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Notes & Tips

Catalytic enzyme activity on a biosensor chip: Combination of surface plasmon resonance and mass spectrometry

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ABSTRACT

Surface plasmon resonance (SPR) as a label-free biosensor technique has become an important tool in drug discovery campaigns during the last couple of years. For good assay performance, it is of high interest to verify the functional activity on the immobilization of the target protein on the chip. This study illustrates the verification of the catalytic activity of the drug target protein PqsD by monitoring substrate conversion as a decrease in SPR signal and product detection by ultra high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS^2). This assay would be applicable to control surface activity of immobilized ligands.

Surface plasmon resonance (SPR)[1] spectroscopy is an outstanding method for the characterization of a variety of molecular interactions. The real-time measurement allows the determination of kinetic parameters, thermodynamics, concentrations, and quantitative characterizations of ligand and analyte interactions [1]. The widespread application ranges, the low consumption of unlabeled protein, the monitoring of binding events, and the high-throughput capability are striking advantages of the SPR technique [2]. Typically, a ligand is permanently immobilized on a sensor surface, and by the adsorption of an analyte a rise in signal can be detected [3]. It is known that the immobilization process affects the activity of a ligand [4], complicating data analysis [5]. Therefore, it is of high interest to verify the ligand activity to obtain good-quality data. This proof is usually performed using an antibody or a known binder [6] providing affinity data. However, activity data are lacking.

In this article, we describe a novel approach for the determination of ligand activity, the investigation of the on-chip enzymatic product formation using mass spectrometry. PqsD was used as an exemplary protein that is discussed as a potential drug target for the development of antivirulence compounds [7]. PqsD is involved in the PQS biosynthetic pathway, a signal molecule of the QS (quorum sensing) communication system in *Pseudomonas aeruginosa* [7–9]. It belongs to the bisubstrate enzymes and catalyzes the biosynthesis of 2-heptyl-4(1*H*)-quinolone (HHQ) [10] by the condensation of anthraniloyl–coenzyme A (ACoA) [11] and β-ketodecanoate (β-K) [12]. HHQ is a precursor of PQS (2-heptyl-3-hydroxy-4(1H)-quinolone), and both interact with their receptor PqsR to control the transcription of PqsR-dependent target genes (e.g., virulence genes) [13].

The principle of product formation measurement is described in Fig. 1A. Immobilized PqsD is incubated successively with its two substrates. First, PqsD is incubated with ACoA, where anthranilic acid binds covalently to Cys112 of the catalytic center in the active site of the protein [14] and the second substrate, β-K, which is subsequently assembled in a condensation reaction with the covalently bound anthranilic acid to form HHQ. These steps are monitored by SPR, and the formed product is determined by mass spectrometry.

After EDC/NHS (*N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide) surface activation, PqsD was covalently immobilized in a carboxymethyl dextran matrix under acidic conditions (10 mM NaOAc, pH 4.5) to a high-density surface (see Supplementary material). The PqsD-loaded sensor was first treated with ACoA (K_D = 1.08 μM, Fig. 1B) for 5 min at 37 °C to allow a covalent binding of anthranilic acid, and excessive material was washed out by the buffer flow (5 min) until β-K (K_D = 2.95 mM, Fig. 1C) was injected for an additional 15 min. By the start of the second injection, the flow-through was collected for the mass spectrometric analysis. The SPR signal was monitored as shown in Fig. 1D. A clear signal increase was observed for ACoA, and it did not decrease back to the baseline level during the dissociation phase (see also Fig. S1 in Supplementary material), demonstrating the covalent binding of anthranilic acid (ΔRU = 15). As a consequence of the β-K injection, the baseline decreased to the initial level (0 RU), indicating the

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[1] *Abbreviations used:* SPR, surface plasmon resonance; HHQ, 2-heptyl-4(1*H*)-quinolone; ACoA, anthraniloyl–coenzyme A; β-K, β-ketodecanoate; UHPLC–MS^2, ultra high-performance liquid chromatography coupled to tandem mass spectrometry.

