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Short Communication

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Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties

Joel Z. Nordin, M.D.[a,1], Yi Lee, BSc[b,1], Pieter Vader, PhD[b], Imre Mäger, PhD[b,c], Henrik J. Johansson, PhD[d], Wolf Heusermann, PhD[e], Oscar P.B. Wiklander, M.D.[a], Mattias Hällbrink, PhD[f], Yiqi Seow, DPhil[g], Jarred J. Bultema, PhD[h], Jonathan Gilthorpe, PhD[i], Tim Davies, MSc[j], Paul J. Fairchild, PhD[j], Susanne Gabrielsson, PhD[h], Nicole C. Meisner-Kober, PhD[e], Janne Lehtiö, PhD[d], C.I. Edvard Smith, M.D., PhD[a], Matthew J.A. Wood, M.D., DPhil[b,*], Samir EL Andaloussi, PhD[a,b,**]

[a]*Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden*
[b]*Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom*
[c]*Institute of Technology, University of Tartu, Tartu, Estonia*
[d]*Cancer Proteomics Mass Spectrometry, Science for Life Laboratory, Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden*
[e]*Novartis Institutes for Biomedical Research, Basel, Switzerland*
[f]*Department of Neurochemistry, Stockholm University, Stockholm, Sweden*
[g]*Molecular Engineering Laboratory, Proteos, Agency for Science, Technology and Research (A*STAR), Singapore*
[h]*Department of Medicine Solna, Translational Immunology Unit, Karolinska Institutet and University Hospital, Stockholm, Sweden*
[i]*Umeå Centre for Molecular Medicine, Umeå University, Umeå, Sweden*
[j]*Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom*

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Abstract

Extracellular vesicles (EVs) are natural nanoparticles that mediate intercellular transfer of RNA and proteins and are of great medical interest; serving as novel biomarkers and potential therapeutic agents. However, there is little consensus on the most appropriate method to isolate high-yield and high-purity EVs from various biological fluids. Here, we describe a systematic comparison between two protocols for EV purification: ultrafiltration with subsequent liquid chromatography (UF-LC) and differential ultracentrifugation (UC). A significantly higher EV yield resulted from UF-LC as compared to UC, without affecting vesicle protein composition. Importantly, we provide novel evidence that, in contrast to UC-purified EVs, the biophysical properties of UF-LC-purified EVs are preserved, leading to a different in vivo biodistribution, with less accumulation in lungs. Finally, we show that UF-LC is scalable and adaptable for EV isolation from complex media types such as stem cell media, which is of huge significance for future clinical applications involving EVs.

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*Correspondence to: M. JA Wood, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom.

**Correspondence to: S. El Andaloussi, Department of Laboratory Medicine, Karolinska Institutet, Huddinge, Sweden.

E-mail addresses: matthew.wood@dpag.ox.ac.uk (M.J.A. Wood), Samir.El-Andaloussi@ki.se (S.E.L. Andaloussi).

[1]These authors contributed equally to the manuscript.

