Biophysical Journal Volume 76 May 1999 2769–2776

A New Principle for Rapid Immunoassay of Proteins Based on In Situ Precipitate-Enhanced Ellipsometry

Markus Robers,* Irma J. A. M. Rensink,# C. Erik Hack,# Lucien A. Aarden,# Chris P. M. Reutelingsperger,* Jan F. C. Glatz,* and Wim Th. Hermens*

*Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, and #Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands

ABSTRACT A new technique is presented that allows measurement of protein concentrations in the picomolar range with an assay time of only 10–20 min. The method is an enzyme-linked immunosorbent assay (ELISA), but uses in-situ ellipsometric measurement of a precipitating enzyme product instead of the usual colorimetric detection of accumulating enzyme product in solution. Quantitative validation was obtained by use of annexin V, a protein with high binding affinity for phosphatidylserine-containing phospholipid membranes, resulting in a transport-limited adsorption rate. This property was exploited to obtain a range of low surface concentrations of annexin V by timed exposures of phospholipid bilayers to known concentrations of annexin V. Using polyvinylchloride (PVC)-coated and silanized silicon slides, various versions of this technique were used for the rapid assay of fatty acid-binding protein (FABP), a recently introduced early marker for acute myocardial infarction with a normal plasma concentration below 1 nmol/l, interleukin 6 (IL-6), a cytokine with normal plasma concentrations below 1 pmol/l, and again, annexin V. A possible future application of the method in the development of a one-step ELISA is discussed.

INTRODUCTION

Detection of proteins by immunosorption techniques was first demonstrated in pioneering studies of Rothen (1947) and Vroman (1969), who combined the principles of protein recognition by specific antibodies and the use of protein adsorption to a solid surface in order to create increased local protein concentrations. The performance of these techniques was much improved by the introduction of enzyme labels (see Avrameas (1992) for a review), and together these principles produced the enzyme-linked immunosorbent assay (ELISA), now generally used for (ultra) sensitive protein determination.

In its sandwich form, this technique is explained in Fig. 1 (*left*). A reservoir, usually a well in a microtiter plate, contains a solid surface covered with a primary "catcher" antibody. The protein to be assayed (the antigen) is added to the reservoir and binds to the catcher. A second "tagging" antibody, conjugated to an enzyme, is then added and binds to the captured protein. Excess unbound conjugate is removed by washing, and "enzymatic amplification" is started by addition of a chromogenic enzyme substrate. Due to continuing substrate conversion, even a small amount of bound enzyme will ultimately become detectable by colorimetric measurement of product formation.

Various modifications of this ELISA technique are used, such as simultaneous addition of antigen and conjugate, or use of rabbit tagging antibodies, which are then recognized by a swine anti-rabbit conjugate (see Methods). Because of some aspecific binding of conjugate to the surface, a low rate of color production will also occur in the absence of antigen. It is attempted to minimize this effect by adsorption of an inert bulk protein, such as albumin or casein, thus "blocking" free surface space between the catcher antibodies. Still, this circumstance limits the observation time for color production, and only the primary incubation time of catcher and antigen can be lengthened at will in order to improve assay sensitivity. For very low protein concentrations, however, antigen adsorption to the surface will also be very slow, and ELISAs in the picomolar range usually take several hours.

An obvious simplification of this technique would be the direct detection of the amount of adsorbing antigen and conjugate, using one of the available techniques for the measurement of thin protein films on solid surfaces, such as ellipsometry, surface plasmon resonance (SPR), or total internal reflection fluorescence (TIRF). Even in a well-stirred solution, however, the maximal mass transfer constant for protein adsorption to a solid surface will not exceed $\sim 5 \times 10^{-3}$ cm/s (Corsel et al., 1986; Willems et al., 1993). If we want to measure a protein concentration of, for instance, 10 pg/ml and also want to restrict the adsorption time to 5 min (300 s), the amount adsorbed would maximally be $5 \times 10^{-3} \times 10 \times 300 = 15$ pg/cm^2. This is far below the detection limit of the mentioned techniques, and amplification of the signal is thus required.

The present study describes a protein assay meeting the requirements of high sensitivity (<100 fmol/l) and short assay times (<20 min). The method is explained in Fig. 1 (*right*) and is based on detection of surface mass by enzymatic amplification, using a precipitating product. The in-

Received for publication 2 November 1998 and in final form 25 February 1999.

Address reprint requests to Dr. Wim Th. Hermens, CARIM, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands. Tel.: +31 43 3881650; Fax: +31 43 3670916; E-mail: w.hermens@carim.unimaas.nl.

