HHS Public Access

Author manuscript

Anal Bioanal Chem. Author manuscript; available in PMC 2021 February 01.

Published in final edited form as:

Anal Bioanal Chem. 2020 February ; 412(5): 1027–1035. doi:10.1007/s00216-019-02342-8.

Polymeric Nanoassemblies for Enrichment and Detection of Peptides and Proteins in Human Breast Milk

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Abstract

Human breast milk is an understudied biological fluid that may be useful for early detection of breast cancer. Methods for enriching and detecting biomarkers in human breast milk, however, are not as well-developed as compared to other biological fluids. In this work, we demonstrate a new enrichment method based on polymeric nanoassemblies that is capable of enhancing the mass spectrometry-based detection of peptides and proteins in human breast milk. In this method, positively-charged nanoassemblies are used to selectively deplete abundant proteins in milk based on electrostatic interactions, which simplifies the mixture and enhances detection of positively-charged peptides and proteins. Negatively-charged nanoassemblies are used in a subsequent enrichment step to further enhance the detection and quantification of trace-level peptides and proteins. Together the depletion and enrichment steps allow model biomarkers to be detected at low nM levels, which are close to instrumental limits of detection. This new method not only demonstrates the ability to detect proteins in human breast milk but also provides an alternative approach for targeted protein detection in complex biological matrices.

Graphical Abstract

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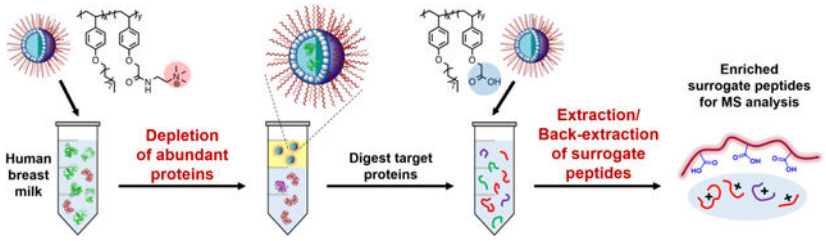
CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.



Keywords

breast milk; nanomaterials; mass spectrometry; protein enrichment; biomarkers

INTRODUCTION

Breast cancer remains a major health concern for women worldwide [1, 2], but early detection and effective treatments at an early stage of the disease can significantly extend the overall survival rates in patients [3]. Despite progress in conventional tools such as mammograms and breast biopsies, a diagnostic challenge still remains due to the lack of sensitivity and specificity of these strategies at the very earliest stages of the disease. Biomarkers, as measurable hallmarks of specific disease states, have been extensively investigated and validated for cancer detection and monitoring since they can (i) cover a broad range of biological species; (ii) provide risk assessment at the earliest stage, even at the premalignant stage; (iii) deal with the high heterogeneity of cancer subtypes; (iv) guide targeted therapies; and (v) help evaluate therapeutic responses [4–6]. Proteins are often excellent biomarkers because their levels of expression or activity directly impact cellular activities [7–9].

Various biological fluids, such as serum, nipple aspirate and breast milk, can be used as sources of breast cancer biomarkers [5, 10–12]. Among these, collection of breast milk is the least invasive, and it provides valuable information about breast tissue, especially in young women and for pregnancy-associated breast cancers [13–15]. Previous work has shown that both specific DNA methylation and differential expression of cancer-associated proteins can be identified in human breast milk (HBM) [16–19]. Driven by these observations, we have begun to investigate methods to enrich peptides and proteins from HBM as such methods could be used as part of an approach to detect breast cancer before the disease is clinically apparent.

To reliably detect and quantify protein biomarkers, various methods have been developed including enzyme linked immunosorbent assay (ELISA), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and proteomics based techniques using mass spectrometry (MS) [3, 20–24]. MS is one of the most promising methods due to its high specificity, high throughput and multiplexed detection capability. However, detecting and quantifying protein biomarkers using MS is a significant challenge due to the inherently low biomarker abundances (usually pg/mL) as well as the complex biological backgrounds. Thus, approaches that can reliably and efficiently simplify complex mixtures and enrich targeted molecules prior to MS measurements are needed.

