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**Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins**

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**Abstract**

Extracellular vesicles (EVs) are phospholipid-based particles endogenously produced by cells. Their natural composition and selective cell interactions make them promising drug carriers. However, in order to harness their properties, efficient exogenous drug encapsulation methods need to be investigated. Here, EVs from various cellular origins (endothelial, cancer and stem cells) were produced and characterised for size and composition. Porphyrins of different hydrophobicities were employed as model drugs and encapsulated into EVs using various passive and active methods (electroporation, saponin, extrusion and dialysis). Hydrophobic compounds loaded very efficiently into EVs and at significantly higher amounts than into standard liposomes composed of phosphocholine and cholesterol using passive incubation. Moreover, loading into EVs significantly increased the cellular uptake by >60% and the photodynamic effect of hydrophobic porphyrins *in vitro* compared to free or liposome encapsulated drug. The active encapsulation techniques, with the saponin-assisted method in particular, allowed an up to 11 fold higher drug loading of hydrophilic porphyrins compared to passive methods. EVs loaded with hydrophilic porphyrins induced a stronger phototoxic effect than free drug in a cancer cell model. Our findings create a firm basis for the development of EVs as smart drug carriers based on straightforward and transferable methods.

**Keywords**

Exosomes, shedding microvesicles, drug loading, porphyrins, photodynamic therapy, high-content imaging

**Graphical abstract**

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**Introduction**

In recent years, extracellular vesicles (EVs) have received growing interest as a promising drug delivery system. EVs are small, cell-derived phospholipid-based bilayer membrane particles decorated with functional surface and membrane proteins [[1](#_ENREF_1)]. It is known that EVs are produced by most – if not all – cells *in vitro* and *in vivo* [[2](#_ENREF_2), [3](#_ENREF_3)]. In the living system, these vesicles serve for transmission of biological signals, transfer of proteins and nucleic acids, and they induce several biological effects [[4](#_ENREF_4), [5](#_ENREF_5)]. There are three major subsets of EVs that can be distinguished according to their cellular origin: exosomes, shedding microvesicles (SMVs) and apoptotic bodies. Exosomes are usually 50-200 nm in size and are derived from multivesicular bodies [[3](#_ENREF_3)] while SMVs are bud directly from the cell’s surface [[6](#_ENREF_6)]. These SMVs can be up to 1 μm in size, but they have also been reported to be in the lower nanometre scale (100-300 nm) [[7](#_ENREF_7), [8](#_ENREF_8)]. Apoptotic bodies are released upon cell death, can be up to several micrometres in size and may contain DNA, histone and organelle fragments. In general, exosomes and SMVs exhibit a naturally derived composition which results in a reduced immunogenicity [[9](#_ENREF_9), [10](#_ENREF_10)] and they can potentially bypass complement activation and coagulation factors giving them better stability in the blood circulation [[11](#_ENREF_11), [12](#_ENREF_12)]. Moreover, off-target effects are less likely when using EVs as they often specifically transit to their target cells [[11](#_ENREF_11), [13](#_ENREF_13)]. Due to these unique properties and their small size, both exosomes and SMVs are promising candidates as drug delivery vehicles.

A few studies have investigated the delivery potential of EVs, mainly for the purpose of RNA-based therapeutics [[14](#_ENREF_14), [15](#_ENREF_15)]. In these studies, engineered donor cells were used to encapsulate nucleic acids endogenously into EVs (*i.e.*, those produced and secreted by the cell). The first proof-of-concept study of exogenous drug loading (loading after separation of EVs from cells) has shown huge potential for exosomes as smart delivery systems [[16](#_ENREF_16)]. Exosomes loaded with siRNA (using electroporation) enhanced drug transport through the blood-brain-barrier resulting in a significant downregulation of an Alzheimer-associated gene in a mouse model. Drug encapsulation by electroporation has recently been investigated and it was suggested that this method may induce siRNA precipitate formation which may affect the drug’s biological activity [[17](#_ENREF_17)]. Therefore, one goal of the present study was to identify whether electroporation would be a viable method for loading of small molecule drugs into EVs. Indeed, efficient loading of small molecule drugs into these cell-derived particles has not been described in detail. Previous studies employ passive incubation for successful incorporation of anti-inflammatory drugs [[18](#_ENREF_18)]. However, additional active loading methods for EVs in particular for encapsulating small molecule drugs would be essential.

In the present manuscript, we examined the drug loading efficiency of EVs derived from different cell types using various active and passive loading methods. The loading methods employed in this study were non-destructive and technically simple to allow application with a broad range of therapeutic molecules. Porphyrins with different degrees of hydrophobicity were selected as model drugs because they can be captured by fluorescent and colorimetric detection, and they induce a strong photodynamic effect through induction of reactive oxygen species (ROS) after light activation [[19](#_ENREF_19), [20](#_ENREF_20)]. In a cancer cell model, porphyrin-loaded EVs showed a massively increased cellular uptake and decreased cell survival after exposition to light compared to the free drug or drug encapsulated in liposomes. These results indicate that EVs are highly versatile entities that can deliver therapeutics to target cells and that innovative, smart and efficient methods of drug-encapsulation are promising as a clinical approach.

