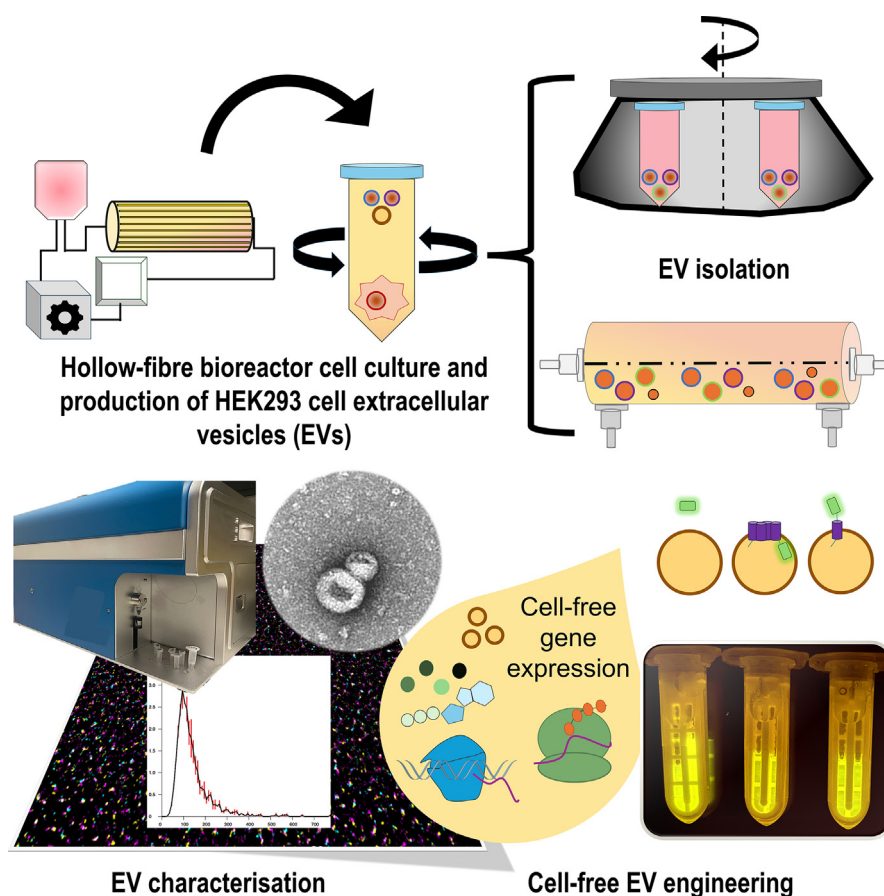


Report

Hollow-fibre biomanufacturing and cell-free engineering of HEK293 extracellular vesicles



Extracellular vesicles are cell-derived particles that can have therapeutic effects. To this end, we developed a cell-free extracellular vesicle engineering workflow that can be used to quickly prototype and functionally test new therapeutic extracellular vesicle designs. We envision that this will help accelerate next-generation extracellular vesicle therapeutics.

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Highlights

Cell-free extracellular vesicle engineering could be utilised to rapidly prototype and test novel biotechnological, vaccine, or therapeutic extracellular vesicles for foundational or translational applications.

To advance this type of extracellular vesicle engineering, we established a full end-to-end cell-free extracellular vesicle engineering workflow.

Our data highlight some of the impacts that hollow-fibre-based cell culture, extracellular vesicle isolation methods, and different cell-free engineering approaches have on cell-free extracellular vesicle engineering workflows.

We also demonstrate several characterisation assays and technologies, including nanoflow cytometry, that can be used to assess cell-free extracellular vesicle engineering efficiency and extracellular vesicle integrity.

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Report

Hollow-fibre biomanufacturing and cell-free engineering of HEK293 extracellular vesicles

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Extracellular vesicles (EVs) are lipid-delimited particles produced by most cell types that can have therapeutic effects. Cell-free gene expression systems can be used to produce membrane proteins *in vitro*, which can integrate with exogenously added EVs. To advance this type of cell-free EV engineering, we established an end-to-end cell-free EV engineering workflow. Firstly, human embryonic kidney (HEK293) cells were cultured within a hollow-fibre bioreactor to generate several batches of HEK293 EVs. Subsequently, these EVs were successfully cell-free engineered with several CD63-based membrane fusion proteins. Nano-flow cytometry analyses revealed that, under optimal conditions, up to 4.83×10^{11} /ml of HEK293 EVs were successfully cell-free engineered to incorporate CD63 I-shaped membrane-insertion topology transmembrane helix 3 (CD63ITM3)-monomeric green lantern membrane fusion proteins. Finally, we demonstrated that cell-free engineered EVs can be functionally assessed using nano-flow cytometry and cell-based assays. We envision that, in the future, cell-free EV engineering workflows could be used to help accelerate future EV discoveries and advancements in EV therapeutics.

Introduction

Extracellular vesicles (EVs) (see [Glossary](#)) are lipid-delimited particles produced by most cell types that have important biological roles in health and disease [1,2]. EVs carry complex molecular cargoes and are present in all biofluids, including blood, urine, saliva, and cerebrospinal fluid. These aspects have accelerated EV disease biomarker studies [3–5]. EVs also hold significant potential for use as vaccines, postbiotics, therapeutics, and drug delivery vehicles for the treatment of cancers, inflammatory or neurological conditions, and infectious diseases [6–9]. Indeed, EVs have several beneficial therapeutic characteristics, including their biocompatibility, capacity to cross the blood–brain barrier, and ability to deliver bioactive molecules to cells [10–12]. Bacterial vesicle-based vaccines, including VA-MENGOC-BC and Bexsero, are currently the most clinically and commercially successful EVs on the market [13–15]. However, this may change, given that more sophisticated EV therapeutics are increasingly being developed from human cells, including mesenchymal stem cells [16–18] and **human embryonic kidney (HEK293) cells** [19–21].

However, whilst promising, the development of next-generation EV therapeutics is not trivial, and many challenges remain [12,22–24]. Additionally, EV-based clinical and *in vivo* biodistribution studies indicate that advancements in EV engineering might also be needed to improve EV cell/tissue targeting and enhance EV therapeutic efficacy [20,25,26]. In relation to these challenges, the global EV research community is also calling for the development of reference materials (EV metrology standards) and potency assays that can be used to robustly characterise the therapeutic modality and efficacy of next-generation EV therapeutics [2,27–29].

Technology readiness

Cell-free systems are a well-established part of the engineering biology toolkit. Indeed, several applications are already at the technology readiness level nine (TRL9) stage, including commercially available cell-free protein synthesis kits, drug screening assays, field-tested medical biosensors, as well as therapeutic antibody manufacturing that has now reached cell-free reaction volume scales of up to 4500 l. However, cell-free extracellular vesicle engineering is currently at the TRL3/4 stage. Whilst our study helps to further advance cell-free extracellular vesicle engineering, key challenges remain. To accelerate technology readiness, improvements in scalable extracellular vesicle isolation methods, increased eukaryotic cell-free protein synthesis yields, and assay automation will likely speed up cell-free extracellular vesicle engineering workflows. Furthermore, artificial intelligence-guided design workflows could be used to rapidly create community accessible libraries of extracellular vesicle membrane protein scaffolds, therapeutic, cell targeting, and other modular elements for use in extracellular vesicle engineering studies. Finally, the development of extracellular vesicle potency assays for functional assessment of cell-free engineered therapeutic extracellular vesicles are needed to progress prototyped designs towards clinical translation. If these challenges are addressed, we envision that cell-free extracellular vesicle engineering will become an important approach in extracellular vesicle biological research and clinical applications.

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We envision that innovative solutions to these challenges may reside at the convergence of EV research with **engineering biology** [13,30,31]. Indeed, EV studies are increasingly adopting synthetic biology methods and technologies to engineer cells, EVs, and their cargoes, including clustered regularly interspaced short palindromic repeats / CRISPR-associated protein 9 (CRISPR/Cas9), SpyCatcher, fusion-protein engineering, hybrid EV/liposome engineering, and artificial intelligence (AI) approaches (e.g., AlphaFold) [26,30,32–35]. However, despite these advancements, it is still the case that cell-based engineering is relatively slow and is encumbered by cell growth and burden dynamics that can extend workflows across several days, weeks, or months [36,37]. In contrast, cell-free synthetic biology encompasses a suite of *in vitro* technologies, flexible engineering approaches, and reaction formats that are rapid, spanning only several hours in duration [38–40]. Essentially, cell-free systems make use of isolated cellular components (proteins, nucleic acids, etc.) or biological processes outside of the cell and can, for example, enable *in vitro* protein production from DNA templates [40–43]. To achieve this, **cell-free gene expression systems** typically make use of isolated cellular transcription and translation machinery [e.g., protein synthesis using recombinant elements (PURE)] [44–46] or whole-cell extracts [47–51]. Given the unique biochemistries and protein synthesis yields of different cell types, various cell-extract-based systems have been developed, each with their own distinctive capabilities [40,52–55]. Cell-free reactions can also be further customised through supplementation with reaction-boosting energy mixes, enzyme cofactors, chaperones, and other application-specific components as required [56–59]. For example, the inclusion of lipid nanostructures (e.g., liposomes, nanodiscs, and bicelles) can facilitate the correct folding and insertion of cell-free-produced membrane proteins [60–63]. Lipid-integrated membrane proteins can then be used for structural biology [64,65], drug screening [59,66], or synthetic cell applications [67,68].

