

Loading of Enzymatic Cargos into Extracellular Vesicles Derived from Lung Cancer Cells

Magdalena Bamburowicz-Klimkowska¹, Monika Ruzycka-Ayoush¹, Alicja Targonska², Agnieszka Stawarska¹, Ireneusz P Grudzinski¹

¹Department of Toxicology and Food Science, Faculty of Pharmacy, Medical University of Warsaw, Warsaw, Poland; ²Laboratory of Molecular Bases of Ageing, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warsaw, Poland

Correspondence: Magdalena Bamburowicz-Klimkowska; Ireneusz P Grudzinski, Email mjbamburowicz@wum.edu.pl; ireneusz.grudzinski@wum.edu.pl

Purpose: Extracellular vesicles (EVs) secreted by cancer cells play a crucial role in tumor progression by facilitating communication within the tumor microenvironment. These EVs carry molecular payloads that promote cancer cell survival, invasion, and metastasis. Recently, EVs have garnered significant interest as potential drug and gene delivery systems in cancer therapy.

Methods: Glucose oxidase (GOX) was uploaded into EVs derived from adenocarcinomic human alveolar basal epithelial cells (A549) and further used as a potent starving agent in pristine A549 cells. In uploading studies, various loading methods such as incubation with and without saponin, freeze-thaw cycles, sonication, and different electroporation setups were tested.

Results: The electroporation technique exhibited the highest efficiency in loading GOX into EVs while maintaining EV integrity. GOX-loaded A549-derived EVs demonstrated significant cytotoxic effects on pristine A549 cells, suggesting that the EVs could effectively deliver enzymatic cargo to target cancer cells.

Conclusion: The results highlight the potential of A549-derived EVs as “Trojan-horse-like” carriers for enzymatic cargo, offering a novel approach for targeting and disrupting metabolic pathways in lung cancer cells. This study presents EVs as promising vehicles for targeted delivery of therapeutic agents in cancer treatment.

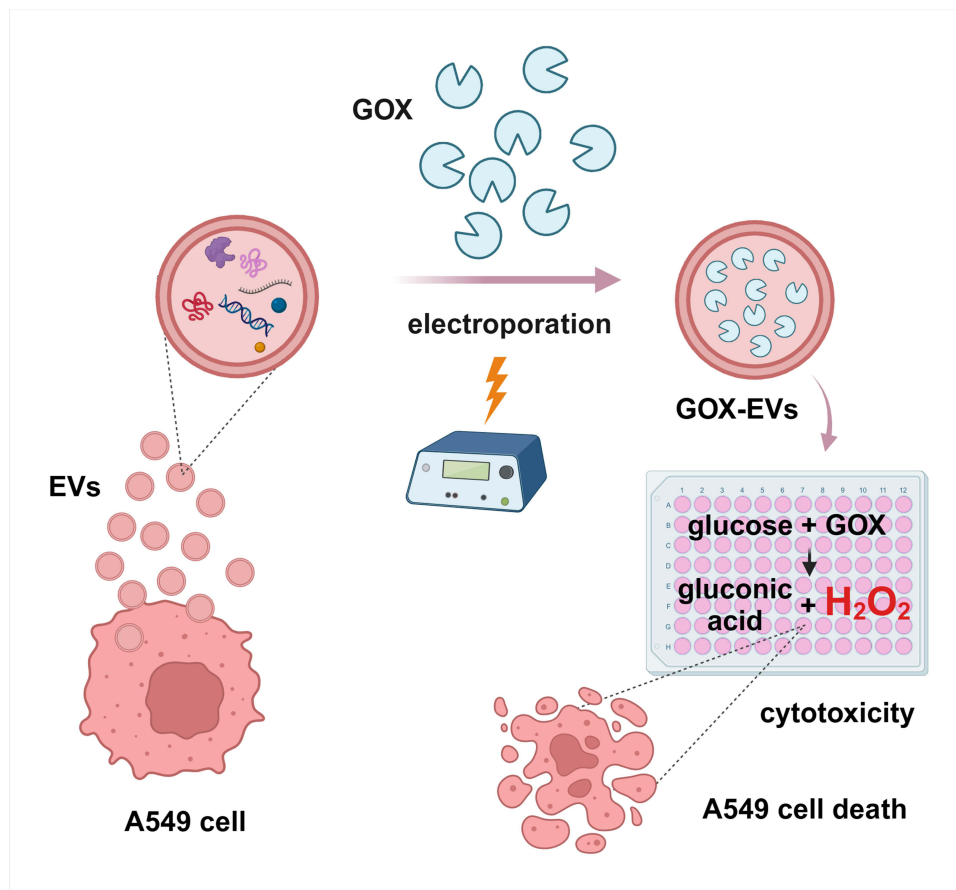
Keywords: extracellular vesicles, glucose oxidase, uploading studies, lung cancer cells, cytotoxicity

Introduction

Extracellular vesicles (EVs) are lipid bilayer-bounded vesicles released by any type of cells. They have the same bilayer wall structure as cells of origin and contain different cargos such as proteins (ex., cytoskeletal proteins, transmembrane proteins, and heat shock proteins), lipids, enzymes (GAPDH, ATPase, pgk1)¹ and various types of nucleic acids as mRNA, noncoding RNA and microRNAs (miRNAs) and, in some cases, DNA.² EVs subclassed as exosomes are nanosized (30–160 nm) membrane-bound vesicles that are secreted into the extracellular environment by many different types of cells including cancer cells.³ They are also found in various body fluids such as breast milk, plasma, saliva, urine, amniotic fluid, semen and others.⁴ EVs are taking part in intercellular communication and signaling, immune surveillance, and even elimination of unwanted products from damaged cells.⁵ They interact with target cells via endocytosis, fusion with the plasma membrane and other mechanisms releasing their payloads.⁶ Cancer cell-derived exosomes, sometime called as oncosomes, typically carry some discrete molecular markers and disease effectors such as mutated onco-proteins, oncogenic transcripts, microRNAs and DNA sequences.³ Once taken up by benign recipient cells, they contribute to horizontal cellular transformation and phenotypic reprogramming, pass through the tumor microenvironment and ultimately lead to malignant cell transformation.^{7,8}

The possibility of using EVs as next-generation drug or gene delivery systems in cancer treatment has recently begun to be widely explored.^{5,9,10} Various studies have shown that exosomes are potential carriers for therapeutic small molecules such as curcumin,¹¹ paclitaxel¹² and doxorubicin.¹³ Different research describe exosome-mediated delivery of large biomolecules such as peptides and proteins,¹⁴ and antigens.¹⁵ Exosomes also seem to be effective carriers of

Graphical Abstract



therapeutic nucleic acids (such as siRNA and miRNA) in gene therapy due to the ability to protect the cargo against degradation processes.^{16,17} Comparing exosomes to synthetic liposomes having a similar structure of the lipid bilayer, bio-derived exosomes are characterized by an increased ability to load biological molecules and greater efficiency in reaching target cells and delivering therapeutic agents when administered intravenously.¹⁸ When using EVs to deliver active molecules, many approaches to the exosomal cargo of various drugs and other bioactive compounds of interest have been explored. There are two approaches to cargo loading into EVs. One is called the pre-loading method (parental cells engineering) when usually drug molecules are encapsulated into EVs by natural sorting process, during EV maturation, before EVs isolation so when they are secreted by EV-producing cells, drugs are already inside the EVs. The second approach is named the post-loading method (direct loading) when drug molecules can be encapsulated into EVs after EV isolation.¹⁰

