CHAPTER IV

Affimers as an alternative to antibodies in an affinity LC-MS assay for quantification of the soluble receptor of advanced glycation end-products (sRAGE) in human serum

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ABSTRACT

Antibodies are indispensable tools in biomedical research, but their size, complexity, and sometimes lack of reproducibility created a need for the development of alternative binders to overcome these limitations. Affimers are a novel class of affinity binders based on a structurally robust protease inhibitor scaffold (i.e. Cystatin A), which are selected by phage display and produced in a rapid and simple *E. coli* protein expression system. These binders have a defined amino acid sequence with defined binding regions and are versatile thereby allowing for easy engineering. Here we present an affimer-based liquid chromatography-mass spectrometry (LC-MS) method for quantification of the soluble Receptor of Advanced Glycation End-products (sRAGE), a promising biomarker for chronic obstructive pulmonary disease (COPD). The method was validated according to European Medicines Agency and U.S. Food and Drug Administration guidelines and enabled quantitation of serum sRAGE between 0.2 and 10 ng/mL. Comparison between the affimer-based method and a previously developed, validated antibody-based method showed good correlation ($R^2 = 0.88$), and indicated that 25% lower sRAGE levels are reported by the affimer-based assay. In conclusion, we show the first-time application of affimers in a quantitative LC-MS method, which supports the potential of affimers as robust alternatives to antibodies.
4.1. INTRODUCTION

Antibodies have found numerous applications in present-day biomedical research owing to their capability of binding antigens with high affinity and specificity. These affinity reagents are for example widely used for the enrichment of target molecules, the detection of target molecules, and the analysis of cells. Antibodies have furthermore become well-rooted in clinical practice as exemplified by the more than 60 therapeutic antibodies reaching the market since the first therapeutic antibody, Muromonab-CD3, received regulatory approval from the United States (U.S.) Food and Drug Administration (FDA) in 1986.

The possibilities and importance of antibodies are beyond dispute though these proteins are not without their limitations. For example, antibodies are large and complex proteins with a molecular weight around 150 kilodalton featuring several disulfide bonds and N-glycosylated asparagine residues. Antibody production accordingly is a challenging and costly process. Their production is furthermore difficult to control as reflected in an increasing number of reports describing reproducibility issues. Consequently, considerable efforts have been expended in recent years to develop non-antibody affinity ligands, which resulted in an impressive number of putative antibody alternatives, including adnectins, affibodies, anticalins, avimers, DARPin, fynomers, knottins, and kunitz domains. Affimers which are derived from the cysteine protease inhibitor Cystatin A, represent another example of antibody alternatives. These innovative affinity ligands can be produced quickly, relatively easily, and without the use of animals. Affimers are small, versatile, and stable proteins which can be engineered to bind target proteins with high affinity and selectively. Examples of their application are still limited in number as affimer technology has only been established recently, though the few available examples do indicate that affimers represent attractive alternatives to antibodies, at least for some applications.

Recently, we reported on the development and validation of an antibody-based immunoaffinity liquid chromatography-mass spectrometry (LC-MS) method for quantification of the soluble Receptor of Advanced Glycation End-products (sRAGE) in human serum. sRAGE has anti-inflammatory properties by acting as a decoy receptor for pro-inflammatory ligands in the lungs and is considered to be a promising biomarker candidate for chronic obstructive pulmonary disease (COPD) on the basis of findings in several large-scale clinical studies. In addition, sRAGE is considered to be an interesting biomarker as well for other diseases including diabetes mellitus, autoimmune diseases, and neurological diseases. The corresponding findings are, however, all based on data obtained with a single sRAGE immunoassay. In our initial report on the antibody-based LC-MS method, we presented data that triggered concerns about the accuracy of the immunoassay. Since our antibody-based LC-MS method relies on antibodies from the vendor of the sRAGE immunoassay, we felt the
need for complementary, antibody-free strategies for enriching sRAGE to support the further development of sRAGE as a biomarker.