0006-3495/99/05/2769/08 $2.00

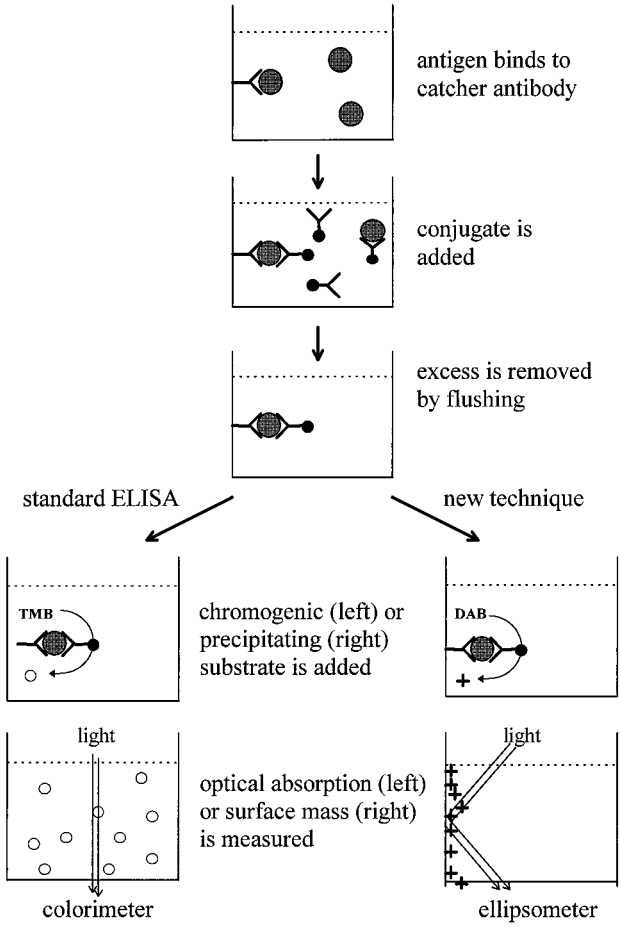

FIGURE 1 Schematic protocols for the standard ELISA technique, and for the new technique, based on precipitate-enhanced ellipsometry.

creasing mass of precipitate on the surface rapidly becomes much larger than the mass of the antigen-conjugate complex, and can be measured in situ by ellipsometry. Because of the local accumulation of enzyme product at the site of its production, this "two-dimensional ELISA" is faster than a normal ELISA, where the product is distributed over a three-dimensional volume.

The method was validated by using transport-limited adsorption of annexin V on phospholipid membranes. In this way, a range of very low but accurately known surface concentrations was obtained. The new technique was compared with existing ELISAs for annexin V and fatty acid-binding protein (FABP), a recently introduced early marker for myocardial necrosis (Glatz et al., 1994). For this comparison, silicon slides were coated with the same material as used for standard ELISA microtiter plates. A last example concerned the rapid assay of a very low (10 pg/ml) concentration of interleukin 6.

MATERIALS AND METHODS

Phospholipids and proteins

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine) and DOPS (1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Conjugates of horseradish peroxidase (HRP) with swine anti-rabbit IgG or with rabbit anti-mouse IgG (2–4 molecules of HRP/IgG molecule) were obtained from DAKO A/S (Glostrup, Denmark). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Recombinant human annexin V was prepared as described in Maurer-Fogy et al. (1988). Polyclonal rabbit and monoclonal mouse antibodies against annexin V were obtained as described in van Heerde (1994). Recombinant human heart-type fatty acid-binding protein (FABP) was a kind gift from Dr. T. Börchers, Institut für Chemo- und Biosensorik, Münster, Germany. Two different monoclonal anti-FABP IgG's, one HRP-conjugated, were prepared as described in Roos et al. (1995), and were a kind gift from Dr. W. Roos, Roche Diagnostic Systems (Basel, Switzerland). Affinity-purified human interleukin 6 (IL-6) was obtained from lipopolysaccharide-stimulated monocyte cultures as described in Brakenhoff et al. (1987). Monoclonal anti-IL-6 (CLB IL-6/16) and biotinylated sheep polyclonal anti-IL-6 were obtained as described in Brakenhof et al. (1990). Streptavidine-poly-HRP was obtained from the CLB (Amsterdam, The Netherlands). Concentrations of annexin V, FABP, and IL-6 were determined by ELISAs as described in van Heerde (1994), Wodzig et al. (1997), and Brakenhoff et al. (1990), respectively. Total assay times for these ELISAs were 120–215 min.

Buffer solutions

For comparability of results, the same buffer solutions and blocking agents as used in the existing ELISAs for the three proteins were used, that is, buffer A = 50 mM Tris-HCl buffer of pH 7.5 with 0.1 M NaCl; buffer B = buffer A with 3 mM $CaCl_2$; buffer C = buffer B with 1 g/l BSA; buffer D = buffer B with 1 g/l skimmed milk powder (Vremini "Excellent," Vreugdenhil BV, Voorthuizen, The Netherlands); buffer E = 0.1 M bicarbonate (Na_2CO_3-$NaHCO_3$) buffer of pH 9.4.

Ellipsometry

Binding of lipids, proteins, and benzidine precipitates to planar surfaces was measured by ellipsometry, as described before (Cuypers et al., 1983; Corsel et al., 1986). Briefly, light from a helium-neon laser (λ = 632.8 nm) first passes a rotatable polarizer P, then a compensator (¼-λ plate), and is reflected by a silicon slide, mounted in a quartz cuvette filled with 5 ml stirred buffer. After reflection, the light passes a second rotatable polarizer, the analyzer A, and is detected by a photodiode. The positions of P and A are adjusted automatically such that the resulting light intensity is kept minimal (null ellipsometry). The angle of incidence on the reflecting slide was 68° and a trapezoidal cuvette was used, allowing perpendicular light passage through the cuvette walls. A full description of system geometry was given in Willems et al. (1993).

Adsorption of organic material on the reflecting slide results in changes ΔP and ΔA. Normally, the adsorbed mass Γ is calculated from these changes by use of the Lorentz-Lorenz relation (Cuypers et al., 1983), but for the optical constants of the silicon slides and the organic adsorbing substances used in the present study, the adsorbed mass Γ could simply be calculated from the relation $\Gamma = 0.085 \times \Delta P$ $\mu g/cm^2$, with ΔP expressed in degrees (Giesen et al., 1993). The minimal detectable change of ΔP was 0.02°, corresponding to 1.7 ng/cm^2. Measurements were performed at room temperature (20 ± 1°C). Washing was performed in ~20 s by rapidly flushing the cuvette with 50 ml buffer under constant stirring, while keeping the fluid level (and volume) of the cuvette constant with a suction pump.

