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Citation for published version:
Sebastian, V, Sancho-Albero, M, Arruebo, M, Perez-Lopez, A, Rubio Ruiz, B, Martin-Duque, P, Unciti-Broceta, A & Santamaría, J 2020, 'Non-destructive production of exosomes loaded with ultrathin Palladium nanosheets for targeted bioorthogonal catalysis', *Nature Protocols*. https://doi.org/10.1038/s41596-020-00406-z

Digital Object Identifier (DOI):
10.1038/s41596-020-00406-z

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Nature Protocols

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Download date: 17. Aug. 2021

Non-destructive production of exosomes loaded with ultrathin Palladium nanosheets for targeted bioorthogonal catalysis

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KEYWORDS Exosomes; Palladium Nanosheets; Bioorthogonal Catalysis; Cell Targeting; Anticancer therapy

EDITORIAL SUMMARY A protocol for producing exosomes loaded with ultrathin Palladium nanosheets for targeted bioorthogonal catalysis. Exosomes are isolated by ultracentrifugation and Pd precursors are loaded in their interior by diffusion and reduced into metallic nanosheets using gas phase CO.

TWEET A new protocol for non-disruptive loading of Pd #nanosheets into #exosomes for targeted #bioorthogonal #catalysis.

COVER TEASER Non-destructive production of Pd-loaded exosomes

Please indicate up to four primary research articles where the protocol has been used and/or developed (including DOI).

1. María Sancho□Albero, Belén Rubio□Ruiz, Ana M. Pérez□López, Víctor Sebastián, Pilar Martín□Duque, Manuel Arruebo, Jesús Santamaría and Asier Unciti□Broceta. Cancer□derived exosomes loaded with ultrathin palladium nanosheets for targeted biorthogonal catalysis. Nature Catalysis. 2019, 2, 864. doi.org/10.1038/s41929□019□0333□4

2. V. Sebastian, Christopher D. Smith and Klavs F. Jensen, Shape□controlled continuous synthesis of metal nanostructures. Nanoscale, 2016, 8, 7534. doi.org/10.1039/C5NR08531D

Abstract

The use of exosomes as selective delivery vehicles of therapeutic agents, such as drugs or hyperthermia-capable nanoparticles is being intensely investigated on account of their preferential tropism towards their parental cells. However, the methods used to introduce a therapeutic load inside exosomes often involve disruption of their membrane, which may jeopardize their targeting capabilities, attributed to their surface integrins. On the other hand, in recent years bioorthogonal catalysis has emerged as a new tool with a myriad of potential applications in medicine. These bioorthogonal processes, often based on Pd-catalyzed chemistry, would benefit from systems capable of delivering the catalyst to target cells. It is therefore highly attractive to combine the targeting capabilities of exosomes and the bioorthogonal potential of Pd nanoparticles to create new therapeutic vectors. In this protocol, we provide detailed information on an efficient procedure to achieve a high load of catalytically active Pd nanosheets inside exosomes, without disrupting their membranes. The protocol involves a multistage process in which exosomes are first harvested, subjected to impregnation with a Pd salt precursor followed by a mild reduction process using gas phase CO which acts as both reducing and growth-directing agent to produce the desired nanosheets. The technology is scalable and the protocol can be conducted by any researcher having basic biology and chemistry skills in about 3 days.

Introduction

Bioorthogonal chemistry provides exciting opportunities in medicine by allowing *in situ* synthesis of functional molecules that are useful as probes to provide information of ongoing biochemical processes and/or drugs capable of modulating physiological or pathological processes.[1,2] As in other fields of chemistry, in a bio-orthogonal scenario the use of catalysis is effective to facilitate multiple cycles of selected chemical reactions and such approach is being widely explored for different applications, giving rise to the subfield of bioorthogonal catalysis[3-6]. In particular, Pd has often been used as the catalyst of choice, given its flexibility to enable a wide variety of reactions (e.g. dealkylations,[7-14] cross-coupling reactions,[7,14-17] ring formation[18], cleavage[19], etc.). The use of intra-tumor Pd-catalyzed chemistry to generate toxic species *in situ* from molecules having low toxicity (e.g. through catalyzed uncaging reactions) would be of enormous interest in oncology, as systemic administration of the drug would be replaced by a localized intra-tumoral production[3-6], potentially reducing side effects by orders of magnitude.[8,12] While proof of concept demonstrations have been achieved *in vitro*, *ex vivo* and *in vivo*,[8,10-13,20-22] the use of bioorthogonal catalytic chemistry *in vivo* faces enormous challenges. The main obstacle to be cleared refers to the fact that the catalyst (e.g. Pd nanoparticles) must be selectively delivered to the target cells or tissues within the organism. A similar problem has been faced for a long time by researchers working on selective drug delivery using nanoscale vectors, with tumors often being the target of the encapsulated drug[22-27]. In response to this challenge, a range of nanoparticle-based drug delivery systems (organic and inorganic, with a variety of stealth covers and targeting moieties) has been developed, although the delivery efficiency is poor: on average, less than one percent of the administered nanoparticle dose reaches the tumor, even for highly sophisticated antibody-grafted nanoparticles.[28]

To address the challenge of delivering Pd catalysts specifically to cancer cells, our laboratories have recently reported the use of cancer-derived exosomes as targeted carrier systems.[29] Exosome-based delivery is raising huge interest as a more effective alternative way to deliver catalysts to tumors.[30-32] Exosomes are nanoscale extracellular entities known since the early 1980s when they were defined as vesicles produced by exfoliation of cell membranes.[33] They are secreted by most cells in the organism and

for a long time it was thought that their main function was to dispose unwanted material out of the cell. Today, it is known that exosomes contain a number of key components from the parental cell including proteins, lipids and nucleic acids, and play a crucial role in cell to cell communication.[34,35] More importantly, there are strong indications that the exosomal membrane may confer unique identification properties to these vesicles that could allow selective targeting towards their cells of origin.[32,36,37] We have developed a method that allows loading a wide variety of cell derived exosomes with Pd nanosheets. The synthesis procedure is based on the in situ reduction of Pd metal precursors previously encapsulated by diffusion within the exosomes. A mild chemical gas-phase methodology was developed to enable the controlled assembled of Pd nanosheets inside exosomes, without compromising their integrity and functionality. Our diffusion-based method for loading the Pd precursor into exosomes allows to solve the main problem when using exosomes as catalytic delivery vectors: to introduce the desired payload inside the exosomes without disrupting the exosomal membrane. Here we present a detailed protocol to create exosome-catalyst hybrids that preserves the inherent properties of exosomes and incorporates the bioorthogonal activity of the catalyst.

Applications of the method

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Comparison with other methods

Conventional methods to introduce metallic nanoparticles inside exosomes include aggressive methods such as electroporation, sonication or thermal shock, and milder methods such as incubation[24]. We studied these procedures in a previous work[24] and concluded that not only these methods showed a poor yield of nanoparticle-loaded exosomes, but also that especially the most severe methods produced significant damage to the exosomes, resulting in impaired morphology and damaged exosomal membranes. In addition, using those previous methods, the ability to control the amount of the catalytic cargo inside the exosomes is limited. We and others obtained improved results by leveraging the physiological biogenesis pathway: in this case, the parental cells are incubated with the desired nanoparticles to allow endocytosis followed by excretion via the exosomal route.[32,38] In this way the exosomes are naturally loaded with the

desired nanoparticles, allowing to preserve the key features of their membrane. However, this method faces important limitations to produce catalyst-loaded exosomes. First, the catalytic nanoparticle itself must be biocompatible: since the cell biological mechanisms are used to generate loaded exosomes, the cell must be alive and completely functional, and the load must be necessarily non-toxic at the doses used. Second, it is not possible to control the load of the catalyst inside the exosomes. The catalyst-loaded exosomes are formed in a complex process: nanoparticles are internalized by endocytosis and enclosed into multivesicular bodies that eventually fuse with the cellular membrane to produce exosomes.[24,39] In contrast, our recently developed method[29] works with exosomes that are harvested and purified from cell cultures, then incubated with the desired catalyst precursor and finally subjected to a mild reduction process to generate the active catalyst. The amount of catalyst inside the exosomes can be tuned by controlling the concentration of the precursor solution and/or the diffusion time.

Limitations

The procedure described here could be applied to the majority of the cells lines, but also to exosomes obtained from any other source, including fluids from the organism (serum, urine, saliva, etc.), explants or concentrates from different tissues. However, along with the critical steps highlighted in the procedure, there are possible limitations to be considered: 1) A multidisciplinary team is necessary to enable the proper culture of cells to isolate exosomes, and then treat them in a well-equipped chemical engineering lab facility to manipulate the toxic CO gas at high pressure. Cryo-Transmission Electron microscopy, Energy Dispersive X-ray Spectroscopy (EDS) analysis, Microwave Plasma Atomic Emission Spectroscopy (MP-AES) and an ultracentrifuge are also required to validate the quality of the resulting Pd modified exosomes for their biomedical use. 2) The exogenous production of exosomes relies on the understanding of exosome biogenesis pathway, since the exosome harvesting and isolation protocols could vary between cell lines from different sources. 3) The size of exosomes produced can be different for different cell lines, this fact may require optimization of the isolation conditions to avoid an inefficient separation. 4) The promising potential of bioorthogonal catalysis in biomedicine inspires new strategies to produce bioactive agents, regulating their activity in living systems. However, to achieve this, it is not

only crucial to nanoengineer biocompatible catalysts and produce customized inactive prodrugs, but also to choose the right vector to deliver abiotic catalysts into designated locations. Then, it is key not to modify the functionality of exosomes with any supporting reagent such as the ones usually considered in wet chemistry procedures, such as reducing agents and stabilizers. The use of those reagents could modify the chemical functionality of exosomes and their preferential tropism to progenitor cells, which would unable the bioactive agent production in targeted therapies.

Experimental design

The entire procedure includes the following stages: cell culture and exosome isolation (Steps 1-21), exosome concentration determination (Steps 22-23), loading of exosomes with Pd precursor by diffusion (Steps 24-28), setup of the high pressure CO reduction system and assembly of Pd nanosheets inside exosomes (Steps 29-51), Pd-exosome characterization (Steps 52-153) and Pd-exosome activity test with a fluorogenic sensor *in vitro* (Steps 167-178) and inside cancer cells (Steps 154-166) (Fig 1). The individual stages are discussed in detail in the following sections.

Cell culture and exosome isolation. Differences in the protein, genetic and lipid contents and surface composition and their relative abundances, can be observed in exosomes secreted from different parental cells[40]. These components do not only determine effective targeting of the exosomes, but also they could potentially interfere with the diffusion of metal precursors.[3] These considerations should be taken into account when adopting this method to other cell types. We have successfully tested our approach in four different cell lines: 1&2) mouse melanoma cell lines with two aggressive states, low metastatic B16-F1 and high metastatic B16F10; 3) non-small cell lung carcinoma (NSCLC) A549 cells and 4) human placenta-derived mesenchymal stem cells (hPMSCs) (see Anticipated Results). Lung cancer is the most common cause of cancer-related death in men and second most common in women after breast cancer[41]. Approximately 80% of lung cancer is NSCLC[4], which has prompted us to also test our approach on a widely used human NSCLC cell line, A549[5] [6] Based on the results with the 4 cell lines shown in the Anticipated Results section, we postulate that our approach can be used with a wider range of exosomes, including coming from many other cell lines and with exosomes isolated from blood and tissues. When

adapting the approach for different cell lines, it is important to consider cell growth rate to optimize the purification and amounts of exosomes obtained. Researcher should take into account whether his/her cell line secretes high amounts of exosomes to calculate the starting cell amount to be seeded. For different and more complex exosome sources such as tissue, blood, urine, etc., additional purification steps might be employed such as sucrose gradient or an immunocapture procedure.

Another determinant factor would be the lipidic membrane composition from each type of exosome. Their membrane might change amongst different sources and the resistance to the procedure (CO, toxicity of the materials etc.) might be crucial. It should be tested for each type of exosome.

Exosome isolation is challenging because the purity and quality of the final exosomes depend on the presence of non-exosomal impurities ("contaminants"), which is strongly affected by the procedure considered for the removal of those impurities. Cells must therefore be tested regularly for mycoplasma contamination before use. Ultracentrifugation is the method used in this protocol (Steps 6-21), since this is the most widely used primary isolation method from conditioned cultured media. However, alternative approaches such as density gradient centrifugation, ultrafiltration, size-exclusion chromatography, charge neutralization-based precipitation, magnetic affinity purification, and the use of microfluidic devices based on different principles[42-44] are also applicable to isolate exosomes from complex biological fluids.

Exosome concentration determination. Following isolation, the exosome content is determined by measuring the total protein concentration (Steps 22-23). This is a key procedure to control the loading of Pd nanosheets inside the exosomes, as well as for their final application as bioorthogonal catalyst.

Loading of exosomes with Pd precursor by diffusion. Our approach uses a passive cargo loading approach based on diffusion (Steps 24-28) to avoid damage to the exosome instead of alternative mechanically disruptive approaches (see Comparison with other methods). This approach relies on the passive diffusion of Pd^{2+} cations through the lipid membrane of exosomes according to the Fick´s law, where the Pd^{2+} concentration gradient is the driving force. Given the small size of the exosomes (short

diffusion length) and the high diffusivity of Pd^{2+} ions, the time required for their diffusion into exosomes is likely to be short, and therefore incubation time or temperature (which has a direct effect on diffusion coefficients) are not the main variables, since the incubation time used is already sufficient to achieve equilibration of Pd^{2+} concentration. Instead, the concentration of Pd^{2+} in the surrounding solution seems to be the main factor to determine the total Pd load in the exosomes that will be achieved upon CO reduction. Despite the differences in the exact composition of the lipidic membrane bilayer structure of the exosomes having the characteristic surface ligands and receptors from source cells; the diffusion-driven approach here described for loading Pd precursors is very versatile and used in all different exosomes.

Setup of the high-pressure carbon monoxide reduction system and assembly of Pd nanosheets inside exosomes. After loading the Pd^{2+} cations, Pd nanosheets are formed inside the exosomes by CO-mediated reduction (Steps 38-51). We chose a soft reducing agent to minimize side reactions during exosome treatment, as most reductants that are capable of reducing Pd^{2+} species could potentially react with functional groups present in biomolecules composing the exosome. We have optimized the procedural conditions for each step of the protocol to promote Pd nanoparticle formation without causing functional defects in the exosomes. In addition, the high pressure of CO assures a high CO solubility in the reaction media, which is key to control the anisotropic shape of Pd nanosheets. This fact is explained because in addition to its reductant role, CO also acts as capping agent since it is selectively chemisorbed on different facets and at active sites of Pd nanocrystals.[45,46] A mild temperature (30 ºC) is used to prevent exosome damage. Finally, CO is a gas which means that it can be easily removed after its use with the aid of inter gas pressure swing cycles. We designed a high-pressure system to manipulate CO, a toxic gas, which requires some basic safety measures to assure its appropriated management.

Pd-exosome characterization. We describe a variety of characterization techniques to determine the functionality of the exosomes (Steps 52-104), Pd loading (Steps 105-110), their stability (Steps 111-114),

toxicity (Steps 115-124), cellular uptake and localization (Steps 125-150), morphology and structure (Steps 151-162).

Pd-exosome activity test with a fluorogenic sensor* in vitro *and inside cells. The catalytic activity of the Pd exosomes is tested under physiological conditions (PBS, 37ºC) using the Palladium sensitive off-on sensor 7-propargyloxy-3H-phenoxazin-3-one (Pro-Res) (Steps 154-166).[29] The small size and lipophilic properties of Pro-Res allows its passive diffusion across exosomal membranes. Once inside the exosome, nonfluorescent Pro-Res undergoes a single Pd-catalyzed dealkylation reaction to produce highly-fluorescent resorufin, which passively diffuses back into the medium. Thereby the reaction monitoring is facilitated by a simple fluorometric analysis of the reaction medium or by time-lapsed imaging. Pro-Res shows negligible fluorescence emission under white LED excitation, while resorufin exhibits >200-fold superior fluorescence intensity at Ex/Em 540 /590 nm.[29] To validate the results, it is essential to perform the same test with unmodified exosomes as a negative catalytic control.

Finally, to validate both the cell targeting and catalytic properties of the exosome-catalyst hybrid, exosomes are incubated with their cells of origin (e.g. A549) at different time-points (from 1 to 24 h) in order to determine the time of maximum internalization of the vector inside the cells. Next, the culture medium is removed and the cells are carefully washed with PBS to eliminate all non-internalized exosomes. Cells are then detached and incubated with a solution of Pro-Res in culture medium. The lipophilic properties of Pro-Res and resorufin facilitate back and forth diffusion of the reagents through the cell and exosome membranes, which allows monitoring the Pd-catalyzed synthesis of resorufin (and the reaction yield) by the fluorometric analysis of the medium (without cells). To validate the results, it is essential to perform the same test with unmodified exosomes as a negative catalytic control. We also recommend including a different cell line as negative target cell control to confirm the preferential tropism of the exosomes.

Materials

Biological materials

▲**CRITICAL** We have successfully used the cell lines listed below in combination with our approach. See the Experimental Design section for guidelines on how to adapt and optimize the approach to different cell lines of interest.

!CAUTION All cell lines should be regularly controlled to guarantee that they are authentic and free of mycoplasma.

- A549, Human lung epithelial non-small carcinoma cells (ATTC, CCL-185; RRID: https://scicrunch.org/resolver/CVCL_0023.). The cell line was authenticated by STR profiling at ECACC and tested for potential mycoplasma contamination every 2 weeks.
- B16-F1 and B16-F10 cell lines (Cell Services Cancer Research-UK; RRID: https://scicrunch.org/resolver/CVCL_0158; https://scicrunch.org/resolver/CVCL_0159). The cell line was tested for mycoplasma contamination every 2 weeks.
- hpMSCs (Cellular Engineering Technologies, CET-Coralville, USA; RRID: https://web.expasy.org/cellosaurus/CVCL_1D55). Cells were subcultured every 5 days to maintain them in exponential growth phase. The cell line was tested for mycoplasma contamination every 2 weeks.

Reagents

▲**CRITICAL** Prepare all solutions with sterile solvents and store them as indicated.

- Glacial acetic acid, > 99 % (Sigma Aldrich, CAS no. 64-19-7). Store at room temperature (20-28°C) in a well-ventilated area. **!CAUTION** Corrosive, flammable liquid and vapor
- Acetone for analysis, ACS, ISO (PanReac AppliChem, CAS no. 67-64-1). Store at room temperature in a well-ventilated area. **!CAUTION** Highly flammable liquid and vapor.
- Air (UN 1002–GAI3X Praxair).

- Ammonium persulfate (APS) for molecular biology for electrophoresis, >99.8 %, (Sigma Aldrich, CAS no. 7727-54-0). Store at room temperature. **!CAUTION** Handle it in laboratory fume hood.
- Analtech Uniplates TLC Silica gel GF 250 µm, 20 x 20 cm (Sigma-Aldrich, cat. no. Z265500).
- Alexa Fluor 488 Phalloidin (ThermoFisher Scientific, cat no. 21833); RRID: https://scicrunch.org/resolver/AB_2532155 **▲CRITICAL** Store at -20 ºC.
- Anti-CD9 antibody [EPR2949] (Abcam, cat no. ab92726; RRID: https://scicrunch.org/resolver/AB_10561589). **▲CRITICAL** Store at -20 ºC.
- Anti-CD63 (for western detection) (Invitrogen, cat no. 10628D; RRID: https://scicrunch.org/resolver/ AB_2532983). **▲CRITICAL** Store at 4 ºC.
- Anti-CD63 Monoclonal (MEM-259) antibody Alexa Fluor 488 (ThermoFisher Scientific, cat no. MA5-18149; RRID: https://scicrunch.org/resolver/ AB_2539523). **▲CRITICAL** Store at 4 ºC.
- Anti-CD81 (B-11) (Santa Cruz Biotechnology, cat no. sc-166029; RRID: https://scicrunch.org/resolver/ AB_2275892). **▲CRITICAL** Store at -20 ºC.
- Anti-ALIX (3A9) mouse (Cell Signal Technology, cat no. mAb 2171; RRID: https://scicrunch.org/resolver/ AB_2299455). **▲CRITICAL** Store at -20 ºC.
- Antibiotic-antimycotic 100x (Biowest-bw, cat no. L0010-100). **▲CRITICAL** Store at -20 ºC.
- Anti-Rabbit IgG Antibody (H+L), HRP Conjugated (Bioss Antibodies, cat no. bs-0296G; RRID: https://scicrunch.org/resolver/ AB_10856484). **▲CRITICAL** Store at 4 ºC.
- Anti-Mouse IgG, HRP Conjugated (Sigma Aldrich, Cat no. A9917; RRID: https://scicrunch.org/resolver/%20AB_258476). **▲CRITICAL** Store at 4 ºC.
- Bovine Serum Albumin (BSA), (Sigma Aldrich, CAS no. 9048-46-8). **▲CRITICAL** Store at 4 ºC.
- Bromophenol Blue sodium salt, (Sigma Aldrich, CAS no. 34725-61-6). Store at room temperature.

