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On-line coupling of surface plasmon resonance optical sensing to size-exclusion chromatography for affinity assessment of antibody samples

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ABSTRACT

Surface plasmon resonance (SPR) is an optical technique that measures biomolecular interactions. Stand-alone SPR cannot distinguish different binding components present in one sample. Moreover, sample matrix components may show non-specific binding to the sensor surface, leading to detection interferences. This study describes the development of coupled size-exclusion chromatography (SEC) SPR sensing for the separation of sample components prior to their on-line bio-interaction analysis. A heterogeneous polyclonal human serum albumin antibody (anti-HSA) sample, which was characterized by proteomics analysis, was used as test sample. The proposed SEC-SPR coupling was optimized by studying system parameters, such as injection volume, flow rate and sample concentration, using immobilized HSA on the sensor chip. Automated switch valves were used for on-line regeneration of the SPR sensor chip in between injections and for potential chromatographic heart cutting experiments, allowing SPR detection of individual components. The performance of the SEC-SPR system was evaluated by the analysis of papain-digested anti-HSA sampled at different incubation time points. The new on-line SEC-SPR methodology allows specific label-free analysis of real-time interactions of eluting antibody sample constituents towards their antigenic target.

1. Introduction

During the last decades, surface plasmon resonance spectroscopy (SPR) has emerged as a fast and sensitive optical technique to study biomolecular affinity interactions [1] and their kinetics [2]. Most SPR instruments use Kretschmann based configurations [3] comprising an optical part with a prism, a metal layer, and a liquid handling part. When p-polarized light is shone through the prism on an electrically conducting metal layer (e.g. gold) in a certain angle, light is absorbed by the free electrons in the metal layer, causing them to resonate in so-called surface plasmons [4]. The angle where the intensity of the reflected light is minimal [5], known as the SPR dip-angle, is sensitive to changes in the dielectric of the metal and surrounding medium, and thus can be used to detect binding of compounds to the metal film surface [6]. Variations in the SPR dip-angle [6] are monitored as function of time yielding a so-called sensorgram [7]. Commonly, a ligand is immobilized on the metal surface and then the potential interaction of specific sample components with the immobilized ligand is monitored by SPR by flushing the sample through a flow channel positioned on the metal sensor.

During direct analysis of biological samples by SPR, interfering components of the sample matrix may non-specifically bind to the sensor surface [8]. Moreover, SPR is not able to discriminate different ligand-binding components present in one sample. Combining a liquid chromatographic (LC) separation with SPR is one way to circumvent the aforementioned drawbacks, allowing assessment of the affinity of individual components with the immobilized ligand. Until now, only a few studies on on-line LC-SPR have been reported [9–12]. In one of the first works, SPR was used as a refractive index detector after LC separation, but no real bind-

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ing events were monitored [9]. In another work, oligosaccharides were separated with a gel permeation column and their affinities towards lectins were monitored with SPR both off- and on-line [10]. A similar approach has been applied for assessment of the interaction between carbohydrates and an immobilized monoclonal oligosaccharide-antibody [11]. However, a relatively low SPR signal was obtained due to the low molecular weight of the carbohydrates and sample dispersion. Carbohydrate detection was actually established largely via refractive index changes in solution and not via actual binding events. A more recent study reported monitoring of electrostatic adsorption of proteins on chemically modified SPR surfaces after size-exclusion chromatography (SEC) [12].

So far, LC-SPR has been confined to either weak affinity [9–11] or non-specific interactions [12].

Our goal was to make a significant step forward by developing an LC-SPR method for probing specific protein interactions with high affinity. As a model system, we used binding of a polyclonal anti-human serum albumin antibody (anti-HSA) with HSA. Initial MS analysis of the anti-HSA sample confirmed presence of several other proteins in the sample, enabling evaluation of the specificity and selectivity of the developed approach. SEC was used for separation since it allows elution of compounds under near-physiological and isocratic conditions. This ensures adequate binding conditions throughout the entire run when the compounds reach the SPR sensor surface where HSA is immobilized. The effect of several chromatographic parameters on the separation of the components as well as their binding to the SPR surface was systematically studied. In order to avoid surface saturation, a number of switch valves was included in the system to allow on-line regeneration of the sensor surface in between injections as well as heart cutting prior to SPR detection. Papain-digested [13] fragments from the antibody preparation were also used as an example of complex mixture, demonstrating further applicability and selectivity of the SEC-SPR system. The papain-digested anti-HSA preparations were sampled at different incubation time points and directly analysed by the SEC-SPR system. This manuscript presents the development of on-line SEC-SPR as a powerful technique to study the binding characteristics of the individual fragments present in complex mixtures.