Preparation of reflecting silicon slides

Silicon wafers from Wacker Chemitronic (n-type, phosphorus-doped) were obtained from Aurel GmbH (Landsberg, Germany) and cut into slides of 4.0 × 0.8 cm. After cutting, the slides were thoroughly cleaned with detergent (Sparkleen, Calgon, Pittsburgh, PA) and deionized water (Milli-Q3 system, Millipore, Etten-Leur, The Netherlands). Thereafter they were

kept for 20 min at 80°C in 30% chromic sulfuric acid (Merck, Darmstadt, Germany), again washed with water and stored in 50% ethanol (Merck). Before use, the slides were again rinsed with detergent and deionized water, resulting in a highly hydrophilic surface. After coverage with either phospholipid or polyvinylchloride, as described below, these slides were used as reflecting surface in the ellipsometer.

Preparation of phospholipid-covered silicon slides

Small unilamellar vesicles were prepared from a nitrogen-dried mixture of 20% DOPS and 80% DOPC, dissolved to a 1 mM dispersion in buffer A, and sonicated for 10 min. Bilayers were formed by immersion of the slide in a stirred 20 μM vesicle suspension in buffer B, and resulted in a phospholipid surface mass of 0.42 $\pm$ 0.02 μg/cm^2 (mean $\pm$ SD, $n = 100$). After 5 min of incubation of the slide in the phospholipid suspension, the remaining unbound vesicles were removed by flushing with buffer B, and the slide was transported to the ellipsometer cuvette in a small holder, filled with buffer in order to prevent exposure of the membrane to air (Giesen et al., 1995).

Preparation of polyvinylchloride-coated silicon slides

Silicon slides were coated with the same material as used in the usual ELISA microtiter plates (Falcon 3912 Micro Test III, Becton Dickinson, Oxnard, CA). The upper parts of these plates were dissolved in cyclohexanone (Merck) to a 2% polyvinylchloride (PVC) solution. Hydrophilic silicon slides were immersed in this solution and then slowly retracted at a speed of 1.5 mm/min, using a mechanical dipping device. They were dried in a stream of hot air for ~1 min and this procedure resulted in uniform PVC layers with a ΔP of 21.5 $\pm$ 1.5 and ΔA = 2.8 $\pm$ 0.3° (mean $\pm$ SD, $n = 60$), as measured by ellipsometry in air. Average thickness and refractive index of these layers were 23.6 nm and 1.434, respectively. Before use, the slides were stored dust-free for ~12 h at room temperature.

Validation by measurement of annexin V adsorbed on phospholipid bilayers

The internal surface of the cuvette was rendered hydrophobic by silanization with Sigmacote (Sigma) and filled with buffer C. Calcium is required for the binding of annexin V to the phospholipid membrane, and albumin, adsorbed on hydrophobic silane, prevents loss of annexin V due to adsorption to the cuvette walls. Initial adsorption rates of annexin V on planar DOPS/DOPC bilayers are transport-limited (Andree et al., 1990) and satisfy the relation $d\Gamma/dt = \mathrm{MTC} \cdot C_{bulk}$, with Γ representing the surface concentration of protein (g/cm^2), C_{bulk} the bulk concentration of protein (g/ml), and MTC the mass transfer constant (cm/s). The latter depends on stirring conditions, buffer viscosity, and the diffusion constant of the protein (Willems et al., 1993). In the present study MTC was determined by ellipsometric measurement of the adsorption rate of a high concentration (450 ng/ml) of annexin V. In view of the time required for the flushing procedure, very short incubations could not be timed exactly, and incubation times of at least 120 s were used.

Low annexin V concentrations (0–1.8 ng/ml) were added to the ellipsometer cuvette and, after 200 s of adsorption to the bilayer, the cuvette was flushed with buffer C. Because the monoclonal anti-annexin V did not recognize phospholipid-bound protein, a two-step procedure was adopted. First, 1 μg/ml of polyclonal rabbit anti-annexin V was added as tagging antibody for 120 s and, after flushing, a second 120 s incubation step with 1 μg/ml swine anti-rabbit IgG/HRP conjugate followed. After flushing with buffer C, 40 μl of a substrate solution containing 3′3-diaminobenzidine (DAB; 360 Da) (Sigma) and H_2O_2 in 0.1 M Tris, pH 7.5, was added to a final concentration of 0.278 mM DAB and 0.834 mM H_2O_2, and the formation of DAB precipitate was measured by ellipsometry.

Determination of annexin V and FABP on PVC-coated slides

PVC-covered silicon slides were coated overnight at 4°C with either 2 μg/ml immunoaffinity-purified polyclonal anti-annexin V or 1 μg/ml monoclonal anti-FABP in buffer E. Thereafter, the slides were washed during 15 s with buffer C and inserted into the ellipsometer cuvette. Annexin V or FABP (0–10 ng/ml) was added to the cuvette and incubated for 10 min at room temperature while stirring. After flushing with buffer D, 1 μg/ml monoclonal anti-annexin V or monoclonal anti-FABP/HRP conjugate was added and incubated for 5 min. Excess antibody was removed by flushing with buffer D and, in the case of annexin V, 5 μg/ml rabbit anti-mouse IgG/HRP conjugate was added and incubated for another 5 min. After final flushing with buffer D, the DAB-H_2O_2 substrate solution was added to the cuvette, and formation of precipitate was measured by ellipsometry.

