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Letter

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Label-Free Biosensing in Complex Media: A Referencing Approach

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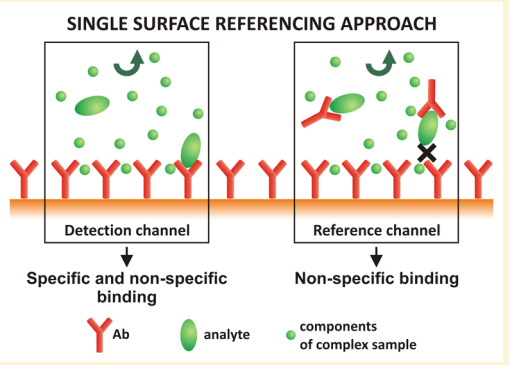
Supporting Information

ABSTRACT: We present a novel approach to reference-compensated label-free affinity biosensing in complex media. Unlike conventional approaches that employ surfaces with different biological functionalities in the detection and reference channels to produce a reference-compensated sensor response, the new approach (referred as to single surface referencing (SSR)) uses a single functionalized surface split into the detection and reference channel to which complex sample (detection channel) and complex sample mixed with biomolecules binding to the analyte and thus inhibiting the binding of the analyte to the functionalized surface (reference channel) is introduced. This approach ensures that (i) only the detection channel captures the analyte and (ii) nonspecific binding incurred in the detection and reference channels are the same. We evaluate this approach in a model biosensing experiment, detection of a cancer biomarker carcinoembryonic antigen (CEA) in blood plasma using antibody against CEA and a surface plasmon resonance (SPR) biosensor. We detect CEA in three different blood plasma samples and demonstrate that this novel referencing approach provides more accurate results and lower biological variability than the conventional referencing.



Label-free optical affinity biosensors measure changes in the refractive index caused by the interaction between immobilized biorecognition elements (e.g., antibodies) on a sensor surface and target analytes in a liquid sample. These biosensors offer high sensitivity and limits of detection down to sub-ng/mL;[1,2] however, they are susceptible to interferences that produce refractive index changes in the proximity of the sensor surface. These interferences include (i) background refractive index changes due to variations in the composition of the sample and fluctuations of temperature and (ii) the binding of nontarget molecules to the sensing surface (i.e., nonspecific binding). To mitigate these interferences, label-free affinity biosensors are typically composed of detection channels that respond to both analyte (producing the specific sensor response) and interferences as well as reference channels that respond to interferences only. Subtracting the response of the reference channel from that of the detection channel produces a reference-compensated sensor response. While the detection channel is typically functionalized with biorecognition elements recognizing the analyte, the reference channel is functionalized either with biorecognition elements against biomolecules not present in a sample[3,4] or with biomolecules minimizing the nonspecific binding (e.g., bovine serum albumin (BSA) or BSA conjugates).[5] Although this dual surface referencing (DSR) allows for accurate compensation for background refractive index changes in simple media (e.g., buffers), they fail to reference out the nonspecific binding from real-world complex samples (e.g., blood plasma).[4,6] This failure is primarily due to two factors: (i) the detection and reference channel employ functionalized surfaces with different physical and chemical characteristics, and (ii) the nonspecific binding properties of the sample are not known in advance, making it difficult to create detection and reference surfaces with the same nonspecific binding response for a given sample. For instance, the nonspecific binding of components from blood plasma to functionalized surfaces has a large dependency on the composition of blood plasma and may differ considerably from patient to patient.[7] Although advanced coatings exhibiting low fouling from complex media have been recently reported,[8,9] functional coatings allowing complete suppression of the nonspecific binding from complex media such as blood plasma have not been developed yet.

In this work we propose a new approach to referencing in label-free optical biosensors that uses a single functionalized surface split into the detection and reference channel (single surface referencing (SSR)) to reliably detect analyte in a complex sample. The potential of this approach is demonstrated in a model biosensing experiment in which the cancer marker carcinoembryonic antigen (CEA) is detected in blood plasma using a surface plasmon resonance (SPR) biosensor.