**Materials and Methods**

**Chemicals**

2,7,12,17-Tetra-tert-butyl-5,10,15,20-tetraaza-21H,23H-porphine (por, Mw 539 Da), 5,10,15,20-Tetrakis(1-methyl-4-pyridinio) porphyrin tetra(p-toluenesulfonate) (TMP, Mw, no counterion 679 Da) and 4,4′,4′′,4′′′-(Porphine-5,10,15,20-tetrayl) tetrakis(benzoic acid) (porBA, Mw 791 Da) (Supplementary Fig. 1) and all chemicals (unless indicated otherwise) were purchased from Sigma-Aldrich (Gilingham, UK).

**Cell culture and EV extraction**

MDA-MB231 breast cancer cells (MDA) were maintained in DMEM supplemented with 10% (*v/v*) FBS and penicillin/streptomycin (P/S). Human umbilical vein endothelial cells (HUVEC) were grown in fully supplemented endothelial growth factor medium EGM™2 with BulletKit™ (Lonza, CC-3156 & CC-4176) with P/S and cultured up to passage 12. Bone-marrow derived human mesenchymal stem cells (hMSC) were expanded in supplemented hMSC growth medium (Promocell, C-28010) with P/S and used up to passage 8. MDAs, HUVECs and hMSCs were incubated at 37°C and 5% CO2, and their medium was changed every 2 days. The human embryonic stem cell (hESC) line H9 (WiCell) was maintained on growth factor reduced Matrigel (BD) coated plates in supplemented mTeSR1 medium (Stem Cell Technologies) according to the manufacturer's instructions. Plates were prepared as follows: Matrigel was defrosted overnight at 4°C and diluted 1:1 in ice cold Advanced DMEM/F12 (Life technologies). For coating, a 1 ml aliquot was slowly defrosted on ice, diluted with 30 ml of Advanced DMEM/F12 and 1 ml/well was dispensed (6 well plate). Plates were then kept at 4°C up to 1 week. Before plating, plates were equilibrated to 37°C. Finally, hESCs were passaged with 1 mg/ml Collagenase IV (Life technologies) and grown in these plates to sub confluence.

To obtain EVs, sub confluent cells (70-80% confluence) were cultured in serum-free medium (typical number of cells): MDAs in DMEM (8 x 106 cells), HUVECs in EBM-2 (5 x 106 cells), hMSCs in αMEM (2 x 106 cells) and hESCs in mTeSR1 (5 x 106 cells) for 48 h. Cells did not show any substantial changes in viability and morphology when cultured under serum-free conditions (Supplementary Fig. 2). The conditioned medium was collected and centrifuged at 500 x *g* (to remove cells) and at 4500 x *g* (to remove apoptotic bodies) for 20 min each. Subsequently, filtered through a 0.1 μm membrane filter (Millipore®, VVLP01300, Durapore® PVDF Membrane Filter) using a syringe pump (Cole Palmer, 100 mL/h) leading to deposition of EVs onto the filter membrane. This membrane was subsequently transferred into a vial with PBS (typically 200 μL), vortexed for 20 s and sonicated for 15 min (sonication bath) to allow detachment of EVs from the membrane. To compare the filtration method with a centrifugation approach, the supernatant was ultracentrifuged (120,000 x *g,* 1 h, 4°C, Beckman Coulter Optima XE90), resuspended in 200 μL PBS and analysed by NTA. All native EVs were stored at 4°C until use and for no longer than 5 days.

**Size distribution, stability and transmission electron microscopy (TEM) of EVs**

Size distribution and particle concentration of EVs were measured by nanoparticle tracking analysis (NTA, NanoSight LM10) recording video frames of 60 s. The stability of EVs was measured upon storage in PBS and at 4°C using dynamic light scattering (Zetasizer, Malvern Instruments).

EVs were incubated on a TEM grid (copper grid with 300 mesh, carbon coated; Electron Microscopy Sciences) and fixed in paraformaldehyde/glutaraldehyde (4/0.2% *v/v*) both for 10 min. Samples were subsequently washed 3x with water and incubated for 10 min with uranylacetate/methylcellulose (2:1). Excess solution was gently wicked off using filter paper and samples were air-dried. TEM images were taken at 80 kV using a JEOL 2010 TEM.

**Flow cytometry**

For surface protein/lipid detection, isolated EVs were diluted 1:10 in PBS [0.1% (*w/v)* BSA] or annexin V binding buffer (Annexin V-PE Apoptosis Detection Kit, abcam) and incubated with annexin V-PE or labelled with anti-human CD40-APC or anti-human CD63-eFluor450® (eBioscience). After 1 h at RT, measurements were made on a 3 laser, 11 colour Fortessa (BD Biosciences) using the following settings: annexin V-PE: filter/bandpass 585/15 nm; anti-CD40/63: filter/band pass 670/14 nm and 450/50 nm (APC and Pacific Blue, respectively). For all samples signal area instead of height was measured to get a more robust signal. All positive staining particles were quantified using the appropriate fluorescence channel. Annexin V-PE diluted in binding buffer or appropriate isotype controls (APC and eFluor450 labelled mouse IgG1 antibodies, eBiosciences) were used as negative controls to determine gating. For each sample, events were collected for 30 s at a flow rate of 12 μL/min using identical settings. For comparison of the different EVs, thresholding was carried out on the fluorescence signal in comparison with the negative controls. All results are displayed as positive events (measured in 30 s) and in comparison with the diluent alone [[21](#_ENREF_21), [22](#_ENREF_22)].

