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Technical note

Peptide-mediated ‘miniprep’ isolation of extracellular vesicles is suitable for high-throughput proteomics

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ABSTRACT

Extracellular vesicles (EVs) are cell-secreted membrane vesicles enclosed by a lipid bilayer derived from endosomes or from the plasma membrane. Since EVs are released into body fluids, and their cargo includes tissue-specific and disease-related molecules, they represent a rich source for disease biomarkers. However, standard ultracentrifugation methods for EV isolation are laborious, time-consuming, and require high inputs. Ghosh and co-workers recently described an isolation method utilizing Heat Shock Protein (HSP)-binding peptide Vn96 to aggregate HSP-decorated EVs, which can be performed at small ‘miniprep’ scale. Based on microscopic, immunoblot, and RNA sequencing analyses this method compared well with ultracentrifugation-mediated EV isolation, but a detailed proteomic comparison was lacking. Therefore, we compared both methods using label-free proteomics of replicate EV isolations from HT-29 cell-conditioned medium. Despite a 30-fold different scale (ultracentrifugation: 60 ml/Vn96-mediated aggregation: 2 ml) both methods yielded comparable numbers of identified proteins (3115/3085), with similar reproducibility of identification (72.5%/75.5%) and spectral count-based quantification (average CV: 31%/27%). EV fractions obtained with either method contained established EV markers and proteins linked to vesicle-related gene ontologies. Thus, Vn96 peptide-mediated aggregation is an advantageous, simple and rapid approach for EV isolation from small biological samples, enabling high-throughput analysis in a biomarker discovery setting.
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Body fluids represent a rich source of disease biomarkers as they pass or perfuse different tissues and can be easily sampled. However, these fluids tend to have a complex composition and exhibit a large dynamic range of protein levels. This has hampered protein biomarker discovery to date. Yet, virtually all biofluids harbor a potential treasure trove in the form of extracellular membrane vesicles (EVs) emanating from cells that, depending on the circumstances, selectively load the vesicles with some of their contents and secrete them into the surroundings [1,2]. EVs are believed to serve intercellular communication and macromolecular shuttling to nearby and distant cells, affecting diverse processes such as those involved in cancer progression [3]. They offer a stabilizing environment for long-distance journeys of their cargo [4], which consists of proteins, nucleic acids and lipids—some of them telltale components that reflect the (state of the) cellular origin of secretion. Thus, EVs carry an informative sub-proteome that is segregated from dominating constituents precluding analysis of whole biofluids. Hence, EVs could be exploited as stockpiles of indicators for pathologic conditions in the parental tissue, a notion for which an encouraging case in point was published recently [5]. Mechanistically, EVs can arise in two ways: ‘exosomes’ are endosome-derived vesicles released into the extracellular space from the lumen of multivesicular bodies, whereas ‘microvesicles’ (also termed ‘ectosomes’) pinch off directly from the plasma membrane and can reach larger sizes [6,7]. As yet there is no ‘gold standard’ EV isolation method [8], and most procedures yield mixtures of vesicles (there being no distinguishing physicochemical features enabling separation of exosomes and ectosomes) and varying amounts of contaminating material. To date, the most commonly used method involves fractionation of biological fluid through differential centrifugation followed by one or more ultracentrifugation steps to collect crude