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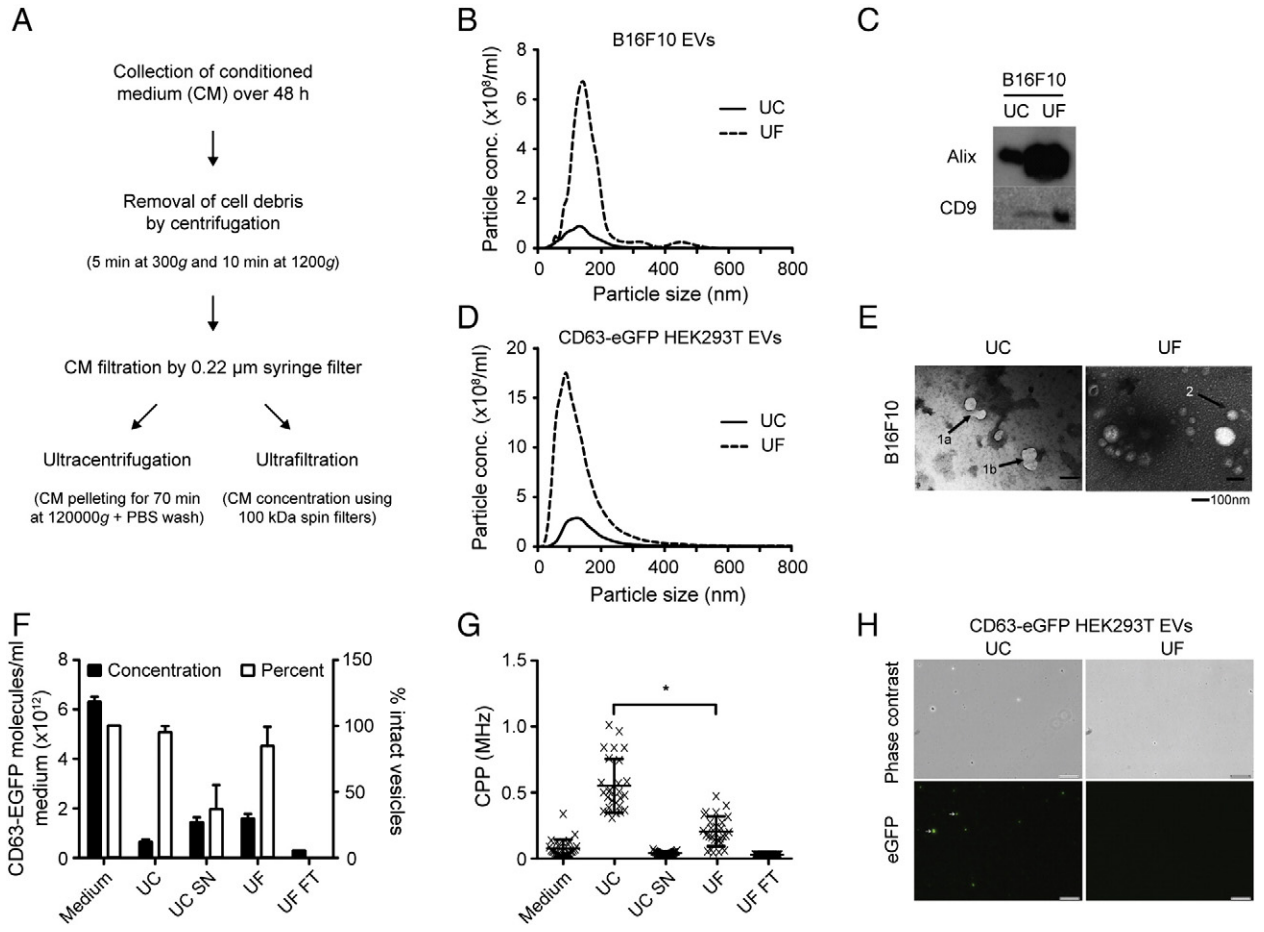

Figure 1. Ultrafiltration allows efficient isolation of intact EVs. **(A)** Chart outlining UF and UC protocols. **(B)** NTA of UF and UC-purified EVs. **(C)** WB of UF and UC-purified EVs. **(D)** NTA (fluorescence mode) of UF and UC-purified eGFP-positive EVs. **(E)** TEM of UF and UC-purified EVs. Arrows 1a: fragmentation, 1b: fusion and 2: intact. **(F)** Absolute concentrations of CD63-eGFP molecules (left y-axis) and percentage of intact vesicles (right y-axis) according to FCS (SN=supernatant, FT=flow-through). **(G)** Molecular brightness for each particle (counts per particle)(CPP) (n=3). *Indicates $p<0.05$, results represent mean+s.d. **(H)** Fluorescence microscopy of CD63-eGFP-positive EVs.

From the Clinical Editor: Recent evidence suggests extracellular vesicles (EVs) as another route of cellular communication. These EVs may be utilized for future therapeutics. In this article, the authors compared ultrafiltration with size-exclusion liquid chromatography (UF-LC) and ultracentrifugation (UC) for EV recovery.

Key words: Biophysical properties; extracellular vesicles; size-exclusion liquid chromatography; ultracentrifugation; ultrafiltration

Intercellular communication, via paracrine factors, is essential for survival of all multicellular organisms. Recent evidence suggests another route of cellular communication: extracellular vesicles (EVs), comprising of nano-sized exosomes, microvesicles and apoptotic bodies.[1] Due to their ability to convey information through RNA and proteins, EVs can influence both physiological[2] and pathophysiology processes.[3,4] Moreover, EVs from mesenchymal stem cells have been exploited for regeneration of injured tissues.[5–7] Hence, EVs are emerging as disease biomarkers and nanotherapeutics.[8]

Despite progress in EV research, the challenge of purifying biologically intact EVs remains. Differential ultracentrifugation (UC)[9] is the classical protocol for EV isolation. However, problems with UC include low and operator-dependent yields.[10] Alternative strategies like immuno-affinity capture,[11] polymer-based precipitation[12] and microfluidics[13] also present shortcomings, e.g. vesicle disruption and co-purification of non-vesicular proteins.[10] Deriving an EV isolation method that enables high recovery of functional EVs in a scalable fashion is therefore essential for EV research.

