Journal of Visualized Experiments

Evaluation of the storage stability of extracellular vesicles

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59584R1
Full Title:	Evaluation of the storage stability of extracellular vesicles
Keywords:	Extracellular vesicles; Exosomes; storage stability; enzyme encapsulation; lyophilisation; freeze-drying; asymmetric flow field-flow fractionation
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Saarbrücken, Germany

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Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Saarbrücken, 4 February 2019

Dear Dr Bajaj,

Re: Revision of manuscript JoVE59584 for the *Journal of Visualized Experiments*

On behalf of my co-authors, I am happy to submit the revised version of our manuscript entitled "Evaluation of the storage stability of extracellular vesicles" JoVE59584.

We have taken great care to make constructive alterations to the manuscript based on the comments made by the reviewers. For your convenience, a detailed point-by-point account of these changes is included in the response to reviewers, including the respective line numbers. All changes are highlighted in green in the text.

We hope that you find this manuscript suitable for publication in *JoVE* and look forward to hearing from you soon.

Please do not hesitate to contact me should you require any further information.

Yours sincerely,

Dr Gregor Fuhrmann
Head Junior Research Group Biogenic Nanotherapeutics
Helmholtz-Institute for Pharmaceutical Research

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TITLE:

Evaluation of the Storage Stability of Extracellular Vesicles

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KEYWORDS:

Extracellular vesicles; exosomes; storage stability; enzyme encapsulation; lyophilization; freeze-drying; asymmetric flow field-flow fractionation

SUMMARY:

Here we present a readily applicable protocol to assess the storage stability of extracellular vesicles, a group of naturally occurring nanoparticles produced by cells. The vesicles are loaded with glucuronidase as a model enzyme and stored under different conditions. After storage, their physicochemical parameters and the activity of the encapsulated enzyme are evaluated.

ABSTRACT:

Extracellular vesicles (EVs) are promising targets in current research, to be used as drugs, drug-carriers, and biomarkers. For their clinical development, not only their pharmaceutical activity is important but also their production needs to be evaluated. In this context, research focuses on the isolation of EVs, their characterization, and their storage. The present manuscript aims at providing a facile procedure for the assessment of the effect of different storage conditions on EVs, without genetic manipulation or specific functional assays. This makes it possible to quickly get a first impression of the stability of EVs under a given storage condition, and EVs derived from different cell sources can be compared easily. The stability measurement is based on the physicochemical parameters of the EVs (size, particle concentration, and morphology) and the preservation of the activity of their cargo. The latter is assessed by the saponin-mediated encapsulation of the enzyme beta-glucuronidase into the EVs. Glucuronidase acts as a surrogate and allows for an easy quantification via the cleavage of a fluorescent reporter molecule. The present protocol could be a tool for researchers in the search for storage conditions that optimally retain EV properties to advance EV research toward clinical application.

INTRODUCTION:

EVs are membrane-bound nanoparticles produced by nearly all cell types. For mammalian cells, EVs can be subdivided into two main groups with distinct production pathways[1,2]. Membrane vesicles, with a size range from roughly 100–1,000 nm, are produced by direct budding from the cell membrane. Exosomes, sized 30–200 nm, are derived from multivesicular bodies formed by inward budding into endosomes that subsequently fuse with the cell membrane to release multiple exosomes at once. The main function of these vesicles is the transport of information between cells[3]. For this purpose, cargos such as RNA, DNA, and proteins are actively sorted into them. EVs can convey a variety of effects on their targets, with implications for both health and disease state. On one side, they mediate positive effects such as tissue regeneration, antigen presentation, or antibiotic effects, which makes them auspicious targets for their development as therapeutics[4,5]. On the other side, EVs can promote tumor vascularization[6], induce bystander effects in stress responses[7], and might play a role in autoimmune diseases[8] and inflammatory diseases[9]. Thus, they might be a key component to a better understanding of many pathological effects. However, the presence of altered EVs in manifold diseases, such as cancer[10–12] and cardiovascular disorders[13], and their easy accessibility in blood and urine makes them ideal biomarkers. Finally, their good biocompatibility[14] and their inherent targeting ability make EVs also interesting for drug delivery[15]. In this manuscript, we describe a protocol for the evaluation of the storage stability of EVs derived from mammalian cells, an important property that is still little investigated.

For the clinical development of EVs, there are still many obstacles to surmount[16], including the evaluation of their therapeutic effects, production, purification, and storage[17]. While -80 °C is widely seen as the gold standard for EV storage[18], the required freezers are expensive, and maintaining the required cold chain from the production to the patient can be challenging. Moreover, some reports indicate that storage at -80 °C still not optimally preserves EVs and induces a loss in EV functionality[19,20]. Other methods, such as freeze-drying[21,22] or spray-drying[23], have been proposed as potential alternatives to the frozen storage of EVs.

The optimal way of assessing storage stability would be to test the EVs in functional assays or by the evaluation of a specific marker, for instance, their antibacterial activity[19]. This is possible when the desired effect of the vesicles is known and when one distinct group of EVs is to be studied. If EVs from different cell sources are to be compared (e.g., for drug encapsulation) or if there is no known functional readout, it is no longer possible to assess changes due to storage in a direct manner.

On the other hand, simply evaluating changes in their physicochemical parameters, such as size, particle recovery, and protein concentration, does not always predict changes in EV activity, as has been shown in a recent patent[20].

Here, we provide a readily applicable protocol for measuring the storage stability of EVs by assessing their physicochemical parameters combined with the activity of an encapsulated beta-glucuronidase enzyme as a surrogate for the cargo of the EVs. The loading of the enzyme is done by saponin incubation, a mild method established with EVs from different sources[21,24,25]. Saponin forms transient pores in the EV membrane, which allows enzyme uptake into the vesicle. As

enzymes are prone to lose their activity if subjected to unfavorable storage conditions, they are an ideal surrogate for the evaluation of the preservation of functional cargoes of the EVs.

We have demonstrated that the application of this protocol to EVs derived from human mesenchymal stem cells (MSCs), human umbilical vein endothelial cells (HUVECs), and human adenocarcinoma alveolar epithelial cells (A549) indeed result in great differences in storage stability between different cell lines, which should be taken into consideration when choosing the EV source[21].

PROTOCOL:

1. Cell culture and the production of cell-conditioned medium

1.1. Generally, cultivate cells under the individual conditions required for the respective cell line.

1.2. Cultivate the cells for 24–72 h in serum-free conditions or in medium containing EV-depleted fetal bovine serum (FBS).

NOTE: If EV-depleted FBS is used, employ a method proven to efficiently deplete the serum, to prevent contamination with bovine serum-derived EVs[26].

1.3. Collect the medium from the flasks. Centrifuge at 300 x *g* for 10 min to pellet the cells. Carefully collect the cell-conditioned medium (CCM), without disturbing the pelleted cells. Preferably, use the CCM directly, or store it overnight at 4 °C.

NOTE: It is always preferable to use freshly produced CCM. If storage for longer time periods cannot be circumvented, all relevant parameters should be recorded in accordance with MISEV2018 guidelines[26], and the potential biases of the results acquired need to be taken into consideration.

1.4. Example protocol for HUVECs

1.4.1. Cultivate HUVEC cells for 120 h in EGM-2 medium containing FBS and other supplements.

1.4.2. Cultivate HUVEC cells for 48 h in EBM-2 basal medium free of any additional supplements.

1.4.3. Collect the medium from the flasks and perform the centrifugation step as indicated above (step 1.3). Typically, use 100 mL of medium for one EV-isolation.

2. Ultracentrifugation of CCM

2.1. Immediately before ultracentrifugation (UC), centrifuge the CCM for 15 min at 3,000 x *g* and 4 °C to remove cell debris and large agglomerates.

2.2. Carefully transfer the supernatant to the UC tubes. If using a fixed angle rotor, mark the orientation of the tubes in the centrifuge to facilitate the retrieval of the EV pellet after the UC. Centrifuge for 2 h at 120,000 x *g*, with a k-factor of 259.4.

2.3. After UC, carefully discard the supernatant using a serological pipet, to avoid the disturbance of the pelleted EVs.

NOTE: The pellet might be invisible.

2.4. Add 200 µL of 0.2 µm-filtered phosphate-buffered saline (PBS) to the first tube and use PBS and the residual supernatant to resuspend the pellet by pipetting up and down. Transfer the resulting EV suspension to the next tube of the respective sample and use it for the resuspension. Proceed this way to resuspend all EVs of the sample in a final volume of approximately 300–350 µL.

2.5. After resuspension, confirm the presence of particles by nanoparticle tracking analysis (NTA). Use the settings optimized for the given EV type, such as the settings below (step 2.5.1).

NOTE: The papers of Gardiner et al.[27] and Vestad et al.[28] contain valuable information on how to optimize the parameters for measuring EVs.

2.5.1. To reproduce the results described below, use instruments (e.g., NanoSight LM14) equipped with a green laser. Record three videos of 30 s with a screen gain of 1.0 and a camera level of 13. For analysis, use a screen gain of 1.0 and a detection threshold of 5.

2.6. Use the pellet immediately, if possible; otherwise, store it at 4 °C overnight.

3. Glucuronidase encapsulation into EVs

3.1. To the resuspended pellet, add beta-glucuronidase (10 mg/mL in PBS) to a final concentration of 1.5 mg/mL and saponin (10 mg/mL in H_2O) to a final concentration of 0.1 mg/mL. Mix well by vortexing for 3 s.

3.2. Incubate for 10 min at room temperature with intermitted mixing by gently flicking the tube. After incubation, directly purify by size-exclusion chromatography (SEC) (see section 5).

NOTE: Do not refreeze glucuronidase samples once thawed, to prevent enzyme degradation due to freezing.

4. Liposome production

4.1. To prepare liposomes for comparison with EVs, dissolve 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in a 2:3 molar

ration in chloroform to a final concentration of 5 mM. Prepare 1 mL aliquots in high-performance liquid chromatography (HPLC) vials and let them dry overnight to form a lipid film.

CAUTION: Chloroform is toxic and suspected to be cancerogenic. Take proper precautions when handling it.

4.2. Rehydrate the lipid-film with 1 mL of PBS containing 1.5 mg/mL glucuronidase. Heat it to 42 °C and vortex for 1 min. Heat the extruder assembly to 42 °C and extrude the lipid suspension 11x through a 200 nm polycarbonate membrane. Directly purify by SEC (see section 5).

5. Purification by SEC

5.1. Prepare the SEC column using the following protocol.

5.1.1. Use only fresh purified water and freshly prepared buffers. Filter all buffers through a 0.2 µm membrane filter and degas them to prevent the formation of air bubbles in the column.

5.1.2. For the preparation of an SEC column, use agarose gel filtration-based matrix (e.g., Sepharose Cl-2b) or another SEC medium suitable to separate EVs and liposomes from protein impurities and excess enzyme. First, remove the 20% EtOH solution the medium is stored in, to prevent air bubble formation in the column. To this end, centrifuge the SEC medium at 3,000 x *g* for 10 min, remove the EtOH, and replace it with degassed water.

5.1.3. Fill a glass column (with an inner diameter of 10 mm) with the SEC medium to the 15 mL mark.

NOTE: Volumes will differ for columns with different dimensions. Make sure to let the gel settle completely.

5.1.4. Before a run, equilibrate the column with at least two column volumes of PBS. To store the column, first wash it with one column volume of water, followed by at least two column volumes of 20% EtOH. After storage, wash the column first with one column volume of water before equilibrating with PBS.

5.1.5. Use up to 400 µL of EV or liposome suspension in one separation. Collect fractions of 1 mL. After SEC, either store the purified EVs (see section 7) or subject them to a glucuronidase assay (see section 6).

5.2. Confirm the separation of EVs and liposomes from contaminating proteins and free glucuronidase. To this end, correlate the particle concentrations of the collected fractions with the protein concentration and the glucuronidase activity.