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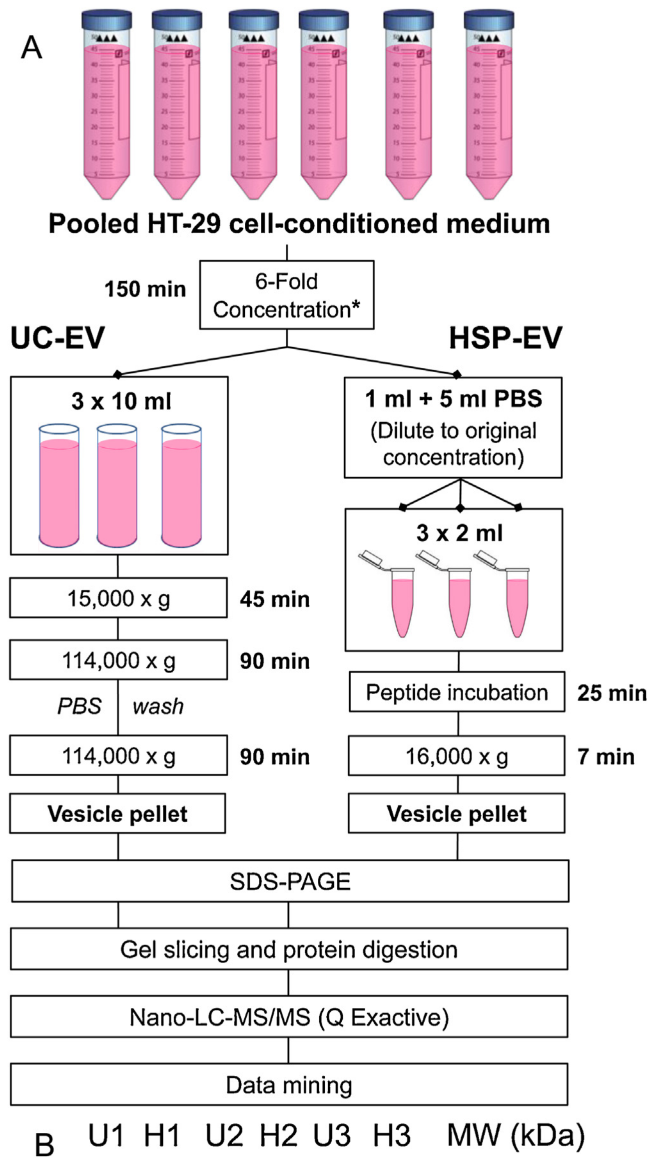

Fig. 1. Proteomic analysis of EVs from HT-29 cell-conditioned medium. (A) General scheme of the workflows for EV isolation by ultracentrifugation (UC-EV, left) and by Vn96-peptide mediated aggregation (HSP-EV, right) as well as common downstream analysis steps. The asterisk in the box denoting "6-fold concentration" indicates that time-consuming concentration was used for both UC-EV and HSP-EV, but only required for UC-EV so as to accomodate samples in ultracentrifugation tubes; it is not needed for the HSP-EV workflow but was included to start with an identical input proteome. (B) Protein gel pattern of all EV fraction replicates (numbered 1–3). U, UC-EV; H, HSP-EV; MW, molecular weight marker.

or purified EVs [9]. Unfortunately, due to sample-, equipment- and tube-specific parameters as well as protocol variations, this procedure suffers from disappointing lab-to-lab reproducibility; furthermore, it requires a large amount of starting material while providing inefficient EV recovery, and high centrifugal forces may affect vesicle quality [10]. Moreover, ultracentrifugation can only be performed with a limited number of samples and is laborious as well as time-consuming (especially when employing buoyant density-based "floatation"), whereas high-throughput screening requires a fast and easy 'miniprep' procedure for small-sized samples. This has impeded application to large clinical cohorts for biomarker discovery. Alternative methods obviating ultracentrifugation exist – some coming in commercial kits – that utilize precipitation with polymers, size exclusion chromatography, ultrafiltration, or (immuno) affinity capture [7,11]. Recently, another method for EV isolation has been described [12], involving the use of a specific peptide (Vn96) that not only exhibits high affinity for 70-kDa heat shock proteins (HSPs), but also has the capacity to aggregate EVs. It was proposed that Vn96 peptides bring down vesicles through their binding of HSPs decorating the EV surface. HSPs have been found to be secreted and bound to the surface of cells and vesicles [13,14], some ranking high among the top 100 most encountered EV components [6] and implicated in MVB loading [15,16]. Importantly, the EV-aggregating propensity of Vn96 peptides allows for a simple single-step collection procedure with centrifugation in a standard centrifuge. Microscopic, western blot and microRNA analyses underscored the value of this new approach for EV isolation from both cell-conditioned media and biofluids [12]. However, a detailed proteomic analysis was lacking.