In this study we describe the application of affimers in an LC-MS method for the quantification of sRAGE in human serum. The affimer-based method met the requirements as stipulated in the European Medicines Agency (EMA) and FDA guidelines on bioanalytical method validation, and its measurements correlated well with those carried out using a previously developed antibody-based LC-MS method. The successful application of affimers in a quantitative sRAGE method is expected to contribute to further elucidating the role of sRAGE in COPD pathophysiology and in facilitating further development for this highly promising COPD biomarker candidate.

4.2. EXPERIMENTAL SECTION

4.2.1. Chemicals and materials
Recombinant human sRAGE (rh-sRAGE; Cat. No. C423; UniProtKB ID ‘Q15109’; Ala23-Ala344 with C-terminal hexa-histidine tag) was purchased from Novoprotein (Summit, NJ, U.S.A.), anti-sRAGE affimers (raised against recombinant human sRAGE from Novoprotein, Cat. No. C423) were produced and supplied by Avacta Life Sciences (Wetherby, U.K.), and stable-isotope-labeled RAGE peptides (i.e. IGEPLVLK* & VLSPQGPPWSVAR*) 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), Trizma base (tris; Cat. No. T6791), and phosphate buffered saline (PBS; 10x; Cat. No. D1408) as well as all other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

4.2.2. Serum samples
Pooled human serum from Seralab (West Sussex, U.K.) was diluted with 10 mM tris-buffered saline, pH 7.5 (TBS Buffer) containing 1% BSA for preparation of the QC-low sample or was fortified with recombinant RAGE at two levels to obtain the QC-medium and QC-high samples. Recovery and spike recovery experiments were carried out using six different sources of human serum from healthy subjects (all from Seralab). Recovery and 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.
4.2.3. Calibrants and internal standard

Lyophilized sRAGE 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 verifying the absence of proteins other than sRAGE using SDS-PAGE and MALDI-TOF MS. The resulting solution was sequentially diluted to 100 ng/mL with 1% BSA in TBS Buffer (Surrogate Matrix), 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 in Surrogate Matrix. The internal standard (IS) stock solution was prepared by mixing equimolar amounts of the two SIL-peptides (supplied as 5 pmol/µL solutions in 5% ACN) and subsequently diluting these peptides to 5 fmol/µL with 1% dimethyl sulfoxide (DMSO) in water.

