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Supramolecular Polymeric Assemblies for the Selective Depletion of Abundant Acidic Proteins in Serum

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Abstract

The analysis of low-level protein biomarkers in serum is precluded by the presence of other highly abundant serum proteins. Hence, the preliminary removal of serum albumin along with other abundant proteins in serum (*i.e.* immunoglobulins, transferrin, haptoglobin, α -2-macroglobulin and apolipoproteins), is often a requirement prior to any biomarker analysis. In this work, we take advantage of the low isoelectric points (pI) of these highly abundant proteins to selectively deplete them from serum by extraction using functionalized amphiphilic polymeric nanoassemblies. The selectivity of extraction is dependent on the pI of the protein and the extraction pH, which holds true even for extremely complex protein mixtures like serum. High extraction capacity is achieved by optimizing the extraction conditions and is found to be comparable to currently available methods for depletion. Depletion of these abundant acidic proteins allows for the enhanced detection of higher pI proteins and enables a three orders of magnitude increase in detection sensitivity for a putative cancer biomarker, demonstrating the utility of these polymeric assemblies for enhancing the analysis of the serum proteome.

Graphical Abstract

Keywords

serum depletion; amphiphilic polymers; reverse micelle nanoassemblies; acidic proteins; serum proteomics; biomarker detection

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Supporting Information

Materials and methods, syntheses and characterization of polymers, and supporting figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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INTRODUCTION

Serum is the most common clinical source of disease biomarkers due to the ease and minimally invasive means of obtaining samples from patients and the fact that it is rich in biomolecules, proteins in particular, that can reflect the overall physiological status of the body. Besides human serum albumin (HSA), which constitutes about 55% of total serum proteins by mass, serum also contains a wide variety of immunoglobulins, signaling peptides and proteins, such as hormones and cytokines, and proteins leaked by tissues into circulation as a result of cell death, damage, abnormal physiology or diseased state.¹ Detection and reliable measurement of low-level proteins is of prime importance for biomarker discovery, early disease diagnosis and disease progression monitoring. However, these efforts are challenged, not only by the sheer complexity of the serum proteome, but also by protein concentrations that vary over ten orders of magnitude. The top six abundant proteins in serum, for instance, comprise about 85% of its total protein content,² and hence dominate the signal in proteomic analyses³ and obscure the presence of low-level putative biomarkers.

In addressing this challenge, various methods have been developed to remove or reduce the concentration of these highly abundant serum proteins to improve the detection of low abundance proteins. Chemical-based strategies that rely on precipitating agents to deplete albumin have been used for a long time,^{4–6} but the selectivity of these precipitation techniques is hard to predict and control. Commercially-available immunoaffinity-based depletion methods that use antibodies to selectively remove albumin and other abundant serum proteins are highly specific and efficient.^{2,7–9} However, these commercially available products tend to be costly and suffer from stability and leakage issues when used under harsher conditions or upon storage for long times. Kits and columns using immuno-depletion strategies also have limited sample loading capacity, usually a few tens of microliters of serum. Ligand affinity-based methods, such as immobilized Cibacron Blue dye for depleting albumin,^{10–12} and immobilized Protein A/Protein G for binding to the Fc region of immunoglobulins,^{13–15} are also commercially available options for selective protein removal. Although less costly and capable of higher sample loading than immuno-depletion methods,⁹ these ligand affinity-based methods are more prone to non-specific binding and tend to dilute the sample through several washing and elution steps.

Newer polymer-based depletion methods have been recently explored as they offer advantages in terms of stability and capacity. Their structure and chemistry can be optimized to achieve high sample loading capacity and sufficient selectivity, while working under harsher conditions. Various strategies for imparting selectivity for binding HSA have been reported, such as functionalization of polymers with albumin-binding ligands¹⁶ and via molecular imprinting.^{17–19} To our knowledge, however, very few, if any, polymers have been developed to simultaneously target a wider range of proteins, thereby limiting their applicability if deeper mining of the serum proteome is desired.