Determination of IL-6 on hydrophobic silicon slides

Silicon slides were first silanized and then covered with antibody by 15-min immersion in 1 mg/l monoclonal anti-IL-6 in buffer E, and subsequent flushing with buffer D. These pretreated slides were mounted in the ellipsometer cuvette, filled with 4 ml stirred buffer D, and exposed for 10 min to IL-6, added directly to the cuvette. The cuvette was then flushed with buffer D and 4 μl biotinylated sheep polyclonal anti-IL-6 was added and allowed to adsorb for 5 min. The cuvette was flushed again with buffer D, and 1.5 ml streptavidine-poly-HRP was added and allowed to adsorb for 10 min. After final flushing with buffer D, the DAB-H_2O_2 substrate solution was added to the cuvette, and the formation of precipitate was measured by ellipsometry.

RESULTS

Validation of the method with annexin V adsorptions on phospholipid bilayers

Formation of a phospholipid bilayer on a hydrophilic silicon slide is presented in Fig. 2. In this example, membrane formation by vesicle adsorption was studied in the ellipsometer cuvette, instead of the usual preparation in a separate vessel as described in the Methods. A bilayer with a phospholipid surface mass of 0.41 μg/cm^2 was formed and remained stable after removal of unbound vesicles by flushing of the cuvette with buffer C. After 800 s, 0.45 μg/ml annexin V was added and the best-fitting straight line for the first 100 s of annexin V adsorption was calculated and indicated in Fig. 2. From the slope of the regression line, divided by the annexin V concentration, a mass transfer constant of MTC = (1.48 $\pm$ 0.02) $\times$ 10^{-3} cm/s (mean $\pm$ SD; $n = 6$) was obtained.

Measurement of low surface concentrations of annexin V is shown in Fig. 3. A phospholipid bilayer was exposed during 200 s to 1.8, 0.9, 0.036, 0.18, 0.036, and 0 ng/ml annexin V in buffer B. The concentrations were chosen such that in 200 s a surface mass of 0.50, 0.25, 0.10, 0.05, and 0.01 ng/cm^2 annexin V adsorbed, as calculated from the value of MTC (see Methods), that is, below direct ellipsometric detection. Flushing was followed by two 120 s incubations in 1 μg/ml rabbit anti-annexin V and 1 μg/ml swine anti-rabbit IgG/HRP conjugate, respectively. After repeated flushing, the DAB/H_2O_2 substrate solution was

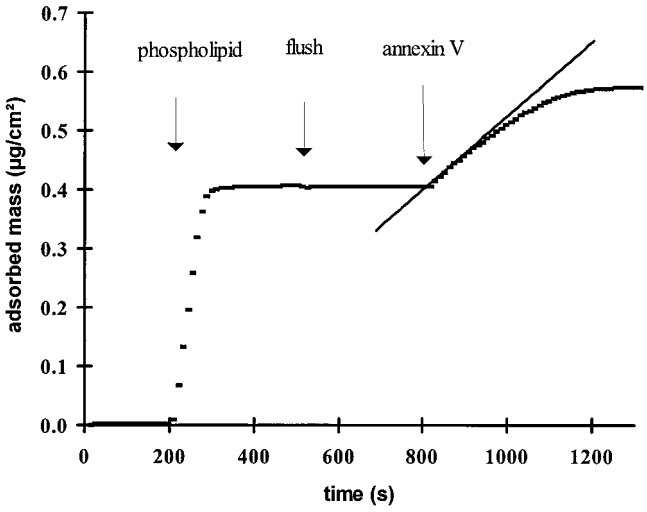

FIGURE 2 Ellipsometric measurement of the formation of a phospholipid bilayer on a hydrophilic silicon slide, and of subsequent binding of annexin V. Bilayer formation started after 200 s, when a suspension of phospholipid vesicles was added. The cuvette was flushed with buffer after 500 s, and 0.45 mg/l annexin V was added after 800 s. The best-fitting straight line for the first 100 s of annexin V adsorption is shown.

added, causing a small change in P. An initial transition phase was apparent, but after ~200 s, steady-state formation of precipitate was observed. Results of these experiments are summarized in Table 1. By using the average polarizer change plus three times the SD for the blank as the detection limit, the lowest surface concentration of annexin V of 0.01 ng/cm^2 could already be detected within 300 s of incubation time.

The lower part of Fig. 3 shows a nonlinear relation between annexin V surface concentrations and rates of precipitate formation. The relatively low rates of precipitate formation in the low range of annexin V concentrations may reflect the two-step character of DAB precipitate formation (Seligman et al., 1968, 1973). Some of the still-soluble product of the first step could be lost to the bulk phase at low HRP activities.

Determination of annexin V and FABP on PVC-coated silicon slides

Formation of DAB precipitate after application of the sandwich principle on PVC-coated silicon slides is shown in Fig. 4 for five protein concentrations between 0 and 10 ng/ml. Effects of varying protein concentrations and incubation times for these assays are shown in Table 2. Using 600-s incubation in 6 ng/ml or 300-s incubation in 12 ng/ml annexin V produced similar results, indicating that for these conditions the assay remained in its linear range. Incubation times and antibody concentrations for the two separate steps in the annexin V assay were not critical, as shown by the results obtained with 600-, 300-, and 120-s incubations.