A key advancement for the engineering biology field was the recognition that cell-free synthetic biology workflows can generate insights comparable to those obtained using cell-based characterisation assays [38–40,48,69]. Indeed, interoperability between cell-free and cell-based systems has enabled cell-free synthetic biology to underpin key advances in molecular biology, metabolic engineering, biosensing, materials research, drug discovery, therapeutic biomanufacturing, and several areas of EV research [24,31,38,40,60,70–74]. For example, a cell-free EV packaging assay was developed that helped uncover a role for Y-box protein 1 in the sorting of specific microRNAs into exosomes [24]. Similar cell-free approaches could also be developed to study other aspects of EV biogenesis and cargo-loading mechanisms [31]. Whilst in other studies, cell-free gene expression systems have been used to produce many different recombinant proteins, including those of relevance to EV biology [31,60,70,71,75].

Inspired by these disparate studies, we previously demonstrated that it is also feasible to produce membrane fusion proteins in cell-free gene expression reactions that can co-localise and integrate with exogenously added HEK293 cell EVs [31]. In the future, we envision that this type of cell-free EV engineering could become an important strategy to rapidly prototype and test many different *in vitro*-engineered EV designs for foundational or translational research aims [31]. We imagine applying cell-free-based EV engineering to rapidly decorate cell-derived or synthetic EVs with libraries of vaccine antigens, therapeutic cargoes, cell-targeting elements, or other elements for testing in downstream functional assays [13,31,76]. Importantly, this would allow for more systematic and engineering biology-based approaches to EV research by making it easier to enable interlaboratory collaboration and data sharing [30]. Furthermore, cell-free-optimised designs might then be transferred into cell-based EV engineering workflows to facilitate final testing and manufacturing scale-up.

To this end, we present progress beyond our earlier pilot study [31] such that the present study advances cell-free EV engineering from concept towards the development of a realised end-to-

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end cell-free EV engineering workflow. Specifically, we demonstrate that hollow-fibre-based cell culture is a feasible and scalable way to generate significant amounts of EVs for cell-free EV engineering applications. Additionally, these data highlight the impacts that different EV isolation methods can have on EV batch characteristics and the presence of contaminating proteins, which can potentially impact cell-free EV engineering performance. We also demonstrate that different membrane protein scaffold designs impact cell-free EV engineering efficiency, and that these observed differences can be readily measured using nano-flow cytometry. Finally, we show that cell-free engineered EVs remain intact and can be utilised in nano-flow cytometry and cell-based assays. Taken together, we anticipate that our cell-free EV engineering workflow will inform future studies and efforts towards the development of novel EV therapeutics.

Results

Hollow-fibre biomanufacturing of HEK293 EVs

Hollow-fibre cell culture systems can facilitate high-density cell culture and scalable EV biomanufacturing that is compliant with good manufacturing practice [17,77,78]. **Hollow-fibre bioreactors** consist of a hollow-fibre cartridge within which cells are cultured, whilst a cell media reservoir, a gas exchanger, and a pump facilitate continuous circulation of cell media throughout the bioreactor (Figure 1A). Once established, cells can remain in continuous culture for several weeks or more, whilst simultaneously the EV-enriched cell-conditioned media can be readily harvested for downstream processing [17,77,78]. To test the suitability of a hollow-fibre bioreactor for EV production, we continuously cultured HEK293 cells for 26 days within a hollow-fibre bioreactor equipped with a 20 kDa molecular weight cut-off cartridge and a 1 l media reservoir that enabled cell culture densities of up to $\sim 10^9$ HEK293 cells. Similar to previous studies, cell-conditioned batches (up to 20 ml each) were harvested from the hollow-fibre cartridge and initially processed (centrifuged) to remove cells and large debris prior to EV isolation (Figure 1A) [3,17,31]. EVs were subsequently isolated from each cell-conditioned media batch using ultracentrifugation (UC) and tangential flow filtration (TFF) approaches (Figure 1A; see also the Materials and Methods section: isolation of HEK293 EVs). Whilst HEK293 EVs were isolated using UC and TFF from the same cell-conditioned media batches, there were differences. Notably, the average protein concentrations of UC-isolated EV samples (0.79 ± 0.09 mg/ml) were higher than TFF-isolated EV samples (0.22 ± 0.03 mg/ml) (Figure 1B), which are likely indicative of co-isolated contaminating proteins.

Post-protein quantification, UC- and TFF-isolated HEK293 EV samples were characterised using a nanoparticle tracking analysis (NTA) system to determine mean EV/nanoparticle sizes and concentrations (Figure 1C–F; Figures S1 and S2 in the Supplemental information online). Mean EV/nanoparticle sizes of UC-isolated EVs were 159.8 ± 6.23 nm, whilst TFF-isolated EV/nanoparticles were on average slightly smaller at 118.2 ± 4.04 nm (Figure 1C), though these size ranges are both consistent with widely reported small EV sizes (<200 nm) [2,27]. EV sizes were also measured using dynamic light scattering (DLS) and were broadly consistent with our NTA data. DLS mean EV/nanoparticle sizes for UC samples were 186 ± 10.09 nm and for TFF samples were 130.2 ± 16.66 nm (Figure S3 in the Supplemental information online). For comparative purposes NTA-quantified EV/nanoparticle concentrations were normalised against sample protein concentration and sample volume differences (UC 200 μ l; TFF 1000 μ l; see Figure S1 for pre-normalised data). Representative NTA-quantified EV size and concentrations for UC- and TFF-isolated EVs are shown in Figure 1E,F, respectively. Normalised mean EV/nanoparticle concentrations of UC-isolated HEK293 EVs were $4.27 \pm 0.86 \times 10^{11}$ /ml and for TFF-isolated samples were $6.39 \pm 1.1 \times 10^{11}$ /ml (Figure 1D). In addition to producing higher EV yields, we and other studies have shown that TFF protocols are simpler, quicker, and more scalable than UC-based EV isolation protocols [79–83]. Nevertheless, both UC and TFF EV isolation methods

Glossary

Cell-free gene expression systems:

a general description of biotechnologies that use cell extracts and/or isolated cellular components to produce proteins outside of a cell (*in vitro*) from engineered DNA templates. In this report article, we used cell-free gene expression systems to engineer HEK293 cell-derived EVs.

Engineering biology:

an interdisciplinary scientific field that aims to design, construct, and assemble cellular components (DNA, proteins, metabolic pathways, etc.) into new biology-based technologies that address industrial applications and societal needs in a responsible manner.

Extracellular vesicles (EVs): lipid-membrane-enclosed particles released from cells that cannot self-replicate. This definition is derived from EV nomenclature guidance, which is recommended by the International Society for Extracellular Vesicles.

Human embryonic kidney (HEK293) cells:

an epithelial cell line that was isolated from the kidney of a human embryo. HEK293 cells are widely used in industrial biotechnology applications.

Hollow-fibre bioreactors: devices that employ porous fibres for culturing cells at high cell densities. Typically, these fibres and the cultured cells are bundled within a compact cartridge, whilst a pump continuously flows cell media through the cartridge in a manner that mimics *in vivo* circulation.

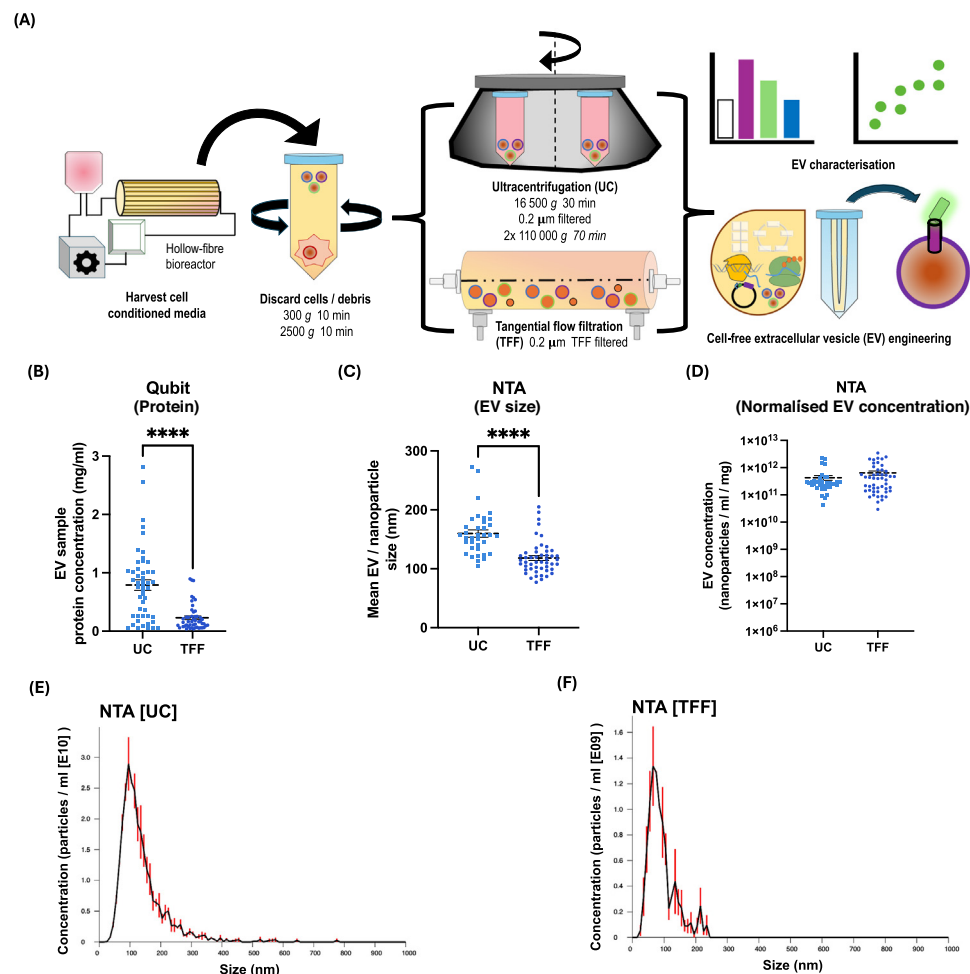


Figure 1. Hollow-fibre biomanufacturing of HEK293 extracellular vesicles (EVs). (A) Overview schematic depicting hollow-fibre HEK293 cell culture, EV isolation and cell-free EV engineering. Forty-nine independent batches of HEK293 cell-conditioned media were harvested from the hollow-fibre bioreactor, and from these, HEK293 EVs were isolated using either ultracentrifugation (UC) or tangential flow filtration (TFF). EV batches were quantified for total protein concentration using (B) Qubit protein assay and nanoparticle tracking analysis (NTA) to quantify (C) EV size and (D) EV concentration, which was normalised according to batch total protein concentration. Representative NTA histograms for (E) UC and (F) TFF-isolated HEK293 EVs. Error bars denote standard error of the mean, $n = 49$ EV batches, P values were obtained using one-way ANOVA with Tukey's multiple comparisons test at the following significance level **** $P < 0.0001$. ANOVA: analysis of variance; HEK293: human embryonic kidney.

generated sufficient HEK293 cell-derived EVs for further downstream characterisation and cell-free EV engineering experiments.

