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Technical Note

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Single Chain Variable Fragment Displaying M13 Phage Library Functionalized Magnetic Microsphere-Based Protein Equalizer for Human Serum Protein Analysis

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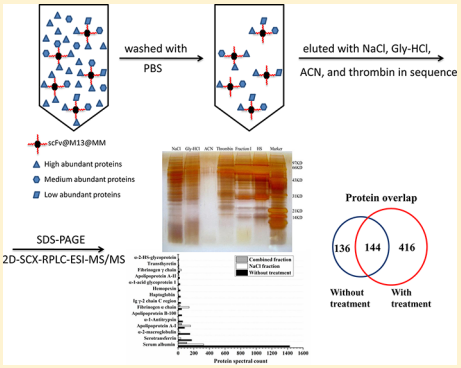
Supporting Information

ABSTRACT: Single chain variable fragment (scFv) displaying the M13 phage library was covalently immobilized on magnetic microspheres and used as a protein equalizer for the treatment of human serum. First, scFv displaying M13 phage library functionalized magnetic microspheres (scFv@M13@MM) was incubated with a human serum sample. Second, captured proteins on scFv@M13@MM were eluted with 2 M NaCl, 50 mM glycine-hydrochloric acid (Gly-HCl), and 20% (v/v) acetonitrile with 0.5% (v/v) trifluoroacetic acid in sequence. Finally, the tightly bonded proteins were released by the treatment with thrombin. The eluates were first analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining. Results indicated that the difference of protein concentration was reduced obviously in NaCl and Gly-HCl fractions compared with untreated human serum sample. The eluates were also digested with trypsin, followed by online 2D-strong cation exchange (SCX)-RPLC−ESI-MS/MS analysis. Results demonstrated that the number of proteins identified from an scFv@M13@MM treated human serum sample was improved 100% compared with that from the untreated sample. In addition, the spectral count of 10 high abundance proteins (serum albumin, serotransferrin, α-2-macroglobulin, α-1-antitrypsin, apolipoprotein B-100, Ig γ-2 chain C region, haptoglobin, hemopexin, α-1-acid glycoprotein 1, and α-2-HS-glycoprotein) decreased evidently after scFv@M13@MM treatment. All these results demonstrate that scFv@M13@MM could efficiently remove high-abundance proteins, reduce the protein concentration difference of human serum, and result in more protein identification.



Searching indicators or biomarkers for diseases is one of the most important issues in clinical proteomics. Among various body fluids, serum is the one of the most favorite clinical samples, because not only it contains most possible biomarkers of diseases,[1,2] but also the collection is minimally invasive.

However, the enormous dynamic range of protein concentration makes serum the most difficult specimen to be dealt with by existing techniques. The few dozen of high-abundance proteins make up 99% of overall contents, which seriously undermine the identification of low-abundance ones.[3−5] Therefore, the decrease of the dynamic range of protein concentration in serum is essential to discover low-abundance ones. Until now, there are two main strategies to decrease the dynamic range of protein concentration in serum, depletion of high-abundance proteins,[6−14] and equalization of protein abundance.[15−28]

For depletion of high-abundance proteins in serum samples, several kinds of immnoaffinity columns have been widely used.[6−13] Recently, Zeng et al.[10] applied CaptureSelect depletion resins (from BAC. B.V.) to remove albumin and immunoglobulin from human serum, followed by high-performance multilectin affinity chromatography fractionation, isoelectric focusing fractionation, in-gel digestion, and reversed-plase liquid chromatography−electrospray ionization-tandem mass spectrometry (RPLC−ESI-MS/MS) analysis. Approximately 80% of the high-abundance protein representing albumin and immunoglobulins were initially removed, beneficial to extend the dynamic range of the serum proteome measurement and find proteins with biological and diagnostic significance. In addition, potential glycan changes in the control and cancer samples were determined. The most obvious advantage of immunoaffinity depletion is good specificity, but Yadav et al.[13] found that some low-abundance proteins could also be removed simultaneously, which might result in loss of interesting low-abundance proteins. In addition, the number of