Determination of IL-6 on hydrophobic silicon slides

Fig. 5 shows the formation of DAB-precipitate after 600 s incubation of anti-IL-6 coated hydrophobic silicon slides in 100 and 10 ng/l IL-6. Biotinylated sheep polyclonal anti-IL-6 and streptavidin-poly HRP were added in two separate steps, before addition of the DAB substrate solution. IL-6 (26 kD) is comparable in molecular size to annexin V (32 kDa), and using the value for MTC as determined for annexin V, the amount of IL-6 adsorbing during 600 s from a 10 pg/ml solution is $1.48.10^{-3} \times 600 \times 10 = 8.9$ pg/cm^2. This implies that a surface mass of <1% of the detection limit of direct ellipsometry could be measured within 5 min.

DISCUSSION

Characteristics of the presented technique

The technique introduced in the present study can be described as a two-dimensional ELISA with the enzyme product remaining confined to the surface, where it is produced, instead of being distributed over a three-dimensional volume. Because of the high sensitivity of ellipsometry for the detection of surface mass, this will generally imply shorter assay times. As an example, let us assume that the enzyme product generated by 1 cm^2 of surface is distributed in a cuvette of 1 ml volume, allowing spectrophotometric measurement of the product concentration with a light path of 1 cm. For a minimal detectable extinction change of $\Delta E = 0.01$, one would have $0.01 = \epsilon \cdot C$, with ϵ the molar extinction coefficient and C the molar concentration. Using the value of ϵ(1 cm; 450 nm) $= 5.9 \times 10^4$ M^{-1} cm^{-1} (Josephy et al., 1982) for tetramethylbenzidine (TMB; M = 313 Da), the soluble analog of DAB, it follows that the

TABLE 1 Polarizer changes in the model study with annexin V

Annexin V Concentrations (ng/ml)	Calculated Surface Mass (ng/cm^2)	Incubation Times (in DAB)		
		300 s	600 s	900 s
0.00	0.00	0.037 ± 0.005	0.046 ± 0.004	0.060 ± 0.006
0.036	0.01	0.056 ± 0.007	0.075 ± 0.009	0.115 ± 0.008
0.18	0.05	0.063 ± 0.007	0.118 ± 0.013	0.192 ± 0.017
0.36	0.10	0.102 ± 0.008	0.236 ± 0.018	0.388 ± 0.023
0.90	0.25	0.258 ± 0.021	0.689 ± 0.058	1.136 ± 0.097
1.80	0.50	0.765 ± 0.070	1.843 ± 0.109	2.953 ± 0.140

Data indicate mean change in polarizer degrees (±SD) of four experiments.

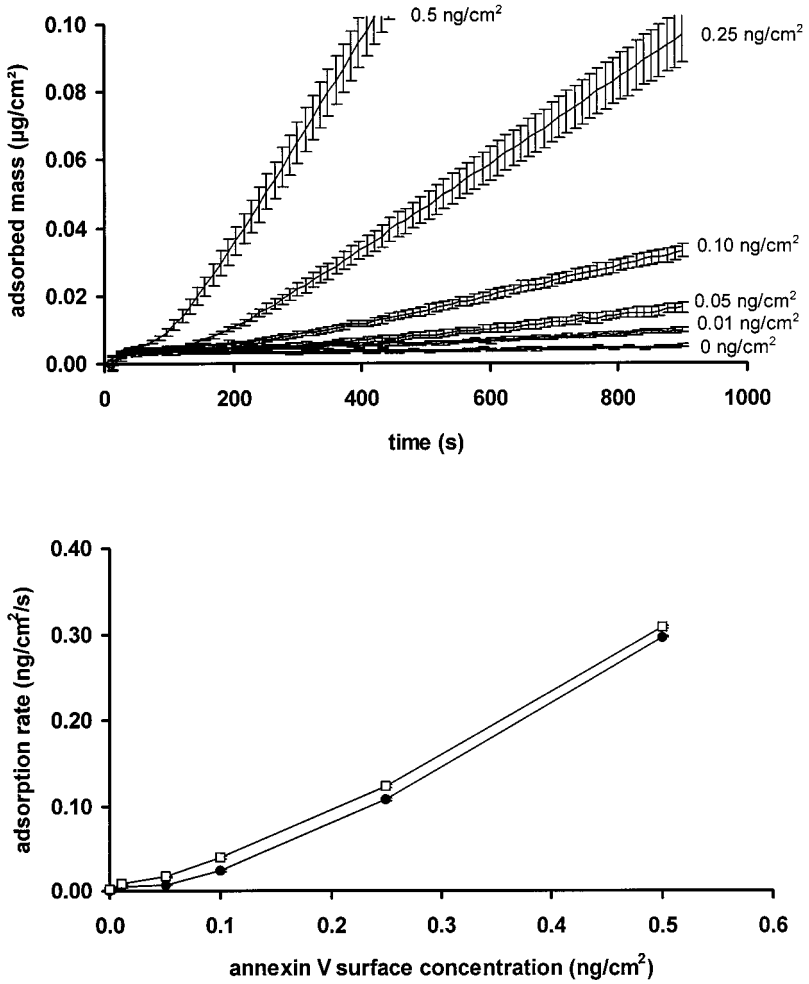

FIGURE 3 *Top:* Measurement of the formation of DAB-precipitate on phospholipid bilayers for the indicated surface concentrations of annexin V. Mean values of four experiments with SD are shown. *Bottom:* Rate of DAB precipitation as a function of annexin V surface concentrations. Results were calculated from the full 900-s observation interval (●), and for the interval between 240 and 300 s (□). SD falls within symbol size.

minimal amount of product detectable would be ~1.7 × 10^{-7} M or 53 ng/ml. If this amount of product had directly precipitated on the surface, ~30 times less would have been detectable by ellipsometry and assay time could have been 30 times shorter. Alternatively, the primary incubation time with antigen could have been 30 times shorter.