4.2.4. Affimer-based sRAGE capture and in-well digestion

(1: plate coating) Microplate wells were coated overnight (room temperature) with 100 µL PBS Buffer containing 0.5 µg of the affimers B7 and G10 (affimers were supplied as 1 mg/mL solutions in citrate buffered saline, pH 6.5 containing 5 mM TCEP and 0.02% sodium azide). (2: plate blocking) After removal of unbound affimers by three washing steps with 300 µL Wash Buffer (0.05% Tween-20 in TBS Buffer), uncoated surface was blocked with 300 µL Blocking Buffer (0.01% BSA in TBS Buffer) for 30 minutes while shaking on a plate shaker (600 RPM; room temperature). (3: sRAGE capture) 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 capture of sRAGE (120 min; 600 RPM; room temperature). (4: disulfide bond reduction) After three washing steps with 300 µL Wash Buffer, 100 µL Digestion Buffer (50 fmol/mL SIL-peptides in 50 mM ammonium bicarbonate (ABC) containing 10 mM TCEP) was added to the wells, and disulfide bonds were reduced following 30 minutes of incubation (600 RPM; room temperature). (5: cysteine alkylation) Thiols were alkylated in 20 mM iodoacetamide (IAM) (5 µL 420 mM IAM in ABC) for 30 minutes in the dark (600 RPM; room temperature) after which non-reacted IAM was quenched with a 0.5 molar excess of DTT (5 µL 210 mM DTT in ABC). (6: trypsin digestion) 100 ng of trypsin was added to each sample, plates were sealed with a 96 well cap mat (Screening Devices B.V., Cat. No. SD964075), and proteins were digested following overnight incubation in an oven kept at 37 °C. Next, plates were briefly centrifuged in a plate centrifuge to spin down droplets and condensation, and the digests were acidified through addition of 5 µL of 25% formic acid (FA).
4.2.5. LC-MS
Analyses were performed with a Waters Ionkey/MS system using an ACQUITY M-Class UPLC and a XEVO TQ-S mass spectrometer (Milford, MA, U.S.A.). Chromatographic separation was achieved on a C18-bonded Waters iKey HSS T3 Separation Device (1.8 μm particles, 100 Å pore size, 150 μm × 100 mm; Cat. No. 186007261) which was kept at 40 °C, using 0.1% FA in H₂O as mobile phase A and 0.1% FA in ACN as mobile phase B. 20 μL of sample was loaded onto a Dionex Acclaim PepMap100 C18 trap column (5 μm particles, 100 Å pore size, 300 μm × 5 mm; Cat. No. 160454) for 2.5 min with 3% B at 20 μL/min. Subsequently, peptides were separated on the analytical column at 3 μL/min with a 10 minute linear gradient from 3 to 33% B, after which the column was cleaned (0.6 min at 60% B and 2.1 min at 95% B) and equilibrated (4.3 min at 3% B). Mass spectrometric detection was performed using the following conditions: ESI positive, capillary voltage 3.5 kV, cone voltage 30 V, source offset 50 V, source temperature 120 °C, cone gas (nitrogen) flow 150 L/h, sheath (nanoflow) gas (nitrogen) flow 0.2 Bar, and collision gas (argon) flow 0.15 mL/min. MRM transitions and settings for IGEPLVLK (selected for quantification) and VLSPQGGGPWDSVAR (selected for confirmation) are presented in Table S-1. The Ionkey/MS system was operated under the Waters MassLynx software suite (version 4.1), and the TargetLynx module of this package was used for data processing.

4.2.6. Method validation
The method was validated according to EMA and FDA guidelines, and the following criteria were addressed: selectivity (e.g. spike recovery and ligand challenge tests), accuracy & precision, recovery, calibration curve, and stability (e.g. 28 days benchtop, 10× freeze-thaw, and 13 days autosampler (10 °C)). For the recovery experiment, samples were fortified with 5 ng/mL sRAGE either before or after sRAGE capture to obtain the pre-capture and post-capture spiked samples, respectively. The sRAGE dilutions for the recovery experiment were prepared in 50 mM ABC since adding Surrogate Matrix to the post-capture spiked samples would introduce excessive BSA to the samples thereby interfering with digestion and LC-MS analysis. sRAGE in Surrogate Matrix was used for the spike-recovery experiments, and the corresponding spiking procedure was similar to that of the pre-capture spiked samples of the recovery experiment. Ligand challenge tests were performed by adding 200 ng of fully-reduced HMGB1 (HMGBiotech, Milano, Italy; Cat. No. HM-116), S100A12 (Novoprotein; Cat. No. C743), serum amyloid A1 (SAA1; Novoprotein; Cat. No. C633), and Nε-(carboxymethyl)lysine-modified bovine serum albumin (CML-BSA; Academy Bio-Medical Co., Houston, TX, U.S.A.; Cat. No. 30P-CML-BS102) to the samples. For recovery and selectivity experiments, samples were incubated for at least 30 minutes following addition of sRAGE or the ligands prior to initiating sRAGE capture.
4.2.7. Method comparison

For method comparison, 40 serum samples were analyzed from a cross-sectional study (NCT00807469) within the University Medical Center Groningen (UMCG). Ethical approval for this study has been granted by the UMCG’s review board (METc 2008/136), and the study adheres to the Declaration of Helsinki. Blood samples were collected as described previously. In all 40 samples, sRAGE was quantified using the affimer-based sRAGE assay as well as with the previously described antibody-based sRAGE assay.

