Mag-Net: Rapid enrichment of membrane-bound particles enables high coverage quantitative analysis of the plasma proteome

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ABSTRACT

Membrane-bound particles in plasma are composed of exosomes, microvesicles, and apoptotic bodies and represent ~1-2% of the total protein composition. Proteomic interrogation of this subset of plasma proteins augments the representation of tissue-specific proteins, representing a “liquid biopsy,” while enabling the detection of proteins that would otherwise be beyond the dynamic range of liquid chromatography-tandem mass spectrometry in unfractionated plasma. We have developed a one-step enrichment strategy (Mag-Net) using hyper-porous strong-anion exchange magnetic microparticles to sieve membrane-bound particles from plasma. The Mag-Net method is robust, reproducible, inexpensive, and requires <100 µL plasma input. Coupled to a quantitative data-independent mass spectrometry analytical strategy, we demonstrate that we can routinely collect results for >37,000 peptides from >4,000 plasma proteins with high precision. We demonstrate excellent quantitative accuracy and analytical reproducibility of the protocol.

INTRODUCTION

The robust quantitative characterization of proteins from plasma is critical to the diagnosis of disease and monitoring response to treatment. Blood plasma is the primary specimen used in the clinical lab, and proteins in plasma hold exceptional promise as they facilitate the ubiquitous sampling of tissues as a “liquid biopsy” while remaining minimally invasive and, in essence, providing a proxy measurement of the tissue. Despite blood plasma being estimated to contain well over 10,000 proteins, most proteomics experiments performed using unfractionated plasma digests are limited to measuring <800 proteins[1,2], mainly due to the immense dynamic range of protein concentrations spanning 10 orders of magnitude -- e.g., the difference in concentration between albumin and IL-6[3]. In other words, 50% of plasma protein by mass is one protein (albumin), and 22 proteins account for 99% of the total protein mass[4,5].

The dynamic range of plasma proteins can be partially mitigated by depleting the most abundant proteins using immunoaffinity subtraction chromatography. Such depletion frequently removes 6-20 of the most abundant proteins in human plasma by about 90-99%[6–8]. Depletion increases the number of detected proteins by decreasing the dynamic range. However, these depletion columns also capture more than just the target antigens – frequently removing the target antigens plus any proteins they bind in plasma. Finally, despite depleting abundant proteins, these proteins are so abundant that even after immunodepleting, the 50 most abundant proteins in plasma can still account for 90% of the total signal in a proteomics experiment[8].

To further overcome the dynamic range challenge, protein depletion can be combined with off-line protein or peptide level fractionation before LC-MS/MS. There have been numerous strategies, including SDS-PAGE, isoelectric focusing, peptide level high-pH reversed-phase fractionation, and peptide level strong cation exchange separations. A downside of these methods is that they have both the disadvantages of immunodepletion and they also result in a major reduction in throughput – some methods resulting in as many as 25-96 fractions per sample[9–11].

Recently, Tognetti et al demonstrated the value of state-of-the-art data independent acquisition methods – both acquisition methods and computational analysis – for plasma proteomics[12]. By combining Agilent affinity removal column human-14, with the acquisition of DIA with a 2-hour HPLC gradient on an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) equipped with a FAIMS Pro device, they could measure 2732 proteins across their samples without additional fractionation. This work was significant as it was the first to demonstrate that >2500 proteins could be measured in plasma across multiple samples using a single LC-MS/MS run per sample.

More recently, Blume et al. reported the enrichment of different subpopulations of the plasma proteome without immunoaffinity depletion using paramagnetic nanoparticles derivatized with various surface chemistry[13]. These data show that using multiple nanoparticles and LC-MS runs can achieve 1500 to 2000 protein groups[13–15]. However, like with prior fractionation experiments, the number of nanoparticles used for enrichment comes at the expense of throughput because of an increase in the number of LC-MS runs needed. Despite these challenges, the excitement and current impact of this nanoparticle technology highlights the promise in using a magnetic bead to capture a subset of the plasma proteome to extend the analysis to lower abundant proteins.

Plasma contains a heterogeneous collection of membrane-bound particles, commonly referred to as extracellular vesicles (EV), which include exosomes, microvesicles, and apoptotic bodies. These membrane-bound particles represent ~1-2% of the total protein composition in plasma and are commonly enriched using a variety of methods, such as ultracentrifugation, size exclusion chromatography, and immunoaffinity capture. Kverneland et al. recently demonstrated that by using an EV fraction prepared by differential ultracentrifugation coupled with the latest data independent acquisition-mass spectrometry methods, they could measure almost 4000 proteins in a single LC-MS/MS run without depletion[16]. Analysis of this enriched fraction by mass spectrometry is effectively a “liquid biopsy,” and significantly improves the range of the proteins measurable in plasma. However, EV isolation by ultracentrifugation is time consuming and requires specialized centrifuge equipment

capable of performing at >100,000 x g, making it difficult to apply to large patient cohorts. To address this limitation, Kverneland et al. used multiple ultracentrifugation steps at 20,000 x g to collect a pellet that is enriched in larger >100 nm diameter membrane-based particles[16]. These data demonstrate the potential of EVs as useful subfraction of the plasma proteome that improves the coverage of biomedically interesting proteins. However, EV enrichment protocols based entirely on density are often contaminated with high density lipoprotein (HDL) particles as they have similar density[17]. These demonstrate a need for an EV enrichment protocol that can minimize contamination with lipoprotein particles and can be performed in a 96-well plate format.

A magnetic bead based chemical affinity purification strategy for the enrichment of EVs from biofluids was recently described by Tao and colleagues[18–20]. This “EVTrap” strategy uses a combination of “hydrophilic and aromatic lipophilic groups” that the authors describe as having high affinity toward lipid-coated EVs[18,19]. The reported methodology is promising and demonstrated significant membrane particle enrichment with minimal contamination relative to competing methods like ultracentrifugation, size exclusion, and polymer precipitation[18]. The use of magnetic beads to capture EVs makes this strategy amendable to automation. Using this simple magnetic bead capture method, they reported the measurement of >1,000 proteins from just 10 µl of plasma and 600-1,000 phosphoproteins from 0.5 mL of plasma[19]. This method has even been used to define putative diagnostic biosignatures from urinary EVs for Parkinson’s disease[20]. The method is promising, however, the EVTrap magnetic beads are not currently commercially available. Thus, the scientific community remains in need of a strategy for the enrichment of EV particles that is broadly accessible to enable assay transferability and harmonization within the community.

Here we describe Mag-Net, a robust, inexpensive, and simple magnetic bead based method to capture EV particles from plasma while simultaneously depleting abundant plasma proteins. The method enriches EVs from plasma using a combination of size and charge. Coupled to a quantitative data-independent mass spectrometry analytical strategy, we demonstrate routine measurement of

>37,000 peptides from >4,000 plasma proteins with high precision. Interestingly, 21.4% proteins measured by Mag-Net have a predicted transmembrane domain by either Philius[21] or DeepTMHMM[22], with an additional 5-10% known to be lipid anchored proteins or peripherally associated membrane proteins, a category of proteins often under-represented in proteomic analyses, particularly from plasma. We characterize the proteins captured by Mag-Net, the physical characteristics of the captured membrane particles, and demonstrate the value of this method for high coverage quantitative analysis of the plasma proteome.

