Mixed alkanethiol based immunosensor for surface plasmon field-enhanced fluorescence spectroscopy in serum

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SUPPORTING INFORMATION

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SUMMARY

Supporting information is given on the type of the used flow cell and flow cell assembling for SPRS and SPFS measurements, used antibody labeling procedure, instrumentation for SPRS and SPFS, including the theoretical background of this methods, mathematical operations and equations used for calculating the LOD, LOQ, and the mass coverage. Furthermore, supportive graphical material of the shown results is included as well for better understanding and to support the discussion in the main paper.
2. Materials and methods

2.2. Flow cell

The 70 µL flow cell (28 mm x 800 mm) similar to the design of Nicole\(^1\) consists of the sensor, a PDMS spacer with a centred hole defining the volume of the flow cell, and a quartz glass cover, supplied with an in- and outlet, assembled in a sandwich manner. The PDMS spacer was casted with a 10:1 mixture of Sylgard 184 and its curing agent. After degassing the PDMS under vacuum, the liquid PDMS was cast into the desired shape and size with a self-made oval polytetrafluorethylen mould and polymerized for 48 h at room temperature. The spacer layer was stored in ultra pure water until use.

2.3. Sensor preparation

![Figure S-1](image)

**Figure S-1.** Schematic representation of the used sensor approach. (1) LaSFN9 glass slide; (2) metal layer consisting of 2 nm Cr covered with 45 nm Ag; (3) mixed binary thiol SAM (11-mercaptoundecanoic acid and 6-mercaptohexanol); (4) randomly bound capture-antibody (Y-shape) and BSA (ellipse-shape).

2.4. Antibody labelling

The labelling of the detection antibodies was performed using the DyLight 649 antibody labelling kit from Thermo Fisher Scientific. Antibody labelling was done according to the manufacturer's instructions and is described briefly as follows: First the antibody solution was adjusted to a concentration of 1 mg/ml with PBS (pH 7.4), and 40 µL borate buffer (0.67 M)
were added to the antibody solution. This solution was transferred into the DyLight reagent tube and incubated for 60 min at room temperature in the dark. After removal of the storage solution of the purification resin, the labeled antibody solution was mixed with 540 µL of the purification resin and centrifuged at 1,000 x g for 45 s (Heraeus Fesco 17, Thermo Fisher Scientific). The purified solution was aliquoted and stored at -20 °C until use.

2.5. Instrumentation

Measurements were performed on a RT2005 SPFS spectrometer from Resonant Technologies GmbH, Germany. The system operates by coupling a p-polarized HeNe laser beam (λ = 633 nm) via a prism (LaSFN9) onto the metal film, evaporated on a glass slide (LaSFN9). The angle of incidence of the laser light is controlled by a goniometer, while the totally internal reflected light from the metal surface at the prism base is detected by a photodiode and recorded as a function of the angle of incidence. The surface plasmon resonance is detected as a minimum in the angle spectrum of the reflected light. At this angle of incidence the laser light’s wave vector and the surface plasmon’s wave vector matching is maximum. Angle dependent measurements by observing changes in the resonance angle (scan mode) as well as kinetic measurements by recording the shift of the resonance angle or changes in reflectivity at a fixed angle of incidence can be performed in the case of SPRS.

Materials adsorbed on metal films result in a change of the dielectric constant (ε_D) at the metal/dielectric interface. By this, the wave vector of the surface plasmon wave changes and therefore the resonance angle (θ), at which the surface plasmon excitation takes place, shifts. The shift of the resonance angle (Δθ = θ_f - θ_b) is proportional to a convolution of the change of the refractive index (ΔN) and the film thickness (Δd).² Because of the penetration depth for very thin films (d ≤ λ/2) one finds:
\[ \Delta \Theta \sim (N_{\text{film}} - N_{\text{dielectric}}) \Delta d \]

The complex refractive index $N$ consists of a real part and an imaginary part which is assigned to energy dissipation, e. g. absorption or scattering.

In SPFS the fluorescence signal of the fluorophore excited by the surface plasmon is recorded via a photomultiplier tube PMT (Resonant Technologies GmbH, Germany) placed perpendicular to the sensor surface. The PMT was combined with a computer controlled laser shutter (Resonant Technologies GmbH, Germany). The shutter was placed in front of the prism to prevent photo bleaching of the sample. In order to distinguish excitation light from fluorescence an interference filter (670FS10-25, LOT Oriel, Germany) was placed in front of the PMT entry window. The fluorescence detected by the PMT produces an electric signal which is registered by a counter and was computer-aided analysed.

