

# PEG Precipitation Followed by Albumin Depletion for Plasma Proteomics Analysis

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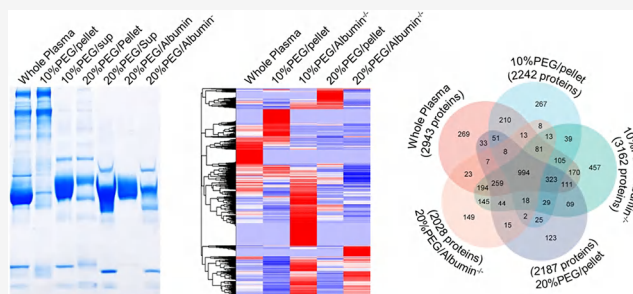


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Supporting Information

**ABSTRACT:** The mass spectrometry (MS)-based blood plasma or serum proteomic analysis is limited by interference from albumin, immunoglobulins, and other highly abundant proteins. We have found that poly(ethylene glycol) (PEG) can efficiently precipitate some of these proteins except albumin. By PEG precipitation followed by albumin depletion, additional proteins and N-glycoproteins can be identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the plasma. In-depth LC-MS/MS proteomic analyses of the whole plasma, its 10% and 20% PEG-precipitated pellets, and albumin-depleted supernatants have profiled 2943, 2242, 3162, 2187, and 2028 proteins respectively, yielding 5040 proteins in total and thus expanding the plasma proteome coverage. Therefore, PEG precipitation and albumin depletion should be used as a general plasma processing method for successful proteomic discoveries of blood biomarkers.



## INTRODUCTION

The plasma or serum proteomics analysis is an important approach for clinical biomarker discoveries to evaluate wellness and diseases of the human body.<sup>1–5</sup> Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has classically been the predominant tool for analyzing proteins in the circulating system.<sup>6–8</sup> Recent powerful high-throughput tools, the proximity extension assays (PEA, by Olink) and the SomaScan (by SomaLogic), measure near 3000 and 7000 proteins in plasma or serum, respectively,<sup>1,5,9,10</sup> providing indispensable techniques that are complementary to the MS-based platform for deep blood plasma proteome profiling.<sup>5,11</sup> However, these antibody- or aptamer-based assays might have issues with the specificity<sup>5,12</sup> and are unable to detect proteins not defined in the libraries of their kits.

The mass spectrometry-based proteomics is an indispensable approach in blood plasma protein profiling at the systemic level, which has been extensively studied with great achievements in the plasma proteome coverage and depth.<sup>5,13–17</sup> With developments in techniques such as extensive peptide fractionation,<sup>18</sup> DMSO in liquid chromatography,<sup>19</sup> sophisticated mass spectrometers,<sup>20</sup> data-independent acquisition and SWATH scan,<sup>21</sup> and other emerging methodologies,<sup>22,23</sup> the LC-MS/MS-based proteomics has experienced an unprecedented advance that is now able to profile over 4000 proteins in the plasma or serum.<sup>16</sup> However, a general issue in this approach is the limited proteome coverage and depth due to interference

from the commonly abundant proteins (albumin, immunoglobulins, complements, etc.).<sup>5,8,11</sup> One of the common methods to overcome this is using specific antibodies or other reagents to deplete these abundant proteins prior to analysis, but this is costly and inconvenient.

Here, we find that poly(ethylene glycol) (PEG), a traditional reagent used in clinical laboratories to precipitate immune complex from the serum<sup>24–27</sup> and now commonly used for precipitation of extracellular vesicles from the plasma,<sup>28,29</sup> is able to precipitate not only immunoglobulins but also other abundant proteins efficiently. By PEG precipitation followed by albumin depletion from the supernatant, additional proteins and glycoproteins can be identified from the plasma, expanding the MS-based proteome coverage and thus extending the proteome depth for successful proteomic discoveries of blood biomarkers.