RESULTS AND DISCUSSION

Overview of the Mag-Net method for plasma proteomics

To improve the dynamic range of plasma proteomics, we endeavored to develop a simple, low-cost, efficient, and automated method capable of enriching membrane-bound particles while simultaneously depleting abundant plasma proteins. MagReSyn® magnetic particles have been demonstrated to have high binding capacity and are compatible with automated LC-MS/MS workflows. The MagReSyn® beads have a hyper-porous polymer matrix that enables biomolecules to penetrate throughout the volume of the particles. Previously, positively charged peptides composed of polylysine (K8, K16) have been shown to bind phospholipids and enable the enrichment of extracellular vesicles[23]. Therefore, using an electrostatic strategy, we tested the MagReSyn® strong anion exchange (SAX) beads, functionalized with quaternary ammonium, for binding to phospholipid bilayer-bound particles and depletion of abundant plasma proteins in plasma. Figure 1 illustrates the Mag-Net workflow. Plasma is incubated with SAX beads in a 4:1 volume ratio and incubated at room temperature with gentle mixing. Unbound plasma components are washed away and depleted. The captured membrane particles are lysed with SDS, and the lysate proteins are reduced, alkylated, and prepared using protein aggregation capture (PAC) sample preparation[24,25]. The proteins were aggregated to the SAX beads using acetonitrile, washed with multiple cycles of acetonitrile and ethanol, and then digested with trypsin. The entire process was performed using a KingFisher Flex magnetic bead robot using two separate cycles as illustrated in Figure 1b.

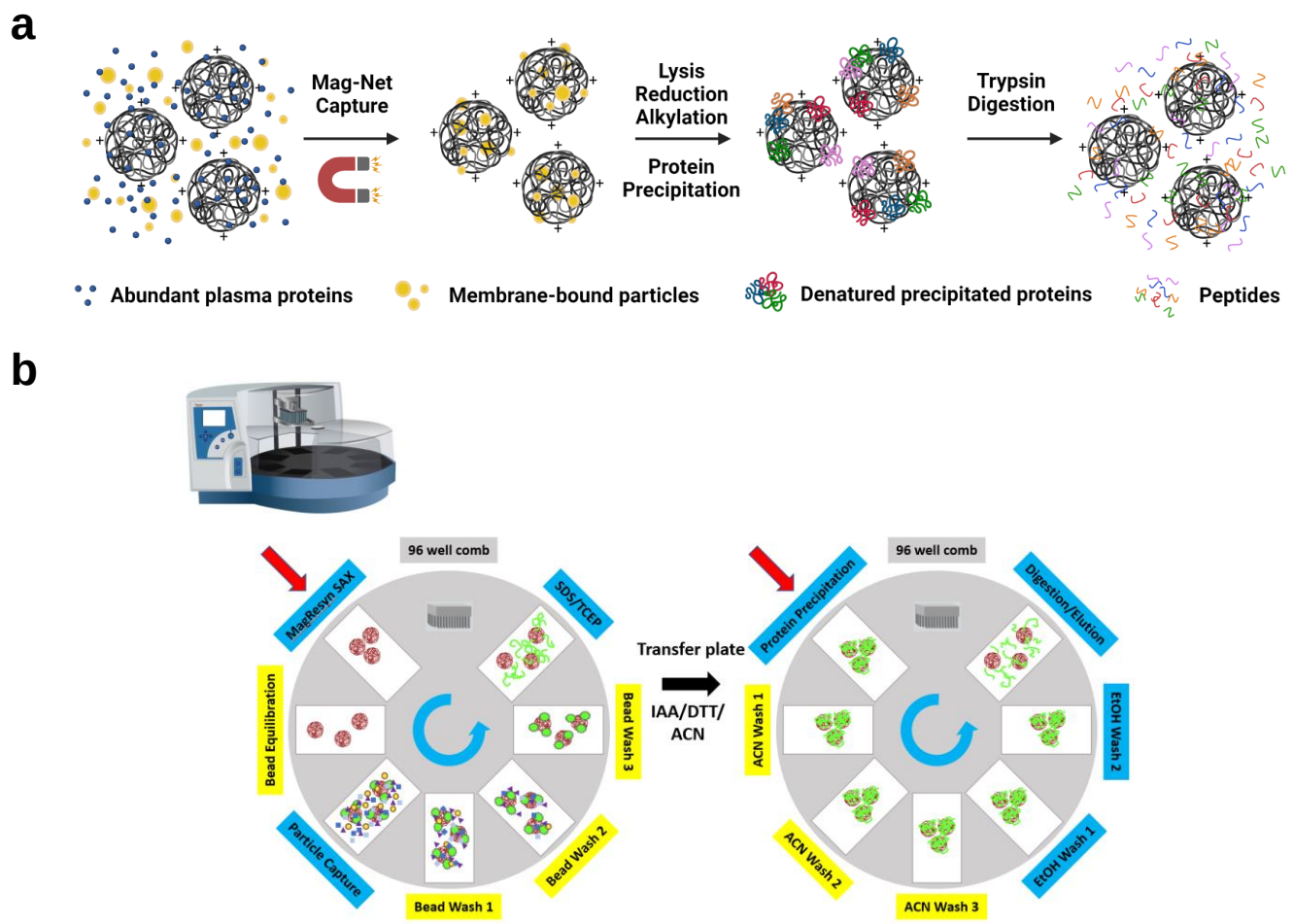

Figure 1. Mag-Net affinity capture workflow. a) SAX magnetic beads are incubated with plasma to sieve and bind negatively charged membrane-bound particles. The beads are immobilized with a magnet and abundant plasma proteins are gently washed away. The membrane particles are lysed on the beads and proteins are reduced and alkylated, precipitated back onto the beads, and then digested with trypsin. Peptides are collected and analyzed using nLC-MS/MS. b) The Kingfisher robot method to automate the Mag-Net workflow.

LC-MS/MS analysis of the Mag-Net enriched sample and the corresponding unfractionated plasma sample using data-independent acquisition mass spectrometry (DIA-MS) resulted in unusually large numbers of peptides and proteins that were detectable regardless of the computational method used to analyze the data (Figure 2). Using EncyclopeDIA v2.12.30 with a chromatogram library generated from six gas phase fractionated LC-MS/MS runs from the Mag-Net

enriched sample, we were able to detect 4,163 protein groups in the enriched fraction and 1,088 protein groups from the corresponding unfractionated total plasma, prepared using standard PAC (Figure 2B, yellow bars). To demonstrate that the protein and peptide detections were not solely due to the use of an on-column chromatogram library generated using the Mag-Net enriched fraction, we also analyzed the data using EncyclopeDIA with a Prosit library generated from the human fasta downloaded (September 2022) from Uniprot with one protein per gene (Figure 2B, blue bars). Additionally, the analysis was also performed using DIA-NN in library free mode with the identical fasta file (Figure 2B, gray bars). The number of peptides (DIA-NN reports precursors instead of peptides) detected in triplicate preparations and analysis of the membrane particle enriched fraction and unfractionated plasma are shown in (Figure 2A). In all cases, the use of the MagReSyn® SAX beads to capture membrane particles prior to digestion resulted in improved coverage of the plasma proteome using a single LC-MS/MS analysis (Figure 2B). Additionally, the use of a chromatogram library generated from the gas phase fractionated analysis of the Mag-Net enriched sample even improved the protein coverage of unfractionated plasma.