4.3. RESULTS AND DISCUSSION

4.3.1. Affimer-based sRAGE enrichment

The affimers were supplied with an added C-terminal cysteine residue (see Figure 1) which allows coupling of the affimers to maleimide-activated solid supports (e.g. microtiter plates, magnetic beads) through formation of stable thioether bonds, thus allowing for controlled orientation for a capture surface. Initially, the performance of the three affimers – A11, B7, and G10 – was assessed on the basis of recombinant human sRAGE (rh-sRAGE) spiked into saline-based buffers (e.g. PBS, TBS) at high concentrations (0.1-1 µg/mL) and by using maleimide-activated microtiter plates (Thermo Fisher Scientific, Cat. No. 15150) or magnetic beads (Cube Biotech, Cat. No. 51201) according to the manufacturer’s instructions. When affimer performance testing was extended to more complex samples (e.g. 1% BSA in TBS, serum) fortified with rh-sRAGE, it was observed that an increased matrix complexity led to decreased signals for rh-sRAGE. Specifically, the relative peak areas for two sRAGE peptides (see data for rh-sRAGE in Figure 2) were around 10 times lower than expected based on samples containing the same amount of sRAGE spiked in buffer only. In addition, it was found that the individual affimers were incapable of enriching endogenous sRAGE from serum (see data for non-spiked serum and the individual affimers in Figure 2). However, endogenous sRAGE was successfully enriched when combining the B7 affimer with at least one of the two other affimers (see data for non-spiked serum and the affimer combinations in Figure 2).

We hypothesized that the B7 affimer may ‘unveil’ the binding sites of the other two affimers, and furthermore reasoned that the observed extraction differences for endogenous and recombinant sRAGE may be due to different ligands being bound to these proteins. Differences in ligand binding may, for example, be explained by varying N-glycans on asparagine residues 25 and 81 of sRAGE, which are proposed to determine the ligands to which sRAGE will bind. Alternatively, it could be possible that one affimer alone does not bind endogenous sRAGE strongly enough to extract it from the complex environment of serum comprising numerous sugars, lipids, electrolytes, metabolites, and proteins. Minor structural differences between the
binding sites of endogenous and recombinant sRAGE may in such a case represent a plausible explanation for the varying extraction behaviors that we observed thereby pointing to a general difficulty when using recombinant proteins as surrogates for the corresponding endogenous proteins. It is also possible that the affimers used in combination create an avidity effect, thus increasing binding efficiency by targeting discontinuous conformational epitopes of sRAGE. In order to rule out any batch-specific effect, we requested the production of a second batch of affimers, which confirmed the need for using a combination of affimer B7 with one of the other two affimers. Corresponding experiments furthermore revealed excellent batch-to-batch consistency for the affimers’ effectiveness in enriching sRAGE from serum (see Figure S-1).

With respect to the affimer binding sites, we aimed to provide an estimate of their locations by means of epitope slicing and epitope extraction experiments for which we used the proteases trypsin and GluC. However, we were unable to map any of the affimer binding sites, which suggests the presence of conformational binding sites. In addition, in other experiments we observed that sRAGE could not be enriched from samples that were acidified and subsequently neutralized, which hints at conformational binding sites as well.

We evaluated the affimers’ performance in a quantitative workflow for serum sRAGE by coating the best performing affimer combination (i.e. B7 and G10, see Figure 2) on adsorptive microtiter plates, which represent a more straightforward alternative to the maleimide-activated solid supports, in analogy to our antibody-based sRAGE method.\textsuperscript{16} The method was optimized with respect to affimer coating, sRAGE enrichment, and protein digestion resulting in the final protocol as outlined in the ‘Experimental’ section (see Table S-2 for details). The resulting method fulfilled all requirements as stipulated in regulatory guidance documents on bioanalytical method validation (see ‘Method Validation’ section below). Still, we acknowledge that elucidating the mechanism behind the multi-affimer requirement would be desirable to exploit the affimers to their full potential. We furthermore acknowledge that calibration materials for complex analytes such as proteins are potential sources of bias as it is implausible that one recombinant protein can adequately represent all different forms of the analyte (so-called ‘proteoforms’ or ‘protein species’)\textsuperscript{27,28} which are present in biological samples.