TABLE 2 Effects of incubation times and protein concentrations for the assays on PVC-covered silicon disks

Protein Incubation		Moab Incubation		Conjugate Incubation		Polarizer Change (degrees) after		
ng/ml	s	µg/ml	s	µg/ml	s	300 s	600 s	900 s
Annexin V								
0	0	2	600	5	300	0.06	0.07	0.09
6	600	1	120	5	300	0.18	0.57	1.11
6	600	1	300	5	300	0.42	1.45	2.22
6	600	1	600	5	300	0.41	1.36	2.14
6	600	3	120	5	300	0.27	1.00	1.80
12	300	1	300	5	120	0.40	0.77	1.02
12	300	1	300	5	300	0.49	1.23	1.68
12	300	1*	300*	5*	300*	0.10	0.19	0.29
12	300	1	300	5	120	0.65	1.30	1.70
12	300	5	120	5	300	0.16	0.56	1.12
12	300	10	120	5	300	0.21	0.65	1.22
FABP								
0	0	—	—	1	1000	0.06	0.07	0.09
10	600	—	—	1	120	0.29	0.85	1.40
10	600	—	—	1	300	0.42	1.25	2.00
10	600	—	—	1	600	0.41	1.25	2.05

* Moab and conjugate added together.

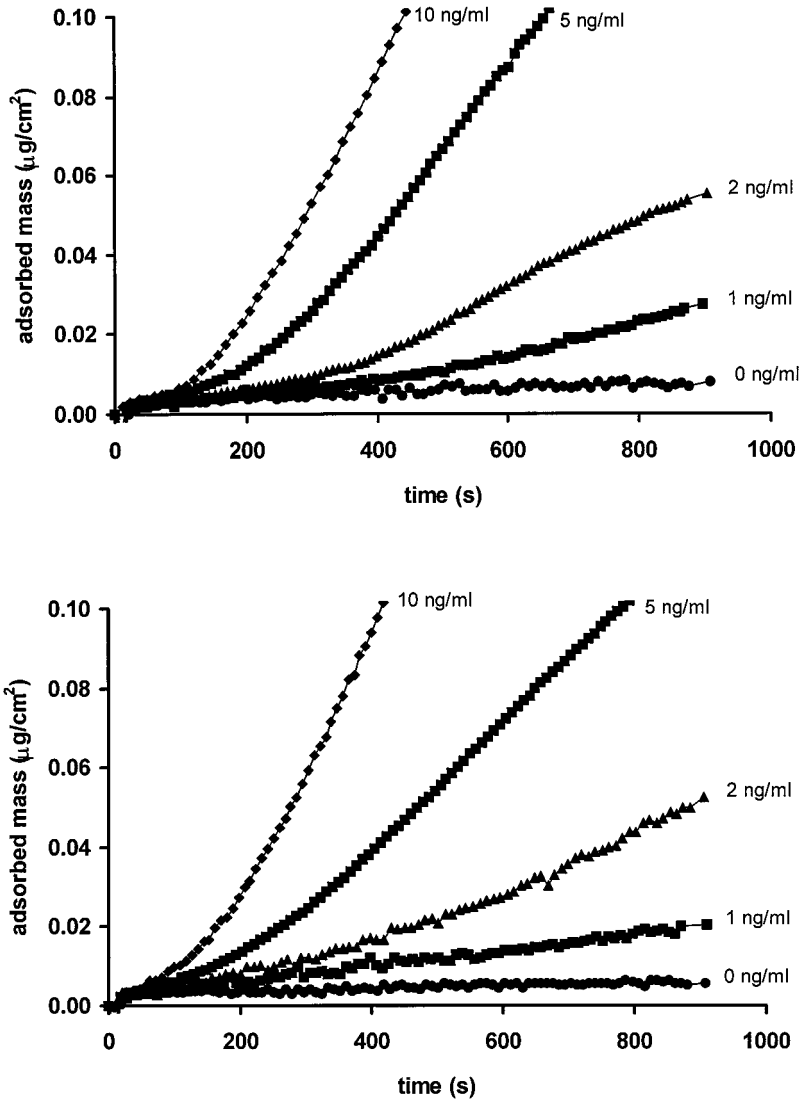

FIGURE 4 Measurement of DAB precipitate formation after adsorption of annexin V (*top*), and FABP (*bottom*) on PVC-coated silicon slides. Proteins were incubated for 600 s, followed by flushing, and 5-min incubations in HRP-labeled conjugate. For annexin V, addition of the conjugate was preceded by 5-min incubation in monoclonal anti-annexin V. The DAB-H_2O_2 substrate solution was added at zero time.

Other techniques for amplified ellipsometry of adsorbing proteins have been described in the literature. Mandenius and Mosbach (1988) obtained 5 to 7× amplification of the ellipsometric signal by coupling antibodies to 12-nm silica particles, and this technique could obviously be extended to gold-labeled antibodies, as used in electron microscopy. A drawback of coupling proteins to heavy labels is the ensuing slow adsorption caused by largely decreased diffusion coefficients. More importantly, however, the continuing precipitate formation in the presented new technique allows amplification by much larger factors, as illustrated by the amount of precipitate of more than 100 times the surface mass of IL-6, formed within 5 min.