Biomarker enrichment methods in HBM are understudied as compared to other biological fluids such as serum or urine. Most methods are based on affinity binding, where antibody- or affinity probe-based particles specifically capture and concentrate biomarkers of interest [25–27]. Antibody-based methods, however, have some drawbacks. Most notably, developing a new antibody is costly and time- and labor-intensive, and antibody enrichment techniques are typically limited in scope. As an alternative to these other methods, we have been investigating supramolecular nanomaterials for enrichment. Our group has developed an approach that uses polymeric supramolecular nanoassemblies to selectively enrich peptides or proteins via a biphasic extraction format, where electrostatic interactions are used for selective and predictable enrichment. Other polymeric materials for protein recognition and separation, such as protein imprinted polymers and functionalized polymeric membranes, have similarly been used [28, 29]. Our amphiphilic polymers are unique in that they self-assemble into reverse micelle-type nanoassemblies in apolar solvents and sequester targeted peptides or proteins based on their isoelectric points (pI) [30–32]. High enrichment selectivity and capacity can be easily achieved by tuning the functional groups on the polymers and varying the extraction conditions [33–35]. Moreover, these materials are easy to synthesize, low-cost, and highly stable as compared to current antibody-based enrichment reagents. Hence, we envision that these materials are capable of selectively enriching biomarkers in the complex matrix of HBM, thereby enhancing their detection using MS. In this work, we describe an analytical method based on these supramolecular nanoassemblies that can (i) selectively deplete abundant proteins in HBM; (ii) effectively enrich peptides and proteins of interest; and (iii) allow the sensitive quantitation of targeted molecules using MS. We predict that this enrichment method would assist the detection of established biomarkers or cancer-related proteins in HBM.

MATERIALS AND METHODS

Materials:

HBM was collected with Institutional Review Board approval at the University of Massachusetts Amherst from a 33 year-old participant nursing her second child. The participant completed a health history questionnaire at the time of milk donation and was asked to pump all the milk in each breast. Milk was stored at −20 degrees C until used for the current analysis. The peptide bradykinin (RPPGFSPFR) was purchased from Bachem (Torrance, CA). Bovine serum albumin (BSA) and cytochrome c, 3-morpholinopropanesulfonic acid (MOPS), urea, dithiothreitol (DTT), and iodoacetamide (IAM) were obtained from Sigma-Aldrich (St. Louis, MO). Tris(hydroxymethyl)aminomethane (Tris), acetic acid, toluene and tetrahydrofuran (THF) were obtained from Fisher Scientific (Fair Lawn, NJ). Deionized water was prepared from a Millipore (Burlington, MA) Simplicity 185 water purification system. Trypsin and Lys-C were purchased from Promega (Madison, WI).

Polymer synthesis and characterization:

Syntheses and characterizations of the random co-polymers were described previously [30, 32]. General details about the synthesis can be found in the Electronic Supplementary Material (ESM).

Collection of breast milk proteins:

We focused on secreted proteins in HBM. HBM samples were first centrifuged at 453 × g for 5 min at room temperature. The lipid layer was then removed from the top of mixture, while the cells were pelleted at the bottom of the centrifuge tube. The supernatant layer containing secreted proteins was collected. The centrifugation process was repeated several times until no further lipid layer or cell pellets were formed. Finally, the whey proteins were used for further experiments.

SDS-PAGE analysis:

HBM aliquots for SDS-PAGE were mixed 1:1 (v/v) with a 2× loading dye containing 3% DTT and then heated at 95 °C for 10 min. 10 μL of the sample was loaded into each well of the gel (4% polyacrylamide stacking gel, 12% polyacrylamide resolving gel). Electrophoretic runs were done at 150 V for 40–50 min in a Tris/glycine/SDS running buffer. Staining was done using a Coomassie Brilliant Blue R-250 solution from Bio-Rad. The gel images were obtained using a photo scanner and analyzed by ImageJ.

Preparation of polymeric nanoassemblies:

Amphiphilic random co-polymers were dispersed in toluene at a final concentration of 2 mg/mL. 1 μL of H_2O was added per 1 mL of the amphiphilic polymer solution to allow for the formation of a water pool inside the nanoassemblies. Sonication was performed until the solution became optically clear.