PRINCIPLE OF THE SSR APPROACH

In the proposed referencing approach, a single surface functionalized with the biorecognition molecule (e.g., antibody, (Ab)) is interfaced with two fluidic channels to form two independent sensing channels (see Figure 1). During the

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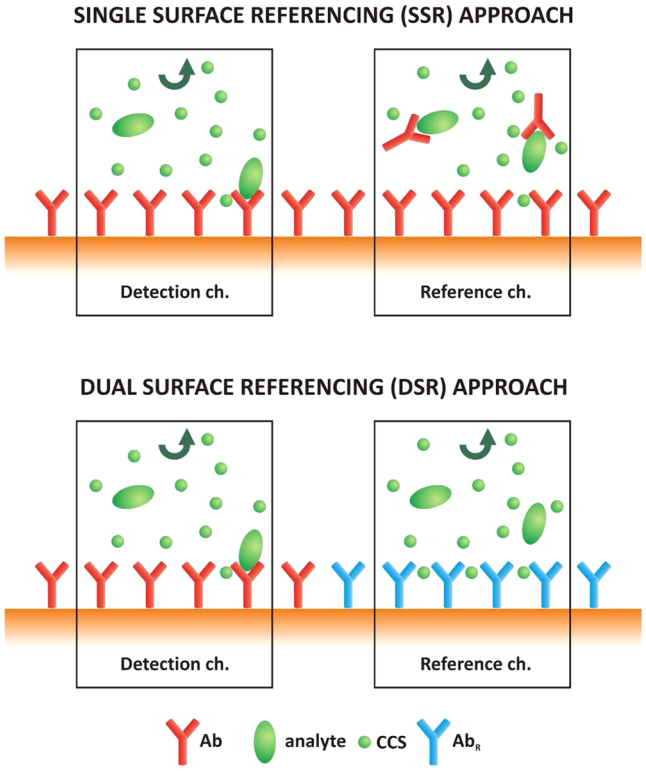

Figure 1. Principle of the SSR and conventional DSR approaches. Ab, Ab_R, and CCS denote antibody against analyte, reference antibody, and components of a complex sample, respectively.

experiment, the first channel (detection channel) is incubated only with the analyte sample while the second channel (reference channel) is incubated with the same analyte sample that has been mixed with an abundance of the antibody. In the reference channel, the antibody binds to the analyte present in the sample and acts to prevent the analyte from binding to the antibody on the sensor surface. While the detection channel responds both to the capture of analyte and nonspecific binding, the reference channel responds to the nonspecific binding only. In contrast to the conventional DSR approach, the level of nonspecific binding in both detection and reference channel is expected to be the same, as the same type of surface is used in both channels. The specific sensor response can therefore be determined by subtracting the response of the reference channel from that of the detection channel. It is worth mentioning that the SSR approach is not limited to antibodies and that any type of biorecognition molecule with high specificity and affinity to the analyte which prevents the analyte from binding to the biorecognition molecules immobilized on the surface of the sensor can be used.

MATERIALS AND INSTRUMENTATION

Reagents. Sodium acetate, KH_2PO_4, Na_2HPO_4, KCl, NaCl, NaOH, and BSA were purchased from Sigma-Aldrich, in molecular biology grade or higher. Antibody (Ab_{CEA}) against CEA and CEA were purchased from Fitzgerald. Reference antibodies against *Salmonella typhimurium* (Ab_{Salm}), horseradish peroxidase (Ab_{HRP}), tetrodotoxin (Ab_{TTX}), and human chorionic gonadotropin (Ab_{hCG}) were obtained from Meridian Life Science, GenWay Biotech, Hawaii Biotechnology Group, and Scripps, respectively. No cross-reactivity with CEA was determined for reference antibodies. Carboxy-terminated [HS−C_{11}−$(EG)_6$−OCH_2−COOH] and hydroxy-terminated [HS−C_{11}−$(EG)_4$−OH] alkanethiols were purchased from Prochimia, Poland. The composition of the phosphate buffer was 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4 at 25 °C. PBS and PBS_{NaCl} consisted of phosphate buffer with the addition of 137 mM and 750 mM NaCl, respectively. PBS_{BSA} and $PBS_{NaCl/BSA}$ buffers were prepared by adding 500 μg/mL BSA to PBS and PBS_{NaCl}, respectively. Normal human plasma (mixed/pooled gender) in sodium citrate (sample P_1) was purchased from Biochemed Services. Additional human plasma samples (samples P_2 and P_3) from two different healthy volunteers were kindly provided by the Institute of Hematology and Blood Transfusion, Prague, Czech Republic. In all the detection experiments reported herein, 50% plasma was used. The plasma samples were prepared by mixing the undiluted (100%) human plasma and PBS with 10 mg/mL BSA in a ratio of 1:1.