**Buoyant density determination**

Isolated EVs were diluted in sucrose (3 mL, 60% *w/v*) in a polyallomer ultracentrifuge tube (14 mL, round bottom, #331374, Beckman Coulter). Subsequently, layers of sucrose (45, 30 and 8% *w/v*, 3 mL each) were added carefully on top. Tubes were centrifuged at 192,000 x *g* for 15 h at 4°C (using a SW40Ti rotor on a Beckman Optima XE 90, Supplementary Fig. 3b and c). Afterwards, fractions of 1.5 mL were collected from top to bottom and their density was determined by differential weighing. Particle distribution and number was determined by NTA as described above.

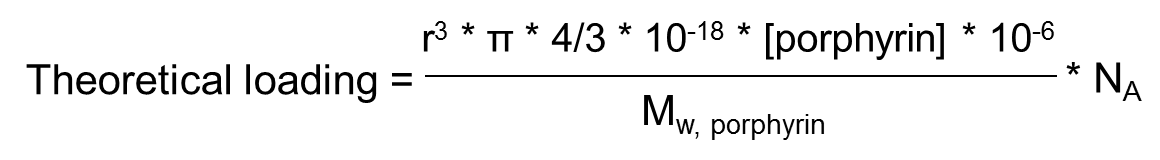
**Lipid composition of EVs**

The lipid composition of EVs was analysed by thin-layer chromatography (TLC) using silica gel plates on aluminium foil (Merck, layer thickness 200 μm) of 6.5 cm running height. Suspensions of EVs in PBS were evaporated at 95°C, and then lipids were dissolved in chloroform and applied onto the TLC plate. For phospholipid analysis, standards (egg phosphatidylethanolamine, PE; egg phosphatidylcholine, PC; Dioleylphosphoserine [18:1, 18:1], PS; and egg sphingomyelin, SM; all Lipoid, Steinhausen, Switzerland) were used (0.5 ng/lane). All plates were run using a horizontal developing chamber containing chloroform/methanol/water (65:25:4) [[23](#_ENREF_23)]. For cholesterol analysis, standards of cholesterol (0.1-0.5 ng/lane) were used. TLC plates were developed using hexane/diethyl ether/glacial acetic acid (90:10:1) [[24](#_ENREF_24)]. After drying, plates were incubated with charring reagent (7.5% Cu-acetate and 2.5% CuSO4 (both *w/v*), and 8% H3PO4 (*v/v*)) and dried for 15 min at 60°C on a heating plate. Afterwards, the temperature was increased to 150°C to allow charring [[23](#_ENREF_23)]. The position and the plot profile of each lane was analysed by ImageJ. For each peak (spot) the area under the curve was measured and used to calculate a relative abundance of each lipid.

**Drug loading of EVs**

Typically, EVs isolated from conditioned media from 2 x 106 cells (hMSC), 5 x 106 cells (HUVEC, hESC) and 8 x 106 cells (MDA) were used for drug loading. For each model compound (por, TMP and porBA, Supplementary Fig. 1), vesicles suspended in 200 μL PBS were mixed with drug (1.5 mg/mL) in an electroporation buffer (21% sucrose *(w/v)*, 1.15 mM KH2PO4 and 25 mM NaCl, pH 7.4). For all drug loading experiments, buffer type, EV and drug concentration were kept constant in order to provide equal conditions.

For passive loading (PV), EVs and drug were incubated at RT for 10 min. Electroporation (EP) was performed in Gene Pulser cuvettes (0.4 cm cell electrode gap, #167-2081) on a BioRad Gene Pulser equipped with a capacitance extender, with conditions selected at 200 Ω, 500 μF, 200 mV and pulse time of 20-30 ms [[16](#_ENREF_16)]. Extrusion (EX) of EVs and drug was conducted at 42°C using a syringe-based hand-held mini-extruder (Avanti Lipids) equipped with a heating block using polycarbonate membranes of 400 nm pore size (track-etch membrane, Whatman). Each sample was extruded 31 times. For saponin-assisted (SP) drug loading, EVs and drug were incubated with 0.1 mg/mL saponin (Sigma-Aldrich, #47036) at RT for 10 min. Hypotonic dialysis (HP) was performed by transferring EVs and drug into dialysis membranes (cellulose ester, molecular weight cut-off 100-500 Da, Spectrum Labs) placed in 200 mL 10 mM phosphate buffer (pH 7.4) and stirred at RT for 4 h. All drug-loaded EVs were purified by size-exclusion chromatography (SEC) using 8 mL (Biorad Polyprep column) of Sephadex G75 (Sigma-Aldrich) and PBS as eluent. EVs typically eluted within 2-4 mL elution volume (Supplementary Fig. 3a). The absorbance at 630 nm (por) or 420 nm (TMP, porBA) and particle concentration (NTA) was determined for each fraction. The loading of EVs was calculated as drug molecules per vesicle. For each drug type, the theoretical loading was calculated assuming that vesicles are spherical particles of 150 nm size (r=75 nm) which are completely filled with drug solution (NA Avogadro constant):

 (Eq. 1)

The loading efficiency for each drug and vesicle combination was defined as n-fold increase compared to the theoretical loading. Drug loaded EVs were used within 24 h of preparation.