Depletion/Extraction procedure:

The general depletion/extraction and back-extraction workflows have been described elsewhere [30, 36], but in short, the following procedure was used. For the protein depletion step, HBM protein aliquots were diluted 5-fold with 100 mM MOPS buffer at a defined pH, which was a pH of 6.0 in this work. The buffered HBM protein sample (1 mL) was vigorously mixed with 400 μL of a nanoassembly solution in toluene for 1 h. After equilibration, the mixture was centrifuged at 12500 rpm for 30 min. Then, the aqueous phase was collected for sequential depletions or proteolytic digestions. For the back-extraction step, 200 μL of THF was mixed with the organic phase from the previous extraction step to disassemble the nanoassemblies, followed by the addition of 200 μL of a 10% acetic acid aqueous solution to release the extracted molecules back into an aqueous phase. The entire mixture was vortexed for 30 min, and then centrifuged at 10000 rpm for 1 h to separate the aqueous layer containing the molecules of interest.

Proteolytic digestion:

500 μL of a HBM protein sample (before or after depletion) were incubated with 2 M urea to denature the proteins. To reduce the disulfide bonds, the sample was incubated with 10 mM DTT for 30 min at room temperature. To alkylate the reduced cysteines, IAM was added at a DTT: IAM molar ratio of 1:4, and the sample was incubated in the dark for 30 min at room temperature. After alkylation, the sample was diluted with 200 mM Tris buffer to reduce the urea concentration to 1 M as well as adjust the pH of solution to 8.0. Protein samples were then digested with 10 μg of trypsin and 0.5 μg of Lys-C. After 24 h of digestion at 37 °C, the

enzyme was separated from the mixture by centrifugation using a 10K NMWL Microcon filter (Millipore, Burlington, MA). The filtrate was then directly analyzed by LC-MS/MS or extracted by nanoassembly solutions for targeted peptide enrichment.

LC-MS analysis:

Protein digests were separated on a Thermo Scientific EASY-nLC 1000 liquid chromatography system (Thermo Scientific, Tewksbury, MA) with an Acclaim PepMap RSLC C18 reverse phase column (75 μm × 15 cm, 2 μm particle size) from Thermo Scientific (Tewksbury, MA). To achieve efficient separation of the proteolytic peptides, a shallow gradient was used where % B (0.1% formic acid in acetonitrile) was increased from 0% to 50% over 90 min. The column was then flushed by increasing to 95% B over 5 min. The column was then cleaned at 95% B for another 20 min. A flow rate of 300 nL/min was used throughout the run. The LC system was coupled with a Thermo Orbitrap Fusion Tribrid (Tewksbury, MA) mass spectrometer. The electrospray ionization source was typically operated at a needle voltage of 2100 V, and the ion transfer tube temperature was set to 300 °C. Tandem mass spectra were collected using CID with a normalized collision energy of 35%. Due to the large number of detectable peaks, an exclusion limit of 60 s was applied after 3 spectra had been collected for any given peak within 15 s. The resolution of the Orbitrap was set to 60000.

Protein identification and quantification:

Raw mass spectral data files were analyzed by Thermo Proteome Discoverer 2.2 software. Spectra were searched against the human proteome database using Sequest HT as the search engine. Variable modifications such as oxidation of methionine and carboxyamidomethylation of cysteine were used in the searches. Trypsin enzyme cleavage was selected, and a precursor mass tolerance of 10 ppm was used. Peptides were identified at high confidence levels based on MS/MS data. To perform the label free quantification of specific proteins before and after depletion, spectral counts were used as an indicator. Four different search engines (X! Tandem, MS-GF+, OMSSA, and MyriMatch) were used to identify the HBM proteins and their corresponding peptides. PeptideShaker [37] (CompOmics) was used to integrate the results from different search engines and export the spectral counts value for label-free quantification of each identified protein.