5.2.1. Assess the particle concentration by NTA (see 2.5)

5.2.2. Assess the protein content by bicinchoninic acid (BCA) assay or another suitable protein quantification assay. Perform the assay according to the manufacturer's protocol.

5.2.3. Assess the glucuronidase activity by glucuronidase assay (see section 6).

5.3. Optionally, assess the EV morphology by transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

5.3.1. For the preparation of TEM samples, add 10 µL of EV suspension to a TEM grid, incubate for 10 min, and then blot away any excess liquid using a filter paper. Perform the fixation for 10 min with 10 µL of 4% paraformaldehyde and blot away any excess. Wash 3x with water. Stain the vesicles by 20 s incubation with 30 µL of 1% phosphor-tungstic acid hydrate. After blotting away the excess, dry the vesicles overnight. Visualize by TEM.

CAUTION: Phospho-tungstic acid is highly caustic; thus, protect skin and eyes.

5.3.2. For SEM, fix the previously prepared TEM samples onto carbon disks and sputter them with a 50 nm thick gold layer. Visualize by SEM.

6. Glucuronidase assay

6.1. To allow a comparison between different samples and storage conditions by correlating particle number and enzyme activity, first measure the particle concentration of the sample by NTA (see step 2.5).

6.2. Prepare a working solution of fluorescein di-β-D-glucuronide by adding 1 µL of the compound (10 mg/mL in H_2O) to 199 µL of PBS. Add 25 µL of this solution to 125 µL of purified EVs to get a final concentration of 8.3 µg/mL. Pipet the sample into a black 96-well plate. Measure time point 0 h with a plate reader, using 480 nm as excitation and 516 nm as emission wavelength.

6.3. Cover the plate tightly (e.g., with transparent plastic foil used for PCR plates) to minimize evaporation and incubate in the dark for 18 h at 37 °C. Measure the fluorescein production using the plate reader parameters listed in step 6.2.

7. Storage of EVs and liposomes

NOTE: For all storage purposes, it is advisable to use low-binding tubes to reduce EV loss due to adsorption.

7.1. Follow the parameters in this section to reproduce the representative results given below. Use samples consisting of 400 µL of EV suspension.

7.1.1. Store at 4 °C or -80 °C or proceed to steps listed below.

7.1.2. Lyophilize the EVs.

7.1.2.1. Add trehalose (40 mg/mL in H_2O) up to a final concentration of 4 mg/mL to the purified EVs. Freeze the samples at -80 °C for at least 1 h.

7.1.2.2. Lyophilize the samples using the following parameters. For main drying, set the shelf temperature to 15 °C and pressure to 0.180 mbar and leave the samples to dry for 46 h. For final drying, set the shelf temperature to 25 °C and pressure to 0.0035 mbar and leave the samples to dry for 2 h. Store the lyophilized samples at 4 °C.

7.1.2.3. To rehydrate the samples, add H_2O, equal to the amount of EV suspension present in the beginning (typically, 400 µL). Do not use any buffer for rehydration.

8. Analysis after storage

8.1. To assess the enzyme activity, first remove the free glucuronidase, which may have leaked from the EVs during storage. Achieve this by an additional step of purification that is carried out either by SEC (see step 5) or asymmetric flow field-flow fractionation (AF4) (see step 8.1.2).

NOTE: Please be advised that both methods lead to a dilution of the EV sample; thus, use sufficient EV concentrations before storage to avoid moving below the NTA quantification limit. Expect a 1:10 dilution of the particles due to SEC or AF4.

8.1.1. For SEC purification, follow the protocol described above (see section 5).

8.1.2. Perform AF4 purification.

8.1.2.1. Set up the instrument using a small channel with a 350 µm spacer and a 30 kD molecular weight cut-off cellulose membrane. Place a 0.1 µm pore size filter between the HPLC pump and the AF4 channel. Use freshly prepared 0.1 µm-filtered PBS as the mobile phase to reduce the particle load and noise in the light-scattering detectors.

8.1.2.2. Detect proteins using a UV detector set to 280 nm. To detect particles, use multiangle light scattering with the laser set to 658 nm.

8.1.2.3. Use the following run method. Pre-focus for 1 min with a focus flow of 1 mL/min; then, inject 300 µL of the sample at a rate of 0.2 mL/min and keep up the focus flow for 10 min. After the injection, elute the sample at 1 mL/min while applying a cross flow that decreases from 2 mL/min to 0.1 mL/min over the course of 8 min. Elute for another 10 min without cross flow. Collect fractions of 1 mL, starting after 12.5 min and continuing until 27 min.

8.1.3. Perform the glucuronidase assay as described above (see section 6).

8.2. To assess the size and concentration, use the NTA as described above (see step 2.5).

8.3. Optionally, perform TEM and SEM to assess the morphology of the EVs after storage (see 5.3).

REPRESENTATIVE RESULTS:

Figure 1 displays the storage characteristics of EVs isolated from HUVECs. EVs were isolated by UC, glucuronidase was encapsulated, and after SEC, the purified EVs were evaluated for their physicochemical properties by NTA. A sample of the vesicles was subsequently subjected to AF4 purification and the glucuronidase activity was measured.

The vesicles were then stored for 7 d at 4 °C or -80 °C and at 4 °C in lyophilized form, in the latter case with the addition of 4% trehalose. After storage, the vesicles were again measured by NTA, and after AF4, the remaining glucuronidase activity per particle was assessed.

The working principle of AF4 is based on the combination of laminar flow and an orthogonal crossflow through the porous membrane at the bottom of the AF4 channel, which differentially affects particles according to their size (**Figure 2**). Larger particles tend to be located closer to the membrane while smaller particles are located further up in the channel. Due to the parabolic flow profile of the laminar flow, particles further away from the membrane travel faster toward the detector, leading to a particle-separation by size. In a typical AF4 experiment, the injected particles are first focused on the membrane, by applying a cross flow without a longitudinal flow through the channel (**Figure 2A**). After injection and focusing, the elution starts by simultaneously applying a cross flow and a longitudinal flow to fractionate and elute the different subsets of particles (**Figure 2B**), which, in our case, were EVs and free glucuronidase.

Figure 3 illustrates the separation of nanoparticles (EVs or liposomes) from contaminating proteins and nonencapsulated glucuronidase. The SEC elution profile of liposomes (**Figure 3A**) purified after their preparation (see section 4 of the protocol) showed the separation of the particles with encapsulated glucuronidase from the free enzyme, detected both through BCA assay and enzyme activity. In the present example, fraction 6 and 7 would be chosen for further experiments as they contained the highest particle concentrations and to prevent possible contaminations with free glucuronidase. AF4 was also successful in separating EVs from free glucuronidase as demonstrated in **Figure 3B**, with a higher degree of separation than SEC, making contamination of the fractions containing vesicles less probable.

In **Figure 4**, a control experiment was performed to ensure that the enzyme activity measured for the vesicles was indeed linked to the encapsulation of glucuronidase into EVs and not caused by enzyme aggregates. These aggregates might have been formed due to the incubation of glucuronidase with saponin and lead to false positive results. To verify the fractions where vesicles would elute, purified EVs without encapsulated glucuronidase were subjected to SEC on the same column as the sample just containing saponin and the enzyme.

When glucuronidase was incubated with saponin and subsequently purified by SEC, no enzyme activity was found in the fractions typically containing EVs. While small amounts of particles were

found to elute at the same time as EVs (making up <0.1% of the particles recovered from a typical EV pellet), there was no correlating enzyme activity. These results indicate that active enzyme recovered in the fractions containing vesicles was encapsulated in them.

Figure 5 compares the recovery of active enzyme after storage with or without additional AF4 purification to remove nonencapsulated dye. With AF4 purification, there is generally a lower recovery of enzyme per particle, with the highest effect for storage at 4 °C, where recovery drops by two-thirds. Thus, omitting this additional purification step can lead to wrong assumptions about the enzyme stability.

Figure 6 shows the effect of lyophilization without a cryoprotectant on vesicles derived from MSCs, A549 cells, and liposomes, compared with freezing at -80 °C. The particles were imaged by TEM and SEM as described above (see step 5.3 of the protocol). The MSCs and A549 EVs did not exhibit big differences in shape in TEM images, comparing the two storage conditions. In the SEM pictures, however, the lyophilized samples displayed aggregates not found in the -80 °C samples. Liposomes also displayed aggregates in the SEM picture, while in TEM, lyophilization appeared to induce a size increase of liposomes. Lyophilization without cryoprotectants also reduced the recovery of intact glucuronidase after storage (**Figure 7**). The addition of trehalose as a cryoprotectant increased the recovery of the active enzyme in a dose-dependent manner.

Figure 8 demonstrates the conversion of fluorescein di-β-D-glucuronide to free fluorescein, taking place in the glucuronidase assay. While the educt was nonfluorescent at 516 nm, fluorescein is highly fluorescent at this wavelength. This allowed for a straightforward enzyme activity assay with high sensitivity.

FIGURE AND TABLE LEGENDS:

Figure 1: Storage stability of HUVEC EVs. (**A**) Particle recovery compared to before storage, (**B**) mean size, and (**C**) the normalized glucuronidase activity per particle of EVs isolated from HUVECs. Vesicles were stored for 7 d at 4 °C and -80 °C and at 4 °C after lyophilization with 4% trehalose. The mean size and particle recovery were measured by NTA; the glucuronidase activity per particle was calculated combining NTA data and the results of the glucuronidase assay and normalized to before storage. Mean ± SD, $n = 3$, $*p < 0.05$ (one-way ANOVA followed by Tukey's post hoc test). Modified from Frank et al.[21].

Figure 2: Working principle of the AF4. (**A**) EVs and free glucuronidase are focused after injection by applying a cross flow. (**B**) Afterward, EVs and free enzyme are eluted separately by combining the flow through the channel with a cross flow. Modified from Frank et al.[21].

Figure 3: Separation of EVs and liposomes from free glucuronidase. (**A**) Representative SEC separation of glucuronidase-loaded liposomes, free glucuronidase, and protein contaminants. The graph displays the particle concentration (red), protein concentration (blue), and glucuronidase activity (green). (**B**) Demonstration of the separation of EVs from free glucuronidase. Untreated EVs, EVs spiked with 0.05 mg/mL glucuronidase, free glucuronidase

(0.5 mg/mL), and EVs loaded with glucuronidase and purified by SEC (EV glucuronidase) were injected and analyzed by UV at 280 nm and 90° light scattering. Free enzyme eluted separately from EVs. Modified from Frank et al.[21].

Figure 4: Control experiments for the purification of EVs from free glucuronidase. 400 µL of native EVs or 400 µL of 1.5 mg/mL glucuronidase in PBS incubated for 10 min with 0.1 mg/mL saponin were purified by SEC. (**A**) Particle concentrations of the collected fractions of vesicles and glucuronidase, respectively, and the enzyme activity of the purified glucuronidase. (**B**) UV absorption at 280 nm measured in the same experiment. The first small peak for glucuronidase corresponds with the grey line in panel **A**.

Figure 5: The effect of additional purification steps after EV storage. Remaining glucuronidase activity per particle compared to d0 with or without an additional AF4 purification step after storage. HUVEC EVs were stored for 7 d at 4 °C or -80 °C and lyophilized with 4% trehalose.

Figure 6: TEM and SEM pictures of EVs. TEM and SEM pictures of EVs from (**A**) MSCs and (**B**) A549 cells and (**C**) liposomes. Samples were stored for 14 d at -80 °C or lyophilized without the addition of trehalose. Arrows indicate the presence of morphologically altered particles in the TEM pictures and aggregates in the SEM pictures. Modified from Frank et al.[21].