Among different techniques such as electroporation, sonication, extrusion, freeze-thaw cycling, incubation, permeabilization, transfection, and hypotonic dialysis used to load different cargos into exosomes,^{9,19} the gold standard for encapsulation of exogenous materials into EVs is the electroporation method, where the electric pulse temporally induces pores of lipid bilayer, and exogenous cargo can be transferred into the inner space of EVs.²⁰ Recently, numerous nanomaterials, drugs, and nucleic acids have been electroporated into exosomes. It needs to be highlighted that the efficiency of drug loading can be significantly increased by electroporation compared to incubation method.²¹ For tumor diagnosis and therapy to obtain gold nanoparticles coated with polymethacrylates and coupled with chlorin-6 loaded exosomes Pan et al used exosomes isolated from the urine of gastric cancer patients. So nanomaterial-loaded exosomes

can be then applied in cancer imaging and photodynamic therapy.²² More recently, Kowalczyk et al uploaded superparamagnetic iron oxide nanoparticles into surface-bioengineered extracellular vesicles and used these biomolecules as targeted MRI contrast agents in human lung cancer xenografts.²³ Chemotherapeutic drugs such as paclitaxel into macrophage-derived exosomes¹ and doxorubicin into immature dendritic cell-derived exosomes²⁴ were also loaded by electroporation. In recent years, electroporation was also utilized to directly load nucleic acids into exosomes. Alvarez-Erviti et al have obtained siRNA-loaded exosomes possessed the ability to knockdown β -secretase 1 in the mouse brain²⁵ and into murine dendritic cell-derived exosomes loaded with shRNA, allowed to decrease α -synuclein aggregation and alleviate dopaminergic neuron loss in Parkinson Disease models.²⁶ At present, chemotherapeutic loaded EVs are described as internalizing in tumor cells to induce cell death,²⁷ improving cytotoxic effect of paclitaxel on LNCaP and PC3 cells,²⁸ inhibiting and improving malignant U-87 cells growth in dose-dependent manner,²⁹ enhancing antiproliferative and anti-inflammatory activity in CFPAC-1 cells,²⁷ and highly efficient targeting to α v-integrin-positive breast cancer cells in vitro and high specific delivery to tumor tissue without overt toxicity in a MDA-MB-231 cancer mouse model.²⁴ Moreover, nucleic acid loaded EVs allowed reduction cell proliferation and invasion, increase apoptosis, and cell cycle arrest with inhibited growth in xenograft tumors in vivo,³⁰ deliver siRNA molecules and induction down-regulation of TPD52 gene expression in SKBR3 breast cancer cells,³¹ and decrease rates of cell migration and proliferation due to exosomes release miR26a from selectively bind HepG2 cells.³²

We demonstrated earlier that glucose oxidase (GOX) immobilized on carbon-encapsulated iron nanoparticles decorated with polyethyleneimine can be utilized as a potent tumor starving agent.³³ GOX is an enzyme that catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide.^{34–36} In cancer therapy, GOX has gained interest due to its unique biochemical properties that can be exploited to selectively breakdown the glycolysis in target cancer cells. Hydrogen peroxide (H_2O_2), the GOX generated byproduct of glucose metabolism, is a pro-oxidative agent that can induce oxidative stress and damage cellular components, leading to cell death.^{37–39} The oxidative stress generated by GOX can push cancer cells beyond their oxidative stress threshold, causing enhanced cytotoxicity.^{36,40} Seeing that GOX consumes glucose, which is a crucial energy source for cancer cells due to their high metabolic demands, depleting glucose can starve cancer cells and inhibit their growth.^{39,41} Direct application of GOX to tumors can locally increase oxidative stress and glucose depletion, leading to cancer cell death while minimizing damage to surrounding healthy tissues.^{37,39,42} GOX is often used in combination with other treatments to enhance their efficacy in cancer cells. For example, combining GOX with chemotherapy or radiotherapy can potentiate the oxidative damage to cancer cells.^{38,42,43} Some strategies involve the co-delivery of GOX and other reactive oxygen species-generating agents or inhibitors of antioxidant defenses in cancer cells to maximize pro-oxidative states.⁴⁴ While GOX can selectively kill cancer cells, it's important to balance GOX activity to avoid excessive damage to normal cells and tissues. Controlled release mechanisms and precise dosing are essential for minimizing such side effects.^{45–47} Ensuring that GOX is delivered specifically to cancer cells without affecting normal tissues is a significant challenge. Advances in nanotechnology and targeted delivery systems are critical to overcoming this hurdle. GOX can be encapsulated into nanoparticles or conjugated to other molecules to enhance its delivery and stability in the body.^{33,36} These delivery systems can improve the targeting of GOX to tumors and protect it from degradation in the bloodstream.⁴⁴

Nowadays researchers are exploring various formulations and delivery methods to improve the stability, targeting, and efficacy of glucose oxidase in cancer therapies.^{36,38,42,48} Advances in nanobiotechnology may enable the engineering of GOX variants with enhanced properties, such as increased stability or improved specificity for cancer cells.³⁸ As enzymes can be targeted to malignant cells in different ways,⁴⁹ in the present study, we examined the GOX loading efficiency of extracellular vesicles derived from adenocarcinomic human alveolar basal epithelial cells (A549). The loading of GOX was conducted using various methods such as incubation with and without saponin, freeze-thaw cycles, sonication, and different electroporation setups. The as-obtained GOX-loaded A549-derived EVs were examined for loading efficiencies, and they were tested for their cytotoxic effects on pristine A549 cells.

Materials And Methods

Isolation, Purification and Characterization of Extracellular Vesicles

Adenocarcinoma human alveolar basal epithelial cells (A549; CCL-185 ATCC) were purchased from ATCC (Manassas, Virginia, USA) and were cultured in the F-12K Medium (Kaighn's Modification of Ham's F-12 Medium; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK) and antibiotics: streptomycin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$; amphotericin B, 1.25 $\mu\text{g}\cdot\text{mL}^{-1}$; gentamicin, 50 $\mu\text{g}\cdot\text{mL}^{-1}$; penicillin, 50 $\text{U}\cdot\text{mL}^{-1}$ (Gibco, Paisley, UK). Prior to exosome isolation, the standard media was replaced with a 10% exosome-depleted FBS media (One ShotTM format, Gibco, Paisley, UK), and A549 cells were incubated for a further 3 days in T225 culturing flasks. Exosome released into exosome-depleted medium was obtained from A549 cells by ultracentrifugation as described earlier by Ruzycka-Ayoush et al and characterized with Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM) and Western Blot analysis.⁵⁰

Loading Glucose Oxidase into Extracellular Vesicles

Glucose oxidase (GOX) from *Aspergillus niger* (SERVA, Rosenheim, Bayern, Germany) was diluted in pH 7.2 phosphate-buffered saline (PBS) to a concentration of 0.5 $\text{mg}\cdot\text{mL}^{-1}$. Extracellular vesicles (EVs), as derived from A549 cells, with a total protein concentration of 0.15 $\text{mg}\cdot\text{mL}^{-1}$ were mixed with 170 μL of the GOX solution.

Method I – Incubation Without Saponin

The GOX and EVs mixture was incubated in the dark at room temperature for 18 hours.

Method II – Incubation with Saponin

Saponin (Sigma-Aldrich, Burlington, Massachusetts, USA) was added to the GOX and EVs mixture to a final concentration of 0.2% and shaken for 20 minutes at room temperature, followed by incubation in the dark for 18 hours at room temperature.

Method III – Freezing and Thawing Cycles

The GOX and EVs mixture was incubated for 0.5 hours at room temperature, then subjected to three freezing cycles at -80°C (0.5 hours) and thawing (up to 1 hour at room temperature).

Method IV – Sonication

The GOX and EVs mixture was sonicated three times on ice with the following settings: 30 cycles of 1-second pulse/1-second pause, amplitude 10, 10%, and frequency 20 kHz.

Method V – Electroporation

The GOX and EVs mixture was electroporated in a 4 mm cuvette under various conditions: (IA) 100V, 10 ms, 1 pulse; (VA) 50V, 10 ms, 1 pulse; (VB) 50V, 10 ms, 2 pulses with a 1-second interval using a BTX ECM 830 electroporation system.

Method Vs – Electroporation with Saponin

The GOX and EVs mixture, along with saponin as described in Method II, was subjected to electroporation as described in Method V.

In all methods, the unpackaged GOX was removed by ultracentrifugation at 100,000 g for 1.5 hours at 4°C . The supernatants were discarded, and the pellets were resuspended in 100 μL of PBS. Electroporation was performed in triplicate for each sample, and the average value was used for further analysis.

