of 2.5 cm × 0.5 cm nitrocellulose strips in the NBT/BCIP solution for 15 min.

2.12. Sensitivity of the method

A non-competitive inhibition ELISA was used to determine the amount of protein adsorbed to polystyrene surfaces and this was compared to proteins bound to surfaces prepared by standard ELISA coating techniques surfaces with respect to fibrinogen. In the case of fibrinogen coated surfaces an 0.1 µg ml^{-1} solution of purified fibrinogen obtained from Calbiochem dissolved in coating buffer, pH 9.6; 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3, and brought to a final volume of 1 l with deionized H_2O. This was incubated overnight, at 4 °C. The plates were then washed with 3 × 300 µl/well of PBS containing 0.5% Tween 20, pH 7.4, dried and blocked with 300 µl/well with 1% BSA and 0.5% Tween 20 in PBS, pH 7.4, for 1 h or more. These were washed once more in wash buffer just prior to use. In fibrinogen adsorbed plates, the same basic procedure was employed except that fibrinogen was prepared in PBS, pH 7.4, only to a concentration of 1.0 mg ml^{-1} for 1 h and all wash and blocking procedures were performed in the absence of Tween 20.

Once plates were prepared in this manner inhibition curves were performed. An ideal antibody concentration was determined by serially diluting stock antibody 1:1 to extinction. Triplicates of these were added 100 µl/well to the plates, incubated for 2 h at 37 °C, and washed 3 × 300 µl in PBS/Tween 20 and shaken dry. Wells were incubated in 100 µl/well volume of an excess (1:2500) of SwaRbIgG-alkaline phosphate for 2 h at 37 °C, washed 3 × 300 µl in PBS/Tween 20 and shaken dry. Wells were incubated with 100 µl/well of 1 mg ml^{-1} p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8 for 20 min. The reaction was stopped by the addition of 50 µl/well of 3 M NaOH. An antibody titration was chosen as 80% maximal binding to surface bound antigen. This represented a high signal that was on the linearly decreasing portion of the curve where inhibition would be proportional to decrease in OD. The inhibition of this binding was done by preincubating concentrations of antibody in free fibrinogen at increasing dilutions beginning with a concentration of 2 mg ml^{-1} final for 1 h at 37 °C prior to adding to the protein treated ELISA plates. The protein bound to the surface was determined as the point at which the binding was 50% of maximum at which point the amount of inhibitor protein is equal to the amount of surface bound protein assuming that the antigenicity of the two molecules is not affected.

2.13. Determination of resolution of the direct imaging method

In order to more precisely determine if images diffuse from the proteins as they are adsorbed to the surface, a silicon wafer was made hydrophobic as previously described. A 1.25 mg ml^{-1} solution of IgG in PBS was applied to filter paper to saturation. A clean rubber stamp was applied to the filter paper for 15 s and then to the silicon flat surface for 15 s. The wafer was rinsed immediately in PBS and then blocked for 1 h in TBS containing 1% BSA. It was washed 3 × 5 min in 20 ml TBS and then incubated in 1:1000 rabbit anti-human IgG containing 0.1% BSA at 37 °C for 2 h. The silicon wafer was washed for 3 × 5 min in TBS and then incubated for 2 h at 37 °C in 1:2500 alkaline phosphatase conjugated swine anti-rabbit IgG containing 1% BSA. The surface was again washed 3×5 min in TBS and rinsed once with deionized water and subjected to imaging.

3. Results

The initial screening of various purified proteins and citrated plasma, all at 10% normal physiological concentrations, against specific antibodies indicated that, overall, the method was effective in establishing positives for purified proteins (Figs 2 and 3). The plasma treated surfaces indicated the presence of all proteins being surveyed for. All purified proteins were positive with the exception of HMWK on hydrophilic silicon. While some chromogenic background was observed, it was minimal and could easily be distinguished from the positives. There were some differences in detection between hydrophilic and hydrophobic silicon surfaces in which the hydrophobic surfaces displayed much higher image intensity.

One significant observation concerned anti-human IgG and anti-human HMWK positive reactions on purified fibrinogen. In the case of anti-human HMWK, the fibrinogen positives were as strong or stronger than images obtained from surfaces treated with purified HMWK. Moreover, the hydrophilic silicon yielded no impression for purified HMWK treated surfaces while fibrinogen and plasma treated surfaces showed strong positives.