Removing a select set of proteins at the same time requires a common property among the proteins. A survey of the properties of the abundant serum proteins reveals that most of them are acidic or low-pI proteins. Taking this into account, we envisioned the use of a polymer that could bind to the abundant proteins based on their isoelectric point (pI). A protein's pI

determines its net charge at a given solution pH; at pH 7, for instance, proteins with pI lower than 7 will be negatively charged. Thus, by virtue of electrostatic interaction, a cationic polymer will have affinity for low-pI proteins. Our group has recently utilized amphiphilic polymers for the selective enrichment of peptides based on their pI values to enhance their detection.^{20–23} We have previously demonstrated how these polymeric materials self-assemble into highly stable nanoassemblies in non-polar solvents^{24–26} and are capable of selectively enriching complementary peptides when used in biphasic extractions of complex mixtures.^{27–29} Herein, we describe investigations into the utility of these materials to extract whole proteins from serum. Using these supramolecular hosts for extracting proteins in this format has several advantages: (i) simple preparation of the nanoassemblies is achieved by mere self-assembly in a non-polar solvent; (ii) the assemblies are highly stable nanocontainers for extraction; (iii) the functional group on the polymer can be easily varied; (iv) selectivity is tunable by simply controlling the extraction pH; and (v) high extraction capacity can be achieved due to multivalent binding. We find that these polymeric assemblies selectively and efficiently deplete abundant acidic proteins from serum and demonstrate how this depletion can improve the detection sensitivity of a cancer biomarker.

EXPERIMENTAL

Materials and reagents.

Pooled normal human serum samples were obtained from Innovative Research (Novi, MI). Protein standards, including bovine serum albumin (BSA, pI 4.7), human serum albumin (HSA, pI 4.7), chicken egg lysozyme (LZ, pI 11), bovine cytochrome c (CC, pI 9.6), β -lactoglobulin (BLG, pI 5.1), bovine carbonic anhydrase (BCA, pI 6.2), human transferrin (TF, pI 6.8), as well as the peptide biomarker bradykinin (RPPGFSPFR, pI 12), were purchased from Sigma-Aldrich (St. Louis, MO). Sequencing-grade trypsin was acquired from Promega (Madison, WI).

Polymer synthesis and characterization.

Synthesis and characterization of the amphiphilic polymers are described in detail in the Supporting Information (SI).

Preparation of reverse micelles.

Reverse micelle-like nanoassemblies of the polymer were prepared by dissolving the appropriate amount of the solid polymer in a corresponding volume of toluene to make the intended concentration (*e.g.* 1 mg/mL or 2 mg/mL solution). The mixture was sonicated for at least 15 minutes to completely dissolve the solid, and 2 μ L of water was added to serve as the “water pool” interior of the assembly. The mixture was sonicated again for at least 15 minutes to yield a clear solution.

General extraction procedure.

An aqueous solution of the proteins (standard protein mixture or serum), buffered at the specified pH using either 100 mM Tris or 100 mM MOPS buffer, was mixed with the prepared polymer assemblies in toluene using a vortex mixer for 30 minutes. After extraction, the mixture is centrifuged at 12500 rpm at 15 °C for 60 minutes to separate the

aqueous from the toluene layer. The bottom aqueous phase is then carefully drawn out and transferred to another tube for sequential extraction or for subsequent analysis (SDS-PAGE or LC-MS/MS analysis). Sequential extraction is done by adding a fresh solution of the polymeric assemblies to the aqueous phase and repeating the extraction procedure. The extracted or bound proteins in the toluene phase can also be subsequently analyzed by transferring them first to an aqueous medium using a back-extraction strategy described previously.³⁰ Briefly, 200 μ L of tetrahydrofuran (THF) is added to the organic phase to disrupt the assemblies, followed by the addition of 200 μ L of 10% acetic acid (pH~2) to neutralize the electrostatic interaction between the polymer and the proteins. The mixture is placed on a vortex mixer for at least 30 minutes, followed by centrifugation at 3000 rpm for 60 minutes to separate the THF+polymer layer and the aqueous layer containing the back-extracted proteins.

