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Blood plasma coagulation studied by surface plasmon resonance

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Abstract. A surface plasmon resonance (SPR) apparatus was used to investigate blood plasma coagulation in real time as a function of thromboplastin and heparin concentrations. The response curves were analyzed by curve fitting to a sigmoid curve equation, followed by extraction of the time constant. Clotting activation by thromboplastin resulted in increased time constant, as compared to spontaneously clotted plasma, in a dose dependent way. Addition of heparin to the thromboplastin-activated plasma counteracted this effect. Atomic force microscopy (AFM) pictures of sensor surfaces dried after completed clotting, revealed differences in fibrin network structures as a function of thromboplastin concentration, and the fiber thickness increased with decreased thromboplastin concentration. The physical reason for the SPR signal observed is ambiguous and is therefore discussed. However, the results summarized in the plots and the fibrin network properties observed by AFM correlate well with present common methods used to analyze blood coagulation. [S1083-3668(00)00901-1]

Keywords: surface plasmon resonance (SPR); blood plasma coagulation; atomic force microscopy (AFM).

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1 Introduction

Surface plasmon resonance is by now a well-established method for the study of biomolecular interactions and thin molecular films.[1] Changes in the refractive index at the interface region between the sensor surface and the sample due to changes in molecular mass of tens of picograms per mm^2 can be accurately and reproducibly detected.[2]

The formation of a clot is the final event in the blood coagulation cascade. Vessel injuries initiate enzymatic reactions that result in the cleavage of fibrinogen to fibrin monomers by thrombin. The fibrin monomer polymerization into a three-dimensional network efficiently blocks the flow through the lesion.[3] The blood clotting time is a frequently used parameter in medical diagnosis and is commonly determined by photometrical or mechanical methods. The determinations are based on the rapid changes in transmittance or viscosity that occurs when fibrin polymerizes. Some authors have proposed the possibility of extracting more diagnostically important information from the response curves than simply the clotting time, as the detailed curve shapes of the clotting curves are dependent on individual properties of the clotting sample.[4–6] However, so far these efforts have had limited success. We observed that the surface plasmon resonance (SPR) signal from blood plasma on a gold sensor surface changed on the time scale of expected clotting and decided therefore to investigate the clotting kinetics with this method by using commercial coagulation accelerators and inhibitors. The aim of the present work was to investigate whether the SPR response curves could provide more information about the blood clotting process than simply the clotting time. To our knowledge polymerization observed by SPR has not been published previously, and we believe that the study of blood coagulation with SPR has a potential for the development of more sophisticated diagnostic tools in medical applications.

2 Materials and Methods

All the SPR experiments were performed on a modified prototype of BIAlite (Biacore AB, Uppsala, Sweden). The detection unit was placed in a horizontal position in order to enable the placement of the sample directly onto the sensor surface. The detection principle has been described before.[2] A schematic of the system is shown in Figure 1. The gold surfaces, 45 nm Au on glass, were from Biacore AB (Uppsala, Swe-

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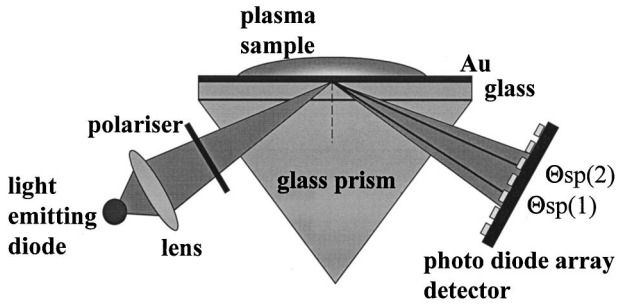

Fig. 1 Principal drawing of the SPR apparatus. Light (λ=760 nm, *p*-polarized) is focused on to the sensor surface and reflected to the photo diode array detector. At the resonance angle, $\Theta sp(1)$, the evanescent field of the light interacts with the surface plasmon of the gold film and the reflected intensity is reduced. Changes in the optical density of the medium in close proximity to the sensor surface changes the resonance angle to $\Theta sp(2)$.