Characterisation of HEK293 EVs

In addition to NTA and DLS analyses, we further characterised several batches of UC- and TFF-isolated HEK293 EVs using minimal information for studies of EVs -compliant methodologies [2]. Firstly, EV and cellular protein markers were assessed in four independent batches of UC- and TFF-isolated HEK293 EVs using an Exo-Check Exosome Antibody dot-blot array (Figure 2A,B; Figure S4 in the Supplemental information online). UC- and TFF-isolated EV batches were visibly

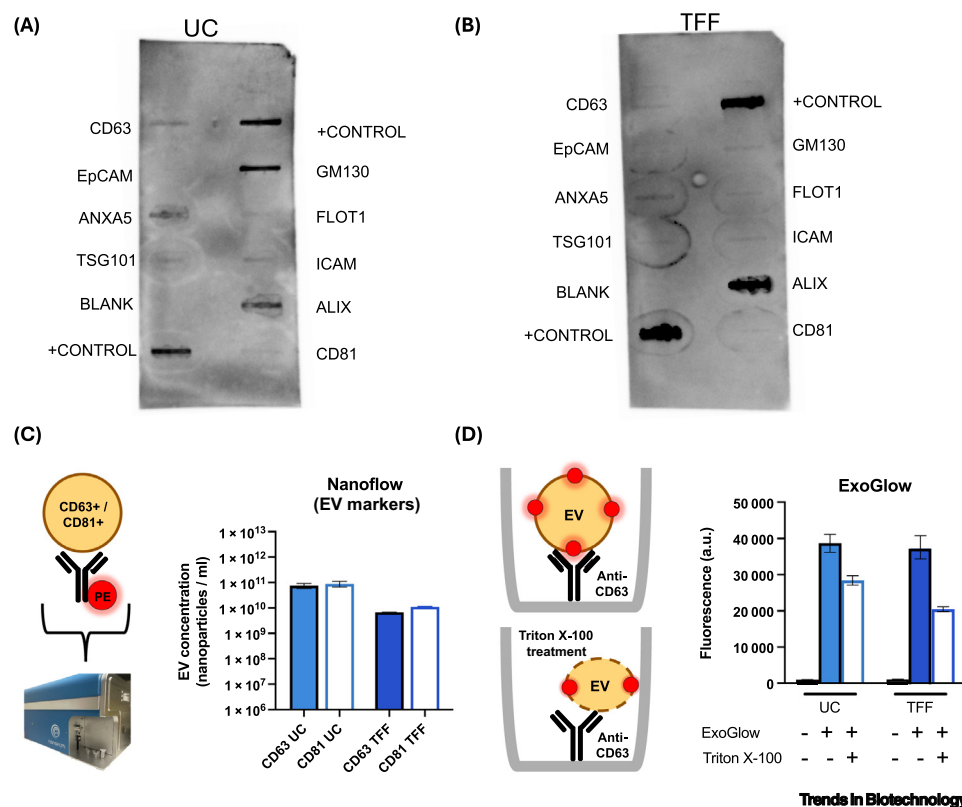


Figure 2. Characterisation of HEK293 extracellular vesicles. Representative Exo-Check Exosome Antibody arrays of (A) ultracentrifugation (UC)- and (B) tangential flow filtration (TFF)-isolated HEK293 extracellular vesicles. (C) Nano-flow cytometry characterisation of CD63- and CD81-positive HEK293 EVs isolated using either UC or TFF, $n = 4$. (D) ExoGlow EV membrane labelling of CD63-positive HEK293 EVs treated with 0 or 0.1% Triton X-100. Assay plates were analysed using a CLARIOstar plate reader (Ex:465-15/Em:635-20), $n = 4$. Error bars denote the standard error of the mean. ALIX: apoptosis-linked gene 2-interacting protein X; ANXA5: Annexin A5; EpCAM: epithelial cell adhesion molecule; EV: extracellular vesicle; FLOT1: flotillin-1; HEK293: human embryonic kidney; ICAM: intercellular adhesion molecule-1; TFF: tangential flow filtration; UC: ultracentrifugation.

positive for several EV markers, including CD63, CD81, and tumour susceptibility gene 101 (TSG101) (Figure 2A,B; Figure S4) [2], whilst all tested UC and TFF EV samples were strongly positive for the EV marker apoptosis-linked gene 2-interacting protein X (ALIX) [2,84] (Figure S4). Finally, we observed that GM130, a *cis*-Golgi matrix protein, was more consistently strongly positive in UC- than TFF-isolated EV samples (Figure S4), suggesting that these samples contain higher levels of cellular protein contaminants. However, this is not unexpected given that previous studies also observed that UC methods invariably produce impure EV samples [79,85,86]. These data are also consistent with our previous observations in which UC samples were generally higher in overall protein concentration than TFF-isolated EV samples (Figure 1B), despite having lower normalised EV concentrations (Figure 1D). Nevertheless, these data confirm that both methods isolate EVs that display several common markers.

Representative EV samples were also imaged using super-resolution microscopy, utilising a microscope equipped for direct stochastic optical reconstruction microscopy. Our images visually confirmed that UC- and TFF-isolated HEK293 EVs were heterogeneously positive for EV marker tetraspanins CD63, CD81, and CD9 (Figure S5 in the Supplemental information online). CD63- and CD81-positive EVs were also detected using nano-flow cytometry (Figure 2C; Figures S6 and S7 in the Supplemental information online). Prior to use, the nano-flow was calibrated

using quality control (QC) and size-reference standard beads to validate machine performance and to calibrate scatter measurements to EV/nanoparticle size (Figure S6). We then stained UC- and TFF-isolated HEK293 EVs with antibodies for CD63 or CD81 and then analysed using the calibrated nano-flow cytometer (Figure 2C; Figures S6 and S7). Mean EV/nanoparticle sizes of UC- (69 ± 0.76 nm) and TFF (78 ± 1.3 nm)-isolated EVs were smaller than our NTA and DLS measurements, though these data are consistent with nano-flow cytometry measurements of HEK293 EVs in prior studies [19,31]. For the final EV characterisation assay, UC- and TFF-isolated HEK293 EVs were immunocaptured using a CD63-antibody coated plate and stained with an ExoGlow membrane labelling dye that stains intact EVs (Figure 2D). As expected, EV-membrane integrity and concomitant ExoGlow dye fluorescence were reduced following Triton X-100 treatment (Figure 2D), thereby indicating that structurally intact CD63-positive EVs were present in the tested UC- and TFF-isolated EV batches. Once characterised, several EV batches were ready for subsequent cell-free EV engineering experiments.

Cell-free EV engineering

For cell-free EV engineering, we constructed a panel of cell-free expression constructs (STAR Methods, Table S1 in the Supplemental information online). This panel included the truncated enhanced green fluorescent protein deGFP [87] and monomeric green lantern (mGL) [88], as reporter constructs deGFP (pRK66) and mGL (pRK67), respectively. Whilst full-length CD63 (tetraspanin) and its truncated variant CD63 I-shaped membrane-insertion topology transmembrane helix 3 (CD63ITM3) were fused using a short amino acid linker with mGL to create the constructs CD63-mGL (pRK68) and CD63ITM3-mGL (pRK69). CD63 and its truncated form CD63ITM3 were identified as suitable membrane proteins for cell-free EV engineering experiments due to their demonstrated associations with exosomes in cell-based EV engineering studies [34]. Additionally, as previously observed, cell-free-produced enhanced green fluorescent protein (EGFP) fused with CD63 (EGFP-CD63) can co-localise with HEK293 EVs [31]. It was therefore anticipated that these new CD63-based membrane fusion proteins would be suitable for cell-free EV engineering.

We first determined whether UC- or TFF-isolated HEK293 EVs can impact HeLa cell-free reaction performance (Figure S8 in the Supplemental information online). Briefly, 10- μ l scale cell-free reactions containing different HEK293 EV concentrations were setup in 384-well plates, and time course fluorescence measurements were used to assess cell-free deGFP production (Figures S8 and S9 in the Supplemental information online). As expected, deGFP cell-free reactions produced $\sim 77 \pm 0.67$ μ g/ml of deGFP, whilst control cell-free reactions containing neither deGFP DNA nor HEK293 EVs produced only low background levels of fluorescence (Figure S8). Interestingly, the inclusion of UC- or TFF-isolated HEK293 EVs modestly improved cell-free reaction performance, though reactions containing low-EV concentrations produced more deGFP than reactions containing high-EV concentrations (Figure S8). These observed differences in cell-free deGFP production yields were most pronounced between cell-free reactions containing low ($\sim 94 \pm 0.68$ μ g/ml) and high ($\sim 83 \pm 1.75$ μ g/ml) concentrations of UC-isolated HEK293 EVs. Similarly to this, we previously observed that the inclusion of UC-isolated HEK293 EVs can negatively impact cell-free protein synthesis yields [31]. In contrast, TFF-isolated HEK293 EVs appear to have less pronounced impacts on cell-free reaction performance than UC-isolated HEK293 EVs (Figure S8), which might be due to the relatively lower levels of protein contaminants in these TFF-isolated EV samples (Figures 1 and 2).