Determination of extraction selectivity and capacity by intact protein LC-MS.

One-mL solutions containing a constant lysozyme concentration (5 μ M) and variable BSA concentrations (5, 10, 20, 40 μ M) were prepared in 100 mM MOPS buffered at pH 7.4, and extracted with the nanoassemblies as described in the general extraction procedure. Aliquots of each solution before and after depletion were analyzed by liquid chromatography (LC) on a reversed phase column (Phenomenex Jupiter® C4 column, 5 μ m, 300 Å, 150 mm $\times$ 1.00 mm) coupled to electrospray ionization mass spectrometry (ESI-MS) on a Bruker Esquire ion trap mass spectrometer to separate and measure the proteins in solution. Extraction selectivity and capacity were determined by comparing the LC-MS chromatogram for each protein before and after extraction. For determining the extraction capacity, a calibration curve for BSA was used to calculate the amount of BSA remaining in solution after extraction and consequently the amount of BSA extracted.

SDS-PAGE analysis.

Samples for SDS-PAGE (fractions from extraction of standard protein mixtures and serum samples) were first mixed 1:1 (v/v) with a 2X loading dye containing 3% dithiothreitol (DTT), and then heated at 95 °C for 10 minutes. Ten μ L of sample were loaded onto each well of the gel (4% polyacrylamide stacking gel, 10% or 12% polyacrylamide resolving gel). Electrophoretic runs were done at 150 V for 40–50 minutes on a 1X Tris/Glycine/SDS running buffer. Staining was done using Coomassie Brilliant Blue R-250 staining solution from Bio-Rad. The gels were imaged on a photo scanner and band intensities were quantified by *ImageJ* analysis using the Analyze -> Gel function.

Protein digestion and LC-MS/MS analysis.

In-gel trypsin digestion of protein bands from SDS-PAGE was done to identify the proteins by peptide mass fingerprinting, while in-solution digestion was performed on aqueous fractions (before and after depletion, and the bound protein fraction) for proteomic analysis. Protocols for both digestion procedures are detailed in the Supporting Information. Protein digests were then analyzed by LC-MS/MS on an Orbitrap Fusion™ mass spectrometer (Thermo Scientific) coupled to an Easy-nanoLC 1000 UHPLC equipped with a FortisBIO C18 nano-flow column (150 mm $\times$ 75 μ m, 1.7 μ m (Fortis Technologies Ltd.)). LC gradient and MS parameters are described in detail in the Supporting Information. Proteome

Discoverer v1.4 (Thermo Scientific) was used as the analysis software for peptide identification based on the MS/MS data. Sequest HT was used as the search engine for identifying peptides and proteins by searching against the FASTA sequence for the Uniprot human proteome database. To get the spectral counts for the selected proteins before and after depletion, the raw MS files were first converted to .mzML files. Using several search engines (X!Tandem, MS-GF+, OMSSA, and MyriMatch), peptides and proteins were identified by searching against the FASTA sequence for the Uniprot human proteome database. PeptideShaker³¹ (Compomics) integrates the results from multiple search engines and gives the value for the spectral counts (SC) for each identified protein, which were then compared for the serum samples before and after depletion (3 replicate samples).

SRM-MS analysis of spiked bradykinin in serum.

The peptide bradykinin with a sequence of RPPGFSPFR (MW=1060 g/mol) was spiked at different concentrations (1, 10, 100, 1000 nM) in serum. One set of spiked serum was left undepleted, while the other set was depleted by three sequential extractions using 400 μ L of 2 mg/mL solution of the positively charged polymeric nanoassemblies. The serum samples were then digested with trypsin. Targeted detection of bradykinin by selected reaction monitoring (SRM) on a Waters TQD triple quadrupole mass spectrometer was employed on the digested serum samples. LC separation of the digest on a Supelco® Discovery C18 (150mm $\times$ 2.1mm, 5 μ m (Sigma-Aldrich)) reversed phase column was done with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in ACN as mobile phase B under the following gradient: 0–5 min (5%B), 5–15 min (5–50%B), 15–20 min (50–95% B), 20–25 min (95% B), 25–25.1min (95–5% B), 25.1–30 min (5% B). The optimized SRM transition for the bradykinin peptide was determined to be the +2 charge for the precursor ion (m/z 530.8) and the y8 product ion (m/z 904.5). SRM for this transition was acquired from 8 to 15 minutes of the LC run with an optimized collision energy of 22 and cone voltage of 44 V. The resulting SRM chromatogram was analyzed and processed using the MassLynx software.