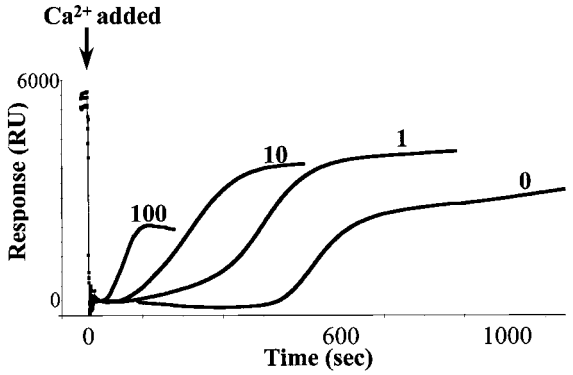

Fig. 2 Typical appearance of time response curves obtained with SPR when blood plasma samples with different thromboplastin concentrations were allowed to coagulate on a gold sensor surface. Time is depicted on the *x* axis and response [in resonance units (RU)] on the *y* axis. The thromboplastin concentrations were as indicated in the figure (AU/ml). Ca^{2+} was added at time=0 s, as indicated (final concentration 10 mM).

den). Prior to use, the surfaces were cleaned in ozone (oxygen plasma) yielding hydrophilic surfaces with contact angle <20°. Citrated plasma was mixed from two apparently healthy donors and stored at −70°C. After thawing, the plasma was kept in refrigerator and used within 4 h. The thromboplastin was from Global Hemostasis Institute (Linköping, Sweden). Its concentration is given in arbitrary units relative to the stock solution, where 100 AU/ml corresponds to a ten times diluted stock solution. $CaCl_2$ (20 mM) was from Sigma, heparin (5000 IU/ml) was from LEO (Denmark), and all reagents were stored refrigerated. All dilutions were made in Hepes buffered saline (10 mM HEPES, 0.15 M NaCl, 0.05% P20).

All the experiments were run at room temperature (22°C). The gold surfaces were cleaned within 1 h prior to use in order to obtain a proper wetting of the sample on the surface. Appropriately diluted thromboplastin, 8 μl, was added to 92 μl of plasma, and 20 μl of the mixture was placed on the sensor surface. The data collection was started immediately. 20 μl of $CaCl_2$ solution was added to the sample and the solution was mixed manually within 15 s. The time point of Ca^{2+} addition was taken as time zero in the data evaluation.

For the atomic force microscopy (AFM) experiments, the Au surfaces were, after completion of clotting, thoroughly cleaned in streaming Milli-Q water and dried in flowing N_2. The measurements were performed within 4 h after the sample preparation and were performed in air on a NanoScope IIIA from Digital Instruments (USA) operated in tapping mode. Nano Probe silicon tips having a cantilever resonance frequency around 300 kHz were used for imaging.

3 Results

Representative original SPR response curves for plasma with different thromboplastin concentrations are shown in Figure 2. The response change is commonly interpreted as a change in refractive index at the sensor surface/liquid interface. A response of 1000 resonance units (RU) corresponds then to a refractive index change (Δn) of approximately 10^{-3}. The time at which the clotting process was initiated, through the addition of Ca^{2+}, is indicated by an arrow in the figure, and the thromboplastin concentration in each sample is indicated.

The steep response fall seen at the point of Ca^{2+} addition is due to dilution of the sample with a solution of lower refractive index. As can be seen in Figure 2, the signal increase from the recalcified plasma sample with no thromboplastin added occurred after more than 500 s, and the sample with 100 AU/ml of thromboplastin added responded in less than 50 s. Also, the slope of the response curve during the increase and the maximum response seemed to be affected by the thromboplastin concentration, so the SPR curves were fitted to a sigmoid curve equation[6] using BIAevaluation software (ver.3.0) from Biacore AB (Uppsala, Sweden):

$$Y(t) = R_{eq}\{1 + \exp[-2k(t - t_{lag})]\}, \qquad (1)$$

where

$Y(t)$ = response at time t,
R_{eq} = maximum response,
k = the time constant of the equation,
t_{lag} = time at which $Y = R_{eq}/2$.

An original curve and the corresponding fitted one is shown in Figure 3. The residuals (|original−fit|) were smaller than 1% of R_{eq}, as determined by the evaluation program. The extracted features; t_{lag}, k, and R_{eq}, are indicated in the figure.