Another significant aspect is the effect of buffer on detectability. In further testing the lack of HMWK positive on hydrophilic silicon, an experiment was run that statistically compared ellipsometric thickness versus image intensity with respect to antibody and wash treatments in which the buffer was alternatively PBS or TBS. In Fig. 4 it is seen that in using TBS as the buffer system hydrophilic silicon shows some imaging in the BSA treated control. The plasma treated surface shows a definite positive but the purified HMWK is negative, as previously observed. When PBS replaces TBS as the buffer, there is virtually no image on the BSA control and both plasma and purified HMWK display equivalent image intensities. The hydrophobic surfaces, however, display minimal differences with respect to the different buffers, perhaps giving slightly stronger image intensities for plasma treated surfaces. Ellipsometric data on these surfaces indicate that

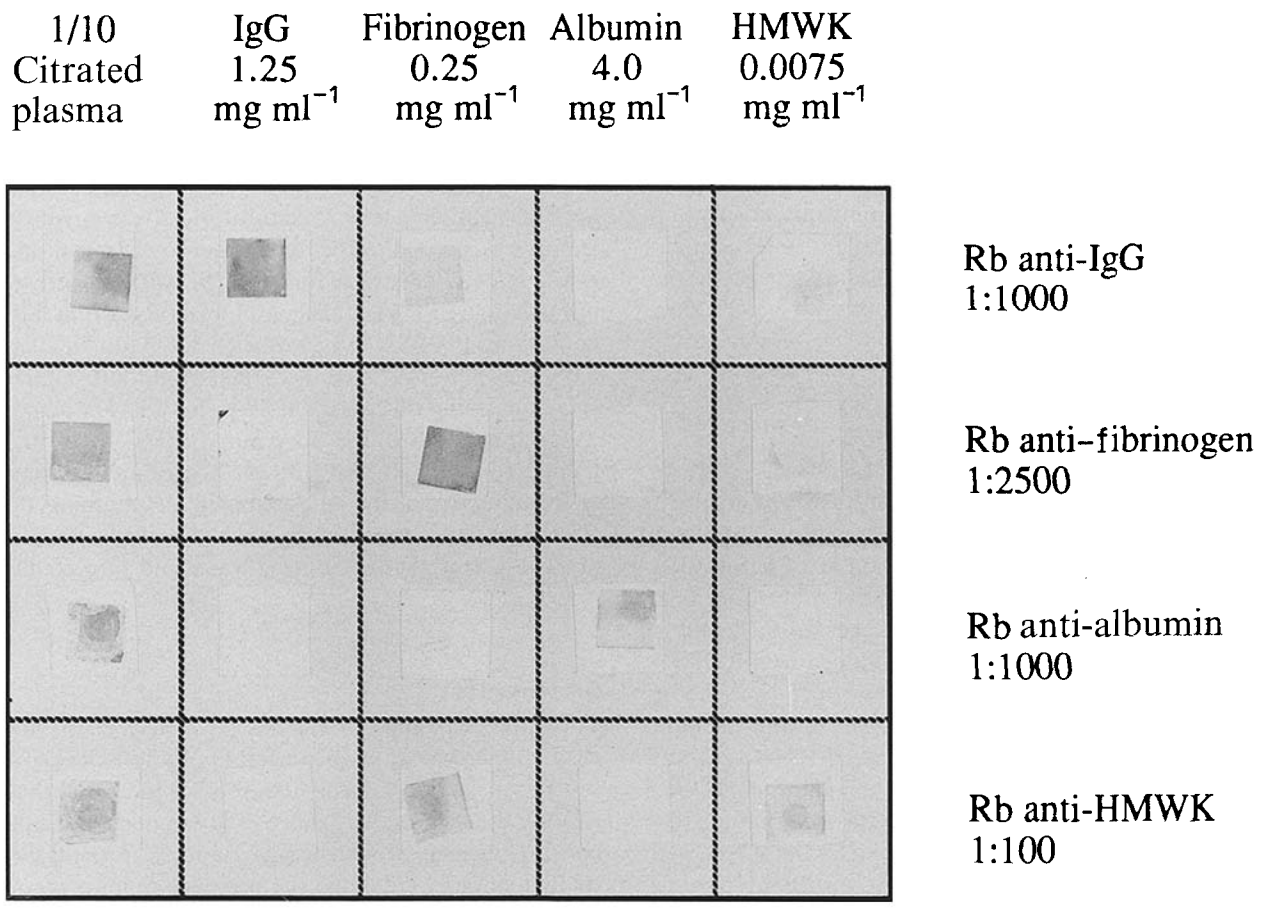

Figure 2 Detection of albumin, IgG, fibrinogen, high molecular weight kininogen as 1/10th purified antigens and 1/10th plasma components on hydrophobic silicon.

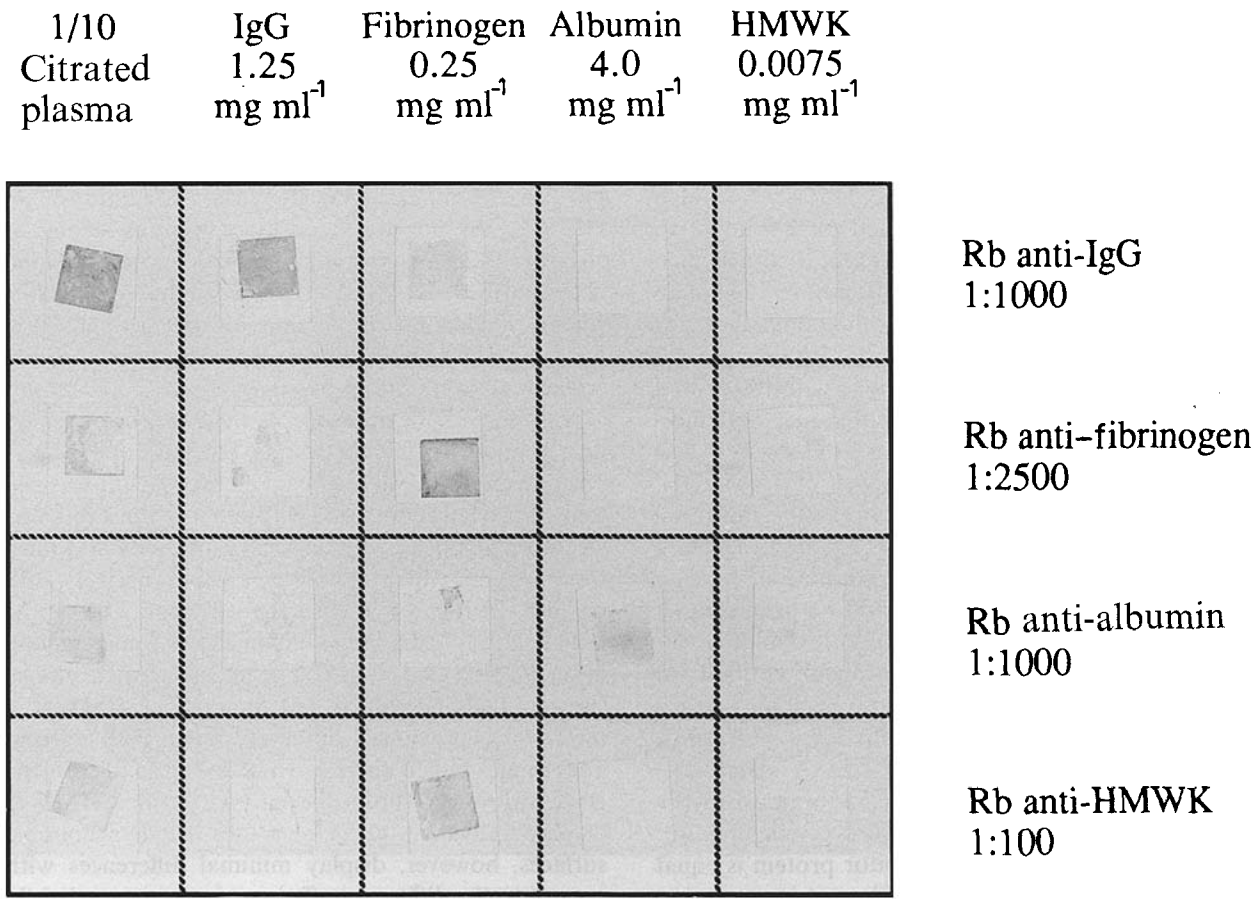

Figure 3 Detection of albumin, IgG, fibrinogen, high molecular weight kininogen as 1/10th purified antigens and 1/10th plasma components on hydrophilic silicon.