RESULTS AND DISCUSSION

Extraction selectivity.

Extraction of complex peptide mixtures using nanoassemblies of a positively charged amphiphilic polymer has been previously shown to enable the enrichment of acidic peptides into the organic phase, enhancing their subsequent detection.²⁷ The polymer used is a styrene-based homopolymer, **P-1** (Figure 1), functionalized with a quaternary ammonium group that makes it positively charged, and a hydrophobic decyl chain that balances the hydrophilicity of the charged group. This property allows the polymer to self-assemble into reverse micelle-like nanostructures upon dissolution in an apolar solvent. In these assemblies, the charged moieties form a hydrophilic core, while the hydrophobic groups are oriented on the outside (Figure 1). Selective binding and extraction of acidic, negatively charged peptides to the nanoassemblies of **P-1** are based on electrostatic interactions,²⁷ which in turn can be tuned by controlling the aqueous phase pH used in the extraction. Because albumin, the most abundant serum protein, has a low pI value, we postulated that the same polymer could be used to selectively bind the protein at a pH higher than its pI,

while leaving higher pI proteins behind. We tested this idea by extracting an aqueous mixture of bovine serum albumin (BSA, pI=4.7) and cytochrome c (CC, pI=9.6) at pH 7.3 using assemblies of **P-1** in toluene. Based on their respective LC-MS peaks before and after extraction, it is clear that the high-pI protein, cytochrome c, remains in aqueous solution after extraction, while BSA has been removed (Figure 2). This result confirms the ability of the polymeric assembly to remove proteins based on their pI.

We also investigated the selective protein extraction capability of a class of random copolymer-based assemblies that we reported recently,²⁶ as they offer synthetic versatility and simplicity as compared to homopolymers. Polymer **P-2** (Figure 1) was designed and synthesized with the same alkylammonium and decyl functional groups as homopolymer **P-1**. For the synthesis, two monomers were used and were polymerized by nitroxide-mediated radical polymerization (Scheme 1). In addition to synthetic ease, we found that polymer **P-2** could be solubilized in toluene at higher concentrations than polymer **P-1**, making it more useful for protein depletion.

Similar to **P-1**, the extraction selectivity of **P-2** was tested on a mixture of BSA and another high-pI protein, lysozyme (LZ, pI=11), and assemblies of this polymer show the same preferential removal of BSA from solution while retaining lysozyme after extraction (Figure S1). We further tested the extraction selectivity of **P-2** using an aqueous mixture of proteins with a range of pI values, including HSA (pI 4.7), BLG (pI 5.1), BCA (pI 6.2), TF (pI 6.8), CC (pI 9.6), and LZ (pI 11). Three sequential extractions using **P-2** were performed at pH 7.4 and aliquots from each fraction were run on an SDS-PAGE gel to visualize which proteins are depleted (Figure 3A). The acidic proteins HSA and BLG were effectively depleted by the polymer after one or two extractions. BCA and TF, which are slightly acidic, were also extracted by the polymer, but to a lesser extent. The inability to fully extract BCA and TF could be for three reasons. First, saturation of the nanoassemblies may have been reached. Second, the assemblies may have a preference for more highly acidic proteins, presumably due to a greater magnitude of negative charges that can bind to the positively charged polymer sites. Third, a small fraction of the BCA and TF molecules have a net neutral or positive charge at pH 7.4, thereby influencing their extraction. Interestingly, we see no decrease in the amount of the basic proteins, CC and LZ, demonstrating the pI-dependent selectivity of extraction. As negative controls, extractions using only toluene (Figure 3B) and using an amphiphilic random copolymer **P-3** (Figure 3C), which has charge-neutral polyethylene glycol (PEG) groups as the hydrophilic functionality (Scheme 1), were also conducted. The control experiment using only toluene for extraction shows that proteins are not extracted into the organic phase without the polymeric nanoassemblies, while the results for extraction using **P-3** prove that (1) the proteins do not get extracted when a charge-neutral amphiphilic polymer is used, and (2) the positively charged quaternary ammonium groups of **P-2** are indeed responsible for the extraction selectivity.