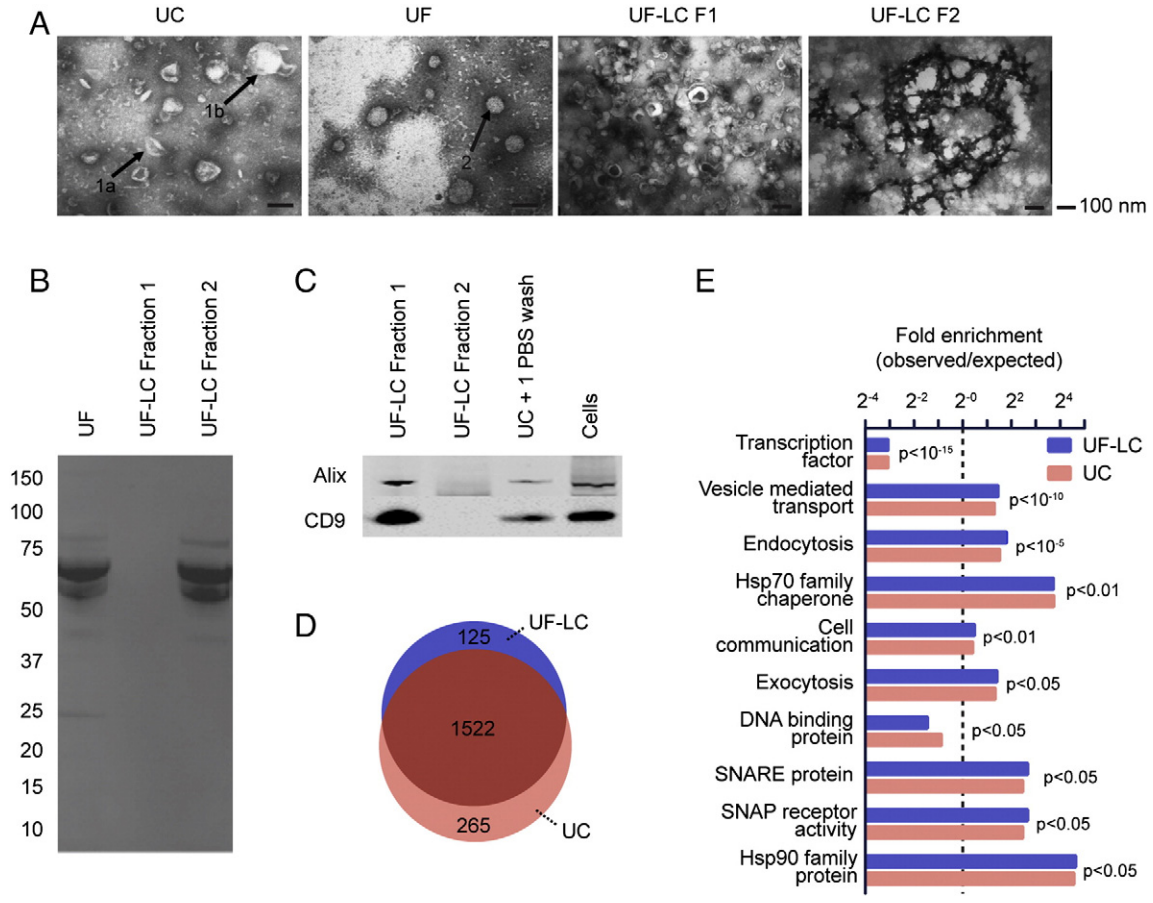

Figure 2. Size-exclusion chromatography separates EVs from contaminants. **(A)** TEM of UF-LC fraction 1 shows intact EVs, whereas fraction 2 contains protein aggregates. **(B)** Total protein staining of UF and UF-LC fractions. **(C)** WB of UF-LC and UC-purified EVs. **(D)** Venn diagram depicting protein overlap for UF-LC and UC-purified EVs. **(E)** Gene ontology (GO) enrichment terms for UF-LC and UC-purified EV proteomes.

Table 1
Protein/vesicle ratio of UC and UF-LC-purified EVs.

Protein/vesicle ratio ($x10^{-15}$ g/particle)	N2a OptiMEM	N2a Prespun	iPSCs
UC	0.25	0.25	0.70
UF-LC	0.074	0.059	0.55

Here, we present a systematic comparison study between a robust EV purification method, ultrafiltration with size-exclusion liquid chromatography (UF-LC), adapted from previous studies,[14–16] and UC. We show that UF-LC permits higher EV recovery with intact biophysical properties.