Multiple reaction monitoring:

Targeted peptides were detected and quantified by multiple reaction monitoring (MRM) on a Waters TQD triple quadrupole mass spectrometer coupled with Waters ACQUITY UPLC system with a Sigma-Aldrich Supelco Discovery C18 reverse phase column (150 mm × 2.1 mm, 5 μm particle size). A gradient was used where % B (0.1% formic acid in acetonitrile) was increased from 5% to 31.5% over 35 min. The column was then flushed by increasing to 95% B over 5 min. The column was then cleaned at 95% B for another 10 min. Finally, the gradient was adjusted back to 5% B over 5 min. A flow rate of 0.2 mL/min was used throughout the run. The optimized MRM parameters are listed in the (ESM Table S1). The MRM data were analyzed by MassLynx software. Peak heights of the target peptides, as measured in MRM, were used to estimate the limit of detection (LOD) based on signal-to-

noise (S/N) ratio of response. The LOD is taken as the analyte concentration corresponding to a S/N ratio that is 3 times the blank S/N ratio.

RESULTS AND DISCUSSION

To design a method for protein quantification in HBM, we first investigated the total protein concentration and the protein composition of the given HBM sample because HBM composition can vary depending on the lactation stage [38–43]. Bradford assays were performed to determine the total protein concentration of each sample (e.g. ESM Fig. S1), and this information was used to choose the amount of the nanoassemblies for depletion of the abundant proteins. The HBM sample was also analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the most abundant protein species in the sample before (ESM Fig. S2) and after depletion. Three of the four most abundant proteins, including serum albumin, caseins, and lactalbumin have low pI values, so they are negatively charged at physiological pH. Therefore, we used nanoassemblies with positively-charged interiors to selectively remove these abundant proteins based on electrostatic interactions, allowing the complex mixture to be simplified for improved detection sensitivity of the target molecules.

A multi-step procedure that included this depletion step was explored as a means of enriching target proteins for detection and quantification by mass spectrometry (Figure 1). After removing the lipids and cells from the HBM sample and adjusting the pH to 6, as described in the experimental section, proteins having pI values < 6 were depleted by nanoassemblies of polymer **P1**, whereas positively charged proteins, including the target proteins, remained in the aqueous phase. The proteins remaining in the aqueous phase were digested at pH 8, and then nanoassemblies of the negatively charged polymer **P2** were used to selectively enrich positively charged peptides into the organic phase. The enriched peptides were released from the organic phase into fresh aqueous phase using THF and acid as described previously [36]. Finally, the enriched and back-extracted peptides were quantified by LC-MS based MRM measurements on a triple quadrupole mass spectrometer.

To identify an efficient protocol for depletion of the abundant negatively-charged proteins, we tested different extents of dilution, numbers of depletion steps, and concentrations and volumes of the nanoassemblies. We found that two sequential depletions of 1 mL of 5-fold diluted HBM (i.e. ~ 2 mg/mL total protein) at pH 6.0 using 400 μL of 2 mg/mL of polymer **P1** provide reliable conditions that are capable of efficiently removing abundant low-pI proteins. As an example, a 5-fold dilution of HBM leads to more effective removal of human serum albumin (pI 4.7), caseins (pI 4.7) and lactalbumin (pI 4.5) after three depletion steps (Figure 2, lanes 6 – 9) than a two-fold dilution (Figure 2, lanes 2 – 5). Different from other abundant proteins, Lactroferrin (pI 8.7), as a high-pI protein with a positive charge, does not decrease under the same conditions, confirming that the depletion step is selective for low pI proteins. Moreover, this observation suggests that the positively charged nanoassemblies of polymer **P1** are not likely to remove other positively charged proteins including target proteins.

To test this idea further, we digested the proteins in the HBM sample before and after depletion and used spectral counts (SC) from LC-MS measurements as a label-free quantification method to assess the relative abundance of proteins in the diluted HBM aliquots before and after depletion. The data for a subset of the detected proteins, with pI values ranging from about 3 to 10, are shown in Figure 3. After depletion with nanoassemblies of **P1**, we observe a decrease in SC for almost all the negatively charged proteins (pI < 6), indicating that these proteins are effectively depleted (Figure 3a). In contrast, almost all of the positively charged proteins (pI > 6) show enhanced SC (Figure 3b) likely because of the reduction of interfering signals from the more abundant proteins that initially suppressed the signal of low-abundance proteins. One protein that is now detectable that was not before depletion is cytochrome c, which was spiked into the HBM at a concentration of 1 μM.