Experiments were performed with serial dilution of thromboplastin and heparin in citrated blood plasma. The thromboplastin concentrations were varied between 0 and 100 AU/ml, and in experiments with heparin the thromboplastin concentration was 100 AU/ml. Heparin was added to final concentrations of up to 2 IU/ml. The k values obtained from curve fitting and feature extraction of the sensorgrams are plotted in Figures 4(a) and 4(b). It can be seen in Figure 4(a) that an increased thromboplastin concentration increased the time constant of the sigmoid curve. In Figure 4(b), the time constant is plotted versus the heparin concentration for samples containing 100 AU/ml thromboplastin. Increased concentra-

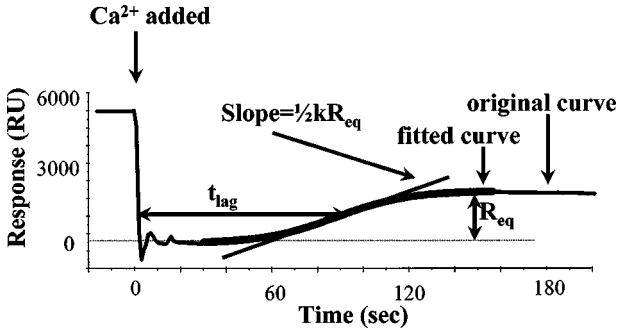

Fig. 3 Original SPR response curve and the corresponding one fitted to Eq. (1). The indicated features were extracted; lag time (t_{lag}), maximum response (R_{eq}), and k, indicated in the figure as slope $= kR_{eq}/2$.

tions of heparin decreased the time constant, and at 1–2 IU/ml the effect of the thromboplastin on k was fully counteracted.

After the completion of the clotting, some surfaces were washed and dried and studied by AFM. Pictures of surfaces on which citrated plasma samples containing 1 and 10 AU/ml thromboplastin were allowed to clot are shown in Figures 5(a) and 5(b), respectively. The thread-like structures are assumed to be fibrin fibers formed on the sensor surface during the clotting process. Apparently, the fibers formed with the lower thromboplastin concentration [1 AU/ml, Figure 5(a)] are thicker, longer, and fewer, than the fibers formed in the presence of a higher thromboplastin concentration [10 AU/ml, Figure 5(b)].

4 Discussion

Surface plasmon resonance is an optical phenomenon of interference between light and matter. Under conditions of total internal reflection using the Kretschmann configuration,[7] reflection is attenuated by the coupling of the evanescent field to the surface plasmon of the metal film electrons. The coupling occurs at a given angle of incidence, the so called resonance angle, which is determined by the wavelength of the light and by the dielectric constants of the prism, metal film, and the medium outside the metal film. The evanescent field outside the gold film decays exponentially and limits the sensing depth to approximately 1 μm. The detection area in the present configuration is approximately 1 mm^2.

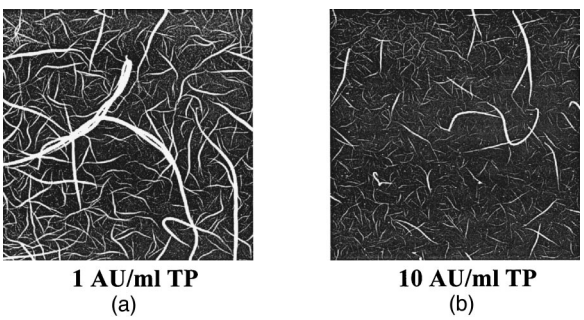

Fig. 5 AFM pictures of fibrinogen network on gold. Blood plasma was allowed to clot with additions of (a) 1 and (b) 10 AU/ml thromboplastin. Scan sizes were 25×25 μm^2, and the gray scale covers 25 nm.