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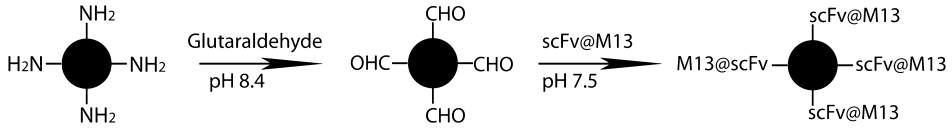

Figure 1. Procedure for immobilization of scFv@M13 phage library on magnetic microspheres.

high-abundance proteins that can be removed is limited, and the cost is usually high. Actually, multidimentional chromatography was also applied for the depletion of high-abundance proteins, even for unknown ones.[14] However, the method is nonspecific, and some low abundance proteins with high UV adsorption might be removed as well.

For the equalization of protein abundance in complex samples, Righetti et al.[16−25] proposed the technique of a protein equalizer by immobilizing combinatorial peptide ligand libraries, comprising dozens of millions of baits on beads or columns, by which proteins were bounded to beads according to the specific interaction with ligands. Once the most abundant proteins saturated their binding sites, the remaining molecules were washed away, while low abundant proteins were progressively enriched on their corresponding binding sites by overloading. Therefore by this technique, the difference of protein concentration in complex samples is reduced. Recently, Tu et al.[28] employed an optimized combinatorial peptide ligand library based protein equalizer to reduce the protein concentration dynamic range of swine plasma and a dual-enzyme, dual-activation strategy to achieve high proteomic coverage. The protein equalizer treatment enriched the lower abundance proteins by >100-fold, and a total of 3421 unique proteins spanning a concentration range of 9−10 orders of magnitude were identified.

Phage display is one of the most efficient techniques to analyze interaction between proteins, DNA/RNA, and proteins as well as small molecules and proteins. A single chain variable fragment (scFv) displaying M13 phage library (scFv@M13) is a powerful tool to screen antibodies against specific targets.[29] For this library, scFv fragments are displayed on the M13 phage surface as fusions to a protein, and there are up to 10^6 kinds of scFv fragments displayed on the M13 phase, which means that, in principle, almost every protein in complex samples could find its specific binding scFv fragment.[30] Furthermore, compared to the peptide library, scFv@M13 not only has strong binding capacity and high specificity toward proteins but also can be reproduced to obtain enough ligands for sample preparation. Therefore, scFv@M13 should be good candidate ligands for protein equalizers. In our previous work,[31] scFv@M13 was successfully immobilized on monolithic cryogel to develop a protein equalizer for the treatment of nephropathy patient urine, by which the number of identified proteins was increased from 142 to 369.

Magnetic microsphere is of high dispersibility, magnetic responsivity, and modifiable surface and has been widely used in proteome analysis.[32−34] In this work, a single chain variable fragment (scFv) displaying M13 phage library was covalently bonded on magnetic microspheres (scFv@M13@MM), to prepare a new kind of protein equalizer for the treatment of human serum. The captured proteins on scFv@M13@MM were eluted by NaCl, glycine-hydrochloric acid (Gly-HCl), and acetonitrile (ACN) solutions followed by thrombin digestion. The eluates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and online two-dimensional-strong cation exchange-reversed phase liquid chromatography−electrospray ionization-tandem mass spectrometry (2D-SCX-RPLC−ESI-MS/MS). Results showed that the number of proteins identified from scFv@M13@MM treated human serum was obviously improved compared with that from the untreated sample, and the abundance of 10 high abundance proteins decreased evidently.

MATERIALS AND METHODS

The three sections of Materials, Incubation of scFv displaying M13 phage library, and Purification of scFv displaying M13 phage library are found in the Supporting Information.