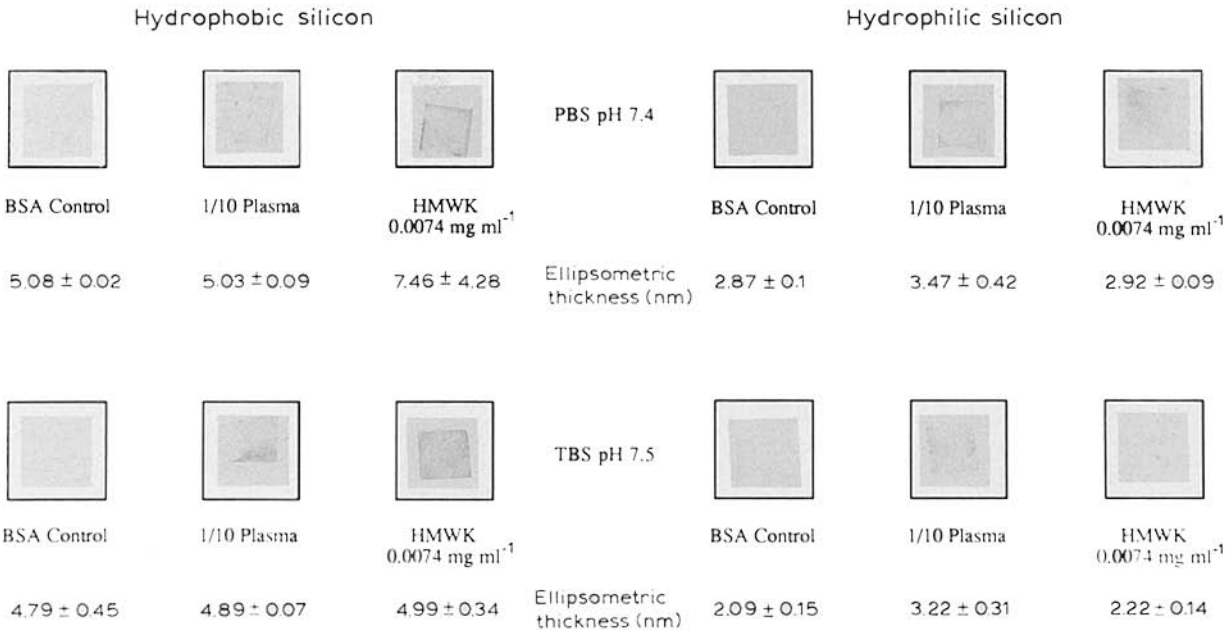

Figure 4 HMWK detection on hydrophobic and hydrophilic silicon using rabbit anti-high molecular weight kininogen in PBS and TBS buffer systems.

there was more protein overall on the hydrophobic surfaces than the hydrophilic surfaces but controls would seem to indicate that this is due more to BSA or other proteins than ellipsometrically detectable HMWK.

The results obtained for HMWK detection by rabbit anti-HMWK also indicate that images obtained, and therefore the antigenic detectability, is not necessarily reflected in the ellipsometric measurements of protein thickness. Regardless of the buffer used there is no statistical difference between control surfaces and the corresponding surfaces treated with pure HMWK at 1/10 physiological concentrations. In the PBS buffer system experiment the images are evident in the HMWK treated surfaces.

In order to determine the extent of the reaction of the anti-HMWK antisera with fibrinogen, ELISA plates were coated with 0.01 mg ml^{-1} of either fibrinogen or HMWK and standard antibody titrations (not shown) were run for the goat anti-HMWK on these two proteins. At the 1:100 antisera concentration there is a distinctive high binding to fibrinogen coated plates which can be titrated to extinction much sooner than the more specific binding to HMWK. The 50% binding to each surface was 1:60 000 for HMWK and 1:850 for fibrinogen for the cross-reactive component but at 1:100 this component competes effectively for antibody binding.