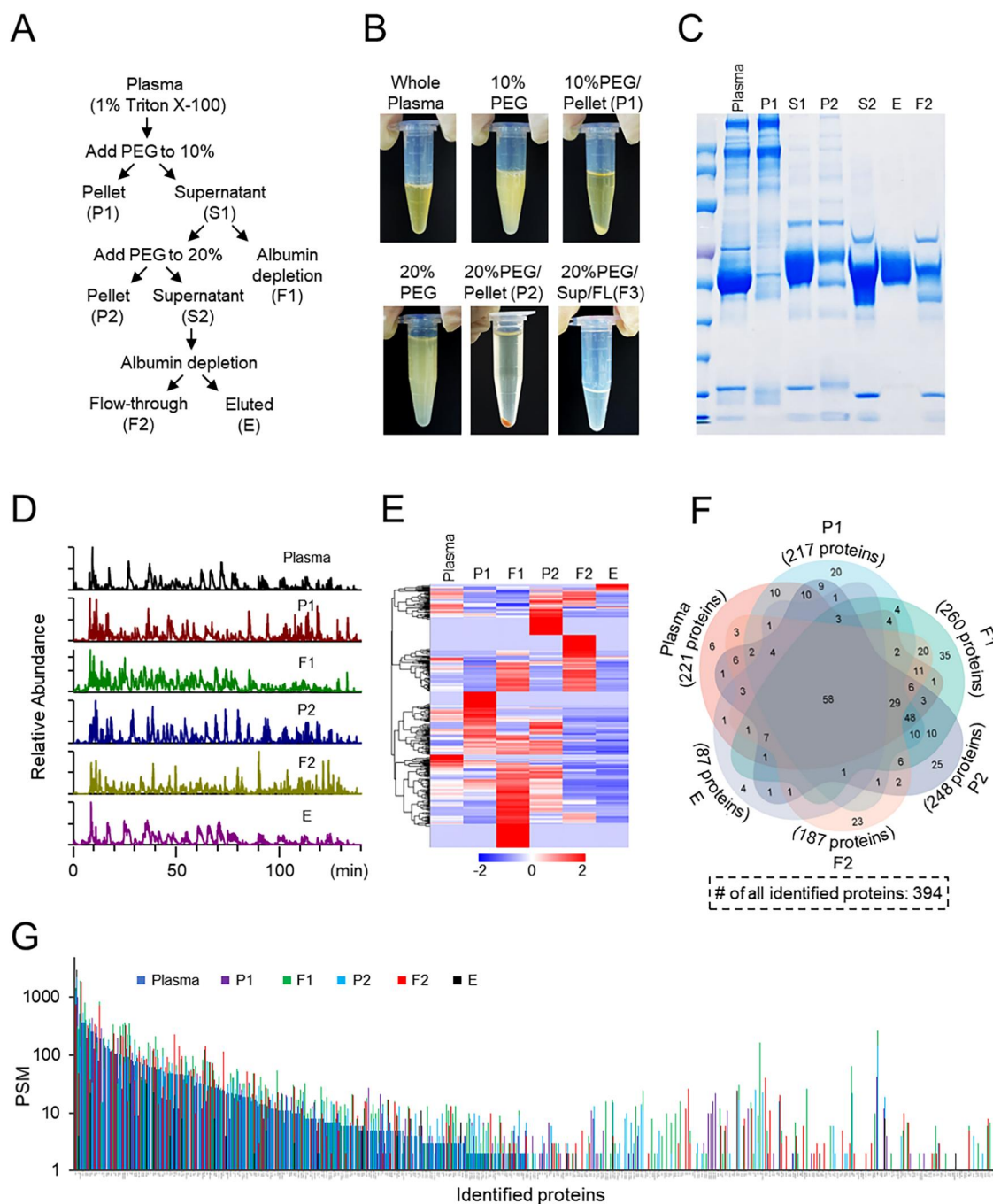
## EXPERIMENTAL SECTION

**Plasma PEG Precipitation.** The plasma with 1% Triton X-100 was mixed with PEG6000 in TBS buffer to 10% for

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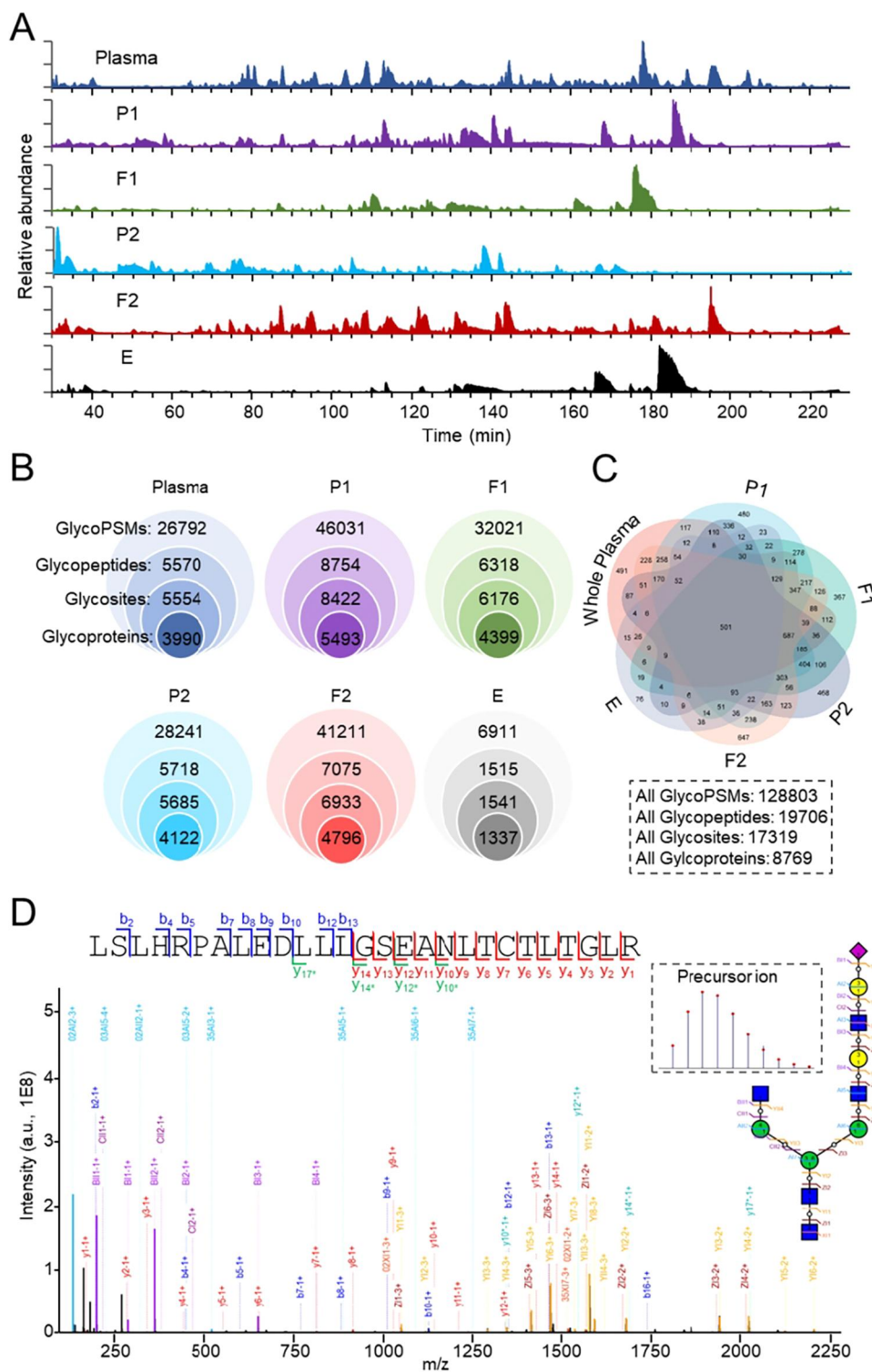


**Figure 1.** Plasma protein extraction by PEG precipitation and albumin depletion. (A) The workflow of plasma extraction. PEG: poly(ethylene glycol) 6000. (B) The samples during each extraction step. (C) SDS-PAGE of fractionated samples during extraction. (D) The liquid chromatographs of the samples during LC-MS/MS analyses. (E) Heatmap of profiled proteins after proteomics analyses. (F) Venn diagram to demonstrate the unique or shared proteins profiled. (G) Comparison of profiled proteins among these samples. The X-axis are all identified proteins sorted by their PSMs from largest to smallest in the whole plasma sample. PSM: peptide-spectrum match, equal to the number of times that a peptide or a protein is sequenced.

precipitation. After brief vortexing and then rotation for 15 min at room temperature, the mixture was centrifuged to separate the pellet and the supernatant. The supernatant was then mixed with PEG6000 to 20% for further precipitation.

**Albumin Depletion.** The supernatant of the plasma precipitated by PEG6000 was loaded to the Cibacron Blue 3G-A agarose column and then incubated by rotation at room temperature for 30 min. After that, the column was briefly and gently centrifuged for the collection of albumin-depleted flow-through. Proteins bound to the column were then eluted.

