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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.

1. 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]. It is known that EVs are produced by most – if not all – cells *in vitro* and *in vivo* [2,3]. In the living system, these vesicles serve for the transmission of biological signals and transfer of proteins and nucleic acids, and they induce several biological effects [4,5]. 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] while SMVs bud directly from the cell's surface [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) [7,8]. 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 [9,10] and they can potentially bypass complement activation and coagulation factors giving them better stability in the blood circulation [11,12]. Moreover, off-target effects are less likely when using EVs as they often specifically transit to their target cells [11,13]. 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 [14,15]. 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]. 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]. 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

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detail. Previous studies employ passive incubation for successful incorporation of anti-inflammatory drugs [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 [19,20]. 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.

2. Materials and methods

2.1. Chemicals

2,7,12,17-Tetra-tert-butyl-5,10,15,20-tetraaza-21H,23H-porphine (por, $M_w = 539$ Da), 5,10,15,20-tetrakis(1-methyl-4-pyridinio) porphyrin tetra(p-toluenesulfonate) (TMP, $M_{w,\ no\ counterion} = 679$ Da) and 4,4′,4″,4‴-(porphine-5,10,15,20-tetrayl) tetrakis(benzoic acid) (porBA, $M_w = 791$ Da) (Supplementary Fig. 1) and all chemicals (unless indicated otherwise) were purchased from Sigma-Aldrich (Gillingham, UK).

2.2. Cell culture and EV extraction

MDA-MB231 breast cancer (MDA) cells were maintained in DMEM supplemented with 10% (*v*/*v*) FBS and penicillin/streptomycin (P/S). Human umbilical vein endothelial cells (HUVECs) 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 (hMSCs) 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% CO_2, 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 and diluted with 30 mL of Advanced DMEM/F12 and 1 mL/well was dispensed (6 well plates). 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 (8×10^6 cells), HUVECs in EBM-2 (5×10^6 cells), hMSCs in αMEM (2×10^6 cells) and hESCs in mTeSR1 (5×10^6 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 ×*g* (to remove cells) and at 4500 ×*g* (to remove apoptotic bodies) for 20 min each and 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 ×*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.

2.3. 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 3× 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.

2.4. 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 [21,22].

2.5. 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 ×*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 were determined by NTA as described above.

2.6. 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;