Optimizing the Electroporation Protocol

Four experimental setups were applied for optimizing the electroporation parameters using a square wave pulse generator (BTX ECM 830 _BTX The electroporation Experts, Waterbeach, Cambridge, UK). To date, different pulses and voltages, pulse number and pulse duration were evaluated (Tables 1–4).

Table 1 The 1st Setup: Optimizing the Pulse and Voltage

	Method 1A	Method 2A	Method 3A	Method 5A
Voltage [V]	100	250	500	750
Pulse duration [msec]	10	10	10	0.1
Number of pulses	1	1	1	1
Ω	22	19	18	18

Table 2 The 2nd Setup: Optimizing the Number of Pulses

	Method 2A	Method 2B	Method 2C	Method 2D
Voltage [V]	250	250	250	250
Pulse duration [msec]	10	10	10	10
Number of pulses	1	2	5	10
Pulse interval [sec]		1	1	1
Ω	22	24	19	23

Table 3 The 3rd Setup: Optimizing the Pulse Duration

	Method 1A	Method 1B	Method 1C	Method 1D
Voltage [V]	100	100	100	100
Pulse duration [ms]	10	5	1	0.10
Number of pulses	1	1	1	3
Pulse interval [s]	–	–	–	1

Table 4 The 5th Setup: The Final Optimization

	Method 1A	Method 4A	Method 4B
Voltage [V]	100	50	50
Pulse duration [ms]	10	10	10
Number of pulses	1	1	2
Pulse interval [s]	–	–	1
Ω	20	18	18

Determination of Glucose Oxidase Loaded into Extracellular Vesicles

To verify the enzymatic cargo (GOX) in extracellular vesicles, both the uploaded and pristine EVs were analyzed for protein (Pierce™ BCA Protein Assay kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA), and flavin adenine dinucleotide (FAD) levels using HPLC with spectrofluorimetric detection. Before protein and FAD quantification, extracellular vesicles were lysed in RIPA buffer (Pierce™ RIPA Buffer, Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the addition of Halt™ protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Protein determination was performed using the BCA method based on the formation of a Biuret complex in alkaline protein solutions with bicinchoninic acid (BCA). The GOX protein was quantified using a microplate protocol. Briefly, 25 µL of each sample was transferred to a 96-well microplate, and then 200 µL of standardized BCA protein assay working reagent was added to each well. The microplate was covered and shaken at 100 rpm while incubating at 37°C for 0.5 h. After incubation, the plates were allowed to cool to room temperature and the absorbance of the samples was measured using a microplate reader (Bio-Tec LX800UV, Agilent, Santa Clara, California, USA) at a wavelength of 562 nm.

Calibration curves were done based on freshly prepared bovine albumin standard solutions with a linear working range from 25 to 1000 µg mL⁻¹. Protein content was analyzed in duplicate, and the average value was used for further analysis.

After lysis, FAD was determined by HPLC with fluorescence detection according to the previously described method with modifications.⁵¹ The excitation and emission wavelengths of the spectrofluorometer were set to 450 and 520 nm, respectively. The injection volume was 10 µL. The analysis was performed on a reversed-phase column (250 x 4 mm) packed with a Nucleosil C18 column (250 x 3.2 mm I.D, 5 µm) (Macherey & Nagel, Düren, Germany). The mobile phase consisted of 20 mM NaH₂PO₄ (pH 2.5):CH₃OH (7:3, v/v). The flow rate was set to 1 mL min⁻¹. HPLC analysis was performed for each sample in duplicate, and the average value was used for further analysis. Five-point calibration curve was linear ranging between 5 and 200 nM FAD with correlation coefficient $r^2 = 0.998$. The detection and quantification limits were also determined at 3.1 nM and 9.5 nM, respectively. The LOD and LOQ values were calculated according to the following equations:

$$LOD = \frac{3\delta}{a}$$

$$LOQ = 3.3LOD$$

where: “δ” is the standard deviation of the current signal intensities at the lowest measurable FAD concentration, whereas “a” is the slope of the calibration curve.

Cytotoxicity of Glucose Oxidase-Loaded Extracellular Vesicles on A549 Cells

The A549 cells were maintained in DMEM medium without glucose (Gibco, Paisley, UK) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Pen/Strep) and maintained in an incubator with humidified atmosphere at 37 °C and 5% CO₂. To facilitate detachment, the cells were incubated with trypsin-EDTA (0.25% trypsin/EDTA solution; Gibco, Paisley, UK) with polyvinylpyrrolidone (PVP, 0.5% wt/vol) for 3–5 minutes. Medium was added and the suspension was centrifuged to remove the trypsin/PVP, before cells were seeded at 1.3×10^4 cells·cm⁻² in Corning CellBind® cell culture flasks. Cytotoxicity of the GOX-loaded extracellular vesicles (GOX-EVs) was investigated on A549 cells by the Alamar Blue assay to measure the metabolic activity of these cells after 24 h exposure to the GOX-EVs bioconstruct at the concentration of 2.81×10^{10} EVs × mL⁻¹, 1.4×10^{10} EVs × mL⁻¹ and 7×10^9 EVs × mL⁻¹, respectively, which is corresponded to FAD levels at 2.48 µM, 1.24 µM and 0.62 µM, respectively. To data, the A549 cells were seeded in 96-well plates at 1.5×10^4 cells/well. The A549 cells treated without GOX-EVs were served as negative control (NC). Three independent experiments were performed, with samples run in duplicate in each experiment. At the end of exposure, the cells were washed twice with PBS and incubated for 3 h with fresh culture medium supplemented with 10% Alamar Blue staining solution. The fluorescence signal of Alamar Blue was detected on a microplate reader (excitation 530 nm, emission 590 nm, FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany). The cell viability was calculated relative to NC cells, after subtracting the blank value (wells with only medium and Alamar Blue solution) from all wells. Possible interference with the assay was investigated by mixing GOX-EVs (2.81×10^{10} particles/mL equal 2.48 µM of FAD) with the 10% Alamar Blue staining solution in medium (without cells). No interference was detected.

NTA Analysis

The mean size and concentration of extracellular vesicles were analyzed using a Nano Sight NS300 (Malvern Panalytical Ltd., Worcestershire, UK) equipped with a 488 nm blue laser. For each measurement, five 30-s videos were captured and analyzed using the built-in Nano Sight Software NTA 3.2. Before measurement, each sample was appropriately diluted (1:4 in PBS). The diagram presenting all steps of EV production and characterization before and after GOX loading is included in Figure 1.

Statistical Analyses

All experiments were performed in triplicate and data were presented as mean $\pm$ standard error of the mean (SD) and analyzed by Statistica software (Version 13.3). The results were tested for parametric assumptions using Shapiro–Wilk and Brown–Forsythe tests. The groups were compared using analysis of variance (ANOVA), and if the assumptions of the analysis of normal distribution of the results within groups were found to be violated the nonparametric test was used to determine significant differences. The results of FAD concentration were analyzed with the post hoc Newman-Keuls test. The mean values were compared among treatments at a 5% ($p < 0.05$) level of significance.