Figure 7: The effect of trehalose on enzyme recovery after lyophilization. Comparison of the enzyme activity after lyophilization for 14 days, with 0%, 1%, and 4% trehalose with the sample before storage (0 days). Modified from Frank et al.[21].

Figure 8: The enzymatic cleavage of fluorescein di-β-D-glucuronide by glucuronidase. In the scheme, the reaction underlying the detection of glucuronidase is explained. Nonfluorescent fluorescein di-β-D-glucuronide is cleaved by glucuronidase. Through the removal of the sugar residues, fluorescein regains its fluorescent properties. The fluorescence measured after the incubation period correlates with the amount of active enzyme present and is the readout of the glucuronidase assay.

DISCUSSION:

In this manuscript, we present a comprehensive protocol to study the stability of EVs under different storage conditions. With the combination of encapsulated glucuronidase as a functional readout and the evaluation of the physicochemical parameters of the EVs, the protocol allows for a straightforward storage stability evaluation of EVs and the comparison of EVs from different cell lines. SEM and TEM as complementary methods allow an insight into changes of the EVs on the single-particle level. The results presented here showed a tendency of the EVs and liposomes to aggregate due to lyophilization without a cryoprotectant (**Figure 6**). However, this was only observed in the SEM experiments. While EM imaging was not conducted with samples that were preserved with trehalose, the literature suggests that this cryoprotectant might indeed reduce the aggregation of EVs[29]. It is also possible to assess, if a given storage condition differentially affects EV size, the recovery rate and encapsulated molecules. Such an effect is exemplified by the results obtained for HUVEC EVs (**Figure 1**). While the particle recovery for 4 °C and -80 °C was

better than for the lyophilized samples, the recovery of active encapsulated glucuronidase was best for the lyophilized samples. Lyophilization of EVs could be the more favorable storage condition, for instance, when analyzing EV biomarkers, where the focus is more on obtaining and preserving intact cargo rather than on intact vesicles.

In their recent paper on the lyophilization of EVs[22], Charoenviriyakul et al. followed a comparable approach. Regarding the physicochemical parameters, they focused on the polydispersity index (PDI) and the zeta potential, as changes in zeta potential can correlate with reduced colloidal stability. However, the zeta potential cannot solely explain observed changes in stability[30], which was also reflected in their results. They also compared the protein and RNA content of stored EVs by polyacrylamide gel electrophoresis. This technique can very well indicate substantial changes in the protein or RNA content of the vesicles. However, it requires much larger amounts of material than the method presented here. To assess the effect of storage on the EV cargo, Charoenviriyakul et al. heterologously expressed an enzyme and DNA species each in their EV producer cell line and monitored their activity and integrity in the EVs. However, such heterologous expression is not suitable if EVs from different sources are to be compared, as it requires a substantial amount of time for each new cell line, while the simple saponin-mediated encapsulation can be readily applied.

It is crucial to remove any nonencapsulated enzyme, as shown in **Figure 3**. EV-encapsulated glucuronidase reacts much slower with its substrate than free enzyme in solution, leading to an overestimation of the encapsulation efficiency. Clean separation is especially important for the analysis after storage, as the storage conditions might affect the activity of free glucuronidase in the sample differently and might lead to leakage from damaged EVs. This was apparent in our experiments (**Figure 5**), as the application of AF4 before the glucuronidase assay managed to remove dye not encapsulated in the vesicles. Thus, it made it possible to get clear results on the effect of the storage methods discussed here, while without AF4, the activity loss due to storage would have been underestimated.

Another important consideration is the isolation and characterization of the EVs to be tested for their stability. Although the described technique enables the comparison of EVs from different sources, this is only possible if all of them are prepared by the same isolation method so the results are not distorted[18,31,32]. In this context, cell culture conditions need to be taken into consideration also, as they can impact the quantity and bioactivity of the isolated EVs[33]. To obtain results that can be compared to other published results, it is advisable to consult the recently updated “Minimal information for studies of extracellular vesicles” that contains guidelines for the harmonization of EV research[26].

In the protocol presented here, we used SEC or AF4 for removing the free enzyme after storage. Other methods could be applied, such as gradient ultracentrifugation, UC, or ultrafiltration[34]. Compared to centrifugal methods, SEC and AF4 are less time-consuming (e.g., roughly 1.5 h for SEC including column equilibration versus up to 16 h or more for gradient UC) and they can completely separate free proteins from the EVs in comparison to normal UC, where there always remains residual supernatant with the pellet. Moreover, SEC[15] and AF4[35] are mild methods, which

induce less shear stress on the EVs. In comparison, the forces imposed upon EVs during UC might lead to alterations of the particles, such as aggregation and alterations in size[34,36].

A disadvantage of SEC and AF4 is the dilution of the samples. Thus, it is required to isolate sufficient amounts of EVs to maintain the concentrations required for NTA measurements after multiple purification steps. EV fractions may be concentrated using centrifugal filters but, depending on the filter material and the protocol, there could be a loss of vesicles[37].

The limitation of the protocol discussed here is that it only monitors the enzyme activity of exogenously encapsulated glucuronidase, neither taking into account encapsulated RNA and DNA nor the EV surface proteins that might be important for functionality[18]. For the research of new EV therapeutics, this technique cannot replace assays on functionality but compliment them and give an indication about vesicle stability. It could be of great use if, for example, EVs derived from biofluids are to be stored for later analysis of their vesicle content.

In the future, the scope of this protocol could be expanded to also include EVs derived from other organisms, for instance, bacterial outer membrane vesicles. Another interesting next step would be to test the encapsulation of nucleic acids by saponin incubation and to also look into the stability of DNA or RNA cargos.

In conclusion, this procedure offers a simple method for the assessment of the storage stability of EVs from various mammalian cell sources by integrating both a physicochemical and a functional readout. This detailed protocol will allow EV researchers a straightforward determination of suitable storage conditions for their vesicles.

ACKNOWLEDGMENTS:

The NanoMatFutur Junior Research program from the Federal Ministry of Education and Research, Germany (grant number 13XP5029A) supported this work. Maximilian Richter was supported by Studienstiftung des Deutschen Volkes (German Academic Scholarship Foundation) through a Ph.D. fellowship.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Stremersch, S., De Smedt, S. C., Raemdonck, K. Therapeutic and diagnostic applications of extracellular vesicles. *Journal of Controlled Release.* **244, Part B**, 167-183, doi:10.1016/j.jconrel.2016.07.054 (2016).

2. Fuhrmann, G., Herrmann, I. K., Stevens, M. M. Cell-derived vesicles for drug therapy and diagnostics: Opportunities and challenges. *Nano Today.* **10** (3), 397-409, doi:10.1016/j.nantod.2015.04.004 (2015).

3. Goes, A., Fuhrmann, G. Biogenic and Biomimetic Carriers as Versatile Transporters To Treat Infections. *ACS Infectious Diseases.* **4** (6), 881-892, doi:10.1021/acsinfecdis.8b00030 (2018).

4. György, B., Hung, M. E., Breakefield, X. O., Leonard, J. N. Therapeutic Applications of

528 Extracellular Vesicles: Clinical Promise and Open Questions. *Annual Review of Pharmacology and*
529 *Toxicology.* **55**, 439-464, doi:10.1146/annurev-pharmtox-010814-124630 (2015).
530 5. Schulz, E. et al. Biocompatible bacteria-derived vesicles show inherent antimicrobial activity.
531 *Journal of Controlled Release.* **290**, 46-55, doi:10.1016/j.jconrel.2018.09.030 (2018).
532 6. Feng, Q. et al. A class of extracellular vesicles from breast cancer cells activates VEGF receptors
533 and tumour angiogenesis. *Nature Communications.* **8**, 14450-14450, doi:10.1038/ncomms14450
534 (2017).
535 7. Bewicke-Copley, F. et al. Extracellular vesicles released following heat stress induce bystander
536 effect in unstressed populations. *Journal of Extracellular Vesicles.* **6**, 1340746 (2017).
537 8. Xu, Y. et al. Macrophages transfer antigens to dendritic cells by releasing exosomes containing
538 dead-cell-associated antigens partially through a ceramide-dependent pathway to enhance
539 CD4(+) T-cell responses. *Immunology.* **149** (2), 157-171, doi:10.1111/imm.12630 (2016).
540 9. Buzas, E. I., György, B., Nagy, G., Falus, A., Gay, S. Emerging role of extracellular vesicles in
541 inflammatory diseases. *Nature Reviews Rheumatology.* **10**, 356, doi:10.1038/nrrheum.2014.19
542 (2014).
543 10. Rajappa, P. et al. Malignant Astrocytic Tumor Progression Potentiated by JAK-mediated
544 Recruitment of Myeloid Cells. *Clinical Cancer Research: An Official Journal of the American*
545 *Association for Cancer Research.* **23** (12), 3109-3119, doi:10.1158/1078-0432.CCR-16-1508
546 (2017).
547 11. Umezu, T. et al. Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances
548 angiogenesis by targeting factor-inhibiting HIF-1. *Blood.* **124** (25), 3748-3757, doi:10.1182/blood-
549 2014-05-576116 (2014).
550 12. Costa-Silva, B. et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in
551 the liver. *Nature Cell Biology.* **17** (6), 816-826, doi:10.1038/ncb3169 (2015).
552 13. Boulanger, C. M., Loyer, X., Rautou, P.-E., Amabile, N. Extracellular vesicles in coronary artery
553 disease. *Nature Reviews Cardiology.* **14**, 259, doi:10.1038/nrcardio.2017.7 (2017).
554 14. Zhu, X. et al. Comprehensive toxicity and immunogenicity studies reveal minimal effects in
555 mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *Journal of*
556 *Extracellular Vesicles.* **6** (1), 1324730, doi:10.1080/20013078.2017.1324730 (2017).
557 15. Vader, P., Mol, E. A., Pasterkamp, G., Schiffelers, R. M. Extracellular vesicles for drug delivery.
558 *Advanced Drug Delivery Reviews.* **106, Part A**, 148-156, doi:10.1016/j.addr.2016.02.006 (2016).
559 16. Ingato, D., Lee, J. U., Sim, S. J., Kwon, Y. J. Good things come in small packages: Overcoming
560 challenges to harness extracellular vesicles for therapeutic delivery. *Journal of Controlled*
561 *Release.* **241**, 174-185, doi:10.1016/j.jconrel.2016.09.016 (2016).
562 17. Gimona, M., Pachler, K., Laner-Plamberger, S., Schallmoser, K., Rohde, E. Manufacturing of
563 Human Extracellular Vesicle-Based Therapeutics for Clinical Use. *International Journal of*
564 *Molecular Sciences.* **18** (6), 1190, doi:10.3390/ijms18061190 (2017).
565 18. Jeyaram, A., Jay, S. M. Preservation and Storage Stability of Extracellular Vesicles for
566 Therapeutic Applications. *The AAPS Journal.* **20** (1), 1, doi:10.1208/s12248-017-0160-y (2017).
567 19. Lőrincz, Á. M. et al. Effect of storage on physical and functional properties of extracellular
568 vesicles derived from neutrophilic granulocytes. *Journal of Extracellular Vesicles.* **3,** 25465-25465,
569 doi:10.3402/jev.v3.25465 (2014).
570 20. Kreke, M., Smith, R., Hanscome, P., Peck, K., Ibrahim, A. Processes for producing stable
571 exosome formulations. WO/2016/090183. Beverly Hills, CA (2016).