Possibility of transport-limited precipitate formation

The formation of precipitate, as shown in Figs. 3–5, could be influenced by a transport limitation of the DAB flux toward the surface. The mass transfer constant for DAB is 2.7×10^{-3} cm/s. [Calculated by multiplication of the value of 1.48×10^{-3} cm/s for annexin V by $(D_{DAB}/D_{annexin\ V})^{2/3}$ (Willems et al., 1993), using the values 7×10^{-7} cm^2/s for the diffusion constant of annexin V ($D_{annexin\ V}$) (Willems et al. 1993) and 17×10^{-7} cm^2/s for the diffusion constant of DAB (D_{DAB}) (Malpiece et al., 1980).] For a DAB concentration of 0.1 mg/ml, as used in the present study, the maximal transport-limited rate of precipitation is then ~270 $ng/cm^2/s$. The highest DAB precipitation rates in the present study were ~3 $ng/cm^2/s$ (Fig. 5), that is, ~1% of the maximal transport rate. It is concluded that the rates of precipitate formation are not influenced by transport limitations.

Assay sensitivity and self-limiting precipitate formation

Comparison of Figs. 3 and 4 shows that the assay for annexin V on phospholipid bilayers was much more sensitive than on PVC-covered slides. The main cause of this

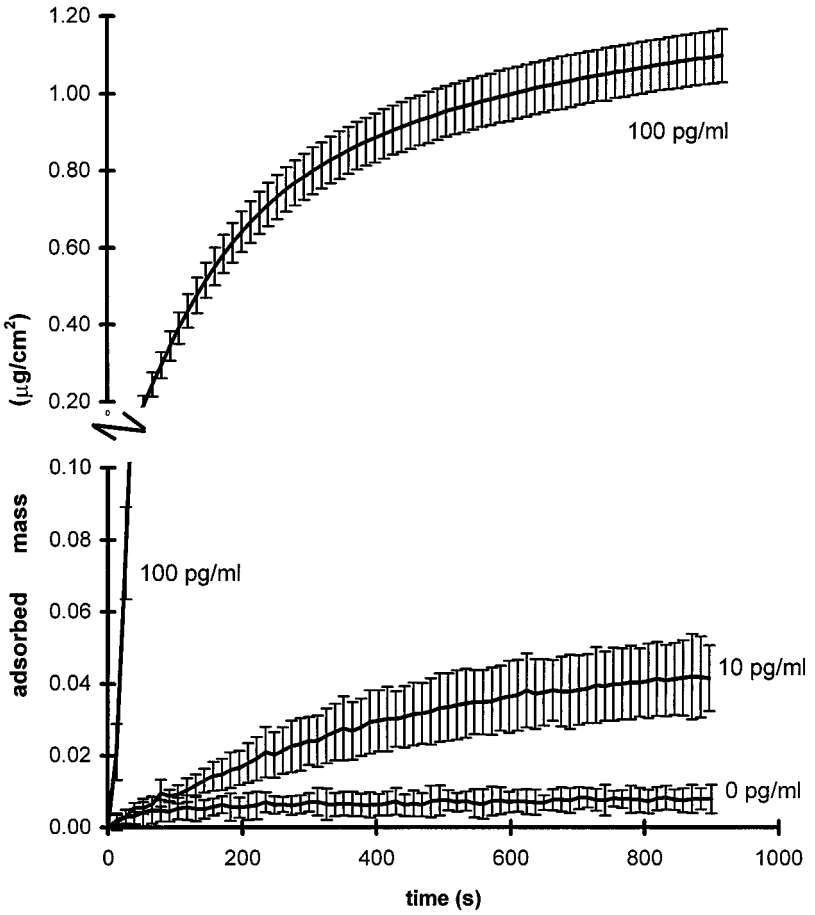

FIGURE 5 Measurement of DAB precipitate formation after adsorption of interleukin 6 on hydrophobic silicon slides. After 600 s of incubation in the indicated IL-6 concentrations, biotinylated sheep polyclonal anti-IL-6 and streptavidine-poly-HRP were used, and precipitate formation was started by addition of DAB-H_2O_2 substrate solution at zero time. Mean values of four experiments with SD are shown.

difference was probably the amount of annexin V adsorbed. While the high-affinity adsorption of annexin V on bilayers proceeds at its maximal, transport-limited rate (Andree et al., 1990), binding by the PVC-adsorbed antibodies may proceed much slower. Use of high-affinity monoclonal catcher antibodies could further improve the results on PVC.

Due to the use of high-affinity catcher and tagging antibodies and a poly-HRP conjugate, the IL-6 assay (see Fig. 5) was also much more sensitive than the assays on PVC-coated slides. However, the nonlinear responses in Fig. 5 suggest that formation of a large amount of precipitate per adsorbed molecule of IL-6 may cause a self-limiting situation, with the HRP molecules becoming increasingly less accessible to DAB substrate. This complicates a quantitative interpretation of the rate of precipitate formation and could necessitate the use of standard curves, as in the usual ELISA procedure.

Importance of in situ measurement

In the measurement of very low protein concentrations, aspecific adsorption of the blocking proteins in ELISA-type assays will generally exceed the small increases of surface mass due to DAB precipitation. This implies that in situ measurement was essential in these measurements. If the slides had been washed before ellipsometric measurement, or washed and dried for processing in air, the small measuring signal would have disappeared in the changes of surface mass of blocking proteins.