- Carbon monoxide (UN 1016 Praxair, 47 BOT 10HA, purity 99.997%).**!CAUTION** acute toxicity (inhalation) - Category 3. Extremely flammable gas.
- Coomassie Brilliant Blue R-250 protein stain powder (Bio-Rad, cat no. 1610400). Store at room temperature. Store at room temperature.
- Developer Kodak Readymatic (VWR, cat no. KODK5023866). **▲CRITICAL** Store at 4 ºC.
- 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; 6674-22-2, Sigma-Aldrich, cat. no. 33482)
- Dichloromethane (DCM; 75-09-2, VWR International Ltd., cat. no. 23354.326). Store at room temperature in a well-ventilated place).**!CAUTION** Causes skin irritation.
- Dimethylformamide (DMF; dry, 68-12-2, VWR International Ltd., cat. no. 43997.K2). Store at room temperature in a well-ventilated place).**!CAUTION** Acute dermal and inhalation toxicity.
- Dimethylsulfoxide (67-68-5, Sigma-Aldrich, cat. no. D2650). Store at room temperature.
- Dimethyl sulfoxide (DMSO) Cell culture grade (PanReac AppliChem, CAS no. 67-68-5). Store at room temperature.
- Distilled, deionized water (ddH_2O).
- DMEM High Glucose w/ Stable glutamine w/ sodium pyruvate (Biowest-bw, cat no. L0103-500). **▲CRITICAL** Store at 4 ºC.
- DraQ5 (Labclinics, cat no. 65-0880-96). **▲CRITICAL** Store at 4 ºC.
- Dulbecco's phosphate buffered saline (DPBS) w/o calcium w/o magnesium (Biowest-bw, cat no. L0616-500). Store at room temperature.
- Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, cat. no. D6546). Store at room temperature.
- Epithelial lung carcinoma A549 cells (ATTC, CCL-185^{TM}).Cryopreserved
- Ethanol (PanReac AppliChem, cat no. 131086.1211). Store at room temperature in a well-ventilated place).**!CAUTION** Flammable liquid and vapor. Causes severe eye irritation.
- Exosome-depleted FBS (Gibco, cat. no. A2720803). **▲CRITICAL** Store at -20 ºC.

- Fetal Bovine Serum (FBS), qualified, E.U.- approved, South America origin (Gibco, REF. 10270-106). ▲**CRITICAL** Store at -20 ºC.
- Fibroblast Growth Factor-basic (FGF-2), Cat No. 100-18C (PeptroTech, USA).
- Fixer, Kodak Readymatic (VWR, cat no. KODK5023874). ▲**CRITICAL** Store at 4 ºC.
- Fluoromount-G (RMS, cat no. 17984-25). Store at room temperature.
- Glycerol pure, pharma grade (PanReac AppliChem, CAS no. 56-81-5). Store at room temperature.
- Glycine (Bio-Rad, cat no. 1610717). Store at room temperature.
- H_2O Milli-Q water (resistivity value: 18.2 MΩ·cm @ 25 °C) produced in a Milli-Q system from Millipore (Bedford, Germany).
- High purity Acrylamide/Bis Solution (40 %, 37.5:1) (Bio-Rad, cat no. 1610148). ▲**CRITICAL** Store at 4 ºC.
- Hydrochloric acid (ACS reagent, 37%, Sigma Aldrich, cat no. 7647-01-0). Store at room temperature in a well-ventilated area. **!CAUTION** Corrosive.
- Laemmli lysis buffer (Sigma Aldrich, cat no. 38733). ▲**CRITICAL** Store at -20 ºC.
- Lysotracker Green DND-26, (Molecular Probes, cat no. L7526). ▲**CRITICAL** Store at -20 ºC.
- L-glutamine 200 mM (Gibco, cat. no. 25030-081). ▲**CRITICAL** Store at -20 ºC.
- Luminata Crescendo Western HRP Substrate (Millipore, cat no. WBLUR0100). ▲**CRITICAL** Store at 4 ºC.
- Magnesium sulphate anhydrous (MgSO4; 7487-88-9, Fisher Scientific, cat. no. 10003812).
- Merck TLC Silica gel 60 F254 plates (Sigma-Aldrich, cat. no. 105554).
- Methanol for analysis, ACS, ISO (PanReac AppliChem, CAS no. 67-56-1). Store at room temperature in a well-ventilated place). **!CAUTION** Flammable liquid and vapor. Causes severe eye irritation
- MycoAlert Mycoplasma Detection kit (Lonza, cat no. LT07-318). Store at room temperature.

- *N, N, N', N'*- Tetramethylethylenediaminde, BioReagent, suitable for electrophoresis 99%. (Sigma Aldrich, CAS no. 110-18-9). Store at room temperature. **!CAUTION** Handle it in laboratory fume hood.
- Nitric acid (Sigma Aldrich, cat no. 7697-37-02). Store at room temperature.
- Nitrogen gas (7727-37-9, Praxair).
- Palladium-sensitive off-on sensor Pro-Res. Prepare it by following the procedure described in Box 1.[29]
- Palladium Standard for AAS (Fluka Analytical, ref:78437). ▲**CRITICAL** Store at 4 ºC.
- Paraformaldehyde (PFA), 4% (w/v) in PBS, (Alfa Aesar, CAS no. J61899). ▲**CRITICAL** Store at 4 ºC.
- Phosphate buffered saline (PBS; Fisher Scientific, cat. no. 10173433).▲**CRITICAL** Store at -20 ºC.
- Pierce BCA Protein Assay Kit (Thermo Scientific, cat no. 23225).
- Potassium tetrachloropalladate (II) (Sigma Aldrich, CAS no. 10025-98-6). Store at room temperature. ▲**CRITICAL** Besides potassium chloropalladate (II), some other organometallic precursors with a high lipophilic character could be used to increase the Pd exosomal retention, but after a previous cytotoxicity and solubility studies.
- Prestained SDS-PAGE Standards (Bio-Rad, cat no. 1610305EDU).
- Protease Inhibitor Cocktail Tablets, PhosSTOP (Roche, cat no. 04906845001). ▲**CRITICAL** Store at 4 ºC.
- Propargyl bromide (106-96-7, Sigma-Aldrich, cat. no. 81831). Store at room temperature.
- Resorufin (635-78-9, Sigma-Aldrich, cat. no. 424455). Store at room temperature.
- RIPA Buffer (Sigma Aldrich, cat no. R0278).
- Saponin from Quillaja Bark pure BC. (PanReac AppliChem, CAS no. 8047-15-2). Store at room temperature.
- Skimmed milk powder (Nestle, ref 8410100002330). ▲**CRITICAL** Store at 4 ºC.

- Sodium dodecyl sulfate (SDS) (Bio-Rad, cat no. 1610301). Store at room temperature.
- Tween 20 (Sigma Aldrich, CAS no. 9005-64-5). Store at room temperature.
- Trypsin (Biowest-bw, cat no. X0915-100). ▲**CRITICAL** Store at -20 ºC.
- Trypsin-EDTA solution (Sigma-Aldrich, cat. no.T4174). ▲**CRITICAL** Store at -20 ºC.
- Transparent fingernail polish (any commercial name and brand is suitable here).
- Tris Base (> 99.8 pure, Bio-Rad, cat no. 1610716). Store at room temperature.
- Tris Buffered Saline (TBS) 10X (Bio-Rad, cat no. 1706435). Store at room temperature.

Equipment

- Adjustable-volume pipettes 2 µL, 10 µL, 200 µL, 1000 µL (Gilson).
- Air gas tank cylinder pressure controller regulator (PRAXAIR model 2K12).
- Amersham Hyperfilm ECL (GE Healthcare cat no 28-9068-36).
- Amersham Hypercassette neutral standard 20x40 cm (GE Heathcare, Life Sciences, RPN11647).
- Beakers, low form. Borosilicate glass 3.3 100 mL (VWR, cat no. VWRI213-1122).
- Biosafety cabinet Telstar Bio II Advance (Telstar).
- BioTek Instrument Synergy HT Microplate Reader (Izasa s/n 208853).
- Centrifuge 5804R (Eppendorf, cat no. 585000320).
- CO gas tank cylinder pressure controller regulator (PRAXAIR model 2K12).
- Confocal microscopes (Spectral Confocal Microscope Leica TCA SP2 and ZEISS LSM800 Confocal Laser Scanning Microscope).
- Cooling device recirculating system (Büchi, model F-100 / F-105).
- DLS Brookhaven 90 Plus Particle size analyzer (Brookhaven Instruments Corporation).
- Eppendorf microcentrifuge tubes low retention (1.5-mL; Thermo Scientific, cat no. 2448).
- Flangeless Ferrule Tefzel™ ethylene tetrafluoroethylene (ETFE), 1/4-28 Flat-Bottom, for 1/16" (Idex Health and Science, Part#P-200X).

- Flangeless Fitting Short, PEEK, 1/4-28 Flat-Bottom for 1/16" OD (Idex Health and Science, Part#XP-235X).
- Forma Steri-Cycle CO_2 cell growth incubator (Fisher Scientific, cat no. 15311085).
- Freezer (Liebher profiline).
- Fridge (Liebher cat no. 7085638-01).
- 2-Gel Tetra and Blotting Module (Bio-Rad cat no. 1660827EDU).
- Glass pipettes (Labbox, cat no. PIPN-230-250).
- Glass plates 1 mm (Bio-Rad cat no. 1653311).
- Heraeus™ Fresco™ 17 Microcentrifuge.
- Heraeus HERAcell® CO_2 incubator set up at 37 °C and 5% CO_2.
- High-angle annular dark-field scanning transmission electron microscope (HAADF-STEM) Tecnai-F30 operated at 300kV with an Energy dispersive x-ray spectroscopy (EDS/EDX) detector.
- High pressure vessel (Model 4749, 23 mL, Parr Instrument Company) with a Teflon liner and a spanner wrench.
- High pressure polyether ether ketone (PEEK) tubing (Outside diameter 1/16” , inside diameter 0.02 “, length 5 feet, Idex Health and Science, Part#1532).
- Immobilon-P Transfer Membrane 0.45 um pore size (Millipore, cat no. IPVH00010).
- JP Selecta 3001208 Ultrasonic Cleaning Bath (Fisher Scientific, cat no. 12022085).
- JP Selecta Precisterm Water Batch (Fisher Scientific, cat no.12027874).
- LAB20ULFNL (20 µL), LAB10ULFNL (10 µL).
- Laboratory film (Labbox, cat no. PRFL-001-001).
- Leica Microscope model DM IL LED (4x, 10x, 20x and 40 x; Leica).
- Magnetic followers 25 x 10 mm (Fisher Scientific, cat. no. 11547772).
- Magnetic hotplate stirrer (VWR International Ltd, cat no. 442-0603).
- Magnetic stirrer/hotplate IKA®-C Mag HS7 (Reference number 0003581200).

- Measuring cylinder hexagonal base, class A, Glassco (Labbox, cat no. MCHA-1K0-001).
- Microcentrifuge Micro Star 17R (VWR, cat no. 521-1647).
- Microcentrifuge (Thermo Scientific, cat. no. 75002410).
- Microscope Slides (VWE, car no. 631-1533).
- Microwave Plasma-Atomic Emission Spectrometer 4100 MP-AES (Agilent Technologies).
- Mini-protean short plates (Bio-Rad cat no. 1653308).
- Mini trans-Blot filter paper (Bio-Rad cat no. 1703932).
- Nanosight NS500 (Malvern Panalytical Cat N 98-4730).
- Neubauer counting chamber. Depth of 0.1 mm (Marienfeld, cat no. 0640110).
- Nunc cell culture 96-Well plates, Nunclon Delta surface (Thermo Fisher, cat no. 167425).
- Nunc cell culture 24-Well plates, Nunclon Delta surface (Thermo Fisher, cat no. 142475).
- PerkinElmer EnVision 2101 multilabel reader (PerkinElmer, cat. no. 2101-0010).
- Petri Nunc dish (10-cm; Thermo Scientific, cat no. 150350).
- Pipette package (maximum volume: 1000, 200, 20 and 10 µL; Eppendorf, cat no. 2231000224).
- Pipette tips (Labclinics, cat no. LAB1000ULFNL (1000 µL), LAB200ULFNL (200 µL).
- Plastic Tubing cutter (Idex Health and Science, Part#A-327).
- PowerPac Basic Power Supply (Bio-Rad cat no. 1645050).
- Professional aluminum foil (Cellofix).
- Rotavapor (Büchi, model R-200).
- Round bottom flask, 25 mL (Quickfit, QFH-082-S).
- Round cover glasses 13 mm (VWR, cat no. MARI0117530).
- Screw extractor in a machine laboratory
- Separating funnel (VWR International Ltd., cat. no. 527-1131).
- Septum stopper (Fisher Scientific, cat. no. CG302401).
- Serological pipets (volume: 25, 10 and 5 mL; VWR, cat no. 7516-090P (25 mL), 612-5541P (10 mL), 612-5523P (5 mL)).

- Spectral Confocal Microscope Leica TCA SP2.
- Stirrer hotplate (Fisher Scientific, cat. no. 10414452).
- Swagelok SS-42XTS4 Ball Valve-3 way.
- Time Lapse microscope (Leica, AF6000 LX).
- ThermoMixer C Eppendorf (VWR International Ltd., cat. no. 460-0222).
- Transmission electron microscope Tecnai-FEI T20 operated at 200 kV.
- Tube polyclear 36 mL ultracentrifuge (Thermo Scientific, cat no. 75000571).
- Tweezers (Ideal-tel, cat no. 7LU1.S).
- U-Slide 8 Well ; Glass Bottom #1.5H (IBIDI, cat no. 80827).
- Unstirred water bath (JB Nova, Grant Instruments).
- Ultracentrifuge Sorval tm WX Ultra Series with AH-629 swinging Bucket Rotor (Thermo Fisher, cat no. 75000100).
- Vortex mixer (VWR, cat no. 444-1372).
- Water-bath sonicator (Fisher Scientific, cat. no. FB15047).

Software

- Gen5 (https://www.biotek.es/es/products/software-robotics-software/gen5-microplate-reader-and-imager-software/).
- Fiji (https://fiji.sc).
- GraphPad (https://www.graphpad.com).
- ZetaPals software (www.brookhaveninstruments.com)
- DigitalMicrograph® (https://www.gatan.com/)

Reagent setup

▲**CRITICAL** Prepare all solutions with sterile media and store them as indicated.

- **Heat-inactivation of FBS**: Incubate the stock FBS bottle during 30 min at 56 ºC. Heat-inactivated FBS can be stored at -80°C for up to one year.

- **Exosome-depleted FBS:** Ultracentrifuge the heat inactivated FBS at 100,000 g during 12 h at 4ºC Exosome-depleted FBS can be stored at -20 ºC for one months or at -80ºC for one year. ▲**CRITICAL** If an ultracentrifuge is not available commercially available exosome-depleted FBS can be used.
- **Supplemented DMEM for hPMSCs.** Supplement 500 mL of DMEM (High Glucose w/ Stable L-glutamine w/ sodium pyruvate) with 10% (v/v) heat-inactivated FBS and with 5 µg mL^{-1} of FGF-2 growth factor. The media can be stored at 4°C for up to one month. If some bacterial or fungal contamination were observed in the cultures, DMEM supplemented with antibiotics and antibiotics is recommended. Add 1 % (v/v) penicillin/streptomycin, 1 % (v/v) amphotericin (Antibiotic-antimycotic) to the DMEM. **?TROUBLESHOOTING**
- **Ultracen media for hpMSCs.** Supplement 500 mL of DMEM (High Glucose w/ Stable L-glutamine w/ sodium pyruvate) with 10% (v/v) heat-inactivated exosome-depleted FBS, and with 5 µg mL^{-1} of FGF-2 growth factor. The media can be stored at 4°C for up to one month. ▲**CRITICAL** If an ultracentrifuge is not available, instead use 50 mL of commercially available exosome-depleted FBS and supplement with 5 mL of L-glutamine. If some bacterial or fungal contamination were observed in the cultures, DMEM supplemented with antibiotics and antibiotics is recommended. Add 1 % (v/v) penicillin/streptomycin, 1 % (v/v) amphotericin (Antibiotic-antimycotic) to the DMEM. **?TROUBLESHOOTING**
- **Supplemented DMEM for cancer cells.** Supplement 500 mL of DMEM (High Glucose w/ Stable L-glutamine w/ sodium pyruvate) with 10% (v/v) heat-inactivated FBS. The media can be stored at 4°C for up to one month. If some bacterial or fungal contamination is observed in the cultures, DMEM supplemented with antibiotics and antibiotics is recommended. Add 1 % (v/v) penicillin/streptomycin, 1 % (v/v) amphotericin (Antibiotic-antimycotic) to the DMEM. **?TROUBLESHOOTING**
- **Ultracen media for cancer cells.** Supplement 500 mL of DMEM (High Glucose w/ Stable glutamine w/ sodium pyruvate) with 10% (v/v) heat-inactivated exosome-depleted FBS. The media can be stored at 4°C for up to one month. ▲**CRITICAL** If an ultracentrifuge is not

available, instead use 50 mL of commercially available exosome-depleted FBS. If some bacterial or fungal contamination were observed in the cultures, DMEM supplemented with antibiotics and antibiotics is recommended. Add 1 % (v/v) penicillin/streptomycin, 1 % (v/v) amphotericin (Antibiotic-antimycotic) to the DMEM. **?TROUBLESHOOTING hpMSCs cell culture.** hpMSCs are cultured in supplemented DMEM cell culture medium for hpMSCs at 37 ºC 3 % O_2 humidified atmosphere under hypoxic conditions. Cells should be subcultured every 5 days to maintain them in exponential growth phase.

- **Human lung epithelial non-small carcinoma cell culture.** Human NSCLCs are cultured in supplemented DMEM cell culture medium for cancer cells at 37 ºC, 5 % humidified atmosphere under normoxic conditions. Cells should be subcultured every 2 days to maintain them in exponential growth phase.
- **B16-F1 and B16-F10 cell culture**. B16-F1 and B16-F10 cells are cultured in supplemented DMEM cell culture medium for cancer cells at 37 ºC 5 % humidified atmosphere under normoxic conditions. Cells should be subcultured every 2 days to maintain them in exponential growth phase.
- **APS 10 % (wt/v) solution.** Dissolve 0.05 g of APS in 500 µL of Milli-Q water. Prepare always freshly and store it at room temperature. **▲CRITICAL STEP** The APS should be freshly prepared.
- **SDS 10 % (wt/v) solution**. Dissolve 0.05 g SDS in 500 µL of Milli-Q water. Store at room temperature for up to one month.
- **Tris-HCl 1.5M solution pH= 6.8.** Add 80 mL of Milli-Q water to 6 g of Tris Base. Adjust the pH to 6.8 with 1N HCL and make up the final volume to 100 mL with Milli-Q water. The Tris-HCl can be stored at room temperature for up to one year.
- **Tris-HCl 1.5M solution pH= 8.8.** Add 80 mL of Milli-Q water to 18.16 g of Tris Base. Adjust the pH to 8.8 with 1N HCL and make up the final volume to 100 mL with Milli-Q water. The Tris-HCl can be stored at room temperature for up to one year.