572 21. Frank, J. et al. Extracellular vesicles protect glucuronidase model enzymes during freeze-
573 drying. *Scientific Reports.* **8** (1), 12377, doi:10.1038/s41598-018-30786-y (2018).
574 22. Charoenviriyakul, C., Takahashi, Y., Nishikawa, M., Takakura, Y. Preservation of exosomes at
575 room temperature using lyophilization. *International Journal of Pharmaceutics.* **553** (1), 1-7,
576 doi:10.1016/j.ijpharm.2018.10.032 (2018).
577 23. Kusuma, G. D. et al. To Protect and to Preserve: Novel Preservation Strategies for Extracellular
578 Vesicles. *Frontiers in Pharmacology.* **9** (1199), doi:10.3389/fphar.2018.01199 (2018).
579 24. Haney, M. J. et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *Journal*
580 *of Controlled Release.* **207**, 18-30, doi:10.1016/j.jconrel.2015.03.033 (2015).
581 25. Fuhrmann, G., Serio, A., Mazo, M., Nair, R., Stevens, M. M. Active loading into extracellular
582 vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *Journal*
583 *of Controlled Release.* **205**, 35-44, doi:10.1016/j.jconrel.2014.11.029 (2015).
584 26. Théry, C. et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a
585 position statement of the International Society for Extracellular Vesicles and update of the
586 MISEV2014 guidelines. *Journal of Extracellular Vesicles.* **7** (1), 1535750,
587 doi:10.1080/20013078.2018.1535750 (2018).
588 27. Gardiner, C., Ferreira, Y. J., Dragovic, R. A., Redman, C. W. G., Sargent, I. L. Extracellular vesicle
589 sizing and enumeration by nanoparticle tracking analysis. *Journal of Extracellular Vesicles.* **2** (1),
590 19671, doi:10.3402/jev.v2i0.19671 (2013).
591 28. Vestad, B. et al. Size and concentration analyses of extracellular vesicles by nanoparticle
592 tracking analysis: a variation study. *Journal of Extracellular Vesicles.* **6** (1), 1344087-1344087,
593 doi:10.1080/20013078.2017.1344087 (2017).
594 29. Bosch, S. et al. Trehalose prevents aggregation of exosomes and cryodamage. *Scientific*
595 *Reports.* **6,** 36162, doi:10.1038/srep36162 (2016).
596 30. Bhattacharjee, S. DLS and zeta potential – What they are and what they are not? *Journal of*
597 *Controlled Release.* **235**, 337-351, doi:10.1016/j.jconrel.2016.06.017 (2016).
598 31. Van Deun, J. et al. The impact of disparate isolation methods for extracellular vesicles on
599 downstream RNA profiling. *Journal of Extracellular Vesicles.* **3** (1), 24858,
600 doi:10.3402/jev.v3.24858 (2014).
601 32. Taylor, D. D., Shah, S. Methods of isolating extracellular vesicles impact down-stream analyses
602 of their cargoes. *Methods.* **87**, 3-10, doi:10.1016/j.ymeth.2015.02.019 (2015).
603 33. Patel, D. B. et al. Impact of cell culture parameters on production and vascularization
604 bioactivity of mesenchymal stem cell-derived extracellular vesicles. *Bioengineering &*
605 *Translational Medicine.* **2** (2), 170-179, doi:10.1002/btm2.10065 (2017).
606 34. Gardiner, C. et al. Techniques used for the isolation and characterization of extracellular
607 vesicles: results of a worldwide survey. *Journal of Extracellular Vesicles.* **5** (1), 32945,
608 doi:10.3402/jev.v5.32945 (2016).
609 35. Zhang, H. et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by
610 asymmetric flow field-flow fractionation. *Nature Cell Biology.* **20** (3), 332-343,
611 doi:10.1038/s41556-018-0040-4 (2018).
612 36. Linares, R., Tan, S., Gounou, C., Arraud, N., Brisson, A. R. High-speed centrifugation induces
613 aggregation of extracellular vesicles. *Journal of Extracellular Vesicles.* **4** (1), 29509,
614 doi:10.3402/jev.v4.29509 (2015).
615 37. Lobb, R. J. et al. Optimized exosome isolation protocol for cell culture supernatant and human

616 plasma. *Journal of Extracellular Vesicles.* **4**, 27031, doi:10.3402/jev.v4.27031 (2015).

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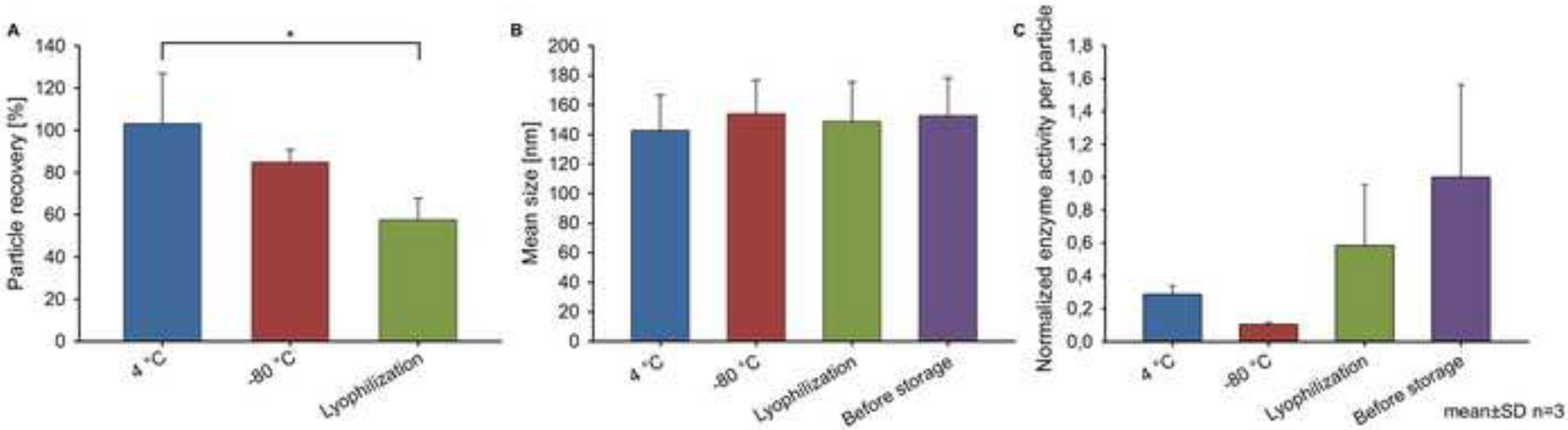

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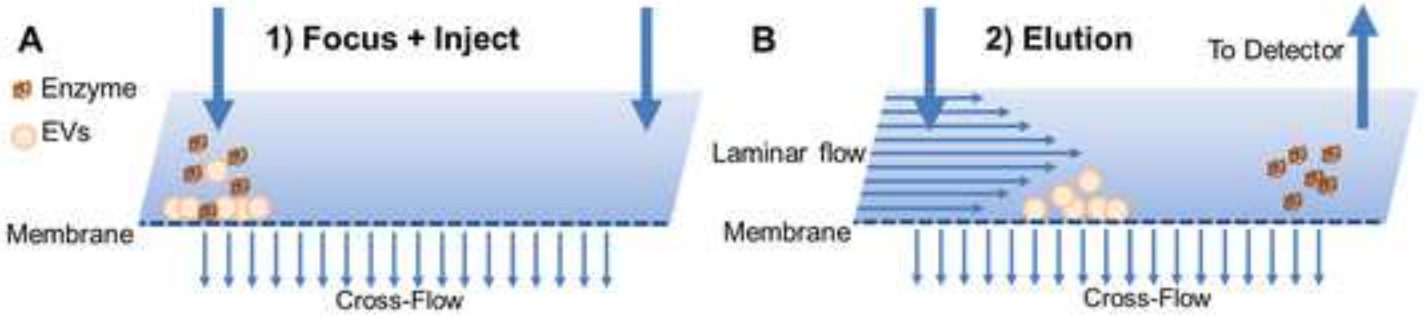

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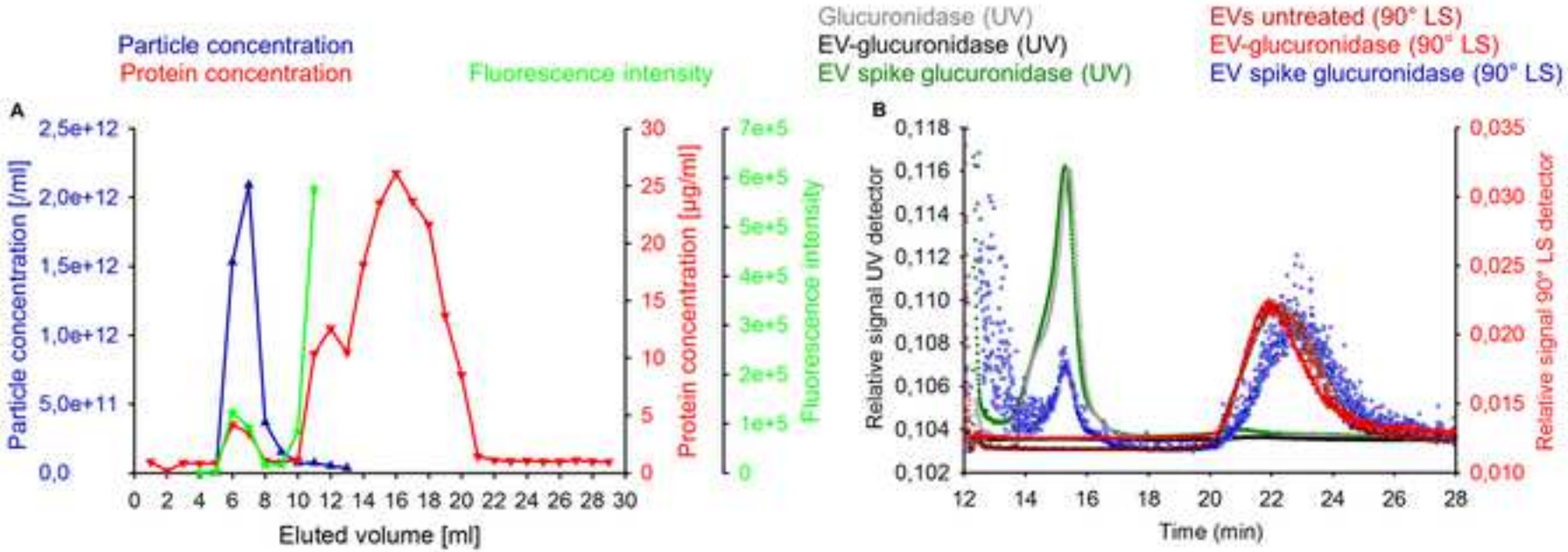