**Mass Spectrometry-Based Proteomics.** The whole plasma, the PEG precipitates, and the albumin-depleted supernatants were precipitated by 80% acetonitrile or 10% TCA/acetone and then dissolved in 8 M urea, followed by trypsin digestion, reduction/alkylation, and C18 desalting. For in-depth analysis, the desalted peptides were further fractionated extensively by RPLC at basic pH into ~100 fractions. The peptide samples were analyzed through a column (50  $\mu$ m ID and 30 cm long, packed with C18, 1.9  $\mu$ m) under a gradient of acetonitrile for ~120 min by the mass spectrometer (QExactive



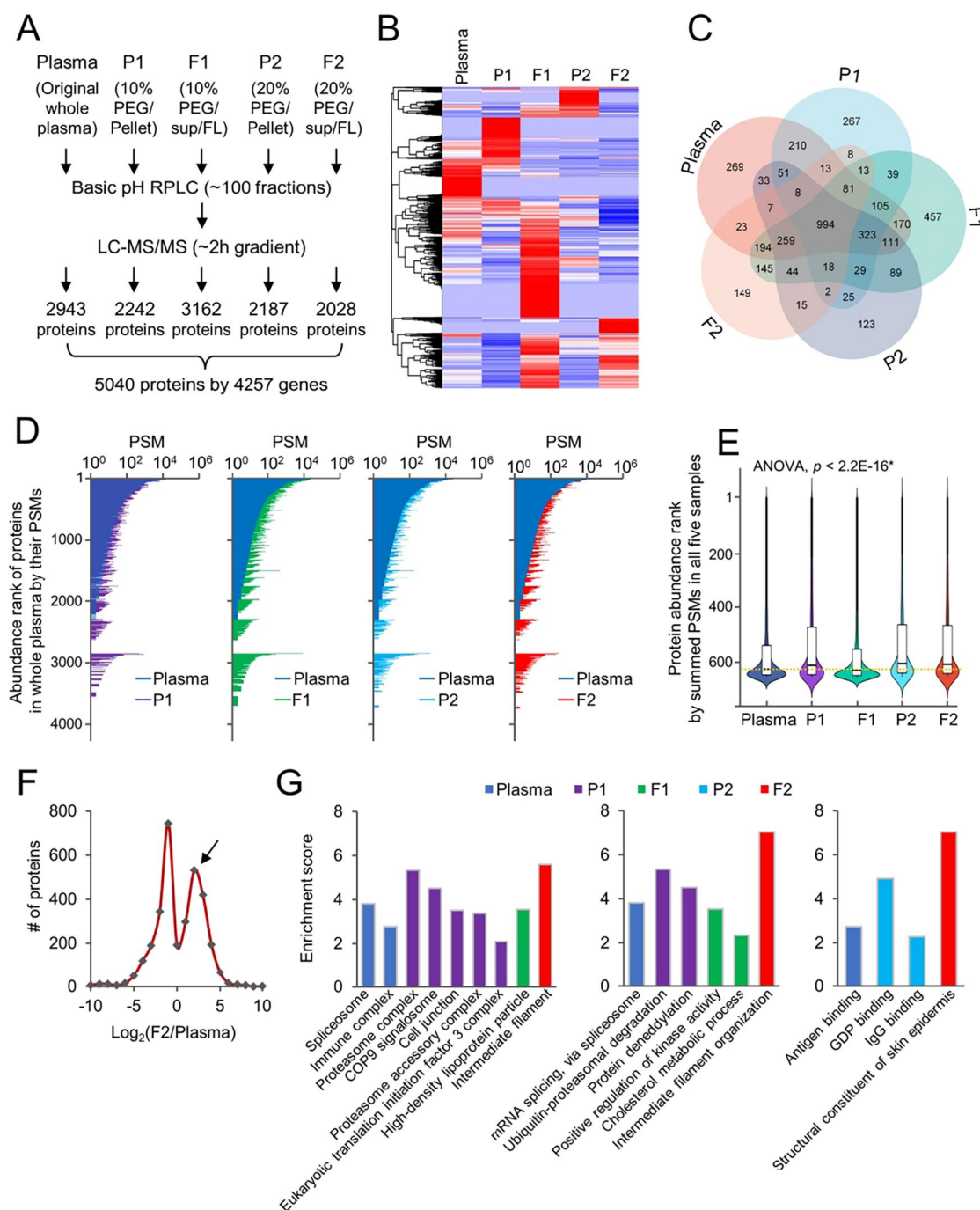
**Figure 2.** Glycoproteomic analyses of the plasma and extracts. (A) The liquid chromatograms of the samples during LC-MS/MS analyses. (B) Glyco-profiles of the original plasma and each isolated fraction. (C) Venn diagram to demonstrate the unique or shared glycosylated proteins identified by MS. (D) An example of spectra of an identified glycopeptide. This glycopeptide is from protein IGHA1 (P01876). Boxed in the dashed line are the isotopic peaks (in blue lines) of the precursor ion with the theoretical intensities (in red dots). Square = N-acetylglucosamine, green circle = mannose, yellow circle = galactose, and purple diamond = N-acetylneuraminic acid.