**Liposome preparation**

Liposomes were prepared by a film hydration/extrusion method [[25](#_ENREF_25)]. In brief, egg PC and cholesterol were dissolved (60/40% molar ratio, total lipid concentration 2.5 mM) with por (1.5 mg/mL final concentration) in chloroform and dried overnight. Lipid films were hydrated in water (0.5 mL) and subsequently extruded 31 times through 200-nm polycarbonate membranes. Liposomes were purified using Sephadex G75 (8 mL) and PBS as eluent. Size distribution, zeta potential and absorbance of liposomes was measured in a similar fashion to drug loaded EVs. Drug loaded liposomes were used within 24 h of preparation.

**Photodynamic effect and cellular uptake of drug-loaded EVs *in vitro***

MDAs were seeded in 96-well plates (20,000 cells/well) in DMEM [10% *(w/v)* FBS, P/S] and allowed to adhere overnight at 37°C and 5% CO2. Stock solutions of drug loaded EVs, unloaded EVs, free drug, liposome encapsulated drug or PBS were diluted 1:2 in medium and incubated with the cells for 4 h in the dark. Afterwards, samples were removed; cells were washed with PBS and mixed with PBS containing 5 mM glucose. The plate was placed on a heating plate (37°C) and wells were irradiated with at 633 nm (HeNe single frequency laser, model 200, Coherent, 1.5 mW,) for 200 or 400 s (corresponding to 0.3 and 0.6 J/well) [[19](#_ENREF_19)]. A control plate was treated similarly but kept in the dark. After irradiation (0 and 18 h) cells were incubated in alamarBlue® (Invitrogen) solution (10% (*v/v*) in DMEM without phenol red) for 3 h at 37°C and 5% CO2. Absorbance readings (570 and 600 nm) of the wells were taken and used to calculate the relative levels of metabolic activity. The metabolic activity of the cells was used as a measure for cell viability. Drug-treated samples were compared to PBS-treated controls.

Fluorescence microscopy measurements were conducted on a Zeiss Observer using a bright field and a fluorescence channel (627 nm). Cells were seeded at 20,000 cells/well in 96-well plates (TPP, Switzerland) as above. After 4 h of incubation with drug-loaded EVs and liposomes, free drug or PBS, cells were washed with PBS and covered with fresh medium. For fluorescence imaging, cells were kept at 37°C and 5% CO2. To analyse cellular uptake, images were taken of each well and fluorescence-positive cells counted (20x magnification). For time series measurements, one image was taken every 20 min until 4 h. Images were processed using ZEN software (Zeiss microscopy) and ImageJ.

**Statistical analysis**

All data are displayed as mean ± standard deviation. One-way analysis of variance followed by a Tukey *post hoc* test was used for pairwise comparisons. For cell viability testing, samples were analysed by Dunnett’s *post hoc* test using untreated cells as control (Sigma Plot). Differences were considered significant at *p*>0.05. Linear regression analysis was conducted using the statistical program SPSS (IBM Corporation). The survival analysis was conducted as reported previously [[26](#_ENREF_26)]. In brief, for each sample, the time point of cell death was counted for 10 randomly selected cells. The cumulative risk for cell death was analysed using R software (and freely available survival analysis scripts). The Kaplan-Meier estimator was used to establish the survival in the different groups and the cumulative risk of death curves were plotted to analyse the difference in survival between the various conditions. Statistical significance of the differences between experimental conditions was determined using the log-rank test. The influence of the vesicles’ loading on cell survival was determined by Cox proportional hazards analysis.

**Results and Discussion**

**Characterisation of EVs indicate their advantageous size distribution and storage stability**

EVs were isolated from various cell lines and analysed by NTA. They showed a size distribution in the small nanometre range (~150 nm) as reported in Fig. 1. No larger particles were observed at sizes >500 nm and an insignificant amount of particles was present in the filtrate indicating that the major population of EVs was retained by the filter. When comparing the filtration method with the well-established ultracentrifugation, no major differences were detected in terms of size, distribution profile (polydispersity) and yield of EVs (Supplementary Fig. 4). Moreover, it was observed that the EVs’ morphology was not impaired by the filtration extraction method, indicating that this filtration protocol is a suitable method for EV isolation. Therefore, all drug loading and cell experiments were conducted using EVs obtained by filtration. Our results indicate that EVs feature an optimal size distribution to take advantage of the enhanced permeation and retention effect to accumulate passively in cancer tissue [[27](#_ENREF_27)]. Moreover, EVs retained their small size for a minimum of 5 days (EVs from MDA and hESC in Supplementary Fig. 5a), and at least 14 days (HUVEC and hMSC) upon storage at 4°C in PBS. Such storage stability is another prerequisite for clinical applicability of nanoformulations. The size evolution robustly correlated with the zeta potential of EVs measured in PBS; a stronger zeta potential was associated with better storage stability of vesicles (Supplementary Fig. 5b). Their size distribution and storage stability make EVs suitable for the development in a pharmaceutical setting.