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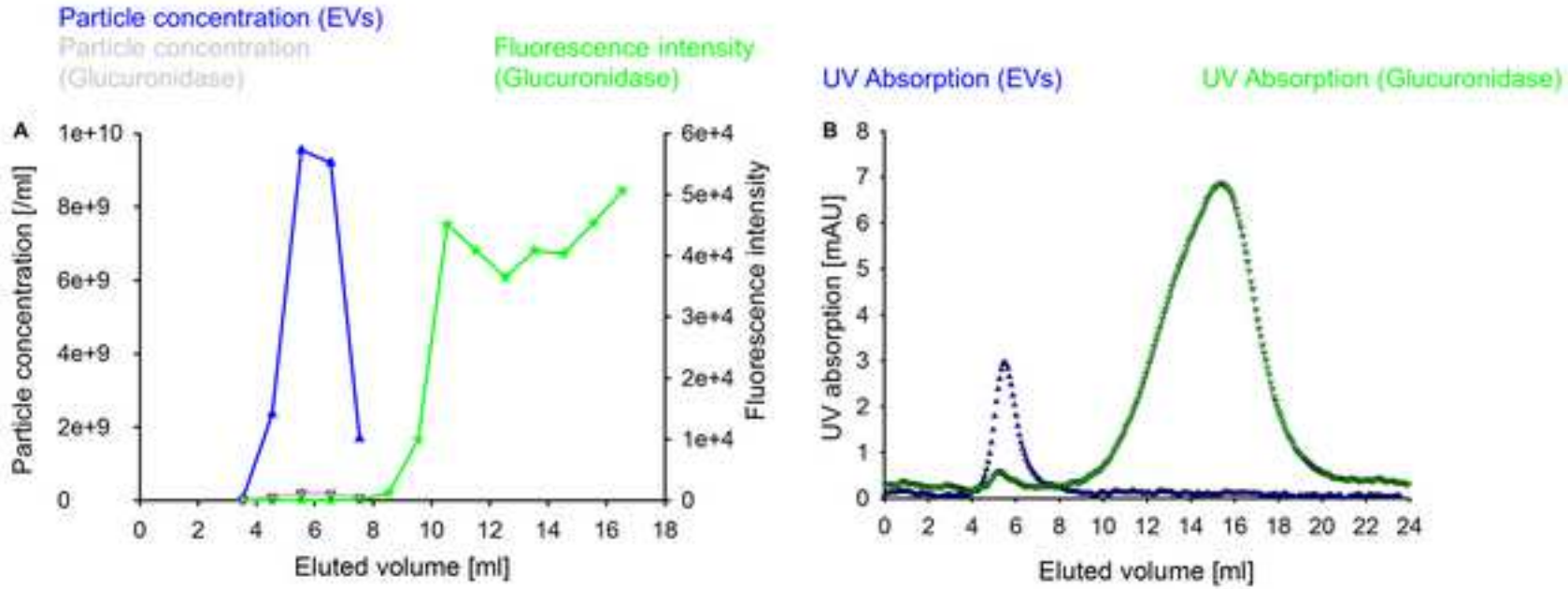

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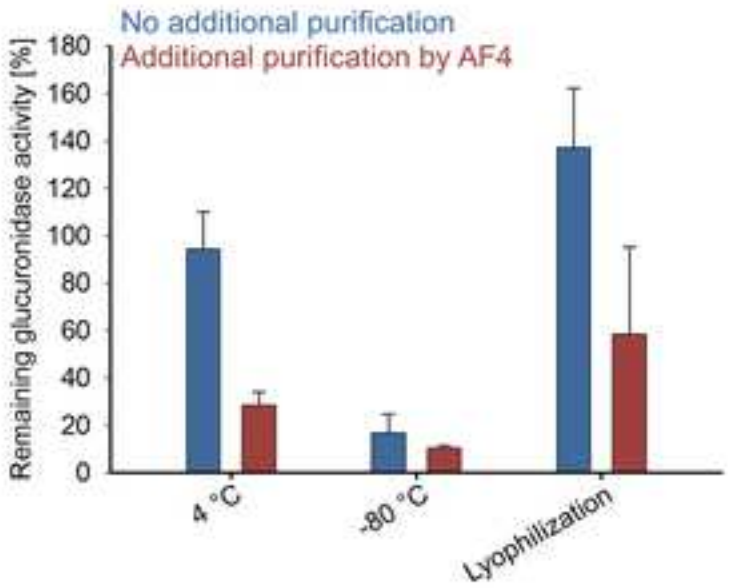

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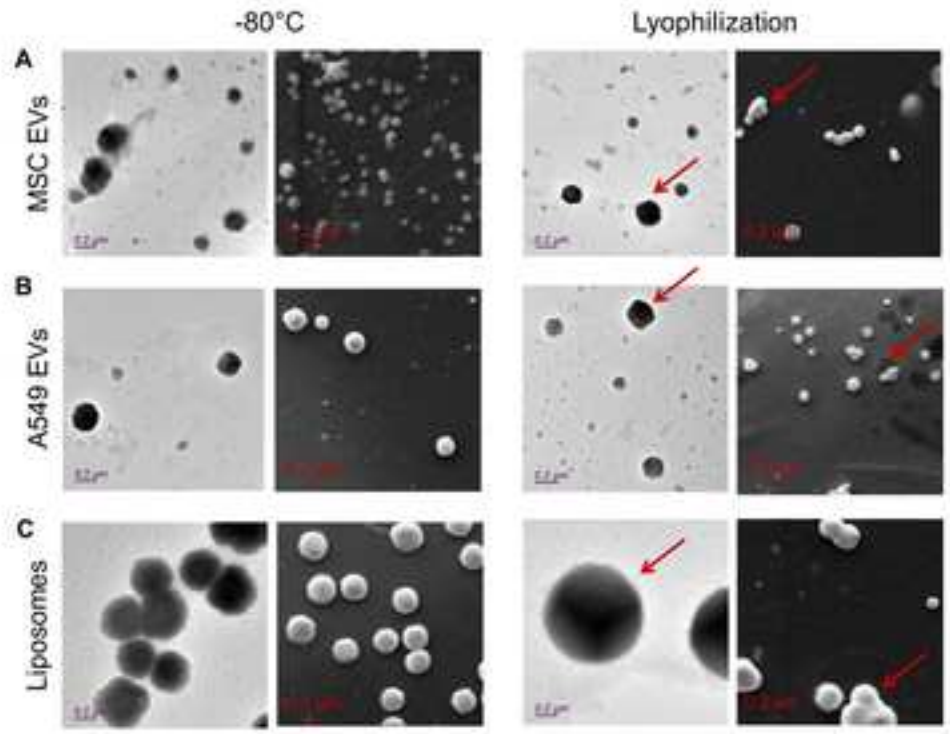

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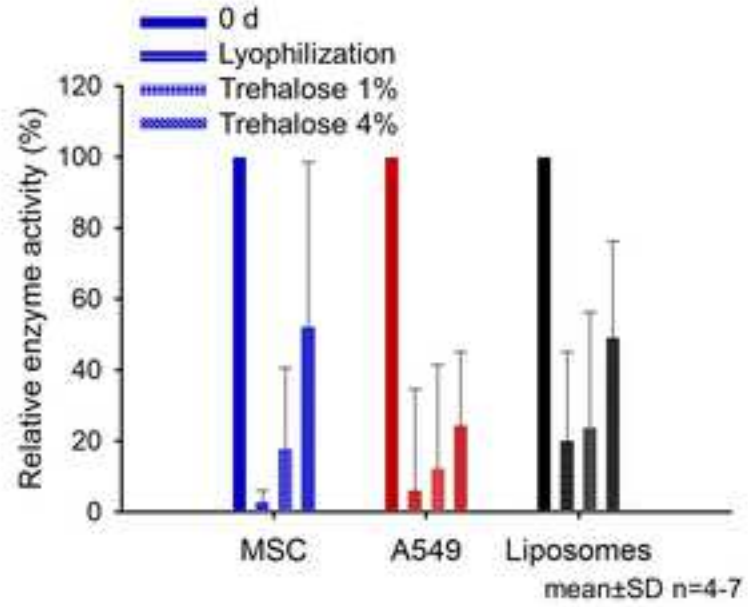

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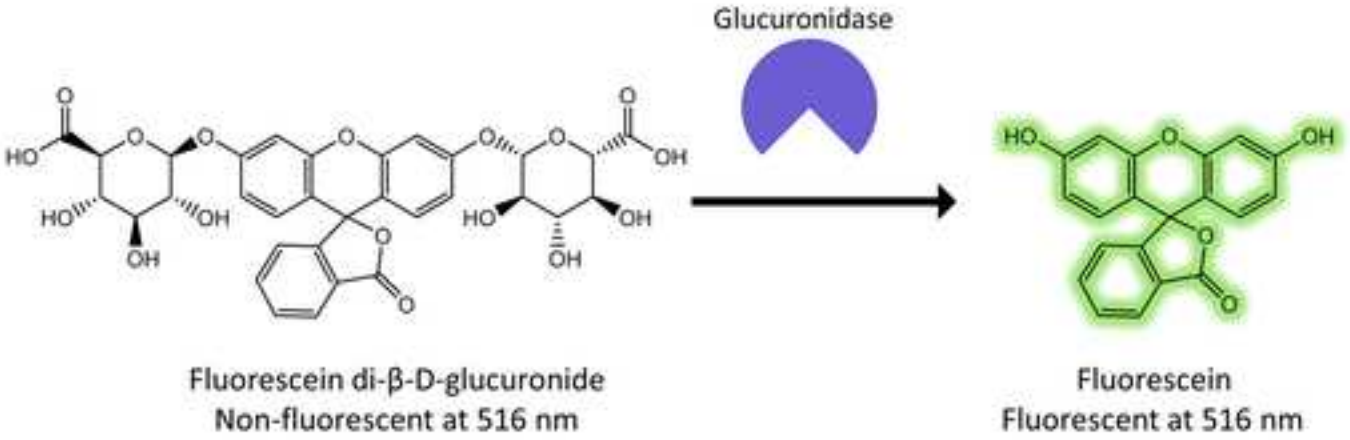

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
1,2 dimyristoyl-sn glycero-3-phospho-choline (DMPC)	Sigma-Aldrich	P2663-25MG
1,2-dipalmitoyl-sn-glycero-3-phospho-choline (DPPC)	Sigma-Aldrich	P4329-25MG
225 cm² cell culture flasks	Corning	431082
30 kDa regenerated cellulose membrane	Wyatt Technology Europe	1854
350 µm spacer	Wyatt Technology Europe	
Automated fraction collector	Thermo Fisher Scientific	
Beta-glucuronidase	Sigma-Aldrich	G7646-100KU
Chloroform	Fisher scientific	C/4966/17
Column oven	Hitachi High-Technologies Europe	
D-(+)-Trehalose dihydrate	Sigma-Aldrich	T9531-10G
DAWN HELEOS II, Multi-angle light scattering detector	Wyatt Technology Europe	
Durapore Membrane filter, PVDF, 0,1 µm, 47 mm	Merck	VVLP04700
EBM-2	Lonza Verviers, S.p.r.	CC-3156
Eclipse dualtec	Wyatt Technology Europe	
EGM-2	Lonza Verviers, S.p.r.	CC-3162
ELISA Plate Sealers	R&D Systems	DY992
Ethanol	Fisher scientific	E/0665DF/17
Extruder Set With Holder/Heating Block	Avanti Polar Lipids	610000-1EA
Filter support	Avanti Polar Lipids	610014-1EA
Fluorescein di-β-D-glucoronide	Thermo Fisher Scientific	F2915
Gibco PBS-tablets+CA10:F36	Thermo Fisher Scientific	18912014
Hettich Universal 320 R	Andreas Hettich GmbH & Co.KG	
Hettich Rotina 420 R	Andreas Hettich GmbH & Co.KG	
HUVEC cells	Lonza Verviers, S.p.r.	C2517A
Kimble FlexColumn 1X30CM	Kimble	420401-1030
Lyophilizer ALPHA 2-4 LSC	Christ	
Microcentrifuge Tubes, Polypropylene	VWR international	525-0255
Nanosight LM14 equipped with a green laser	Malvern Pananalytical	
Nanosight-software version 3.1	Malvern Pananalytical	

Nucleopore 200 nm track-etch polycarbonate membranes	Whatman/GE Healthcare	110406
PEEK Inline filter holder	Wyatt Technology Europe	
Phosphotungstic acid hydrate	Sigma-Aldrich	79690-25G
Polycarbonate bottles for ultracentrifugation	Beckman Coulter	355622
QuantiPro BCA Assay Kit	Sigma-Aldrich	QPBCA-1KT
Saponin	Sigma-Aldrich	47036
Scanning electron microscopy Zeiss EVO HD 15	Carl Zeiss AG	
Sepharose Cl-2b	GE Healthcare	17014001
SEM copper grids with carbon film	Plano	S160-4
Small AF4 channel	Wyatt Technology Europe	
Sputter-coater Q150R ES	Quorum Technologies	
Transmission electron microscopy JEOL JEM 2011	Oxford Instruments	
Type 45 Ti ultracentrifugation rotor	Beckman Coulter	339160
Ultimate 3000 Dionex autosampler	Thermo Fisher Scientific	
Ultimate 3000 Dionex isocratic pump	Thermo Fisher Scientific	
Ultimate 3000 Dionex online vacuum degasser	Thermo Fisher Scientific	
Ultracentrifuge OptimaTM L-90 K	Beckman Coulter	
UV detector	Thermo Fisher Scientific	
Whatman 0.2 µm pore size mixed cellulose filter	Whatman/GE Healthcare	10401712

Comments/Description

Used with 25 ml of medium

Used for the preparation of buffers for AF4
Endothelial Cell Growth basal medium, used for the serum free culture of HUVEC cells

Endothelial Cell Growth medium, used for the normal culture of HUVEC cells
used for sealing of 96-well plates for the glucuronidase assay

used for liposome preparation

Used for pelleting cells at 300 g
Used for pelleting larger debris at 3000 g

the tubes used for all EV-handling, found to be more favorable than comparable products from other suppliers regarding parti

used for liposome preparation

Used for the filtration of all buffers used with the EVs and in SEC

cle recovery

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Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please correct the title of the manuscript in the word doc.