Results and Discussion

Glucose oxidase, an enzyme known for its ability to catalyze the oxidation of glucose to hydrogen peroxide and gluconic acid, has significant potential in biomedical applications.^{41,45,52} The encapsulation of GOX into extracellular vesicles offers numerous advantages, including enhanced stability, targeted delivery, and protection from immune system recognition.^{36,37,42} Exosomes, which are nano-sized extracellular vesicle subtypes, offer a promising platform for the encapsulation and delivery of therapeutic agent, including enzymes, due to their natural ability to transport biomolecules across cellular barriers.^{1,3,18} Several methods have been employed to load GOX into exosomes, each with its own advantages and limitations.^{44,45,52,53} One of the most commonly used methods is the passive loading method, in which GOX is introduced into extracellular vesicles during the incubation process.^{38,54} However, the passive loading method may lead to relatively low encapsulation efficiency and variable enzyme activity. The incubation method, both with and without saponin, was the least invasive, favoring the preservation of EV integrity and functionality. However, loading efficiency was low without permeabilizing agents.^{55–57} The addition of saponin improved enzyme penetration into the EVs but carried a risk of partial membrane disruption, potentially compromising vesicle stability. The freeze-thaw cycle provided a simple approach to increase EV membrane permeability, allowing enzyme entry without chemical additives. Nevertheless, repeated freeze-thawing could cause aggregation or damage to EVs and potential loss of GOX activity due to enzyme denaturation.^{55–57} Sonication, which uses ultrasonic waves to temporarily disrupt membrane structure, enabled higher loading efficiency compared to incubation methods. At the same time, excessive sonication could damage both EVs and the enzyme, negatively affecting the biological properties of the carriers and GOX activity.^{55–57} In contrast, we applied some active loading techniques such as electroporation or membrane disruption, which increase loading efficiency. Electroporation involves applying a discrete electric pulse to the mixture of extracellular vesicles and the

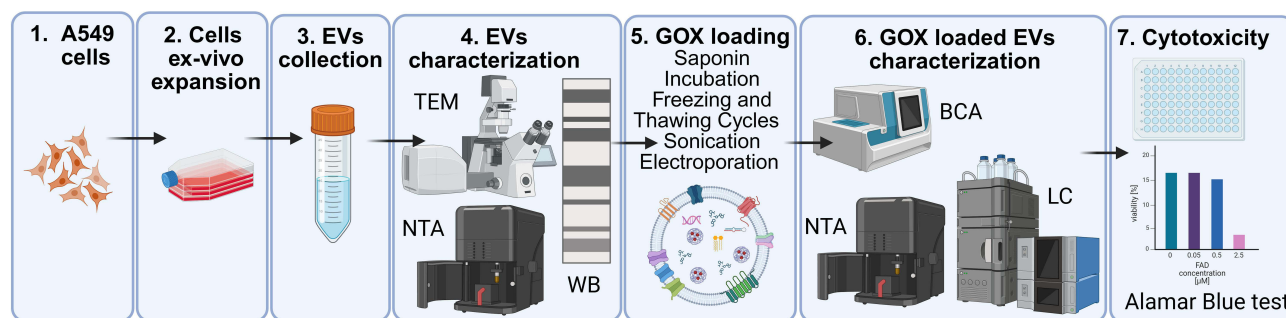


Figure 1 Schematic diagram illustrating EVs production, characterization and loading processes. Sections 1–4 show a schematic diagram illustrating the EV production and characterization according to the methods described by Ruzycka,⁵⁰ while sections 5–7 show the steps developed and described in the present study.

enzyme, temporarily disrupting the vesicular membrane structure, allowing the enzyme to enter the vesicles. This method allows for higher loading capacity and better control over the amount of enzyme encapsulated. Electroporation showed the greatest potential by creating transient pores in the EV membrane through short electric pulses, facilitating efficient enzyme loading. However, if not properly optimized, electroporation may lead to loss of vesicle integrity or reduced enzyme activity due to enzyme denaturation or EV aggregation, ultimately compromising their functionality.^{55–57} Another promising strategy was the use of chemical or lipid-based agents that facilitate the fusion of the enzymatic cargo within the EV membrane. These methods generally involve modifying the EVs surface to improve interaction with the enzyme or to enable the incorporation of the enzyme into the vesicles during their formation.^{56,58} However, this process required optimization, as excessive electric field exposure could.

In the present studies, extracellular vesicles derived from A549 cells were isolated and characterized as previously described.⁵⁰ The conditioned culture media were subjected to sequential centrifugation and ultracentrifugation. All the as-collected EVs were characterized by TEM, which revealed their typical morphology and the spherical lipid bilayer vesicle structure that defines EVs. NTA was employed to determine the size and concentration of EV samples. Additionally, protein concentration and zeta potential were measured and described elsewhere.⁵⁰ The as-collected EVs were used in uploading studies using GOX as an enzymatic cargo applying four different setups. The GOX-EV constructs were analyzed for protein and FAD levels, and they were characterized for size and concentration. Studies evidenced that the average size of GOX-EV constructs ranged from 104.7 ± 64.4 nm to 149.8 ± 70.5 after 18 h incubation and electroporation, respectively (Figure 2A). There were no significant differences in EV sizes ($n = 3$). The GOX-EVs yield the concentration of $3.5 \times 10^9 \pm 1.03 \times 10^9$ EVs mL^{-1} due to incubation processes. The lower concentration of GOX-EVs was recorded after electroporation in the presence of saponin, and it was found at $2.6 \times 10^8 \pm 6.9 \times 10^7$ EVs $\times \text{mL}^{-1}$ ($n = 3$) (Figure 2A). The protein concentration in the mixture after the loading process, as well as after ultracentrifugation in the supernatant and isolated EVs, is shown in Figure 2B. The protein analysis of GOX-loaded EVs showed that the method I, III and IV loaded EVs at the lowest protein concentration, while the method II, V and Vs loaded EVs at the highest one. It was found that the protein level for the saponin-assisted method was inadequately high (Figure 2B and C).

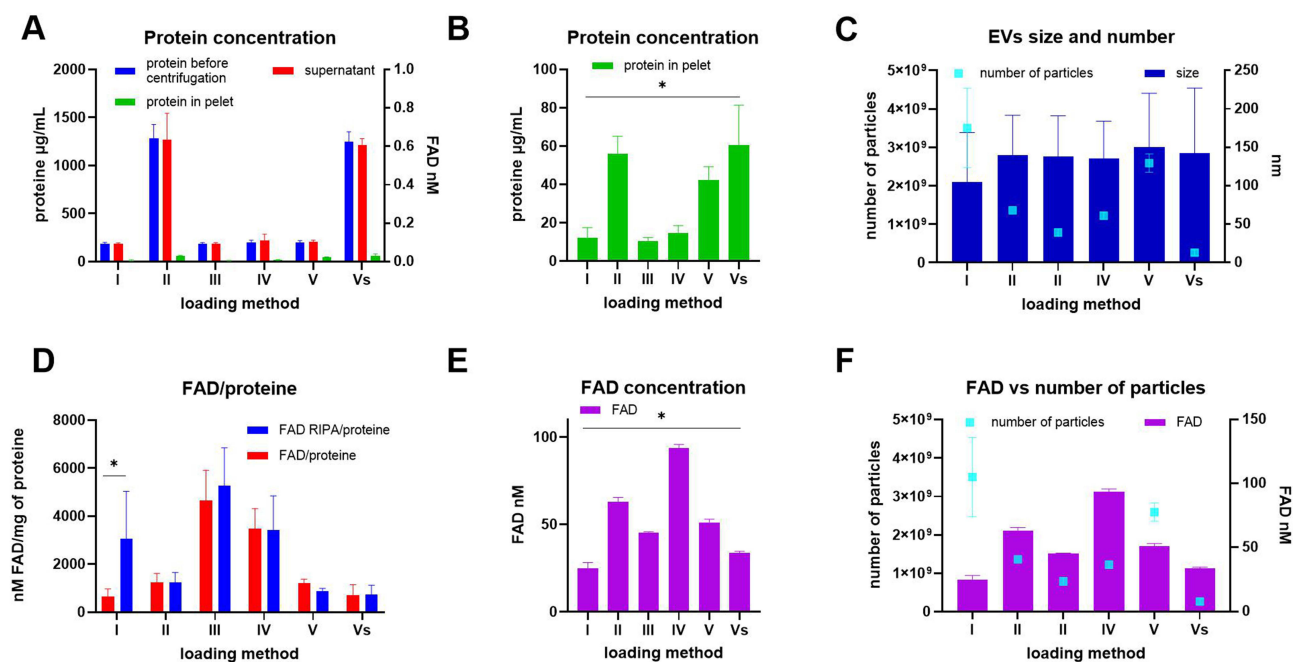


Figure 2 Characterization of protein levels, EV size, and FAD concentration in extracellular vesicles loaded with glucose oxidase using different methods. (A) Protein levels in the sample before ultracentrifugation and after the separation of EVs, in the supernatant and pellet post-ultracentrifugation. (B) Protein levels in the pellet after ultracentrifugation. (C) Extracellular vesicle (EV) size and number after GOX loading. (D) FAD per protein in GOX-loaded EVs with and without RIPA buffer. (E) FAD in EVs loaded with GOX using different methods, depending on protein content. (F) FAD in EVs loaded with GOX using different methods, depending on the number of EVs in the sample. Data presented as mean $\pm$ standard error of the mean (SD), $n=3$, (*) indicates a statistically significant difference $p < 0.05$.