Given these data, future studies could explore whether improved TFF methods, the addition of an extra 0.22- μ m filtration step [77], or other non-UC-based EV isolation methods are able to generate purer EV preparations [86] that have more consistent impacts on cell-free reaction performance. Indeed, EVs can contain chaperones, enzymes, and other molecules that can

impact cell-free metabolic processes and protein synthesis [31]. Future studies could potentially make use of proteomics, metabolomics, and other EV characterisation data to identify which components impact cell-free reaction performance and then utilise these insights to further advance cell-free EV engineering [19,30,31]. Nevertheless, in the present study, both UC- and TFF-isolated HEK293 EVs positively impacted cell-free reaction performance and were used in cell-free EV engineering reactions.

Cell-free engineering of HEK293 EVs was carried out within semi-continuous dialysis-mode cell-free reactions (Figure 3; Figure S11 in the Supplemental information online), using a protocol adapted from our previous study [31]. Essentially, reactions were setup for cell-free production of mGL, CD63-mGL, or CD63ITM3-mGL proteins in the presence of UC- or TFF-isolated HEK293 EVs, whilst control reactions included both UC- and TFF-isolated HEK293 EVs but did not include a cell-free expression construct. Post-incubation, visual inspections (Figure 3A), and endpoint fluorescence measurements (Figure 3B) were indicative that mGL, CD63-mGL,

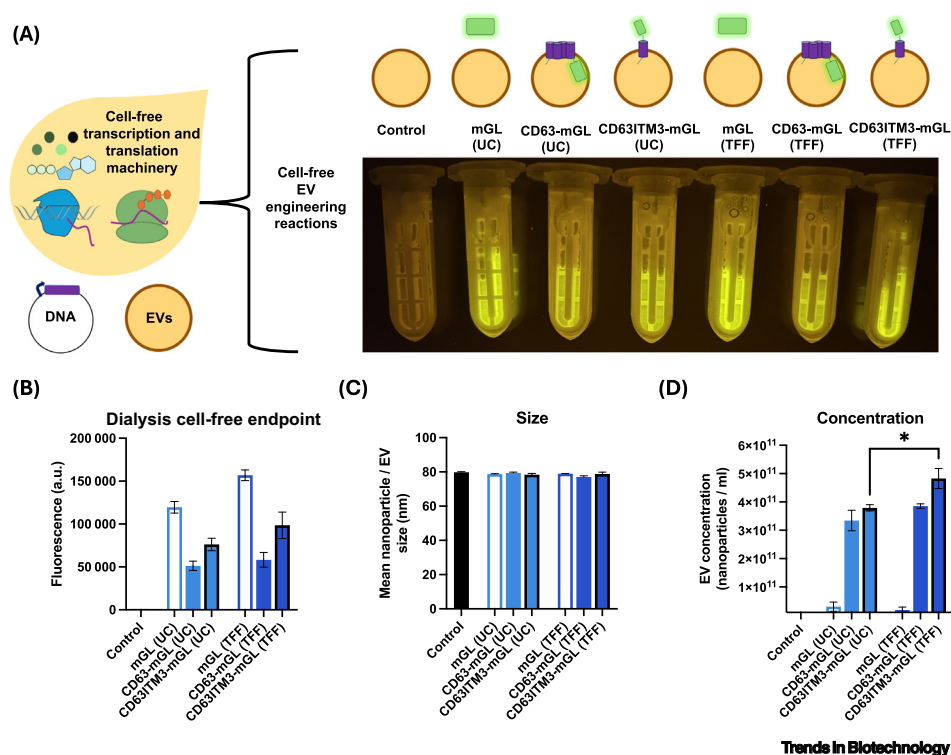


Figure 3. Cell-free engineering of HEK293 extracellular vesicles (EVs). (A) Schematic of ultracentrifugation (UC) and tangential flow filtration (TFF)-isolated HEK293 EVs in control (no DNA), monomeric green lantern (mGL), CD63-mGL membrane fusion protein, or truncated CD63ITM3-mGL membrane fusion protein-producing cell-free EV engineering reactions. Representative 60 μ l scale 'semi-continuous dialysis mode' cell-free transcription-translation reactions were placed on a BluPAD LED transilluminator (Bio-Helix) and imaged as shown. (B) Endpoint (22 h) fluorescence measurements of control (no DNA), mGL, CD63-mGL, or CD63ITM3-mGL protein production in 60 μ l scale semi-continuous dialysis mode cell-free EV engineering reactions, containing UC- or TFF-isolated HEK293 EVs. Endpoint samples were analysed using a CLARIOstar plate reader (Ex:483-14/Em:530-30). Nano-flow cytometry analysis of (C) mean nanoparticle/EV size and (D) concentration of green-fluorescent positive cell-free engineered HEK293 nanoparticles/EVs within the indicated semi-continuous dialysis mode cell-free gene expression reactions. Error bars denote standard error of the mean, *n* = 3 independent cell-free reactions, *P* value was obtained using one-way ANOVA with Tukey's multiple comparisons test at the following significance level * *P* < 0.05. ANOVA: analysis of variance; CD63ITM3: CD63 I-shaped membrane-insertion topology transmembrane helix 3; HEK293: human embryonic kidney; LED: light emitting diode.

and CD63ITM3-mGL proteins were successfully produced in their respective cell-free reactions. Whilst transmission electron microscopy (TEM) visually confirmed that cell-free engineered EVs remained intact within their respective reactions (Figure S10 in the Supplemental information online).

These cell-free EV engineering reactions were subsequently processed using micro-size exclusion chromatography (SEC) columns and prepared for nano-flow cytometry analyses, which confirmed the presence of nanoparticles/EVs in all samples (Figure 3C). Gated nano-flow cytometry events in all CD63-mGL and CD63ITM3-mGL cell-free EV engineering reactions indicate relatively high concentrations of strongly green fluorescent (FITC-H channel) nanoparticles/EVs (Figure 3D; Figure S11). Interestingly, higher concentrations of green fluorescent positive nanoparticles/EVs were present in cell-free reactions containing TFF than UC-isolated HEK293 EVs (Figure 3D). Indeed, the highest concentrations of green fluorescent nanoparticles/EVs were found in CD63ITM3-mGL TFF cell-free reactions, which were $\sim 4.83 \pm 0.35 \times 10^{11}$ /ml (Figure 3D). The negative control reactions showed no fluorescence and only $\sim 0.4\%$ of nanoparticles/EVs were green fluorescent in mGL cell-free EV engineering reactions (Figure 3D; Figure S11). Consistent with these results, an anti-GFP-AF647 antibody displayed negligible staining of nanoparticles/EVs from control or mGL cell-free reactions (Figure S11). Likewise, our observations of minimal mGL EV-surface aggregation/adsorption are also consistent with our previous cell-free EV engineering study [31].

To further investigate cell-free EV engineering efficiencies, we also adapted our previous EV co-localisation and membrane protein topology assay [31] (Figure S11). The assay's purpose was to determine membrane protein topology, given that correctly EV membrane-integrated CD63-mGL would be green fluorescent (FITC-H) but could potentially render mGL inaccessible to GFP antibody staining (anti-GFP-AF647), whilst EVs that have integrated CD63ITM3-mGL will be green fluorescent in a manner that might also be GFP antibody accessible (Figure S11A). To this end, a quadrant-based nano-flow cytometry gating strategy was developed to categorise recorded events according to their mGL (FITC-H) and GFP antibody fluorescence signals (anti-GFP-AF647) (Figure S11B,C). These data were used to quantify cell-free EV engineering efficiencies of correctly integrated CD63-mGL (P4) and CD63ITM3-mGL (P1 + P4) membrane fusion proteins with UC- and TFF-isolated EVs (Figure S11D). Interestingly, whilst we observed relatively high concentrations of strongly green fluorescent (FITC-H) nanoparticles/EVs in our cell-free EV engineering reactions (Figure 3D; Figure S11), the percentage of events (nanoparticles/EVs) corresponding to correctly integrated membrane proteins was generally low (Figure S11D). However, CD63ITM3-mGL co-localised and EV integrated more efficiently than CD63-mGL (Figure S11D). This is unsurprising given the simpler single transmembrane architecture of CD63ITM3 compared with full-length CD63. Nevertheless, UC- and TFF-isolated EVs were successfully cell-free engineered with CD63-mGL and CD63ITM3-mGL. These data also offer an important insight into the development of additional membrane protein scaffolds that could further improve cell-free EV engineering capabilities and efficiencies.

TFF-isolated HEK293 EVs were cell-free engineered in additional control, mGL, and CD63ITM3-mGL reactions for use in a nano-flow cytometry-based EV integrity assay. Essentially, we stained these additional cell-free reactions/EVs with Cell Tracker Deep Red (CTDR) dye, then processed these samples using SEC micro-columns to isolate the EVs prior to nano-flow cytometry analysis (Figure S12 in the Supplemental information online). CTDR is a membrane-permeable dye that contains a succinimidyl ester reactive group, which reacts with the amine groups present on intracellular [89] and EV luminal cargo proteins [90]. Upon covalently binding to protein, CTDR becomes anchored, and as such, the dye can accumulate within live cells or intact EVs that contain cargo proteins [89–91]. Therefore, by adopting a quadrant-based nano-flow cytometry gating strategy for the detection of mGL/CD63ITM3-mGL and CTDR dye fluorescence, we

were able to detect intact CD63ITM3-mGL cell-free engineered HEK293 EVs [P1; $\sim 4.3 \pm 0.09 \times 10^{11}/\text{ml}$] that have likely retained their original HEK293 cell-derived protein cargoes (Figure S12). Meanwhile, nanoparticles/EVs that negatively stained for CTDR dye (P3 and P4) likely contained little to no luminal cargo proteins or were potentially leaky/not fully intact [90,91]. Importantly, these data, along with our TEM imaging (Figure S10), indicate that cell-free engineered HEK293 EVs and their luminal protein cargoes remain largely intact.