To elaborate on these basic findings, here we set out to evaluate the suitability of Vn96 peptide-mediated EV isolation as a tool to produce EV minipreps for global proteomics and biomarker discovery. Specifically, using cancer cell-conditioned medium (harboring a 'secretome') as input material we compared the HSP-targeting EV isolation method (abbreviated: HSP-EV) with a standard ultracentrifugation method yielding a $100,000 \times g$ pellet (abbreviated: UC-EV). Our focus was on the capture of established EV proteome constituents, with a special emphasis on reproducibility of protein identification and quantification.

For an overview of the workflow, see Fig. 1(A). To compare HSP-EV and UC-EV methods, we used either method to process three aliquots of a single, pooled sample containing crude serum-free conditioned medium from HT-29 colorectal cancer cells. As input we used 2 ml for HSP-EV (corresponding to $\sim 10^7$ cells, miniprep scale), and 60 ml for UC-EV (normal scale), thus at the same time assessing the value of HSP-EV as an EV isolation modality that should capture enough of the UC-EV 'standard' proteome, as well as testing the possibility of downscaling. Fractions were analyzed by a combination of one-dimensional gel electrophoresis and nanoscale liquid chromatography coupled to tandem mass spectrometry (GeLC–MS/MS) [17]. Fig. 1(B) shows the protein gel patterns for all EV fractions. The major band at 3–4 kDa in HSP-EV fractions corresponds to the Vn96 peptide that was added for EV isolation. For proteomic analyses, gel lanes were cut into five consecutive slices, and each slice was cut up further and in-gel digested with trypsin [18]. Extracted tryptic peptides were separated by reversed-phase nano-LC and analyzed on-line with a Q Exactive mass spectrometer as described previously [19,20]. Further experimental details can be found in Supplementary methods.

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A total of 3443 unique proteins (3495 protein groups) were identified across all EV fractions, with some 3100 proteins identified per preparation method (Fig. 2(A); see Supplementary Table 1 for raw database search results and Supplementary Table 2 for annotated data). The majority (>80%) was identified in at least one replicate with both methods. An inspection of the data for a selection of established exosome/EV markers including CD63, CD81, TSG101 and PDCD6IP/ALIX showed that both methods captured all of these proteins (Fig. 2(B)), with the UC-EV method giving somewhat higher yields, but not proportional to the 30-fold difference in input between the two methods. Supplementary Fig. 1 shows boxplots for a broader selection of proteins often associated with EVs and vesicle transport as well as some putative cargo. We have also assessed the overlap of the proteins in our data set with publicly available EV protein databases ExoCarta, Vesiclepedia, and EVpedia [21–23](see Supplementary methods and Supplementary Fig. 2), which showed that less than 5% are not present in at least one of these repositories. Furthermore, the UC-EV and HSP-EV proteomes contained 80% of the HT-29 EV proteome published by Choi et al. [24], covering 95% of their tetraspanin-centered subnetwork (data not shown). Overlap analysis of technical replicates indicated that with both isolation methods a significant proportion of proteins were identified in all three replicates (73% for UC-EV and 76% for HSP-EV, Fig. 2(C)). This indicates good reproducibility of protein identification, an important prerequisite for global discovery proteomics. Reproducibility of protein quantification was also similar for both methods. As a quantitative proxy for protein abundance we used spectral counts, i.e. the total number of identified MS/MS spectra linked to a protein [17]. Among triplicates, the average CV% of spectral counts was 31% versus 27% for UC-EV versus HSP-EV (median of 26% versus 23%, mode of 16% versus 5%; Supplementary Table 2). Scatter plots and correlations can be found in Supplementary Fig. 3. Of note, spectral counts for proteins identified with both methods also showed a good correlation (Pearson correlation coefficient $r = 0.88$, Fig. 2(D)). Using immunoblot analysis, we further verified enrichment of key EV markers in a typical EV fraction obtained with the HSP-EV method (Fig. 2(E)). The markers are easily detected in the EV fraction whereas they are below the detection limit (CD63) or marginally visible (CD81) in the corresponding supernatant after EV isolation. To further characterize our UC-EV and HSP-EV fractions, we performed gene ontology mining of the DAVID database [25] using proteins that passed a median spectral count cutoff (Supplementary Table 3; Supplementary Fig. 4 shows top-scoring term clusters filtered for enrichment score and FDR). The results further supported both the vesicular content and the similarity of UC-EV and HSP-EV samples. Top ontologies included terms related to vesicle structure (membrane-bounded, lumen, membrane coat) and function (protein localization, protein transport), proteins important for intracellular vesicle transport and sorting (cytoskeletal elements, small GTPases), proteins important for vesicle-environment interaction and homing (junction/adhesion molecules), as well as putative cargo that especially seemed to encompass gene expression-related molecules (i.e., ribosomal/translational, proteasomal and mRNA splicing components) in addition to, e.g., glycolytic enzymes. FunRich, a gene ontology mining tool which uses a dedicated database focusing on exosome/EV-related proteins [26] identified 'exosomes' and 'lysosome' as the most significant cellular components (Supplementary Fig. 4). Our ontology analyses indicate a high similarity between UC-EV and HSP-EV preparations, although some differences can be discerned, which is not unexpected as the UV-EV method removes a subset of large EVs in the intermediate-speed spin.

