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A fully validated liquid chromatography-mass spectrometry method for the quantification of the soluble receptor of advanced glycation end-products (sRAGE) in serum using immunopurification in a 96-well plate format

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ABSTRACT

The study of proteins is central to unraveling (patho)physiological processes and has contributed greatly to our understanding of biological systems. Corresponding studies often employ procedures to enrich proteins from their biological matrix using antibodies or other affinity binders coupled to beads with a large surface area and a correspondingly high binding capacity. Striving for maximal binding capacity may, however, not always be required or desirable, for example for proteins of low abundance. Here we describe a simplified immunoprecipitation in 96-well ELISA format (IPE) approach for fast and easy enrichment of proteins. The applicability of this approach for enriching low-abundant proteins was demonstrated by an IPE-based quantitative workflow using liquid chromatography-mass spectrometry (LC-MS) for the soluble Receptor of Advanced Glycation End-products (sRAGE), a promising biomarker in chronic obstructive pulmonary disease (COPD). The method was validated according to U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines and enabled accurate quantitation of sRAGE between 0.1 and 10 ng/mL in 50 µL serum. The assay showed substantial correlation with the two most commonly-used sRAGE immunoassays (ELISAs) (R^2-values between 0.7 and 0.8). However, the LC-MS method reported 2–4 times higher sRAGE levels compared to the ELISAs, which is largely due to a suboptimal amount of capturing antibody and/or calibration strategy used by the immunoassays. In conclusion, our simplified IPE approach proved to be an efficient strategy for enriching the low-abundant protein sRAGE from serum and may provide an easy to use platform for enriching other (low-abundant) proteins from complex, biological matrices.

1. Introduction

Studying proteins in complex, biological matrices is a common feature of (bio)medical research and has contributed considerably to our current understanding of life processes. For studies on protein expression, structure, and interactions, a variety of analytical techniques is being deployed including gel electrophoresis, liquid chromatography and mass spectrometry, which may all require specific protein enrichment procedures depending on the aim of a study and/or the protein(s) of interest [1,2]. Immunopurification is an eminent example of such enrichment techniques and uses specific 'bait' proteins to selectively bind and purify the targets of interest (*e.g.* antigens) [3]. Antibodies are generally used to capture proteins or even protein complexes, though conversely, antigens may also serve as baits to capture antibodies which

Abbreviations: BSA, bovine serum albumin; CML-BSA, Nε-(carboxymethyl)lysine-modified bovine serum albumin; COPD, chronic obstructive pulmonary disease; CV, coefficient of variation; EMA, European Medicines Agency; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; HMGB1, high mobility group box 1 protein; IPE, immunoprecipitation in 96-well ELISA format; LC-MS, liquid chromatography–mass spectrometry; LLOQ, lower limit of quantification; PBS, phosphate-buffered saline; PTM, post translational modification; QC, quality control; S100A12, protein S100-A12; SAA1, serum amyloid A1; SIL, stable-isotope-labeled; SNP, single nucleotide polymorphism; (s)RAGE, (soluble) receptor of advanced glycation end-products

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may be valuable for analyzing autoantibodies [4,5]. The former approach is a widely-used application of antibodies in basic and applied scientific research, and has contributed to the conception of antibodies being the 'workhorses' of (bio)medical experiments [6].

Besides bait proteins, immunopurification strategies require a (solid) support to which a bait protein is or can be coupled thereby allowing to separate the bait/target-complex from the original matrix [7]. Examples of such supports comprise gel-based (*e.g.* agarose) and magnetic beads each having specific advantages and disadvantages in terms of binding capacity, protocol flexibility and throughput as well as the degree and extent of non-specific binding [8]. As alternative to bead-based supports, (adsorptive) microtiter plates commonly used for immunoassays, have also been employed for immunoaffinity enrichment purposes [9–12]. Some hybrid assays based on immunoaffinity enrichment and digestion in microtiter plates followed by LC-MS detection demonstrated efficient enrichment of low abundant proteins [9,10]. A similar approach (referred to as immunoprecipitation in 96-well ELISA format, or IPE) showed matching capabilities, though IPE features a decoupled enrichment and digestion strategy and thereby also allows to study intact proteins, for example by top-down proteomics or Western Blot analysis [11]. This approach utilizes microtiter plates coated with Protein (A/)G to which antibodies are bound and covalently coupled with disuccinimidyl suberate (DSS). Although this methodology has many potential applications, examples of such applications are absent in literature, which may be because IPE's advantages compared to (magnetic) beads-based alternatives are less pronounced due to its dependence on Protein (A/)G-coated plates.