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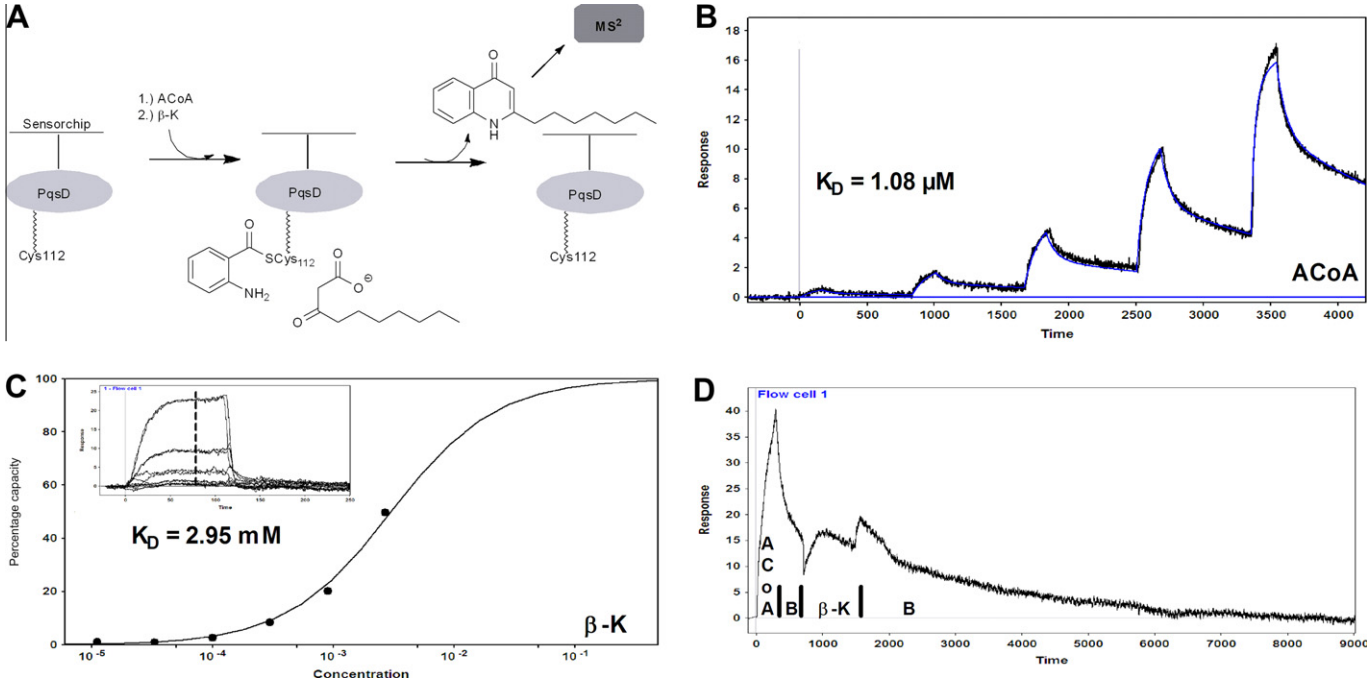

Fig.1. (A) Schematic view to detect surface activity of an enzyme immobilized on a biosensor chip. (B) Binding affinity for ACoA at 12 °C: Simulated data for a kinetic titration of ACoA binding to PqsD. The data set was simulated using $K_A = 1.11 \times 10^2\ M^{-1}\ s^{-1}$, $K_D = 1.12 \times 10^{-4}\ s^{-1}$, and analyte concentrations of 0.62, 1.85, 5.55, 16.67, and 50 µM. An association phase of 180 s and a dissociation phase of 600 s were used for the simulated data set. (C) Binding affinity for β-K. Inset: Overlay of sensorgrams for β-K measured in duplicates at 12 °C. Double-referenced data read-out is indicated by the dashed line. Analyte concentrations of 11.1, 33.3, 100.0, 300.0, 900.0, and 2700.0 µM, an association phase of 150 s and dissociation phase of 300 s were used. Fit of the duplicates of β-K equilibrium response data from the PqsD surface to a 1:1 interaction is shown. (D) Sensorgram of surface activity determination for PqsD immobilized on the surface. Injections are indicated in the figure: 5 µM ACoA for 5 min and 10 µM β-K for 15 min. The sensorgram is blank surface referenced. B, bufferflow.