In comparing the antigenic detectability of purified proteins versus its presence in plasma, differences are immediately apparent. In determinations of the presence of IgG on silicon gradients in Fig. 5, the image appears to be more intense at the hydrophobic end but it maintains this intensity at all but the most extreme hydrophilic points, where it is reduced by half. In plasma, however, the cutoff point is much more abrupt indicating a much greater loss of antigenicity and possibly the removal of IgG on the hydrophilic side.

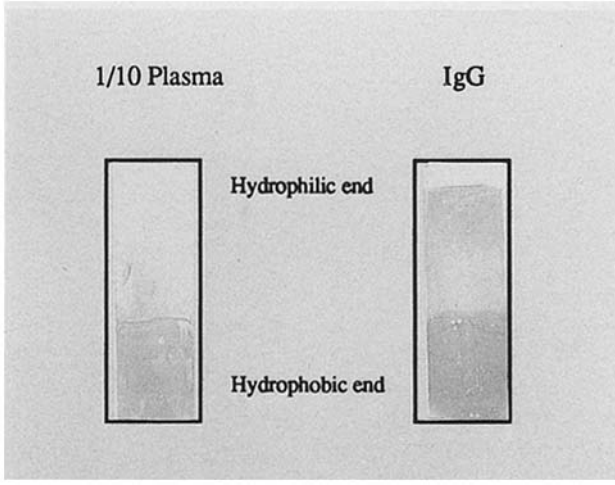

Figure 5 IgG detection on silicon gradients as a purified protein and a plasma component.

The adsorption studies of NBT by nitrocellulose failed to generate a value for saturation of the support by the substrate. Instead a relationship was determined based upon the relative concentration and the total surface area. As indicated in Fig. 6, the relationship was linear at all concentrations tested and found to be 48.3 ± 3.2 μg of NBT adsorbed per cm^2 per mg ml^{-1} initial concentration.

In determination of the limit resolution of images, we found that the images picked up off the surface were distinct to the eye (Fig. 7). Under the microscope the edge of the image could be determined as having a limit of resolution of 10 micrometres.

Non-competitive inhibition studies of fibrinogen coated and fibrinogen adsorbed polystyrene were performed. Fig. 8 establishes a 50% inhibition of antibody binding to fibrinogen adsorbed plates to be at a concentration of free inhibitory fibrinogen of 50 ng per 100 μl. This implies that, barring antigenic

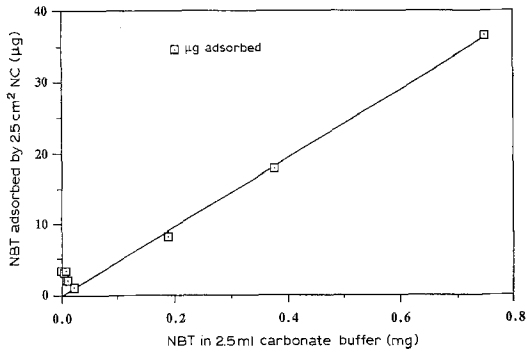

Figure 6 Graph of NBT adsorption of nitrocellulose as a function of initial concentration.

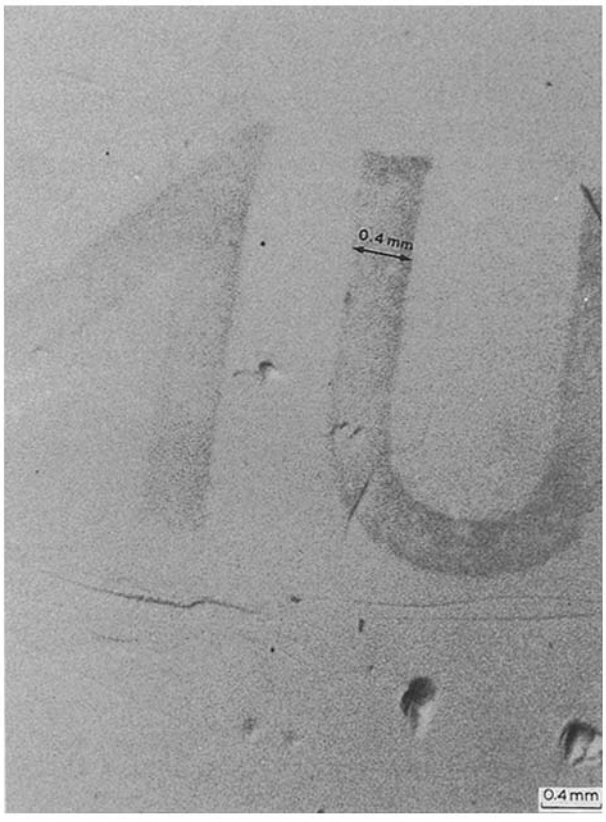

Figure 7 Enlarged immunoblot image derived from IgG stamped onto hydrophilic silicon surface.

