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Quantitative proteomic analysis for evaluating affinity isolation of extracellular vesicles

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ABSTRACT

Absolute quantification with mass spectrometry and isotope labeled internal standards has found broad applications in biomedical research. In the present research, it was used for developing and evaluating a new affinity-based approach to isolate extracellular vesicles (EVs) from human plasma. First, a phage display peptide library was screened against EVs as a bait and absolute quantification of multiple proteins helped to select the best bait available. Then, absolute quantification was used to evaluate the efficiency of affinity chromatography on peptide-Sepharose. In summary, we have demonstrated that peptides with affinity to EVs selected from phage library screening can be valuable ligands for EVs isolation.

Significance: Extracellular vesicles (EVs) have an important role in intercellular communication for all cell types. This makes EVs a promising new type of therapeutics capable to deliver drugs to specific sites with no off-target side effects. However, their isolation, and correct assignment of their biological function and properties remains an obscure field of research. In this study, we proposed to use MRM quantitation of a pattern of EVs and non-EVs proteins to develop a purification protocol based on affinity peptides selected from phage library screening. MRM quantification of EVs proteins can also help in identifying those that are subpopulation specific markers for further target-specific isolation.

1. Introduction

Extracellular vesicles (EVs) released by cells are nano-sized vesicles that play a role in intercellular communication, have promising potential as diagnostic markers of disease conditions, and are considered as unique site-specific drug delivery systems without off-target side effects [1–4]. Detailed studies of their properties require well-purified EVs samples, which remains a challenge because of the low-abundance and high heterogeneity of EVs subpopulations. Traditional isolation techniques such as precipitation, differential ultracentrifugation and size exclusion chromatography (SEC) [5–7], result in partially purified EVs preparations and can only be used as initial steps of isolation.

In addition to traditional isolation techniques, various affinity-based isolation methods for EVs have been proposed. There are multiple reports of successful isolation of different subpopulations of EVs based on immunocapture techniques [reviewed in 6, 7]. However, immuno-based isolations include an elution step with glycine buffer at pH 2.2 that might alter the properties of isolated EVs. Apart from immunocapture techniques, other EV-binding molecules have been used to isolate EVs with a non-destructive release of bound EVs. The list of these EV-binding molecules includes aptamers, lectins, heparin, Tim4 proteins targeting exposed phosphatidylserine, TiO_2 resin targeting phosphopeptides, and vinceremin (V_n), a specific class of peptides targeting heat shock proteins [6–8].

In the present study, we capitalized on idea of affinity peptides to isolate EVs and propose the use of a phage display method for their selection. Assay development included using partially purified EVs as bait for screening of a phage display peptide library and identifying those peptides with random sequence which can recognize EVs. Once identified, the selected peptides could be used for developing EVs isolation.

There are two critical steps in the proposed approach: i) selection of the purest possible EVs preparation to be used as a bait for peptide library screening, and ii) quantitative evaluation of the protocol of EVs

Abbreviations: α-2-MG, alpha-2-macroglobulin; EV, extracellular vesicle; MRM, multiple reaction monitoring; QconCATs, quantification concatamer; SEC, size exclusion chromatography.

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isolation. Both these goals can be achieved using multiple reaction monitoring (MRM) assay [9,10], also called selected reaction monitoring (SRM) assay [11,12].

2. Materials and methods

2.1. Preparation of EVs for phage display screening

An aliquot (20 mL) of pooled male plasma K2EDTA (BioreclamationIVT, Westbury, NY) was centrifuged at 2000 x g_n for 10 min and the supernatant was subjected to 12,000 x g_n centrifugation for 10 min. The pellet was discarded and the supernatant was further centrifuged at 106000 x g_n for 60 min using a Beckman TLA-55 rotor and TL-100 ultracentrifuge. The pellet was dissolved in 1.0 mL of PBS and centrifuged again at 106000 x g_n for 60 min. The PBS washed pellet (abbreviated as 106 K-EVs) was re-dissolved in 0.1 mL of PBS. The 106 K EVs was then loaded on a Superdex 200 Increase 10/300 GL column equilibrated in PBS. For size exclusion chromatography (SEC), an AKTA FPLC (Amersham Biosciences, Piscataway, NJ, USA) was used. The flow rate was 0.4 mL/min and 0.5 mL fractions were collected. Void volume fractions (A5, A6, A7, and A8) were used for the MRM assay and the A5 fraction was designated as the best EVs fraction for phage display screening (see section 3.1).