While depleting the abundant HBM proteins can improve the detectability of low abundance proteins, further improving the detectability of trace-level proteins and peptides can be achieved via a subsequent enrichment step. For the purposes of this study, we focused on the enrichment of positively-charged proteins using nanoassemblies of polymer **P2**. Surrogate peptides from the target protein can be extracted into the organic phase based on electrostatic interactions that simplify the mixture, which also concentrates them because of the smaller volume of the organic solvent. Then, an efficient back-extraction step can be used to release the enriched peptides for quantitation by MRM in an LC -MS experiment.

To test the effectiveness of this approach, we spiked two molecules, bradykinin and cytochrome c, into HBM, using the surrogate peptide TGPNLHGLFGR for detection of cytochrome c. In aqueous solutions, the limit of detection (LOD) for both peptides is approximately 6 nM, whereas neither is detectable when spiked into HBM at μM concentrations due to the complexity of the sample. If the abundant HBM proteins are depleted with nanoassemblies of polymer **P1**, and the subsequently digested samples are enriched by nanoassemblies of polymer **P2**, bradykinin and cytochrome c can be detected in HBM at concentrations of 10 nM and 7 nM, respectively, which is close to the instrument LOD for these biomolecules (Fig. 4 and ESM Fig. S4). To investigate the contribution of each sample preparation step to the detection sensitivity, LODs with and without depletion and enrichment steps were determined (Table 1). The data in Table 1 indicate that the depletion step lowers the LOD more extensively than the enrichment step, but evidently the depletion and enrichment steps work synergistically to lower the LOD close to the instrument LOD. The ability to both remove interfering highly abundant low pI proteins, together with selective enrichment of a subset of high pI proteins evidently simplifies the mixture sufficiently to facilitate detection of the high pI biomolecules of interest, which are cytochrome c and bradykinin in this case. The two-step extraction protocol described here is an encouraging proof-of-concept for detecting higher pI molecules in HBM that is relatively unique compared to typical biological fluids used for biomarker detection. We predict that the methods developed in this work could achieve even lower LODs when coupled with more sensitive instrumentation.

Conclusion

In summary, we have described a new method using supramolecular nanoassemblies to enrich and quantify peptides and proteins in HBM, opening up the possibility of targeted detection of trace-level proteins in HBM, an understudied biological fluid. Abundant proteins in HBM can be efficiently depleted using positively charged nanoassemblies to remove low pI proteins, thereby simplifying HBM samples and enhancing detection of low-abundance positively charged proteins. Selective extraction/back-extraction enables further enrichment and sensitive detection of peptides and surrogate peptides from target proteins. Overall, this method allows for at least over 2 orders of magnitude improvement in detection sensitivity for target proteins in HBM. When combined with MRM in LC-MS experiments, the described method provides advantageous quantification capability, offering an alternative approach to antibody-based methods for assessment of proteins in HBM. The polymeric nanoassemblies used in this work also offer other features such as tunable structures, low-cost and high stability, allowing them to have great potential for biomarker and other biomolecules detection in complex mixtures.

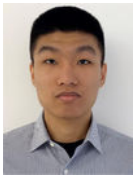
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health grant R01 CA 169140. M.A.C. Serrano was partially supported through the UMass Chemistry-Biology Interface (CBI) Fellowship Program (NIH NRSA T32 GM008515).

Biographies



Bo Zhao received his Ph.D. degree in chemistry from University of Massachusetts Amherst under the guidance of Prof. Richard Vachet and Prof. S. Thayumanavan. He is currently a senior scientist in the Protein Analytics Department at AbbVie Bioresearch Center in Worcester, MA. His research is focused on detection of proteins in biological mixtures and characterization of protein structures using mass spectrometry.

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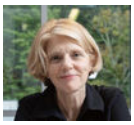
protein and amphiphiles based on supramolecular assemblies through covalent or noncovalent approaches for biomedical applications.

Mahalia Serrano, M.A.C., obtained her PhD in chemistry from the University of Massachusetts Amherst, with interests in utilizing functional materials for sensitive detection of biomarkers and method development in mass spectrometry. Currently, she is a scientist at the Mass Spectrometry Center of Excellence under Biologics Development at Bristol-Myers Squibb.