HF-X, Thermo) in the data-dependent acquisition mode. In the in-depth analysis, the fractions were run individually without concatenation. The MS raw data files are processed by the software Proteome Discoverer with the Sequest HT searching

engine against the human proteome database (UP000005640, Uniprot).

**N-Glycoproteomics Analysis.** The original plasma and its isolated samples were precipitated by acetonitrile or TCA as above and then dissolved in 8 M urea. After quantification,





**Figure 3.** In-depth proteomic analysis of the plasma and the four fractions. (A) Workflow of the deep analysis by peptide extensive fractionation. FL: the flow-through after albumin depletion. (B) Heatmap of proteins profiled in the proteomic analyses. (C) The Venn diagram to demonstrate the unique or shared proteins identified. (D) Comparison of levels of proteins profiled in each fraction with those of proteins in the original whole plasma. The Y-axis is the sequence of all proteins sorted by their PSMs in the original whole plasma sample. PSMs of “1” are not shown as they are zeros under the logarithmic scale. (E) Analysis of protein abundance rank among the original whole plasma and the four fractions. (F) Enrichment analysis of the F2 fraction. The score of each protein is calculated as  $\text{Log}_2[(\text{PSM}_{\text{F2}} + 1)/(\text{PSM}_{\text{Plasma}} + 1)]$ . (G) Cellular component, biological process, and molecular function analyses of the enriched proteins in the plasma and the four fractions. Top hits are shown.

reduction, and alkylation, the protein samples were digested by trypsin overnight. After being desalted by C18, the peptides were dried, resuspended, and loaded to a zic-HILIC column for enrichment of *N*-glycopeptides. In LC-MS/MS, the enriched peptides were separated through the column (75  $\mu\text{m}$  ID and 75 cm long, packed with C18, 5  $\mu\text{m}$ , 300 Å) by a gradient of acetonitrile (9–40%) for ~200 min and scanned by the mass

spectrometer (Q Exactive MS, Thermo). The MS raw data were searched and processed by the software GPseeker3.<sup>30</sup>

**Data Analysis.** The number of PSM (peptide-spectrum match) was used to quantify protein levels. In the enrichment analysis, each protein in different samples was calculated as  $\text{log}_2[(\text{PSM}_{\text{sample 1}} + 1)/(\text{PSM}_{\text{sample 2}} + 1)]$ . Protein abundance ranks were determined by their sorted PSMs from the report<sup>17</sup> or absolute concentrations provided by the human protein

atlas.<sup>31</sup> Cellular component, biological processes, molecular function, and protein domain enrichment analyses were performed by the online DAVID tool provided by Pubmed.

More details and other information are provided in the [Supporting Information](#).

## RESULTS

**Plasma PEG Precipitation and Albumin Depletion and Proteomic Analyses.** We found that PEG6000 could selectively precipitate immunoglobulins and other abundant proteins except albumins ([Figure S1A–C](#)), and therefore utilized the commonly used Cibacron Blue column to remove albumins ([Figure S2A–C](#)) and further optimized the experimental conditions ([Figure S3A–F](#)). In this PEG precipitation and albumin depletion strategy, the original whole plasma sample was first added with 1% Triton X-100 to release associated proteins and those in extracellular vesicles, and then mixed with an equal amount of 20% PEG6000 to 10% for precipitation (P1). The supernatant (S1) was either filtered by the Cibacron Blue column to deplete albumin (F1) or further precipitated by 20% PEG6000 into the pellet (P2) and the supernatant (S2). S2 was filtered through the Cibacron Blue column, yielding the albumin-depleted flow-through (F2) and the eluate (E) ([Figure 1A,B](#)). SDS-PAGE of these samples clearly showed that 10% PEG6000 precipitated many large abundant proteins, except albumin ([Figure 1C](#)). Further precipitation of 20% PEG6000 led to more precipitation of these proteins ([Figure 1C](#)). Finally, the Cibacron Blue column filtered the majority of albumin from the supernatant of plasma precipitated by 20% PEG6000 ([Figure 1C](#)).