SPR Biosensor. A laboratory SPR biosensor (Plasmon IV) developed at the Institute of Photonics and Electronics, Prague, Czech Republic, was used in this study.[10] We employed a dispersionless microfluidics system, which allows for the delivery of the sample directly to the SPR chip without the effects of intra- and inter-sample dispersion.[11] SPR chips used in this study were prepared by coating BK7 glass substrates with thin layers of titanium (1−2 nm) and gold (48 nm) via e-beam evaporation. Initially, the surface of an SPR chip was functionalized with a self-assembled monolayer (SAM) of mixed carboxy- and hydroxy-terminated alkanethiols. Antibodies (Ab_{CEA}, Ab_{Salm}, Ab_{HRP}, Ab_{TTX}, or Ab_{hCG}) and blocking BSA protein were then covalently attached to a SAM of alkanethiols as described previously.[12] Prior to the detection experiment, 10 mM NaOH and $PBS_{NaCl/BSA}$ were injected to remove any remaining noncovalently bound antibodies from the surface of an SPR chip. In all SPR experiments, the flow rate and the temperature were set to 20 μL/min and 25 °C, respectively.

REFERENCE-COMPENSATED BIOSENSING

Experiments. We performed reference-compensated detection of CEA in PBS_{BSA} buffer and three different plasma samples using both the SSR approach as well as the conventional DSR approach with four different reference antibodies. In order to determine the concentration of free Ab_{CEA} to be used in the SSR approach, we measured the sensor response to the binding of a rather high concentration of CEA (1000 ng/mL) that was incubated with varying concentrations of free Ab_{CEA}. It was concluded that 5000 ng/mL Ab_{CEA} is sufficient to prevent 1000 ng/mL CEA from binding to the surface of SPR chip and that both Ab_{CEA} and Ab_{CEA}−CEA complex do not nonspecifically bind to the SPR chip functionalized with Ab_{CEA} (Supporting Information).

In the SSR approach, CEA at a concentration of 200 or 500 ng/mL in either PBS_{BSA} or 50% plasma (P_1, P_2, and P_3) samples was introduced into the detection channel functionalized with Ab_{CEA}. At the same time, the same CEA concentrations were mixed with 5000 ng/mL Ab_{CEA} (in the same PBS_{BSA} or in plasma sample) and introduced into the reference channel, also functionalized with Ab_{CEA}. In the DSR experiment, CEA at a concentration of 200 or 500 ng/mL in either PBS_{BSA} or plasma was injected into both the detection channel functionalized with Ab_{CEA} as well as into the reference channels that had been functionalized with Ab_{Salm}, Ab_{HRP}, Ab_{TTX}, or Ab_{hCG}. Finally, in order to reduce the nonspecific

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binding, the channels which were exposed to plasma samples were flushed with $PBS_{NaCl/BSA}$.