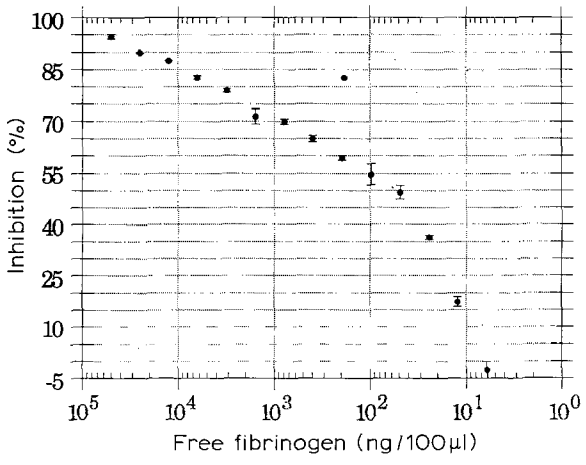

Figure 8 Imaging sensitivity from surfaces in which anti-fibrinogen is inhibited from binding surface adsorbed fibrinogen.

differences in the free and coated fibrinogen, the amount of fibrinogen on the surface is equal to the amount in solution. The surface area covered by 100 µl is calculated to be 0.877 cm^2 which would give a surface coating of 57 ng cm^{-2}. Imaging was taken from alternate row wells on the same plate and shows good impressions on these surfaces even though the surface area being tested represented 34.4% of the 50 ng exposed to ELISA testing implying that impressions were generated from 17.2 ng. In addition, impressions can be interpreted even with 80% inhibition of antibody to surface adsorbed material making it as sensitive as the ELISA in antigenic detection. Fibrinogen coated plates in a parallel ELISA experiment (data not shown) also gave a value of 57 ng/fibrinogen cm^2.

4. Discussion

The technique presented in this paper is based on principles used for most enzyme immunoassays but is novel in that the enzymatic substrate reaction takes place on a support separate from the system under investigation. All protein adsorption and subsequent immunodetection steps take place directly on the surface. Any alterations to the initial protein adsorption conditions are limited to activities of the components as they exist on the surface, the specific antisera (IgG) used to analyse the surface components, the blocking agents or the washes employed to remove non-adhering components. After treating surfaces with the proteins, surfaces are blocked with BSA or other appropriate agents. Between incubation steps surfaces are rinsed in TBS or PBS in order to flush away excess non-binding proteins.

The second antibody is a specific alkaline phosphatase conjugated molecule. The substrates are Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate which, in combination, have been reported to give the most intense images [9]. These reagents imbed the support which is overlaid directly on the surface. The colorimetric breakdown product is immobilized in a position corresponding to that of the enzyme on the surface. After 20 min the reaction is stopped by washing three times in deionized water and dried. Images do not appear to diffuse or fade. The adherent proteins, their relationship to the surface and one another, the antisera specific to the target proteins, and the alkaline phosphatase conjugated anti-antisera remain at the analysed surface.

The initial assays employed the use of TBS buffer for washes as well as primary and secondary antibody reactions with target protein molecules. Subsequent experiments also looked at the effects of using PBS as a buffer throughout the procedure. The first rationale was to harmonize experimental conditions to those employed in previous ellipsometric studies. A more important reason was initial results indicating a reduced detectability of HMWK on hydrophilic silicon. The change from TBS, pH 7.5, to PBS, pH 7.4, led to an improvement in imaging intensity for HMWK (Fig. 4) but this appeared to be coupled with a reduction in the detection of other molecules particularly