- **Native loading buffer:** Mix 1 mL Tris-HCl 0.5M pH=6.8 with 2 mL Glycerol, 1 mL Bromophenol Blue and 4.9 mL of Milli-Q water. The native loading buffer can be stored at -20 ºC for up to one year.
- **Running buffer.** Mix 200 mL of methanol with 800 mL of Milli-Q water, 14.4 g of glycine, 3.03 g of Tris Base, and 1 g of SDS. The running buffer can be stored at room temperature for up to 2 weeks.
- Transfer buffer. Mix 200 mL of ethanol with 800 mL of Milli-Q water 14.4 g of glycine and 3.03 g of Tris Base. The transfer buffer can be stored at room temperature for up to 2 weeks.
- **TBS 5% (w/v) milk solution**. Dissolve 5 g powder milk in 100 mL TBS 1x. The milk solution should be prepared fresh each time.
- **Tween-TBS (TTBS) solution.** Mix 90 µL Tween 20 With 180 mL TBS 1x. The TTBS solution can be stored at room temperature for up to 4 weeks.
- **Coomassie blue staining solution.** Dissolve 0.025 % (w/v) Coomassie Brilliant Blue R-50 with 45 % (v/v) Methanol and 6 % (v/v) Acetic Acid. The Coomassie blue staining solution can be stored at 4ºC for up to one year.
- **De-staining solution.** Mix 10 % (v/v) Methanol and 30 % (v/v) Acetic Acid. The de-staining solution can be stored at 4ºC for up to one year.
- **BSA 1 % (w/v) in PBS.** Dissolve 1 g of BSA in 100 mL of PBS. The BSA solution should be prepared fresh.
- **Saponin 0.1 % (w/v) in PBS + BSA.** Dissolve 0.05 g of saponin in 50 mL of BSA 1 % (w/v) in PBS. The saponin solution should be prepared fresh.
- **Protease inhibitor in RIPA buffer.** Dissolve 1 tablet in 10 mL RIPA buffer. The protease inhibitor solution should be prepared fresh.
- **Potassium tetrachloropalladate (II) exosome reagent.**
    1. Prepare a potassium tetrachloropalladate (II) 30 mM stock solution by dissolving 10 mg of potassium tetrachloropalladate (II) in 1 mL of PBS. This stock solution should be

prepared each time. ▲CRITICAL. Do not use a metallic lab spatula when potassium tetrachloropalladate (II) powder is weighed to avoid metal leaching. A plastic spatula can be made by cutting the tip of a plastic pipette.

2. Dilute 60 µL of the potassium tetrachloropalladate (II) stock 30mM solution with 970 µL of PBS to obtain 1mL of a 1.84 mM solution. The potassium tetrachloropalladate (II) solution is prepared fresh.

- **100 mM resorufin stock solution in DMSO.** Dissolve 21.3 mg of resorufin in 1 mL of DMSO. The resorufin stock solution can be stored at - 20 ºC for up to 12 months.
- **Resorufin solution in PBS.** Dilute 1 µL of 100 mM resorufin in DMSO with 1 mL of PBS. The resorufin solution in PBS can be stored at 4ºC for up to 1 week.
- **100 mM Pro-Res stock solution in DMSO.** Dissolve 25.1 mg of Pro-Res in 1 mL of DMSO. The Pro-Res stock solution can be stored at - 20 ºC for up to 12 months.
- **Pro-Res solution in PBS.** Dilute 1 µL of 100 mM Pro-Res in DMSO with 1 mL of PBS. The Pro-Res solution in PBS can be stored at 4ºC for up to 1 week.

?TROUBLESHOOTING.

Procedure

Cell culture preparation ● Timing 48-120 h

▲CRITICAL For any other cell line, exosome amounts, and timings should be adapted. For exosome isolation from A549 cell culture it is crucial to culture the cells under optimal conditions before the exosome purification procedure according to the following steps:

1. Maintain cell cultures (5 petri dishes P100) under normoxic conditions until they reach 80 % of confluence.
2. Aspirate the cell culture media carefully with a Pasteur pipette and a vacuum pump.
3. Wash the cells twice with 6 mL of PBS. ▲CRITICAL STEP Do not add the PBS directly to the cell monolayer in order to prevent cell detachment from the surface of the petri dish.

4. Add 6 mL of Ultracen cell media to each petri dish.

5. Maintain the cell cultures under normoxic conditions for 48 h. ▲CRITICAL STEP When using other cell lines, test various incubation times to find optimal conditions for exosome production.

Exosome isolation ● **Timing 5 h 30 min**

6. Take the supernatants (total volume = 30 mL) from all the petri dishes and add them in a 50 mL falcon tube. ▲CRITICAL STEP Be careful not to remove the cells.

7. Centrifuge the falcon tube at 2,000 g during 20 min at 4 ºC (Fig.2).

8. Carefully collect the supernatant with a pipette and transfer it to a polyclear 36 mL ultracentrifuge tube. Discard the pellet.

9. Use PBS to fill another ultracentrifuge tube in order to balance the weight and stabilize the ultracentrifuge.

10. Introduce the ultracentrifuge tubes in the adapters and close them with the proper cap. !CAUTION Label the cap of each tube with a number to avoid any confusion.

11. Assemble the tubes in the AH-629 rotor of the centrifuge.

12. Introduce the rotor in the centrifuge and close the door of the ultracentrifuge.

13. Press de vacuum button.

14. Set the centrifuge speed, time and temperature at 10,000 g, 30 min and 4 ºC, respectively, in order to eliminate the organelles and the cellular debris (Fig.2). ▲CRITICAL STEP Acceleration and deceleration profiles must be set at 9 and 5, respectively.

15. Carefully collect the supernatant with a pipette and transfer it into a clean ultracentrifuge tube and discard the pellet.

16. Set the centrifuge speed, time and temperature at 100,000 g, 2 h and 4 ºC, respectively (Fig.2). ▲CRITICAL STEP Acceleration and deceleration profiles must be set at 9 and 5, respectively.

17. Discard the supernatant with the aid of a pipette.

18. Resuspend the exosomal pellet in 33 mL of PBS with a 25 mL pipette and transfer it to a clean ultracentrifuge tube.

19. Centrifuge the sample again at 100,000 g during 2 h at 4 ºC in order to eliminate the serum proteins (Fig.2). ▲**CRITICAL STEP** Acceleration and deceleration profiles must be set at 9 and 5, respectively.

20. Discard de supernatant.

21. Resuspend the pellet in 1 mL of PBS and take the exosome unquantified fraction to store or for characterization. ■ **Pause point** The exosomes can be stored for long periods at -20 ºC for 3 months or -80ºC up to 2 years, avoiding freeze and thaw cycles. ▲ **CRITICAL STEP** Store exosomes for use in step 117 as a non-toxic control.

Exosome quantification ● **Timing 45 min**

22. Quantify exosome fraction by total protein amount with the BCA kit. Prepare the BSA standards and the BC working reagent and follow the microplate procedure as indicated by the manufacturer. Example of total protein quantification is discussed in the Anticipated Results section in Fig.5 .

23. Read the absorbance at 562 nm with the microplate reader using the Gen5 software. **?TROUBLESHOOTING.**

Pd loading into exosomes ● **Timing 14h**

24. Take a volume containing 30 µg of exosomes (according to the concentration in µg/mL determined by using the BCA kit in Step 23) and resuspend them in 29 mL of PBS.

25. Add 1 mL of the 1.84 mM potassium tetrachloropalladate (II) PBS solution to the exosomes dispersion (total volume 30 mL) to obtain a 0.06 mM solution.

26. Incubate the exosomes with the potassium tetrachloropalladate (II) solution overnight at room temperature (Fig.2).

27. Pellet the exosomes by ultracentrifugation at 100,000 g during 2 h at 4 ºC (Fig.2). Discard the supernatant, containing the non-internalized potassium chloropalladate (II).

28. Resuspend the exosomes loaded with the potassium chloropalladate (II) in 1 mL of PBS.

■**PAUSE POINT** It would be highly recommended to continue at this stage of the protocol.

Setup of the high-pressure CO reduction system ● Timing 4h

! CAUTION Carbon monoxide is listed as highly flammable, colorless, odorless, tasteless and is an acutely toxic gas because binds to myoglobin and mitochondrial cytochrome oxidase. Carbon monoxide easily combines with hemoglobin to form carboxyhemoglobin which can cause hypoxic stress in healthy individuals as a result of the reduced oxygen-carrying capacity of the blood. According to OSHA (https://www.osha.gov/dts/sltc/methods/inorganic/id209/id209.html), the Permissible Exposure Limit (REL) in an 8-hour time-weighted average (TWA) is 35 ppm and the short-term exposure limits (15 min) is 100 ppm. It is strongly recommended that operators familiarize themselves with safety measures (https://www.cdc.gov/niosh/topics/cocomp/default.html) and use a portable CO detector with threshold alarm set at the TWA value.

29. Use a screw extractor in a machine laboratory to set a male 1/4"-28 Thread Size port in the cap of the Teflon-liner in the high-pressure vessel.

30. Cut the PEEK tubing with a tubing-cutter. ▲**CRITICAL STEP** Improper tube handling and cutting are the main causes of tube fitting failure.

31. Introduce the short flangeless PEEK fitting and the flangeless ferrule in the PEEK tubing.

32. Plug the tubing in the female 1/4"-28 Thread port and finger tight. ▲**CRITICAL STEP** Ferrule is required to seal the tubing tightly in the port.

33. Plug the PEEK tubing to a 3-way valve (inlet). ▲**CRITICAL STEP** The 3-way valve must be piped correctly. Identify the inlet, outlet CO/air and outlet exhaust gas. Add a schematic diagram to help with the inlet/outlet identification.

34. Connect the 3-way valve (CO/Air outlet) to the proper gas tank cylinder pressure controller regulator. Select the CO cylinder to reduce the Pd cations or Air cylinder to desorb the solubilized CO and quench the redox process.

35. Connect the 3-way valve (exhaust outlet) to an exhaust pipe which should be connected to the exhaust duct.

36. Properly secure cylinders in a well ventilated and protected area away from heat, flames, and the sun. **! CAUTION** It is recommended to introduce the set-up in a laboratory fume hood for safe handling. Fit the set-up with a CO gas sensor connected to alarms to alert in the event of a leak, or exhaust system failure. **▲CRITICAL STEP** Support the gas cylinders to the fume hood by using chains or belts.

37. Check the correct function of the set-up and under-tightened tubing fittings. **▲CRITICAL STEP.** The integrity of the set-up should be examined at the highest pressure using soapy water. The soapy water is applied to the connectors with a pipette. The appearance of bubbles indicates the locations of leaks. Check first the integrity of the high pressure system with air and then with CO to minimize CO release and safety concerns. **! CAUTION** Switch the CO gas sensor on before opening the CO valve and set a caution sign to limit the exposure. **■PAUSE POINT** The high-pressure system is ready to be used but a gas leak check should be performed daily.

Pd reduction in the high-pressure CO reduction system ● Timing 60 min

38. Fill a glass beaker (500 mL capacity) with 200 mL of water and warm it up to 30ºC in the magnetic stirrer/hotplate. The stirring rate profile needs to be set at 3 (medium speed) in the hot water bath (Fig 3).

39. Introduce the exosomes (the vial with a volume of 1 mL) prepared in step 28 in the Teflon-liner. **▲CRITICAL STEP** The Teflon-liner cap modified in step 29 is inserted in the high pressure vessel. The force required to seal the liner is applied by tightening the vessel stainless-steel cap with the spanner wrench

40. Tight the high-pressure connections and introduce the high-pressure vessel into the hot water bath.

41. Switch the CO gas sensor on, exhaust outlet (3-way valve) is in the exhaust duct and the glass sash window is down (Fig 3). **! CAUTION** Keep eye contact with these three components of the high pressure system to safely operate.

42. Open the CO gas cylinder valve (turn the valve handwheel anti-clockwise 2 or 3 turns) and set the CO gas tank cylinder pressure controller regulator to 6 bar. **! CAUTION** The 3-way valve must shut off the CO gas outlet while opening the gas exhaust outlet.

43. Close the CO gas cylinder valve to check if the CO pressure is stable and there is no CO gas leaking in the system. If the CO pressure is stable for 5 min, go to step 44. **?TROUBLESHOOTING.**

44. Open the CO gas cylinder valve and flush the high pressure vessel with CO. **▲CRITICAL STEP** The CO gas outlet in the 3-way valve must be opened and closed for 5 cycles to assure the proper gas atmosphere inside the high pressure vessel and remove the air initially located at the high-pressure system.

45. Run the CO mediated redox reaction for 40 minutes to reduce Pd^{2+} cations to Pd nanosheets.

46. Close the CO gas cylinder. The 3-way valve must shut off the CO gas outlet while opening the gas exhaust outlet (Fig 3).

47. Disconnect the CO gas outlet at the 3-way valve. Set the CO gas tank cylinder pressure controller regulator to 6 bar.

48. Connect the Air gas outlet at the 3-way valve and open the air gas cylinder valve (turn the valve handwheel anti-clockwise 2 or 3 turns). Set the air gas tank cylinder pressure controller regulator to 6 bar. **▲CRITICAL STEP** The 3-way valve must shut off the gas exhaust outlet while opening the Air gas outlet (Fig 3).

49. Flush the high pressure vessel with air. CO solubilized in the exosome solution is desorbed by a pressure swing scrub with air. The air gas outlet in the 3-way valve must be opened and closed for 5 cycles to assure the proper removal of the CO absorbed. **▲CRITICAL STEP** This step assures to stop the redox process and the safe handling/opening of the high-pressure vessel.

50. Open the high-pressure vessel and collect the exosome dispersion. **?TROUBLESHOOTING**

■PAUSE POINT The treated Pd-exosome dispersion can be stored a 5 ºC for at least 1 week. The Pd exosomes may also be stored in the freezer at – 20 °C for up to 2 months without losing their catalytic activity. Exosome disruption happens after several freeze& thaw cycles. Ideally, Pd exosomes should be stored in aliquots and defrosted just before use to avoid multiple freeze& thaw steps.

Western Blot of exosomes to determine the alteration of membrane proteins ● Timing 3 days

51. Clean carefully the 1.0 mm Western blot glasses with ethanol in order to eliminate dust.

52. Assemble the support for the polymerization of the gels.

53. Prepare the 12 % (wt/v) and the 8 % (wt/v) running gels (depending on the size of the protein of interest) for the conventional or the native Western blot, respectively, using the recipes indicated in table 1. **▲CRITICAL STEP** The APS should be freshly prepared. **▲CRITICAL STEP** For the native Western blot, SDS must not be added. The reagents must be added according to the following order:

- 1.5 M Tris-HCl, pH=8.8
- Acryl/Bisacrylamide.
- H_2O.
- SDS
- **▲CRITICAL STEP:** The addition of APS and TEMED should be done as fast as possible.

Table 1. Preparation of the running and the stacking gels for Western blot.

	Running gel 0.37 M Tris, pH=8.8		**Stacking gel 1.12 M Tris, pH=6.8**
Crosslinking	**12 % (wt/v)**	**8 % (wt/v)**	**4 % (wt/v)**
H_2O	1.75 mL	2.75 mL	1.606 mL
1.5 M Tris-HCl, pH=8.8/	1.25 mL	1.25 mL	625 µL

(to prepare the running gel) 0.5 M Tris-HCl, pH=6.8 (to prepare the stacking gel)			
10 % (w/v) SDS	50 µL	50 µL	25 µL
Acryl/Bisacrylamide 40 % (w/v)	1.5 mL	1 mL	244 µL
10 % APS (w/v)	25 µL	25 µL	23.5 µL
TEMED	2.5 µL	2.5 µL	2.5 µL
Total volume	4.5 mL	4.5 mL	2.5 mL

54. Add the running gel into the space between the glasses assemblage with a Pasteur pipette and fill ¾ of the volume. ▲**CRITICAL STEP** The upper 2 cm should not be filled in order to have enough space for the stacking gel. Remove any bubbles as much as possible.

55. Add isopropanol in the space between the glasses on top of the running gel in order to homogenize the top of the running gel and to prevent the gel from drying.

56. Allow the gel to polymerize for 45 min at room temperature.

57. Remove the isopropanol, wash with Milli-Q water and remove the water completely with a soaking paper or any other tissue paper.

58. Prepare the stacking gel as indicated in table 1. ▲**CRITICAL STEP** The APS should be freshly prepared. ▲**CRITICAL STEP** To perform the native Western blot assay, SDS must not be added. The reagents must be added according to the following order:

- 0.5 M Tris-HCl, pH 6.8
- Acryl/Bisacrylamide.
- H_2O.
- SDS
- ▲**CRITICAL STEP:** Quickly add the APS and the TEMED.

59. Add the stacking gel into the space between the glasses assemblage with a Pasteur pipette and fill ¾ of the volume. ▲**CRITICAL STEP** Avoid the formation of bubbles and remove them as much as possible.

60. Introduce the Western blot comb and allow the gel to polymerize for 45 min at room temperature.

61. Cover the gel with a humidified paper with Milli-Q water and with aluminum foil.

■**PAUSE POINT** The gel can be used directly or kept at 4 ºC for the next day.

62. Take 30 µg of the Pd-exosome dispersion from Step 50 (according to the concentration in µg/mL determined using the BCA kit in Step 23) and add 80 µL of RIPA buffer with protease inhibitors.

63. Keep the sample on ice for 15 min.

64. Centrifuge the sample at 17,000 g during 10 min at 4 ºC.

65. Transfer the supernatant where the exosomal proteins are contained to a clean tube and discard the pellet.

66. Add cold acetone to the exosomal fraction at 1:1 (v:v) ratio.

67. Keep it at -20 ºC for 2 h in order to precipitate the exosomal proteins.

68. Centrifuge the sample at 17,000 g during 10 min at 4 ºC.

69. Carefully remove the acetone with a micropipette and allow the sample to dry.

70. Resuspend the pellet (containing the exosomal proteins) in 10 µL of PBS.

71. Add 10 µL of room temperature Laemmly buffer to the sample. ▲**CRITICAL STEP** For the native Western blot, mix the sample with the native loading buffer (1:2, v:v sample: native loading buffer) and directly proceed to Step 74 (without boiling or denaturing the sample).

72. Boil the samples in the sample buffer at 90 ºC during 10 minutes.

73. Immediately, incubate it at 4 ºC during 5 min. ■**PAUSE POINT** Samples might be kept at -20 ºC for any other day.

74. Prepare the running buffer 1x according to the Reagent Setup.

75. Add the running buffer in the space between the two gels.

76. Short spin the sample in the microcentrifuge.

77. Directly load the sample in the acrylamide gel along with the molecular weight markers (2 uL).

78. Take out the Western blot comb, wash the wells and assembly them in the Western blot running tank.

79. Connect the chamber to the electrophoresis power source and run the gel at 100 V for 2h.

80. To verify if the protein separation has been performed properly, incubate the separating gel with the Coomassie blue staining solution overnight.

81. Remove the Coomassie blue staining solution and de-stain the gel in the de-staining solution for 20 min. If the separation of the proteins has not been carried out properly, go to step 51 and prepare the new gels and samples. Consider adjusting the crosslinking of the gels as well as the electrophoresis time in order to optimize the separation of the target protein. **?TROUBLESHOOTING.**

82. Prepare the transfer buffer 1x according to the Reagent Setup. **▲CRITICAL STEP** Transfer buffer must be prepared always fresh.

83. Measure the gel dimensions, cut a nitrocellulose membrane and two filter papers of the same dimensions.

84. Assemble the transfer folders sandwich, the blot membrane, the filter paper and place the gel in the chamber. Make sure no bubbles are trapped into the sandwich. **▲CRITICAL STEP** The blot membrane should be on the cathode side and the gel on the anode side. **▲CRITICAL STEP** Maintain always the blot membrane, the filter paper, the gels and the transfer folders humidified with the transfer buffer. **▲CRITICAL STEP** If needed, activate previously the blot membrane with methanol. **?TROUBLESHOOTING**

85. Place the transfer cassette in the wet tank transfer system.

86. Add the transfer buffer.

87. Place an ice block in the tank.

88. Add a magnetic stirrer in the chamber and put it under stirring at 200 rpm.

89. Connect the chamber to the electrophoresis power supply and transfer the proteins at 350 mA constant voltage for 4h. **▲CRITICAL STEP** For the native western blot, the transference should be performed at 300 mV during 12 h at 4 ºC.