Finally, we tested our cell-free engineered EVs within an *in vitro* pancreatic cancer cell viability assay. Pancreatic carcinoma cell line 1 (PANC-1) cells were seeded into 96-well plates and treated with Dulbecco's phosphate-buffered saline (DPBS), 3% dimethyl sulfoxide (DMSO) (v/v), or TFF-isolated and cell-free engineered HEK293 EVs as indicated (Materials and Methods section; Figure 13A,B in the Supplemental information online). At 4 h post treatment, the PANC-1 cells were imaged using an EVOS fluorescent microscope. We observed faint green fluorescence signals that co-localised with PANC-1 cells treated with CD63ITM3-mGL cell-free engineered EVs (Figure S13A), which indicate association as HEK293 EVs have previously been shown to co-localise with PANC-1 cells [92,93]. PANC-1 cell viability was assessed at 24 h post treatment. As expected, cytotoxic effects were observed with 3% DMSO treatment [94] but not in PANC-1 cells treated with control (DPBS) or cell-free engineered HEK293 EVs (Figure S13B) [19,93]. Though in the future, cell-free engineered EVs with cytotoxic or pro-apoptotic peptides [95] could feasibly be screened with this approach. We also separately seeded PANC-1 cells within a 24-well plate and treated these cells with DPBS or CTDR-stained cell-free engineered HEK293 EVs as indicated (Materials and Methods section; Figure S13C). These additional microscopy data also show co-localisation of cell-free engineered HEK293 EVs (CD63ITM3-mGL) with PANC-1 cells (Figure S13C), thus highlighting the future potential for *in vitro* screening of cell-free engineered EVs.

Discussion

Beneficially, cell-free gene expression reactions are compatible with cytotoxic peptides and other molecules, including unnatural amino acids, xeno nucleic acids, and other reagents, which could facilitate future explorations of more diverse EV therapeutic design spaces than is currently possible [31,38,72]. Cell-free reactions are also open, meaning that they can be supplemented with chaperones, enzymes, energy-molecules, reducing agents, EVs, and other components to augment their intrinsic cellular or recombinant/PURE-based biochemistries [40,44]. Increased adoption of cell-free EV engineering workflows could have other advantages as well. For example, the possible future creation of community-generated libraries of cell-free expression constructs incorporating many different EV membrane fusion proteins, therapeutic cargoes, cell-targeting elements, or other modular elements could empower interlaboratory data sharing and insights, thereby accelerating EV research [30,31,49]. Similar efforts in other application spaces, including metabolic engineering, biosensors, and therapeutic protein biomanufacturing, have already benefited from such approaches [38,48,50,96,97]. Furthermore, cell-free reaction formats are also highly flexible (batch/dialysis-mode, etc.) and scalable including reports of reaction volumes in excess of several hundred litres [40,72,98].

Whilst technical challenges remain, the cell-free synthetic biology field has made great strides towards improving cell-free protein synthesis yields, batch-to-batch consistency, whilst also reducing reaction costs [40,57,72,98]. Typical trade-offs between different cell-free systems are also narrowing. For example, cell-free production of proteins with post-translational modifications is possible in both eukaryotic and bacterial cell-extract-based expression systems [38,40,50]. Likewise, AI and model-based design-of-experiments approaches have already led to improvements in energy utilisation in bacterial cell extract-based cell-free systems that could

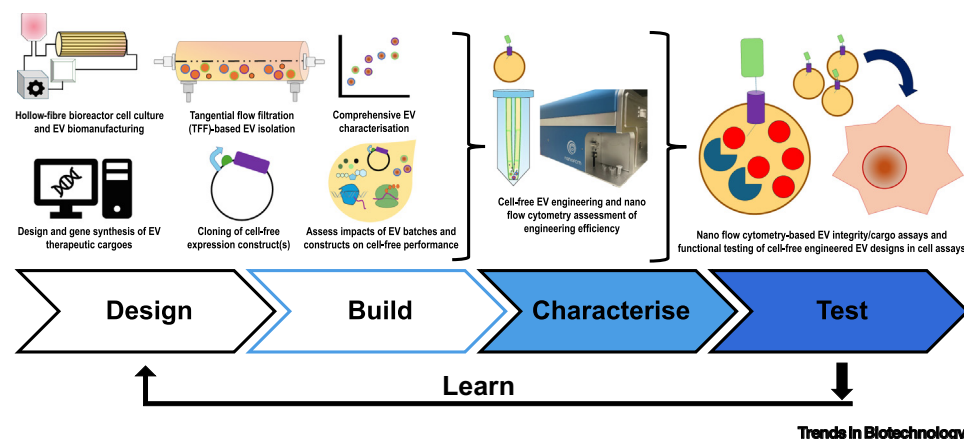


Figure 4. Cell-free extracellular vesicle (EV) engineering workflow. The diagram depicts the key stages of an EV prototyping workflow in which EVs are manufactured, cell-free engineered with therapeutic or other elements, characterised, and then tested in functional assays. These insights can then be used to iterate future therapeutic EV designs, thereby completing the design cycle.

conceivably inspire future advancements in eukaryotic cell-free reaction performance [31,53,72]. Interestingly, clues on how to boost eukaryotic cell-free protein synthesis yields might also come from exploring EV-associated components and their impacts on cell-free reaction performance (Figure S8).

Interdisciplinary research approaches spanning both the EV and engineering biology fields could facilitate other novel scientific avenues and application areas. For example, in the near-term, foundational studies on cell-free reaction energy utilisation, further experimental interrogations of EV cargo loading mechanisms, or the development of EV metrology standards could be carried out [13,24,30,31]. Whilst in the longer-term, cell-free EV engineering could conceivably underpin innovations towards ‘smart’ EVs that have sophisticated biosensing capabilities and therapeutic modalities [13,30,31]. The capabilities and advantages of cell-free EV engineering are also a means to expand upon and complement other exogenous EV engineering approaches, including sonication, extrusion, or electroporation-based methods for loading EVs with therapeutic molecules [31]. Likewise, we also anticipate that liposome/EV hybrids or fully synthetic EVs could conceivably be cell-free engineered using our workflow (Figure 4) [31,35]. In combination, these different EV engineering approaches and the possibilities for future interdisciplinary research across the EV and cell-free synthetic biology research fields could have far-reaching impacts on our future understanding of EV biology (e.g., cargo loading mechanisms [24]), the possibilities for next-generation EV therapeutics, and the speed at which they are prototyped [30,31].

Concluding remarks

In recent years, the EV field has increasingly adopted engineering biology approaches, and these include nascent applications of cell-free synthetic biology technologies in EV research. Cell-free synthetic biology encompasses several different *in vitro* technologies that, in recent years, have evolved into highly powerful prototyping platforms that have helped accelerate biotechnological development. Extrapolating these trends, we envision that it will likely become increasingly feasible to also use cell-free gene expression-based workflows to *in vitro* prototype, characterise and functionally test cell-free engineered EVs (see Outstanding questions).

Here, we sought to further advance these types of cell-free EV engineering approaches from concept towards the development of a realised end-to-end cell-free EV engineering workflow,

Outstanding questions

Are extracellular vesicles from different cell types or disease states easier to cell-free engineer than others?

Can insights into how different extracellular vesicle co-isolation contaminants impact cell-free reactions lead to the discovery of new accessory proteins or metabolites that can further boost cell-free protein synthesis yields?

Can cell-free extracellular vesicle engineering be used to generate sufficient extracellular vesicle for downstream potency assays?

Do frozen and/or lyophilised cell-free engineered extracellular vesicles remain intact and functional over long time periods?

Can insights from cell-free extracellular vesicle engineering be applied to adjacent fields, such as the development and engineering of synthetic cells?

summarised in [Figure 4](#). We envision that future cell-free EV engineering workflows could be employed to accelerate progress in understanding EV biology and the development of EV translational applications. In doing so, cell-free EV engineering could complement other engineering approaches and help accelerate the development of next-generation EV therapeutics for the future benefit of human health.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Dialysis-mode cell-free EV engineering reactions
 - Nano-flow cytometry analysis of cell-free engineered EVs
 - Transmission electron microscopy (TEM)
 - EV integrity and cargo protein staining assay
 - Cell viability assay

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to, and will be fulfilled by, the lead contact, Prof. Paul Freemont (p.freemont@imperial.ac.uk).

Materials availability

Plasmids generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

Primer sequences are available in the Methods section, whilst protein sequences are publicly available in the supplementary material (Table S1 in the Supplemental information online).

This report article does not include original code.

Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

Author contributions

R.J.R.K.: conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualisation, writing—original draft, and writing—review and editing. A.J.W.: conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, and writing—review and editing. A.H.: data curation, formal analysis, investigation, methodology, and writing—review and editing. P.S.F.:

conceptualisation, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualisation, writing—original draft, and writing—review and editing.

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Declaration of interests

The authors declare no competing interests.