Characterization of the proteins and particles enriched by Mag-Net

We performed a differential analysis of the proteins enriched in the membrane particle fraction versus the matched unfractionated plasma sample. In the analysis of the same sample prepared and measured six times, three times as unfractionated plasma and three times using the Mag-Net protocol, 4675 protein groups were detected. Of these, 2,877 proteins were enriched (BH corrected p-value ≤ 0.05 and >1.5 fold change) in the membrane particle fraction, and 413 proteins were depleted from the unfractionated plasma (Figure 2C, Supplementary Figure S1). Statistical enrichment analysis using the Enrichr tool[26,27] against Jensen COMPARTMENTS resource[28] revealed that the majority of the enriched proteins were proteins classically localized to membrane-bound vesicles, extracellular vesicles, exosomes, and extracellular organelles (Figure 2D)

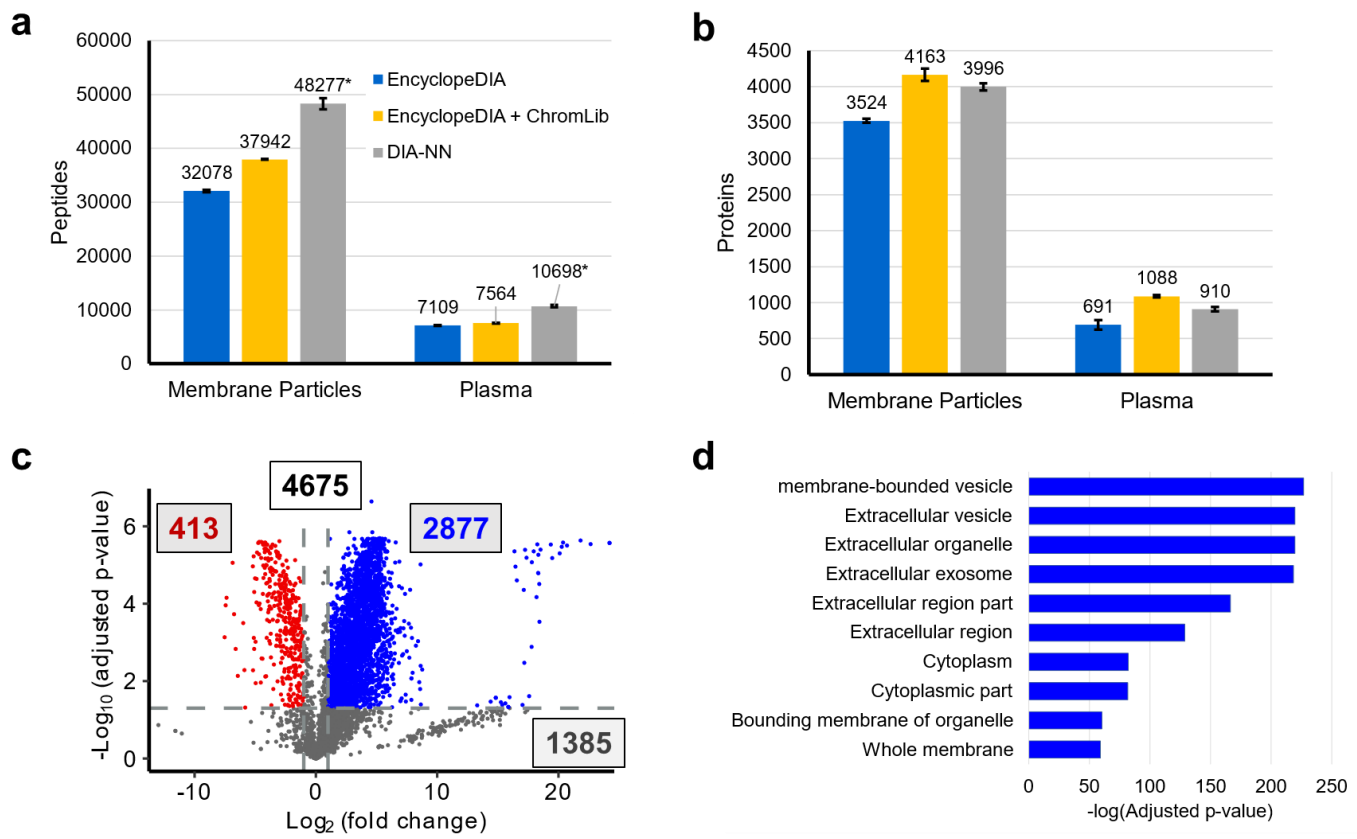

Figure 2. DIA-MS/MS analysis of the peptides from enriched membrane particles and unfractionated plasma. a) The number of peptides detected from three preparations of the same plasma sample at a 1% FDR with three different analysis strategies. The different analysis strategies were the use of EncyclopeDIA with a Prosit library (blue), EncyclopeDIA with a chromatogram library generated from gas phase fractionation (yellow), and DIA-NN in library free mode. EncyclopeDIA reports peptides detected and DIA-NN reports peptide precursors. The Mag-Net protocol detected a minimum of 451% more peptides than using the same analysis with unfractionated plasma. b) Proteins detected at a 1% FDR with the three different analysis strategies. The use of EncyclopeDIA with an on-column chromatogram library returned the most peptides and proteins and, thus, was used for all further analysis. c) Differential analysis of proteins between the enriched membrane particles (blue) and the same unfractionated plasma sample (red). d) The 2877 proteins that were enriched in the Mag-Net protocol were used to assess the enrichment of Jensen COMPARTMENTS using the Enrichr tool.

To confirm the fraction captured by Mag-Net is enriched in extracellular vesicles, we calculated the fold enrichment of known extracellular vesicle proteins relative to the matched unfractionated plasma sample (Figure 3A). We used the known exosome marker proteins CD9, CD63, PDCD6IP (aka Alix), FLOT1, FLOT2, TSG101, SDCBP (aka Syntenin-1) and NCAM1[29–33]. NCAM has been used in the past for the immuno-enrichment and detection of neuronal derived exosomes from plasma or serum[34–37]. We used the proteins CD40, SEPTIN2, and ATP5F1A as markers of

microvesicles[30,31,33,38,39]. The proteins HSP90B1 (aka Endoplasmin, GRP-94, or tumor rejection antigen 1) and ANXA5 (aka Annexin A5) were used as markers of cellular debris or apoptotic bodies[29,30,33,40,41]. Most of these markers were enriched >16 fold over their abundance in plasma and the canonical exosome markers CD9, CD63, Syntenin-1, and NCAM1 are all enriched >30-fold. This enrichment is a significant underestimate as many of these proteins are not detectable in plasma without enrichment and, thus, the quantity was estimated by integrating the background in the plasma sample where the peptide signal should be based on the Mag-Net sample. For example, the exosome marker CD9 had peptides that were enriched from 14.3 to 608.6-fold. The CD9 peptide with the lowest enrichment (DEPQRETLK) had a large background from a chemical interference in plasma, thus, an underestimate of the enrichment. We also confirmed the depletion of classic plasma and lipoprotein particle (LPP) markers from the Mag-Net fraction. The proteins albumin (ALB), serrotransferrin (TF), alpha-2-macroglobulin (A2M), alpha-1-acid glycoprotein 1 (ORM1), haptoglobin (HP), and hemoglobin (HBA1 and HBB) were all depleted by ~95% -- a degree of depletion that is equal or exceeds antibody depletion columns[4].