90. Disassemble the transfer cassette and take the nitrocellulose membrane.

91. Block the nitrocellulose membrane with 5 % (w/v) TBS powder milk overnight at 4 ºC. **▲CRITICAL STEP** If the blot is not properly blocked, unspecific interactions with the antibodies could occur. If the blot has been blocked for less time, high background on the blot could be observed after its development **?TROUBLESHOOTING.**

92. Wash the membrane with TTBS during 10 min. If the washing steps are not performed properly, unspecific interactions and impurities could be observed in the western blot. **?TROUBLESHOOTING.**

93. Repeat step 92 three times.

94. Add the primary antibody for 1.5 h at the concentration shown in the table below. **▲CRITICAL STEP** The antibody dilutions should be made according to the manufacturer's recommended ratio. The antibodies may be incubated with the blot for 1-3 h at 4 ºC or room temperature depending on the antibody quality and performance, also following the manufacturer's instructions. Some examples of the antibodies that can be employed to characterize the exosomes by Western blot are shown in the table below.

Antibody	Target	Clone	Supplier	RRID	Dilution	Buffer	Host species
Anti-CD9	CD9	EPR2949	Abcam	AB_10561589	1/2000	TTBS 5 % (w/v) milk	Rabbit
Anti-CD63	CD63	Ts63	Invitrogen	AB_2532983)	1/250	TTBS 5 % (w/v) milk	Mouse
Anti-CD81	CD81	B-11	Santa Cruz Biotechnology	AB_2275892).	1/500	TTBS 5 % (w/v) milk	Mouse

Anti-ALIX	Alix	3A9	Cell Signaling	AB_2299455).	1/1000	TTBS 5 % (w/v) milk	Mouse
Anti-Rabbit-HRP	IgG rabbit	Polyclonal	Bioss Antibodies	AB_10856484).	1/1000	TTBS 1 (w/v) %BSA	Goat
Anti-Mouse HRP	IgG mouse	Polyclonal	Sigma Aldrich	AB_258476).	1/12000	TTBS 1 (w/v) %BSA	Goat

95. Wash and block the membrane with TTBS during 15 min. **▲CRITICAL STEP** If the washing steps are not performed properly, unspecific interactions and impurities could be observed in the western blot. **?TROUBLESHOOTING.**

96. Repeat step 95 three times.

97. Add the secondary antibody and incubate for 1 h and 30 min.

98. Wash the membrane with TTBS during 20 min.

99. Repeat step 98 three times. **▲CRITICAL STEP** The minimum washing time is 1 h. If the washing steps are not performed properly, unspecific interactions and impurities could be observed in the western blot. **?TROUBLESHOOTING**

100. Add the chemiluminescent substrate to the blot (HRP Luminata Crescendo reagent) following the manufacturer´s instructions.

101. Introduce the blot membrane into the hypercassette.

102. Reveal the membrane in a room with red light with the developer and fixer. Example Western blots are shown in Anticipated Results section (Fig.10) . **?TROUBLESHOOTING.**

Quantification of Pd content in exosomes● Timing 4h

▲CRITICAL Quantification of the Pd content in exosomes is key before carrying out the *in vitro* catalytic study in Steps 103-108.

103. Pipette 100 uL of the Pd loaded exosomes dispersion from Step 50 into a clean tube.

104. Add 150 µL of aqua regia prepared by adding 1/3 (v/v) of HNO_3 and 2/3 (v/v) of HCl.

105. Keep the sample for chemical digestion during 1h at room temperature.

106. Add 1750 uL of distilled water (final concentration of Aqua regia 10 % (v/v)).

107. Prepare the Pd standard solutions at different concentration levels (0 ppm, 0,125 ppm, 0,250 ppm, 0,5 ppm, 1 ppm, 3 ppm, 5 ppm and 10 ppm) with 10 % of aqua regia.

108. Calibrate the Microwave Plasma-Atomic Emission Spectrometer (MP-AES) equipment with the Pd standard solutions and proceed with the analysis of the samples following manufacturer instructions. If the Pd loading within the exosomes were very low and below the detection limit of the MP-AES an ICP-OES could be used instead but generally high yield is obtained.

Stability analysis of the exosomes ● **Timing 10 min/sample**

▲**CRITICAL** It is important to determine that the exosome stability was not affected along the purification process. In this section, exosome integrity is analyzed by evaluation of the particle size as well as their zeta-potential.

109. ***Size analysis (Steps 109-110):*** As the number of particles at this stage is unknown, usually from the Pd loaded exosome fraction from Step 50 make a dilution 1µl in 1ml or 0.5 ml of PBS (1:500) and they might be further diluted to 1:1000 or 1:2000 depending on the sample concentration, adjusting to the equipment requirements. Usually, concentration of exosomes range between 10^6-10^9particles/mL.

110. Use a Nanosight to determine the exosome size and perform concentration quantification following the manufacturer instructions. Exosomes should have a particle size between 80-150nm, depending on the cell source of origin.

?TROUBLESHOOTING

111. ***Zeta potential analysis (Steps 111-112):*** Take the Pd loaded exosome sample from Step 110 and introduce it in the DLS apparatus.

112. Connect properly the electrode and measure their surface charge at different time-points, following the manufacturer instructions. Exosomes carry a net negative surface charge under

physiological conditions due to the glycosylated proteins intercalated within the lipid bilayer and the inter- and intramolecular H-bonding, and ion adsorption from the electrolytes present in solution.

Cytotoxicity evaluation ● Timing 34 h

▲**CRITICAL** It is important to have at least one confluent P100 plate of the cells of interest to test, for the evaluation of the toxicity and the internalization of the exosomes into cells.

113. Remove the cell culture media from a 100% confluent P100 plate and detach the cells with 3 mL trypsin-EDTA solution, neutralizing them with 3 volumes (9ml) of PBS or cell culture medium. Count the cells with a Neubauer chamber.
114. Prepare a cell suspension in DMEM at 1500 cells/100 µL.
115. Add 1500 cells (100 µL) to each well in a 96-well plate.
116. Place the 96-well plate in the cell incubator for 24 h.
117. Add 0.2, 0.4 and 0.6 µg of Pd loaded exosomes (from Step 50) per 100 µL. ▲**CRITICAL STEP** It is important to have wells of untreated cells (control cells) to be used as 100 % viability and cells incubated with unmodified exosomes to be used as controls. ▲ **CRITICAL STEP** Use untreated exosomes from Step 21 as a non-toxic control.
118. Incubate the cells for 6 h.
119. Remove the media containing the non-internalized exosomes from the wells.
120. Wash twice the cells with 100 uL PBS buffer.
121. Add Cell-Blue reagent following the manufacturer´s instructions.
122. Read the fluorescence in the plate reader following the manufacturer's instructions. **?TROUBLESHOOTING** …

Preparation of the cells for the confocal microscopy analysis ● Timing 27-50 h

123. Remove the cell culture media from a totally confluent P100 plate and detach the cells with 3 mL trypsin-EDTA solution, neutralizing them with 3 volumes (9mL) of PBS or cell culture medium. Count the cells with a Neubauer chamber.

124. Seed the cells at a cell density of 15,000 cells onto 20 mm cover slips deposited onto a 24-well plate.

125. Incubate the cells for 24 h under standard culture conditions.

126. Add 1 µg (according to the concentration in µg/mL determined using the BCA kit in Step 23) of Pd-loaded exosomes from Step 50 to each well diluted in 500 µL Ultracen media.

127. Incubate the exosomes for 1, 2, 4, 6 and 24 h under standard culture conditions.

128. Optional: In order to label the lysosomal compartments, add lysotracker to the cells following the manufacturer´s instructions (75 nM of lysotracker for 1h).

129. Remove media containing the non-internalized exosomes with a Pasteur pipette and a vacuum pump.

130. Wash the cover slips three times with PBS buffer.

131. Fix the cells with 500 µL 4 % (w/v) PFA during 30 min at room temperature.

132. Add 500 µL of PBS to the fixed cells. ■ **PAUSE POINT** The cells can be stored for 24 or 48 h in the fridge before running the immunocytochemistry. In the case of lysosome labelled cells, pass directly to step 142 for nuclei staining.

Immunochemistry of cells for confocal imaging ● **Timing 1h 30 min**

▲**CRITICAL** Before the permeabilization and the staining of the cells, a humidified chamber must be prepared with a Tupperware covered with filter paper and parafilm (Fig. 4a).

133. Dilute the phalloidin 1/200 in 0.1 % (w/v) Saponin in PBS + BSA solution. Prepare the required volume considering 50 µL of the solution for each sample. .▲**CRITICAL STEP** In addition to the visualization of the exosomes loaded with Pd inside the cytosol by labelling the cell nuclei and their actin filaments, also the endosomal and the lysosomal pathways should be labelled in order to confirm that the exosomes loaded with the Pd are not localized in the lysosomal

compartments. In order to label the endosomal pathway, add 0.16 µL of the CD63-Alexa-488 antibody to 50 µL of 1 % (w/v) Saponin in PBS + BSA for each coverslip.

134. Deposit one droplet of 50 µL phalloidin or CD63-Alexa-488 solution for each coverslip on the parafilm placed inside the humidified chamber (Fig. 4b).

135. Carefully take the coverslips from the 24 well plate with the tweezers and wash the coverslips from Step 131 by introducing them three times with the tweezers in a beaker containing 100 mL of PBA + BSA 1 % (w/v).

136. Permeabilize the cells deposited onto the coverslip by introducing it three times with the tweezers in a beaker containing 100 mL of 0.1 % (w/v) Saponin in PBS + BSA.

137. Deposit the coverslips into the droplets from Step 133. **▲CRITICAL STEP** The cell monolayer must be in contact with the droplet containing the fluorescent label (Fig. 4c).

138. Incubate the coverslips for 1 h in the dark and at room temperature.

139. Take a microscope slide.

140. Deposit 5 µL of Fluoromount-G with DraQ5 for each coverslip on the slide (Fig. 4d).

141. Carefully take the coverslips with the tweezers, place them back in a 24 well plate and wash them by introducing them with the tweezers in a beaker with 100 mL of PBS + 1% (w/v) BSA

142. Wash the coverslips by introducing them with the tweezers in a beaker with 100 mL of Milli-Q water. **▲CRITICAL STEP** Last washing step must be done with Milli-Q water in order to eliminate the salts from the PBS.

143. Deposit the coverslips onto the DraQ5 droplets from Step 139 .**▲CRITICAL STEP** The cell monolayer must be in contact with the droplet (Fig. 4e).

144. Dry the coverslips overnight in the dark.

145. Fix the coverslips onto the slips by placing transparent fingernail polish at the edges. **■ PAUSE POINT** Fixed coverslips can be stored at -20 ºC in the dark for up to 12 months.

Confocal imaging ● Timing 2- 4 h

▲**CRITICAL** In order to know the optimal incubation time to perform the catalytic experiments inside the cells, the internalization of the exosomes loaded with the Pd nanosheets are evaluated by confocal microscopy in this section.

146. Image the coverslips from Step 145 by confocal microscopy with a ×63 oil immersed N.A. 1.40 objective. Use the reflection of the incident light at Ex/Em of 488/490 nm to directly determine the location of Pd-Exosome^{A549}. Create Z-stack orthogonal projections to enable the visualization of the exosomes inside cells cytosol. The table below summarizes the excitation and emission wavelengths used for all the antibodies or molecular probes employed in this protocol. Example images are discussed in the Anticipated Results section.

Probe	Target	Excitation wavelength (nm)	Emission wavelength (nm)
Anti-CD63	Endosomal pathway	490	525
Lysotracker	Lysosomes	504	511
Phalloidin	Actin filaments	495	518
DraQ5	Nuclei	647	670
Palladium NPs	Pd exosomes	Reflection	Reflection

▲**CRITICAL STEP** Before carrying out the experiments, it is crucial to check the absence of signal or background coming from other channels to set the acquisition settings. Moreover, it is recommended to work in sequential mode to guarantee the absence of channel overlapping. By working in sequential mode, the confocal microscope will take all the images from the Z-stack channel and channel separately, rather than simultaneously. By checking the absence of overlapping between channels and working in sequential mode, we guarantee that all the signal emitted in each channel came from this specific excitation wavelength and not from the overlap with other channels.

?TROUBLESHOOTING.

Electron microscopy imaging ● **Timing 12 h**

▲**CRITICAL** This section describes how to perform Cryo-TEM or HAADF-STEM imaging to study the morphology of Pd loaded exosomes ^{A549} as well as the location of the produced Pd nanosheets at high CO pressure conditions.

147. Prepare a thin film vitrified specimen using the Pd loaded exosomes form Step 50 with a Vitrobot (FEI) in melting ethane. ▲**CRITICAL STEP** Electron microscopy grid preparation of cryogenically immobilized Pd-Exosomes ^{A549} samples requires an extremely rapid sample freezing to achieve a vitreous state.
148. Maintain the sample holder with the specimen grid under cryogenic conditions with liquid nitrogen.
149. Carry out Cryo-TEM and Cryo HAADF-STEM imaging[47-49] using a T20-FEI and F30-FEI microscope, respectively. Perform Energy dispersive X-ray spectroscopy (EDS/EDX) analysis to determine the presence of Pd nanosheets inside the exosomes. Example data are discussed in the Anticipated Results section. **?TROUBLESHOOTING.**

***In vitro* catalytic study** ● **Timing 24 h**

▲**CRITICAL** This section discusses the *in vitro* conversion of non-fluorescent Pro-Res into highly fluorescent resorufin by Pd exosomes in PBS (pH=7.2-7.4, isotonicity). This is an essential step to verify the catalytic properties of the exosomes before moving to cellular assays.

150. Add 0.5, 1 and 2 µg of Pd loaded exosomes from Step 50 to a 500 µL solution of Pro-Res in PBS (100 µM) in a microcentrifuge tube (1.5 mL).
151. Sonicate the exosomal suspension for 30 s to avoid agglomeration.
152. Use a 500 µL solution of 100 µM resorufin in PBS and a 500 µL solution of 100 µM Pro-Res in PBS as controls. ▲ **CRITICAL STEP** Measurements have to be done at least in triplicate.
153. Shake the reactions in a Thermomixer (0.821 g) for 16 h at 37 °C.

154. Sediment the exosomes by centrifugation (17,000 g, 5 min at room temperature) and collect the supernatant.

▲ CRITICAL STEP Carefully collect the supernatant without removing the pellet of exosomes.

155. Measure the solution fluorescence intensity (corresponding to resorufin) on a PerkinElmer EnVision 2101 multilabel reader (Ex/Em: 540 /590 nm) and calculate the percentage of conversion by comparing the fluorescence intensity of resorufin in PBS (100 µM, equivalent to full conversion). Example data are discussed in the Anticipated Results section.

▲ CRITICAL STEP Resorufin fluorescence maximum excitation/emission is 550nm/585nm.

?TROUBLESHOOTING.

Time lapse imaging ● **Timing 25 h**

▲ CRITICAL This section discusses how to perform time lapse imaging in order to illustrate the fast conversion rate of the prodrug to the drug in the presence of the exosomes loaded with the Pd.

156. Add 1 µg of Pd loaded exosomes from Step 50 to 20 µM solution of Pro-Res in PBS (0.2 mL) in a u-slice 8 well Ibidi plate.

157. Incubate them for 24 h while visualizing under the time lapse microscope. Take frames every 15 min for 24 h in differential interference contrast (DIC) mode and under red fluorescence emission (Ex/em 560/630 nm) with a 20x objective.

158. Create videos using ImageJ software. Example data are discussed in the Anticipated Results section (Fig.12). **?TROUBLESHOOTING.**

Intracellular catalytic study ● **Timing 48 h**

▲ CRITICAL Before entering more complex functional assays, an intracellular catalytic study is essential to validate the capacity of the exosomes to penetrate cells and remain catalytically active inside them.

159. Starting with 80% confluent T75 flask for adherent cells of A549 cells, remove the 15 mL media and detach the cells with 1 mL trypsin-EDTA solution and dilute them in complete DMEM media (9 mL).

160. Seed the cells (100 µL per well containing 1,500 cells) in each well of a 96-well plate and incubate them at 37 °C and 5% CO_2 for 24 h.

161. Remove media from the wells and add 100 µL of a freshly-prepared suspension of Pd loaded exosomes (from Step 50) in Ultracen media (0.5 µg of exosomes per 100 µL of media) to each well and incubate at 37 °C and 5% CO_2 for 6 h.

162. ▲ **CRITICAL STEP** Before cell treatment, it is important to sonicate the exosomes suspension for 30 s to avoid agglomeration. Remove media from the wells and carefully wash the cells twice with 100 µL of PBS buffer per well.

163. Remove the PBS and add 50 µL of trypsin-EDTA solution to each well in order to detach the cells. Deactivate the trypsin by adding 50 µL of 10% (v/v) FBS in PBS buffer.

164. Collect the cells in microcentrifuge tubes (1.5 mL) and centrifuge at 17,000 g for 5 min at room temperature.

165. Remove the supernatant and suspend the cells in 100 µL of a 100 µM solution of Pro-Res in PBS.

▲ **CRITICAL STEP** Carefully collect the supernatants without removing the pellet of cells.

166. Shake the mixture at 0,821 g and 37 °C in a Thermomixer for 16 h.

167. Analyze fluorescence intensity in a PerkinElmer EnVision 2101 multilabel reader (Ex/Em: 540 n /590 nm). Example data are discussed in the Anticipated Results section. **?TROUBLESHOOTING.**

Troubleshooting

Troubleshooting guide can be found in Table 2.

Table 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Reagent setup	Precipitation of stock solutions.	Low temperature affects the solubility of the reagents.	Warm up the stock solutions to 37 ºC before use or prepare a fresh one in case the precipitates were still in solution.
Reagent setup	Unreliable results	Bacterial contamination of culture media	Use media supplemented with antibiotics and antimitotic: 1 % (v/v) penicillin/streptomycin, 1 % (v/v) amphotericin (Antibiotic-antimycotic) as described in the Reagent setup section in order to incubate the cells and during the performance of the experiment.
5	The exosome yield is not high enough to achieve the targeted amount	Some cell lines secrete a reduced number of exosomes under the same confluent conditions.	If the number of exosomes were lower than expected, it would be recommended the scale up of the starting plates or the concentration of the exosomes with one of the many commercially available column-based exosome purification kits.
43	CO pressure is not stable in the high pressure vessel when the CO gas cylinder is closed.	There is a CO gas leak in the high pressure system.	Revise the correct function of the set-up and potential under-tightened tubing fittings. Apply soapy water to the connectors with a pipette. The appearance of bubbles indicates the locations of the leaks.

50	Exosomes agglomerate.	This could be related to the upstream ultracentrifugation treatment	Use an ultrasound bath to disperse the exosomes (sonication time = 1 min)
81	The proteins are not properly separated in the gel.	Sample is not properly denatured, the crosslinking of the gels is not properly adjusted to the size of the protein of interest, or the pH of the gels is not suitable	Go to step 51 and prepare new gels and a new sample. Alternatively, adjust the crosslinking of the gels as well as the electrophoresis time in order to optimize the separation of the target protein.
84	The membrane is not activated	The membrane needs to be incubated with methanol.	Introduce the membrane blot in methanol during 1 minute before assembling the transfer sandwich.
86	Gas bubbles are trapped in the transfer sandwich.	Glass and filter paper are not properly assembled and/or humidified.	Roll a pipette tip several times over the sandwich to push the air bubbles out.
91	Unspecific bands are	The membrane is not properly	Blocked the blot again as described in Step 91 and proceed with antibody incubation and detection as

	observed in the blot.	blocked.	described in Steps 92-102.
92,95, 99	Unspecific signals are observed in the blot as black spots or non-specific bands of the target proteins.	The membrane is not properly washed.	Increase the washing steps and the washing time.
102	If the results on the WB were not as expected.	Western-blotting is a long technique that requires many difficult manipulation processes.	Many issues could happen on the different steps along the days. The mostly common problems found and how to resolve them could be found on the manufacturer´s web page (https://www.bio-rad.com/en-us/applications-technologies/troubleshooting-western-blots-with-western-blot-doctor?ID=MIW4HR15) or in some publications (doi: 10.4103/1947-2714.100998)
110	Size distribution curve is not homogenous.	Sample concentration could not be appropriated.	Modify the sample concentration to optimize the distribution curve according to the manufacturer´s instructions
122	Toxicity observed with Pd Exo	Possible sample contamination or degradation of Pd	If the unmodified exosome control shows also toxicity, discard culture media and prepare a fresh one to repeat the experiment.

