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Letter

Adsorptive Microtiter Plates As Solid Supports in Affinity Purification Workflows

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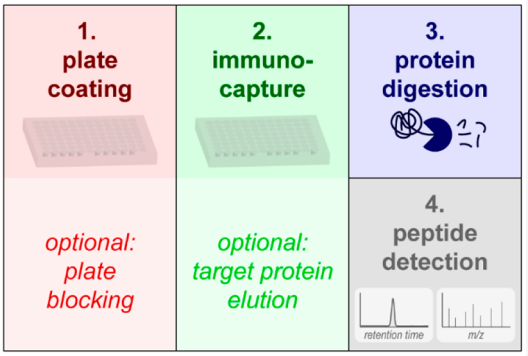
ABSTRACT: Affinity ligands such as antibodies are widely used in (bio)medical research for purifying proteins from complex biological samples. These ligands are generally immobilized onto solid supports which facilitate the separation of a captured protein from the sample matrix. Adsorptive microtiter plates are commonly used as solid supports prior to immunochemical detection (e.g., immunoassays) but hardly ever prior to liquid chromatography−mass spectrometry (LC-MS-)-based detection. Here, we describe the use of adsorptive microtiter plates for protein enrichment prior to LC-MS detection, and we discuss opportunities and challenges of corresponding workflows, based on examples of targeted (i.e., soluble receptor for advanced glycation end-products (sRAGE) in human serum) and discovery-based workflows (i.e., transcription factor p65 (NF-κB) in lysed murine RAW 264.7 macrophages and peptidyl-prolyl cis−trans isomerase FKBP5 (FKBP5) in lysed human A549 alveolar basal epithelial cells). Thereby, we aim to highlight the potential usefulness of adsorptive microtiter plates in affinity purification workflows prior to LC-MS detection, which could increase their usage in mass spectrometry-based protein research.



Affinity purification (AP)—a technique that uses affinity ligands (e.g., antibodies, aptamers) to selectively bind and purify a target of interest (e.g., proteins) from sample matrices like plasma, serum, and cell or tissue extracts—has become a standard tool in biological and medical research. Central to this technique is the coupling of affinity ligands to a solid support which is needed for separating the affinity ligands from the sample matrix once the target is bound. As an alternative to widely used bead-based materials, adsorptive microtiter plates may be used as a solid support given that antibodies, for example, can be easily immobilized onto these plates through passive adsorption. This principle forms the basis of most immunoassays, a standard tool in (bio)medical research, and it is surprising that the same principle is hardly ever adopted for affinity purification purposes in liquid chromatography−mass spectrometry (LC-MS)-based workflows.

Early reports on the use of adsorptive microtiter plates as solid supports in sequential AP and LC-MS workflows, dating back to around 10 years ago, demonstrated the applicability of such workflows for quantifying low abundance serum proteins (e.g., prostate-specific antigen (PSA), pro-gastrin-releasing peptide (ProGRP)).[1,2] These methods included an overnight incubation step to let antibodies (approximately 0.5−1.0 μg per well) bind to a microtiter plate. The plate was then used for AP purposes either directly[1] or after treating it with a "blocking agent"[2] to cover uncoated plastic surface with the aim of reducing nonspecific binding. After removing the unbound fraction and washing the plate with a saline solution[1]/buffer,[2] an in-well tryptic digestion procedure was carried out to digest the target proteins, which were then still bound to the antibodies. Such a procedure gives rise to peptides that are unique for the protein of interest, so-called "proteotypic peptides", which can subsequently be measured by LC-MS.

Despite the seemingly great potential of sequential AP and LC-MS workflows based on affinity ligands coupled to microtiter plates through passive adsorption, hardly any publication described their use since publication of the original PSA and ProGRP methods. The surface area and maximum volume that can be used and thereby the capacity of microtiter plates admittedly is limited as compared to bead-based solid supports, which may represent a shortcoming for some applications. However, a large surface area may not always be required, for example, for low abundance proteins, or could even be undesirable by favoring nonspecific binding. It is thus not surprising that both the early methods[1,2] and also a recent

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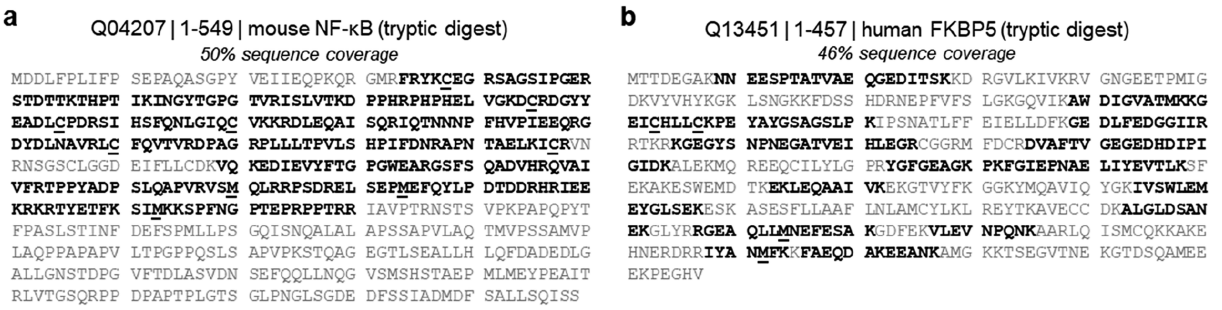

Figure 1. (A) Overview of transcription factor p65 (NF-κB) peptides identified in microtiter plate-mediated immunoprecipitation samples from lysed murine RAW 264.7 leukemic macrophages. (B) Overview of peptidyl-prolyl cis−trans isomerase FKBP5 (FKBP5) peptides identified in microtiter plate-mediated immunoprecipitation samples from lysed human A549 adenocarcinomic alveolar basal epithelial cells. In both subfigures, identified peptides are displayed in bold while amino acids which were found to be modified (e.g., carbamidomethylated cysteine (C) residues, oxidized methionine (M) residues) are underlined.