Kathleen Arcaro is a Professor of environmental toxicology and cancer biology at the University of Massachusetts Amherst. She studies breastmilk to understand how breast cancer develops and how to prevent it. Her ongoing research includes a study of DNA methylation and somatic mutations in sloughed epithelial cells from the milk of women with a germline mutation in the *BRCA* gene, and a clinical trial assessing the effects of a diet rich in fruits and vegetable on biomarkers of breast cancer risk in breastmilk. http://www.breastmilkresearch.org

S. “Thai” Thayumanavan is a Distinguished Professor of Chemistry at the University of Massachusetts Amherst. He is also the Scientific Director of the Center for Bioactive Delivery at the Institute for Applied Life Sciences at the University. His research work involves the design and syntheses of new macromolecules, especially polymers, to obtain novel responsive supramolecular nanoassemblies. These responsive nanoassemblies are being pursued for applications in the delivery of small molecules and biologics, in addition to imaging, sensing, and diagnostics applications.

Richard W. Vachet is a Professor of Chemistry at the University of Massachusetts Amherst. His research interests include the study of protein aggregation, the use of nanomaterials as

novel enrichment methods, the imaging of nanomaterials in biological and environmental systems.

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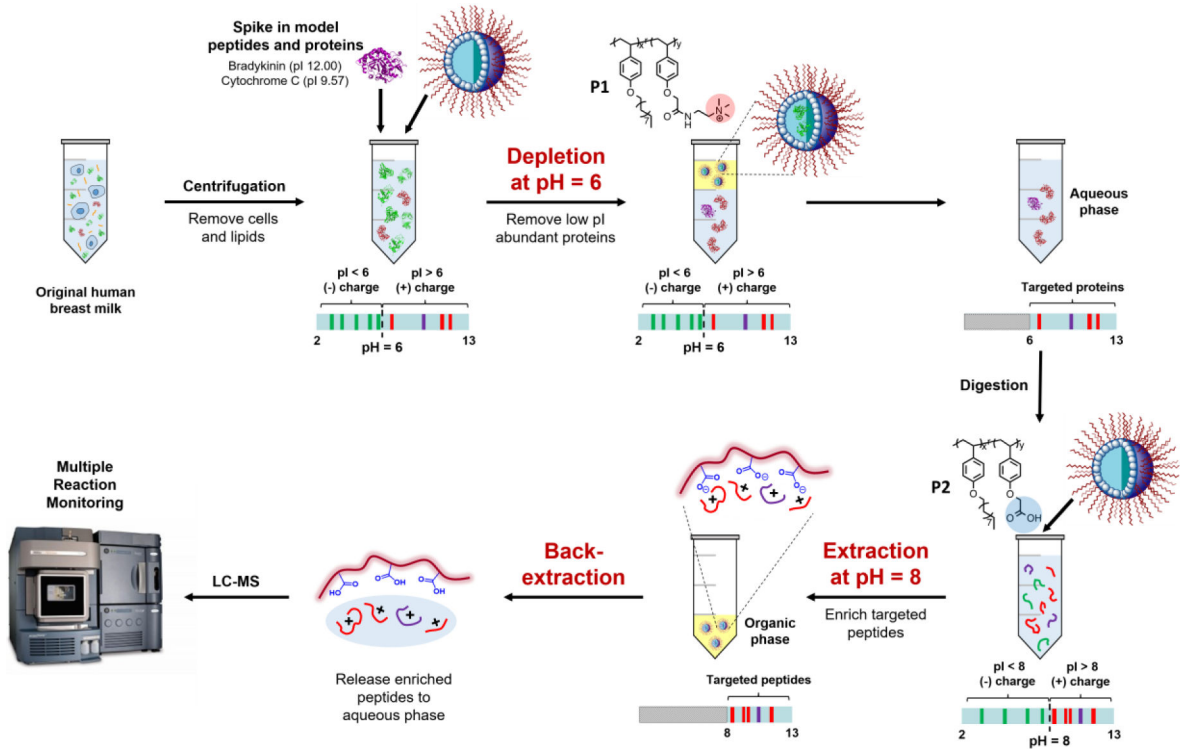

Fig. 1.