Extraction efficiency and capacity.

To assess the extraction efficiency and capacity of **P-2**, the optimum extraction time needed for depletion was first determined. By monitoring the amount of HSA remaining after various extraction times, we found that 30 minutes is needed to achieve efficient extraction

without affecting the selectivity (Figure S2). The extraction capacity for **P-2** was then determined based on how much albumin can be extracted by a given amount of polymer. BSA solutions (1 mL) buffered at pH 7.4 were prepared at varying concentrations and then were extracted using the polymeric nanoassemblies. The BSA concentration remaining in the aqueous phase after extraction was measured by LC-MS and was used to back-calculate the concentration of BSA that was extracted (Figure 4A). We initially used 200 μ L of a 1 mg/mL solution of **P-2** for extraction and the capacity was about 10 μ M. At a 2 mg/mL polymer concentration, the extraction capacity increased to about 20 μ M of BSA. To increase the capacity even more, we doubled the volume of polymer solution used for extraction to 400 μ L. This further improved the capacity to about 40 μ M BSA, or the equivalent of 3.3 mg serum albumin extracted per mg of polymer, which is comparable to, if not better than other depletion methods based on polymers.¹⁷ This relatively high extraction capacity can be attributed to the polycationic nature of the nanoassembly hosts, which allows for multiple binding sites with the protein. Our recent findings with peptide extractions reveal that increasing the charge density on the nanoassemblies increases their extraction capacity but only up to a certain point, after which the assemblies become unstable and lose some of their binding capacity.²⁵ It is possible that the optimum charge density has not yet been reached in this case and that the capacity may be increased even more by tuning the polymer structure (*i.e.* the ratio of charged moieties and the hydrophilic-lipophilic balance). Structure-property optimization is of great interest, but it is beyond the scope of the current work.

To determine the polymer's capacity to deplete acidic proteins in serum, we quantified the amount of proteins extracted from serum using the Bradford assay. A three-step depletion using 200 μ L of 2 mg/mL polymer was able to extract 57% of the total proteins, while 400 μ L of the same polymer concentration extracted 76% of the total proteins, which correlates well with the total proteins measured in the bound protein fraction (Figure 4B). A side-by-side comparison with an immuno-based depletion method reveals that a comparable depletion capacity (in terms of total proteins depleted) can be achieved by our polymer after a three-step extraction. It should be noted, however, that the sample loading capacity for the immunodepletion column is limited to 50 μ L of serum, whereas our method can allow for higher sample loading capacity.

Depletion of abundant acidic proteins in serum.

Upon demonstrating its extraction selectivity and capacity in protein mixtures, nanoassemblies of **P-2** were employed for depleting the abundant low-pI serum proteins. Because HSA is so high in concentration in serum (about 50 mg/mL), it is the main target protein for depletion and the first protein that we examined. To check if the pI-dependent selectivity still applies to serum extractions, several standard proteins were spiked into serum at high enough concentrations to be visualized on a gel. Serum samples were then subjected to sequential extractions and the proteins remaining after each extraction step were analyzed by SDS-PAGE. Figure 5 shows that the extraction selectivity holds true even in a complex protein sample like serum. HSA, being an acidic protein, is clearly being depleted based on its fading gel band intensity, while the basic proteins, CC and LZ, remain unchanged throughout 5 sequential extractions. Interestingly, while the selectivity remains

effective at both pH 6.0 and pH 7.4, we found that HSA is more efficiently depleted at pH 6.0 than at pH 7.4 (Figures 5B and 5C), presumably because fewer proteins get extracted along with HSA and compete for binding to the polymer at pH 6.0 than at pH 7.4.