		Exo	If the unmodified exosome control does not show toxicity, analyze whether the structural integrity of the Pd Exo has been altered and prepare them again if required.
146	Confocal imaging results are not the expected	Fluorescence microscopy is a complex technique and many steps are required to take images from the Z-stack at different wavelengths	This technique requires proficiency. Troubleshooting guides for many of the common errors are provided by the manufacturers such as https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/fluorescence/troubleshoot/
149	Electron microscopy imaging does not enable to observe the features of exosomes and Pd nanosheets	Concentration of exosomes in the area of electron microscopy analysis is low or water ice crystals are localized in the TEM grid	A proper Cryo-TEM specimen preparation minimize the presence of water ice crystals, which could have similar dimensions than exosomes. However the geometry of those crystals is different from the one of exosomes. If the density of exosomes in the area of analysis were low, it is recommended to analyze different areas since the drop casting to prepare the specimen could not be homogenous.
155	Fluorescence results are not as expected	Pd-Exosomes could be damaged or reagent	If positive control yield the targeted fluoresce intensity and the Pd loaded exosomes do not show any catalytic activity it is recommended to use a new

		concentration is not proper.	batch of Pd-exosomes to avoid a feasible structural damage of exosomes during their preservation or manipulation.
158	Time-lapse imaging results are not as expected	Pd-Exosomes could be damaged or reagent concentration is not proper.	If positive control yield the targeted fluoresce intensity and the Pd loaded exosomes do not show any catalytic activity it is recommended to use a new batch of Pd-exosomes to avoid a feasible structural damage of exosomes during their preservation or manipulation.
167	Intracellular catalytic results are not as expected	Fluorescence signal is not detected	Increase the incubation time of Pd-loaded exosomes (step 161). If fluoresce issues still continue, it is recommended to use a new batch of Pd-exosomes to avoid a feasible structural damage of exosomes during their preservation or manipulation.

Box 1: Synthesis of Pro-Res. Timing 24 h.

Reagents

! CAUTION Reagents and organic solvents might be toxic and/or corrosive. Use appropriate personal protection equipment.

- 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; 6674-22-2, Sigma Aldrich, cat. no. 33482)
- Dichloromethane (DCM-CH_2Cl_2; 75-09-2, VWR International Ltd., cat. no. 23354.326)
- Dimethylformamide (DMF; dry, 68-12-2, VWR International Ltd., cat. no. 43997.K2)
- Magnesium sulphate anhydrous ($MgSO_4$, 7487-88-9, Fisher Scientific, cat. no. 10003812)
- Methanol (MeOH; 67-56-1, VWR International Ltd., cat. no. 20847.307)
- Propargyl bromide (106-96-7, Sigma-Aldrich, cat. no. 81831)
- Resorufin (635-78-9, Sigma-Aldrich, cat. no. 424455)

Consumables

- Whatman® filter paper (Sigma Aldrich, cat. no. WHA2200150)
- Merck TLC Silica gel 60 F254 plates (Sigma Aldrich, cat. no. 1055540001)
- Analtech 01013 Preparative Uniplates, 20x20cm/Silica G/1000mm Thick/15um (Sigma Aldrich, cat. no. Z265500)

Equipment

- Round-bottom flask 25 mL with septum stopper and magnetic stirring bar
- Magnetic plate stirrer
- Büchi® Rotavapor® RII evaporator
- Büchner funnel 90 mm
- Büchner flask 500 mL
- TLC viewing cabinet
- Aldrich® TLC developing tank
- Rectangular TLC developing tank for preparative chromatography

Procedure

1. Dissolve Resorufin (300 mg, 1.4 mmol) in dry DMF (5 mL) in a 25-mL round-bottom flask sealed with a septum stopper under N_2 atmosphere.
2. Add 1,8-diazabicyclo[5.4.0]undec-7-ene (390 µL, 2.8 mmol). **! CAUTION** 1,8-diazabicyclo[5.4.0]undec-7-ene is toxic and corrosive. Use appropriate personal protection equipment.
3. Prepare a solution of propargyl bromide (240 µL, 2.8 mmol) in dry DMF (0.5 mL). **! CAUTION** Propargyl bromide is highly flammable, toxic and corrosive. Use appropriate personal protection equipment.

▲**CRITICAL STEP** Water can react with propargyl bromide under basic conditions and consume the reagent. It is then critical to carry out the reaction under anhydrous conditions to improve the yield.

4. Add propargyl bromide solution (740 µL) to the reaction mixture and stir at room temperature for 18 h. ▲**CRITICAL STEP** Reaction needs to be monitored by thin-layer chromatography (TLC). Take a small aliquot of the reaction mixture and add methanol to dilute it. The final compound has a clearly different colour (yellow) than resorufin (pink) in the TLC (eluent mixture 5% (v/v) MeOH in CH_2Cl_2). Rf = 0.35 (Pro-Res); Rf = 0.01 (Resorufin).
5. Remove the solvent using a rotary evaporator and add DCM to get a sticky precipitated solid.
6. Filter the mixture to remove the sticky solid and wash the filtrate with H_2O (2 x 10 mL) in a separating funnel.
7. Dry the organic fraction (DCM) using $MgSO_4$ and remove the solvent using a rotary evaporator.

   The crude can be purified via semipreparative TLC chromatography (2.5% (v/v) MeOH in CH_2Cl_2) to yield the final compound as an orange solid. (75 mg, 21% yield). The final compound can be stored at 4°C for up to 12 months.

PAUSE POINT

End of Box 1

Timing

Steps 1-5 Cell culture preparation: 48-120 h

Steps 6-21 Exosome isolation: 5 h 30 min

Steps 22-23 Exosome quantification: 45 min

Steps 24-28 Pd loading into exosomes: 14h

Steps 29-37 Setup of the high-pressure CO reduction system: 4h

Steps 38-50 Pd reduction in the high-pressure CO reduction system: 60 min

Steps 51-102 Western Blot of exosomes to determine the alteration of membrane proteins: 3 days

Step 103-108 Quantification of Pd content in exosomes:4h

Steps 109-112 Stability analysis of the exosomes:10 min/sample

Steps 113-122 Cytotoxicity evaluation: 34 h

Steps 123-132 Preparation the cells for confocal microscopy: 27-50 h

Steps 133-145 Immunochemistry of cells for confocal imaging: 1h 30 min

Step 146 Confocal imaging: 2- 4 h

Steps 147-149 Electron microscopy imaging: 12 h

Steps 150-155 *In vitro* catalytic study: 24 h

Steps 156-158 Time Lapse Imaging: 25 h

Steps 159-167 Intracellular catalytic study: 48 h

Box 1 Synthesis of Pro-Res: 24 h

Anticipated results

Exosome harvesting

Before attempting the synthesis of Pd nanosheets within exosomes it is crucial to have a high enough amount of exosomes from the cells cultures. Figure 5 depicts the normalized amount of exosomes (µg of exosomes quantified as total protein content obtained by BCA assay) produced by a million of A549, B16-F1, B16-F10 and hpMSCs when they were cultured *in vitro*.

Pd loading into exosomes

It is important to determine if Pd nanosheets are properly loaded into exosomes. Considering the dimensions of exosomes (100-140 nm) and Pd nanosheets (thickness= 1.4 nm) we use cryo-electron microscopy to verify the native morphology of the exosomes and to determine the Pd nanosheet distribution within exosomes. Cryo-Transmission Electron Microscopy (Cryo TEM) and Cryo High-Angle Annular Dark field-Scanning Transmission Electron Microscopy (Cryo HAADF-STEM) are well suited to confirm that Pd nanosheets are grown inside exosomes and that the morphology of the exosomes does not suffer alterations (Fig. 6 a). CO has dual role in the formation of Pd nanosheets: 1) it functions as

a reducing agent and 2) it serves as a selective capping agent. CO has a preferential adsorption to basal {111} planes of Pd nanocrystals, favoring an anisotropic growth with a sheet shape. The anisotropic growth of Pd nanosheets inside exosomes can be observed in the HAADF-STEM inset image depicted in Fig. 5a. and in Fig. 6 b. Due to the ultrathin geometry of this nanosheets (1.4 nm), they are clearly observed (high contrast) when they are perpendicularly oriented to the TEM grid. Energy Dispersive X-ray Spectroscopy (EDS) analysis can be used to confirm the chemical load of exosomes since PBS salts can also crystallize in the TEM grids and other artefacts could arise from the TEM sample preparation, exhibiting high contrast. Then, the presence of artefacts can draw wrong conclusions and a chemical analysis is key to discard them (Fig. 6c). Fig. 7 depicts representative TEM images of Pd nanoengineered exosomes derived from mouse melanoma cell lines with two aggressive states, low metastatic B16-F1 and high metastatic B16F10 and human placenta-derived mesenchymal stem cells (hPMSCs). In all cases it can be observed that Pd nanosheets were successfully assembled inside the exosomes.

Once the presence of Pd nanosheets into exosomes is confirmed, it is crucial to determine the biocompatibility and the optimal time-point of cellular uptake of the Pd loaded exosomes. The performance of cell viability assays at different concentrations of Pd loaded exosomes is required to determine the optimal concentration range to perform cell-based studies (Fig. 8). On the other hand, the efficiency of the catalytic reaction depends on the localization of the Pd loaded exosomes in the different compartments of the cell (in the first steps of the endosomal pathway or even, in the lysosomal compartments). Confocal microscopy of the Pd nanosheets in combination with immunocytochemistry of actin/nuclei and markers of the endosomal pathway/lysosomal compartments will allow to establish the incubation time required for Pd loaded exosomes and cells to fulfill the following requirements: 1) have the maximum amount of Pd loaded exosomes inside the cells to have a maximum efficient bioorthogonal reaction and 2) Having the Pd loaded exosomes in the endosomal pathway rather than in the lysosomal compartments. Orthogonal projections of Fig 9 and Supplementary Video_1 demonstrate the presence of the Pd loaded exosomes inside the cell cytoplasm. Moreover, purple pixels of Fig. 10a indicated with a

yellow arrow evidences the localization of the Pd loaded exosomes in the endosomal pathway rather than in the lysosomal compartments after 6 h of incubation (Fig. 10b).

Exosomes characterization- Size and functionality

Fig. 11a shows NTA results of control exosomes and Pd-loaded exosomes derived from A549 cells. Both types of exosomes revealed a typical exosome diameter of approximately 100 nm. In order to obtain high quality NTA exosomal measurement, the parameters obtained by the equipment have to be optimized according to manufacturer´s indications.

Western blot analysis of Pd-loaded exosomes indicates the expression of CD81 and ALIX proteins as shown in Fig. 11b. CD81 is a protein from the tetraspanin family of around 26 kDa. ALIX is another typical exosomal protein of approximately 96 kDa. Both proteins are involved on endocytosis and on the exosome/ multivesicular body biogenesis pathways.

Finally, Fig. 11c includes a phase analysis light scattering of Pd loaded exosomes. When the electrode is connected with the sample, a voltage is applied to the cell and the movement of the particles under the influence of the applied electric field is registered by the instrument providing a phase change in light scattered produced by particles moving under the influence of an applied electric field. This phase change is directly proportional to the electrophoretic mobility of the exosomes and thus, to their zeta potential.

Pd exosomes as catalytic devices

As observed in Fig. 12a, treatment of Pd exosomes with Pro-Res under physiologically-relevant conditions (37 °C, pH 7.4, isotonicity) results in a dramatic increase of the fluorescence emission of the solution. After 16 h of incubation, the 100 μM Pro-Res solution containing Pd exosomes at concentrations of 0.2 μg per 100 μL (or higher) reaches fluorescence intensity values near or equal to those of a 100 μM resorufin solution, indicating full conversion efficacy. These results confirm the catalytic properties of the exosome-catalyst hybrids. The ex vitro catalytic properties of the Pd exosomes were also evaluated by time-lapse microscopy. Fig. 13 includes three sequences recorded during 24 h of different conditions (using Pro-Res as negative control, Res as positive control and finally Pro-Res incubated with the catalytic exosomes). This figure corroborates the activation of the Pro-Res mediated by the catalytic

exosomes and the subsequent appearance of the fluorescence. On the contrary, when Pro-Res was incubated in absence of the Pd exosomes, no fluorescence was observed, even when the reaction time was augmented.

To test their capacity to catalyze bioorthogonal reactions inside cells, we treated A549 cells with Pd loaded exosomes for 6 h and. After elimination of non-internalized exosomes, cells were incubated with Pro-Res and we monitored the fluorescence intensity of the medium. After 24 h, the fluorescence intensity reached approx. 21% of the value of a 100 μM resorufin solution, confirming the capacity of the intracellular Pd devices to convert Pro-Res into resorufin (Fig. 12b). While the obtained value is lower to that found *in vitro*, it is important to note that only a fraction of the Pd exosomes incubated with A549 cells are effectively internalized. This conversion value it would be more than sufficient to generate bioactive levels of a pharmacological agent from inactivated prodrugs or probes. In the field of bioorthogonal prodrug activation, most prodrug strategies aim to achieve over 100-fold difference in activity between the prodrug (inactive) and drug (active). Considering the hypothetical situation in which a prodrug is inactive at 100 μM and the parented drug displays high anticancer activity at 10 μM, a 20 % conversion of 100 μM of the inactive prodrug would generate 20 μM of the anticancer drug, leading to a very potent antitumor effect. Once the intracellular catalytic properties of the Pd exosomes are confirmed, they are ready to be used for more complex studies.

Acknowledgements:

We gratefully acknowledge financial support from the ERC Advanced Grant CADENCE (grant no. ERC-2016-ADG-742684) and the EPSRC (Healthcare Technology Challenge award no. EP/N021134/1). M.S.-A. thanks the Spanish Government for a FPU PhD research fellowship. B.R.-R. thanks the EC (grant no. H2020-MSCA-IF-2014–658833). VS. thanks the financial support of Ministerio de Ciencia, Innovación y Universidades, Programa Retos Investigación, Proyecto REF: RTI2018-099019-A-I00.M.A. thanks the financial support of the ERC Consolidator Grant programme (grant no. ERC-2013-CoG-614715). P.M-D also thanks Instituto de Salud Carlos III (PI19/01007).We also thank CIBER-BBN, an initiative funded

by the VI National R&D&i Plan 2008–2011 financed by the Instituto de Salud Carlos III and by Fondo Europeo de Desarrollo Regional (Feder) "Una manera de hacer Europa", with the assistance of the European Regional Development Fund. This study is also partially funded by the Aragon Government (T57_17R p) cofounded by Feder 2014–2020 "Building Europe from Aragon".

Author contributions

M.S.-A., B.R.-R., A.M.P.-L., P.M.-D. and V.S. prepared and characterised the materials, planned and performed the experiments and analysed the data; V.S., P.M.-D., M.A., A.U.-B. and J.S. planned and supervised the research, analysed the data and contributed to the manuscript writing; V.S. and M.A. conceived the research. V.S. designed and coordinated the research. All the authors checked the manuscript

Competing interests

I declare the authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the interpretation of the article.

Data availability statement

The main data supporting the examples of this protocol are available within the article and its Supplementary Information files. Extra data are available from the corresponding author upon reasonable request. The source data underlying Figs. 5,8 and 12 are provided as source data files.

References

1. Sletten, E.M. & Bertozzi, C.R. Bioorthogonal Chemistry: Fishing for Selectivity in a Sea of Functionality. *Angew Chem Int Edit* **48**, 6974-6998 (2009).
2. Devaraj, N.K. The Future of Bioorthogonal Chemistry. *Acs Central Sci.***4**, 952-959 (2018).
3. Luan, X., *et al.* Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacologica Sinica* **38**, 754-763 (2017).
4. Cooper, J.R., *et al.* Long Term Culture of the A549 Cancer Cell Line Promotes Multilamellar Body Formation and Differentiation towards an Alveolar Type II Pneumocyte Phenotype. *PloS one* **11**, e0164438-e0164438 (2016).
5. Lieber, M., Todaro, G., Smith, B., Szakal, A. & Nelson-Rees, W. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *International Journal of Cancer* **17**, 62-70 (1976).

6. Giard, D.J., *et al.* In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors2. *JNCI: Journal of the National Cancer Institute* **51**, 1417-1423 (1973).
7. Yusop, R.M., Unciti-Broceta, A., Johansson, E.M.V., Sanchez-Martin, R.M. & Bradley, M. Palladium-mediated intracellular chemistry. *Nature Chemistry* **3**, 239-243 (2011).
8. Weiss, J.T., *et al.* Extracellular palladium-catalysed dealkylation of 5-fluoro-1-propargyl-uracil as a bioorthogonally activated prodrug approach. *Nature Communications* **5**(2014).
9. Li, J., *et al.* Palladium-triggered deprotection chemistry for protein activation in living cells. *Nature Chemistry* **6**, 352-361 (2014).
10. Weiss, J.T., *et al.* Development and Bioorthogonal Activation of Palladium-Labile Prodrugs of Gemcitabine. *Journal of Medicinal Chemistry* **57**, 5395-5404 (2014).
11. Rubio-Ruiz, B., Weiss, J.T. & Unciti-Broceta, A. Efficient Palladium-Triggered Release of Vorinostat from a Bioorthogonal Precursor. *Journal of Medicinal Chemistry* **59**, 9974-9980 (2016).
12. Bray, T.L., *et al.* Bright insights into palladium-triggered local chemotherapy. *Chemical Science* **9**, 7354-7361 (2018).
13. Adam, C., *et al.* Bioorthogonal Uncaging of the Active Metabolite of Irinotecan by Palladium-Functionalized Microdevices. *Chemistry-a European Journal* **24**, 16783-16790 (2018).
14. Stenton, B.J., Oliveira, B.L., Matos, M.J., Sinatra, L. & Bernardes, G.J.L. A thioether-directed palladium-cleavable linker for targeted bioorthogonal drug decaging. *Chemical Science* **9**, 4185-4189 (2018).
15. Li, N., Lim, R.K.V., Edwardraja, S. & Lin, Q. Copper-Free Sonogashira Cross-Coupling for Functionalization of Alkyne-Encoded Proteins in Aqueous Medium and in Bacterial Cells. *Journal of the American Chemical Society* **133**, 15316-15319 (2011).
16. Spicer, C.D., Triemer, T. & Davis, B.G. Palladium-Mediated Cell-Surface Labeling. *Journal of the American Chemical Society* **134**, 800-803 (2012).
17. Destito, P., *et al.* Hollow nanoreactors for Pd-catalyzed Suzuki-Miyaura coupling and O-propargyl cleavage reactions in bio-relevant aqueous media. *Chemical Science* **10**, 2598-2603 (2019).
18. Michel, B.W., Lippert, A.R. & Chang, C.J. A Reaction-Based Fluorescent Probe for Selective Imaging of Carbon Monoxide in Living Cells Using a Palladium-Mediated Carbonylation. *Journal of the American Chemical Society* **134**, 15668-15671 (2012).
19. Mann, G., Satish, G., Meledin, R., Vamisetti, G.B. & Brik, A. Palladium-Mediated Cleavage of Proteins with Thiazolidine-Modified Backbone in Live Cells. *Angewandte Chemie-International Edition* **58**, 13540-13549 (2019).
20. Wang, F.M., Zhang, Y., Du, Z., Ren, J.S. & Qu, X.G. Designed heterogeneous palladium catalysts for reversible light-controlled bioorthogonal catalysis in living cells. *Nature Communications* **9**(2018).
21. Miller, M.A., *et al.* Nano-palladium is a cellular catalyst for in vivo chemistry. *Nature Communications* **8**(2017).
22. Hoop, M., *et al.* Mobile Magnetic Nanocatalysts for Bioorthogonal Targeted Cancer Therapy. *Advanced Functional Materials* **28**, 1705920 (2018).
23. Li, X., *et al.* Superior antitumor efficiency of cisplatin-loaded nanoparticles by intratumoral delivery with decreased tumor metabolism rate. *European Journal of Pharmaceutics and Biopharmaceutics* **70**, 726-734 (2008).
24. Sancho-Albero, M., *et al.* Efficient encapsulation of theranostic nanoparticles in cell-derived exosomes: leveraging the exosomal biogenesis pathway to obtain hollow gold nanoparticle-hybrids. *Nanoscale* **11**, 18825-18836 (2019).