![Figure 1](image)

**Figure 1.** Amino acid sequence of the affimer scaffold in which the added C-terminal cysteine residue and a hexahistidine-tag as well as the sequences of the inserted loops relevant for sRAGE binding are indicated.
Figure 2. Performance evaluation of the individual affimers A11, B7, and G10 (indicated as A, B, and G, respectively) as well as all affimer combinations based on enrichment of endogenous sRAGE and/or recombinant human sRAGE (rh-sRAGE) from 1% BSA in TBS (BSA) and human serum, which contained endogenous sRAGE at a level of approximately 1 ng/mL. The figure shows the mean relative peak areas (plus standard deviations; N = 3) of the proteotypic sRAGE peptides IGEPLVLK (in black; selected as quantifier peptide) and VLSPQGGGPWDSVAR (in grey; selected as qualifier peptide due to its deamidation-sensitive ‘QG’ sequence motive and due to a single nucleotide polymorphism leading to substitution of the C-terminal arginine by a cysteine (e.g. rs116828224) occurring in 0.6% of the population).

4.3.2. Quantitative assay development

We aimed to develop an alternative for our previously developed antibody-based sRAGE LC-MS assay, and accordingly adopted the same internal standard quantification approach using stable-isotope-labelled (SIL) peptides. Details on the selection of the SIL-peptides can be found in the corresponding publication.16

Affimer titration experiments indicated that 0.5 µg of the affimers is sufficient for the reliable and reproducible enrichment of endogenous sRAGE across the entire concentration range that is relevant for sRAGE quantification in human serum (see Figure S-2). For preparation of the calibration curves, we tested 1% BSA in TBS and fetal calf serum (FCS) as surrogate matrices given that analyte-free, authentic matrix could not be obtained. The slopes of calibration curves prepared by spiking recombinant sRAGE in the BSA-based matrix and in human serum were similar as judged from the overlapping 95% confidence intervals (see Figure S-3). Enrichment of sRAGE from FCS was, however, slightly, yet significantly less efficient compared to serum (see Figure S-3). Consequently, the suitability of 1% BSA in TBS as surrogate matrix was tested further during method validation. Corresponding spike-recovery experiments yielded an acceptable bias of ± 15% (see the ‘Method validation’ section below), and we therefore employed 1% BSA in TBS as surrogate matrix for preparation of calibrants in analogy to our antibody-based sRAGE LC-MS assay.
4.3.3. Method validation

Table 1 features a concise summary of the validation results while a full overview is given in the Tables S-3 to S-15 (Supporting Information). Accurate quantification of sRAGE was demonstrated for a 1/x weighted linear calibration model using 7 non-zero standards between 0.2 ng/mL (LLOQ: CV & bias < 20%) and 10 ng/mL. We did not reach the LLOQ of our previously developed antibody-based sRAGE LC-MS method (i.e. 0.1 ng/mL), since the 0.1 ng/mL calibration standard in the third accuracy and precision run deviated too much from the predicted sRAGE level (see Table S-16). This result was the sole obstacle to demonstrating an LLOQ of 0.1 ng/mL (see Table S-17), and we therefore did include a summary of the validation results based on an LLOQ of 0.1 ng/mL in the Supporting Information (see Table S-18). Levels of background noise were furthermore comparable for both methods, and peak areas were similar too, as is exemplified by the LLOQ selected ion chromatograms presented in Figure 3. With respect to the required LLOQ, we can state that we did not observe sRAGE levels below 0.4 ng/mL in around 1,000 samples from various clinical studies, and we therefore consider that an LLOQ of 0.2 ng/mL will not affect the applicability of the affimer-based method.