2.2. Phage display screening

The Ph.D.-12 phage display peptide library kit was from New England BioLabs (Ipswich, MA, USA), catalog # E8110S. The M13 phage was propagated in *E. coli* host strain ER2738 (included in the kit) in Luria-Bertani (LB) medium containing 20 μg/mL tetracycline (Sigma-Aldrich, St. Louis, MO).

For phage panning, the A5 fraction was added in several wells of a 96-well plate and incubated at 4 °C overnight. PBS was used instead of the A5 fraction as a control sample. All subsequent steps were carried out with gentle orbital shaking at room temperature. First, wells were washed with 200 μL of PBS for 10 min and PBS was replaced with 200 μL of 5 mg/mL BSA/PBS for 2 h. Then, each well was loaded with 150 μL of 15-fold diluted Ph.D.-12 phage display peptide library in 5 mg/mL BSA/PBS. After 2 h of incubation, each well was washed 5 times with 200 μL of 0.01% Tween 20/PBS for 10 min each washing. Finally, the bound phage was eluted with 150 μL of 1 mg/mL BSA/0.2 mol/L glycine-HCl (pH 2.2) for exactly 10 min and transferred to clean autoclaved conical tubes containing 22 μL of 1 mol/L Tris-HCl (pH 9.1).

For phage amplification, 1 μL of ER2738 *E. coli* cells (stock solution is included in the Ph.D-12 library kit) was added to 3 mL of LB/tetracycline medium. Cells were grown overnight at 250 rpm shaking at 37 °C. The next morning, 200 μL of overnight culture and 100 μL of eluted/neutralized phage were added to 20 mL of LB/tetracycline medium and incubated for exactly 4.5 h at 37 °C with 250 rpm shaking. Then, the cell culture was centrifuged at 12000 x g_n for 10 min at 4 °C. Supernatant was transferred to a new tube and centrifuged again at 12000 x g_n for 10 min at 4 °C. After the second centrifugation, the supernatant was transferred to a new tube and treated with 1:6 volume of 20% PEG/2.5 mol/L NaCl overnight at 4 °C. The next morning, the precipitated phage was pulled down by centrifugation at 12000 x g_n for 20 min at 4 °C and re-suspended in 0.5 mL of PBS. This amplified phage was then ready for DNA purification.

2.3. Phage DNA purification

Amplified phage (150 μL) was mixed with 25 μL of 20% PEG/2.5 mol/L NaCl, incubated at room temperature for 30 min and centrifuged at 12000 x g_n for 20 min at 4 °C. The pellet was re-suspended in 100 μL of 10 mmol/L Tris-HCl (pH 8.0)/1 mmol/L EDTA/4 mol/L sodium iodide, mixed with 250 μL of ice-cold ethanol and incubated at room temperature for 30 min. After incubation, the mixture was centrifuged at 12000 x g_n for 10 min at 4 °C. The pellet was re-suspended in 200 μL of ice-cold 70% (*v*/v) ethanol and centrifuged at 12000 x g_n for 10 min at 4 °C. The final pellet was re-suspended in 20 μL of 10 mmol/L Tris-HCl (pH 8.0)/1 mmol/L EDTA. To further purify phage DNA, DNA Clean & Concentrator-5 kit (Zymo Research, catalog # D4003) was used with minor modifications to the manufacturer recommended protocol. Essentially, 20 μL of phage DNA were mixed with 140 μL of provided DNA Binding Buffer and loaded on a Zymo-Spin column in a collection tube. After centrifugation at 12,000 x g_n for 30 s, the column was washed twice with 200 μL of provided DNA Wash Buffer by 12,000 x g_n for 30 s centrifugations. Finally, the column was transferred into a new 1.5 mL microcentrifuge tube and phage DNA was eluted with 30 μL of provided DNA Elution Buffer by 12,000 x g_n for 30 s centrifugation. At this stage, the phage DNA had an A260/A280 ratio equal to 1.9 to 2.0 and was ready for sequencing.