fibrinogen, being studied. Pluzek and Ramlau [9] have indicated that buffers other than PBS do not inhibit the reaction in the actual enzymatic reaction step. In this work we have used a carbonate buffer in the development step. What appears evident in this study, however, is that the choice of buffer prior to this step can also have an impact on the images observed. The exact explanation of this has not been determined as yet but other authors [10] have indicated that fibrinogen detectability may be due at least in part to preferential binding to surfaces in the presence of TBS. Giaever and Keese [11] also indicate and confirm findings by Trurnit [12] that phosphate and borate based buffers can, under certain conditions interfere with adsorption and may also cause some proteins to desorb. If this is the case, HMWK detection may benefit at the expense of fibrinogen and other proteins. In addition, antibodies are often pH and buffer dependent with respect to their binding abilities and results may reflect these differences. The overall consideration is that very significant differences in observations can result from the selection of buffers and may in fact bias interpretation of the results.

Furthermore, there are indications, to be presented separately elsewhere [13], that the BSA blocking steps normally used in immunodetection techniques may influence the detectability of certain proteins, which may or may not be buffer dependent. In addition to buffer considerations, preliminary tests indicate that the selection of support for the substrate may also affect the observed results. Although this aspect has not been thoroughly explored by the authors, initial experiments used nitrocellulose membranes, 0.20 μm, obtained from Pharmacia LKB, Sweden for the results presented in this paper. When an alternate, Nitro-Plus™ hybridization transfer membrane obtained from Micron Separations Inc., USA, was used images were extremely weak. Immobilon™ PVDF, a product of Millipore USA, after pretreatment with methanol, gave reasonable images for IgG on hydrophobic silicon. Other factors which may contribute to differences in detection are competition or displacement of target molecules by other components of mixtures, possible proteolytic activities in plasma protein systems or loss of antigenicity due to other factors.

Evidence that the immunoassay causes little, if any, desorption of the protein/antisera complex from the surface is given by the observation that detailed duplicate images can be obtained from a single surface (data not shown).

This method presents new information about localization since large relatively flat surfaces, which may vary in surface composition, can be mapped in one step for the protein under investigation. The overall specificity of the method is good, yielding only anti-IgG and anti-HMWK cross-reactivities with fibrinogen. Experiments presented here address two separate anti-HMWK sources and indicate that some component in purified fibrinogen reacts with these antibodies. In the case of the anti-IgG reactivity, the observation has been made that the fibrinogen used in initial experiments was later found to contain contaminants. Other authors such as Boisson-Vidal *et al.* [14] have noted that IgG is one of several trace contaminants of this material. Indications are that HMWK is also a contaminating component of both fibrinogen sources used in these assays (J. Brash, personal communication). As the contaminant detection would seem to indicate, the sensitivity of this technique is very high and small amounts of a specific protein can be identified on a surface dominated by a complex milieu of other proteins.

The method may have certain limitations if surface adsorbed proteins are antigenically masked by conformational changes or association with the surface or other components. These same factors, however, may equally contribute to their immunodetection. It is also possible that displacement by protein components or analytical techniques can affect observations. The limit of sensitivity for target molecules, however, compares very favourably with ELISA as shown in parallel imaging experiments performed on polystyrene ELISA plates. In fact the ELISA of surface adsorbed (Fig. 8) and surface coated plates (not shown) were essentially equivalent and are of the same order of magnitude as other proteins bound to a variety of ELISA supports [15]. The authors indicate that coating conditions, while having a pH optimum of 9.6, are predominantly a function of time and temperature. The major difference between adsorption and coating is dependent upon these parameters and the susceptibility to removal by detergents is altered perhaps by the residence time effects as suggested by Bohnert and Horbett [16].

Indications are that the level of sensitivity of direct imaging is much greater than ellipsometry since detection is restricted to antigenicity. In ellipsometry, the detection of surface thickness is the parameter under consideration and background levels must be calculated and assumed to be consistent throughout all experimental steps. One observation demonstrated here is that there may not be direct correlations between ellipsometric thickness and the antigenically detectability of target molecules (Fig. 4). If the target protein and detecting antibody thicknesses are small relative to the background, ellipsometry may indicate little or no thickness changes while the direct imaging method yields a strong positive.