Schematic representation of the workflow for quantifying targeted peptides/proteins in HBM based on sequential extractions using polymeric supramolecular assemblies. Peptides of interest are shown in purple.

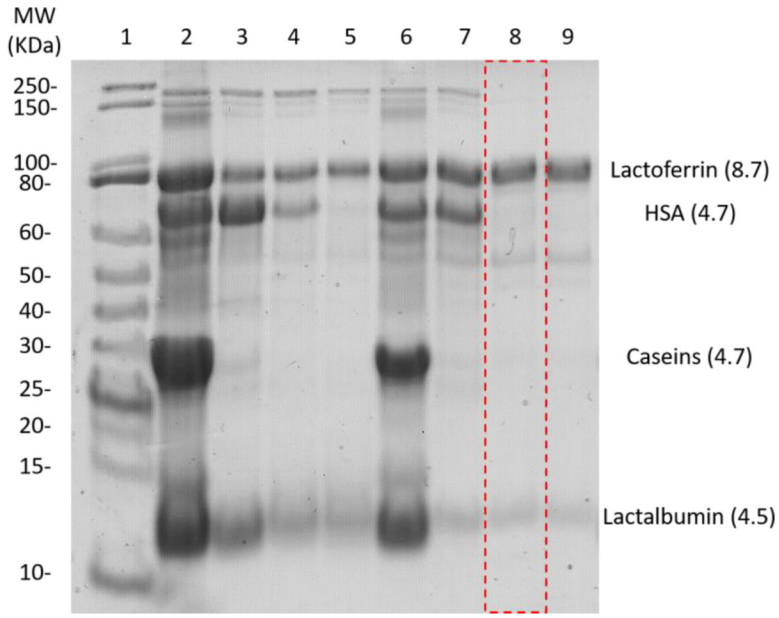

Fig. 2.

SDS-PAGE analysis of HBM before and after depletions using positively charged polymer **P1**. Lane 1: Molecular weight marker; Lane 2: HBM after a two-fold dilution; Lanes 3–5: Two-fold diluted HBM after 1, 2, and 3 depletion steps, respectively; Lane 6: HBM after a five-fold dilution; Lanes 7–9: Five-fold diluted HBM after 1, 2, and 3 depletion steps, respectively. The red dashed rectangle indicates the results from the optimal depletion conditions.

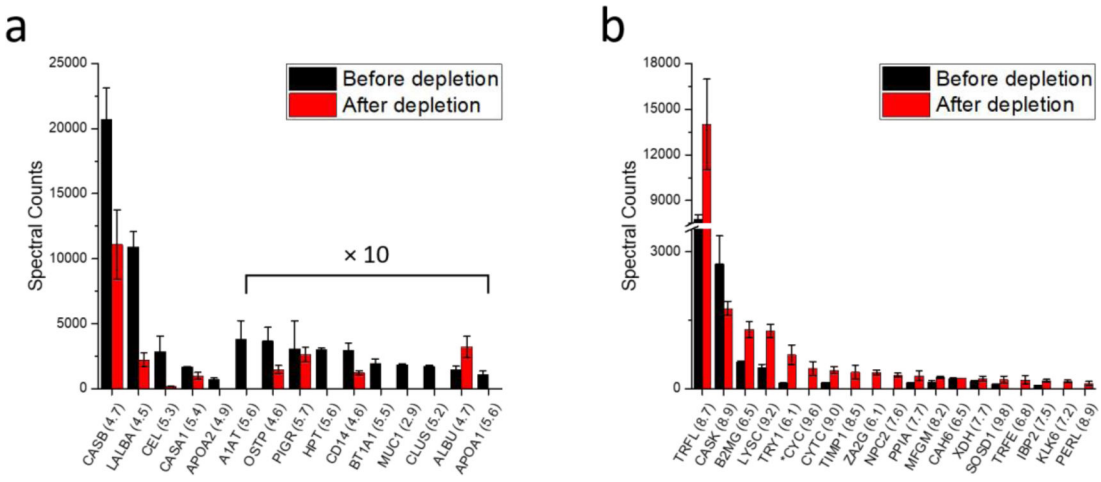

Fig. 3.