Immobilization of scFv@M13. The procedure for immobilization of scFv@M13 is shown in Figure 1. The magnetic microspheres with amine groups (100 mg) were washed with ethanol and then equilibrated with 50 mmol/L phosphate buffer (pH 8.4), followed by the reaction with 10% (v/v) glutaraldehyde for 6 h at room temperature with an occasional vortex. The particles were washed with phosphate buffer (pH 8.4). Subsequently, scFv@M13 coupling solution (10^{10} pfu/mL) in PBS (pH 7.5) was added into the vial with magnetic microspheres and reacted for 12 h at room temperature. Then, 0.75% glycine with 1% $NaCNBH_3$ was added and reacted for 6 h. Finally, scFv@M13@MM were washed with sterile water and PBS and stored at 4 °C before use.

Sample Treatment. The diluted human serum (1 mL, 20 mg proteins) in optimized buffer, PBS (pH 7.4), was loaded on 100 mg of scFv@M13@MM. After incubation, the supernatant was collected (fraction I). Subsequently, scFv@M13@MM were washed with PBS to remove unbounded proteins, followed by protein elution with 2 M NaCl (NaCl fraction), 50 mM Gly-HCl buffer (pH 2.5) (Gly-HCl fraction), and 20% ACN with 0.5% trifluoroacetic acid (TFA) (ACN fraction) in sequence. Finally, scFv@M13@MM were saturated with thrombin solution (0.01 U/μL), and the supernatant was collected (thrombin fraction). All collected fractions were first analyzed by polyacrylamide gel with silver staining and further 2D-SCX-RPLC−ESI-MS/MS. The Gly-HCl fraction, ACN fraction, and thrombin fraction were pooled (combined fraction) for LC−MS analysis.

Protein Digestion and 2D-SCX-RPLC−ESI-MS/MS Analysis. In total, 20 μg of proteins from the NaCl fraction, combined fraction, and untreated human serum were lyophilized and redissolved in 40 μL of 50 mM NH_4HCO_3 with 1 M urea buffer, respectively, followed by denaturation, reduction, alkylation, and trypsin digestion. After desalted and lyophilized, protein digests were redissolved in 20 μL of 0.1% formic acid (FA) and analyzed by 2D-SCX-RPLC−ESI-MS/MS using a quaternary Surveyor pump (Thermo-Fisher, San Jose, CA) coupled with LTQ, similar with a previous report.[35] The details about protein digestion and LC−MS analysis are illustrated in the Supporting Information.

Database Search and Data Analysis. The protein identification was performed using BioWorks Software 3.3.1 for LTQ with a SEQUEST search program. The details for database searching are listed in the Supporting Information.

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After database searching, to control the FDR and reduce the apparent redundancy in protein identification, our results were aligned by a software tool (BuildSummary)[36] using the following criterions. The ΔCn value was set at 0.1, and Xcorr values were adjusted to ensure the FDR of peptide identification is less than 1%.

RESULTS AND DISCUSSION

Characterization of scFv@M13@MM. M13 phage was filaments of ~1 μm long with a diameter of approximately 6 nm. Therefore, it is more difficult to immobilize on microspheres than peptides and proteins. In this work, as shown in Figure 1, glutaraldehyde was used as a spacer to immobilize the scFv@M13 on magnetic microspheres, to reduce steric hindrance. To maintain the activity of scFv@M13 during immobilization, PBS (pH 7.5) was used as the buffer. The number of scFv@M13 immobilized on magnetic microspheres was about 3×10^{10}, measured by a M13 phage library titer before and after immobilization. There are about 10^6 kinds of scFv fragments in applied scFv displaying the M13 phage library, thus the copy number of each kind of displayed scFv fragment should be about 3×10^4.

Choice of Elution Buffer. Different from the peptide library equalizer, the choice of elution buffer for scFv@M13@MM should meet two requirements. One is to keep the original structure of the scFv@M13, so that displayed scFv fragments could not be lost, and the other is to elute the nonspecifically and specifically bound proteins completely.