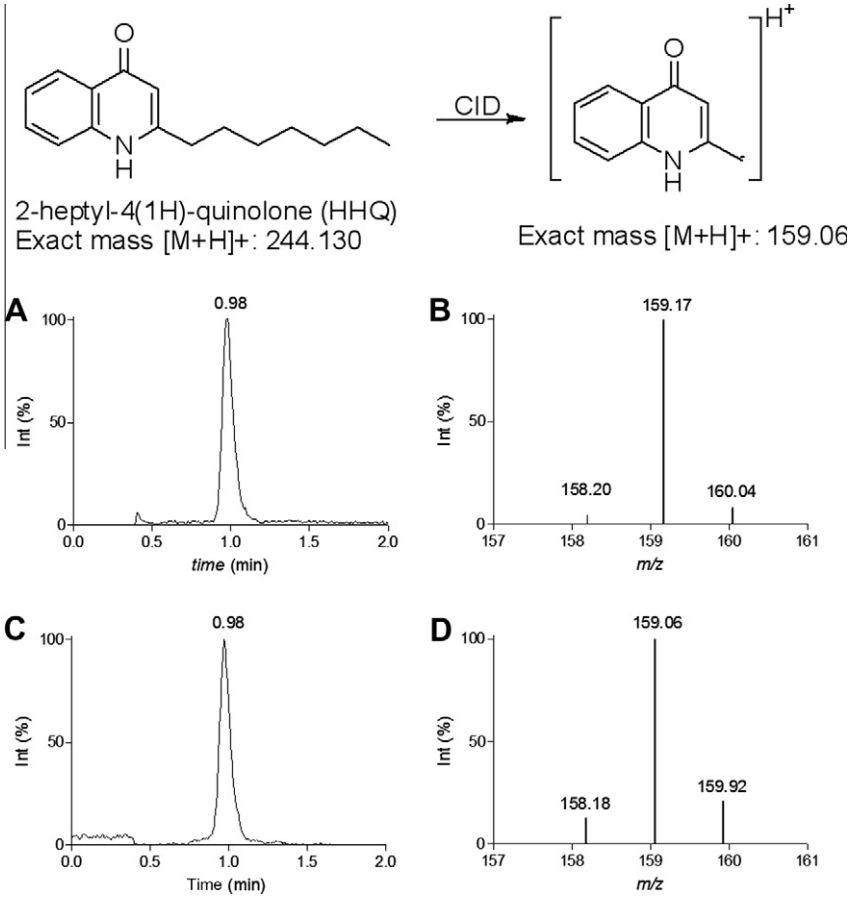

Fig.2. Fragmentation pattern of HHQ [15]. CID, collision-induced decay. (A) UHPLC–MS^2 analysis of synthetic HHQ reference. (B) UHPLC–MS^2 fragmentation pattern of synthetic HHQ reference at retention time of 0.98 min. (C) UHPLC–MS^2 analysis of HHQ formed on-chip. (D) UHPLC–MS^2 fragmentation pattern of HHQ formed on-chip at retention time of 0.98 min. All data for the mass spectroscopy were obtained on a Thermo Fisher TSQ Quantum Access Max mass spectrometer.

elimination of HHQ from the active site by the condensation of the two substrates. The flow-through was analyzed to prove the product formation (HHQ) by ultra high-performance liquid chromatography coupled to mass spectrometry (UHPLC–MS^2), which confirmed HHQ formation (147 pmol/pmol PqsD) on the PqsD sensor by comparison of retention time and fragmentation pattern [15] with the synthetic reference [12] (Fig. 2).

Thus, we provided direct experimental proof that PqsD, immobilized on a sensor surface, is catalytically active. For comparison of the activity of PqsD under nonimmobilized conditions, experiments were also performed with the enzyme in solution. PqsD was incubated in the same substrate order and time as in the biosensor experiments and was quantified for HHQ formation. Here, the turnover rate is approximately 2-fold higher compared with the activity of the immobilized enzyme (307 pmol/pmol PqsD).

Furthermore, ligand stability on the biosensor over time was demonstrated by measuring product formation over a time period of 16 days, resulting in quantifiable amounts of HHQ until day 5. After 16 days, HHQ signals were still detectable. The results demonstrate that the ligand immobilization onto a surface does not limit the functionality of the ligand, confirming the use of SPR as a label-free technology. Showing evidence of the catalytic activity of an immobilized enzyme before starting intensive SPR studies should be regarded as one of the initial steps in SPR assay development and would be very helpful for data analysis; hence, it also provides information of the binding site's accessibility of the immobilized ligand.

In conclusion, we have succeeded in demonstrating real-time monitoring of an on-chip enzymatic reaction by the combination of SPR and mass spectrometry. The current study provides useful information about functional activity and stability of the exemplary protein PqsD on the surface of a chip and illustrates an improved alternative for biosensor surface evaluation. It can be used instead of an antibody or a known binder as positive control. Demonstrating product formation, we have shown that an immobilization process is not necessarily detrimental for activity of an immobilized ligand. Therefore, the on-chip reaction is a method that can be applied to further activity monitoring of potential target proteins that are to be examined by SPR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2012.05.024.

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