25. Balivada, S., *et al.* A/C magnetic hyperthermia of melanoma mediated by iron(0)/iron oxide core/shell magnetic nanoparticles: a mouse study. *BMC Cancer* **10**, 119-119 (2010).
26. Shukla, R., *et al.* Laminin receptor specific therapeutic gold nanoparticles (198AuNP-EGCg) show efficacy in treating prostate cancer. *Proc Natl Acad Sci U S A* **109**, 12426-12431 (2012).
27. Podesta, J.E., *et al.* Antitumor Activity and Prolonged Survival by Carbon-Nanotube-Mediated Therapeutic siRNA Silencing in a Human Lung Xenograft Model. *Small* **5**, 1176-1185 (2009).
28. Wilhelm, S., *et al.* Analysis of nanoparticle delivery to tumours. *Nature Reviews Materials* **1**(2016).
29. Sancho-Albero, M., *et al.* Cancer-derived exosomes loaded with ultrathin palladium nanosheets for targeted bioorthogonal catalysis. *Nature Catalysis* **2**, 864-872 (2019).
30. Yong, T., *et al.* Tumor exosome-based nanoparticles are efficient drug carriers for chemotherapy. *Nature Communications* **10**, 3838 (2019).
31. Darband, S.G., *et al.* Exosomes: natural nanoparticles as bio shuttles for RNAi delivery. *Journal of Controlled Release* **289**, 158-170 (2018).
32. Sancho-Albero, M., *et al.* Exosome origin determines cell targeting and the transfer of therapeutic nanoparticles towards target cells. *Journal of Nanobiotechnology* **17**(2019).
33. Trams, E.G., Lauter, C.J., Norman Salem, Jr. & Heine, U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **645**, 63-70 (1981).
34. Tkach, M. & Théry, C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell* **164**, 1226-1232 (2016).
35. Harding, C.V., Heuser, J.E. & Stahl, P.D. Exosomes: looking back three decades and into the future. *The Journal of cell biology* **200**, 367-371 (2013).
36. Gould, S.J., Booth, A.M. & Hildreth, J.E.K. The Trojan exosome hypothesis. *Proceedings of the National Academy of Sciences* **100**, 10592-10597 (2003).
37. Gourlay, J., *et al.* The emergent role of exosomes in glioma. *Journal of Clinical Neuroscience* **35**(2016).
38. Alhasan, A.H., Patel, P.C., Choi, C.H.J. & Mirkin, C.A. Exosome Encased Spherical Nucleic Acid Gold Nanoparticle Conjugates as Potent MicroRNA Regulation Agents. *Small* **10**, 186-192 (2014).
39. Hessvik, N.P. & Llorente, A. Current knowledge on exosome biogenesis and release. *Cellular and molecular life sciences : CMLS* **75**, 193-208 (2018).
40. Doyle, L.M. & Wang, M.Z. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* **8**(2019).
41. World Cancer Report 2020. http://publications.iarc.fr/586.Access-5th April 2020
42. Théry, C., *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* **7**, 1535750 (2018).
43. Ramirez, M.I., *et al.* Technical challenges of working with extracellular vesicles. *Nanoscale* **10**, 881-906 (2018).
44. Konoshenko, M.Y., Lekchnov, E.A., Vlassov, A.V. & Laktionov, P.P. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *BioMed Research International* **2018**, 8545347 (2018).
45. Sebastian, V., Smith, C.D. & Jensen, K.F. Shape-controlled continuous synthesis of metal nanostructures. *Nanoscale* **8**, 7534-7543 (2016).

46. Herrer, L., *et al.* High surface coverage of a self-assembled monolayer by in situ synthesis of palladium nanodeposits. *Nanoscale* **9**, 13281-13290 (2017).
47. Kuntsche, J., Horst, J.C. & Bunjes, H. Cryogenic transmission electron microscopy (cryo-TEM) for studying the morphology of colloidal drug delivery systems. *International Journal of Pharmaceutics* **417**, 120-137 (2011).
48. Ilett, M., Brydson, R., Brown, A. & Hondow, N. Cryo-analytical STEM of frozen, aqueous dispersions of nanoparticles. *Micron* **120**, 35-42 (2019).
49. Elad, N., Bellapadrona, G., Houben, L., Sagi, I. & Elbaum, M. Detection of isolated protein-bound metal ions by single-particle cryo-STEM. *Proceedings of the National Academy of Sciences* **114**, 11139 (2017).

Figure legends

Fig 1| A schematic description of the production of Pd-exosomes for targeted bioorthogonal catalysis.

Fig 2| A schematic of the exosome isolation and Pd^{2+} loading. (a) schematic overview of the exosome isolation procedure. (b) Schematic overview of the Pd-Exosome production. Exos = exosomes.

Fig 3| A schematic of the high-pressure CO reduction system and summary of the 3-way valve positions to produce Pd-exosomes

Fig 4| Preparation and Fixation of cells on coverslips for inmuno-labeling of cells.(**a)** Preparation of the humidified chamber with the parafilm and the humidified filter paper. (**b)** 50 µL droplet containing the phalloidin. (**c)** Coverslip deposited onto the phalloidin droplet and incubated during 1 h for actin staining. (**d)** Deposition of 5 µL of Fluoromount-G with DraQ5 on the slip. (**e)** Incubation of the coverslips with the DraQ5 for nuclei staining.

Fig. 5| Total protein exosomes quantification by BCA assay for A549, B16-F1, B16-F10 and hpMSCs.

Fig 6| Electron microscopy analysis of Pd loaded exosomes (**a)**- Cryo High-Angle Annular Dark field-Scanning Transmission Electron Microscopy (Cryo HAADF-STEM) images of Pd loaded exosomes. Inset, a high magnification Cryo HAADF-STEM image to show the Pd nanosheet thickness. Scale bar = 50 nm. Scale bar inset= 5 nm. S1 and S2 areas marked in dashed red line are the locations where the chemical analysis (EDX) was performed for elemental identification; (**b)** High magnification Cryo-TEM image of Pd nanosheets loaded within the exosome. Scale bar= 5 nm. Scheme of the Pd nanoheets with

different 3-D orientation to understand the features depicted in the TEM Image-(**c)-** Energy dispersive X-ray spectra from areas S1 and S2 marked in dashed red line in (a) corroborating the presence of Pd.

Fig 7| Representative TEM images of Pd nanoengineered exosomes. Different images are derived from: Tumor cells (B16-F1 and B16-F10) and Human placenta-derived mesenchymal stem cells (hPMSCs).Scale bar=20 nm

Fig 8| Cell viability assays (A549 cells) at different concentrations of Pd loaded exosomes.

Fig 9|. Confocal images of A549 cells incubated with Pd-loaded exosomes during 24 hours. Green channel corresponds to actin filaments labelled with phalloidin, blue channel shows the nuclei stained with draQ5 and red channel shows the Pd-loaded exosomes visualized directly by reflection. Scale bar 50 µm. Scale bar zoom 12.5 µm. Orthogonal projections derived from confocal images of A549 cells reveal red sports of exosomes agglomerates inside cell cytoplasm. Scale bar 50 µm. Scale bar zoom 5 µm

Fig 10.| Confocal microscopy images of cells incubated with Pd loaded exosomes. (**a)-** The endosomal route is labelled with CD63 (blue) and the Pd loaded exosomes are directly observed by reflection (red). Purple pixels (indicated with a yellow arrow) evidence the localization of the Pd loaded exosomes in the endosomal pathway. (**b)-** Lysosomes are labelled with lysotracker (blue) and Pd loaded exosomes are directly observed by reflection (red). The majority of the Pd loaded exosomes are not colocalizing with the lysosomal compartment. Scale bars 5 µm. Scale bar zoom images 0.5 µm

Fig. 11| Exosome characterization. (a) NTA measurements of exosomes size and concentration of empty (right) and Pd loaded (left) vesicles. (b) Western Blot of CD81 and ALIX proteins expressed in A549 derived exosomes. (c) Phase change analysis obtained during zeta potential measurements of Pd loaded exosomes.

Fig 12|**.** Pd-mediated conversion of non-fluorescent Pro-Res to fluorescent resorufin. (**a)**, *In vitro* catalytic study in PBS at 37 °C. Fluorescent analyses (λex/em = 540/590 nm) were performed after 16 h incubation

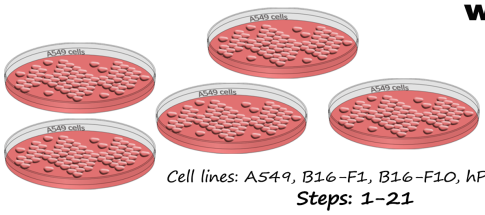
of Pro-Res (100 μM) with Pd exosomes (0.1, 0.2 and 0.4 μg per 100 μL). Fluorescence intensity of Pro-Res in the absence of Pd exosomes (100 μM, green) and Resorufin (100 μM, purple) were used as negative and positive controls, respectively. Error bars= ± 1 SD (n = 3); ANOVA, *** P< 0.001. **(b)**, *Intracellular* catalytic study. Fluorescent analysis (λex/em = 540/590 nm) was performed after 24 h incubation of Pro-Res (100 μM) with A549 cells pre-treated with Pd exosomes (0.2 and 0.5 μg per 100 μL). Fluorescence intensity of untreated A549 cells directly incubated with Pro-Res (100 μM, green) and Resorufin (100 μM, purple) were used as negative and positive controls, respectively. Error bars= ±1 SD (n = 3); ANOVA, * P< 0.05, *** P< 0.001.

Fig. 12| Time lapse frame sequence during 24 h of Pro-Res (negative control), Res (positive control) and Pro-Res with the catalytic Pd-exosomes. The appearance a clear red fluorescent signal (compared with the Res) when the Pro-Res was incubated with the Pd-exosomes was clearly visible. The samples were visualized under a 20x objective of a Leica AF6000 LX microscope.

Supplementary Information

- Supplementary Video 1: Confocal Z-stack sequence of A549 cells incubated with Pd-ExosA549. Actin is labelled with phalloidin (green), nuclei are stained with Draq5 (blue) and the Pd-Exos were directly visualized by reflection (red)

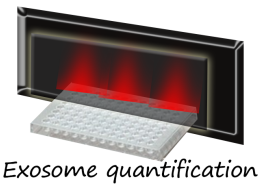
Non destructive production of exosomes loaded with ultrathin Palladium nanosheets for targeted bioorthogonal catalysis



Cell lines: A549, B16-F1, B16-F10, hPMSCs

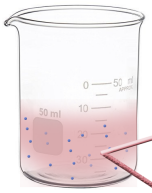
Steps: 1-21

Cell culture and exosome isolation



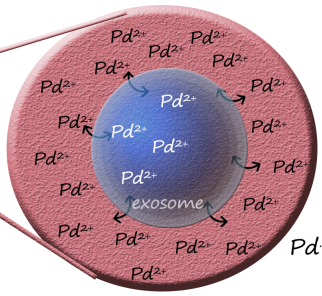
Exosome quantification

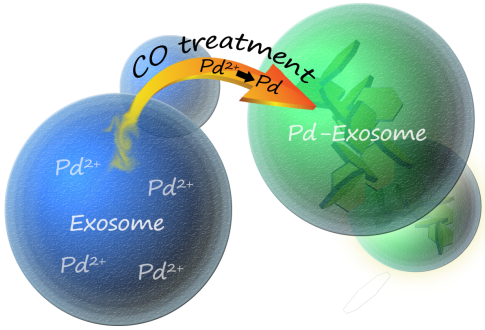
Steps: 22-23



Pd^{2+} loading in Exosomes

Steps: 24-28





Pd^{2+} reduction in a high pressure CO system: assembly of Pd nanosheets into exosomes

Steps: 29-50

Pd:exosomes characterization: Western Blot

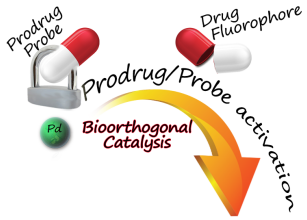
Steps: 51-102

Pd:exosomes characterization: Quantification of Pd content, Stability and toxicity

Steps: 103-122

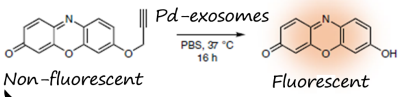
Pd:exosomes characterization: Confocal and electronic microscopy

Steps: 123-149



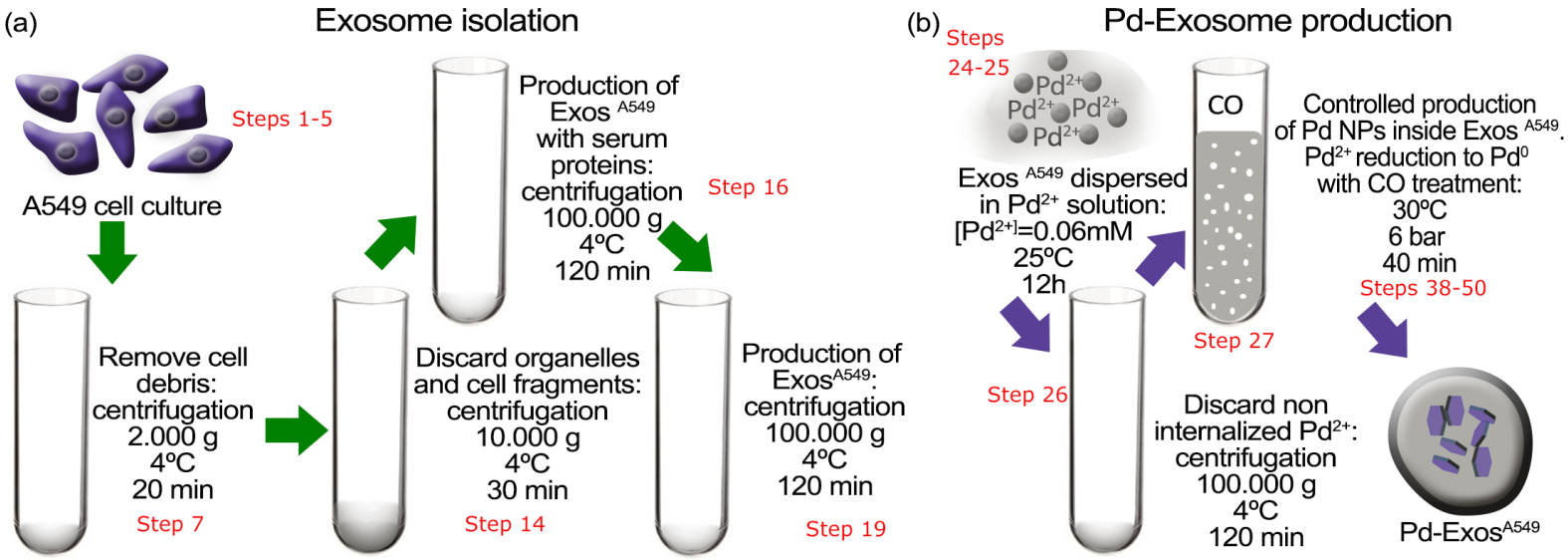
Intracellular catalytic study

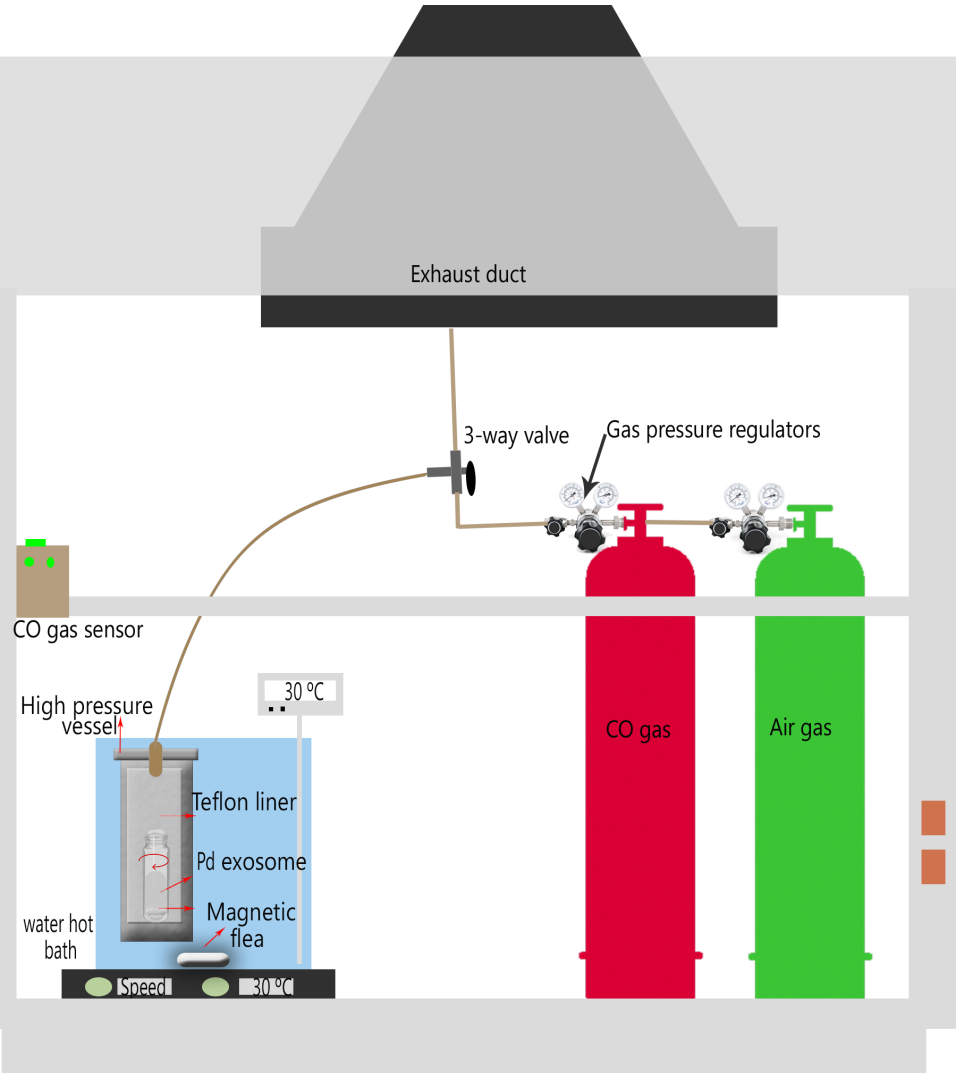
Steps: 156-167



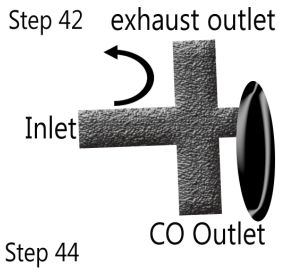
In-vitro catalytic study

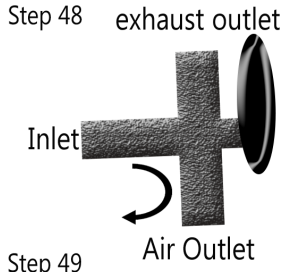
Steps: 150-155

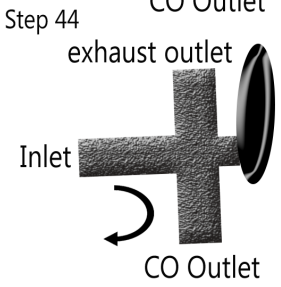

(a)
Exosome isolation
Steps 1-5
A549 cell culture
Remove cell debris: centrifugation 2.000 g 4ºC 20 min
Step 7
Discard organelles and cell fragments: centrifugation 10.000 g 4ºC 30 min
Step 14
Production of Exos A549 with serum proteins: centrifugation 100.000 g 4ºC 120 min
Step 16
Production of Exos A549: centrifugation 100.000 g 4ºC 120 min
Step 19
(b)
Pd-Exosome production
Steps 24-25
Pd2+
Exos A549 dispersed in Pd2+ solution: [Pd2+]=0.06mM 25ºC 12h
Step 26
CO
Step 27
Controlled production of Pd NPs inside Exos A549. Pd2+ reduction to Pd0 with CO treatment: 30ºC 6 bar 40 min
Steps 38-50
Discard non internalized Pd2+: centrifugation 100.000 g 4ºC 120 min
Pd-Exos A549

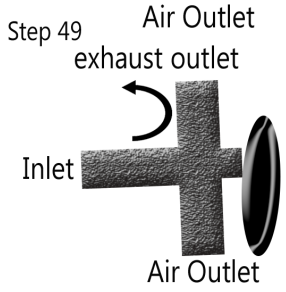


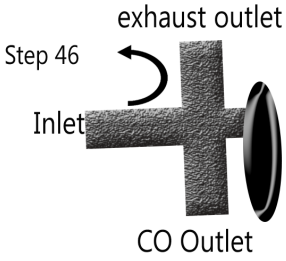
3-way valve operation for CO and Air tretarment