Exosomes and SMVs are the most common types of EVs produced by cells in culture. Due to their different biogenesis and characteristics, a uniform discrimination of EVs obtained from cells in culture is vital for their use in a pharmaceutical setting. This is particularly important because most vesicle preparations are heterogeneous due to their natural origin. Exosomes and SMVs can generally be distinguished by their content in PS. Both types of vesicles expose PS, but in SMVs PS is usually enriched [[28](#_ENREF_28)]. Although no exclusive markers for the identification of exosomes or SMVs exist, there are surface proteins which are more frequently found in either of them. [[3](#_ENREF_3)]. A common exosomal marker is CD63, while SMVs oftentimes carry CD40 on their surface [[3](#_ENREF_3), [8](#_ENREF_8)]. EVs obtained in this study were thus analysed by flow cytometry for the presence of specific markers and PS on the vesicle membrane. Although the general detection limit is 300 nm for most flow cytometers based on light scattering, when using fluorochrome-labelled specific antibodies or isotype control antibodies, it is possible to detect EVs using fluorescence threshold triggering [[8](#_ENREF_8), [22](#_ENREF_22)]. Therefore, PS was detected after binding of Annexin V-PE while fluorochrome-labelled antibodies (anti-CD40 and anti-CD63) were used to analyse for surface proteins. These measurements facilitated a relative comparison of marker abundance between the different types of EVs. It can be seen in Fig. 1 that vesicles from MDA, hESCs hMSC carried a smaller amount of PS in their membrane compared to HUVECs. However, PS is heavily enriched in vesicles that are bud directly from the cell surface (SMVs) [[2](#_ENREF_2), [7](#_ENREF_7)]. All EVs were positive for the exosomal marker CD63 (Fig. 1), though HUVEC vesicles had the smallest abundance of this protein. None of the EVs exhibited discernible levels of antiCD40 expression (common SMV-marker). Another parameter to differentiate between exosomes and SMVs is their floating density in a sucrose gradient. Vesicles from MDA, hMSC and HUVECs showed a buoyant density of 1.09 – 1.11 g/mL (Supplementary Table 1) matching literature values for exosomes (1.10 – 1.21 g/mL, according to ExoCarta database). Thus, EVs from these cells are primarily of exosomal nature. These findings are corroborated by the typical cup-shape morphology of vesicles in TEM (Fig. 1 insets). Although hESC particles had a smaller buoyant density (1.05 g/mL), the presence of the exosomal marker CD63 and the cup-shape of vesicles was indicative of mainly exosomal origin of EVs.

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**Figure 1. Size distribution profile (left), transmission electron microscopy images (insets) and flow cytometry analysis (right) of EVs from various cell lines** (average maximum size and typical cell number to obtain EVs): (**a**) MDA-MB231 (157 ± 9 nm, 10 x 106 cells), (**b**) human umbilical vein endothelial cells (159 ± 8 nm, 2 x 106 cells), (**c**) human embryonic stem cells (208 ± 8 nm, 5 x 106 cells) and (**d**) human mesenchymal stem cells (164 ± 23 nm, 6 x 106 cells). Plots show average spectra from *n* = 3. The relative abundance of phosphatidylserine (annexin V stain) and protein markers (CD40 and 63) on the surface of EVs was analysed by flow cytometry using fluorophore-labelled antibodies. Indicated are the number of events measured in 30 s (*n* = 3-5).

**Loading efficiency into EVs is mainly influenced by the drug’s hydrophobicity but can substantially be improved by electroporation**

One of the major challenges associated with EVs as a promising drug delivery system is their capability for efficient drug loading. However, the use of EVs as carriers for small molecule drugs has not been investigated in detail. The few reported studies on the use of EVs as carriers for small molecule drugs used a co-incubation of EVs with drugs of interest [[18](#_ENREF_18)]. To our knowledge, active loading methods for EVs are yet to be reported. Porphyrins were selected as model drugs due to ease of detection (by UV-Vis spectroscopy) and their use for photodynamic therapy [[29](#_ENREF_29)]. Porphyrins with different degrees of hydrophobicity were used (Supplementary Fig. 1): por is relatively hydrophobic, porBA has intermediate water-solubility and TMP is relatively hydrophilic. First, encapsulation of porphyrins in the different EVs was performed by passive incubation or electroporation. As shown in Fig. 2a, the hydrophobic por incorporated very well into EVs independent of the loading method.

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**Figure 2. Drug loading of EVs from various cell lines.** Loading of (**a**) relatively hydrophobic por, (**b**) intermediate porBA, and (**c**) relatively hydrophilic TMP into EVs using passive incubation or electroporation. The chemical structure of each porphyrin is shown. For each drug, the theoretical loading is displayed as a dashed line. This value is calculated based on the simplified assumption that EVs of 200 nm size are round-shaped spheres which are completely filled with drug solution (Eq. 1). *n =* 3-9, \**p* = 0.02 *vs*. passive (Linear regression, *R2*= 0.475). In (**d**) the loading efficiency for each type of EV was calculated and expressed as n-fold increase compared to the theoretical loading. For each drug the loading for the best method was used (passive loading for por and electroporation for porBA and TMP).