In our experiments, after incubation with human serum, scFv@M13@MM were first washed with PBS (pH 7.4) for at least three times to remove unbounded proteins. Then 2 M NaCl, 50 mM Gly-HCl (pH 2.5), and 20% ACN with 0.5% TFA was used to elute bounded proteins from the scFv@M13@MM, respectively. NaCl solution is a classical washing buffer to destroy ion−ion dominating interaction, helpful to keep not only the native forms of proteins but also the integrity of scFv@M13. Gly-HCl is another commonly used washing buffer that could disrupt tenacious interaction possibly related to conformational structure, such as hydrogen bond and hydrophobic interaction without destroying scFv@M13 integrity.[37] Low-concentration ACN was used to destroy hydrophobic interactions. Finally, thrombin solution was used for cleaving the specific external fragmentation on scFv@M13, ensuring proteins with high binding capacity with ligands to be released completely.[38]

Evaluation of scFv@M13@MM for Treatment of Human Serum. All fractions and human serum without treatment were first analyzed by SDS-PAGE with silver staining. As shown in S-Figure 1 in the Supporting Information, a huge band of albumin is observed in untreated human serum (HS lane), which is also observed in fraction I (I lane). In NaCl, Gly-HCl, ACN, and thrombin fractions, the abundance of albumin is dramatically decreased. Except albumin, the abundance of other several high-abundance bands in the HS lane also decreased simultaneously after treatment with scFv@M13@MM.

We also analyzed the NaCl fraction, combined fraction, and human serum without treatment with the online SCX-RPLC−ESI-MS/MS system. The spectral counts of 15 high-abundance proteins were analyzed. As shown in Figure 2, after treatment with scFv@M13@MM, the spectral count of albumin decreased from 1 400 to 300 in the NaCl fraction and 100 in the combined fraction. In addition, the spectral count of other 9

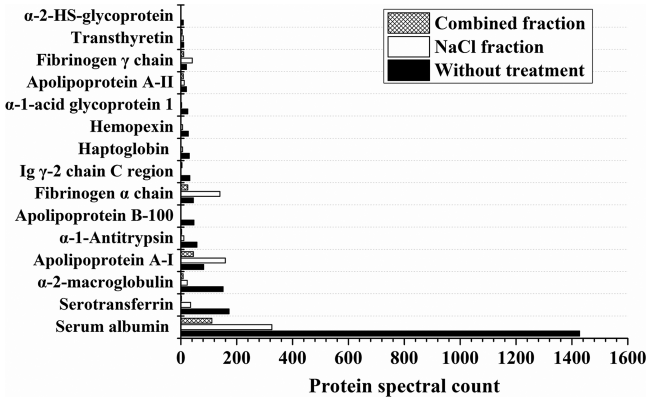

Figure 2. Protein spectral counts of identified 15 high-abundance human serum proteins from untreated human serum, NaCl, and combined fractions after 2D-SCX-RPLC−ESI-MS/MS analysis.

high-abundance proteins (serotransferrin, α-2-macroglobulin, α-1-antitrypsin, apolipoprotein B-100, Ig γ-2 chain C region, haptoglobin, hemopexin, α-1-acid glycoprotein 1, α-2-HS-glycoprotein) also evidently decreased simultaneously. Results from SDS-PAGE and SCX-RPLC−ESI-MS/MS agree well, which confirms that scFv@M13@MM are efficient for removal of high-abundance proteins in human serum.

When we carefully compared the SDS-PAGE results from scFv@M13@MM treated and untreated human serum, several new bands were observed in NaCl, Gly-HCl, and thrombin fractions (S-Figure 1 in the Supporting Information). After SCX-RPLC−ESI-MS/MS analysis, 560 proteins were identified from the NaCl fraction (439 proteins) and combined fraction (332 proteins), while only 280 proteins were found from untreated human serum, shown in Table 1. Therefore, the

Table 1. Number of Identified Proteins from Human Serum without and with the Treatment by scFv@M13@MM

	with treatment by protein equalizer				
		combined fraction			
without treatment	NaCl fraction	Gly-HCl fraction	ACN fraction	thrombin fraction	total proteins
280	439		332		560

identified protein IDs were improved 100% after scFv@M13@MM treatment, which might be due to the efficient depletion of high-abundance proteins (especially human serum albumin) and the reduction of the protein concentration difference.