Our response: The title has been corrected.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Our response: The summary has been rephrased to represent our protocol more clearly.

Modified: l. 28/29: "Here we present a readily applicable protocol to assess the storage stability of extracellular vesicles, a group of naturally occurring nanoparticles produced by cells."

4. Please define all abbreviations during the first-time use.

Our response: Omitted and redundant definitions have been corrected.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Our protocol: The protocol, has been revised to comply with the guidelines stated above and under bullet points 6, 7 and 8.

6. The Protocol should contain only action items that direct the reader to do something.
7. The Protocol should be made up almost entirely of discrete steps, containing hard experimental steps, graphical user interface, button clicks, knob turns etc. without large paragraphs of text between sections.
8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
9. 2.5 Please explain how to perform this step? What to look for? How is the procedure done?

Our response: The most important parameters for performing a Nanosight measurement are given under 2.5.1. As describing all details of the optimization and measuring process would be beyond the scope of the present manuscript, we have added references to two papers dealing with this topic in detail.

Modified and added: l. 148-151: "Use the settings optimized for the given EV-type, such as the settings below (2.5.1).

NOTE: The papers of Gardiner et al.(Gardiner, Ferreira et al. 2013) and Vestad et al.(Vestad, Llorente et al. 2017) contain valuable information on how to optimize Nanosight parameters for measuring EVs."

10. 3.1: Importance of beta glucuronidase?

Our response: In our protocol we encapsulate the enzyme beta-glucuronidase into EVs to act as a surrogate of actual EV-cargos during storage experiments. A loss of activity during storages indicates conditions unfavorable for the enzyme and thus also for other cargos of the EVs.

11. Please ensure that there is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Our response: We have highlighted the parts of the protocol we think should be represented in the video to convey a clear and cohesive story. This now sums up to 2.75 pages.

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Our response: We have uploaded a .docx file containing the Creative Commons Attribution 4.0 International Public License.

13. Table 1: Please label the columns A, B, C, D.

Our response: Table 1 has been replaced with a figure during the review process.

14. Please alphabetically sort the materials table.

Our response: The materials table has been alphabetically sorted.

Reviewers' comments:

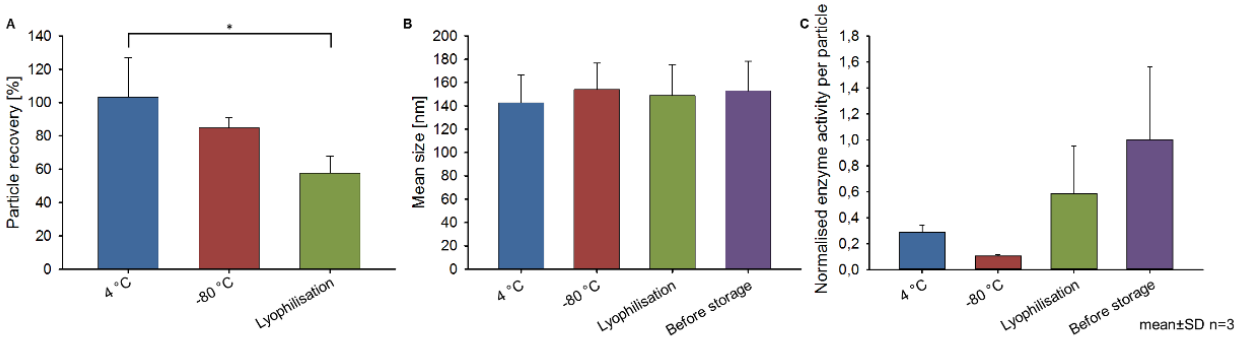
Reviewer #1:
Manuscript Summary:
The manuscript is very well written. Although the literature about extracellular vesicles (EV) is rapidly increasing, they authors have written a very concise and conceivable introduction, thereby stressing the aim of this protocol: evaluating storage conditions of EVs. The authors outline in the manuscript that storage of EVs between +4 and -80°C can cause alterations in

particle size and "activity" as measured by enzyme encapsulation. Apart from these readouts, they also use SEM and TEM to look at EVs morphologically. They among showed that lyophilisation is not necessarily a good storage option since particle aggregates could be observed in the absence of cryoprotectant. In summary, the authors provided a comprehensive protocol and discussion.

Major Concerns:
There are no major concerns.

Minor Concerns:
- Figure 1: Could the authors comment whether the changes in particle recovery, mean size and enzyme activity is statistically significant ? For instance, can one say that enzyme activity is significantly reduced at 4°C, -80°C and/or under lyophilised conditions compared to "before storage" ? In this figure, any statistical significance should be included.

> Our response: Reviewing our data we found significant differences for figure 1A. The figure and its legend have been changed accordingly.
>
> Modified: l. 379/380: "Mean ± SD, n=3, *p<0.05 (one-way ANOVA followed by Tukey post-hoc test)."



- Figure 1: Was particle recovery, EV size and enzyme activity measured in HUVECs because of a special reasons? Why not the other used cell lines in the article?

> Our response: HUVEC cells were chosen as an example for two reasons. Firstly, in our hands they were easier to culture in sufficient quantities, compared to mesenchymal stromal stem cells. Secondly, HUVECs already found some application as EV-producers in literature (e.g.: Kurachi et al. (DOI: 10.1371/journal.pone.0159158); Piffoux et al. (DOI: 10.1021/acsnano.8b02053); Lamichhane et al. (DOI: 10.1021/acs.molpharmaceut.5b00364)) and therefore to us seemed relevant and interesting to explore.

- How pure in the EV fraction and how does purity, or contaminations respectively, affect storage? Since there are different procedures to isolate EVs that might result in different purities (contaminants), it would be interesting to hear from the authors what they think about that.

Our response: The purity of the isolated EVs is always a great concern. Different methods of EV-isolation lead to different purity. Ultracentrifugation alone will always leave co-isolated protein agglomerates and residual conditioned medium, which might greatly affect downstream analysis. These contaminants can be efficiently removed by our SEC technique, using a stationary phase with a high MW cut-off, which is also shown by analyzing the SEC fractions using particle tracking and protein quantification (see Figure 3A). As our samples are derived from cell culture as opposed to plasma-samples, contamination of the EV-containing SEC-fractions with lipoproteins, as discussed in literature, is not an issue. Such samples would potentially be better purified by gradient ultracentrifugation, where the different buoyant densities of EVs and lipoproteins lead to their separation. As one important part of our protocol is measuring the particle concentration before and after storage the presence of significant amounts of particulate impurities in the same size range as the EVs would be problematic.

- Figure 4: Could the authors comment more on Figure 4. It looks that EVs from MSC and A549 have different sizes. Where is the aggregate in the SEM of the A549 picture? Is the arrow really pointing to an aggregate? It looks similar to the other structures.

Our response: Regarding EV-size, it is true that there seem to be size differences between MSC and A549 EVs. It is very much possible that these two cell types preferably produce differently sized vesicles. However, EVs are always a polydisperse mixture of vesicles in the size range between 30 and 1000 nm and it is possible that the EM pictures do not completely reflect the size-distribution of the EVs. Regarding the arrows, it indeed seems as if most A549 EVs in the SEM picture of the lyophilized sample have formed aggregates, which is especially clear for the particle on the left of the red arrow. Moreover, it has been clarified that arrows in the TEM pictures point to morphologically altered EVs, while they point to aggregates in the SEM pictures.

Modified: l. 409-411: "Arrows indicate the presence of morphologically altered particles in the TEM pictures and aggregates in the SEM pictures."

- Is it possible to exploit also another catalytic activity as functional readout instead of encapsulate glucuronidase? No encapsulation?

Our response: Due to the exogenous encapsulation of glucuronidase that works well with EVs from different cell lines, our protocol can be universally applied. To the authors' knowledge there is no widely distributed endogenous enzyme encapsulated in EVs from different parent cells, whose catalytic activity could be used to analyze and compare the storage stability of EVs. In case there is a specific catalytic activity known for EVs produced by the cell line of interest, it is of course preferable to use this as a marker for assessing storage stability.

Reviewer #2:

General comments: In this written protocol (that will be complemented with video) the authors describe a valuable method to assess functionality of EV after conservation. The topic is very relevant to characterize the processes involved in isolation and storage of EVs. Below are a few minor considerations that should be considered in the final version.

Specific comments:
L297-307: Although very clearly explained, I wonder about the relevance of this section and/or whether it is positioned in the right location. These seem very theoretical considerations, and I wonder if they are necessary. Researchers with access to AF4 would likely know most of what is described. In comparison, the manuscript does not provide much details on the fluorescent dye used to study the enzyme activity. Given the importance of the functional test (and why it justifies the use of glucuronidase as a model enzyme), I suggest the authors write a few lines on it, notably what type of signal is expected (increase/decrease or shift in ex/em wavelength).

Our response: This part of the article and the instructions under 8.1.2 are aimed at serving the purpose of introducing people completely unfamiliar with AF4 with the basic workings of the instrument and giving people with access to an AF4-instrument a basic protocol for starting out with vesicle purification. We would thus prefer to keep this section as is. Regarding the enzymatic reaction, we added another figure with the reaction scheme and in the legend explain the expected observations in more detail.

Added: l. 367-370: "Figure 8 demonstrates the conversion of fluorescein di-β-D-glucuronide to free fluorescein taking place in the glucuronidase assay. While the educt is non-fluorescent at 516 nm, fluorescein is highly fluorescent at this wavelength. This allows for a straightforward enzyme-activity assay with high sensitivity."
l. 417-422: "**Figure 8: The enzymatic cleavage of fluorescein di-β-D-glucuronide by glucuronidase**
In the scheme, the reaction underlying the detection of glucuronidase is explained. Non-fluorescent fluorescein di-β-D-glucuronide is cleaved by glucuronidase. Through the removal of the sugar residues fluorescein regains its fluorescent properties. The fluorescence measured after the incubation period correlates with the amount of active enzyme present and is the read-out of the glucuronidase assay.

Figure 8

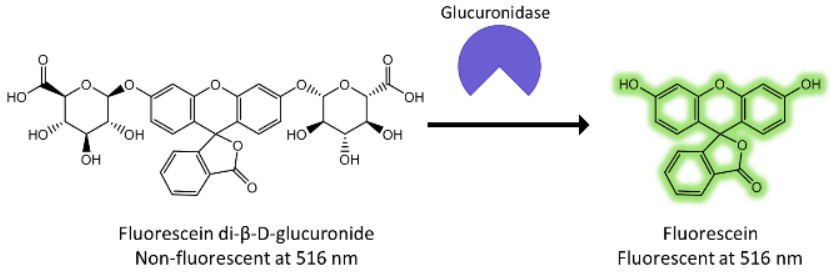


Figure 1, could benefit from identifying the type of measurement on each panel (above the histograms), to emphasize in one glimpse that the proposed method is complementary (and more sensitive to changes) to other common techniques (DLS (or NTA) and NTA for the first two panels). Also, the caption could also emphasize that, by naming the techniques, instead of repeating the information seen on the Y-axis.