2.4. Next generation sequencing

We used the Genohub website (https://genohub.com/) to identify a next generation sequencing (NGS) service. The submitted samples of phage DNA were subjected to two step PCR amplifications using nested primers followed by adaptor addition. Quality control of the final pooled library was performed using Qubit, Agilent 2100 Bioanalyzer and qPCR. Sequencing was completed on an Illumina MiSeq 300 cycle flow cell. Read length was 2 × 150 bp PE. The FastQ was analyzed by merging left and right reads using FLASH and counted for unique DNA sequences. The insert sequences were translated to amino acid sequence using EMBOSS Transseq tool. For various samples, the delivered number of unique peptide sequences with their frequency was in a range of 70,000 to 130,000. Finally, peptide sequences found in the control sample (only PBS) were subtracted from peptide sequences found in the A5 fraction sample.

2.5. Affinity isolation of plasma EVs

To generate an affinity resin for EVs purification, the peptides selected after NGS sequencing were synthetized with Lys extension at the C-terminus (Biomatik USA, Wilmington, DE, USA) and immobilized on CNBr-activated Sepharose. 0.5 g of CNBr-activated Sepharose 4B (GE Healthcare, Uppsala, Sweden) was washed with 150 mL of 1 mmol/L HCl on glass filter and then quickly equilibrated with 100 mmol/L $NaHCO_3$. The Sepharose was immediately transferred to 5 mL of 100 mmol/L $NaHCO_3$ containing 20 mg of selected peptide and incubated with orbital shaking overnight at room temperature. The next morning, the OD_{280} reading indicated that all 20 mg of peptide was immobilized, which provided approximately 12 mg of peptide/mL of Sepharose density for the final peptide-Sepharose. Peptide-Sepharose was washed with 50 mmol/L TrisHCl (pH 8.0) and kept at 4 °C.

The workflow diagram for EVs isolation is shown in Fig. S1 (Supplementary Material). The isolation of EVs from 200 mL of pooled male plasma K2EDTA (BioreclamationIVT, Westbury, NY, USA) started with 2000 x g_n centrifugation for 20 min at 4 °C. The supernatant was further centrifuged at 106000 x g_n for 60 min at 4 °C using a Beckman 70Ti rotor and XL-90 ultracentrifuge. The pellet was resuspended in 300 μL of PBS and called 106 K-EVs. An aliquot (200 μL) of 106 K-EVs was used for further purification by SEC on a Superdex 200 Increase 10/300 GL column in 10 mmol/L phosphate buffer (pH 7.4). The flow rate was 0.4 mL/min and 0.5 mL fractions were collected. Void volume fractions were combined and called SEC-EVs. SEC-EVs were used for total protein measurement and MRM assay. Two mL of SEC-EVs were used for affinity purification of EVs on peptide-Sepharose. SEC-EVs were loaded on a 1 mL column of peptide-Sepharose that was equilibrated with 10 mmol/L phosphate buffer (pH 7.4). The column was washed with 10 mmol/L phosphate buffer (pH 7.4)/150 mmol/L NaCl and eluted with 10 mmol/L phosphate buffer (pH 7.4)/400 mmol/L NaCl. The elution fraction was called peptide-EVs and used for total protein measurement and MRM

assay.

2.6. Dynamic light scattering

Dynamic light-scattering (DLS) measurements were performed for SEC-EVs and peptide EVs samples using a Malvern Zetasizer Nano series, ZEN3600 and analyzed using Malvern Zetasizer 7.10 software (Malvern Instruments Ltd., Worcestershire, UK). All samples were analyzed in triplicate.