Supplemental information

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STAR★METHODS

KEY RESOURCES TABLE

Reagent or Resource	Source	Identifier
Antibodies		
CD63-PE	MACS Miltenyi biotec, Germany	#130-118-077
CD81-PE	MACS Miltenyi biotec, Germany	#130-118-342
GFP-AF647	R&D Systems, MN, USA	#FAB42401R
Bacterial and virus strains		
<i>E. coli</i> JM109	Promega, UK	#L2005
pT7CFE1-Chis	ThermoFisher Scientific, USA	#88860 & [31] pRK63
deGFP	This study	pRK66
mGL	This study	pRK67
CD63-mGL	This study	pRK68
CD63ITM3-mGL	This study	pRK69
Chemicals, peptides, and recombinant proteins		
EGFP	ChromoTek, Manchester, UK	#EGFP
Dulbecco's phosphate-buffered saline (DPBS)	ThermoFisher Scientific, MA, USA	#14190144
Fluorobrite Dulbecco's Modified Eagle Medium (DMEM)	ThermoFisher Scientific, USA	#A1896702
DMEM with high glucose and GlutaMAX	ThermoFisher Scientific, USA	#31966021
10% Exosome-Depleted Fetal Bovine Serum (FBS)	ThermoFisher Scientific	#A25904DG
10% Chemically Defined Medium for High Density Cell Culture	FiberCell Systems, Inc., MD, USA	#CDMHD
Critical commercial assays		
In-Fusion cloning	Takara Bio, USA	#638948
High MWCO (20 kD @ 50%) hydrophilic polysulfone fiber	FiberCell Systems, Inc., MD, USA	#C2011
Microkros 20cm 0.2 µm PES 0.5 mm mll XFLL sterile (hollow fibre module)	Repligen, MA, USA	#C02-P20U-05-S
Plasmid <i>Plus</i> Midi Kit	Qiagen, Germany	#12943
Qubit Protein Assay Kit	ThermoFisher Scientific, MA, USA	#Q33211
Exo-Check Exosome Antibody Array Kit	System Biosciences, USA	#EXORAY200B-4
EV Profiler 2: Tetraspanin Profiling reagent kit	Oxford Nanoimaging, UK	ONI EV Profiler 2
CD63 EV capture immunoplate	HansaBioMed Life Sciences, Tallin, Estonia	#HBM-POC-CC/T1
ExoGlow-Membrane EV labelling solution	System Biosciences, USA	#EXOGM600A-1
1-Step Human High-Yield Mini IVT Kit	ThermoFisher Scientific, USA	#88891
CellTracker Deep Red (CTDR)	ThermoFisher Scientific, USA	#C34565
CellTiter-Fluor Cell Viability Assay	Promega, UK	#G6080
Experimental models: Cell lines		
HEK293	ATCC, USA	#CRL-1573
PANC-1	ATCC, USA	#CRL-1469
Oligonucleotides		
>CF_F GCGGCCGCACTCGAGCACC	IDT, USA	RK036
>CF_R ATGGGTGGTGGCCATATTATCATCG	IDT, USA	RK037

(continued on next page)

(continued)

Reagent or Resource	Source	Identifier
Software and algorithms		
GraphPad Prism	Dotmatics, MA, USA	Software v10
SnapGene	Dotmatics, MA, USA	Software v8.1.1
Fiji	www.imagej.net	Software v1.54p
Other		
FiberCell systems duet pump	FiberCell Systems, Inc., MD, USA	#P3202
Flow NanoAnalyzer (Nano-flow cytometer)	NanoFCM, China	www.nanofcm.com
NanoSight NS300 NTA equipped with a NanoSight Sample Assistant	Malvern Panalytical, UK	www.malvernpanalytical.com
Zetasizer Nano ZS	Malvern Panalytical, UK	www.malvernpanalytical.com
Nanoimager super-resolution microscope	Oxford Nanoimaging, UK	www.oni.bio
CLARIOstar plate reader	BMG, UK	www.bmglabtech.com
NanoClean Model 1070	Fischione, USA	www.fischione.com
FEI Tecnai T12 Spirit	ThermoFisher Scientific, USA	www.thermofisher.com
EVOS Flouid fluorescent microscope	ThermoFisher Scientific, USA	www.thermofisher.com
Zeiss Axio Observer wide field microscope	Carl Zeiss AG, Germany	www.zeiss.de

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains and growth conditions

Plasmid constructs and strains used in this study are listed in the key resources table. *Escherichia coli* JM109 (#L2005, Promega, UK) was used for both cloning and generation of cell-free gene expression plasmids. For plasmid recovery *E. coli* strains were grown in Luria-Bertani (LB) medium supplemented with 100 µg/ml Ampicillin (w/v final concentration) and cultured at 37 °C with shaking (200 rpm).

METHOD DETAILS

Strain and plasmid construction

pT7CFE1-Chis (pRK63, #88860, ThermoFisher Scientific, USA) was commercially sourced from the 1-Step Human High-Yield Mini IVT Kit (#88891; ThermoFisher Scientific) and was transformed into competent *E. coli* JM109 (Promega) to create strain pRK63. The choice of pT7CFE1-Chis was due to the plasmid also encoding a T7 RNA polymerase promoter (pT7) and an internal ribosomal entry site (IRES) from the encephalomyocarditis virus (EMCV), that can facilitate cell-free gene expression of cloned inserts in HeLa and other eukaryotic-based cell-free systems [31,49,50]. For deGFP (pRK66), mGL (pRK67), CD63-mGL (pRK68) and CD63ITM3-mGL (pRK69) cell-free expression plasmids the inserts encoding the relevant proteins were designed using SnapGene cloning software (v8.1.1, Dotmatics, MA, USA) and then ordered as dsDNA gene synthesis fragments (gBlocks, IDT, USA). The plasmid backbone (pT7CFE1-Chis) was amplified and linearised using primer pair RK036 (CF_F) and RK037 (CF_R). PCR products were processed through agarose gel electrophoresis (1% w/v) and subsequently gel purified using the Monarch DNA gel extraction kit (#T1020, New England Biolabs, USA). Finally, In-Fusion cloning (Takara Bio, USA) of linearised pT7CFE1-Chis with the appropriate gBlocks (deGFP, mGL, CD63-mGL or CD63ITM3-mGL) followed by transformation into *E. coli* JM109 (Promega) was used to clone the indicated cell-free expression plasmids. Protein sequences are indicated in Table S1. Constructs were verified using the whole-plasmid sequencing service provided by Full Circle Labs Ltd (London, UK).

To generate plasmids for cell-free experiments, glycerol stocks of the appropriate strains were used to inoculate 100 ml Luria-Bertani (LB) medium cultures supplemented with 100 µg/ml Ampicillin (final concentration), and these cultures were incubated overnight at 37 °C with shaking (200 rpm). Plasmid DNA was isolated using a Qiagen Plasmid PlusMidi Kit (#12943, Qiagen, Germany) and then ethanol precipitated prior to usage within the indicated cell-free reactions.

Hollow-fibre cell culture

HEK293 cells (#CRL-1573, ATCC, USA) were cultured, as previously described [3,31], within a hollow-fibre bioreactor (FiberCell Systems, Inc., MD, USA), which was configured with a 20 kDa molecular weight cut-off cartridge (#C2011, FiberCell Systems,

Inc.) to enable high cell density culture (up to $\sim 10^9$ HEK293 cells) and the concentration of secreted HEK293 cell products (>20 kDa proteins and extracellular vesicles [EVs]) within the cartridge. Prior to cell seeding, the hollow-fibre cartridge was sequentially primed, for 24 h each, with Dulbecco's phosphate-buffered saline (DPBS, 1X; #14190144, ThermoFisher Scientific, MA, USA), then serum-free Fluorobrite Dulbecco's Modified Eagle Medium (DMEM, #A1896702, ThermoFisher Scientific, USA) and finally Fluorobrite DMEM (#A1896702, ThermoFisher Scientific), supplemented with 10% Exosome-Depleted Fetal Bovine Serum (FBS, #A25904DG, ThermoFisher Scientific). Exosome-depleted FBS was only used during priming.

Once primed, the cartridge was seeded with 1×10^8 adherent HEK293 cells that were cultured with Fluorobrite DMEM (#A1896702, ThermoFisher Scientific). Cell growth rate/viability was indirectly evaluated through daily monitoring of the glucose level of the cell culture medium (#GC001000, FiberCell Systems, Inc., MD, USA). The cell culture medium was changed when the glucose levels were below half the original level. Once glucose consumption levels significantly increased (50% glucose consumed within 24 h) the cell culture media was changed to Fluorobrite DMEM (#A1896702, ThermoFisher Scientific), supplemented with 10% Chemically Defined Medium for High-Density Cell Culture (#CDMHD, FiberCell Systems). Conditioned media (~ 20 ml volume sample) was harvested from the hollow-fibre cartridge each day. However, once maximum cell density was reached, and cell glucose consumption exceeded 1.5 g per day then two conditioned media samples (~ 20 ml volume) were harvested from the hollow-fibre cartridge each day.

Isolation of HEK293 extracellular vesicles

HEK293 cells were cultured in a hollow-fibre bioreactor as described above and the harvested cell-conditioned media batches were split into two, such that one 5 ml aliquot was used to isolate extracellular vesicles using a previously described ultracentrifugation (UC) protocol [31,99] and the other 5 ml aliquot was used to isolate extracellular vesicles using tangential flow filtration (TFF). Briefly, 5 ml UC samples were first centrifuged at 300g for 10 minutes. The resultant supernatant was collected then centrifuged at 2,500g for 10 minutes. The supernatant was again collected and then further centrifuged at 16,500g for 30 minutes at 4°C with a Thermo Scientific Sorvall wX+ Ultra Series ultracentrifuge and T-647.5 fixed angle rotor. The supernatants were subsequently filtered using a 0.2 μ m filter and then ultracentrifuged at 110,000g for 70 minutes at 4°C. The resultant pellet was washed with 0.05 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific), re-suspended in 0.05 μ m filtered DPBS and then ultracentrifuged at 110,000g for 70 minutes at 4°C. Post-centrifugation, the supernatant was discarded, and the pellet containing extracellular vesicles was re-suspended into 200 μ l of 0.05 μ m filtered DPBS. In contrast, the 5 ml tangential flow filtration samples were first centrifuged at 300g for 10 minutes, then the supernatants were further centrifuged at 2,500g for 10 minutes to help remove cells and large debris. Prior to sample TFF-processing the sterile 0.2 μ m tangential flow filtration modules (MicroKros #C02-P20U-05-S, Repligen, MA, USA) were sequentially pre-wetted prior to use with ethanol (20 % v/v) and DPBS (1X; #14190144; 0.05 μ m filtered). Finally, centrifugation and TFF-processed cell media samples were normalised to 1 ml sample volumes.