Methods

EVs were isolated by either UF or UC; UF samples were subsequently loaded on a Sephacryl column for size-exclusion fractionation. EV properties were evaluated using molecular (western blotting (WB), nanoparticle tracking analysis (NTA), LC/MS/MS) and biophysical (transmission electron microscopy (TEM), fluorescence microscopy, fluorescence correlation spectroscopy (FCS), total internal reflection fluorescence microscopy (TIRF) and DiR-imaging) analyses (see supplementary data for details).

Results

UF-LC allows high-yield isolation of biophysically intact EVs

OptiMEM conditioned medium was processed by UC or UF (Figure 1, *A*). According to NTA, more particles with similar size distribution were recovered after UF than after UC. Correspondingly, levels of vesicle markers (Alix and CD9) were higher in UF than UC samples. This finding was consistent across different cell lines and with CD63-eGFP labeled EVs (Figure 1, *B-D* and Supplementary figure S1A).

TEM revealed EVs with rounded and cup-shaped morphology in both samples. Occasionally, UC-purified EVs appeared disrupted or fused (Figure 1, *E*), an observation not seen with UF-purified EVs. FCS was employed for more quantitative analysis of EV integrity. In FCS, EV hydrodynamic radius, concentration and changes in biophysical properties (e.g. fusion or fragmentation) were determined by measuring diffusion and intensity of CD63-eGFP positive EVs. From these readings, 25% of the total CD63-eGFP molecules were recovered using UF, 2.5-fold more than using UC, with larger EV radius for UC. The

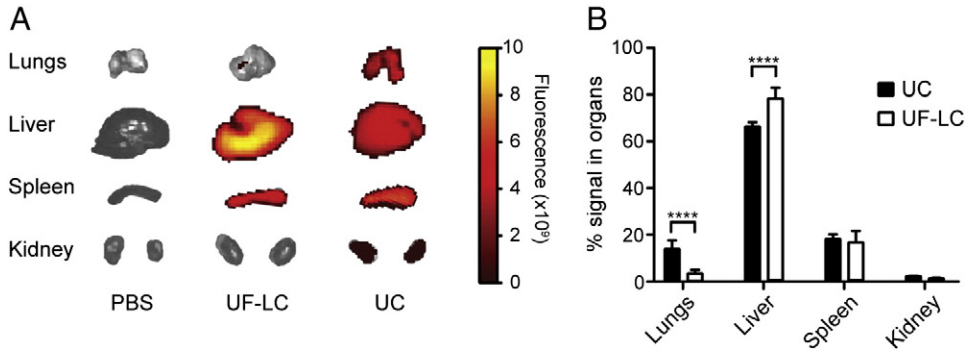

Figure 3. Biodistribution of UF-LC-purified EVs. **(A)** Representative IVIS images of organs from Balb/c mice 24 h post injection of DiR-labeled EVs. **(B)** Biodistribution of UC and UF-LC DiR-labeled EVs. ****Indicates p<0.0001% in comparison to the same organ in the corresponding group (n=5), results represent mean+s.d.