The SPFS setup is controlled by WasPlas 3.0 software. Optical thickness calculations of the adsorbed layers were performed by using the Fresnel equations and the transfer matrix algorithm (Winspall 3.02 shareware from the Max Planck-Institute for Polymer Research, Germany).

### 2.7. Data analysis

The background noise ($I_{\text{background}}$), mainly caused by incomplete sensor recovery, was subtracted from the resulting fluorescence signal after binding of the fluorescence labeled detection antibody ($I_{\text{detection}}$), resulting in $\Delta I_{\text{Fl}}$. ($I_{\text{Fl}} = I_{\text{detection}} - I_{\text{background}}$). The mean values of $\Delta I_{\text{Fl}}$ of each concentration were plotted against the concentration with their maximum error for measurements in PBS (number of measurements ($m$) = 2 in PBS) and with standard deviation for serum ($m = 3$). The resulting concentration response curves were then fitted applying the Hill
equation \( y = \frac{[L]^n}{K_d} + [L] \), were \( L \) is the free unbound compound, \( K_d \) the apparent dissociation constant and \( n \) the Hill-coefficient) by using OriginPro 8.1G. The Hill equation describes the relationship between absorption of a compound to its binding sites and the influence of bound compounds about the binding characteristic of the compound.

The LOD was calculated using the equation (S-Eq. 1) from Naushad et al.\(^4\)

\[
LOD = \frac{3.3 \times SD}{S} \quad (S - \text{Eq. 1})
\]

As the standard deviation (SD) the blank response after the CRP addition was taken, including the background signal after every sensor regeneration step, and before the use of the detection antibody. The slope (S) was taken from the respective calibration curve recorded in PBS and serum.

The LOQ was calculated similar to the LOD by considering tenfold of the SD of the blank response after CRP addition.

In order to get information about the sensitivity of the sensor, it is also of interest to have quantitative data about the mass coverage available, especially of CRP-binding onto the capture antibody. Therefore, the mass coverage of the sensor was calculated using Fejter’s formula (S-Eq. 2) as described by Duque et al.\(^5\)

\[
M = d_A \frac{(n_A - n_{sol})}{dn/dc} \quad (S - \text{Eq. 2})
\]

For the calculation of the mass coverage (\( M \)) the optical thickness (\( d_A \)) in nm of the bound capture antibody and of CRP, the refractive indices of the adsorbed layer (\( n_A \)) and the cover media (\( n_{sol} = 1.33 \)) were used. The refractive index of the proteins was chosen to be 1.45 according to Özkumur et al.\(^6\) 0.182 g/cm\(^3\) was used as the refractive index/concentration increment, \( dn/dc \), of the proteins.\(^7\)\(^8\) Mass coverage by CRP was calculated after addition of 25 \( \mu \)g/mL CRP.
3. Results and Discussion

3.1. Pre-tests

Figure S-2. SPRS-scans including fits (--) of the SAM (■) and after saturating the surface with CRP (o). The insert shows a magnification of the particular resonance angles.

Figure S-3. SPRS-scans including fits (--) of the SAM (■), the BSA saturated SAM (x), and after the addition 100 µg/mL CRP (o). The insert shows a magnification of the particular resonance angles.
**Figure S-4.** SPRS scans including fits taken after SAM formation (■), saturation of the surface with BSA (X) and after adding serum (Δ) to the sensor. The insert shows a magnification of the particular resonance angles.

### 3.2. Sensor preparation

**Figure S-5.** Kinetic profile of the sensor preparation followed *in situ* with SPRS. The capture-antibody (AB) solution was injected after the activation step with EDC/NHS (not shown), followed by a rinsing step with PBS. The surface was passivated and saturated with BSA, followed by a final rinsing procedure with PBS.
3.3. CRP measurements

**Figure S-6.** Simultaneous measurement of SPRS (●)- and SPFS (X)-kinetics of the time dependent response of the CRP-sensor in CRP-free serum. The CRP-free serum sample was first added and circulated for 5 min., followed by a rinsing step with PBS, until no changes could be observed with SPRS. The fluorescence-labeled detection-antibody was then introduced. After equilibrium was reached the sensor was rinsed, resulting in a full recovery of the sensor, observed by the decline of the SPR-angle and the fluorescence signal returning to its original levels shown before injection of the detection antibody.

**Figure S-7.** Fluorescence intensity investigation at 0.1 µg/mL (●), 1 µg/mL (Δ), 2 µg/mL (x), 5µg/mL (o), 10 µg/mL (◊) and 25 µg/mL (+) CRP in serum. Also shown is a SPRS-scan taken at CRP-concentration of 25 µg/mL.

References