Another aspect related to in situ measurement is the possible development of a one-step ELISA. Often, ELISA protocols are shortened by simultaneous addition of antigen and conjugate, but separation of bound and free (enzyme) label remains necessary, a common feature of heterogenous immunoassays. The efficiency and reproducibility of this separation procedure determines to a large extent the precision and sensitivity of the assay. By using the described technique, this separation step could, in principle, be omitted. Formation of DAB aggregates in the solution causes loss of light intensity, due to increasing light absorption in the buffer, but does not interfere with the ellipsometric measurement of surface mass. Some precipitation on the surface of DAB aggregates formed in solution may occur, but preliminary experiments, with a 10 times lower DAB concentration, have shown that such adsorption is slow, probably because of slow diffusion of large aggregates. Because the present study focused on direct comparison with existing ELISA techniques, elimination of excess conjugate in the final washing step was included, and the one-step ELISA was not further investigated.

REFERENCES

Andree, H. A. M., Ch. P. M. Reutelingsperger, R. Hauptmann, H. C. Hemker, W. Th. Hermens, and G. M. Willems. 1990. Binding of vascular anticoagulant-alpha (VAC-alpha) to planar phospholipid bilayers. *J. Biol. Chem.* 265:4923–4928.

Avrameas, S. 1992. Amplification systems in immunoenzymatic techniques. *J. Immunol. Methods*. 150:23–32.

Brakenhoff, J. P. J., E. R. de Groot, R. F. Evers, H. Pannekoek, and L. A. Aarden. 1987. Molecular cloning and expression of hybridoma growth factor in *Escherichia coli*. *J. Immunol.* 139:4116–4121.

Brakenhoff, J. P. J., M. Hart, E. R. de Groot, F. Di Padova, and L. A. Aarden. 1990. Structure-function analysis of human IL-6. Epitope mapping of neutralizing monoclonal antibodies with amino- and carboxy-terminal deletion mutants. *J. Immunol.* 145;561–568.

Corsel, J. W., G. M. Willems, J. M. M. Kop, P. A. Cuypers, and W. Th. Hermens. 1986. The role of intrinsic binding rate and transport rate in the adsorption of prothrombin, albumin and fibrinogen to phospholipid bilayers. *J. Colloid Interface Sci.* 111:544–545.

Cuypers, P. A., J. W. Corsel, M. P. Janssen, J. M. M. Kop, W. Th. Hermens, and H. C. Hemker. 1983. The adsorption of prothrombin to phosphatidylserine multilayers, quantitated by ellipsometry. *J. Biol. Chem.* 258:2426–2431.

Giesen, P. L. A., H. C. Hemker, and W. Th. Hermens. 1995. Production of thrombin as a probe for mixing of phospholipids in membranes on solid supports. *Biochim. Biophys. Acta.* 1237:43–48.

Giesen, P. L. A., G. M. Willems, H. C. Hemker, M. C. A. Stuart, and W. Th. Hermens. 1993. Monitoring of unbound protein in vesicle suspensions with off-null ellipsometry. *Biochim. Biophys. Acta.* 1147:125–131.

Glatz, J. F. C., A. H. Kleine, F. A. van Nieuwenhoven, W. T. Hermens, M. P. van Dieijen-Visser, and G. J. van der Vusse. 1994. Fatty acid binding protein as a plasma marker for the estimation of myocardial infarct size in humans. *Br. Heart J.* 71:135–140.

Josephy, P. D., Th. Eling, and R. P. Mason. 1982. The horseradish peroxidase-catalyzed oxidation of 3,5,3′,5′-tetramethylbenzidine. *J. Biol. Chem.* 257:3669–3675.

Malpiece, Y., M. Sharan, J-N. Barbotin, P. Personne, and D. Thomas. 1980. Theoretical and experimental study of an immobilized bienzyme system. *J. Biol. Chem.* 255;6883–6890.

Mandenius, C. F., and K. Mosbach. 1988. Detection of biospecific interactions using amplified ellipsometry. *Anal. Biochem.* 170:68–72.

Maurer-Fogy, I., Ch. P. M. Reutelingsperger, J. Pieters, G. Bodo, C. Stratowa, and R. Hauptmann. 1988. Cloning and expression of cDNA for human vascular anticoagulant, a calcium-dependent phospholipid-binding protein. *Eur. J. Biochem.* 174:585–592.

Roos, W., E. Eymann, M. Symannek, J. Duppenthaler, K. W. H. Wodzig, M. Pelsers, and J. F. C. Glatz. 1995. Monoclonal antibodies to human heart fatty acid-binding protein. *J. Immunol. Methods.* 183:149–153.

Rothen, A. 1947. Immunological reactions between films of antigen and antibody molecules. *J. Biol. Chem.* 168:75–97.

Seligman, A. M., M. J. Karnovsky, H. L. Wasserkrug, and J. S. Hanker. 1968. Nondroplet ultra-structural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB). *J. Cell. Biol.* 38:1–14.

Seligman, A. M., W. A. Shannon, Y. Hoshino, and R. E. Plapinger. 1973. Some important principles in 3,3′-diaminobenzidine ultrastructural cytochemistry. *J. Histochem. Cytochem.* 21:756–758.

Van Heerde, W. 1994. Annexin V, localization, plasma levels and anticoagulant properties. PhD. Thesis, Utrecht University, Utrecht, The Netherlands.

Vroman, L., and A. L. Adams. 1969. Identification of adsorbed protein films by exposure to antisera and water vapor. *J. Biomed. Mater. Res.* 3:669–671.

Willems, G. M., P. L. A. Giesen, and W. Th. Hermens. 1993. Adsorption and conversion of prothrombin on a rotating disc. *Blood.* 82:497–504.

Wodzig, K. W. H., M. M. A. L. Pelsers, G. J. van der Vusse, W. Roos, and J. F. C. Glatz. 1997. One-step enzyme-linked immunosorbent assay (ELISA) for serum fatty acid-binding protein. *Ann. Clin. Biochem.* 34:263–268.