Results. Figure 2 shows the response of the detection and reference channels to CEA (500 ng/mL) in both PBS_{BSA} and

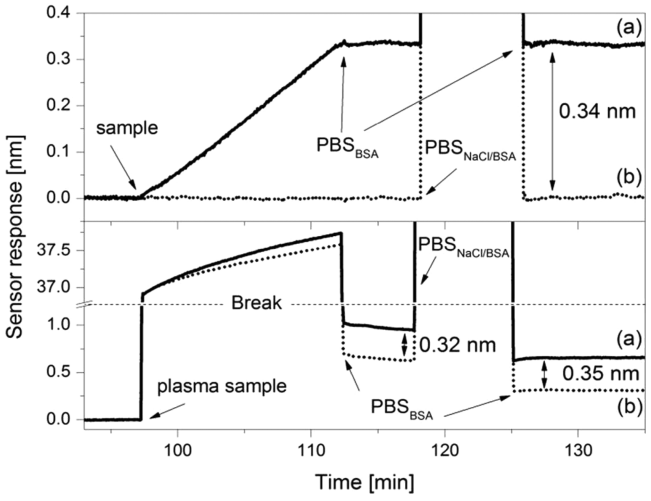

Figure 2. Reference-compensated detection of CEA in PBS_{BSA} (upper graph) and 50% human blood plasma P_1 (lower graph) for the SSR approach. Curve (a) denotes the response to the concentration of CEA of 500 ng/mL (detection channel). Curve (b) presents the sensor response to the mixture of 500 ng/mL CEA with an addition of 5000 ng/mL Ab_{CEA} (reference channel).

50% human blood plasma (P_1) in the SSR approach. The nonspecific binding observed in the reference channel in plasma P_1 before and after the washing step (0.62 and 0.32 nm) was equal to 180% and 94% of the specific response to CEA observed in buffer. The reference-compensated sensor response obtained in plasma (0.32 and 0.35 nm before and after the washing step, respectively) showed good agreement with the value obtained in PBS_{BSA} (0.34 nm). The reference-compensated sensor response for CEA at 200 ng/mL in 50% human blood plasma P_1 (0.13 and 0.15 nm before and after the washing step, respectively) was again close to the value determined in PBS_{BSA} (0.15 nm) (data not shown). Detection experiments performed in PBS_{BSA} using the DSR approach yielded sensor responses of 0.36 and 0.14 nm for concentrations of CEA of 500 and 200 ng/mL, respectively (see the Supporting Information). These results suggest that the SSR and DSR approaches provide similar results when used for detection in simple solutions (e.g., PBS_{BSA}).

The relative deviation of the reference-compensated sensor response from the sensor response obtained in PBS_{BSA}, defined as $|SR_{comp} - SR_{PBS(BSA)}|/SR_{PBS(BSA)}$, was determined for the two referencing approaches (SR_{comp} denotes the sensor response in the complex sample obtained with either the SSR or DSR approach; $SR_{PBS(BSA)}$ is the sensor response obtained with the DSR approach in PBS_{BSA}). The relative deviations for two different concentrations of CEA and three different plasma samples are presented in Figure 3 (the data are also presented in Table S-3 within the Supporting Information). The relative deviation of the sensor response for 200 ng/mL CEA obtained after the washing step with $PBS_{NaCl/BSA}$ ranged from 4 to 17% for the SSR approach as opposed to the DSR approach, which had relative deviation ranges of 38−44% (Ab-Salm), 115−273% (Ab-hCG), 23−35% (Ab-HRP), and 16−44% (Ab-TTX). For 500 ng/mL CEA, the respective relative deviation values were