Tests were performed with saponin alone, which confirmed significant interference of saponin in the protein content analysis by the BCA method (data not shown). To correct the interference of saponin, the FAD concentration in each GOX loading method was converted to protein quantity. Note that the flavin adenine dinucleotide is a noncovalently bonded coenzyme that is present in each subunit of GOX and acts as a redox carrier in catalysis.³⁴ Methods using saponin (II and Vs) are characterized by a low FAD concentration, indicating low loading efficiency of GOX with these methods (Figure 2D). To check whether it is necessary to break down the vesicle wall before flavine determining, FAD content in the samples was tested in a two different mixture with and without RIPA buffer. Although in five out of six cases no significant differences were observed in the content of FAD determined with and without the use of RIPA buffer (Figure 2D), the remaining analyses were performed with RIPA buffer with the addition of Halt™ protease inhibitor cocktail. The results of the FAD content in EVs loaded with GOX using different methods depending on the protein content and the number of particles in the sample is shown in Figure 2E and F.

After analyzing the results regarding protein content, FAD, particle size, and particle number, the electroporation procedure was optimized. The first step in optimizing the GOX loading process into EVs involved investigating the effect of the applied pulse voltage. Pulses with the following voltages were applied: 100, 250, 500, and 750 V. In the studies, no statistically significant differences were observed in either protein concentration or FAD in EVs across the tested voltages (Figure 3A–D). The particle sizes produced by different electroporation methods did not differ significantly, as shown in Figure 3E. To complement these findings, we have provided the data summarizing GOX loading efficiencies for the different electroporation setups (Figure 3F). Based on the analysis of the NTA results, where no significant differences in vesicle concentration were observed, but differences in the size distributions of the EV population in the samples were noted, a decision was made to investigate the effect of the number of applied pulses using a voltage of 250 V. This observation is consistent with previous studies, which have shown that the voltage applied during electroporation can influence the number of particles, the size distribution, and the integrity of vesicles. For instance, a study by Pomatto et al⁵⁹ observed that applying higher voltages generally increases the number of EVs but also leads to a more heterogeneous size distribution, which can reduce the quality of the EV preparation. Additionally, lower voltages might result in better-controlled sizes but with less efficiency in cargo loading.⁶⁰

In the second step, with a constant voltage of 250 V and a pulse duration of 10 ms, different numbers of pulses (ranging from 1 to 10) were applied. Under the outlined electroporation conditions, a statistically significant decrease in protein concentration and FAD was observed with the increasing number of applied pulses, along with an unfavorable size distribution of the EVs, as shown in Figure 3B. This observation aligns with the general understanding from electroporation studies that excessive pulses can lead to irreversible damage to EVs, causing alterations in their structural integrity and cargo release.²¹ Moreover, the adverse effects of pulse numbers on particle size distribution and cargo loading efficiency are well-documented in the literature, where an optimal range of pulses is recommended for preserving the functionality of EVs while ensuring sufficient cargo encapsulation.

Next, the impact of pulse duration on the efficiency of GOX loading into EVs was examined. With a constant voltage of 100 V in a single pulse, four different pulse durations were tested: 0.1, 1, 5, and 10 ms. Under the same conditions, pulse duration had no effect on protein concentration; however, a statistically insignificant, yet noticeable, decrease in FAD concentration was observed, proportional to the shortening of the pulse duration, as shown in Figure 3C. In the case of optimizing pulse duration, it should be noted that the shortest pulse, lasting 0.1 ms, was applied three times. Pulse duration is a crucial factor for determining the extent of membrane permeabilization, which in turn affects the efficiency of cargo loading into EVs. However, the study also noted that excessive pulse durations could lead to irreversible damage to both the EV membrane and the encapsulated cargo.^{5,21}

In the final step, the optimal method identified based on the analysis of the results from the previous steps was further optimized. In this step, both 100 V and 50 V voltages, as well as the number of pulses (one or two with a 1-second interval), were optimized (Figure 3D). The decision to further optimize the voltage (100 V and 50 V) and the number of pulses is consistent with the literature, which suggests that iterative optimization is key to achieving the best cargo loading efficiency while minimizing damage to the EVs.⁶¹

The best results for protein and FAD concentrations, as well as vesicle concentration and size distribution, were obtained using electroporation with method 1A, which involved a single 10 ms pulse at 100 V. This is consistent with

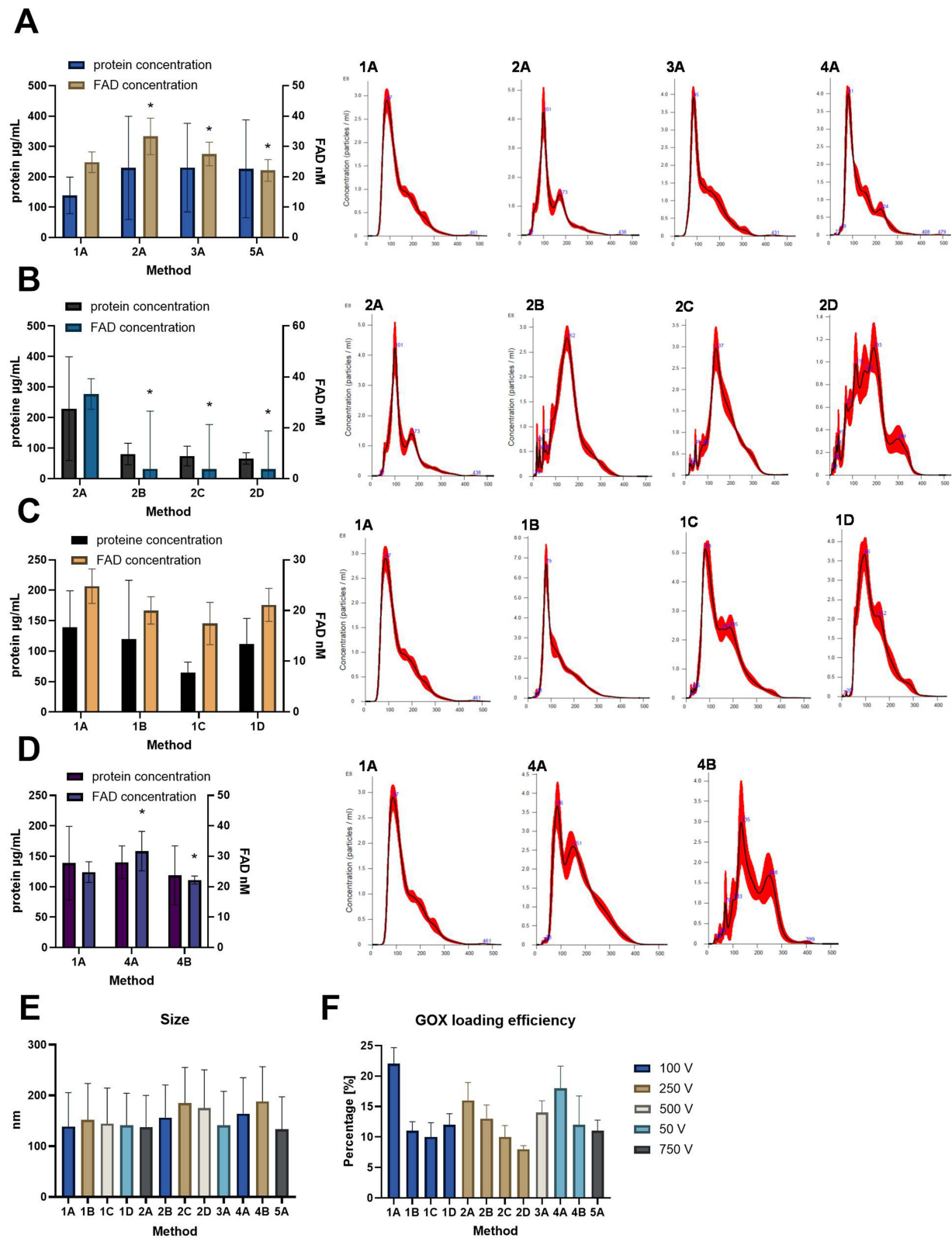


Figure 3 (A–D) Protein and FAD concentration after GOX electroporation into EVs according to the different methods and representative NTA results for EVs loaded with GOX due to electroporation methods. **(E)** Particle size distribution of EVs obtained after electroporation. **(F)** GOX loading efficiencies achieved by different electroporation setups. Data presented as mean $\pm$ standard error of the mean (SD), $n=3$, (*) indicates a statistically significant difference $p < 0.05$. Concentrations and size distributions of GOX-loaded EVs are shown as representative NTA plots. The description of the methods in terms of the parameters applied to electroporation can be found in the [Tables 1–4](#) in the Materials and Methods section.