Identification of serum proteins depleted.

It is apparent that HSA is not the only protein being depleted by the polymer in serum. To determine the identity of the major proteins that are being depleted, an SDS-PAGE gel was run for each fraction: serum before depletion, after each depletion step, and the bound protein fraction (Figure 6). Fractions from serum depleted using the anti-HSA/anti-IgG spin column depletion kit was also run side-by-side our polymer-based depletion method for comparison and as a positive control. The prominent bands were excised and subjected to in-gel tryptic digestion for protein identification by LC-MS peptide mass fingerprinting. The bands were identified based on their apparent MW on the gel and the unique peptides detected from their in-gel tryptic digest as summarized in Table 1.

The depleted proteins were found to be the abundant acidic proteins in serum, with pI values less than the extraction pH of 7.4, except perhaps for IgG whose pI is variable due to the presence of many different isoforms and glycosylation types. Still, some forms of IgG fall within the range of our predicted selectivity. This supports our hypothesis that the abundant low-pI proteins will be preferentially depleted from serum by the polymer. Interestingly, band 1, corresponding to α -2-macroglobulin, was not detected in any of the serum fractions but was prominent in the polymer-bound fraction. It is possible that additional proteins have bound to the polymer but were not detected by SDS-PAGE. Therefore, to get an idea of all the proteins that were depleted, a more in-depth analysis of the bound protein fraction was carried out by tryptic digestion followed by bottom-up proteomic analysis using LC-MS/MS. Listed in Table 2 are the proteins identified in the digest, with their calculated pI values. We see the same seven proteins identified in the gel, along with other proteins that have pI values less than or around 7.4 (except again for IgG as explained above), confirming the selectivity of extraction.

Effect of depletion on detection sensitivity.

Depletion of the highly abundant proteins reduces the extremely wide dynamic range of protein concentration in serum and hopefully improves the detection sensitivity for other proteins and peptides. To demonstrate the effect of our depletion strategy on the detection of other proteins, proteomic analysis of serum was done before and after depletion, and the abundances for a select set of proteins were compared. Spectral counting, a form of label-free LC-MS/MS relative quantitation, was used as a relative measure of protein abundance.^{32–34} A comparison of spectral counts of the top abundant serum proteins before and after depletion at pH 6.0 was made for three replicate serum samples, with LZ spiked as a control (Figure 7). After depletion, a decrease in abundance of low-pI proteins and an increase in abundance of high-pI proteins were observed. This observation is most evident for the spiked basic protein, LZ, suggesting that this depletion strategy could be particularly advantageous for enhancing the detection of higher-pI proteins.

The effect of abundant proteins on sensitivity is even more pronounced in the realm of biomarker detection. This effect is because biomarkers are typically present in ng/mL levels in serum and the abundant proteins typically overwhelm the signal to mask the detection of these low-level proteins. To further demonstrate the effect of our depletion strategy on sensitivity, a putative breast cancer peptide biomarker, bradykinin³⁵ (RPPGFSPFR, MW 1060 g/mol, pI 12) was spiked at varying known concentrations in serum. The abundant proteins were depleted using our method in one set, while the other set was left undepleted. Detection of the spiked bradykinin peptide in each sample was done by SRM measurements on a triple quadrupole mass spectrometer.

Bradykinin was undetectable in any of the spiked serum samples where depletion of abundant proteins was not done (Figure 8A). On the other hand, bradykinin was detectable down to 10 ng/mL in the spiked serum samples where our depletion strategy was employed prior to analysis (Figure 8B), corresponding to at least three orders of magnitude increase in sensitivity compared to the undepleted serum. This underscores the need for abundant protein depletion in trying to dig deeper into the serum proteome, especially for biomarker detection. Even a method as sensitive as SRM was not able to detect the high spike in the presence of these highly abundant interfering proteins. With the use of these polymeric nanomaterials, however, we were able to demonstrate a significant increase in detection sensitivity to levels that are of clinical utility.