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In summary, we have shown that Vn96-mediated EV isolation enables reproducible analysis of the EV proteome. In terms of identified proteins and associated gene ontologies, the HSP-EV

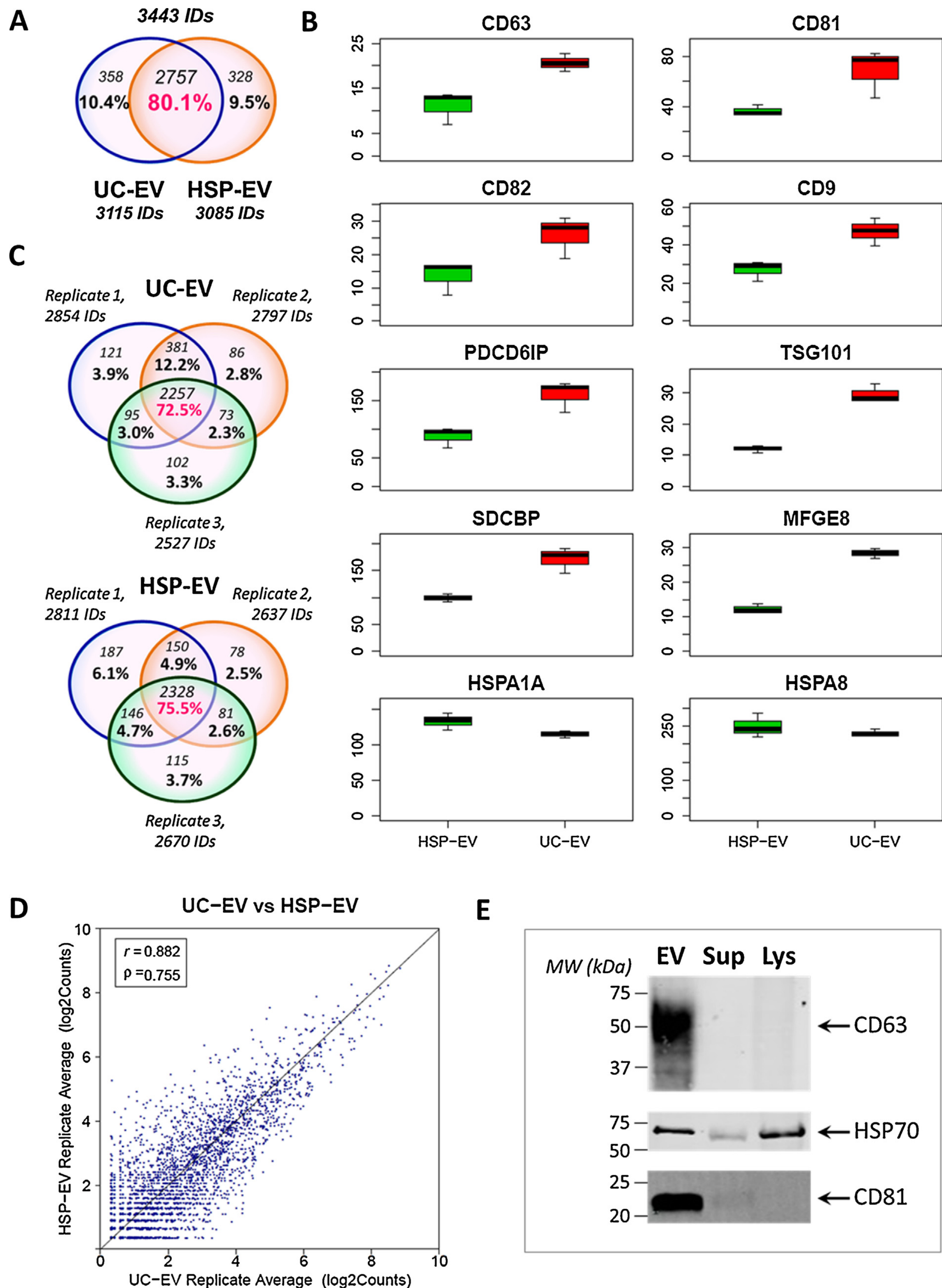

Fig. 2. Comparison of UC-EV and HSP-EV methods for isolation of extracellular vesicles. (A) Venn diagram showing large overlap of the proteomes identified in EV fractions isolated with the UC-EV methods or the HSP-EV method. (B) Boxplots normalized spectral counts for a selection of exosome/EV markers identified with both methods (green: HSP-EV, red: UC-EV). (C) Venn diagrams showing reproducibility of UC-EV and HSP-EV methods as assessed by enumeration of identifications shared among replicates. (D) Scatter plot showing correlation between average normalized spectral counts (log 2-transformed) for proteins identified in UC-EV fractions and in HSP-EV fractions, respectively. Inset shows Pearson (r) and Spearman (ρ) correlation coefficients. E. Immunoblot validation of the capture of classical exosome/EV markers CD63 and CD81 as well as HSP70 from HT-29 cell-conditioned medium by the HSP-EV method. EV, extracellular vesicle fraction; Sup, EV-depleted supernatant; Lys, HT-29 cell lysate.