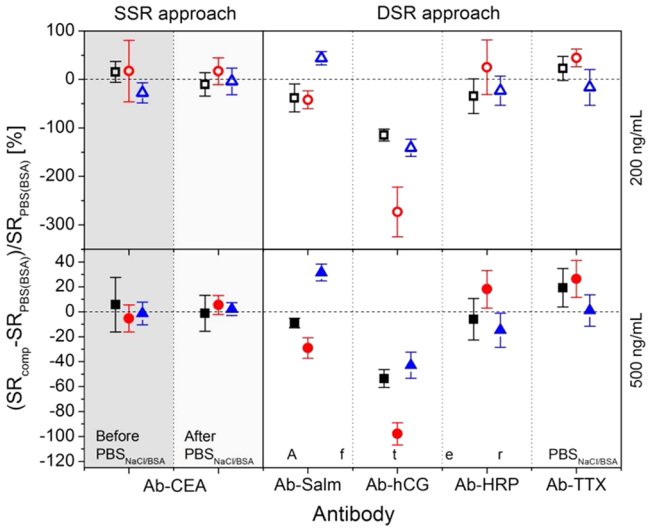

Figure 3. Normalized difference between the reference-compensated sensor response in plasma and PBS_{BSA} for three different plasma samples P_1, P_2 and P_3 (squares, circles, and triangles, respectively) and two different concentrations of CEA, 200 ng/mL (upper plot, open symbols) and 500 ng/mL (lower plot, solid symbols). Relative deviation values before and after the washing step are shown for the SSR approach; only the values after the washing step are shown for the DSR approach. The error bars are calculated as a relative standard deviation of at least three independent experiments.

found to range from 1 to 5% for the SSR approach and from 9 to 32% (Ab-Salm), 43 to 98% (Ab-hCG), 7 to 18% (Ab-HRP), and 1 to 26% (Ab-TTX) for the DSR approach. These results suggest that the SSR approach improves the relative deviation by at least 2.1 and 3.3 times for CEA concentrations of 200 and 500 ng/mL, respectively. If no washing step was employed, higher deviations of the sensor response from that obtained in buffer were observed. In spite of this decrease in accuracy (observed mainly for the 200 ng/mL CEA data), the SSR without the washing step was still found to provide a lower relative deviation than the DSR approach (with or without the washing step). It should be noted that the DSR approach employing Ab_{hCG} produces consistently lower responses in plasma samples than in PBS_{BSA}. We believe that this discrepancy may be in part attributed to the interaction between the plasma components and the reference antibody immobilized on the sensor surface.[4]

We also observed that the *absolute* deviations obtained using the SSR approach for the two concentrations of CEA were approximately equal. This suggests that the nonspecific binding properties of the functionalized surfaces were not affected by the CEA molecules bound to the detection surface and that the accuracy of the referencing is limited mainly by the imperfections between the detection and reference surfaces.

The biological variability of the sensor response was calculated for each referencing approach as a standard deviation of the normalized differences of the sensor response obtained in three different plasma samples P1, P2, and P3 (the data are also presented in Table S-4 in the Supporting Information). The biological variability of the sensor response obtained after the washing step was 14.2% and 3.3% for the SSR approach, 48.4% and 30.9% for DSR and Ab-Salm, 85% and 29.2% for DSR and Ab-hCG, 31.7 and 17.2% for DSR and Ab-HRP, and 30.7% and 12.9% for DSR and Ab-TTX, for CEA concentrations of 200 and 500 ng/mL, respectively. The SSR approach thus provided

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better biological variability of the sensor response than the DSR approach by a factor of 2.2−6.0 and 3.9−9.3 for CEA concentrations of 200 and 500 ng/mL, respectively, when the washing step was used. A similar difference in biological variability of the sensor response was observed when no washing step was performed. In the SSR approach, the ratio of the biological variability for 200 and 500 ng/mL CEA was the same before and after the washing step (in contrast to the DSR approach). This suggests that the biological variability of the sensor response in the SSR approach is determined in a large part by differences in the composition of plasma samples and that the nonspecific binding properties of the detection and reference surfaces are quite similar.

■ CONCLUSIONS

We demonstrated a new referencing approach for label-free detection of biomolecular analytes in complex samples. We evaluated this approach for the detection of the cancer biomarker CEA in blood plasma using antibody reactive against CEA using an SPR biosensor. We have demonstrated that the SSR approach provides more accurate results and lower biological variability than the conventional referencing approaches that rely on reference surfaces functionalized with different antibodies. This novel approach can be readily adapted for the use in other label-free biosensor technologies and analysis of other real-world complex samples.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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