A complication in the enrichment of plasma EVs are that lipoprotein particles are frequently co-enriched with EV fractions[17]. Lipoprotein particles are of similar size and density as extracellular vesicles, so EV enrichment methods using these physicochemical properties are particularly susceptible to contamination from lipoprotein particles. Mag-Net benefits from the MagReSyn® bead technology that facilitates the penetration of membrane particles and biomolecules throughout the volume of the polymer matrix. Thus, Mag-Net uses a combination of a size-based “netting” and charge-based binding from the quaternary ammonium surface chemistry. Using Mag-Net, lipoproteins are depleted by ~80%, as determined for fold depletion of lipoprotein protein groups (Figure 3b). Interestingly, APOB, the lipoprotein present in low density lipoprotein (LDL) and very low density lipoprotein (VLDL) particles and haswhich the closest diameter to extracellular vesicles, is depleted the most. The surface charge of EVs, where the surface is mostly phospholipid, has been shown to

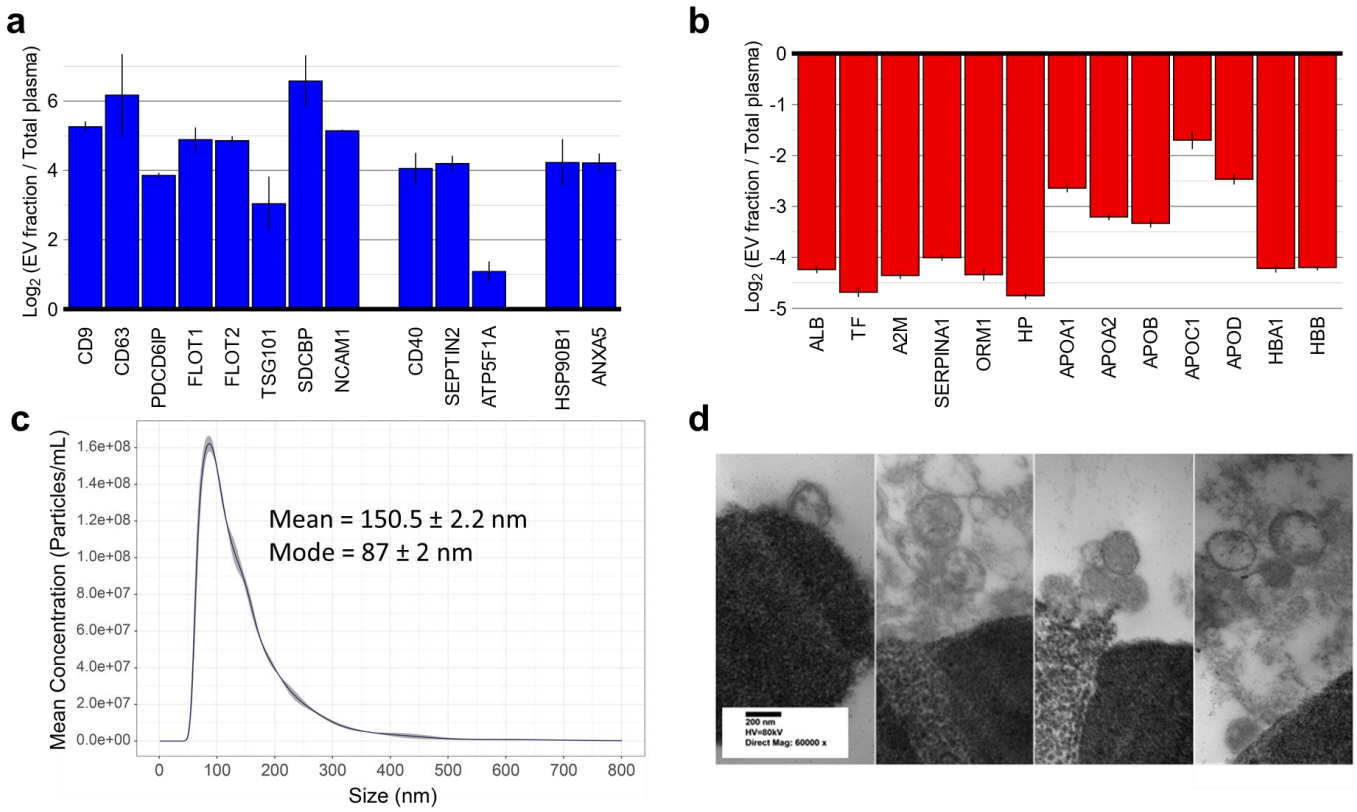

Figure 3. Characterization of Mag-Net enriched membrane particles. a) Fold enrichment of protein groups compared to total plasma. b) Fold depletion of protein groups compared to total plasma c) Nanosight particle count d) micrographs of captured membrane particles on bead surfaces using transmission electron microscopy.

be significantly different from that of lipoprotein particles. At the binding pH of 6.3, as performed in our analysis, the surface of lipoprotein particles will be significantly less negative than the EVs[42] and therefore have significantly less affinity for the quaternary ammonium functionalized beads.

To verify that Mag-Net binds intact membrane particles we performed analyses to assess the particle size and morphology. After capture of particles to the MagReSyn® SAX bead we released the particles using salt and analyzed the fraction in triplicate using Nanoparticle Tracking Analysis (NTA) (Figure 3C). The most frequent particle size was 87 nm, with a mean size of 150 nm. Based on the distribution of the measured particle sizes, most were of a size that is typical of exosome vesicles. However, NTA also confirmed that there are particles of size typically associated with microvesicles – confirming the results from the extracellular vesicle marker protein analysis (Figure 3A). Transmission

electron microscopy (TEM) was also performed on the MagReSyn® beads and their captured membrane particles (Figure 3D). The ultrastructure images show vesicles with lipid bilayers captured on the surfaces of the MagReSyn® beads.

The Mag-Net protocol is unusual in that it combines the vesicle capture step and protein aggregation capture digestion step using a single magnetic bead (Figure 1). To assess whether the improved plasma protein coverage was because SAX beads were used in place of hydroxyl beads during the PAC digestion, we performed identical PAC digestion protocols with both beads. Supplementary Figure S2 shows a volcano plot of the protein differential abundance of the same plasma sample digested with the same protocol using either hydroxyl versus SAX functionalized magnetic beads. There are very few proteins with differential abundance between the two beads confirming that the improved recovery was not because of the beads used during the PAC step. Importantly, these data also show that the SAX beads do not perform worse than the hydroxyl beads for PAC digestion.