SUMMARY AND CONCLUSIONS

In this work, we show that reverse micelle-like nanoassemblies of amphiphilic polymers serve as functional materials for selectively depleting low-pI proteins from complex mixtures. By functionalizing the polymer with positively charged quaternary ammonium groups, selective binding of negatively charged proteins is achieved. The extraction selectivity is predictable based on the extraction pH and the protein's pI value, and holds true even in extremely complex protein mixtures like serum. The extraction capacity can be maximized by increasing the polymer concentration and volume used for extraction, from which we were able to achieve comparable capacities to currently available methods. We demonstrated the application of this strategy to effectively deplete the abundant acidic proteins in serum, most especially HSA and other apolipoproteins, allowing for the enhanced detection of higher pI proteins. Furthermore, depletion of serum using our approach allowed for three orders of magnitude increase in detection sensitivity for a cancer biomarker. The utility of these materials for improving the analysis of serum and the detection of biomarkers offer a cheaper, tunable, and more stable alternative to costly antibody-based methods. The depletion strategy is currently most effective when the biomarker of interest in serum is a high-pI protein as there is low risk of protein loss during depletion using the positively charged polymer. To broaden the applicability of this method to other types of biomarkers, including low-pI ones, we will investigate incorporation of protein-specific ligands into the polymer to more specifically remove certain high abundance proteins in serum. Ligands with high affinity for HSA,^{36,37} for instance, could be incorporated onto the random copolymer design to specifically extract HSA, thereby simplifying serum and leaving behind other low-pI proteins. In recent work from our group using a comparable polymer design, we demonstrated such an approach is potentially

feasible as we were able to selectively transport proteins across an immiscible solvent interface using a protein-specific ligand.³⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of amphiphilic homopolymer **P-1** and random copolymer **P-2** and a cartoon representation of a positively charged reverse micelle-like nanoassembly encapsulating negatively charged proteins.

Figure 2.
Total ion chromatogram (TIC) from LC-MS of a mixture of 8 μ M each of bovine serum albumin (BSA) and cytochrome c (CC) before and after extraction using 1 mg/mL of **P-1** in toluene.

Figure 3.

SDS-PAGE of a protein mixture before extraction and after each extraction step using nanoassemblies of **P-2** (A), using toluene alone (B), and using nanoassemblies of **P-3** (C). One mL containing 0.25 mg/mL of each protein was extracted with 200 μ L of 2 mg/mL of either **P-2** or **P-3** in toluene or 200 μ L of toluene alone. Band intensities (normalized to the initial intensity) of each protein in each fraction/lane were measured by *ImageJ* and graphed accordingly as shown at the bottom of each gel image. pI values of each protein are indicated in parentheses. Proteins used: TF=transferrin, HSA=human serum albumin, BCA=bovine carbonic anhydrase, BLG= β -lactoglobulin, CC=cytochrome c, and LZ=lysozyme.

Figure 4.

(A) Determination of extraction capacity of **P-2**. Increasing concentrations of BSA (1 mL volume) were extracted with nanoassemblies of **P-2** with the indicated polymer concentration and volume. Deviation from the theoretical (100% extraction) line indicates that capacity has been reached as some BSA is left in solution. **(B)** Total protein content of serum (measured by the Bradford assay) before and after depletion using different methods. For all three methods, 50 μ L of serum is the loading volume. Polymer depletion was carried out using the indicated polymer volume and concentration at pH 7.4, the same pH as the equilibration buffer for the ProteoPrep® immunoaffinity depletion method. Percentages on top of each bar indicate the percent of total proteins depleted after each step.

Figure 5.