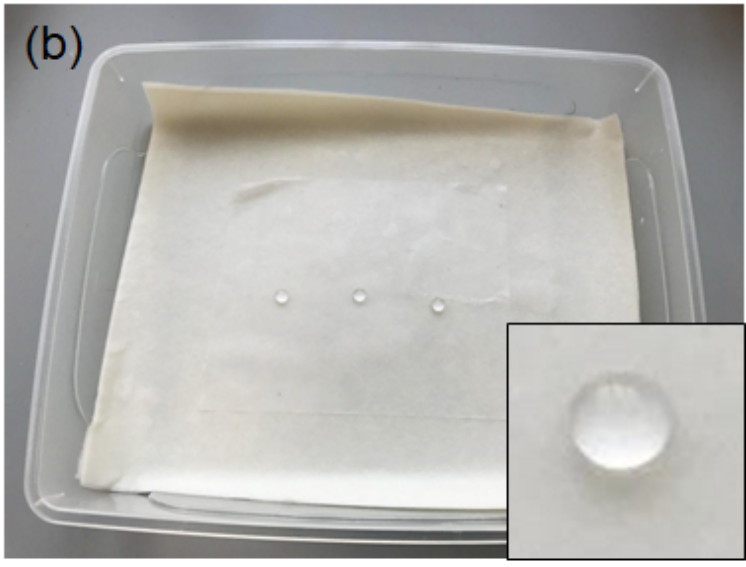


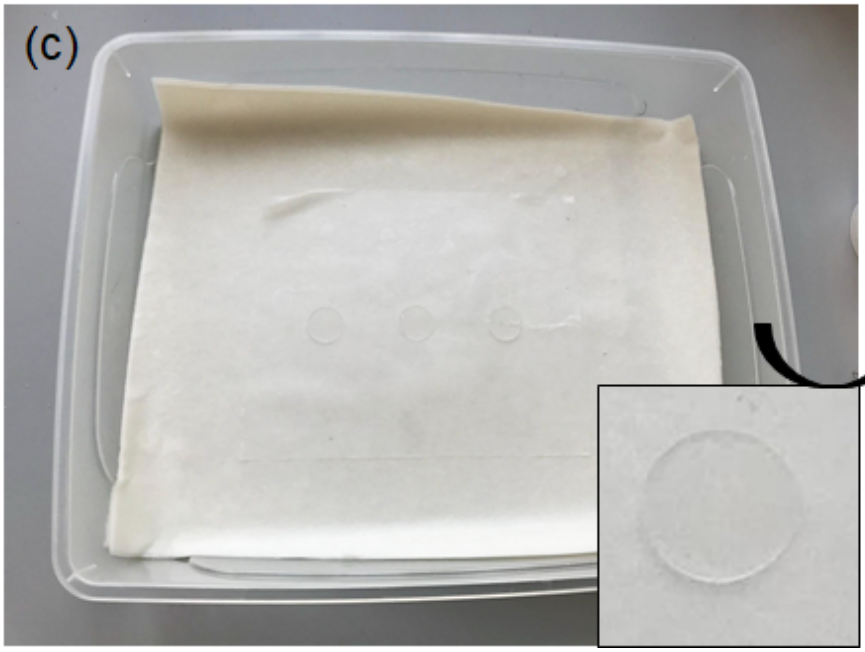




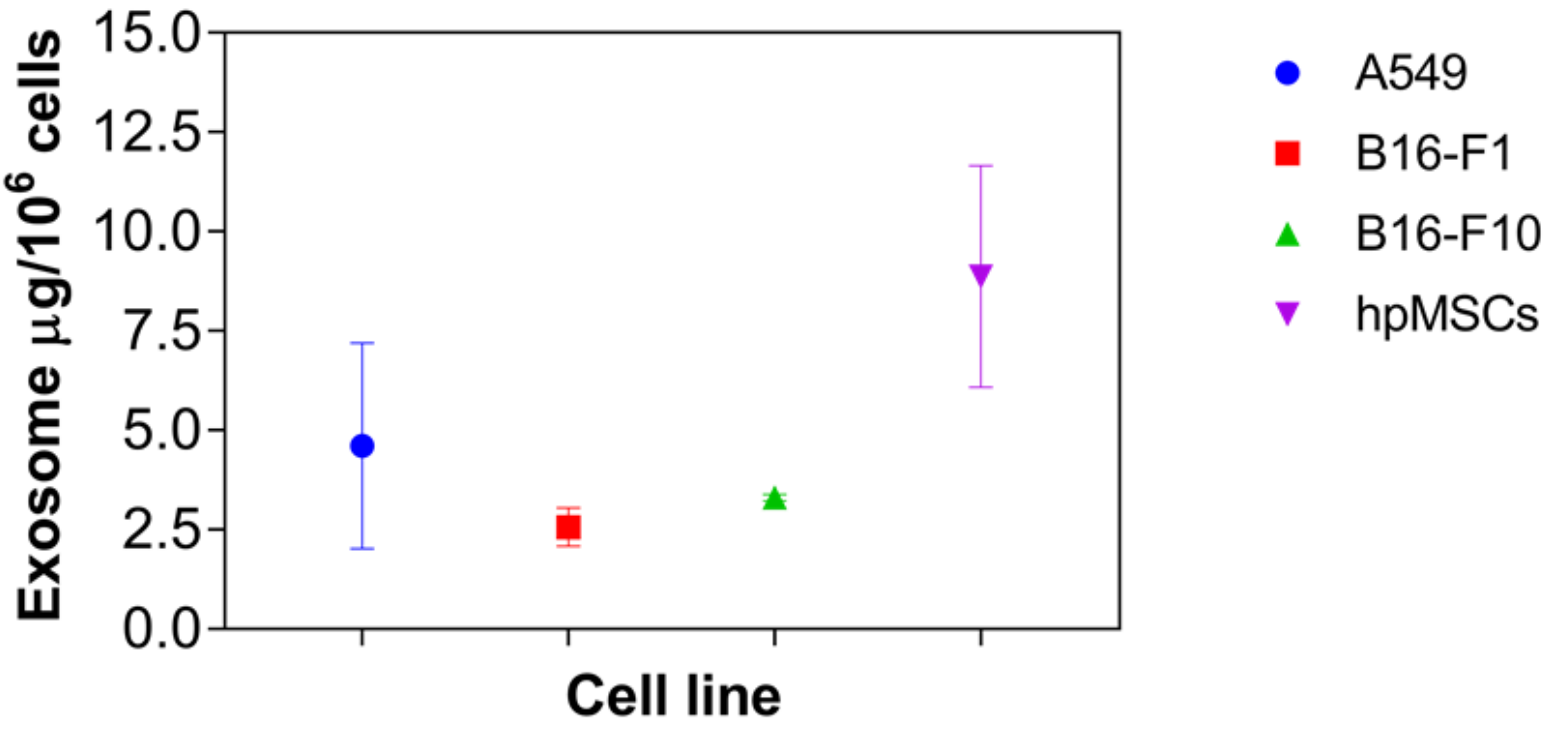


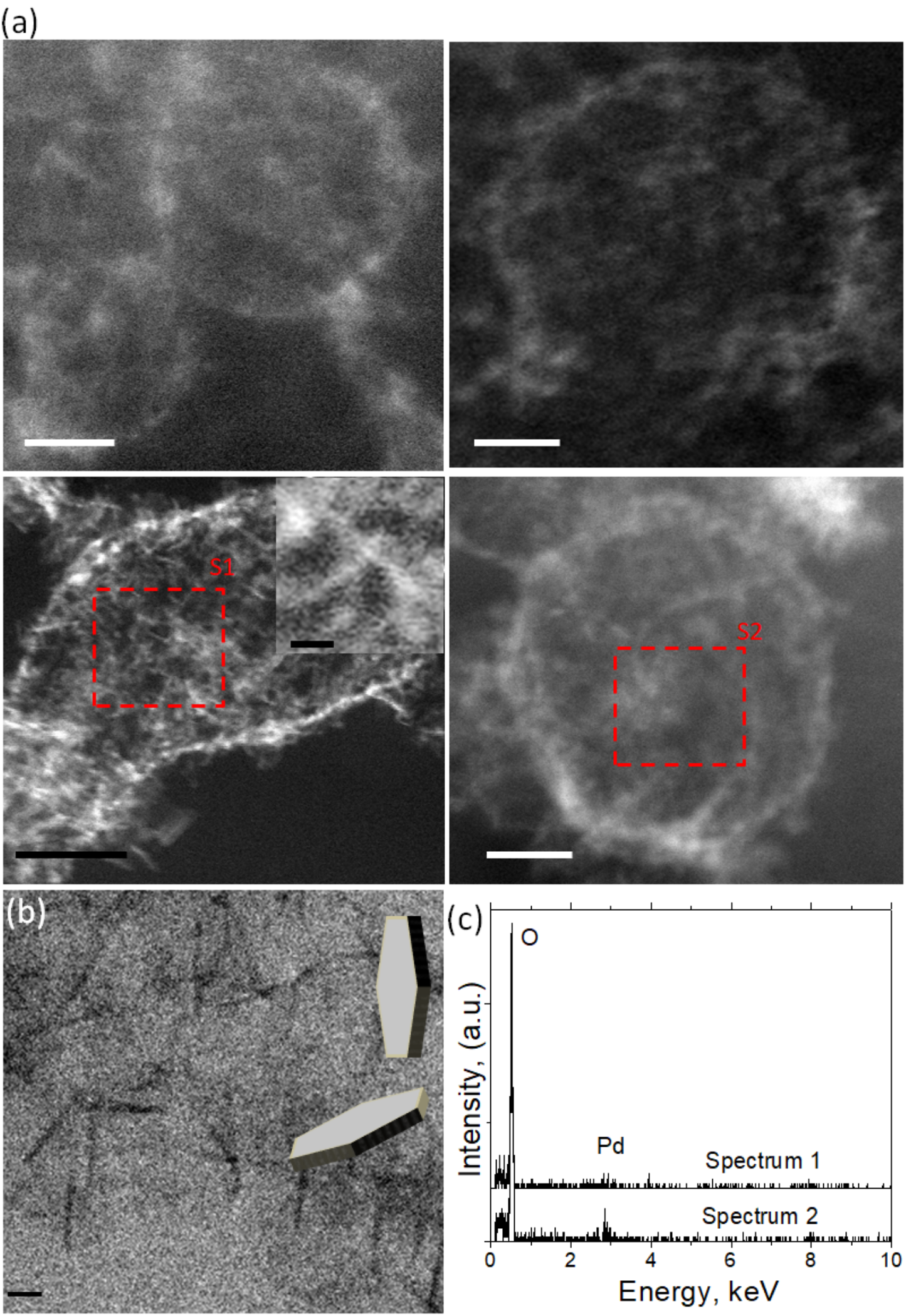




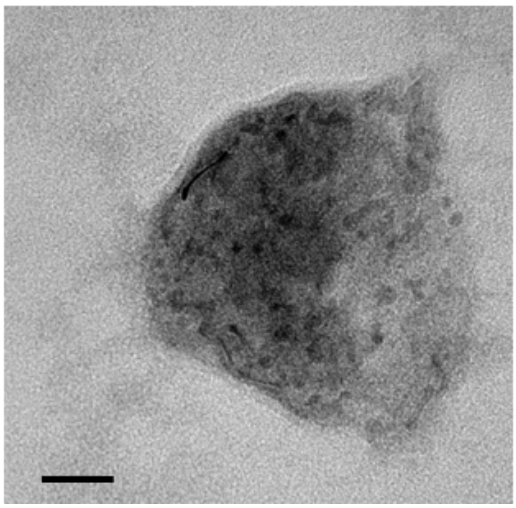


Incubate in the chamber for 1 h in darkness

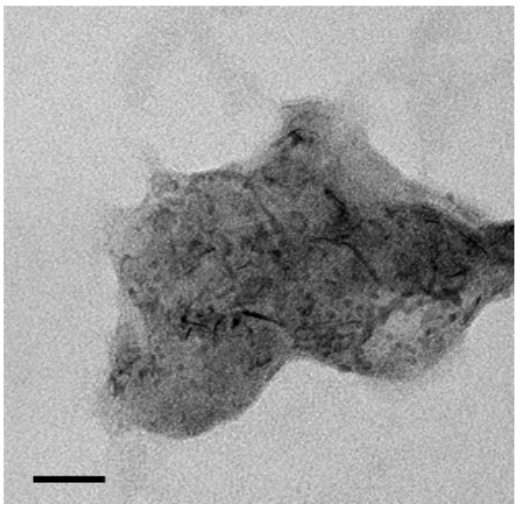

Exosome µg/10^6 cells
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Cell line
A549
B16-F1
B16-F10
hpMSCs


(a)
S1
S2
(b)
(c)
O
Pd
Spectrum 1
Spectrum 2
Intensity, (a.u.)
0
2
4
6
8
10
Energy, keV