proteome is largely comparable to that of crude EVs isolated by a standard ultracentrifugation procedure (UC-EV), including classical exosome/EV markers. The latter are detected in HSP-EV fractions at a level that is only about twofold lower than that observed in UC-EV preparations. This result is remarkable considering the 30-fold lower sample input used for HSP-EV isolation. While we do not claim quantitative recovery or high purity of EVs, realizing that it yields inevitably crude and heterogeneous EV fractions, we conclude that the small-scale HSP-EV isolation method captures a sufficient amount and diversity of the EV proteome to render it useful for clinically relevant comparisons. With multiple methods for EV isolation available, the specific method used depends on the question and application for which it is most appropriate [8]. Indeed, the specific cellular or vesicular origin of biomarkers is not relevant to their diagnostic use [27]. Further exploring the HSP-EV method with body fluids like blood is therefore warranted. Given the ease-of-use, simplicity, and unparalleled speed of this procedure (which is much less time-consuming than UC-EV) this would open a novel avenue of EV miniprep isolation in a clinical setting. This may greatly facilitate biomarker discovery efforts in cancer research as well as similar endeavors for other diseases. Moreover, it could pave the way for novel noninvasive applications.

Conflict of interest

We have received the ME™ kit from New England Peptide at a reduced price for beta test purposes.

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