2. Materials and methods

2.1. Chemicals and reagents

Human serum albumin (HSA), anti-human albumin antibody produced in rabbit, phosphate buffered saline (PBS), papain from papaya latex, 2-(*N*-morpholino)ethanesulfonic (MES) monohydrate, ethanolamine hydrochloride, L-cysteine, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), iodoacetamide (IAM) and ammonium bicarbonate were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium azide (toxic, should be discarded in hazardous waste) and sodium sulfate were obtained from Mallinckrodt Baker B.V. (Deventer, the Netherlands). Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Acros Organics (New Jersey, USA). β-mercaptoethanol was obtained from Merck (Darmstadt, Germany). Acetonitrile LC–MS grade and formic acid ULC–MS grade were purchased from Biosolve B.V. (Valkenswaard, The Netherlands). Trypsin sequencing grade, modified, was purchased from Roche (Mannheim, Germany). Deionized water was produced by a Milli-Q purification system from Millipore (Amsterdam, The Netherlands).

2.2. Separation of polyclonal anti-HSA samples by SEC with UV detection

Separation of the antibody preparations was carried out on a Shimadzu LC system equipped with an LC-20AB binary system pump, a UV detector SPD-20A and an autosampler SIL-20AC ('s Hertogenbosch, The Netherlands). The TSK gel super SW2000 and SW3000 (4.6 × 300 mm) SEC columns were from TOSOH Bioscience (Griesheim, Germany). Separations were done in isocratic mode using 0.01 M phosphate buffer (pH 7.4) with 2.7 mM potassium chloride, 0.137 M sodium chloride and 0.05% sodium azide as mobile phase at a flow rate of 0.15 mL/min with SW3000 and 0.1 mL/min with SW2000. Samples were prepared in mobile phase and kept in the cooled autosampler at 10 °C before injection; 20 μL of each sample was injected. Elution was monitored using UV absorption at 280 nm. For characterization of the eluting fractions by intact protein analysis (see Section 2.4.1) and by a proteomics approach after tryptic digestion (see Section 2.4.2), separated fractions were collected manually and subsequently freeze-dried with a Speed-Vac freeze dryer from Labconco (Abcoude, The Netherlands).

2.3. Sample digestion preparations

2.3.1. Papain digestion of polyclonal anti-HSA

2.3.1.1. Time series analysis. Papain digestion of polyclonal HSA antibody (200 μg/mL) to produce Fab and Fc fragments was performed in digestion buffer containing 0.1 mM tris-HCl, 2 mM EDTA, and 10 mM cysteine (pH 7.2). Papain was first activated in the digestion buffer at 37 °C for 10 min. The polyclonal HSA antibody was added to the papain digestion buffer with a final papain:antibody ratio of 1.25 (w/w). The solution was then incubated at 37 °C at 5 min, 30 min, 1 h and 2 h of papain digestion and directly injected on the SEC column. Digested antibody preparations were analyzed with a SW3000 column which is suitable for antibodies, their fragments, and other large biomolecules. The analysis was done at a flow rate of 150 μL/min with a 20 μL injection volume (Section 2.6).

2.3.1.2. Heart-cutting of fab fraction. 100–1000 μg/mL antibody samples were incubated for 2 h under digestion conditions and were analyzed with SW3000 volumn. Using an external switch valve and a pump, only the Fab fraction was directed to the surface of SPR (Section 2.6).

2.3.1.3. Intact protein analysis. For intact protein analysis, 200 μg/mL antibody samples were incubated for 2 h under digestion conditions. Afterwards, 20 μg/mL (100 μL total volume) of polyclonal HSA antibody samples and papain digested antibody samples were freeze-dried overnight with a Speed-Vac freeze dryer and subsequently re-dissolved in ACN/H2O (50/50 (v/v), 100 μL) for MS anlysis (Section 2.4.1).

2.3.2. Tryptic digestion of antibody fractions

Collected and freeze-dried antibody fractions, as described in Section 2.2, were re-dissolved in 100 μL of MQ water. To remove salts from these samples, they were transferred to 3 kDa spin filters (Millipore corporation, Billerica, Massachusetts, USA) which centrifuged for approximately 60 min at 14,000 rpm, until more than 90% of the solution passed through the filter. Proteins in the concentrate were then re-dissolved with 100 μL Milli-Q water and transferred to Eppendorf tubes. These samples were again freeze-dried and re-dissolved in 25 μL of 25 mM ammonium bicarbonate (pH 8.2). After addition of 1.5 μL 0.5% (v/v) β-mercaptoethanol, the solutions were incubated at 90 °C for 5 min followed by cooling to room temperature. Then, 3 μL of 100 mM IAM was added to the solutions, and the mixtures were incubated in the dark at room