Furthermore, the molecular weight (MW) and p*I* distribution of identified proteins with and without scFv@M13@MM treatment was also determined. As shown in S-Figure 2 in the Supporting Information, MW and p*I* distributions are similar before and after treatment. It is worth mentioning that an improved number of proteins is obtained in all MW and p*I* ranges after scFv@M13@MM treatment, which demonstrates that there is no evident bias for protein treatment.

In addition, the overlap of protein identifications from untreated and treated serum, as well as NaCl and combined fractions, was evaluated. As shown in Figure 3A, about 51% (144) of proteins identified in untreated human serum were also found in the scFv@M13@MM treated sample. However, 49% (136) of proteins identified in untreated sample disappeared in the treated sample, which might be due to the protein loss caused by the protein desalting or the uncertainties

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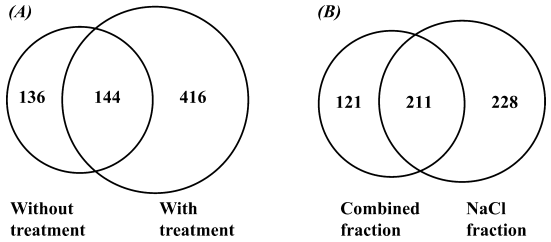

Figure 3. Overlap of identified proteins from treated and untreated human serum (A) and from NaCl and combined fractions (B) after 2D-SCX-RPLC−ESI-MS/MS analysis.

of the MS identification. In addition, 416 hidden proteins could be identified in the treated serum, which might benefit from the decrease of the protein concentration difference after treated by scFv@M13@MM. As shown in Figure 3B, 560 proteins were totally identified from NaCl and combined fractions, and only 211 proteins were identified in both conditions, which indicate that some extent complementarity exists for NaCl and other three reagents used for protein elution from scFv@M13@MM.

Comparisons with Other Techniques. We first compared with our previous work,[31] in which scFv@M13 was immobilized on monolithic cryogel to develop a protein equalizer for the analysis of urinary proteins. Novelty aspects of this work over previous one include three points. First, the loading capacity was improved more than 1 order of magnitude. For monolithic cryogel based equalizer, 60 μg of proteins could be bound on 1 mL of materials (~1 g). For magnetic particles based equalizer, ~1 mg of proteins could be captured by 1 g of particles. Second, magnetic microspheres were used for immobilization, which makes the operation very easy in Eppendorf tubes, and further easily performed in a 96-well plate for high-throughput sample preparation. Third, the volume of applied materials was dramatically reduced (<100 μL for 100 mg of magnetic microspheres; 5 mL for monolithic cryogel), which makes the volume of eluates from materials reduced about 1 order of magnitude, leading to obviously less time and labor consumed. In addition, reduced eluate volume is also valuable for low-abundance proteins analysis because of reduced potential sample loss.

We also compared our technique with immnoaffinity columns and combinatorial peptide ligands library. Compared with immunoaffinity columns,[10] scFv@M13@MM could produce not only comparable depletion performance of serum albumin and immunoglobulin but also efficient depletion of eight other high-abundance proteins simultaneously. For combinatorial peptide ligand libraries, we referred to a paper for human urinary proteins analysis.[39] The results showed the number of proteins from a treated human urine sample was improved 186% compared with that from an untreated one. For scFv@M13@MM, the number of proteins from the treated human serum sample was improved 100% compared with that from the untreated one. Considering the evidently higher complexity and wider dynamic range of human serum compared with urine, the performance of scFv@M13@MM is good enough for real sample analysis.

CONCLUSIONS

The scFv displaying M13 phage library was immobilized on magnetic microspheres and used as a protein equalizer for the treatment of human serum. Results demonstrate that 10 high-abundance proteins could be removed efficiently with the protein equalizer, and the number of identified proteins from the protein equalizer treated human serum could be improved 100% compared with that from the untreated sample, which demonstrates that the developed protein equalizer is of high potential for improving the human serum proteome analysis, leading to the discovery of candidate biomarkers or drug targets.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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