Our response: We have modified the caption of the figure to make the type of measurement and the calculation of the results clear. As the type of measurement for panel (C) (calculation of the enzyme activity per particle from NTA- and glucuronidase assay-data, normalized to the activity per particle before storage) cannot be concisely

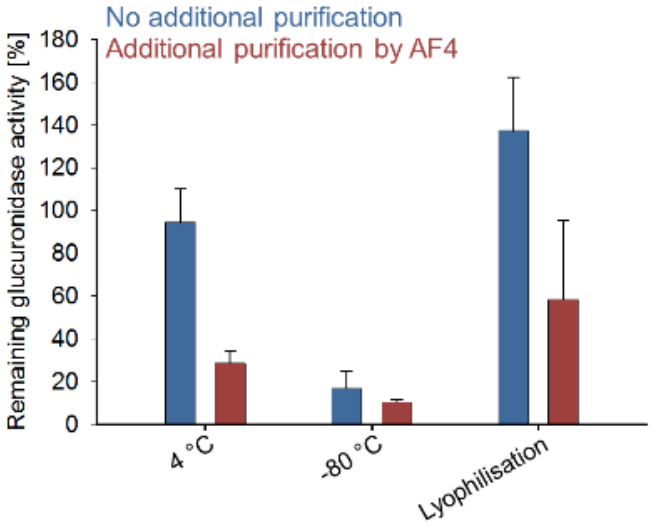
subsumed without omitting important information, we opted to omit the labelling of the panels to keep the look of the figure consistent.

Added: l. 377-379: “Mean size and particle recovery were measured by NTA, the glucuronidase activity per particle was calculated combining NTA-data and the results of the glucuronidase assay and normalized to before storage.”

Table 1 It is striking that the authors decided to present these results in a table, although they are somewhat comparable in nature to those presented in figure 5 as a histogram (that is, an activity after different storage conditions). The authors should consider making the presentation more uniform (I understand that the 137% might look surprising on a histogram, but it doesn't go unnoticed in the table either - and the authors explain it very well in the discussion).

Our response: As suggested, we have replaced the table with a histogram.

Added: Figure 5



Minor comments:
L137 : for clarity, I suggest using the words "immediately/promptly" instead of "directly" which can convey multiple meanings.

Our response: We have changed the sentence as suggested.

Changed: l. 157: “2.6 Use the pellet immediately if possible, otherwise it may be stored at 4 °C overnight.”

L316: the authors write "making contamination of the fractions containing vesicles less of an issue". Although I understand the intended meaning, I believe contamination is as much an issue (that is, it will interfere with the test IF fractions are contaminated), I suggest writing "less probable".

Our response: We have changed the sentence as suggested.

Changed: l. 336/337: "(…) making contamination of the fractions containing vesicles less probable."

L434: For clarity, I suggest replacing e.g. by "for example" and "biofluid-derived EVs" by "EVs derived from biofluids".

Our response: We have changed the sentence as suggested

Changed; l. 487/488: "It could be of great use if for example EVs derived from biofluids are to be stored for later analysis of their vesicle content."

Reviewer #3:

In this manuscript, Richter et al have described protocols for testing storage conditions of extracellular vesicles (EV). This is an important unmet need in the EV field, as researchers really don't know what conditions are optimal (or even acceptable) for storage of EVs. As the field expands we desperately need to answer these kinds of basic but fundamental questions. Overall I feel the results are sound and the work only needs some minor modifications.

In the introduction it would be good to example some more of the biological roles of EVs, such as playing roles in angiogenesis (PMID: 28205552), the immune response (PMID: 27278624) and stress response (PMID: 28717426).

Our response: As suggested, we included more examples for other biological roles of EVs and revised this section of the introduction.

Added: l. 60-65: "On the other side, EVs can promote tumor-vascularization (Feng, Zhang et al. 2017), induce bystander effects in stress response (Bewicke-Copley, Mulcahy et al. 2017) and might play a role in autoimmune diseases (Xu, Liu et al. 2016) and inflammatory diseases (Buzas, György et al. 2014). Thus, they might be a key component to a better understanding of many pathological effects. However, the presence of altered EVs in manifold diseases, such as cancer (Umezu, Tadokoro et al. 2014, Costa-Silva, Aiello et al. 2015, Rajappa, Cobb et al. 2017) and cardiovascular disorders (Boulanger, Loyer et al. 2017) , and their easy accessibility in blood and urine makes them ideal biomarkers."

The authors should go into more depth to discuss the caveat that the approach is based on the exogenous addition of an enzyme via saponin treatment of the EVs, and is not an assay for the endogenous activity of the EVs. Is it possible that saponin causes some structural or physical changes that alter their activity? How do those compare to endogenous assays of function such as that described in other papers (e.g. PMID: 28880029)?

Our response: In the introduction and the discussion we discuss how glucuronidase is only a surrogate for the native EV cargo (l.: 87) and indicate the limitations of our protocol (l.: 483-485). Furthermore, we underline that using an endogenous marker

would be advantageous and that our protocol cannot replace doing functional assays to evaluate EVs for eventual clinical application (line: 76/77; line: 485-487). In cases however, where such an endogenous marker is not known or when EVs from different cell lines need to be screened for their storage stability, our protocol provides a directly applicable way to evaluate EV-storage. To further emphasize the fact that our method is based on exogenous loading of an enzyme, we modified our discussion as indicated below.

While structural changes due to saponin incubation are of course a concern, two separate studies so far have shown that EV-activity was not compromised due to saponin-treatment and the treated EVs were able to efficiently deliver encapsulated porphyrins to tumor cells *in vitro* (Fuhrmann et al. DOI: 10.1016/j.jconrel.2014.11.029) and catalase to the brain of a Parkinson's disease mouse model respectively (Haney et al. DOI: 10.1016/j.jconrel.2015.03.033). Both these studies did not find significant changes in EV size or zeta potential upon saponin treatment.

Added: l. 483/484: "The limitation of our protocol is that it only monitors enzyme activity of exogenously encapsulated glucuronidase, (…)"

Step 1.3 suggests it is possible to use CCM directly or store overnight at 4oC or up to two weeks at -80oC. Given this is a paper about storage of EVs I feel there should be a bit more discussion and justification of this point. What is the effect of freezing the conditioned media on the EVs it contains? Similarly for step 2.6 - how stable are the EVs overnight at 4oC?

Our response: For storage at 4 °C overnight our own published data indicates that there is no change in EV-size or particle concentration due to overnight storage (Frank et al. (DOI: 10.1038/s41598-018-30786-y). While we did not observe any obvious differences between EVs isolated from fresh cell culture supernatant and supernatant stored at -80 °C, no systematic studies on this question were performed. Therefore, we thank the reviewer for voicing concerns about this bold statement, as it gives us the opportunity to rephrase it, to better convey that the immediate usage of the conditioned medium always should be the preferred alternative. Only in cases, where storage prior to EV-isolation cannot be circumvented, the supernatant should be stored, on the condition that all relevant parameters are recorded in accordance with the recently published MISEV2018 guidelines and potential biases are taken into consideration analyzing the data. We have modified our manuscript as indicated below.

Added/modified: l. 111-117: "Use CCM preferably directly or store over-night at 4 °C.

NOTE: It is always preferable to use freshly produced CCM. If storage for longer time periods cannot be circumvented, all relevant parameters should be recorded in accordance with MISEV2018 guidelines(Théry, Witwer et al. 2018) and the potential biases of the results acquired need to be taken into consideration."

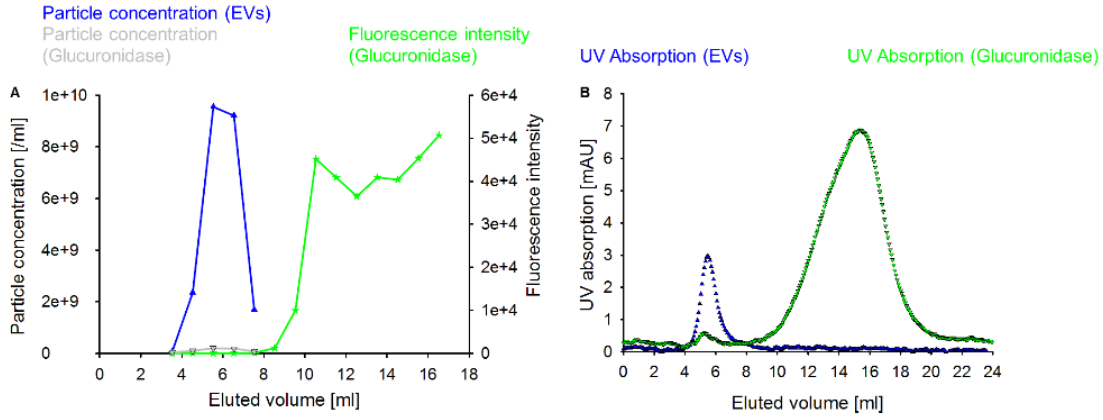
Do the authors have any specific evidence that the EVs have genuinely taken up the enzyme (and therefore EVs are being measured for 'activity'? Or could it be that enzyme aggregates are being measured? This potential caveat should be discussed.

Our response: We have included another figure into the manuscript to demonstrate that pure saponin-treated glucuronidase does not form aggregates that would co-elute with EVs (figure 4). In the absence of EVs, glucuronidase was incubated with saponin and purified by SEC. While some particles were found to elute at the same time vesicles would do, they did not exhibit any enzyme activity. Thus, we see it as unlikely that active enzyme-aggregates would be measured instead of glucuronidase-loaded EVs.

Added: l. 339-349: "In figure 4, a control experiment was performed to ensure that the enzyme activity measured for the vesicles is indeed linked to the encapsulation of glucuronidase into EVs and not caused by enzyme aggregates. These aggregates might be formed due to the incubation of glucuronidase with saponin and lead to false positive results. To verify the fractions were vesicles would elute, purified EVs without encapsulated glucuronidase were subjected to SEC on the same column as the sample just containing saponin and the enzyme.
When glucuronidase is incubated with saponin and subsequently purified by SEC, no enzyme activity is found in the fractions typically containing EVs. While small amounts of particles were found to elute at the same time as EVs (making up <0.1% of the particles recovered from a typical EV-pellet), there was no correlating enzyme activity. These results indicate that active enzyme recovered in the fractions containing vesicles is encapsulated in them."

l. 396-401: "**Figure 4: Control experiments for the purification of EVs from free glucuronidase**
400 µl of native EVs or 400 µl of 1.5 mg/ml glucuronidase in PBS incubated for 10 min with 0.1 mg/ml saponin were purified by SEC. (A) Particle concentrations of the collected fractions of vesicles and glucuronidase respectively and the enzyme activity of the purified glucuronidase. (B) UV absorption at 280 nm measured in the same experiment. The first small peak for glucuronidase corresponds with the grey line in (A)."

Figure 4



Reviewer #4:

Manuscript Summary: The manuscript called "evaluation of the storage stability of extracellular vesicles" compares storage conditions that are used all over the EV-world. Namely, storage at 4°C, -80°C and lyophilisation. The authors focus is on EVs with

encapsulated enzyme that have been purified by ultrafiltration and SEC. They compare size, particle concentration and morphology as well as particle enzyme content. Used technics are NTA, AF4, TEM and SEM.

Minor Concerns: The protocol is comprehensive and neatly written. The following comments are minor and can be easily edited by the author. I have one suggestion for an additional experiment, but even without this experiment the protocol and results are exciting and very interesting to repeat for researchers within the EV world.

Line 59: Can author give more literature examples for other diseases than cancer?