2.7. ^{15}N-labeled internal standards for quantitative proteomic analysis

The design, expression, purification, and characterization of ^{15}N-labeled EXO1, EXO2, LP1, LP2, GP1 and GP2 quantitative concatamers (QconCATs) have been previously described in detail [13–15]. These QconCATs were designed for a broad EV analysis and carry the ability to quantify a total of 44 proteins. However not all of them can be quantified in every specific MRM assay. For those proteins that were quantified in this study, three optimal MRM transitions per each Q-peptide are shown in Table S1 (Supplementary Material).

2.8. Sample processing for mass spectrometry

The protein samples in 50 mmol/L NH_4HCO_3 were supplemented with 10 mmol/L dithiothreitol and ^{15}N-labeled EXO1, EXO2, LP1, LP2, GP1 and GP2 QconCATs (from 1 to 5 pmol each). After incubation for 60 min at room temperature, samples were treated with 30 mmol/L iodoacetamide for another 60 min in the dark and precipitated with chloroform/methanol [16]. The pellets obtained from precipitation were sonicated in 100 μL of 50 mmol/L NH_4HCO_3/0.1% RapiGest and treated with trypsin (1:5 *w*/w) for 15 h at 37 °C. After trypsinolysis, the samples were acidified with 0.5% trifluoroacetic acid for 30 min at 37 °C and centrifuged at 16000 x g_n for 30 min to collect the supernatant. Finally, samples were dried in a vacuum centrifuge (Eppendorf AG, Hamburg, Germany).

2.9. MRM assay

Instrumental analyses were performed on an Agilent 6490 iFunnel Triple Quadrupole LC/MS system (Santa Clara, CA, USA) equipped with an Agilent 1200 HPLC system (Santa Clara, CA, USA). Dried peptides were reconstituted in 3% acetonitrile/ 0.1% formic acid (volume fraction) in water. Separation was performed on an Agilent Zorbax Eclipse Plus C18 RRHD column (2.1 mm × 50 mm, 1.8 μm particle). Peptides were eluted at a flow rate of 200 μL/min using the following gradient of solvent B in solvent A: 3% B for 3 min, 3% to 30% B in 30 min, 30% to 50% B in 5 min, and 50% to 3% B in 5 min. Solvent A was water containing 0.1% formic acid (volume fraction) and solvent B was acetonitrile containing 0.1% formic acid (volume fraction). The acquisition method on an Agilent 6490 iFunnel Triple Quadrupole mass spectrometer used the following parameters in positive mode: fragmentor 380 V, collision energy 20 V, dwell time 100 ms, cell accelerator 4 V, electron multiplier 500 V, and capillary voltage 3500 V.

2.10. Data analysis

Every transition measured per peptide was taken as an individual measurement. MRM peak area integration was performed using Skyline (University of Washington). As an example, the Skyline peak profiles for representative proteins are shown in Fig. S2 (Supplemental Material). Excel was used to calculate peak area ratios. Peak integration was manually inspected and adjusted, if necessary. The peak ratios from transitions were averaged to yield the peptide ratios. Three transitions were measured per each peptide and data are represented as the mean ± SD.

3. Results and discussion

3.1. Selection of affinity peptides for EVs isolation

Fig. 1 shows a separation of 106 K EVs on Superdex 200 Increase 10/300 GL column. Because of size, the EVs are expected to be eluted in the void volume. However, some large plasma proteins, such as alpha-2-macroglobulin (A2M) or complexes of heat shock proteins, can be also eluted in the void volume. In addition, lipoprotein particles are commonly detected in the void volume of SEC separation. For a successful selection of affinity peptides, we needed the EVs sample used as bait for phage display screening to be as pure as possible. Therefore, we collected the void volume as 4 individual fractions (A5, A6, A7, and A8) and used MRM quantitation to calculate molar ratios for common plasma proteins *versus* common EVs proteins (Table 1). HSP90AB1, A2M, and apolipoprotein A-I (apo A-I) were selected as representative abundant plasma proteins while moesin, flotillin-1, TSG101, and EHD4 were selected as representative EVs proteins [13]. MRM assay with ^{15}N-labeled QconCATs that carry peptides for absolute quantitation of the proteins listed above allowed us to measure the pmoles of individual proteins in every fraction of the void volume and to present the data as a molar ratio for each EVs protein against HSP90AB1, A2M, and apo A-I, respectively (Table 1). It is clearly evident that all ratios in the fraction A5 are the most favorable to EVs proteins and that MRM quantitation facilitated finding fraction A5 as the purest EVs sample after SEC. Accordingly, the A5 fraction was selected for further phage display screening.