To analyze what proteins are in these extracted fractions and whether the removal of those abundant proteins by PEG precipitation and albumin depletion has increased the number of identified proteins, we performed mass spectrometric analyses on these samples. The liquid chromatographs during the LC-MS/MS analyses of these six samples showed distinct patterns ([Figure 1D](#)), consistent with the previous SDS-PAGE results, suggesting an efficient protein partition. The heatmap analysis of the proteomic results demonstrated subsets of enriched proteins in each sample, especially the PEG precipitates and albumin-depleted supernatants, suggesting that the PEG precipitation and albumin depletion processes enriched particular subsets of proteins ([Figure 1E](#)). Indeed, albumin was present in the pellets P1 and P2 at relatively lower levels but largely distributed in the final eluate E, and the immunoglobulins (IGHG2, IGHM), complements C3, C4B, and C5, and fibrinogens (FGA, FGB) were enriched in P1, while other proteins such as Serotransferrin, Ceruloplasmin, Hemopexin, and apolipoproteins (APOA1, APOA4) displayed more proportion in the filtered supernatant samples (F1 and F2) ([Figure S4](#)).

Although there were six proteins in the original whole plasma not detected by any of other five fractions, each of these fractions identified extra proteins, including 20 in P1, 35 in F1, 25 in P2, 23 in F2, and 4 in E fractions, leading to an increased number of identified proteins from 221 in the whole plasma to 394 overall ([Figure 1F](#)). Consistently, many proteins displayed a greater number of PSMs in the isolated fractions than in the whole plasma, suggesting increased detectability in isolated fractions ([Figure 1G](#)). It is also notable that there were an extra number of enriched proteins in the F1 and F2 fractions as demonstrated by the enrichment analysis ([Figure S5](#)),<sup>32</sup> strongly suggesting increased detectability of certain proteins after PEG precipitation and albumin removal.

**Plasma PEG Precipitation and Albumin Depletion for N-Glycoproteomics Analyses.** Because plasma proteins are generally secreted and are often glycosylated during its synthesis and sorting, plasma glycoproteomics is important in blood biomarker discovery,<sup>33–36</sup> but it is also affected by commonly abundant and glycosylated proteins. We therefore speculated that PEG precipitation and albumin depletion might also facilitate plasma glycoproteome analysis.

In the N-glycoproteomics analysis, the liquid chromatographic trends of the LC-MS/MS analysis of the original plasma (Plasma), the two PEG precipitates (P1 and P2) and albumin-depleted supernatants (F1 and F2), and the final eluate were clearly different from one another ([Figure 2A](#)), suggesting efficient protein partitioning again in these fractions. Among the results, 3990 glycoproteins were identified in the whole plasma and 5493, 4399, 4122, 4796, and 1337 glycoproteins were identified in the samples of P1, F1, P2, F2, and E, respectively ([Figure 2B](#)). Overall, 8769 glycoproteins were identified in these six samples with 491, 480, 367, 468, 647, and 76 proteins uniquely identified in each sample ([Figure 2C](#)). We also selected some canonical proteins to see whether the peptide-spectrum match was in question. One of these proteins, IGHA1 (immunoglobulin heavy constant  $\alpha$  1), the constant region of immunoglobulin heavy chains showed decently matched isotopic envelopes of the precursor ions and comprehensively annotated fragment ions from both the peptide backbone and N-glycan moieties ([Figure 2D](#)).

**In-Depth Proteomic Analyses of the Plasma PEG-Precipitated Pellets and Albumin-Depleted Supernatants.** Further in-depth proteomic analyses by peptide extensive fractionation led to identification of 2943, 2242, 3162, 2187, and 2028 proteins, respectively, yielding 5040 proteins encoded by 4257 genes overall ([Figures 3A](#) and [S6, Table S1](#)). The heatmap analysis demonstrated distinct subsets of proteins enriched in the plasma and the two PEG precipitates and albumin-depleted supernatants ([Figure 3B](#)), with 269, 267, 457, 123, and 149 proteins uniquely identified in each ([Figure 3C](#)). To be more confident, we removed proteins with PSMs (peptide-spectrum match, equal to the number of times that a protein or a peptide is sequenced) of only “1” (detected once) from each of these samples and performed heatmap analysis. It showed distinct subsets of enriched proteins again in each sample ([Figure S7A](#)) with 170, 178, 403, 98, and 101 proteins uniquely in these five samples, respectively ([Figure S7B](#)). These results suggest that PEG precipitation and albumin depletion are able to identify more proteins significantly, expanding the plasma proteome coverage substantially.