SDS-PAGE analysis of serum spiked with CC (A) and LZ (B and C) to show the pI-dependent selectivity of extraction in serum. Five sequential extractions using 400 μ L of 2 mg/mL of **P-2** were performed at pH 6.0 for (A) and (B), and at pH 7.4 for (C). Changes in protein band intensity after each depletion step (relative to before extraction) were measured using *ImageJ* software and illustrated in a graph below each gel image. pI of spiked proteins: CC (pI 9.6), LZ (pI 11).

Figure 6.

SDS-PAGE analysis of serum before and after depletion using the ProteoPrep® immunodepletion kit and by multi-step depletion using nanoassemblies of **P-2** at pH 7.4. Bound proteins from the ProteoPrep column were eluted according to the manufacturer's protocol. Bound proteins from the polymer depletion were back-extracted from the organic phase by the addition of THF and 10% acetic acid. Prominent bands that get depleted are numbered in red and were carefully excised for digestion and LC-MS analysis.

Figure 7.

Comparison of spectral counts (SC) of selected serum proteins before and after depletion (3 sequential extractions using 400 μ L of 2 mg/mL **P-2**). Normalized spectral counts account for the length of the protein by dividing its SC value by the number of amino acid residues. Calculated pI values of each protein are indicated in parentheses.

Figure 8.

SRM chromatograms for bradykinin in serum samples where no depletion was done **(A)**, and with depletion prior to analysis **(B)**. m/z 530.8 $\rightarrow$ 904.5 transition corresponds to the precursor ion (+2 charge state) and product ion (y_8 ion) of bradykinin, respectively, that was monitored in SRM.

Scheme 1. Synthesis of amphiphilic random copolymers **P-2** and **P-3**.

Table 1.

Protein identification of bands in SDS-PAGE gel of serum (from Figure 6).

Gel Band	1	2	3	4	5	6	7
Protein ID	α -2-macroglobulin	transferrin	serum albumin	IgG heavy chain	haptoglobin	IgG light chain	Apolipoprotein A-1
MW, kDa	163	77	69	52	45	25	28
pI	5.4	6.8	4.7	6–8.5 (variable)	6.1	6–8.5 (variable)	5.6
# Peptides belonging to protein	10	53	66	17	19	8	33
% Coverage	7.5%	57.3%	73.9%	45%	50.9%	46%	72.7%

Table 2.

Proteomic analysis of the polymer-bound fraction.

PROTEIN	pI	#Peptides identified	% Coverage	Protein Score (Proteome Discoverer)
Albumin	4.7	31	37.8	679.93
Apolipoprotein B-100	6.6	59	11.1	462.84
Complement C3	5.7	41	16.5	367.80
Apolipoprotein A-I	5.6	24	56.2	322.60
Apolipoprotein A-IV	5.3–5.4	28	44.2	306.21
Serotransferrin	6.8	20	19.5	253.73
Serpin peptidase inhibitor, clade A	5.6	19	29.4	224.24
Complement C4-A	6.7	24	11.9	205.05
Complement C4-B	7.3	24	12.2	204.38
Alpha-2-macroglobulin	5.4	22	13.8	185.35
Inter-alpha-trypsin inhibitor heavy chain H2	7.0	18	15.7	159.61
Inter-alpha (Globulin) inhibitor H4	6.9	11	11.6	111.50
Apolipoprotein A-II	4.6	7	51.9	104.15
Clusterin	6.3	11	18.3	101.24
Apolipoprotein E	5.7	11	45.0	100.72
Antithrombin-III	6.7	13	26.1	95.49
Ig gamma-1 chain C region	6–8.5	9	15.2	87.09
Inter-alpha-trypsin inhibitor heavy chain HI	7.6	6	8.1	77.44
Hemopexin	6.4	8	11.7	72.94
Haptoglobin	6.1	7	15.3	62.32
Apolipoprotein C-I	7.5	7	49.4	60.38
Heparin cofactor 2	6.9	9	15.2	55.11
Ig kappa chain C region	6–8.5	2	12.6	54.72
Protein IGKV3–11	6.0	2	12.5	51.43
Ig gamma-3 chain C region	6–8.5	5	11.4	50.66
Apolipoprotein C-III	4.7–5.1	1	16.2	49.54