The Ph.D.-12 phage display peptide library (New England BioLabs, Ipswich, MA, USA) consists of M13 filamentous bacteriophage, on which five copies of a 12-amino-acid linear random peptide sequence are expressed as N-terminal fusions to the minor coat protein pIII of the phage. Conventional multi-rounds screening with clone picking for individual sequencing can bias clone diversity due to the growth advantage of individual clones [17]. To avoid this problem, we performed a single-round screening protocol using A5 fraction as a bait followed by NGS of the whole pool of clones. NGS for this screening resulted in the 142,486 unique peptide sequences with their appearance frequency. After subtraction of sequences found in the blank sample (panning without A5 fraction), the final list of peptide sequences decreased to 123,063 arranged in order of their appearance frequency. The first most abundant sequence was found 71 times. The appearance frequency of the remaining peptides declined quickly. At the level of approximately the first 1000 peptides, it dropped to 3 and was considered as non-specific. In other words, only 1000 peptides out of the total 123,063 were further analyzed. Once the list of highest appearance peptides was obtained, the MEME Suite (http://meme-suite.org) was used in search of common motifs. Finally, based on appearance and motif search, we have selected 3 peptides for further development of EVs isolation protocol. This includes LPSINHYSFPQA (P1), SLPGQRADRSWP (P2), and DMPILVPYHTPHALRDFP (P3). P1 and P2 are 12-amino acid residue peptides from the actual phage display library screening while P3 is a

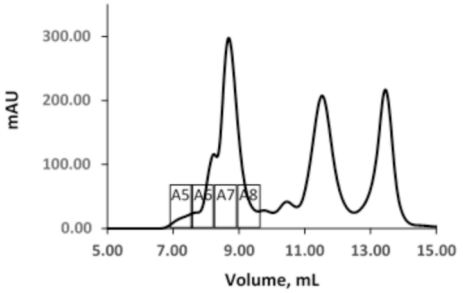

Fig. 1. SEC separation of 106 K-EVs. Individual fractions (A5, A6, A7, and A8) collected for analysis are marked with boxes.

Table 1
Molar protein ratios in the SEC fractions.

Proteins	SEC fractions			
	A5	A6	A7	A8
HSP90AB1/moesin	0.07	0.14	0.24	0.53
HSP90AB1/flotillin-1	0.33	0.31	0.78	1.29
HSP90AB1/TSG101	0.67	0.56	1.17	2.25
HSP90AB1/EHD4	0.8	0.63	1.75	nd
α-2-MG/moesin	0.06	0.53	1.07	1.76
α-2-MG/flotillin-1	0.25	1.19	3.44	4.29
α-2-MG /TSG101	0.50	2.11	5.17	7.50
α-2-MG /EHD4	0.60	2.38	7.75	nd
apo A-I/moesin	3	36	110	330
apo A-I/flotillin-1	11.6	80	348	802
apo A-I/TSG101	23	142	522	1404
apo A-I/EHD4	28	160	783	nd

Total pmoles amount of HSP90AB1, α-2-MG, and apo A-I was divided by total pmoles amount of moesin, flotillin-1, TSG101, and EHD4, respectively to obtain molar protein ratios in A5, A6, A7, and A8 void volume fractions. Nd stands for not detected.

combination of two common motifs found using MEME Suite.

3.2. Affinity isolation of EVs

Selected affinity peptides (P1, P2, and P3) were synthetized with Lys extension at the C-terminus and immobilized on the CNBr-activated Sepharose to generate an affinity resin for EVs isolation (P1-Sepharose, P2-Sepharose, and P3-Sepharose). SEC-EVs sample was used as a starting sample for affinity chromatography and final sample eluted from peptide-Agarose was called peptide-EVs. We would like to emphasize that 10 mmol/L phosphate buffer (pH 7.4)/400 mmol/L NaCl was sufficient to elute attached EVs and these mild elution conditions were unlikely to affect EVs integrity.