To examine whether the PEG precipitation and albumin depletion extended the depth of the plasma proteome, we sorted proteins in the original plasma by their PSMs from the largest to the smallest (PSM = 1) and compared PSMs of these proteins in each of the four fractions. The results showed that all four isolated samples (P1, F1, P2, and F2) displayed proteins that were lowly or not present in the whole plasma ([Figure 3D](#)), suggesting that these proteins were enriched and some new ones became detectable. We also ranked proteins of the entire analyses by their summed PSMs in all five samples. The ranks of proteins in each of these samples demonstrated a significant difference in which the F1 fraction had a lower average of protein abundance ranks than the other three fractions, suggesting an extended depth ([Figure 3E](#)). As many abundant proteins and albumin were largely removed in the F2 fraction, some proteins were expected to be significantly enriched. Indeed, using the



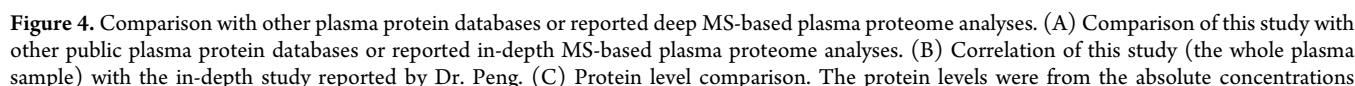


Figure 4. continued

provided by the HPA (Human Protein Atlas) database. (D) A list of newly identified proteins ( $\text{PSM} \geq 2$  in at least one of these five samples) after comparison to the six data sets with their PSMs in the whole plasma and four other extracted fractions in this study.

enrichment analysis method,<sup>32</sup> we found a projection at the enriched side of the frequency curve of the  $\log_2(\text{F2/Plasma})$  (appeared as a shoulder in the right side), suggesting a large number of extra proteins were enriched particularly by the PEG precipitation and albumin depletion processing (Figure 3F).

To find out what subsets of proteins were particularly enriched in each of these samples so that a particular plasma fraction could be selected for the detection of these proteins, we performed functional annotation analyses on the enriched proteins in each sample. In biological processes, the enriched proteins in the whole plasma were more linked to spliceosome and immune complex and the enriched proteins in the P1, F1, and F2 samples were associated with other biological processes while the enriched proteins in the P2 sample showed no significant association with any biological processes (Figure 3G). Enriched proteins in these samples also showed significant associations with different cellular components and molecular functions (Figure 3G). These data might be informative when proteins from particular biological processes, cellular sources, or with particular functions are to be studied in the plasma.

**Comparison of This Study with Public Databases and Reported Proteomic Analyses.** We compared our study with public human plasma databases including the Human Plasma Proteome Project,<sup>5</sup> Human body fluid proteome, and human protein atlas<sup>31,37,38</sup> and also with some MS-based in-depth human plasma or serum proteomics studies.<sup>17,39,40</sup> There were 600 proteins identified in our study that were not recorded in these public databases or reported in the three reported studies (Table S2 and Figure 4 A).

We also compared our study with the report by Dr. Peng's group since our proteomics analysis pipeline was similar to theirs.<sup>17</sup> Our results of the whole plasma sample showed a high correlation with their data (Figure 4B). We then examined the depth of our study together with the other three MS-based plasma proteomics based on the absolute concentrations of plasma proteins available from the Human Protein Atlas database and found the averaged concentration of proteins identified in this study was similar to Dr. Peng's and Dr. Carr's studies, but lower than Dr. Kuster's report (Figure 4C), suggesting a comparable depth of our study with the leading studies.

It is notable that in this study, there were 160 novel proteins not recorded in these public databases and reported studies, providing additional proteins that could be present in the human plasma (Figure 4D).

## DISCUSSION

In this study, we used PEG precipitation, followed by albumin depletion, to extract the blood plasma sample for improved proteome analyses. This strategy expanded the coverage of both whole proteome and N-glycoproteome of the plasma. By in-depth analysis, it increased the number of identified proteins from 2943 proteins in the original plasma to 5040 proteins when the plasma was fractioned by this strategy.