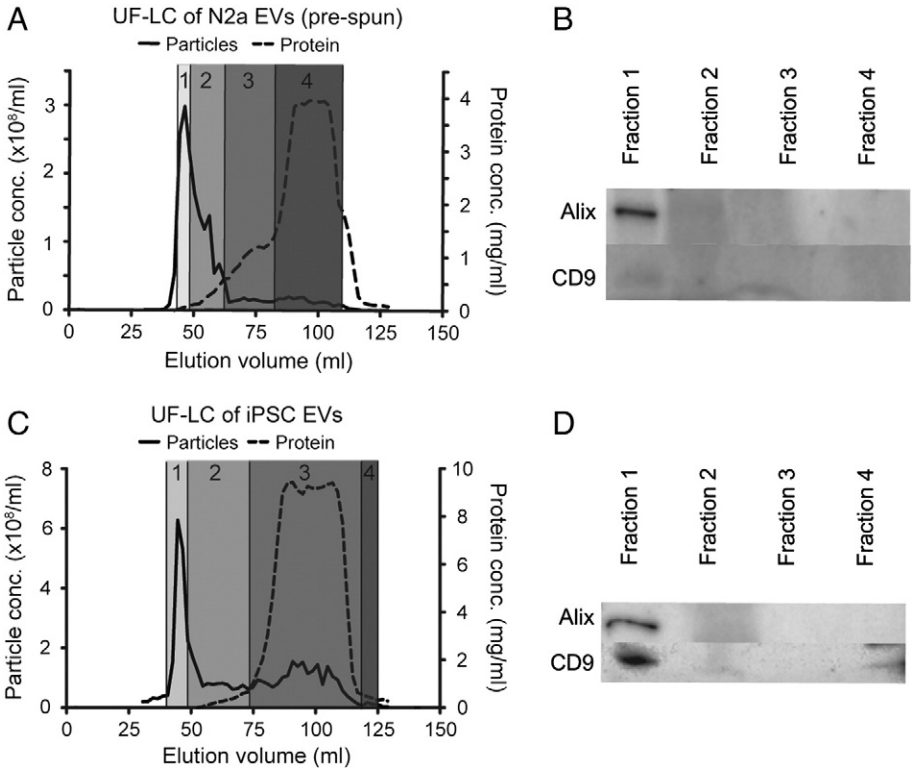

Figure 4. UF-LC EV isolation from complex media types. Graph showing the concentration of particles (x10^8/ml) (left y-axis) and protein concentration (mg/ml) (right y-axis) across the eluted volume after UF-LC for **(A)** N2a pre-spun conditioned media and **(C)** iPSC conditioned media. WB of **(B)** N2a and **(D)** iPSC EVs.

brightness of individual particles (number of CD63-eGFP molecules per vesicle) was higher in UC than UF, suggesting vesicle fusion. Further, disintegration with NP-40 decreased the diameter of CD63-eGFP-positive material to 2 nm for both UC and UF-purified EVs, the putative size of free CD63-eGFP.[17] After UC, only 10% of total vesicles were recovered in the pellet; while in the supernatant, only 38% of the vesicles were intact while the remaining 62% of the eGFP-positive material was 2 nm. This is indicative of vesicle disruption, since disrupted vesicles release their CD63-eGFP into the supernatant as free CD63-eGFP (Figure 1, *F-G* and Supplementary figure S1C). Moreover, fluorescence microscopy of EVs in suspension revealed aggregates only in UC samples (Figure 1, *H*).

Since protein complexes were co-isolated using UF (Supplementary figure S2A-C), size-exclusion LC was used to fractionate OptiMEM conditioned media. Two distinct fractions were detected (Supplementary figure S2D-E), with vesicles and non-vesicular proteins found exclusively in fraction 1 and fraction 2, respectively (Figure 2, *A-B*). UF-LC presented with consistently high EV recovery rates (70% ±19%), along with higher EV marker expression as compared to UC samples (Figure 2, *C* and Supplementary figure S2F-G). LC/MS/MS of EVs purified by both methods presented a good correlation in overall protein expression and gene ontology annotations (Figure 2, *D-E*, Supplementary figure S3A-E and Supplementary Table S1), although the protein/vesicle ratio was lower in UF-LC

than for UC-purified EVs (Table 1). TIRF imaging further corroborated the presence of aggregates/fusion of EVs only in UC samples (Supplementary figure S4A-B).

UF-LC-purified EVs demonstrate different in vivo biodistribution compared to UC-purified EVs

Based on the distinct differences observed in EV integrity, we speculated that this would influence EV biodistribution. DiR-labeled EVs were injected intravenously and mice were imaged 24 h later. UC-purified EVs showed a 4.6 times ($p<0.0001$) stronger signal in lungs compared to UF-LC-purified EVs (Figure 3, *A-B*) while the reverse was seen in the liver.

UF-LC can be extended to complex biological fluids

After subjecting conditioned pre-spun and stem cell media to UF-LC, EV markers were again solely detected in fraction 1, which corresponds to the peak of particles (Figure 4, *A-F* and Supplementary figure S5A-D). Moreover, UF-LC samples consistently had a lower protein/vesicle ratio than UC samples (Table 1).

Discussion

Research in EVs has recently received increasing attention, however, one major roadblock is the lack of a scalable technique permitting efficient purification of EVs. Here we report the first systematic comparison study comparing both biochemical and biophysical aspects of UF-LC and UC-purified EVs. NTA, WB and state-of-the-art LC/MS/MS demonstrated that UF-LC generated EVs with the same proteome as UC. Furthermore, our protein/vesicle ratio was consistently lower in UF-LC than UC samples, suggesting higher EV purity.[18] TEM, FCS, TIRF and fluorescence microscopy data suggest that EVs fuse, disrupt and aggregate during the UC isolation process, an aspect that is circumvented with UF-LC. We postulate that these large EV aggregates account for the accumulation in the lungs in our *in vivo* experiments. Another finding from this study is that UF-LC can be adapted for EV isolation from stem cell media. We believe that using UF-LC for EV isolation will allow researchers to venture into new avenues aimed at unraveling EV biological functions.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2015.01.003.

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