To evaluate the “netting” capture ability of the MagReSyn® bead chemistry the Mag-Net vesicle capture protocol was also performed with the hydroxyl beads. The hydroxyl bead chemistry was chosen because those beads have an identical hyper-porous polymer matrix chemistry but do not have the net cationic charge of the quaternary ammonium functionalized SAX beads. Supplementary Figure S3 is a volcano plot of the same plasma sample digested using PAC with hydroxyl beads versus performing the membrane capture protocol using the hydroxyl beads. Surprisingly, the uncharged hydroxyl functionalized beads also enriched proteins known to be present in membrane bound vesicles and depleted abundant plasma proteins. To further understand the effect, we performed a differential analysis between the proteins enriched with the Mag-Net protocol using hydroxyl versus SAX beads (Supplementary Figure S3). In total, there were 3,334 protein groups that were differentially abundant between the two bead types. While it was clear that the hydroxyl bead enriches for membrane bound particles when compared with an unenriched plasma

digest, there were significantly more proteins enriched with the Mag-Net protocol performed using the SAX versus the hydroxyl beads – 2,992 and 342 proteins respectively (Supplementary Figure S4). Using the GO Cellular Component enrichment terms of the proteins enriched by the respective bead chemistry, the SAX beads enriched proteins (2,992) were derived from intracellular membranes, secretory granules, endosome membranes, etc. In contrast, the 342 proteins specific to the hydroxyl beads were from the lipoprotein particles, chylomicron particles, extracellular matrix, etc. This further confirms the importance of charge in the Mag-Net protocol in that it enables the enrichment of membrane vesicles while minimizing the contamination from much more abundant lipoprotein particles.

The challenges of the dynamic range of plasma have been described in detail previously[3,5]. We performed an analysis to evaluate the effect of Mag-Net on the dynamic range of plasma. The signal of each peptide was measured using the average of three replicate measurements performed using PAC total plasma digestion (Figure 4A) and the Mag-Net extracellular vesicle capture method (Figure 4B). If a peptide was not detected in a specific LC-MS run, the data from the runs where it was detected was used to provide boundaries to compute a background subtracted area where the peptide would be located if it were present. The peptide areas were normalized by equalizing the total ion current (TIC) between runs and the protein abundance calculated by summing the peptides mapping to the protein sequence. Figure 4 illustrates the significant enrichment of the EV marker proteins (blue) and the depletion of the abundant plasma proteins (red). The signal plotted for the protein abundance in the total plasma sample spans ~10 logs (Figure 4A). It is important to point out that measuring the abundance for many of the proteins in the total plasma sample is only possible by matching the peptide signal from the respective measurement using the Mag-Net protocol. Despite the classic plasma proteins being depleted by ~95% following Mag-Net (Figure 4B), those proteins are still some of the most abundant proteins in sample. This observation is analogous to what has been reported with the use of plasma depletion columns[8]. However, a notable difference is that the

Mag-Net protocol depletes any protein not associated with or within a membrane particle. Overall, Mag-Net significantly compresses the dynamic range of proteins in plasma without eliminating proteins.

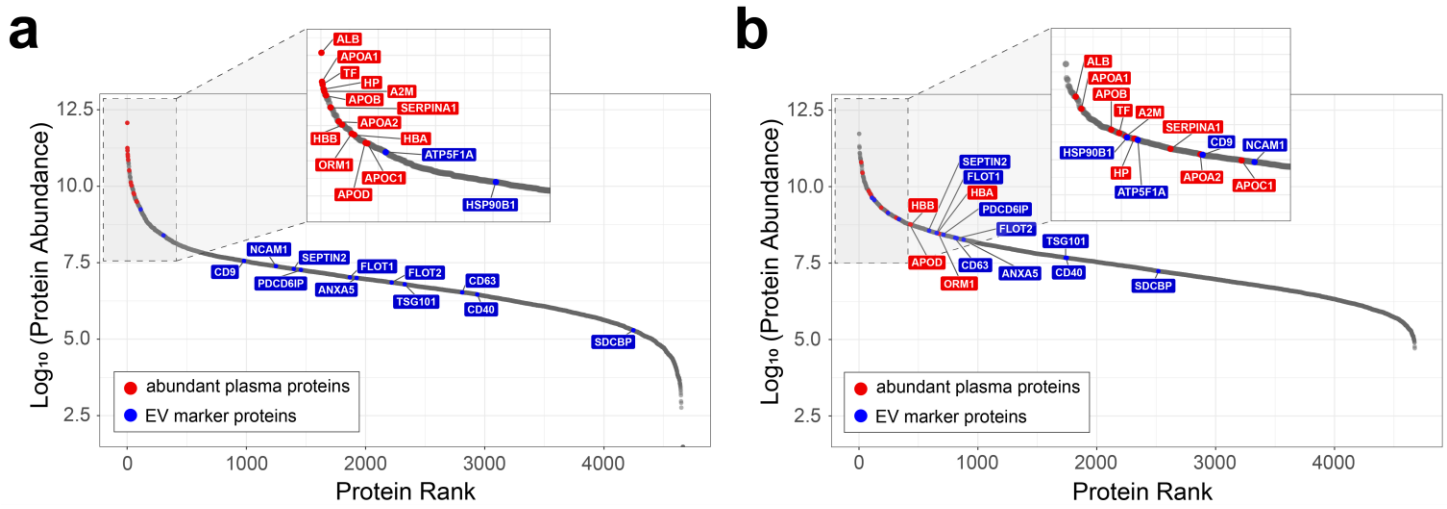

Figure 4. Dynamic range of the plasma proteome. The peptide signal was measured using the average of triplicate measurements performed using PAC total plasma digestion (A) and the Mag-Net extracellular vesicle capture method (B). If a peptide was not detected in a specific LC-MS run, the data from the runs where it was detected was used to provide boundaries to compute a background subtracted area where the peptide would be located if it were present. The peptide areas were normalized by equalizing the total ion current (TIC) between runs and the protein abundance calculated by summing the peptides mapping to a parsimonious protein group. The EV marker proteins are shown in blue and the classic abundant plasma proteins in red. The signal plotted for the protein abundance in the total plasma sample spans ~10 logs. Despite the classic plasma proteins being depleted by ~95% following Mag-Net, those proteins are still some of the most abundant proteins in sample.

Assessment of quantitative accuracy and precision of samples prepared by Mag-Net.

To confirm the peptide measurements following enrichment using the Mag-Net reflected accurate quantities, we generated and analyzed matrix-matched calibration curves[43,44]. Three separate and independent matrix-matched calibration curves were made from the same human plasma sample diluted with chicken plasma. The calibration curve was generated by mixing human plasma with chicken plasma at 14 different volumetric ratios. The three separate matrix matched

calibration curves (14 dilution levels x 3 = 42 samples) were subjected to the Mag-Net membrane particle capture and digestion. Each calibration curve was acquired using our standard DIA LC-MS protocol with runs ordered from lowest human concentration to highest concentration. Several system suitability runs were performed as blanks between each calibration curve. LC-MS data were subjected to our normal analysis pipeline. The peptides that were conserved between human and chicken were removed and the peptide background subtracted peak areas determined using Skyline[45].