![Figure 3. Selected Ion Chromatograms of the y7⁻ (quantifier), y5⁻ (qualifier 1), and y6⁻ (qualifier 2) fragments of the sRAGE-derived proteotypic peptide IGEPLVLK at 0.1 ng/mL in Surrogate Matrix obtained by (A) the affimer-based method and (B) the antibody-based method.](image)

The y-axes of the three MRM traces were linked and scaled to the highest observed signal observed in these traces. The presented peak areas represent the average values for all samples which were measured for the lower limit of quantification (LLOQ) determination. No statistically significant differences (p < 0.05, two-tailed Student’s t-test) between both methods were observed for all three fragments.
Evaluation of accuracy and precision revealed acceptable biases and CVs (within ± 15%), which were slightly higher for the QC-low compared to the QC-medium and QC-high samples. The recovery of the affimer-based enrichment procedure was high (>90%) and precise (CVs <10%) when considering the average of duplicate measurements as well as when based on the individual replicates. Furthermore, assessment of sample stability after 28 days of storage on the benchtop (room temperature) and upon 10 freeze-thaw cycles indicated that sRAGE is a rather stable biomarker, based on the sites that are recognized by the affimers and the proteotypic peptide that is used for sRAGE quantification by LC-MS.

Table 1. Summary of validation data.

|                   | QC-low | QC-medium | QC-high |
|-------------------|--------|-----------|---------|
|                   | CV     | bias b    | CV      | bias b  | CV     | bias b  |
| accuracy & precision (3 runs, in 6-fold) | run 1  | 10%       | -4%     | 4%      | -3%    | 5%      | 1%     |
|                   | run 2  | 11%       | -4%     | 6%      | 1%     | 7%      | -1%    |
|                   | run 3  | 5%        | 8%      | 8%      | 3%     | 7%      | 0%     |
| autosampler stability 10 °C (13 days, in 3-fold) | | 5%       | -14%    | 14%     | -1%    |
| bench-top stability room temperature (28 days, in 3-fold) | | 7%       | -15%    | 6%      | -11%   |
| freeze-thaw stability -20 °C (10 cycles, in 3-fold) | | 11%      | -13%    | 7%      | -1%    |

| recovery (6 different serum samples, in 1- or 2-fold) | replicate 1 | replicate 2 | average of replicates |
|------------------------------------------------------|-------------|-------------|-----------------------|
| recovery                                             | recovery    | CV          | recovery              | CV          | recovery  | CV        |
|                                                      | 91%         | 9%          | 97%                   | 8%          | 94%       | 4%        |

| spike recovery (6 different serum samples, in 1- or 2-fold) | replicate 1 | replicate 2 | average of replicates |
|------------------------------------------------------------|-------------|-------------|-----------------------|
| bias (spike recovery)                                      | bias        | bias        | bias                  |
| limit of detection                                        | 10%         | 10%         | 10%                   |
| lipemic sample (spike) recovery (in 2-fold)               | 95%         | -2%         |                        |
| hemolytic sample (spike) recovery (in 2-fold)             | 91%         | 7%          |                        |

| 0.2 ng/mL calibrant                                       | CV          | bias       | 0.2 ng/mL calibrant    | CV          | bias       |
|----------------------------------------------------------|-------------|------------|------------------------|-------------|------------|
| HMGB1 challenge (4 µg/mL, in 5-fold)                     | 13%         | -6%        | SAA1 challenge (4 µg/mL, in 5-fold) | 9%          | 15%        |
| S100A12 challenge (4 µg/mL, in 5-fold)                    | 11%         | 7%         | CML-BSA challenge (4 µg/mL, in 5-fold) | 12%         | 2%         |

*An extensive summary of the validation results is presented in Tables S-3 to S-15 (Supporting Information).