Indeed, EV’s encapsulation efficiency was at least twofold higher than for standard liposomes (PC/cholesterol 60:40 molar ratio, Supplementary Fig. 6) and exceeded the theoretical loading by two orders of magnitude (dashed lines in Fig. 2 and 2d). Together with the findings that por loading increased the EV’s zeta potential (Supplementary Fig. 7a), it may be assumed that the hydrophobic compound integrated into the vesicle membrane. The ability of EVs to solubilise otherwise water-insoluble drugs is of great advantage as most newly developed active substances are poorly water-soluble, which represents a major hurdle when formulating them for clinical applications [[30](#_ENREF_30)].

Despite the fact that TMP-encapsulation is rather low compared to por (Fig. 2b), the theoretical loading was exceeded for vesicles from MDA and hESC using electroporation. In addition, it appeared that EVs with stronger zeta potential (*i.e.*, from MDA and hESC) tended to have a higher loading efficiency than vesicles with lower zeta potential (hMSC and HUVEC EVs). However, differences in zeta potential and TMP loading for the various EVs were generally not significant. For the intermediate porBA, the overall loading efficiency was better than for TMP. As detailed in Fig. 2c, the electroporation significantly improved the encapsulation efficiency for all EVs compared to passive incubation. Electroporation has previously been used to improve loading of exogenous siRNA into exosomes [[16](#_ENREF_16)], but recently an alternative mechanism has been put forward [[17](#_ENREF_17)]. It was shown that electroporation potentially induced precipitation of RNA and thus could mask the loading efficiency of biological drugs into exosomes. In this study, no precipitation or particle formation was observed when the drug was electroporated without EVs (Supplementary Fig. 3a). Therefore, in our hands electroporation seems to represent a robust method to improve loading of small molecules into EVs. Finally, electroporation did not substantially impact on the EV’s size and shape (Supplementary Fig. 7b and e). Drug loaded EVs could also be purified using a sucrose gradient as described previously (Supplementary Fig. 2a and b) [[31](#_ENREF_31)]

The loading efficiency into EVs appeared to be dependent on the hydrophobic nature of the drug and the loading method, while the chemical lipid composition of the vesicles was also important. MDA and hMSC EVs, which in general have comparable loading profiles for most drugs and methods investigated in this study, were found to have a similar lipid composition as revealed by TLC (Supplementary Table 2). However, high or low content of cholesterol (typically known to increase the bilayer rigidity) does not substantially impact the loading profiles of EVs. Vesicles have also been shown to harbor a manifold variety of surface and membrane proteins in their lipid bilayer, which also influences the EV’s physical properties [[32](#_ENREF_32)].

**Encapsulation into vesicles significantly improved phototoxicity and cellular uptake of porphyrins *in vitro***

Following drug-loading experiments, the delivery potential of EVs was analysed *in vitro.* To do so, vesicles (with or without porphyrins) or free drug were incubated with MDA cells for 4 h, followed by laser irradiation at 633 nm to induce reactive oxygen species (ROS) formation and cell death. This photodynamic effect was assessed by cell viability analysis 0 and 18 h after laser treatment. As evident from Fig. 3a and Supplementary Fig 8a, por encapsulated EVs induced a significant reduction in cell viability, upon laser irradiation, while the free drug or por loaded liposomes (Supplementary Fig. 6c) did not have any substantial effect (1 μM drug per each well). In particular, MDA-derived vesicles appear to be highly efficient because they also induce long term toxicity (18 h after laser irradiation) for por. The drug concentration at which vesicle-assisted phototoxicity occurred was comparable or much lower than that for other photosensitising drugs under comparable conditions [[19](#_ENREF_19)]. MDA EVs loaded with TMP also increased cell death, but no statistical differences were detected (Supplementary Fig. 8b). TMP is particularly hydrophilic and therefore its encapsulation efficiency and ability to be incorporated into cells is known to be limited [[29](#_ENREF_29)]. Nevertheless, EVs from MDA were the only vesicles which significantly improved the phototoxicity of porBA (Supplementary Fig. 8). Vesicles from other cell lines loaded with porBA showed a trend of increasing cell death but no statistical difference was observed – with respect to the limited drug concentration which could be incubated with the cells (0.1 μM). Neither EVs alone nor free drug substantially impaired the cell viability. Due to their cancer cell origin, EVs from MDAs were also tested for toxicity on endothelial cells. Unloaded vesicles from MDA cells did not show any toxicity when incubated with HUVECs (Supplementary Fig. 9).