Figure 5 illustrates the analysis of a subset of the matrix-matched calibration curve[43]. The area of

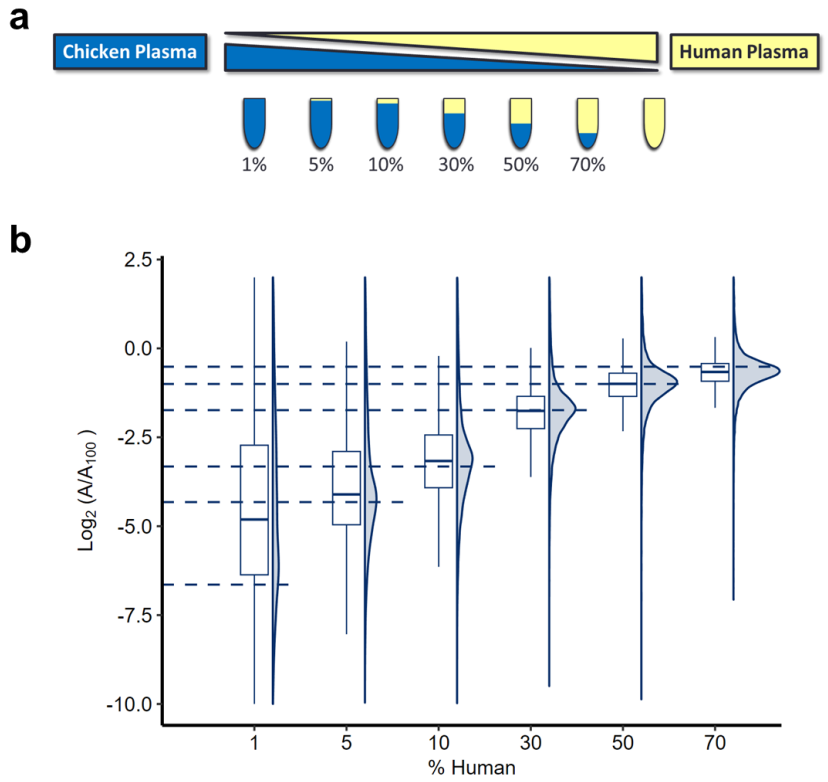

Figure 5. Summary of the matrix matched calibration curve data. A) Schematic of the volumetric mixing of human plasma with chicken plasma for 7 of the dilution points. The percentage represents the percent volume of human plasma where the remainder represents chicken plasma. The mixed plasma samples were enriched and digested using the Mag-Net protocol. Following peak detection with EncyclopeDIA, peptides conserved between human and chicken were removed. The peptide background subtracted peak areas were determined using Skyline and the ratio of the peptide area relative to the area from the 100% undiluted human sample. B) The measured Log_2 area ratios versus the expected % human plasma.

each peptide was divided by the area of the same peptide from the 100% undiluted human plasma

sample. The measured Log_2 area ratios were plotted versus the expected % human plasma (Figure 5B). The distribution of the measured ratios is shown as both a box plot and a density plot. The mode of measured peptide relative abundance reflects the expected value based on the volumetric dilution prior to enrichment, sample preparation, and measurement. The median peptide ratios of the 1% human sample does underestimate the expected dilution. This indicates that a subset of the peptides is below the lower limit of quantification (LLOQ) at that dilution level.

We can also use these matrix matched calibration curve data to calculate the LLOQ and the limit of detection (LOD) for each individual protein group and peptide. Supplementary Figure S5 contains representative plots of the calibration curves for two common EV markers (CD9 and NCAM1) and also an abundant LDL protein marker (APOB). All three of these proteins have excellent quantitative linearity and an impressive LLOQ of 1.3%, 3.3% and 2.0% human plasma for CD9, NCAM1, and APOB respectively. Interestingly, even APOB, which is not enriched, but depleted by Mag-Net shows promising quantitative figures of merit. While every protein and peptide is different, a Skyline document containing the complete matrix matched calibration curve data is available at Panorama Public.

The measured peptide abundances are highly correlated between different preparations and analysis of the same sample. Figure 6a shows a plot of the RAW unadjusted log2 peak area of the same peptide between runs. Replicate samples have Pearson correlation coefficients ranging from 0.978 to 0.985. The same data can also be assessed using a distribution of the coefficient of variation (CV) between the four replicates. The mean (μ) CV of the RAW unadjusted areas between four preparations is 26.85%. After normalization, the median CV improved to 24.75%. Imputation of missing data values and peptide grouping did not significantly alter the CV. As highlighted previously[46], protein grouping further improved the median CV to 20.89%

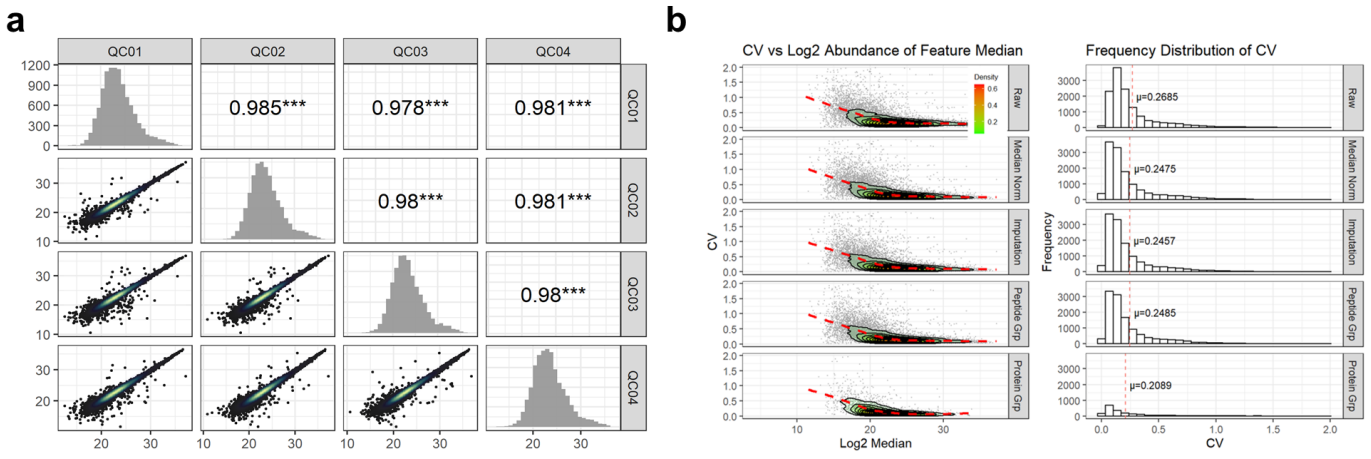

Figure 6. Reproducibility of different Mag-Net preparations of the same pooled sample. A pooled plasma sample was prepared four separated times and peptides quantified by data independent acquisition. a) The log2 peptide abundances measured between 4 separate preparations and measurement of the same sample. The lowest Pearson correlation coefficient between pairs was 0.978. b) Effect of different data processing steps on the coefficient of variation of different peptide and protein quantities. The top row shows the RAW peptide intensities, followed by the data with median normalization, the effect of imputation, peptide grouping and protein grouping on the last row.