The soluble Receptor of Advanced Glycation End-products (sRAGE) is a potential biomarker for chronic obstructive pulmonary disease (COPD) and an example of a clinically relevant protein of low abundance [13]. sRAGE is formed after proteolytic cleavage of membrane-bound RAGE, a pattern recognition receptor involved in pro-inflammatory signaling pathways [14]. In addition, sRAGE can be formed upon alternative splicing of the *AGER* gene thereby leading to a RAGE splice variant known as endogenous secretory RAGE (esRAGE) [15]. Circulating RAGE has anti-inflammatory properties by acting as a decoy receptor for pro-inflammatory RAGE ligands and also by inhibiting homo-dimerization of membrane-bound RAGE which is presumed to be essential for RAGE activation [16–18]. In several (large-scale) biomarker studies, sRAGE was put forward as useful biomarker in COPD, particularly with respect to the presence and progression of emphysema, and sRAGE was consequently considered for biomarker qualification by the U.S. Food and Drug Administration (FDA) [19,20]. However, current knowledge about sRAGE is strongly depending on measurements with "research-grade" enzyme-linked immunosorbent assays (ELISA) from a single vendor, and appropriately validated assays are lacking [19]. Furthermore, it is known that sRAGE is regulated by alternative splicing and post translational modifications, including proteolytic cleavage and N-linked glycosylation [21]. Circulating RAGE thus likely comprises a series of related proteins (also referred to as 'protein species' or 'proteoforms') with potentially different functions, activities or ligand specificities [21–23]. It is therefore not only essential that sRAGE assays for clinical use are adequately validated, but these assays also need to be adequately characterized with respect to the exact forms of circulating RAGE that are being quantified.

In this study, we present an efficient, fast, and easy to use enrichment strategy for proteins in complex matrices on the basis of antibodies directly immobilized on high affinity microtiter plates. This methodology was combined with liquid chromatography-mass spectrometry (LC-MS) for quantifying sRAGE in human serum based on specific peptides in its N-terminal region which is essential for the binding of most RAGE ligands [14,24]. The method was validated according to FDA and European Medicines Agency (EMA) guidelines with a lower limit of quantification (LLOQ) of 0.1 ng/mL [25,26]. The validated LC-MS assay for sRAGE is expected to contribute to the development of sRAGE as biomarker in COPD.

2. Experimental

2.1. Chemicals and materials

Recombinant human RAGE encompassing the extracellular domain of this protein (Cat. No. C423; UniProtKB ID 'Q15109'; Ala23-Ala344 with C-terminal hexa-histidine tag) was purchased from Novoprotein (Summit, NJ, U.S.A.), monoclonal anti-RAGE antibody (Cat. No. MAB11451; clone 176902) was obtained from R&D Systems (Abingdon, U.K.), and stable-isotope-labeled RAGE peptides (*i.e.* IGEPLVL**K***& VLSPQGGGPWDSVA**R***) were synthesized by Pepscan Presto (Lelystad, The Netherlands). Acetonitrile (ACN; LC-MS grade) was obtained from Biosolve (Valkenswaard, The Netherlands) and sequencing grade modified trypsin was purchased from Promega (Madison, WI, U.S.A.). Nunc-Immuno™ MicroWell™ 96 wells plates with MaxiSorp™ coating (Cat. No. M9410), bovine serum albumin (BSA; Cat. No. A7638), and phosphate buffered saline (PBS; 10 × ; Cat. No. D1408) as well as all other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Serum samples

Serum was obtained from healthy volunteers and was pooled for preparation of the quality control (QC-)samples. Pooled serum was used directly as QC-medium sample, diluted eight times with 1% BSA in 1 × PBS, pH 7.4 (Surrogate Matrix) for preparation of the QC-low sample, or fortified with 5 ng/mL recombinant RAGE to obtain the QC-high sample. Recovery and spike recovery experiments were carried out using six different sources of human serum from (seemingly) healthy subjects (all from Seralab). Spike recovery experiments were furthermore performed on a lipemic serum sample (triglyceride content > 150 mg/dL; obtained from Seralab) and a hemolytic sample which was prepared by adding freeze-thawed whole blood (2%) to human serum.

2.3. Calibrants and internal standard

Lyophilized RAGE was dissolved in Milli-Q water to obtain a 200 µg/mL solution (based on the quantity as declared by the supplier) which was diluted to 100 µg/mL with 1 × PBS, pH 7.4 (PBS Buffer) after checking protein purity by SDS-PAGE and MALDI-TOF MS, which did not reveal the presence of proteins other than sRAGE. The resulting stock solution was sequentially diluted to 100 ng/mL with Surrogate Matrix (see Serum samples section above), and calibration samples were prepared at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 8.0 and 10.0 ng/mL. For the internal standard (IS), SIL-peptides (supplied as 5 pmol/µL solutions in 5% ACN) were mixed 1:1 and diluted to 5 fmol/µL with 1% dimethyl sulfoxide (DMSO) in water.

2.4. Simplified IPE protocol

(**1: plate coating**) The plate was coated using 100 µL aliquots of PBS Buffer containing 0.5 µg of antibody (from a 200 µg/mL stock solution; antibody was reconstituted in PBS Buffer) which were added to microplate wells, followed by overnight incubation at room temperature. (**2: plate blocking**) After removal of unbound antibody by three washing steps with 300 µL Wash Buffer (0.05% Tween-20 in PBS Buffer), uncoated surface was blocked with 300 µL Surrogate Matrix for 60 min while shaking on a plate shaker (600 RPM; room temperature). (**3: immunocapture**) Wells were washed three times with 300 µL Wash Buffer, and 100 µL of Sample Solution (for which 60 µL of serum was pre-mixed with 60 µL Surrogate Matrix to allow quantitative transfer of Sample Solution) was added to the wells for the immunocapture of sRAGE (120 min; 600 RPM; room temperature). (**4: analyte collection**) The wells were washed three times with 300 µL Wash Buffer, and sRAGE was eluted from the antibodies with 100 µL 0.1% aqueous trifluoroacetic acid (TFA) solution (10 min; 600 RPM; room temperature).