Subsequently, we assessed whether increased phototoxicity of por loaded vesicles resulted from their improved cellular uptake of EVs. For this, MDAs were incubated at similar conditions as described above and imaged by fluorescence microscopy. We found that uptake of por (1 μM final concentration of por encapsulated in EVs) encapsulated in EVs was significantly higher (>60%) compared to the free drug (Fig. 3c and d). Interestingly, por loaded liposomes (PC/cholesterol 60:40 mol%) showed a significantly lower cellular uptake compared to EVs. Although uptake of neutral/weakly negative charged liposomes (zetapotential -5.0 ± 3.9 and -8.4 ± 2.2 mV for unloaded and loaded liposomes, respectively) is known to be generally less [[33](#_ENREF_33)], these results also indicate that solubilisation of hydrophobic drugs alone did not necessarily improve their cellular uptake. Liposome composition was based on the observation that both PC and cholesterol are most abundant in EVs from MDAs and HUVECs (Supplementary Table 2). Moreover, liposomes of similar composition are commonly used for drug formulation [[34](#_ENREF_34), [35](#_ENREF_35)]. The improved photodynamic effect thus could be correlated with increased cellular uptake of por loaded EVs. Moreover, the drug uptake could be increased to 92.8% and 98.1% when cells were incubated with EVs from MDAs and HUVECs with a higher drug concentration (4 μM, Fig. 3c and e-g). A higher drug concentration also reduced the cell viability more efficiently, even 18 h after laser treatment for MDApor (Fig. 3b). No comparable cellular uptake was observed when cells were incubated at very high concentrations of free drug (40 μM, Fig. 3c).

It was noticed that during fluorescence microscopy imaging, por positive cells underwent cell death due to the light exposure of the microscope, but not PBS-treated cells (Supplementary Videos 1-3). Therefore, we applied high-content imaging to analyse whether uptake of por loaded EVs or free drug had an impact on the overall cumulative risk to undergo cell death upon illumination. Cells were again incubated with por or EV-encapsulated por and imaged under the fluorescence microscope every 20 min for up to 4h. As seen in Fig. 4, uptake of drug-loaded EVs is correlated to a significant risk of cell death. There is no difference between loaded vesicles from MDAs or HUVECs for the lower drug concentration (1 μM, Fig. 4a). However, at higher drug ratios (4 μM), vesicles from HUVECs are more efficient in inducing cell death even though cell uptake is similar for both EV types (Fig. 3c). When analysing cells from the same sample which were por positive or negative, there is a clear indication that por uptake is associated to an increased cumulative hazard and cell death (Fig. 4b). Moreover, incubation of cells with increasing amounts of free drug did not increase the hazard ratio for cells to undergo death (Supplementary Fig. 10). These important findings further support that EVs are highly efficient drug carriers capable of delivering porphyrins into cancer cells to induce the drug’s photodynamic activity more efficiently than the free drug.

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**Figure 3**. **Phototoxicity and cellular uptake of porphyrin-loaded EVs.** MDAs were incubated with por loaded into EVs from MDAs or HUVECs or free drug for 4 h at (**a**) 1 μM and (**b**) 4 μM drug concentration. Cell viability (cell metabolism) was assessed using alamarBlue® assay 0 and 18 h after laser irradiation at 633 nm. *n =* 3-9, \**p*<0.05 (ANOVA followed by Dunnett’s *post-hoc* test). (**c**) Cellular drug uptake of por quantified after fluorescence microscopy of MDAs treated with (**d,e**) por loaded EVs (from MDAs) [(**d**) 1 and (**e**) 4 μM drug concentration], (**f**) free por and (**g**) por loaded into liposomes (both 4 μM). *n =* 3-9, \**p*<0.05 (ANOVA followed by Tukey *post-hoc* test). Exemplary cells from the dashed squares were enlarged.



**Figure 4. Cell survival and cumulative hazard after incubation with drug loaded EVs.** MDAs were incubated with PBS, free por, por encapsulated in EVs (from MDAs or HUVECs) and native (untreated) vesicles. Their survival and (**a**) cumulative hazard for cell death during 4 h of fluorescence microscopy was analysed (\*\*\**p*<0.00001, *n* = 40-60 cells). In (**b**) the cumulative hazard of death for cells (from the same sample) positive or negative for drug after incubation with EV-encapsulated por is shown (\*\*\**p*<0.00001, *n* = 40-60 cells). For ease of analysis samples with EVs from MDAS and HUVEC were pooled. Cells which received (**c**) MDApor 4 μM, (**d**), HUVECpor 4 μM or (**e**) PBS were imaged for a period of 4 h. Cells undergoing cell death are indicated by arrows.

**Saponin treatment enhanced porphyrin loading into EVs without compromising their drug delivery abilities**

Loading of hydrophobic porphyrin into EVs significantly improved cellular uptake and therapeutic effect of the drug (Fig. 3 and 4). Next, it was assessed whether other loading methods could improve uptake of more hydrophilic compounds into EVs and impact on their delivery potential. As a model compound porBA (intermediate hydrophobicity) was selected because it was earlier demonstrated that active loading methods substantially increased the encapsulation efficiency for this drug (Fig. 2). According to the data in Fig. 5a, the loading efficiency of porBA into MDAs EVs was dramatically increased by co-incubation with 0.01% (*w/v*) saponin or by hypotonic dialysis (both >11 fold) but not by extrusion.