Extracellular vesicle characterisation

Qubit protein assay. The protein concentrations of ultracentrifugation (UC) and tangential flow filtration (TFF) isolated EV samples were determined, according to the manufactures' guidance, using a Qubit 3 Fluorometer (Thermo Fisher Scientific, MA, USA) and a Qubit Protein Assay Kit (#Q33211, Thermo Fisher Scientific, MA, USA).

Nanoparticle tracking analysis (NTA)

UC-isolated HEK293 cell EVs were diluted (1:2000) and TFF-isolated HEK293 cell EVs were diluted (1:500) within particle-free DPBS (0.2 μ m filtered; 1X; #14190144, Thermo Fisher Scientific), gently pipetted for 10 s and then aliquoted into a deep-well, 96-well plate. Samples from the 96-well plate were injected into a NanoSight NS300 NTA instrument equipped with a NanoSight Sample Assistant (Malvern Instruments, UK) autosampler system. The NTA images were recorded and analysed to obtain the concentration and distribution of the sample particles. Additional software version and measurement settings are shown in Figure S2.

Dynamic light scattering (DLS)

UC and TFF-isolated HEK293 EVs were diluted (1:100) within particle-free DPBS (1X; 0.2 μ m filtered; #14190144, Thermo Fisher Scientific), gently mixed by pipetting and transferred to a ZEN2112 quartz cuvette (Malvern Panalytical, Malvern, UK). Three technical replicates per sample were measured at 25 °C on a Malvern Zetasizer Nano ZS (Malvern Panalytical).

Exo-Check Exosome Antibody Array

Ultracentrifugation and tangential flow filtration isolated HEK293 EVs were lysed and processed, according to the manufacturer's guidance, and analysed for the presence of a panel of EV markers using the Exo-Check Exosome Antibody Array Kit (#EXORAY200B-4, System Biosciences, USA). The Exo-Check array included 12 dot blot lines that incorporated antibodies specific for the EV markers CD63, CD81, ALIX, FLOT1, ICAM, EpCam, ANXA5 and TSG101, as well as a cellular contamination control (GM130 cis-Golgi marker) and several assay positive or negative [blank] controls. The dot blot array was developed using Immobilon Crescendo Western HRP substrate (#WBLUR0500, Millipore/Merck, Darmstadt, Germany) and imaged using a ChemiDoc imaging system (Bio-Rad Laboratories Inc., USA).

Super-resolution microscopy

Ultracentrifugation and tangential flow filtration-isolated HEK293 extracellular vesicles were prepared and stained for super-resolution microscopy using an EV Profiler 2: Tetraspanin Profiling reagent kit for CD63, CD81 and CD9 tetraspanins, according to manufacturer's recommendations (Oxford Nanoimaging [ONI], UK). EV samples were imaged using direct stochastic optical reconstruction microscopy (dSTORM), using a Nanoimager super-resolution microscope (Oxford Nanoimaging [ONI]) using laser channels (CD63-561, CD81-647 & CD9-488) and settings shown in Figure S5. Resultant images were processed and analysed using the CODI online platform (Oxford Nanoimaging [ONI] <https://alto.codi.bio/>).

Nano-flow cytometry analysis of EV markers

100 µl EV staining samples were setup including 3 µl of ultracentrifugation or 3 µl of tangential flow filtration isolated HEK293 extracellular vesicles, 0 µl or 5 µl of either CD63-PE (#130-118-077, MACS Miltenyi biotec, Germany) or CD81-PE (#130-118-342, MACS Miltenyi biotec, Germany) and 92 µl or 97 µl of 0.2 µm filtered DPBS (1X; #14190144, ThermoFisher Scientific). EV staining samples were pipette mixed and incubated at room temperature (21°C) for 30 minutes, with 350 rpm shaking (Thermo Mixer C, Eppendorf, Germany). Post staining, these 100 µl EV staining samples were processed using Vesi-SEC micro size exclusion chromatography columns (Vesiculab, Nottingham, UK) to remove unbound antibody. Briefly, Vesi-SEC micro columns were centrifuged at 1,000g for 1 minute to remove storage buffer, then post-centrifugation were placed into clean 1.5 ml tubes. 100 µl EV staining samples were loaded into the prepared Vesi-SEC micro columns and incubated for 1 minute. Post incubation, samples were centrifuged at 1,000g for 1 minute and the eluates (stained EV samples) were diluted 1:825 (UC) or 1:297 (TFF) within 0.2 µm filtered DPBS (1X; #14190144, ThermoFisher Scientific) ready for nano-flow cytometry analysis (Flow NanoAnalyzer, NanoFCM, China). The Flow NanoAnalyzer was calibrated using QC Beads (250 nm SiNP Dual laser QC Beads NanoFCM #QS2503) and size reference standard beads (Silica nanospheres, NanoFCM #S16M-Exo 68-155 nm) were used to calibrate the size distribution of EVs, whilst 0.2 µm filtered DPBS (1X; #14190144, ThermoFisher Scientific) was used as a blank control. Standards and samples were recorded for 1 minute using excitation laser 488 nm, SSC (488/10 nm) and PE (580/40 nm) filters. Data analysis (including sample thresholding to remove background and detector noise signals) was carried out according to the manufacturer's recommendations and best practices [100], using Flow NanoAnalyzer software (v2.0).

ExoGlow membrane EV labelling

Control and EV samples were setup for ExoGlow labelling with 0 µl or 5 µl of HEK293 extracellular vesicles (2.5×10^{10} /ml of either ultracentrifugation or tangential flow filtration-isolated EVs as indicated) and 81 µl or 86 µl of either 0.2 µm filtered DPBS (1X; #14190144, ThermoFisher Scientific) or 0.2 µm filtered DPBS-T (DPBS with 0.1% [v/v] Triton X-100). These samples were incubated for 10 minutes at room temperature (21°C) and then added to appropriate wells in an CD63 EV capture immunoplate (#HBM-POC-CC/T1, HansaBioMed Life Sciences, Tallin, Estonia). Appropriate wells were stained with either 0 µl or 14 µl of ExoGlow-Membrane EV labelling solution (2 µl labelling dye and 12 µl reaction buffer; #EXOGM600A-1, System Biosciences), gently pipette mixed and incubated at room temperature (21°C) in the dark for 60 minutes with 100 rpm shaking (Thermo Mixer C, Eppendorf, Germany). Post incubation, wells were gently washed twice with 100 µl of 0.2 µm filtered DPBS (1X; #14190144, ThermoFisher Scientific) and then filled with 100 µl of 0.2 µm filtered DPBS (1X; #14190144, ThermoFisher Scientific). Plates were measured on a CLARIOstar plate reader (BMG, UK) with the following settings - excitation 465-15 nm, 548.8 nm dichroic filter and emission 635-20 nm with 1800 gain.

Cell-free time course reactions

Cell-free time course reactions (10 µl) were prepared using a 1-Step Human High-Yield Mini IVT Kit (#88891; ThermoFisher Scientific, USA) and consisted of the following components: 2 µl Reaction Mix, 1 µl Accessory Proteins, 5 µl HeLa cell lysate, 0 or 400 ng of plasmid DNA (deGFP), 0 µl, 0.3 µl [Low] or 0.6 µl [High] of HEK293 cell EVs (2.5×10^{10} /ml of either ultracentrifugation [UC] or tangential

flow filtration [TFF] isolated HEK293 EVs as indicated) and nuclease free water to top up the reactions to 10 μ l (final volume). These 10 μ l cell-free time course reactions were pipetted into individual wells of 384-well plates (Griener bio-one, NC, USA) and measured using a CLARIOstar plate reader (BMG, UK) with the following settings: 30°C incubation temperature, excitation laser 483-14 nm, 502.5 dichroic and emission filter 530–30 nm with 1000 gain. Plates were sealed and incubated within the plate reader at 30°C. Sample plates were shaken (500 rpm) for 5 seconds prior to each 10 min reading cycle during the 10 h time course. Endpoint measurements were taken at 200 minutes and were used, along with a GFP calibration curve, to calculate GFP yields in the respective cell-free time course reactions. The GFP calibration curve was adapted from previous studies [3,48]. Briefly, EGFP (#EGFP; ChromoTek, Manchester, UK) was serially diluted 0–1 mg/ml, aliquoted as 10 μ l volumes in technical quadruplicate into appropriate wells of a 384-well plate (#781096, Greiner Bio-One, Kremsmünster, Austria) and measured using a CLARIOstar plate reader (excitation laser 483-14 nm, 502.5 nm dichroic and emission filter 530–30 nm with 1000 gain). These data were used to plot the GFP calibration curve (Figure S9) using GraphPad Prism v10 software.