The images obtained thus far have only been resolved laterally to 10 micrometres (Fig. 7). This is quite reasonable on a macroscopic scale and is an improvement from the ellipsometric data presented to date. Diffusion of adherent protein from the surface from the initial point of application was minimal or absent. It is acknowledged, however, that the methods employed in this determination are still somewhat crude. This can probably be improved upon but there may remain practical limitations based on diffusion through the remaining substrate laden liquid interface between the protein bound onto the surface and the nitrocellulose support. The information can be regarded as representative since molecules remain in their surface conformational state and can be analysed under physiological conditions.

The immunoassay can be applied to a variety of surfaces which may have been subjected to differential

modifications and to any molecule able to elicit an immune response. In this paper the method has been applied to flat hydrophobic and hydrophilic silicon surfaces in order to compare results to those obtained with ellipsometry. In experiments presented elsewhere [17], the method has been applied to various polymers as well as to chemically modified gold surfaces explanted from cardiac muscle (not shown). Individual proteins, protein mixtures, and blood plasma have also been studied to determine their interactions on surfaces and the method promises to offer new information regarding surface displacement and protein–protein interactions.

Most significantly, the method permits permanent two-dimensional visual representations of direct adherent protein surface composition, with good resolution, that fills a niche in surface analysis not previously satisfied by other methods. Its purpose is to aid and broaden interpretations of surface–protein interactions studied by other surface analytical methods.

Acknowledgements

This work was supported by the Swedish Biomaterial Consortium founded by the Swedish National Board for Industrial and Technical Development (NUTEK) and the Swedish Natural Science Research Council (NFR).

References

1. K. MERRITT, C. R. EDWARDS and S. A. BROWN, *J. Biomed. Mater. Res.* **22** (1988) 99.
2. B. WÄLIVAARA, A. ASKENDAL, H. ELWING, I. LUNDSTRÖM and P. TENGVALL, *ibid.* **26** (1992) 1205.
3. P. TENGVALL, M. LESTELIUS, B. LIEDBERG and I. LUNDSTRÖM, *Langmuir* **8** (1992) 1236.
4. H. ELWING, S. WELIN, A. ASKENDAL, U. NILSSON and I. LUNDSTRÖM, *J. Colloid Interface Sci.* **119** (1987) 203.
5. A. L. ADAMS, M. KLINGS, G. C. FISCHER and L. VROMAN, *J. Immunol. Meth.* **3** (1973) 227.
6. J. D. ANDRADE and V. HLADY, *Ann. New York Acad. Sci.* **516** (1987) 158.
7. P. W. MÜLLER-ESTERL, *Meth. Enz. Anal.* **9** (1986) 304.
8. O. J. BJERRUM and N. H. H. HEEGAARD (eds) "CRC handbook of immunoblotting of proteins" (CRC Press, Boca Raton, FL, 1988).
9. K.-L. PLUZEK and J. RAMLAU, in "CRC handbook of immunoblotting of proteins", Vol. 1, edited by O. J. Bjerrum and N. H. H. Heegaard (CRC Press, Boca Raton, FL, 1988) p. 177.
10. S. M. SLACK and T. A. HORBETT, *J. Biomater. Sci., Poly. Edn.* **2** (1991) 227.
11. I. GIAEVER and C. R. KEESE, in "Proteins at interfaces: physicochemical and biochemical studies", edited by J. L. Brash and T. A. Horbett (American Chemical Society, Washington, DC, 1987) p. 582.
12. H. J. TRURNIT, *Science* **III** (1950) 1.
13. P. H. WARKENTIN, B. WALIVAARA, I. LUNDSTRÖM and P. TENGUALL, in preparation.
14. C. BOISSON-VIDAL, J. JOZEFONVICZ and J. L. BRASH, *J. Biomed. Mater. Res.* **25** (1991) 67.
15. D. M. KEMENY and S. J. CHALLACOMBE, in "ELISA and other solid phase immunoassays", edited by D. M. Kemeny and S. J. Challacombe (John Wiley and Sons, Essex, 1988) p. 31.
16. J. L. BOHNERT and T. A. HORBETT, *J. Colloid Interface Sci.* **111** (1986) 363.
17. P. H. WARKENTIN, H. ELWING, P. TENGVALL and I. LUNDSTRÖM, Transactions of the Fourth World Biomaterials Congress, Berlin (1992) p. 195.

Received 19 October
and accepted 18 December 1992