method[3] utilizing a sequential microtiter plate-based AP and in-well digestion sample preparation procedure target low abundance serum proteins in the high pg/mL to low ng/mL range. Nonetheless, the application of such a procedure can be extended to somewhat higher abundance proteins, as was recently demonstrated by means of a validated method for quantification of surfactant protein D (SPD) in human serum at clinically relevant levels between 5 and 500 ng/mL.[4] Moreover, it should be taken into account for higher abundance proteins that the dilution of samples can offer the opportunity to match AP surface area to the levels of a target protein of interest, as is also done for many commercial immunoassays.

The sequential microtiter plate-based AP and in-well digestion procedure holds considerable potential for specific MS-based protein analysis experiments; however, for some applications it has been described that including an elution step after the AP procedure is beneficial.[5] Such a step yields an eluate containing the purified protein which may then be studied in its intact form, for example, by MS or Western Blotting. Eluted proteins may also be digested and studied by means of their protein-specific peptides, which could improve the accessibility toward proteases as compared to an in-well digestion procedure.[5] In this respect, the LC-MS method for quantification of the soluble receptor for advanced glycation end-products (sRAGE), which was developed in our group, represents a notable example of a method that includes such an elution step.[6] This method was validated according to regulatory guidelines and has already been applied to over 1000 samples from several clinical cohorts.[7−9] For comparison purposes, we also developed a variant of this method omitting the elution step and including an in-well digestion procedure similar to the previously reported PSA and ProGRP workflows (see Supplementary Method S1). This method could readily be validated according to regulatory guidelines (see Supplementary Tables S1−S7) and was comparable with regard to detection sensitivity to our original method for this particular protein. Thus, neither an absence of codigested affinity protein which could yield less complex samples nor a presence of codigested affinity protein which could serve as a carrier to prevent a loss of low abundance targets seemed to favor quantification of sRAGE.

It should be taken into account that the above-mentioned examples include targeted workflows which only detect a few protein-specific peptides using selected (SRM)[2−4,6] or parallel reaction monitoring (PRM)[1] MS operation modes to achieve optimal analytical sensitivity. The applicability of microtiter plate-based AP is, however, not limited to such applications and may be extended to discovery-based proteomics experiments. In fact, we identified the transcription factor p65 (NF-κB) with 50% sequence coverage in a murine cell lysate (see Figure 1A) and peptidyl-prolyl cis−trans isomerase FKBP5 (FKBP5) with 46% sequence coverage in a human cell lysate (see Figure 1B) on the basis of microtiter plate-based AP followed by data dependent acquisition (DDA) LC-MS analysis (see Supplementary Methods S2 and S3). On the basis of these examples of two inflammatory proteins which are of particular interest to us and our collaborators, we demonstrated that the surface area of microtiter plates can match the detection sensitivities of high-resolution mass spectrometers used in present-day discovery-based proteomics workflows. Furthermore, these examples highlight that microtiter plate-based AP is not only applicable to blood-based samples but also to cell lysates, as we showed for NF-κB in lung-derived cells and FKBP5 in hematopoietic cells in which their abundance was expected to be rather high.

Adsorptive microtiter plate-based solid supports have advantages (e.g., low cost, easy to setup and use, high throughput) and disadvantages (e.g., low capacity, relatively high elution volume), just like bead-based supports have. These plates can thus not replace but will rather complement the solid supports that are currently being employed for AP purposes in biomedical research. In this regard, it is interesting to note that adsorptive microtiter plates have not only been used in combination with antibodies, but also with a different type of affinity ligand, so-called "affimers" (i.e., a novel class of affinity binders based on the protease inhibitor cystatin A[10]) for a quantitative LC-MS method.[3] Furthermore, the principle of enriching proteins from a complex mixture using antibodies coated on adsorptive microtiter plates was recently adopted for the purpose of studying the heterogeneity of proteins that are measured as one single protein by an immunoassay.[4] Specifically, a microtiter plate coated with anti-SPD antibodies was used to enrich SPD from human serum prior to LC-MS-based protein identification. Different SPD forms (so-called "proteoforms"[11] or "protein species"[12]) were found to be enriched on these plates, including SPD forms with modified amino acids (e.g., hydroxylated lysine residues) due to post-translational modifications and substituted amino acids due to single nucleotide polymorphisms.[4] Altogether, these examples demonstrate the versatility and applicability of adsorptive microtiter plates as solid supports in AP workflows and ought

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to provide incentives for biomedical researchers to employ these plates not only for conventional immunoassays but also for workflows using LC-MS as a protein detection technique.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00623.

Microtiter plate-based LC-MS method for serum sRAGE quantification (Method S1); microtiter plate-based detection method for NF-κB in murine RAW 264.7 cell lysates (Method S2); microtiter plate-based detection method for FKBP5 in human A549 cell lysates (Method S3); validation data of the microtiter plate-based LC-MS method for serum sRAGE quantification, including calibration curve results (Table S1); accuracy and precision results for the lower limit of quantification determination (Table S2); accuracy and precision results for the low concentration (Table S3), medium concentration (Table S4), and high concentration (Table 5) validation samples; recovery results (Table S6); and spike recovery results (Table S7) (PDF)

Accession Codes

Experimental data are available in the ProteomeXchange (DDA) and PASSEL (SRM) repositories under accession codes "PXD027530" and "PASS01666", respectively.

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Notes

The authors declare no competing financial interest.

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