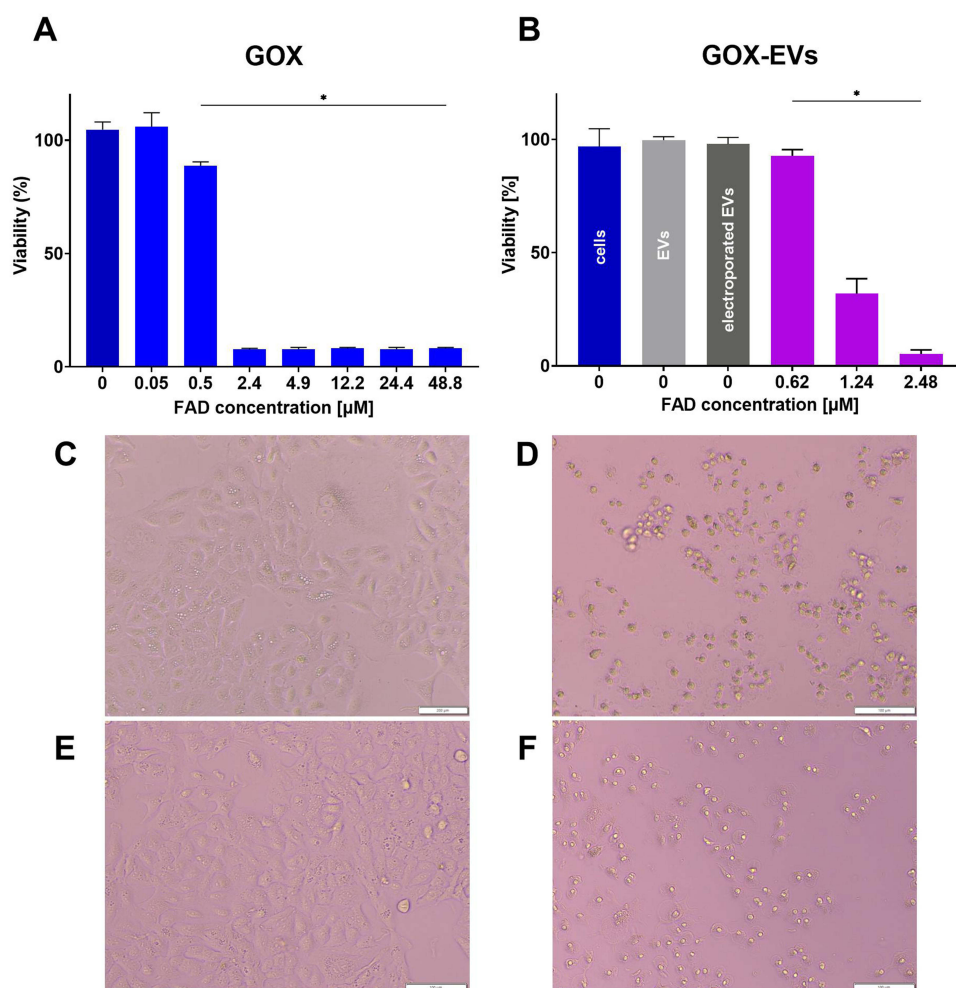


Figure 4 Cytotoxicity of GOX and GOX-loaded extracellular vesicles on pristine lung cancer cells (A549), determined by the Alamar Blue assay. EVs were derived from A549 cells and loaded with GOX using the IA method. The description of the methods in terms of the parameters applied to electroporation can be found in the [Tables 1–4](#) in the Materials and Methods section. **(A)** GOX cytotoxicity, **(B)** GOX-loaded EVs cytotoxicity, data presented as mean $\pm$ standard error of the mean (SD), $n=3$, (*) indicates a statistically significant difference $p < 0.05$. **(C)** Morphology of A549 control cells, **(D)** Morphology of A549 cells after exposure to GOX at the highest concentrations, expressed in terms of FAD, **(E)** Morphology of A549 cells exposed to GOX-loaded EVs at the lowest concentrations, expressed in terms of FAD, **(F)** Morphology of A549 cells exposed to GOX-loaded EVs at the highest concentrations, expressed in terms of FAD.

prior research that has found that lower voltages and moderate pulse durations can optimize cargo loading without compromising vesicle functionality.^{5,61} This method was then applied to load GOX into EVs to investigate their cytotoxicity.

The cytotoxic effects of pure GOX and GOX-loaded EVs were examined using A549 cells. As shown in [Figure 4A](#), the GOX enzyme diminished cell viability with FAD ranging from 2.4 M and higher. Pristine extracellular vesicles without GOX (no FAD) had no effect on cell viability compared to the loaded ones ([Figure 4B](#)). Additionally, pristine EVs without GOX (no FAD) after electroporation also had no impact on cell viability ([Figure 4B](#)). The increasing amount of GOX-loaded EVs, as estimated by FAD levels, caused a concentration-dependent and statistically significant decrease in the viability of lung cancer cells ([Figure 4B](#)). Studies also showed that the GOX-loaded EVs induced noticeable changes in cell shape and morphometry ([Figure 4C–F](#)). The obtained results indicate the cytotoxic-like functionality of GOX-loaded EVs produced through the electroporation method.

Conclusions

Methods for exogenous loading of extracellular vesicles are the use of membrane penetration or other strategies to directly load therapeutic cargos into EVs. We explored different methods such as incubation with and without saponin, freeze-thaw cycles,

sonication, and electroporation for loading glucose oxidase into EVs of the lung cancer cells (A549) origin. We found that the electroporation method stands out as a highly effective technique for achieving optimal GOX loading into lung cancer cell-derived EVs with minimal damages to the vesicles or enzyme denaturation. The experimental steps for electroporation include adjusting pulse voltage, pulse number, and pulse duration, which are consistent with findings from the literature, where these parameters significantly influence the quality and quantity of cargo loaded EVs.⁶² Note that the optimal protocol is required to ensure high loading efficiency while maintaining the integrity and functionality of the EVs.^{20,61} While direct studies on the loading of enzymatic cargos into EVs via electroporation are limited in modern literature, the present data suggest that electroporation is a promising method for loading GOX into extracellular vesicles. The resulting GOX-loaded A549-derived EVs produced serious cytotoxic effects on pristine A549 cells, indicating the great potential of this method in obtaining enzyme-loaded extracellular vesicles.

Acknowledgments

The authors are gratefully Malgorzata Sikorska for her skillful technical assistances. Graphical abstract was created with BioRender™ (academic license agreement).

Funding

The work was financially supported by the Programme “Applied Research” through TEPCAN project granted under the Norwegian Financial Mechanisms 2014 – 2021 / POLNOR 2019 (EEA and Norway Grants), Thematic areas: Welfare, health and care (NCBR Funding No. NOR/POLNOR/TEPCAN/0057/2019-00).

Disclosure

No conflict of interest was declared by the authors of this paper.