CONCLUSION

The Mag-Net protocol is a simple yet powerful approach for the analysis of the plasma proteome. The enrichment of proteins within EVs enables the detection of proteins that would otherwise be beyond the dynamic range of LC-MS/MS in unfractionated plasma. Mag-Net benefits from the MagReSyn® bead technology that facilitates the penetration of membrane particles and biomolecules throughout the volume of the matrix. Thus, Mag-Net uses both a size based “netting” and charge-based binding from the quaternary ammonium surface chemistry. Mag-Net enables the selective enrichment of vesicles in the presence of similar size lipoprotein particles.

Combined with a KingFisher magnetic bead robot, Mag-Net enables the automated membrane particle capture, lysis, and subsequent aggregation of vesicle proteins on a single bead. Performing PAC on the same beads used for the vesicle enrichment results in excellent recovery and minimal losses since efficient elution of the vesicles from the beads is unnecessary. Instead, peptides are

released from the beads via tryptic digestion. Because samples are prepared in a 96-well format, up to 96 samples can be prepared in parallel. The Mag-Net protocol is particularly well suited for high throughput applications requiring the preparation of many plasma samples for analysis by LC-MS/MS.

The Mag-Net protocol enables the reproducible and accurate measurement of >37,000 peptides and >4,000 proteins from human plasma when combined with data independent acquisition mass spectrometry. The correlation coefficients from pairwise comparisons of the same sample prepared and measured multiple times was >0.978. The median CV of peptide and protein measurements between preparations was 24.75% and 20.89% respectively. The peptide measurements showed excellent accuracy for many peptides up to 100:1 dilution using a matrix matched calibration curve. We believe this simple, robuse, and cost effective solution will enable the identification of novel markers of disease and the corresponding response to therapy.

METHODS

Plasma samples. Plasma samples from 17 individual healthy donors collected in K_2EDTA vials were purchased from Innovative Research (IPLASK2E2ML, Innovative Research Inc, Novi, MI). Samples were aliquoted and stored at -80°C.

Membrane particle enrichment coupled with protein aggregation capture (Mag-Net Method). Membrane particle capture from plasma and protein aggregation capture (PAC) steps were performed on a Thermo Scientific™ KingFisher Flex. The combination of the membrane particle capture with PAC and digestion is referred to as Mag-Net (Note: Eight plates were designated for each of two Kingfisher (KF) cycles as follows. KF Cycle 1: Plate 1: SAX beads, Plate 2: Bead Equilibration, Plate 3: Membrane Particle Capture, Plate 4-6: Wash, Plate 7: Solubilization/Reduction, Position 8: 96 well comb. KF Cycle 2: Plate 1: Same as Plate 7 of KF Cycle 1, Plate 2-4: Acetonitrile Wash, Plate 5-6: Ethanol Wash, Plate 7: Trypsin Digestion/Peptide Elution.) Briefly, HALT cocktail (protease and phosphatase inhibitors, Thermo Fisher Scientific) was added to plasma (20-100 µL) and then mixed 1:1 by volume with Binding Buffer (BB, 100 mM Bis-Tris Propane, pH 6.3, 150 mM NaCl). MagReSyn® strong anion exchange or MagResyn® Hydroxyl magnetic microparticles (ReSyn Biosciences) were first equilibrated 2 times in Equilibration/Wash Buffer (WB, 50 mM Bis Tris Propane, pH 6.5, 150 mM NaCl) with gentle agitation and then combined in a 1:4 ratio (volume beads to volume starting plasma) with the plasma:BB sample for 45 minutes at room temperature. The beads were washed with WB 3 times for 5 minutes with gentle agitation. The enriched membrane particles on the beads were then solubilized and reduced in 50 mM Tris, pH 8.5/1% SDS/10 mM Tris (2-carboxyethyl) phosphine (TCEP) with 800 ng enolase standard added as a process control. Following reduction, the plate was removed from the Kingfisher. Samples were alkylated with 15 mM iodoaceamide in the dark for 30 minutes and then quenched with 10 mM DTT for 15 minutes. Total unfractionated plasma (1 µL) was prepared in parallel (reduction, alkylation, and quenched) and added to the Kingfisher plate as a control for enrichment. The samples were processed using protein

aggregation capture with minor modifications. Briefly, the samples were adjusted to 70% acetonitrile, mixed, and then incubated for 10 minutes at room temperature to precipitate proteins onto the bead surface. The beads were washed 3 times in 95% acetonitrile and 2 times in 70% ethanol for 2.5 minutes each on magnet. Samples were digested for 1 hour at 47 ˚C in 100 mM ammonium bicarbonate with Thermo Scientific™ Pierce™ porcine trypsin at a ratio of 20:1 trypsin to protein. The digestion was quenched to 0.5% trifluoracetic acid and spiked with Pierce Retention Time Calibrant (PRTC) peptide cocktail (Thermo Fisher Scientific) to a final concentration of 50 fmol/µL. Peptide digests were then stored at -80°C until LC-MS/MS analysis.

Matched matrix calibration sample preparation. Human plasma was diluted with chicken plasma by volumetric mixing for 14 human:chicken dilution points (100:0, 70:30, 50:50, 30:70, 10:90, 7:93, 5:95, 3:97, 1:99, 0.7:99.3, 0.5:99.5, 0.3:99.7, 0.1:99.9, 0:100). Membrane particles were enriched using the Mag-Net Method for each dilution and performed in triplicate.

Nanoparticle tracking analysis. Membrane particles were eluted from SAX beads in Elution Buffer (25 mM Bis Tris Propane, pH 6.5, 1 M NaCl, 0.1% Tween 20) and diluted in PBS (first 1:50 and then an additional dilution of either 1:25 or 1:50). Diluted samples were analyzed using the Nanosight NS300 (Malvern Panalytical Ltd). The instrument was calibrated using a NIST standardized 100 nm latex bead and 5% CV rate. Using a manual injection method, videos were captured for each sample at 21.7°C. Particle counts were determined using the Nanosight Nanoparticle Tracking Analysis (NTA) software 2.3 (Malvern Panalytical Ltd) with a detection threshold of 3-Multi.

Transmission electron microscopy (TEM). The samples (membrane particles bound to SAX beads) were fixed in 2% glutaraldehyde/0.1 M sodium cacodylate, pH 7.2 overnight at 4°C, and then postfixed in 2% OsO_4 buffered with 100 mM sodium cacodylate, pH 7.2 overnight at room temperature. The samples were then washed with water, dehydrated in an increasing ethanol series (30%, 50%, 60%, 70%, 80%, 90%, 98% for 10 minutes each followed by 100% x 3 for 10 minutes) and then infiltrated stepwise from ethanol into Spurr's resin (2:1, 1:1, 1:2 for 30 min each followed by

100% Spurr's x 3 overnight) and then embedded and baked at 60°C for 2 days. Ultra-thin sections were cut from each sample, collected on grids, and imaged on a JEOL 1230 Transmission Electron Microscope (Peabody, MA).