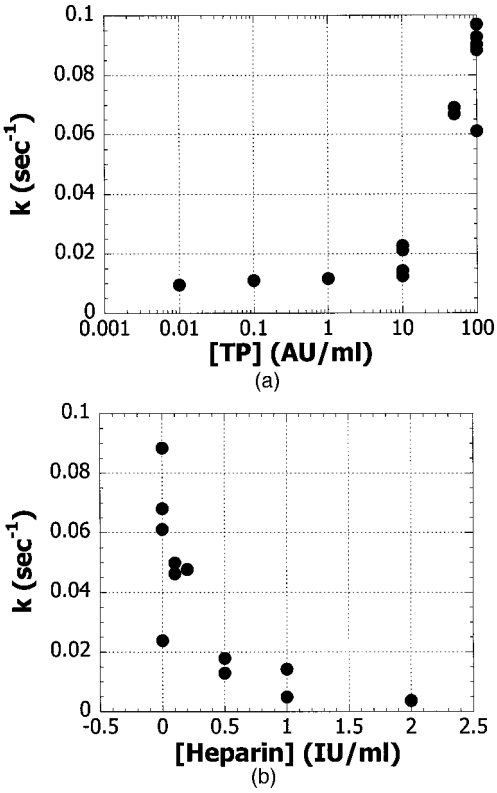

Fig. 4 Plots of the k values extracted after curve fitting. (a) k vs thromboplastin (TP) concentration and (b) k vs heparin concentration for samples containing 100 AU/ml TP.

In the present experiments, the observed changes in the resonance signals are due to reorganization of the sample material, as no new material was added after time zero. Proteins have greater refractive index ($n = 1.5–1.7$)[8] than water ($n = 1.3$) and an SPR signal increase is thus expected from accumulation of proteins on the sensor surface. Since the response curves changed in a thromboplastin (and heparin) concentration dependent manner, the curves are interpreted as representing the clotting process. An important feature of a blood or blood plasma clot is the three-dimensional fibrin network formed, into which a number of other blood factors are incorporated. Since the same kind of response curves can be obtained with SPR on a pure fibrinogen-thrombin (another coagulation activator) system (results not shown), the main contributor to the SPR signal resulting from clotting blood plasma is supposed to be the fibrin network formation. At this stage it is, however, not possible to distinguish between (1) fibrin polymerization on the surface, (2) fibrin polymerization in bulk and sedimentation of fragments, (3) fibrin polymerization in bulk and sedimentation of a completed clot, or combinations of the three. In the case of alternative (1), the slope of the response curve would be interpreted as the coagulation of the plasma sample, i.e., a real-time detection of fibrinogen polymerization into fibrin fibers, and the lag time would likewise be a reflection of the clotting time. Assuming alternative (2), the curve slope would be interpreted as the rate of sedimentation of polymerized fibrin to the surface, and the observed lag time would be a reflection of the time it takes before sufficiently large fragments have been formed, rather

than the polymerization as such. For model (3), the curve slope would be the sedimentation rate of the completed clot and the lag time would reflect the time needed for completion of the clotting process.

The lag time of the response was thromboplastin dependent, as can be seen from Figure 2. Clotting was observed by eye at roughly the times of the slope of the curves seen in Figure 2. Without favoring any one of the above-mentioned models, k is in the following text supposed to represent the time constant of the coagulation process. It was observed that the plot of k value versus lag time overlapped for samples with and without heparin (not shown), indicating that the clotting process follows the same kinetics whether it is accelerated by thromboplastin or delayed by heparin. This is in contradiction to the results presented by others,[9] stating that k is a parameter more sensitive to the clotting kinetics than the lag time. The role of the detection method and the fitting equation needs to be investigated further.

Addition of 0–100 AU/ml thromboplastin to citrated plasma resulted in coagulation times of 700–50 s, i.e., the clotting time decreased with increasing thromboplastin concentration (Figure 2). Citrated plasma (no Ca^{2+} added) with 100 AU/ml thromboplastin gave no response above the baseline until after approximately 1000 s. This may exclude precipitation as an important signal source in the present study. As the same batch of plasma was used in all the experiments, the observed properties can be interpreted in terms of thromboplastin interactions with plasma components equal in all samples. Also, the polymerization of polyacrylamide on plain gold sensor surfaces has been observed to give comparable response curves (results not shown). This supports the interpretation that the SPR response originates from a polymerization process.