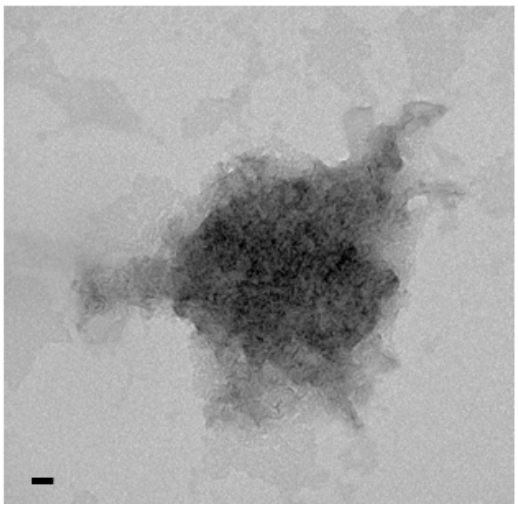
Melanoma cell line B16-F1

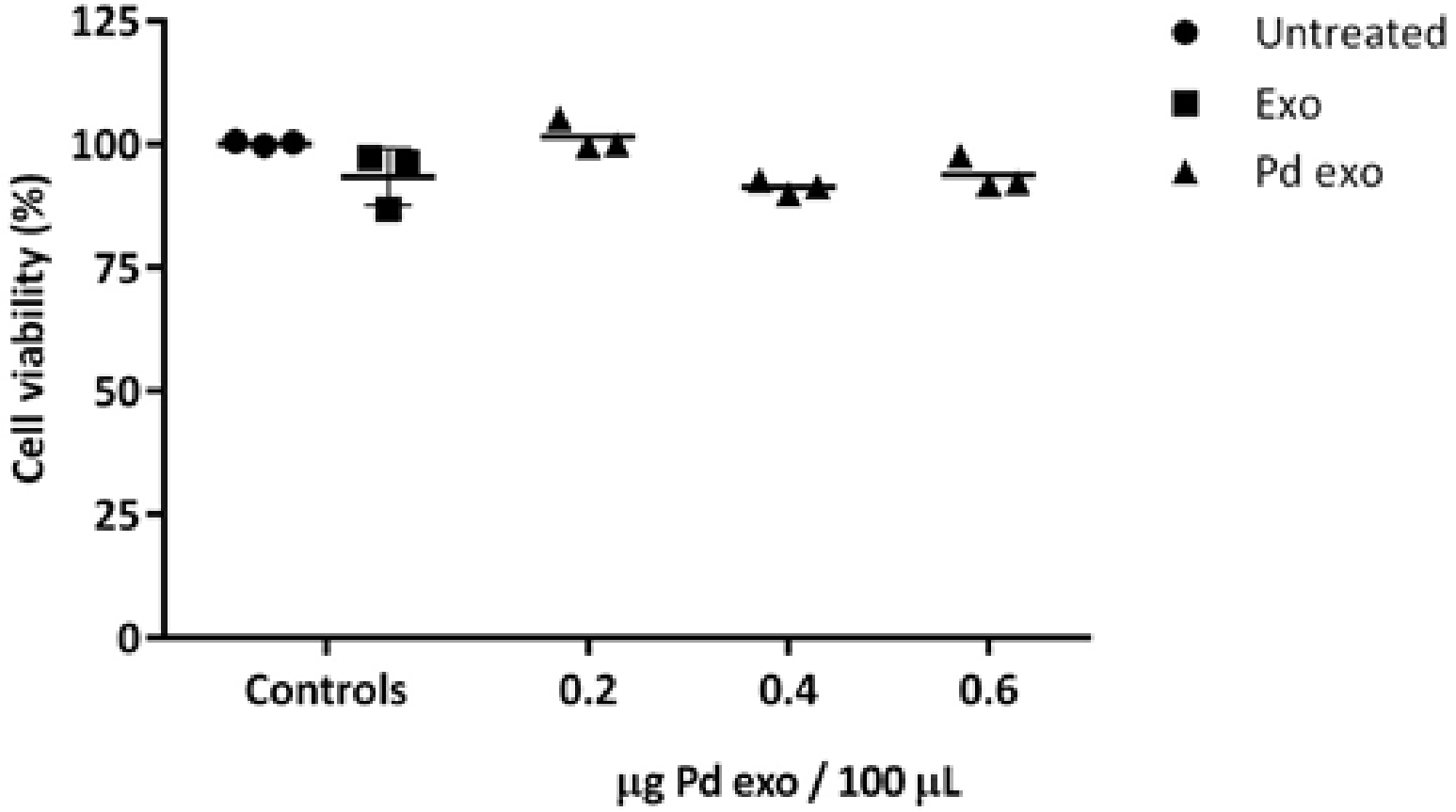


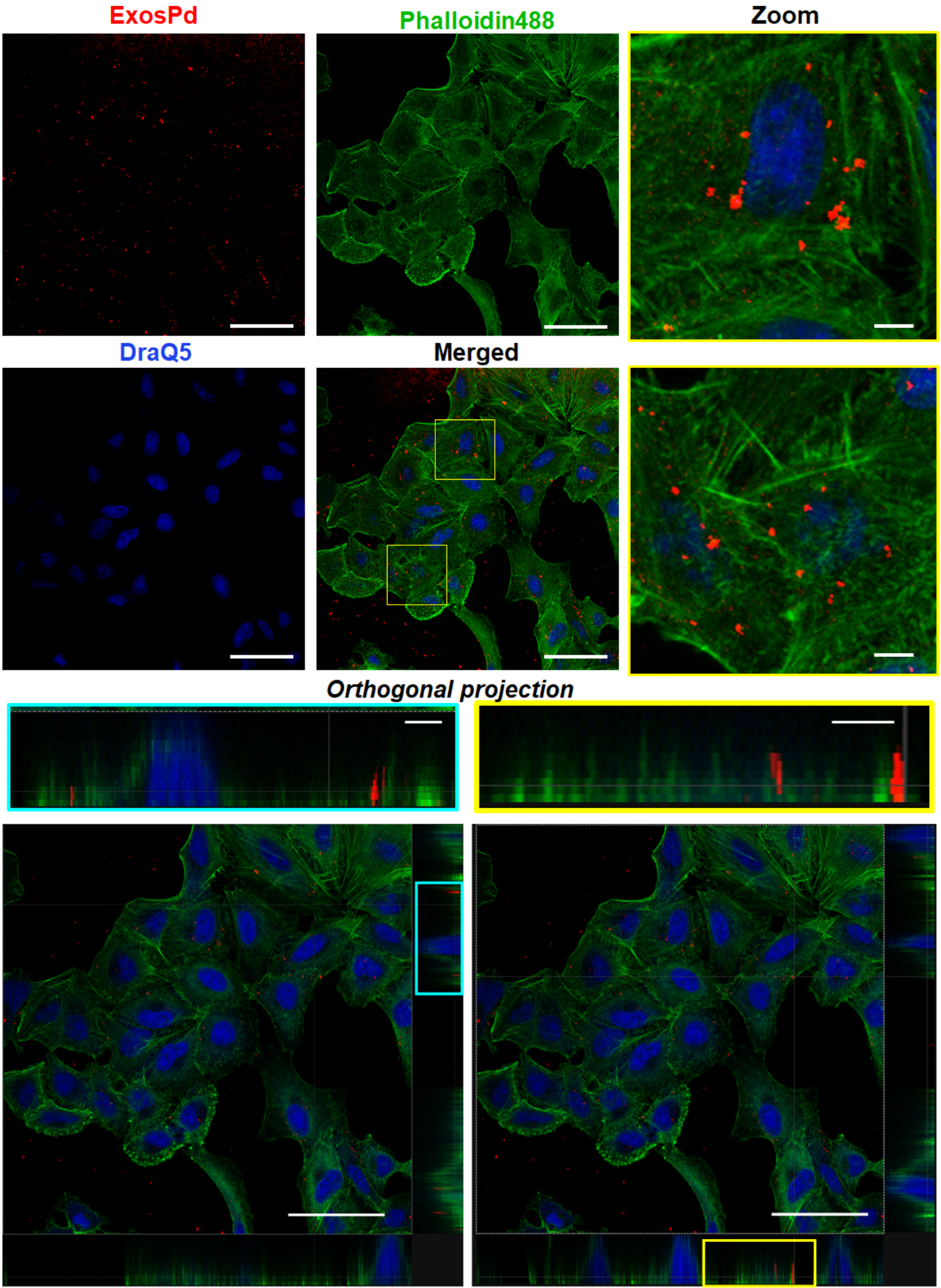
Melanoma cell line B16-F10

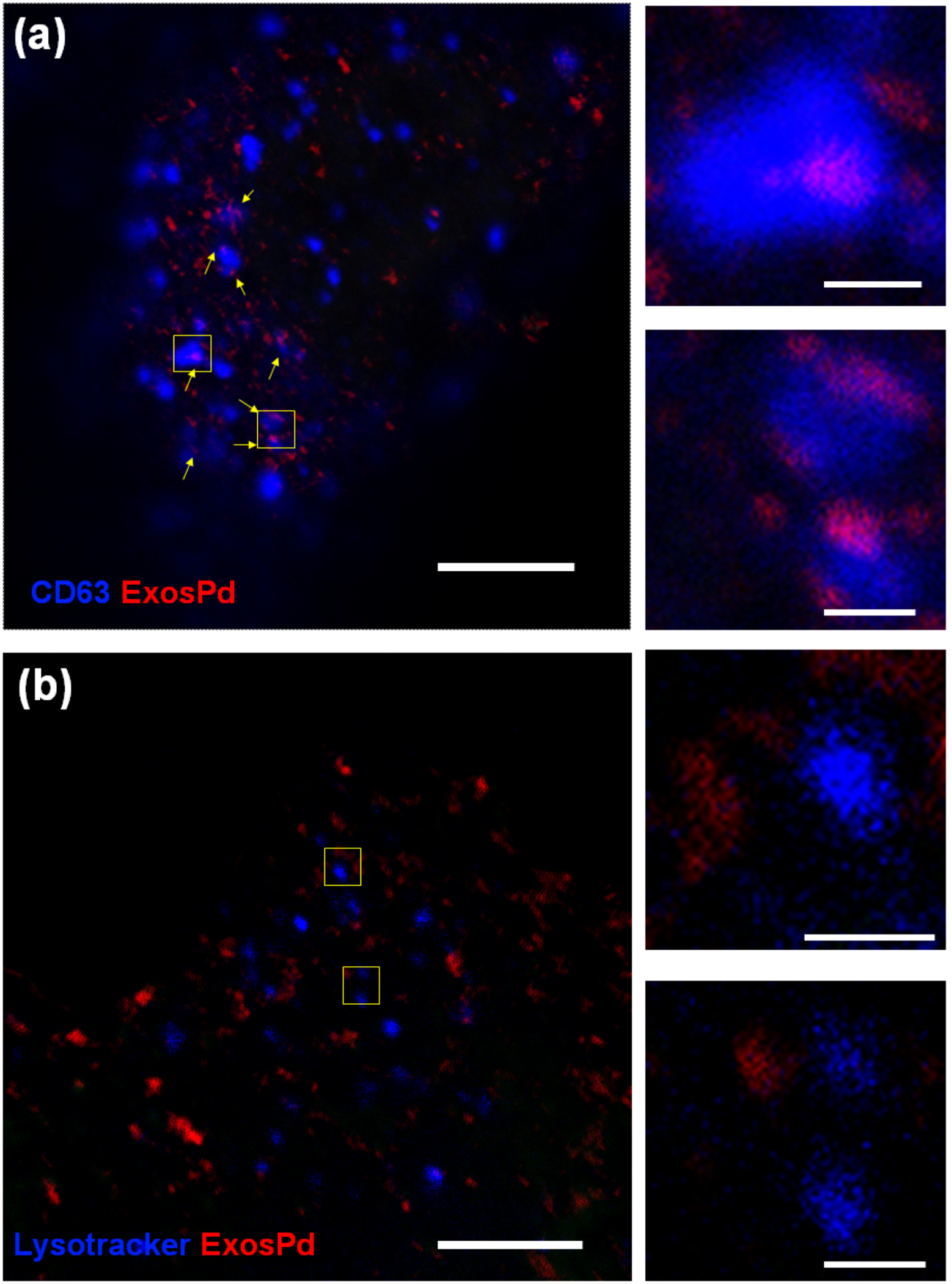


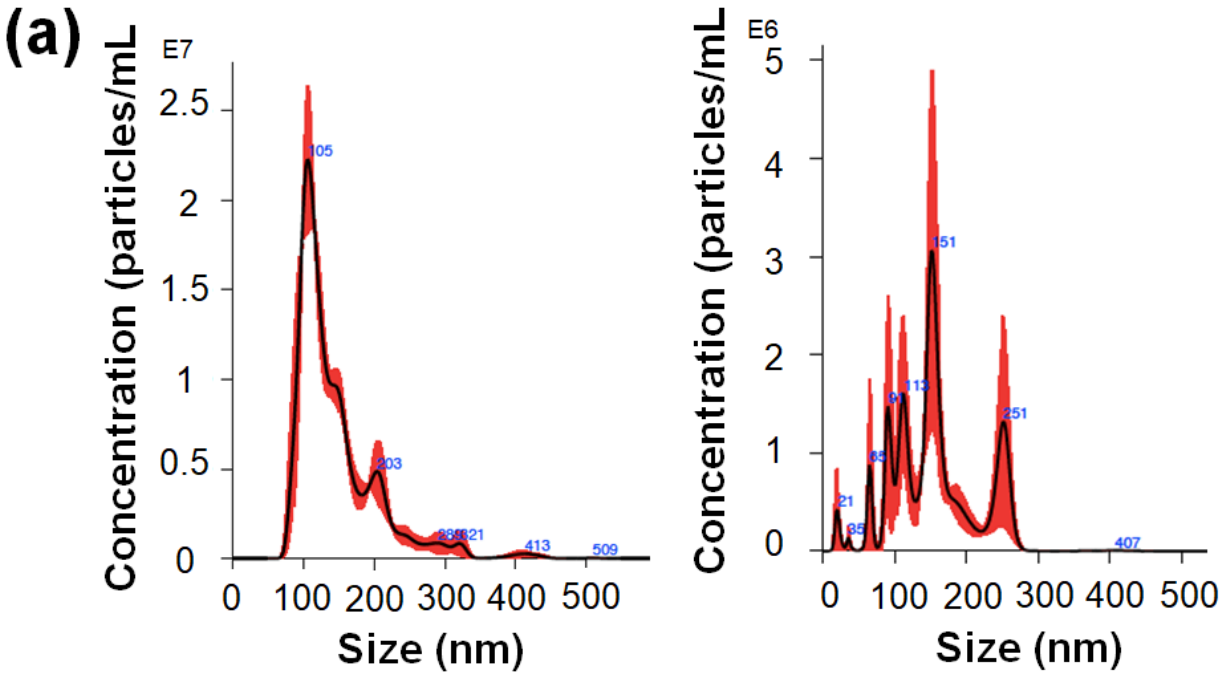
Human placenta-derived mesenchymal stem cells (hPMSCs)

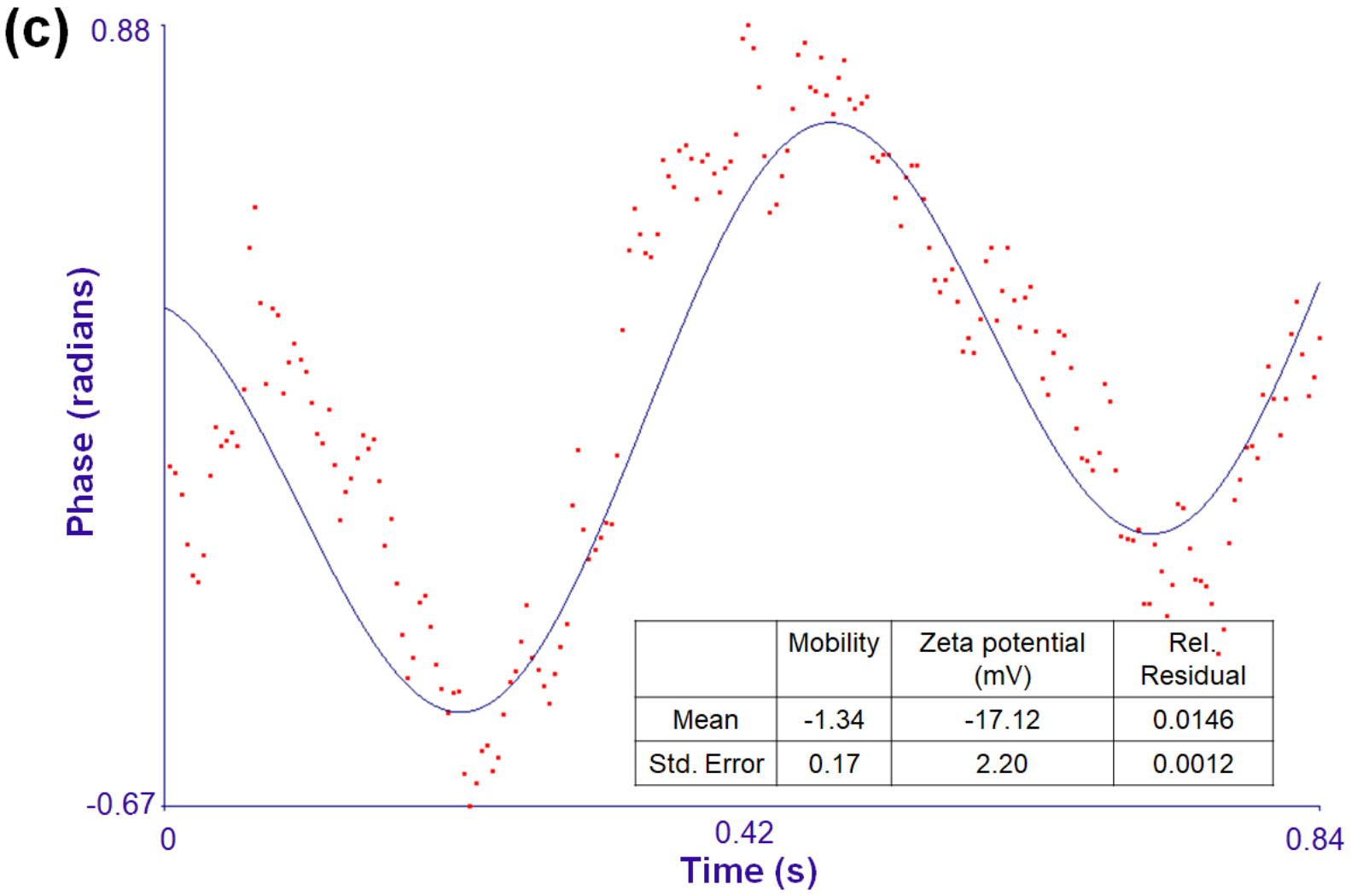



125
100
75
50
25
0
Cell viability (%)
Controls
0.2
0.4
0.6
µg Pd exo / 100 µL
Untreated
Exo
Pd exo

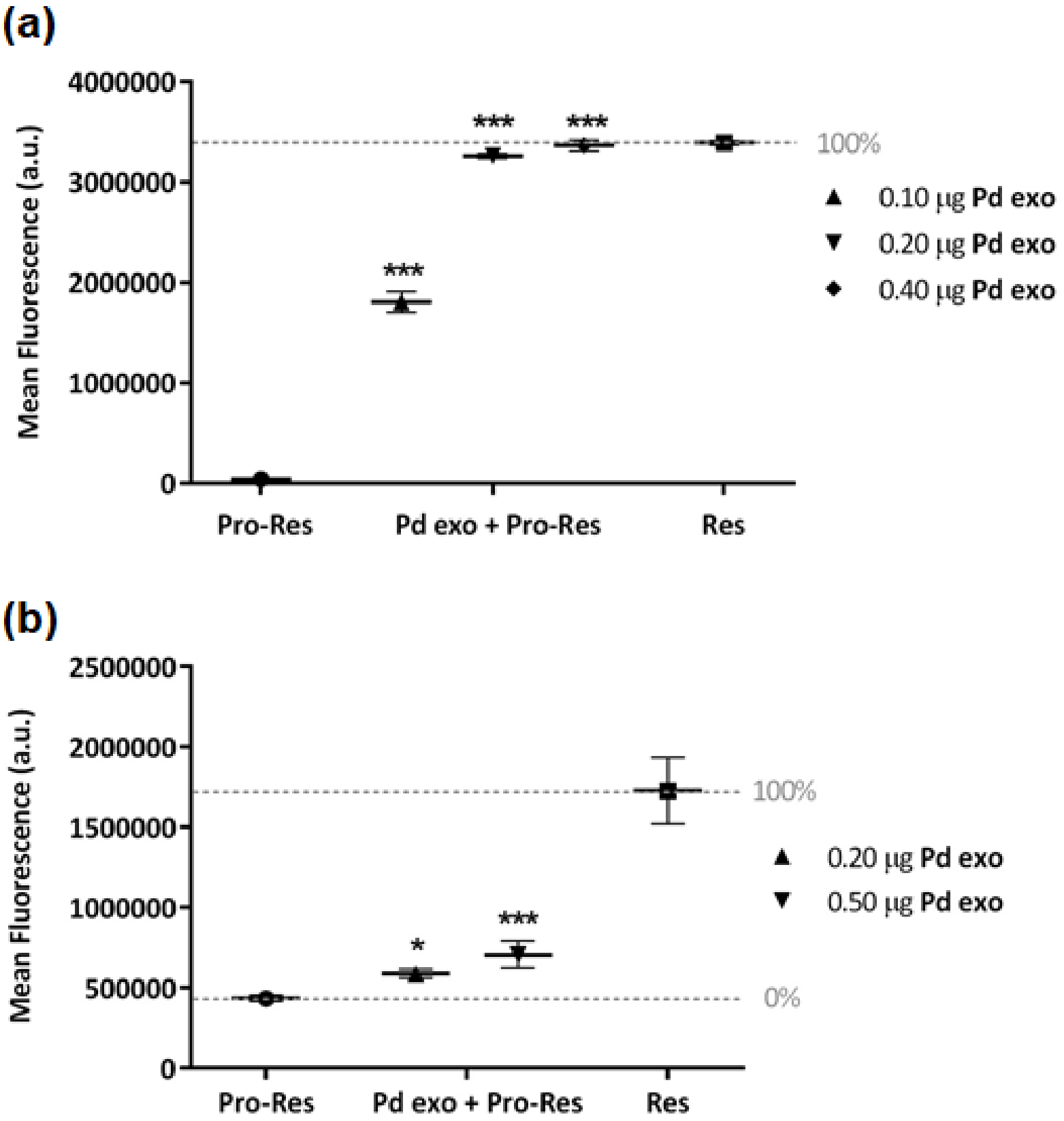

ExosPd
Phalloidin488
Zoom
DraQ5
Merged
Orthogonal projection

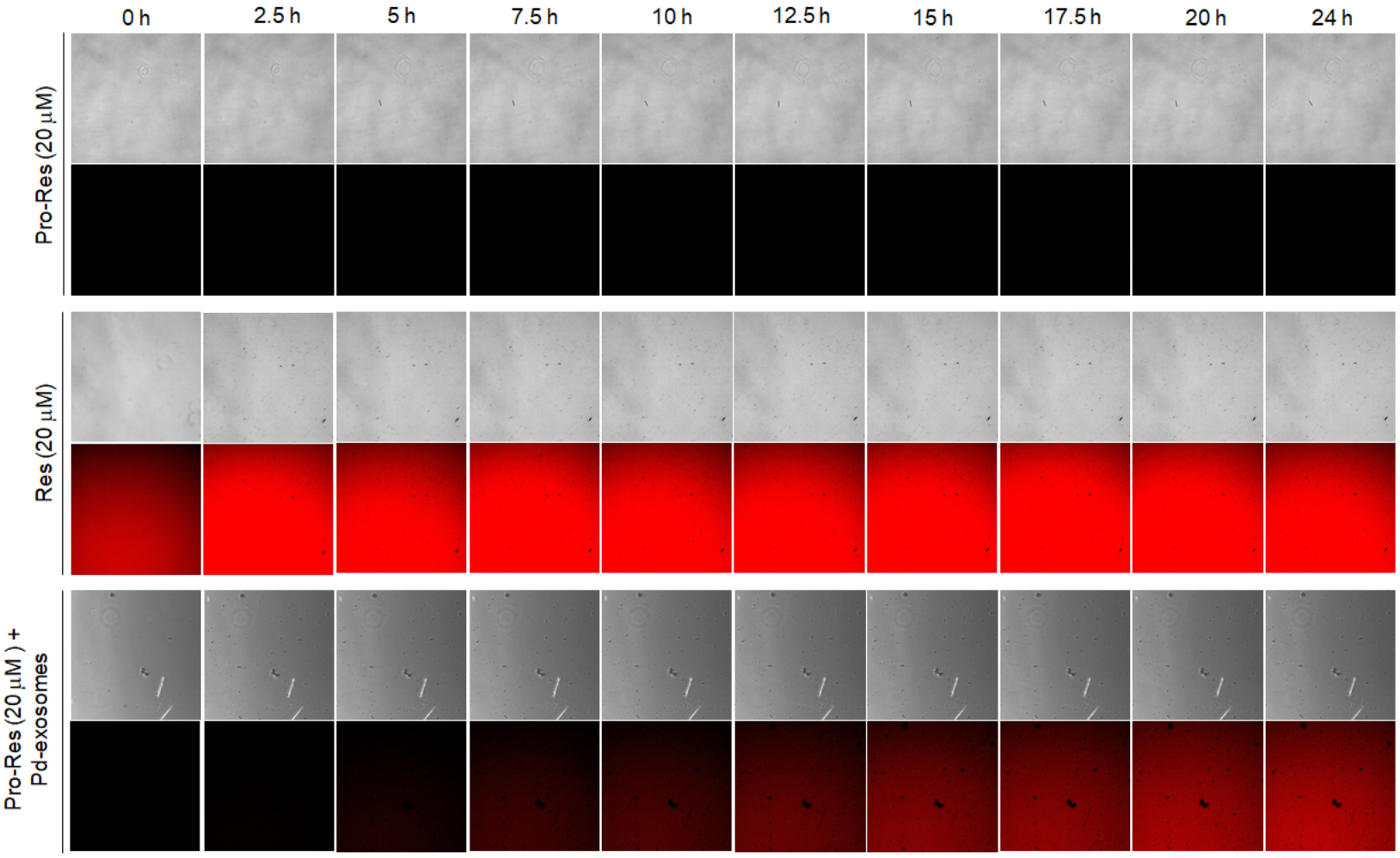

(a)
CD63 ExosPd
(b)
Lysotracker ExosPd





	Mobility	Zeta potential (mV)	Rel. Residual
Mean	-1.34	-17.12	0.0146
Std. Error	0.17	2.20	0.0012


(a)
Mean Fluorescence (a.u.)
4000000
3000000
2000000
1000000
0
***
***
***
100%
0.10 µg Pd exo
0.20 µg Pd exo
0.40 µg Pd exo
Pro-Res
Pd exo + Pro-Res
Res
(b)
Mean Fluorescence (a.u.)
2500000
2000000
1500000
1000000
500000
0
*
***
100%
0%
0.20 µg Pd exo
0.50 µg Pd exo
Pro-Res
Pd exo + Pro-Res
Res


0 h
2.5 h
5 h
7.5 h
10 h
12.5 h
15 h
17.5 h
20 h
24 h
Pro-Res (20 μM)
Res (20 μM)
Pro-Res (20 μM) + Pd-exosomes