PEG works through steric mechanisms such as volume exclusion and attractive depletion in which it sequesters surrounding water molecules from proteins,<sup>41</sup> forcing them to

aggregate. Therefore, proteins with lower solubility such as those in larger sizes, complexed, or bearing particular domains might be more easily precipitated. Indeed, proteins enriched in the PEG-precipitated pellets and supernatants showed significant differences in their length and hydrophobicity (Figure S8A,B). Besides, a higher percentage of proteins in the pellet from 10% PEG precipitation can be polymerized or complexed (e.g., immunoglobins, apolipoproteins) (Figure S8C); enriched proteins in each PEG isolate demonstrated distinct enrichments in their domains (Figure S8D).

Besides binding to albumin through specific interactions, Cibacron Blue also binds to other proteins via ionic attraction to some extent.<sup>42</sup> This is also true in our data, where proteins with lower isoelectric points were retained after filtration by the Cibacron Blue column (Figure S8E).

It is conceivable that proteins with lower solubility and higher isoelectric points will probably be subject to unselective loss during PEG precipitation and albumin depletion. To minimize this, we tightly controlled the amount of protein loading on the column. Indeed, in our study, the majority of proteins commonly measured in clinical laboratories were not markedly reduced (Figure S2C), and we believe that the residual proteins will still be detected by the in-depth proteomic analysis and it is less interfered from those removed abundant proteins. This was also confirmed by in-depth analyses in which many tumor biomarker proteins (e.g., B2M, KIT, MSLN, etc.) were enriched in F1 or other fractions (Figure S9).

Our strategy of plasma extraction by PEG precipitation and albumin depletion had led to prominent enrichment of a subset of proteins (Figures 3F, S5, and S9), which would greatly increase the chances of successful clinical blood biomarker discoveries. Besides, this strategy would be very useful in TMT (tandem-mass-tag)-based quantitative MS proteomics where increased product ion intensity in MS2 is essential to generate sufficient reporter ions for accurate quantitation. Our strategy would certainly enrich subsets of proteins for increased signal strength during TMT analysis.

Although isolation of plasma into several fractions for in-depth MS-based proteomic analyses individually might increase the workload, these samples might be selectively combined for analysis (Figure S10). Besides, according to the results, precipitation by 10% PEG6000 followed by albumin depletion (F1) has yielded the highest number of identified proteins in our study consistently and should thus be preferentially considered rather than analyzed the whole plasma directly. It might also be efficient to combine the fractions (e.g., F1 and F2) in different ratios for a single analysis. With more sophisticated systems and methodologies (such as the data-independent acquisition mode),<sup>43–45</sup> the number of identified proteins is expected to be significantly increased. In addition, for targeted protein analysis, the in-depth plasma proteome data sets of this study suggest particular plasma fractioning methods for their increased detectability.

## CONCLUSIONS

Overall, in addition to other reported MS-based plasma or serum proteomics approaches,<sup>23,46,47</sup> our simple and cost-



effective PEG precipitation and Cibacron Blue albumin depletion strategy is also efficient in improving plasma proteome analysis. The data of this study also provide a resource for detecting proteins of interest in particular plasma fractions.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

All MS raw data and search results have been deposited to the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) via the iProX partner repository<sup>48,49</sup> with the data set identifier PXD060235.

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.5c00593>.


Experimental details; protein level changes in plasma after precipitation by polyethylene glycol 6000 (PEG); plasma albumin depletion by the Cibacron Blue 3G-A agarose column; enrichment analysis of the F1 and F2 samples; summaries of in-depth proteomic analysis of the plasma and its four fractions; tumor protein markers detected in the plasma and the fractions in this study (Figures S1–S9) (PDF)

Five in-depth fractionated plasma proteome data sets (Table S1) (XLSX)

List of plasma proteins from public databases and reported studies (Table S2) (XLSX)

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### Author Contributions

<sup>○</sup>Y.W., M.C., Y.S., and K.J. contributed equally to this work. B.B. conceived and supervised this project. Y.W. and M.C. were involved in the initial preliminary studies of this project. Y.S.,



K.J., T.L., and X.Z. made significant contributions to the proteomics analysis pipeline of this project and analyzed these samples. H.Z., J.S., L.D., Y.C., S.L., D.C., H.S., J.P., and Q.Z. were involved in the entire projects. T.Z. and C.C. contributed to the data analysis. Y.W., M.C., Y.S., and K.J. were involved in the manuscript drafting and B.B. completed it.

## Notes

The authors declare no competing financial interest.

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