**Figure 5**. Encapsulation efficiency and photodynamic activity of porBA into EVs from MDA cells. (**a**) Different loading methods were applied to load the drug into EVs and (**b**) the photodynamic effect of porBA loaded EVs towards MDA cells was analysed after laser irradiation *in vitro* (0.1 μM porBA)*.* *n =* 3-9, \**p*<0.05 (ANOVA followed by Tukey (in a) or Dunnett’s (in b) *post-hoc* test). The theoretical loading is displayed as a dashed line in (**a**). (**c**) Cellular uptake of porBA-loaded vesicles into MDA cells by incubation with different drug concentrations, and analysed by (**d**) fluorescence imaging and in comparison to free drug (0.1 μM porBA). *n =* 3-6, \**p*<0.05 *vs* free drug (ANOVA followed by Tukey *post-hoc* test).

Saponin treatment did not alter the size distribution or zeta potential of loaded EVs (Supplementary Fig. 7b and c). On the other hand, the extrusion and hypotonic dialysis induced a peak broadening and shift in size distribution, while the appearance of shoulders is indicative of alteration in the vesicle population (Supplementary Fig. 7b). Furthermore, changes in zeta potential were observed for EVs after extrusion compared to any of the other loading methods (Supplementary Fig. 7c). When analysing cellular uptake of EV encapsulated porBA, a significant increase in drug uptake (~8%) was observed for EVs loaded by the saponin-assisted method or electroporation compared to the free drug (>2%) (Fig. 5c). In contrast, when porBA was encapsulated into vesicles by hypotonic dialysis, the overall number of porphyrin-positive cells was much lower. It should be noted that for cell experiments with porBA the overall drug concentration was much lower than for por (0.1 compared to 1 μM) due a generally lower encapsulation efficiency of the intermediate porBA.

During *in vitro* experiments, EVs loaded with the saponin method displayed comparable phototoxicity to those loaded by electroporation and extrusion. According to Fig. 5b, a significant reduction of MDA cell viability was observed immediately after laser irradiation. Worthy of note is that EVs treated with extrusion induced cell death also when kept in the dark. Although the trend was not significant for all time points, these findings matched earlier results that extrusion appeared to induce changes in the vesicle’s constitution and their delivery activity. EVs loaded by hypotonic dialysis failed to have any substantial impact on porBA’s phototoxicity (Fig. 5b), which correlated with the poor cellular uptake results (Fig. 5c).

Finally, we analysed whether increased drug concentration or prolonged laser irradiation could improve cellular uptake and photodynamic activity of porBA loaded EVs. Vesicles with porBA were prepared using the hypotonic dialysis and saponin-assisted method and incubated for 4 h with MDA cells at 0.25 μM. Although an increase in drug concentration did not result in an increased cellular uptake (Fig.5c) or phototoxicity immediately following laser irradiation (Supplementary Fig. 11a), EVs loaded with the saponin method tended to induce a reduction in cell viability both 0 and 18 h after laser treatment. Moreover, longer laser irradiation (400 s instead of 200 s, Supplementary Fig. 11b) – even at lower drug concentration (0.1 μM) – significantly decreased cell survival after treatment with saponin and electroporation samples. In all cases, drug-loaded EVs prepared by the various methods were non-toxic when the cells were kept in the dark. These data suggest that loading of hydrophilic compounds into EVs could be adjusted through different active loading methods without compromising the EV’s biological/delivery functionalities.

**Conclusions**

Cell-derived vesicles are interesting new entities that possess great potential as smart drug carriers. Recent studies have investigated their chemical composition and cellular interaction, but efficient drug loading techniques have to date not been investigated systematically. In this manuscript, we provide evidence that model drugs with different degrees of hydrophobicity (*i.e.,* porphyrins) can be loaded into EVs from various cell types using active encapsulation techniques such as electroporation, saponin treatment or hypotonic dialysis without significantly impair the vesicles’ constitution and functionality. It should also be noted that porphyrins provide multimodality of detection by fluorescence (for diagnostic purposes) and photodynamic therapy. By choosing the right type of porphyrin (*i.e*., excitation maximum close to the near-infrared range), drug-loaded EVs could also be employed for non-invasive *in vivo* imaging [[20](#_ENREF_20), [37](#_ENREF_37)]. Por may be a potential candidate for these measurements because of its excitation around 630 nm.

The methods presented herein are both straightforward and easily applicable to other drugs and vesicles. To the best of our knowledge, this is the first head-to-head analysis using various (active) loading strategies to encapsulate small molecule drugs into EVs. This encapsulation has resulted in significant improved cell uptake and therapeutic effect of phototoxic porphyrins *in vitro*. Future work should consist of more complex *in vitro* characterisation of EVs (*e.g.*, in a co-culture cell model) and subsequent *in vivo* experiments including toxicity and efficacy studies of porphyrin-loaded vesicles. The present work creates a basis for further explorations of the drug delivery abilities of EVs and aims to stimulate their further development in a pharmaceutical setting*.*

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**Author contributions**

G.F. designed and conceived the study, conducted and analysed all experiments and wrote the paper; A.S. conducted and analysed the fluorescence microscopy experiments and co-wrote the paper; M.M. cultured hESCs, acquired conditioned medium and revised the paper; R.N. helped with the study design and TEM imaging and revised the paper; M.M.S. supervised the work, discussed the results, and revised the paper. The authors declare no competing financial interest.

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