b The average value of measured concentrations during the precision and accuracy experiments was used as nominal concentration.
To assess the selectivity of the sRAGE method, spike-recovery and ligand challenge testing experiments were carried out. The spike-recovery experiments which included six different sources of serum, a lipemic sample, and a hemolytic sample, revealed acceptable biases within ± 15%. Ligand challenge testing was performed by fortifying 0.2 ng/mL calibrants with >10,000-times molar excesses of high mobility group box 1 protein (HMGB1) which is the most studied and characterized RAGE ligand, S100 calcium-binding protein A12 (S100A12) and serum amyloid A1 (SAA1) as examples of damage-associated molecular pattern (DAMP) proteins known to bind RAGE, or Ne-(carboxymethyl)lysine-modified bovine serum albumin (CML-BSA), which we included as surrogate for advanced glycation end-product-modified proteins. Ultimately, none of the tested ligands affected the measured sRAGE levels.

**Figure 4.** Comparison between the affimer-based sRAGE LC-MS assay and a previously developed antibody-based LC-MS method for sRAGE quantification using (A) linear regression and (B) the Bland-Altman plot.

### 4.3.4. Method comparison

Agreement between the affimer-based LC-MS sRAGE assay and the previously developed antibody-based LC-MS sRAGE assay was assessed on the basis of 40 clinical samples using linear regression and Bland-Altman plots (see Figure 4). Comparison between both methods revealed good correlation ($R^2 = 0.88$) but also showed that around 25% lower sRAGE levels were reported by the affimer-based assay. Given that the same sRAGE stock was used for preparation of the calibrants for both methods, the observed difference likely represents a biology-based bias rather than a calibration-related artifact. While the reason for this slight bias remains unclear at present, it is conceivable that the different affinity ligands enrich a different
subset of sRAGE proteoforms or that sRAGE molecules bound to specific sRAGE ligands are enriched with different efficiencies by the two methods. Thereby, these data emphasize the need to differentiate between sRAGE proteoforms and to study the ‘sRAGE ligandome’ in order to gain a better insight in the biological role and clinical biomarker potential of this protein.

4.4. **CONCLUSIONS**

Affimers are attractive alternatives to antibodies for binding target proteins with high affinity and specificity. Their exceptional thermal and chemical stability and the fact that affimers can be produced easily in bacterial cultures (*E. coli*) in a reproducible and scalable manner are clear advantages towards their use as reagents in clinical and bioanalytical assays. In our study we showed the application of affimers in an LC-MS-based method for quantification of the low-abundant biomarker sRAGE in human serum. The novel method was validated according to EMA and FDA guidelines and enabled quantification of serum sRAGE at clinically relevant levels between 0.2 and 10 ng/mL. Moreover, the method showed good correlation with a previously developed, fully validated, antibody-based LC-MS method for serum sRAGE quantification, although 25% lower sRAGE levels were reported by the affimer-based method. In conclusion, affimers are small and versatile affinity ligands with significant potential for biomedical applications as alternatives to antibodies.
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4.6. SUPPORTING INFORMATION

The Tables S-3 to S-18 can be found in the online version of the Supporting Information which is available on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00414.

![QC-medium serum sample](image)

**Figure S-1.** Equivalence testing of two different affimer batches based on fivefold analysis of the QC-medium serum sample. Batch 1, produced in January/February 2016, had been stored at -20 °C for two years and had undergone numerous freeze-thaw cycles. Batch 2, produced in January/February 2018, was placed in the fridge (2-8 °C) upon arrival and was kept refrigerated until the experiments were carried out (4 days after arrival). The figure shows the mean relative peak areas (plus standard deviations; N = 5) of the proteotypic sRAGE peptides IGEPLVLK (in black) and VLSPQGGGPWDSVAR (in grey). No statistically significant differences (p < 0.05, two-tailed Student’s t-test) were observed between the batches.
Figure S-2. Results of affimer titration experiments using (A) QC-low and (B) QC-high samples (N = 2).