References

- Vader P, Breakefield XO, Wood MJA. Extracellular vesicles: emerging targets for cancer therapy. *Trends Mol Med*. 2014;20(7):385–393. doi:10.1016/j.molmed.2014.03.002
- Ronquist KG, Ronquist G, Carlsson L, Larsson A. Human prostasomes contain chromosomal DNA. *Prostate*. 2009;69(7):737–743. doi:10.1002/pros.20921
- Mohamed AH, Abaza T, Youssef YA, et al. Extracellular vesicles: from intracellular trafficking molecules to fully fortified delivery vehicles for cancer therapeutics. *Nanoscale Adv*. 2025;7(4):934–962. doi:10.1039/d4na00393d
- Colombo M, Moita C, van Niel G, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013;126(24):5553–5565. doi:10.1242/jcs.128868
- Dimik M, Abeysinghe P, Logan J, Mitchell M. The exosome: a review of current therapeutic roles and capabilities in human reproduction. *Drug Delivery Transl Res*. 2023;13(2):473–502. doi:10.1007/s13346-022-01225-3
- Kim J-KL, Ji-Young J, Yoon-Kyung J, Chul W. Extracellular vesicles as an emerging paradigm of cell-to-cell communication in stem cell biology. *J Stem Cell Res Ther*. 2014;4(5):1–5. doi:10.4172/2157-7633.1000206
- Rak J. Extracellular vesicles - biomarkers and effectors of the cellular interactome in cancer. *Front Pharmacol*. 2013;421. doi:10.3389/fphar.2013.00021
- Ciardiello C, Migliorino R, Leone A, Budillon A. Large extracellular vesicles: size matters in tumor progression. *Cytokine Growth Factor Rev*. 2020;51:69–74. doi:10.1016/j.cytogfr.2019.12.007
- Wang J, Chen D, Ho EA. Challenges in the development and establishment of exosome-based drug delivery systems. *J Control Release*. 2021;329:894–906. doi:10.1016/j.jconrel.2020.10.020
- Ferreira D, Moreira JN, Rodrigues LR. New advances in exosome-based targeted drug delivery systems. *Crit Rev Oncol Hematol*. 2022;172:103628. doi:10.1016/j.critrevonc.2022.103628
- Oskouie MN, Moghaddam NSA, Butler AE, Zamani P, Sahebkar A. Therapeutic use of curcumin-encapsulated and curcumin-primed exosomes. *J Cell Physiol*. 2019;234(6):8182–8191. doi:10.1002/jcp.27615
- Kim MS, Haney MJ, Zhao YL, et al. Engineering macrophage-derived exosomes for targeted paclitaxel delivery to pulmonary metastases: in vitro and in vivo evaluations. *Nanomed Nanotechnol Biol Med*. 2018;14(1):195–204. doi:10.1016/j.nano.2017.09.011
- Qian RJ, Jing BP, Jiang DW, et al. Multi-antitumor therapy and synchronous imaging monitoring based on exosome. *Eur J Nucl Med Mol Imaging*. 2022;49(8):2668–2681. doi:10.1007/s00259-022-05696-x
- Haney MJ, Klyachko NL, Zhao YL, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release*. 2015;207:18–30. doi:10.1016/j.jconrel.2015.03.033
- Leone DA, Rees AJ, Kain R. Dendritic cells and routing cargo into exosomes. *Immunol Cell Biol*. 2018;96(7):683–693. doi:10.1111/imcb.12170
- Wan Z, Zhao LB, Lu F, et al. Mononuclear phagocyte system blockade improves therapeutic exosome delivery to the myocardium. *Theranostics*. 2020;10(1):218–230. doi:10.7150/thno.38198

17. Kim G, Kim M, Lee Y, Byun JW, Hwang DW, Lee M. Systemic delivery of microRNA-21 antisense oligonucleotides to the brain using T7-peptide decorated exosomes. *J Control Release*. 2020;317:273–281. doi:10.1016/j.jconrel.2019.11.009
18. Song H, Liu B, Dong B, et al. Exosome-based delivery of natural products in cancer therapy. *Front Cell Develop Biol*. 2021;9650426. doi:10.3389/fcell.2021.650426
19. Xi XM, Chen M, Xia SJ, Lu R. Drug loading techniques for exosome-based drug delivery systems. *Pharmazie*. 2021;76(2–3):61–67. doi:10.1691/ph.2021.0128
20. Danilushkina AA, Emene CC, Barlev NA, Gomzikova MO. Strategies for engineering of extracellular vesicles. *Int J Mol Sci*. 2023;24(17):13247. doi:10.3390/ijms241713247
21. Rankin-Turner S, Vader P, O'Driscoll L, Giebel B, Heaney LM, Davies OG. A call for the standardised reporting of factors affecting the exogenous loading of extracellular vesicles with therapeutic cargos. *Adv Drug Delivery Rev*. 2021;173:479–491. doi:10.1016/j.addr.2021.04.012
22. Pan SJ, Pei LJ, Zhang AM, et al. Passion fruit-like exosome-PMA/Au-BSA@Ce6 nanovehicles for real-time fluorescence imaging and enhanced targeted photodynamic therapy with deep penetration and superior retention behavior in tumor. *Biomaterials*. 2020;230119606. doi:10.1016/j.biomaterials.2019.119606
23. Kowalczyk A, Dziubak D, Kasprzak A, et al. Surface-bioengineered extracellular vesicles seeking molecular biotargets in lung cancer cells. *ACS Appl Mater Interfaces*. 2024;16(25):31997–32016. doi:10.1021/acsami.4c04265
24. Tian YH, Li SP, Song J, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials*. 2014;35(7):2383–2390. doi:10.1016/j.biomaterials.2013.11.083
25. Alvarez-Erviti L, Seow YQ, Yin HF, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature Biotechnol*. 2011;29(4):341–U179. doi:10.1038/nbt.1807
26. Izco M, Blesa J, Schleef M, et al. Systemic exosomal delivery of shRNA minicircles prevents parkinsonian pathology. *Mol Ther*. 2019;27(12):2111–2122. doi:10.1016/j.ymthe.2019.08.010
27. Altanerova U, Jakubecova J, Benejova K, et al. Prodrug suicide gene therapy for cancer targeted intracellular by mesenchymal stem cell exosomes. *Int J Cancer*. 2019;144(4):897–908. doi:10.1002/ijc.31792
28. Saari H, Lazaro-Ibanez E, Viitala T, Vuorimaa-Laukkanen E, Siljander P, Yliperttula M. Microvesicle- and exosome-mediated drug delivery enhances the cytotoxicity of Paclitaxel in autologous prostate cancer cells. *J Control Release*. 2015;220:727–737. doi:10.1016/j.jconrel.2015.09.031
29. Yang TZ, Martin P, Fogarty B, et al. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in danio rerio. *Pharm Res*. 2015;32(6):2003–2014. doi:10.1007/s11095-014-1593-y
30. Cooper JM, Wiklander PBO, Nordin JZ, et al. Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov Disord*. 2014;29(12):1476–1485. doi:10.1002/mds.25978
31. Limoni SK, Moghadam MF, Moazzeni SM, Gomari H, Salimi F. Engineered exosomes for targeted transfer of sirna to her2 positive breast cancer cells. *Appl Biochem Biotechnol*. 2019;187(1):352–364. doi:10.1007/s12010-018-2813-4
32. Liang GF, Kan S, Zhu YL, Feng SY, Feng WP, Gao SG. Engineered exosome-mediated delivery of functionally active miR-26a and its enhanced suppression effect in HepG2 cells. *Int J Nanomed*. 2018;13:585–599. doi:10.2147/ijn.S154458
33. Bamburowicz-Klimkowska M, Kasprzak A, Bystrzejewski M, Poplawska M, Sobczak K, Grudzinski IP. Characteristics of glucose oxidase immobilized on carbon-encapsulated iron nanoparticles decorated with polyethyleneimine. *Polym Bull*. 2021. doi:10.1007/s00289-022-04125-1
34. Bankar SB, Bule MV, Singhal RS, Ananthanarayan L. Glucose oxidase - An overview. *Biotechnol Adv*. 2009;27(4):489–501. doi:10.1016/j.biotechadv.2009.04.003
35. Leskovic V, Trivic S, Wohlfahrt G, Kandrac J, Pericin D. Glucose oxidase from *Aspergillus Niger*: the mechanism of action with molecular oxygen, quinones, and one-electron acceptors. *Int J Biochem Biotechnol*. 2005;37(4):731–750. doi:10.1016/j.biocel.2004.10.014
36. Wang M, Wang DM, Chen Q, Li CX, Li ZQ, Lin J. Recent advances in glucose-oxidase-based nanocomposites for tumor therapy. *Small*. 2019;15(51):1903895. doi:10.1002/sml.201903895
37. Fu LH, Qi C, Hu YR, Lin J, Huang P. Glucose oxidase-instructed multimodal synergistic cancer therapy. *Adv Mater*. 2019;31(21):1808325. doi:10.1002/adma.201808325
38. Wang CH, Yang JX, Dong CY, Shi S. Glucose oxidase-related cancer therapies. *Adv Ther*. 2020;3(10):2000110. doi:10.1002/adtp.202000110
39. Yu JT, Wei ZX, Li Q, et al. Advanced cancer starvation therapy by simultaneous deprivation of lactate and glucose using a MOF nanoplatfrom. *Adv Sci*. 2021;8(19):2101467. doi:10.1002/advs.202101467
40. Sosa V, Moliné T, Somoza R, Paciucci R, Kondoh H, Lleonart ME. Oxidative stress and cancer: an overview. *Ageing Res Rev*. 2013;12(1):376–390. doi:10.1016/j.arr.2012.10.004
41. Agrawal S, Singh GK, Tiwari S. Focused starvation of tumor cells using glucose oxidase: a comprehensive review. *Int J Biol Macromol*. 2024;281136444. doi:10.1016/j.ijbiomac.2024.136444
42. Fu LH, Qi C, Lin J, Huang P. Catalytic chemistry of glucose oxidase in cancer diagnosis and treatment. *Chem Soc Rev*. 2018;47(17):6454–6472. doi:10.1039/c7cs00891k
43. Zhong ZH, Liu C, Xu YT, et al. γ -Fe₂O₃ loading mitoxantrone and glucose oxidase for pH-responsive chemo/chemodynamic/photothermal synergistic cancer therapy. *Adv Healthcare Mater*. 2022;11(11):2102632. doi:10.1002/adhm.202102632
44. Mu D, Wen D, Li Y, Zhong L, Zhao JY, Zhou SB. Renal clearable magnetic nanoreporter for colorimetric urinalysis of tumor. *ACS Biomater Sci Eng*. 2023;9(8):5039–5050. doi:10.1021/acsbiomaterials.3c00821
45. Hang LF, Zhang T, Wen H, et al. Rational design of non-toxic GOx-based biocatalytic nanoreactor for multimodal synergistic therapy and tumor metastasis suppression. *Theranostics*. 2021;11(20):10001–10011. doi:10.7150/thno.65399
46. Lee SY, Park J, Jeong D, et al. Ferrocene and glucose oxidase-installed multifunctional hydrogel reactors for local cancer therapy. *J Control Release*. 2022;349:617–633. doi:10.1016/j.jconrel.2022.07.017
47. Zhu HM, Cao GD, Fu YK, et al. ATP-responsive hollow nanocapsules for DOX/GOx delivery to enable tumor inhibition with suppressed P-glycoprotein. *Nano Res*. 2021;14(1):222–231. doi:10.1007/s12274-020-3071-7
48. He RX, Yang PD, Liu AX, et al. Cascade strategy for glucose oxidase-based synergistic cancer therapy using nanomaterials. *J Mat Chem B*. 2023;11(41):9798–9839. doi:10.1039/d3tb01325a
49. Bamburowicz-Klimkowska M, Poplawska M, Grudzinski IP. Nanocomposites as biomolecules delivery agents in nanomedicine. ReviewPaper. *J Nanobiotechnol*. 2019;17(1):48. doi:10.1186/s12951-019-0479-x