The correlation between the time constant and the thromboplastin concentration is shown in Figure 4(a). Blombäck and co-workers have proposed that the fibrin network structure is determined by the initial coagulation rate of the sample, which is completely determined by the activator (in the referenced paper thrombin) and fibrinogen concentrations.[10] According to Blombäck et al.,[10] a higher activator concentration gives a greater number of so called nucleation sites from which the fibrinogen monomers can start the polymerization, thus giving a higher polymerization rate than at a lower activator concentration. At a constant fibrinogen concentration the activator concentration determines the number of nucleation sites. In the proceeding polymerization, fibrin monomers are incorporated into the initially formed network structure. Coagulation with a long lag time is thus characterized by few initial nucleation sites. This leads to thicker fibers in the mature gel, whereas a short coagulation time is characterized by many nucleation sites and thinner fibers. The polymerization rate, which according to Blombäck leads to different fiber thicknesses, would not necessarily affect the density of accumulated material within the volume detected by our instrument. However, recruitment of fibrinogen towards the surface as the polymerization proceeds and fibrinogen is consumed, could explain the increased maximum responses obtained at longer response times (Figure 2), and could be interpreted in terms of either more fiber strands or thicker strands formed.

The samples with the higher thromboplastin concentrations had a shorter lag time and greater slope, as illustrated in Figure 2, and visualized equivalently as an increased k, plotted in Figure 4(a). Our results agree nicely with the hypothesis that more nucleation sites lead to a shorter coagulation time and to a higher coagulation rate. Heparin binding to thrombin has been shown to alter the thrombin affinity/activity towards fibrinogen.[11] Doses of heparin decreased the time constant, as shown in Figure 4(b), but the lag time to slope ratio was not significantly different from that of the samples with only thromboplastin (not shown).

A tendency of increased R_{eq} with decreased slope was observed (results not shown), indicating greater mass depositions on the sensor surface at the longer coagulation times. However, there were many data points deviating, especially from samples of low thromboplastin concentration or high thromboplastin and high heparin concentrations. It has been observed by others that, below a critical concentration of thrombin, the trend with fewer and thicker fibers with decreasing thrombin concentration is reversed and the fibers become thin and the number increases again.[12] A general observation was that nonthromboplastin samples had a lower R_{eq} than expected with respect to the slope (Figure 2). It can be speculated whether R_{eq} is a parameter more sensitive to experimental variations than the time constant. Factors not controlled in the presented results are, e.g., salt concentration and volume of water retained in the completed clot. In physical terms, the details of the response curves depend on the mass per unit volume, surface area of the clot, its water and ion binding capacity, and packing and arrangement on the sensor surface. The significance of R_{eq} will be explored in future studies with SPR.

The atomic force microscopy pictures in Figures 5(a) and 5(b) show the network structures that remain on the sensor surfaces after completion of clotting (with 1 and 10 AU/ml thromboplastin added, respectively) after thorough rinsing in distilled water followed by drying in nitrogen gas. The structures seen in Figure 5(b) (10 AU/ml thromboplastin) are thinner and shorter than those seen in Figure 5(a) (1 AU/ml thromboplastin). The AFM results agree well with confocal microscopy pictures of plasma provoked to clot by thrombin additions as presented by Blombäck et al.,[10] which show that the fibrin strands grow thicker and longer with increased clotting time. The interpretation of the observed increased SPR maximum response with decreased time constant as fiber formation from fewer nucleation sites that leads to fewer, but thicker and longer fibers is supported by these pictures. Cheng et al.[13] have used a quartz crystal microbalance (QCM) to study the coagulation time of plasma as a function of thromboplastin concentrations between 5 and 22 U/ml. The QCM is comparable to SPR in that it is sensitive to depositions on a sensor surface and the results were in good agreement with ours.

In conclusion, we have in this work reported that blood plasma coagulation that is activated by thromboplastin can be detected by SPR. The details of the interaction between the sensor surface and the plasma sample are ambiguous, but it is probable that the observed SPR responses somewhat reflect the polymerization of fibrinogen into a three-dimensional network of fibrin. The obtained response curves are comparable to response curves obtained by other methods and the sensi-

tivity is high. By use of appropriate computer software, the response curves could be evaluated and relations of the rate constant *k* to the concentrations of thromboplastin and heparin were visualized. These relations together with the presented AFM pictures support the hypothesis that decreased time constant (equivalent to a longer lag time and a slower deposition rate) leads to thicker fibers in the network. The proposed method may be a valuable tool for further studies of blood plasma coagulation and polymerization processes in general.

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