Figure S-3. Evaluation of sRAGE recovery from human serum (endogenous sRAGE level approximately 1 ng/mL), 1% BSA in PBS (BSA), and fetal calf serum (FCS) using linear regression (N = 1).
| Peptide         | Precursor ion m/z | Product ion m/z | Dwell time (s) | Cone voltage (V) | Collision Energy (V) |
|-----------------|-------------------|-----------------|----------------|------------------|----------------------|
| IGEPLVLK        | 434.79 - [M+2H]^{2+} | 755.47 - y7^{2+} | 0.05           | 45               | 16                   |
|                 |                   | 569.40 - y5^{2+} | 0.05           | 45               | 16                   |
|                 |                   | 698.44 - y6^{2+} | 0.05           | 45               | 14                   |
| IGEPLVL[K]^{a}  | 438.79 - [M+2H]^{2+} | 763.48 - y7^{2+} | 0.05           | 45               | 16                   |
|                 |                   | 577.42 - y5^{1+} | 0.05           | 45               | 16                   |
|                 |                   | 706.46 - y6^{1+} | 0.05           | 45               | 14                   |
| VLSPQGGGPWDSVAR | 763.39 - [M+2H]^{2+} | 613.80 - y12^{2+} | 0.05           | 60               | 24                   |
|                 |                   | 657.32 - y13^{2+} | 0.05           | 60               | 24                   |
|                 |                   | 1001.48 - y10^{1+} | 0.05          | 60               | 32                   |
| VLSPQGGGPWDSVA[R]^{a} | 768.40 - [M+2H]^{2+} | 618.80 - y12^{2+} | 0.05           | 60               | 24                   |
|                 |                   | 662.32 - y13^{2+} | 0.05           | 60               | 24                   |
|                 |                   | 1011.49 - y10^{1+} | 0.05           | 60               | 32                   |

^{a} 13C and 15N labelled arginine (R) or lysine (K). Underlined product ions represent the quantifier ions.
Table S-2. Summary of method optimization experiments and outcomes.

| Parameter | Testing conditions/range | Outcome |
|-----------|--------------------------|---------|
| **Plate coating:** | | |
| Affimer amount | 0.1 – 1 µg per sample | see Figure S-2 |
| Coating buffer | 10 mM PBS & 10 mM TBS | PBS ≥ TBS |
| Coating duration | 4 hours & overnight | overnight >> 4 hours |
| Coating temperature | room temperature & 37 °C | room temperature >> 37 °C |
| **Plate blocking:** | | |
| BSA concentration | 0.01% – 1% | 0.01% = 0.1% = 1% |
| Blocking duration | 30 – 120 minutes | 30 minutes = 60 minutes = 120 minutes |
| **Plate washing:** | | |
| Wash buffer | PBS & TBS | TBS > PBS |
| Wash buffer strength | 10 mM – 1,000 mM | 10 mM = 100 mM = 1,000 mM |
| Wash buffer pH | 6.5 – 8.5 | 6.5 = 7.0 = 7.5 = 8.0 = 8.5 |
| Detergent addition | 0.005% – 0.1% Tween-20 | 0.05% > 0.1% > 0.01% >> 0.005% |
| Additional detergent-free wash step | with & without extra wash step with TBS | added wash is not beneficial |
| **sRAGE capture:** | | |
| Incubation duration | 10 – 120 minutes | ≥ 90 minutes is required |
| **sRAGE denaturation/proteolysis:** | | |
| Digestion strategy | indirect digestion & in-well digestion | in-well digestion is a faster alternative |
| Reducing agent | DTT (60 °C) & TCEP (25 °C) | TCEP (25 °C) > TCEP (60 °C) > DTT (60 °C) |
| Protease | trypsin & trypsin/Lys-C | trypsin > trypsin/Lys-C |

* sRAGE denaturation and proteolysis performed after eluting sRAGE from the affimers with 0.1% aqueous TFA, evaporating the samples to dryness in a vacuum centrifuge, and reconstitution of proteins in digestion buffer (following the simplified immunoprecipitation in 96-well ELISA format (IPE) strategy as previously developed for the antibody-based sRAGE method).
Protein

identification

colorization

quantification