To evaluate the efficiency of peptide-Sepharose in EVs isolation, MRM assay was used to quantify a total of 26 proteins in SEC-EVs and peptide-EVs samples. In other words, pmoles of selected protein/mg of total protein was measured before and after chromatography on the peptide-Sepharose to see whether this affinity chromatography indeed enriched sample with EVs proteins. Preliminary parallel runs for P1-Sepharose, P2-Sepharose, and P3-Sepharose showed promising results only for P3-Sepharose, and that was the only resin further used for optimizing EVs isolation. Data for a representative EVs isolation on the P3-Sepharose are summarized in Table 2 and arranged in the four groups of proteins: 10 EVs proteins, 3 molecular chaperons, 2 common plasma proteins, and 11 apolipoproteins. Concentrations of all EVs proteins after affinity chromatography increased in a range of 5–9 times in comparison to the concentrations before chromatography. For example, moesin increased 4.8-times from 77.8 pmoles/mg of total protein in the sample before chromatography (SEC-EVs) to 373.4 pmoles/mg of total protein in the sample after chromatography (peptide-EVs) while EHD4 increased 9.4-times from 6.7 pmoles/mg of total protein to 63.0 pmoles/mg of total proteins.

All three other protein groups showed decreased concentrations after chromatography. For example, molecular chaperons dropped approximately 3–5 times. Albumin and A2M decreased 14- and 11-times, respectively. Apo A and apo C proteins decreased approximately 6–8 times with apo C-II went lower than detection level, if any. Apo B proteins decreased approximately 15-times. Apo E and apo J decreased 6- and 9-times, respectively.

Overall, MRM quantification turns out to be a powerful tool to assess efficiency of chromatography, but it is also important to underline that MRM quantification of multiple groups of proteins brings up interesting observations pertaining to important questions, such as the identity of EVs proteins and heterogeneity of EVs samples.

Identity of EVs proteins remains a topic for discussion. Membrane proteins seem like natural candidates since the only source of membrane proteins circulating in plasma is vesicles. For soluble proteins, the assignment to EVs is more complicated. Albumin and molecular chaperons could be EVs proteins, although it is obvious that their non-EVs concentrations in plasma samples are many orders of magnitude higher than concentrations of EVs. The fact that their concentrations were lower than concentrations of EVs proteins after 3-step isolation (differential ultracentrifugation, SEC, and P3-Sepharose) is very positive in terms of evaluating this isolation in general. In addition, small amounts of albumin and molecular chaperons left in the peptide-EVs sample may be also interpreted as evidence that these proteins are indeed a portion of EVs.

It is hard to find a functional connection between EVs and proteins like A2M or apolipoproteins. A2M is a 720 kDa tetramer bound together by -S-S- bonds and the largest non-immune protein complex in plasma. Apolipoproteins constitute various lipoprotein particles. Both A2M and lipoprotein particles overlap well in size with EVs and, because of their high abundance, always remain a major contamination of any EVs preparation. Table 2 shows that after affinity chromatography on P3-Agarose, the concentrations of these proteins can be brought down to be comparable or even lower than concentrations of EVs proteins. This warrants more research in further applications of peptides with affinity to EVs in their isolation.

Apart from the efficiency of chromatography in EVs isolation, MRM quantification allows an assessment of an important question of heterogeneity of EVs and probability of a selective isolation of EVs subpopulations. It was mentioned above that chromatography on P3-Agarose enriched EHD4 9.4-times while moesin in the same sample was enriched only 4.8-times (Table 2). This difference may be interpreted as a preferable enrichment of a specific EVs subpopulation. Further indirect support of this interpretation came from DLS analysis of SEC-EVs and peptide-EVs samples (Fig. 2). Both samples are represented

Table 2
Affinity isolation of EVs.