Comparison of the relative abundance of a subset of the detected proteins in HBM before and after depletion using the optimal depletion conditions identified in Figure 2. The relative abundance of (a) selected negatively charged proteins (pI < 6) and (b) selected positively charged proteins (pI > 6) was assessed by spectral counts as described in the experimental section. The identities of the proteins in both (a) and (b) are indicated in Table S2.

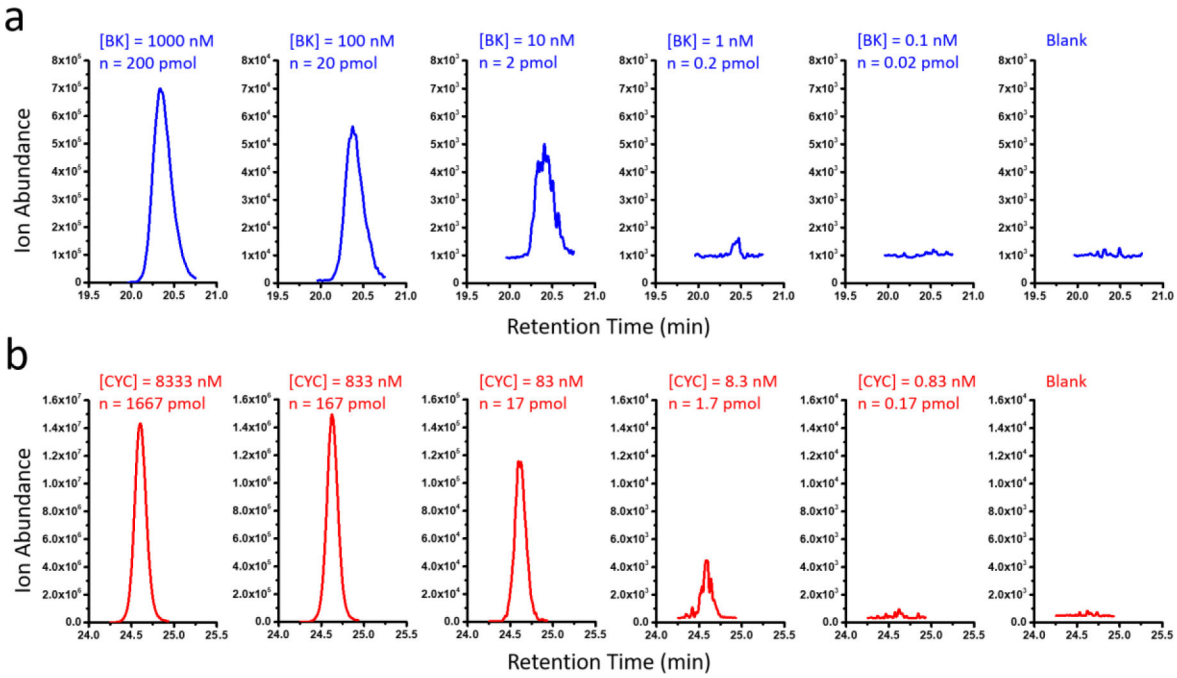

Figure 4.

Example MRM chromatograms of (a) RPPGFSPFR and (b) TGPNLHGLFGR in HBM after depletions and extraction/back-extraction. (The mole quantity (n) that is indicated represents the amount of bradykinin (BK) and cytochrome c (CYC) that was spiked in HBM before depletion.)

Table 1

MRM LOD of peptides RPPGFSPFR and TGPNLHGLFGR (from cytochrome c) in HBM using different sample preparation conditions. (Note that the first entry represents the LODs of pure peptides.)

Sample preparation			Limit of detection (nM)	
Breast milk	**Depletion**	**Extraction/Back-extraction**	**RPPGFSPFR (pl 12.0)**	**TGPNLHGLFGR (pl 9.4)**
−	−	−	6 ± 4	6 ± 6
+	−	−	> 1000	> 800
+	−	+	> 1000	200 ± 70
+	+	−	50 ± 20	30 ± 3
+	+	+	10 ± 3	7 ± 2