Liquid chromatography and mass spectrometry. One µg of each sample with 150 femtomole of PRTC was loaded onto a 30 cm fused silica picofrit (New Objective) 75 µm column and 3.5 cm 150 µm fused silica Kasil1 (PQ Corporation) frit trap loaded with 3 µm Reprosil-Pur C18 (Dr. Maisch) reverse-phase resin analyzed with a Thermo Easy-nLC 1200. The PRTC mixture is used to assess system suitability before and during analysis. Four of these system suitability runs are analyzed prior to any sample analysis and then after every six sample runs another system suitability run is analyzed. Buffer A was 0.1% formic acid in water and buffer B was 0.1% formic acid in 80% acetonitrile. The 40-minute system suitability gradient consists of a 0 to 16% B in 5 minutes, 16 to 35% B in 20 minutes, 35 to 75% B in 1 minute, 75 to 100% B in 5 minutes, followed by a wash of 9 minutes and a 30-minute column equilibration. The 110-minute sample LC gradient consists of a 2 to 7% for 1 minute, 7 to 14% B in 35 minutes, 14 to 40% B in 55 minutes, 40 to 60% B in 5 minutes, 60 to 98% B in 5 minutes, followed by a 9-minute wash and a 30-minute column equilibration. Peptides were eluted from the column with a 50°C heated source (CorSolutions) and electrosprayed into a Thermo Orbitrap Eclipse Mass Spectrometer with the application of a distal 2 kV spray voltage.

For each batch of samples, we collected a chromatogram library using gas phase fractionation from 6 independent injections using a pool of all samples within the batch. These gas phase fractionation runs collected tandem mass spectrometry data each covering a different portion of the total m/z range. For each injection MS1 spectra were collected with a mass range of 100 *m/z* (400-500 *m/z*, 500-600 *m/z*…900-1000 *m/z*). MS2 spectra were collected using the tMSn scan function with a loop count of 25 at 30,000 resolution, a normalized AGC target of 1000%, maximum injection time set to Auto, and 27% normalized collision energy. Each MS2 spectrum was collected using a 4 m/z isolation window with the edges placed in peptide forbidden zones as described previously[47,48]. In alternating

cycles of data independent acquisition, the location of the isolation windows were offset by 50% so that the data could be computationally demultiplexed to 2 m/z[49]. The chromatogram library data is used to quantify proteins from individual sample runs.

Each sample for quantitative analysis was run once using consist of a cycle of one 30,000 resolution full-scan mass spectrum with a mass range of 395-1005 m/z, AGC target of 4e5, maximum injection time set to Auto. MS2 spectra were collected using the tMSn scan function, loop count of 75 at 30,000 resolution, normalized AGC target of 800%, maximum injection time set to Auto (54 ms), and 27% normalized collision energy. Each MS2 spectrum was collected using staggered 12 m/z isolation windows with optimized window placements[47]. Application of the mass spectrometer and LC solvent gradients are controlled by the ThermoFisher Xcalibur

Peptide detection and quantitative signal processing. Thermo RAW files were converted to mzML format using Proteowizard (version 3.0.23063) using vendor peak picking and demultiplexing with the settings of “overlap_only” and Mass Error = 10.0 ppm[49]. On column chromatogram libraries were created using the data from the six gas phase fractionated “narrow window” DIA runs of the pooled reference as described previously[47,48]. These narrow windows were analyzed using EncyclopeDIA (v2.12.30 with version 2 scoring) with the default settings (10 ppm tolerances, trypsin digestion, HCD b- and y-ions) of a Prosit predicted spectra library based the Uniprot human canonical FASTA (September 2022). The results from this analysis from each batch were saved as a “Chromatogram Library" in EncyclopeDIA’s eLib format where the predicted intensities and iRT of the Prosit library were replaced with the empirically measured intensities and RT from the gas phase fractionated LC-MS/MS data. The “wide window” DIA runs were analyzed using EncyclopeDIA (v2.12.30 with version 2 scoring) requiring a minimum of 4 co-varying and interference free quantitative transitions and filtering peptides with q-value ≤ 0.01 using Percolator 3.01. After analyzing each file individually, EncyclopeDIA was used to generate a “Quant Report" which stores all detected peptides, integration boundaries, quantitative transitions, and statistical metrics from all

runs in an eLib format. The Quant Report eLib library is imported into Skyline-daily with the human uniprot FASTA as the background proteome to map peptides to proteins, perform peak integration, manual evaluation, and report generation. A csv file of peptide level total area fragments (TAFs) for each replicate was exported from Skyline using the custom reporting capabilities of the document grid. To examine enrichment and depletion relative to total, the group comparison document grid was used to export a csv file of protein-level average ratios, maximum/minimum ratios, and p-values. Protein-level matrix-matched calibration curve data was exported as a csv file of protein abundance for each replicate.

DATA AVAILABILITY

All mass spectrometry RAW datafiles, demultiplexed mzML intermediate files, EncyclopeDIA output, and final Skyline documents are available in Panorama Public at https://panoramaweb.org/Mag-Net.url. The dataset is registered through the ProteomeXchange with the unique identifier (PENDING).

R scripts used to produce figures in the manuscript and the methods for the KingFisher robot are available at https://github.com/uw-maccosslab/Mag-Net

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AUTHOR CONTRIBUTIONS

CCW and MJM conceived the study and designed the experiments. CCW developed and optimized the Mag-Net method. PN, SB, IG, SS and JJ provided expertise, manufactured non-commercial MagReSyn® bead formulations, contributed to scientific interpretation, and assisted with inter-laboratory method evaluation. KT, JP, DP, GM, and MR contributed to data analysis and generated data figures. AH contributed scientific interpretation. EH and EP provided technical support. CCW and MJM wrote the manuscript.

COMPETING FINANCIAL INTERESTS

Ireshyn Govender, Stoyan Stoychev and Justin Jordaan are employed by ReSyn Biosciences, proprietors of MagReSyn® technology. The MacCoss Lab at the University of Washington has a sponsored research agreement with Thermo Fisher Scientific, the manufacturer of the mass spectrometry instrumentation used in this research. Additionally, Michael MacCoss is a paid consultant for Thermo Fisher Scientific.

SUPPLEMENTARY DATA (Mag-Net EV Method Supplement.docx):

- Figure S1: Volcano plot of human plasma versus Mag-Net enriched EVs with representative markers highlighted.
- Figure S2: Volcano plot comparing the use of MagReSyn Hydroxyl versus SAX beads for protein aggregation capture (PAC) digestion.
- Figure S3: Volcano plot comparing the preparation of total plasma using PAC with Hydroxyl beads versus the use of Mag-Net membrane particle capture with Hydroxyl beads.

- Figure S4: Volcano plot comparing the difference between the use of MagResyn Hydroxyl versus SAX beads for Mag-Net.
- Figure S5: Example matrix matched calibration curves for the EV marker proteins CD9 and NCAM1 and the (V)LDL marker APOB.
- Figure S6: Assessment of plasma freeze thaw cycles on the relative peptide abundance.

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