Proteins	SEC-EVs (pmoles/mg)	Peptide-EVs (pmoles/mg)
moesin	77.8 ± 16.3	373.4 ± 85.9
flotillin-1	12.6 ± 2.1	79.4 ± 16.7
TSG101	7.1 ± 0.9	46.2 ± 8.8
EHD4	6.7 ± 1.1	63.0 ± 15.1
integrin beta-3	55.3 ± 6.6	431.3 ± 60.4
integrin alpha-IIb	59.7 ± 7.2	453.7 ± 10.2
platelet GP Ib alpha	8.9 ± 1.5	73.0 ± 13.1
platelet GP Ib beta	8.6 ± 1.5	71.4 ± 12.8
platelet GP V	9.1 ± 1.7	53.7 ± 11.3
platelet GP IX	8.3 ± 1.6	58.1 ± 12.8
HSP90AA1	13.4 ± 1.2	4.3 ± 0.4
HSP90AB1	18.2 ± 2.2	4.2 ± 0.6
HSPA8	30.8 ± 2.8	5.8 ± 0.5
albumin	39.4 ± 5.9	2.8 ± 0.4
α-2-MG	18.5 ± 3.1	1.7 ± 0.2
apo A-I	708 ± 155	112 ± 26
apo A-II	16.9 ± 2.5	2.3 ± 0.3
apo A-IV	11.8 ± 3.1	1.6 ± 0.4
apo C-I	87.2 ± 23.5	14,1 ± 3.5
apo C-II	5.4 ± 0.8	nd
apo C-III	27.2 ± 7.9	1.7 ± 0.5
apo C-IV	51.7 ± 17.6	6.8 ± 1.9
apo B100/B48	59.8 ± 7.2	3.9 ± 0.5
apo B-100	57.1 ± 6.9	3.9 ± 0.5
apo E	24.5 ± 4.4	4.0 ± 0.6
apo J	59.8 ± 7.2	6.4 ± 0.7

MRM assay was used to quantify several group of proteins before (SEC-EVs) and after (peptide-EVs) separation on the P3-Sepharose. Calculations for all proteins (except apo C-IV) were done base on three transitions per peptide and two peptides per protein (Supplementary Material, Table S1). Data are shown in pmoles of targeted protein per mg of total protein with a SD for two biological replicates and three analytical injections. Nd stands for not detected.

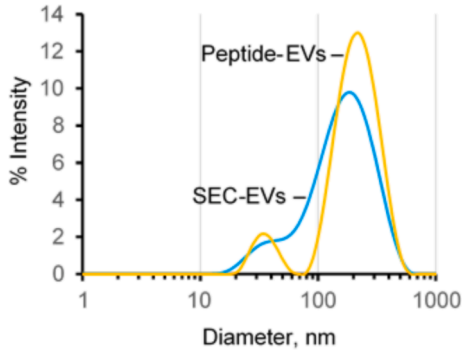

Fig. 2. DLS analysis of SEC-EVs and peptide-EVs. The size distribution of particles is presented by intensity.

by broad peaks pointing to their polydispersity, however the maximum of peptide-EVs peak (225 nm) in comparison to maximum of SEC-EVs peak (180 nm) shifted 45 nm pointing to a partial enrichment of a specific subpopulation of EVs after chromatography on the P3-Agarose.

4. Conclusions

Protein quantification using MRM assay was a critical approach in developing and evaluating the affinity isolation method for plasma EVs. Overall, we have demonstrated that affinity peptides selected from phage library screening can be valuable ligands to isolate EVs under mild elution conditions.

Authors' contribution

I.V.T. designed the research and performed MRM experiments. A.N. performed phage library screening. T.W. expressed and purified Qcon-CATs. I.V.T., A.N., and T.W. analyzed results and wrote the paper.

Declaration of Competing Interest

All authors have no conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgments

We thank Dr. Alexander Marin for the help with DLS experiments. Certain commercial materials, instruments, and equipment are identified in this manuscript in order to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials, instruments, or equipment identified are necessarily the best available for the purpose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104359.

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