> Our response: As suggested, more examples have been added to the introduction and section dealing with the roles of EVs in diseases has been revised.
>
> Added: l. 60-65: "On the other side, EVs can promote tumor-vascularization (Feng, Zhang et al. 2017), induce bystander effects in stress response (Bewicke-Copley, Mulcahy et al. 2017) and might play a role in autoimmune diseases (Xu, Liu et al. 2016) and inflammatory diseases (Buzas, György et al. 2014). Thus, they might be a key component to a better understanding of many pathological effects. However, the presence of altered EVs in manifold diseases, such as cancer (Umezu, Tadokoro et al. 2014, Costa-Silva, Aiello et al. 2015, Rajappa, Cobb et al. 2017) and cardiovascular disorders (Boulanger, Loyer et al. 2017) , and their easy accessibility in blood and urine makes them ideal biomarkers."

Line 99: Do the authors deplete their EV-depleted fetal bovine serum in house? If so, please add details on how to accomplish.

> Our response: For all our experiments, we cultured our cells in serum free medium for EV-production, instead of using EV-depleted medium. Medium containing native, non-EV-depleted FBS is only used in the beginning of the culture and later exchanged with serum free medium for EV-collection. Thus, we do not have an in house method for FBS-depletion. We added a note, pointing at the MISEV2018 guidelines, as they contain a detailed discussion on how FBS-depletion can be achieved.
>
> Added: l. 107/108: "NOTE: If EV-depleted fetal bovine serum is used, employ a method proven to efficiently deplete the serum, to prevent contamination with bovine serum derived EVs(Théry, Witwer et al. 2018)."

Line 318: Have samples been purified with SEC column as well? If so, before or after storage?

> Our response: The SEC-purification took place before storage, to remove non-encapsulated glucuronidase and protein contaminants from the EVs.

Line 337: In figure Mean Size is (B) and particle recovery is (A) - need to be switched around.

> Our response: Thank you for pointing this out. The figure legend has been changed.

Modified: l. 375: “Particle recovery compared to before storage (A), mean size (B), (…)”

Figure 1: For lyophilized form, can author add at which temperature vesicles were stored?

Our response: The storage temperature was added in the manuscript.

Added: l. 314: The vesicles were then stored for 7 d at 4 or -80 °C and at 4 °C in lyophilized form, (…)

l. 376/377: “Vesicles were stored for 7 d at 4 and -80 °C and 4 °C after lyophilisation with 4% trehalose.”

Figure 3: Can author explain a bit better the AF4 graph: why does the black line show no peaks while blue line does? Does the grey line have two peaks or just one (hardly visible)?

Our response: Thanks to the reviewer’s comment, we have noticed a mistake in our figure. The blue line was mislabeled as the UV-signal, while in truth it was the light-scattering signal. We corrected the mistake and also added the UV signal for EVs spiked with glucuronidase. The light-scattering signal for the glucuronidase-spiked EVs now makes more sense, as light scattering does not only measure EV-sized particles, but is also able to detect proteins such as glucuronidase. As this mistake is also present in our previous work (Frank, Richter et al. 2018), from where the figure was adapted, it will also be corrected there.

Regarding the peaks of the Glucuronidase (UV)-line (grey), it appears to have very small peak in the region of 23-24 min. We found a similar occurrence when we subjected glucuronidase without EVs to SEC to see, when the enzyme would elute (Figure 4). There we also found particles eluting in the fractions where we would normally recover the EVs, but did not find any enzymatic activity associated with the particles. The same thing seems to have been the case in our AF4-run. The concentration of these particles was very low, below 0.1% of the total amount of particles that we recover from a typical EV-pellet and is thus only of minute concern regarding our particle measurements.

Added: l. 339-349: “In figure 4, a control experiment was performed to ensure that the enzyme activity measured for the vesicles is indeed linked to the encapsulation of glucuronidase into EVs and not caused by enzyme aggregates. These aggregates might be formed due to the incubation of glucuronidase with saponin and lead to false positive results. To verify the fractions were vesicles would elute, purified EVs without encapsulated glucuronidase were subjected to SEC on the same column as the sample just containing saponin and the enzyme.
When glucuronidase is incubated with saponin and subsequently purified by SEC, no enzyme activity is found in the fractions typically containing EVs. While small amounts of particles were found to elute at the same time as EVs (making up <0.1% of the particles recovered from a typical EV-pellet), there was no correlating enzyme activity. These results indicate that active enzyme recovered in the fractions containing vesicles is encapsulated in them.”

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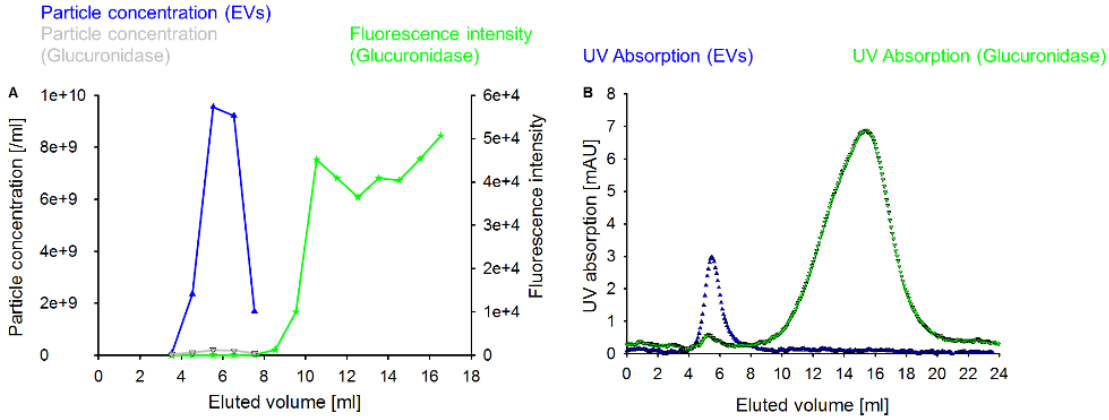
Figure 4



Line 318: Is SEC purification done before or after storage? In case it is done before, could positive impact on enzyme activity compared to after purification by AF4 be explained by enzyme that leaked out of EVs and is still floating in media?

Our response: The SEC purification is done before storage. After storage we do another step of SEC or alternatively AF4 to remove enzyme floating in the media, present due to leakage or the destruction of vesicles (step 8.1 in the protocol). Thus, the positive impact on enzyme recovery observed in the samples where no additional purification after storage has been done, is probably indeed linked to free enzyme in the medium.

Line 402-403: Can author explain how they found that enzyme reacts slower when encapsulated and how that impacts the overestimation of encapsulation?

Our response: As the substrate for the encapsulated glucuronidase is Fluorescein di-β-D-glucuronide, a quite hydrophilic molecule, diffusion through the EV membrane is slow. Thus, if the enzyme is encapsulated in the EVs, the reaction cannot proceed at the same speed as if the enzyme was free in the medium. During the 16 h incubation period of the glucuronidase assay, we assumed that it takes longer for the Fluorescein di-β-D-glucuronide to diffuse into the EVs, than for it to react with glucuronidase that is freely floating in the medium.

Line 424-425: The author states that UC can lead to alterations of the particles. Additionally it does usually not lead to a complete purification of EVs from contaminants of conditioned media. Can these two facts also have impact on enzyme encapsulation? Could enzyme be formulated with remaining contaminants from conditioned media into particles of similar size

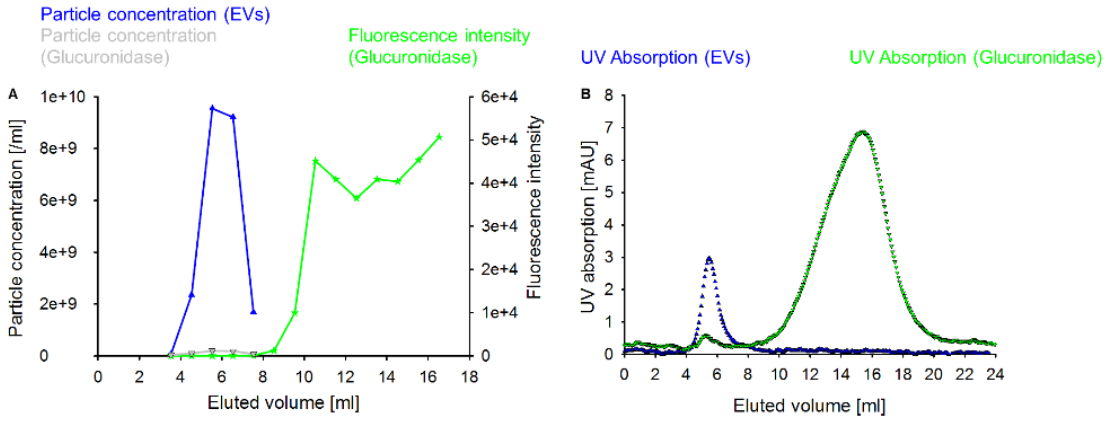
to EVs? Maybe comparison to another set of experiments where EVs have been concentrated with ultrafiltration and purification via SEC before encapsulation of enzyme would be an asset to the existing results. Though the already collected results are very interesting on themselves and can definitely be published as such also without the suggested additional experiment.

Our response: The proposed additional experiment would indeed be an interesting addition to our paper, however it cannot be readily conducted using our experimental setup. During our SEC-purification, dilution of our samples by approximately ten-fold takes place. Thus, performing the glucuronidase-encapsulation after this purification step may lead to a great decrease in encapsulation efficiency, which would make downstream analysis of the EVs very challenging. Moreover, it is not possible to do the reverse experiment of incubating glucuronidase with just the contaminants from the conditioned medium, as we cannot obtain just the cell-derived impurities without EVs. The caveat that saponin incubation could lead to the formation of glucuronidase-agglomerates has been addressed by including another experiment in the manuscript (Figure 4). Here, just the enzyme and saponin were purified by SEC and the enzyme activity and protein concentration in the fractions measured. As mentioned in our comment above, there were some particles found in the sample just containing glucuronidase and saponin, which eluted in the fractions EVs are typically found in. They did not exhibit any enzyme activity.

Added: l. 339-349: "In figure 4, a control experiment was performed to ensure that the enzyme activity measured for the vesicles is indeed linked to the encapsulation of glucuronidase into EVs and not caused by enzyme aggregates. These aggregates might be formed due to the incubation of glucuronidase with saponin and lead to false positive results. To verify the fractions were vesicles would elute, purified EVs without encapsulated glucuronidase were subjected to SEC on the same column as the sample just containing saponin and the enzyme.
When glucuronidase is incubated with saponin and subsequently purified by SEC, no enzyme activity is found in the fractions typically containing EVs. While small amounts of particles were found to elute at the same time as EVs (making up <0.1% of the particles recovered from a typical EV-pellet), there was no correlating enzyme activity. These results indicate that active enzyme recovered in the fractions containing vesicles is encapsulated in them."

l. 396-401: "**Figure 4: Control experiments for the purification of EVs from free glucuronidase**
400 µl of purified EVs without encapsulated glucuronidase or 400 µl of 1.5 mg/ml glucuronidase in PBS incubated for 10 min with 0.1 mg/ml saponin were purified by SEC. (A) Particle concentrations of the collected fractions of vesicles and glucuronidase respectively and the enzyme activity of the purified glucuronidase. (B) UV absorption at 280 nm measured in the same experiment. The first small peak for glucuronidase corresponds with the grey line in (A)."

Figure 4


Particle concentration (EVs)
Particle concentration (Glucuronidase)
Fluorescence intensity (Glucuronidase)
A
Particle concentration [/ml]
Fluorescence intensity
Eluted volume [ml]
UV Absorption (EVs)
UV Absorption (Glucuronidase)
B
UV absorption [mAU]
Eluted volume [ml]

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