50. Ruzycka-Ayoush M, Nowicka AM, Kowalczyk A, et al. Exosomes derived from lung cancer cells: isolation, characterization, and stability studies. *Eur J Pharm Sci*. 2023;181106369. doi:10.1016/j.ejps.2022.106369
51. Petteys BJ, Frank EL. Rapid determination of vitamin B₂ (riboflavin) in plasma by HPLC. *Clin Chim Acta*. 2011;412(1–2):38–43. doi:10.1016/j.cca.2010.08.037
52. Jiang QS, Xiao YC, Hong AN, et al. Highly stable Fe/Co-TPY-MIL-88(NH₂) METAL-ORGANIC FRAMEwork (MOF) in enzymatic cascade reactions for chemiluminescence-based detection of extracellular vesicles. *ACS Sens*. 2023;8(4):1658–1666. doi:10.1021/acssensors.2c02791
53. Wu H, Xing H, Wu MC, Shen F, Chen Y, Yang T. Extracellular-vesicles delivered tumor-specific sequential nanocatalysts can be used for MRI-informed nanocatalytic therapy of hepatocellular carcinoma. *Theranostics*. 2021;11(1):64–78. doi:10.7150/thno.46124
54. Li S, Wang QH, Jia Z, et al. Recent advances in glucose oxidase-based nanocarriers for tumor targeting therapy. *Heliyon*. 2023;9(10):e20407. doi:10.1016/j.heliyon.2023.e20407
55. Fu SY, Wang Y, Xia XH, Zheng JLC. Exosome engineering: current progress in cargo loading and targeted delivery. *Nanoimpact*. 2020;20100261. doi:10.1016/j.impact.2020.100261
56. Zeng HF, Guo SS, Ren XC, Wu ZK, Liu SW, Yao XA. Current strategies for exosome cargo loading and targeting delivery. *Cells*. 2023;12(10):1416. doi:10.3390/cells12101416
57. Fu W, Li TT, Chen HB, Zhu S, Zhou CK. Research progress in exosome-based nanoscale drug carriers in tumor therapies. *Front Oncol*. 2022;12919279. doi:10.3389/fonc.2022.919279
58. Weng ZJ, Zhang BW, Wu CZ, et al. Therapeutic roles of mesenchymal stem cell-derived extracellular vesicles in cancer. *J Hematol Oncol*. 2021;14(1):136. doi:10.1186/s13045-021-01141-y
59. Pomatto MAC, Bussolati B, D'Antico S, et al. Improved loading of plasma-derived extracellular vesicles to encapsulate antitumor miRNAs. *Mol Ther-Methods Clin Devel*. 2019;13:133–144. doi:10.1016/j.omtm.2019.01.001
60. Zuppone S, Zarovni N, Noguchi K, et al. Novel loading protocol combines highly efficient encapsulation of exogenous therapeutic toxin with preservation of extracellular vesicles properties, uptake and cargo activity. *Discover Nano*. 2024;19(1):76. doi:10.1186/s11671-024-04022-8
61. Brezgin S, Danilik O, Yudaeva A, et al. Basic guide for approaching drug delivery with extracellular vesicles. *Int J Mol Sci*. 2024;25(19):10401. doi:10.3390/ijms251910401
62. Zhuo ZW, Wang JH, Luo YJ, et al. Targeted extracellular vesicle delivery systems employing superparamagnetic iron oxide nanoparticles. *Acta Biomater*. 2021;134:13–31. doi:10.1016/j.actbio.2021.07.027

International Journal of Nanomedicine

Publish your work in this journal

The International Journal of Nanomedicine is an international, peer-reviewed journal focusing on the application of nanotechnology in diagnostics, therapeutics, and drug delivery systems throughout the biomedical field. This journal is indexed on PubMed Central, MedLine, CAS, SciSearch®, Current Contents®/Clinical Medicine, Journal Citation Reports/Science Edition, EMBase, Scopus and the Elsevier Bibliographic databases. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/international-journal-of-nanomedicine-journal>

Dovepress
Taylor & Francis Group