Dialysis-mode cell-free EV engineering reactions

Cell-free reaction master mixes (60 μ l) were prepared using a 1-Step Human High-Yield Mini IVT Kit (#88891; ThermoFisher Scientific, USA) and consisted of the following components: 12 μ l Reaction Mix, 6 μ l Accessory Proteins, 30 μ l HeLa cell lysate, 0 or 2.4 μ g of plasmid DNA (mGL, CD63-mGL or CD63ITM3-mGL), 3.6 μ l of HEK293 EVs (2.5×10^{10} /ml of either control [1.8 μ l UC + 1.8 μ l TFF], ultracentrifugation [UC] or tangential flow filtration [TFF] isolated HEK293 EVs as indicated and nuclease free water to top up the master mixes to 60 μ l. These 60 μ l master mixes were aliquoted into a micro-dialysis cassette (10kDa MWCO, ThermoFisher Scientific/Pierce #88260) that was inserted into a 2 ml microtube pre-filled with 1.4 ml of Dialysis Buffer (from 1-Step Human High-Yield Mini IVT Kit #88891). Cell-free dialysis reactions were incubated in an Eppendorf Thermo Mixer C at 30°C with 950 rpm shaking for 22h. Post-incubation these dialysis-mode cell-free EV engineering reactions were pipetted out from the dialysis cassettes and into clean 1.5 ml microtubes. 10 μ l of these master mixes were subsequently aliquoted into individual wells of 384-well plates (Griener bio-one, NC, USA) and endpoint mGL fluorescence was measured using a CLARIOstar plate reader (BMG, UK) with the following settings: preheated to 30°C, excitation 483-14 nm, 502.5 dichroic and emission 530–30 nm with 1500 gain. The remaining samples were analysed using nano-flow cytometry as described below.

Nano-flow cytometry analysis of cell-free engineered EVs

Control and cell-free engineered HEK293 extracellular vesicles were antibody stained *in situ* (i.e. within their respective cell-free reactions) in staining reactions including 10 μ l of the indicated cell-free EV engineering reaction (Control, mGL, CD63-mGL or CD63ITM3-mGL with UC and/or TFF-isolated HEK293 cell EVs), 90 μ l of 0.2 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific) and 0.5 μ l GFP-AF647 conjugated antibody (0.1 μ g; #FAB42401R, R&D Systems, MN, USA), totalling 100.5 μ l (final volume). These EV staining samples were pipette mixed and incubated at room temperature (21°C) for 30 minutes with 350 rpm shaking (Thermo Mixer C, Eppendorf, Germany). Post-incubation, these EV staining samples were processed using Vesi-SEC micro size exclusion chromatography columns (Vesiculab, Nottingham, UK) to remove unbound antibody and to enrich for EVs. Briefly, Vesi-SEC micro columns were centrifuged at 1,000g for 1 minute to remove storage buffer, then the 100.5 μ l antibody-stained cell-free engineered EV samples were loaded into the prepared Vesi-SEC micro columns, incubated for 1 minute and centrifuged at 1,000g for 1 minute to elute stained and non-antibody stained cell-free engineered EVs. Eluted EVs were diluted 1:16,000 within 0.2 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific) ready for nano-flow cytometry analysis (Flow NanoAnalyzer, NanoFCM, China). The Flow NanoAnalyzer was again calibrated using QC Beads (250 nm SiNP Dual laser QC Beads NanoFCM #QS2503) and size reference standard beads (Silica nanospheres, NanoFCM #S16M-Exo 68-155 nm), whilst 0.2 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific) was used as a blank control. Standards and samples were recorded for 1 minute using 488 nm and 638 nm excitation lasers and several filters SSC (488/10 nm), FITC (525/40 nm) and AF647 (670/30 nm). Data analysis, including sample thresholding to remove background and detector noise signals was carried out according to the manufacturer's recommendations and best practices [100] using Flow NanoAnalyzer software (v2.0). Where indicated a quadrant gating strategy was used to identify cell-free engineered EVs (mGL +ve) and GFP antibody stained EVs (AF647 +ve).

Transmission electron microscopy (TEM)

TEM imaging was adapted from a previous protocol [101]. Briefly, 4 μ L of the indicated UC, TFF or cell-free engineered EV samples were deposited onto 300 mesh continuous carbon support grids (Agar Scientific, UK) that were previously glow-discharged by plasma for 90s using a NanoClean Model 1070 (Fischione, USA). After 1 min of sample adsorption, excess buffer was blotted off,

and the grids were briefly washed and incubated with 2% uranyl acetate (v/v) for 55 s. Following this, excess liquid was blotted off, and the grids were left to dry at room temperature prior to imaging. Samples were screened on an FEI Tecnai T12 Spirit (ThermoFisher Scientific) with the accelerating voltage of 120 kV and TEM images were acquired at the following magnifications, 11Kx, 30Kx and 50Kx, on a FEI Eagle 2K CMOS camera.

EV integrity and cargo protein staining assay

Cell-free reaction master mixes (60 μ l) were prepared using a 1-Step Human High-Yield Mini IVT Kit (#88891; ThermoFisher Scientific, USA) and consisted of the following components: 12 μ l Reaction Mix, 6 μ l Accessory Proteins, 30 μ l HeLa cell lysate, 0 or 2.4 μ g of plasmid DNA (mGL, or CD63ITM3-mGL), 3.6 μ l of TFF-isolated HEK293 EVs (2.5×10^{10} /ml) and nuclease free water to top up the master mixes to 60 μ l. These 60 μ l master mixes were aliquoted into a micro-dialysis cassette (10kDa MWCO, ThermoFisher Scientific/Pierce #88260) that was inserted into a 2 ml microtube pre-filled with 1.4 ml of Dialysis Buffer (from 1-Step Human High-Yield Mini IVT Kit #88891). Cell-free dialysis reactions were incubated in an Eppendorf Thermo Mixer C at 30°C with 950 rpm shaking for 22h. Post-incubation, these dialysis-mode cell-free EV engineering reactions were pipetted out from the dialysis cassettes and into clean 1.5 ml microtubes. Control and cell-free engineered HEK293 EVs were stained *in situ* (i.e. within their respective cell-free reactions) in staining reactions including 36 μ l of the indicated cell-free EV engineering reaction (Control [No DNA], mGL, or CD63ITM3-mGL with TFF-isolated HEK293 cell EVs), 72.9 or 74 μ l of 0.2 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific) and 0 or 1.1 μ l of CellTracker Deep Red dye (CTDR, 500 nM final concentration; #C34565, ThermoFisher Scientific), totalling 110 μ l (final volume). These EV staining samples were pipette mixed and incubated at room temperature (21°C) for 30 minutes with 350 rpm shaking (Thermo Mixer C, Eppendorf, Germany). Post-incubation, these EV staining samples were processed using Vesi-SEC columns (Vesiculab) to remove unbound dye and to enrich for EVs. Briefly, Vesi-SEC columns were centrifuged at 1,000g for 1 minute to remove storage buffer, then the 110 μ l samples were loaded into the prepared Vesi-SEC columns, incubated for 1 minute and centrifuged at 1,000g for 1 minute to elute the CTDR-dye stained and non-stained cell-free engineered EVs. Eluted EVs were diluted 1:9800 within 0.2 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific) ready for nano-flow cytometry analysis (Flow NanoAnalyzer, NanoFCM). The Flow NanoAnalyzer was again calibrated using QC Beads (250 nm SiNP Dual laser QC Beads NanoFCM #QS2503) and size reference standard beads (Silica nanospheres, NanoFCM #S16M-Exo 68-155 nm), whilst 0.2 μ m filtered DPBS (1X; #14190144, ThermoFisher Scientific) was used as a blank control. Standards and samples were recorded for 1 minute using 488 nm and 638 nm excitation lasers and several filters SSC (488/10 nm), FITC/mGL (525/40 nm) and CTDR (670/30 nm). Data analysis, including sample thresholding to remove background and detector noise signals was carried out according to the manufacturer's recommendations and best practices [100] using Flow NanoAnalyzer software (v2.0). Where indicated a quadrant gating strategy was used to identify intact cell-free engineered EVs (CD63ITM3-mGL and CTDR +ve [P1]).

Cell viability assay

PANC-1, pancreatic ductal adenocarcinoma cells (#CRL-1469, ATCC) were cultured in DMEM with high glucose and GlutaMAX (#31966021, ThermoFisher Scientific) supplemented with 10% Exosome-Depleted FBS (#A25904DG, ThermoFisher Scientific) at 37°C and 5% CO₂. PANC-1 cells were seeded into 96-well plates at a density of 5,000 cells and 100 μ l media per well. Plated PANC-1 cells were cultured overnight and the next day the cell conditioned media were replaced with 100 μ l of fresh media/well (DMEM with high glucose + 10% Exosome-Depleted FBS). The plated PANC-1 cells were subsequently treated with either 10 μ l DPBS (1X; #14190144, ThermoFisher Scientific), 3% dimethyl sulfoxide (DMSO) (v/v) or 10 μ l of the indicated cell-free EV engineering reactions that were prepared and processed using Vesi-SEC columns (Vesiculab) as described above. At 4 h post treatment the cells were imaged using an EVOS Flويد cell imaging station fluorescent microscope (#E2616-FLD2-046, ThermoFisher Scientific) equipped with a 20X objective, transmitted light and GFP (Ex: 482/18 nm, Em: 532/59 nm) channels. At 24 h post treatment PANC-1 cell viability was assessed using the CellTiter-Fluor Cell Viability Assay (#G6080, Promega) according to the manufacturer's recommendations.

Separately, 65,000 PANC-1 cells were seeded into a 24 well plate within 1 ml of media/well (DMEM with high glucose and GlutaMAX [#31966021] supplemented with 10% Exosome-Depleted FBS [#A25904DG]) and were incubated overnight at 37°C and 5% CO₂. The next day PANC-1 cells were washed, then 500 μ l of fresh media was added and finally the PANC-1 cells were treated with either 100 μ l of DPBS (1X; #14190144) or 100 μ l of the indicated cell-free EV engineering reactions that were prepared, CTDR-stained and processed using Vesi-SEC columns (Vesiculab) as described above. At 2 h post treatment the cells were imaged using an Axio Observer wide field microscope (Zeiss AG, Germany) equipped with a 10X objective, Differential Interference Contrast (DIC), GFP (Ex: 470/40 nm, Em: 525/50 nm) and CTDR (Ex: 640/30 nm, Em: 690/50 nm) channels. Images were processed using Fiji v1.54p [102].