Glucose oxidase virus-based nanoreactors for smart breast cancer therapy

Pedro Gama[1], Patrcica Juarez[2], Ana Rodríguez-Hernández[1], and Rafael Vazquez-Duhalt[1]

[1]Universidad Nacional Autónoma de México Centro de Nanociencias y Nanotecnología
[2]Centro de Investigacion Cientifica y de Educacion Superior de Ensenada

May 5, 2023

Abstract

Background Breast cancer is the most common malignant tumor disease and the leading cause of female mortality. The evolution of nanomaterials science opens the opportunity to improve traditional cancer therapies, enhancing therapy efficiency and reducing side effects. Methods and major results Herein, protein cages conceived as enzymatic nanoreactors were designed and produced by using virus-like nanoparticles (VLPs) from Brome Mosaic Virus (BMV) and containing the catalytic activity of glucose oxidase enzyme (GOx). The GOx enzyme was encapsulated into the BMV capsid (VLP-GOx), and the resulting enzymatic nanoreactors were coated with human serum albumin (VLP-GOx@HSA) for breast tumor cell targeting. The effect of the synthesized GOx nanoreactors on breast tumor cell lines was studied in vitro. Both nanoreactor preparations VLP-GOx and VLP-GOx@HSA showed to be highly cytotoxic for breast tumor cell cultures. Cytotoxicity for human embryonic kidney cells was also found. The monitoring of nanoreactors treatement on triple negative breast cancer cells showed an evident production of oxygen by the catalase antioxidant enzyme induced by the high production of hydrogen peroxide from GOx activity. Conclusions and implications The nanoreactors containing GOx activity are fully suitable for cytotoxicity generation in tumor cells. The HSA functionalization of the VLP-GOx nanoreactors could result in a prevailing strategy to improve selective cancer targeting. The GOx containing enzymatic nanoreactors seems to be an interesting alternative to improve the current cancer therapy. In vivo studies are on going to reinforce the effectiveness of this treatment strategy.

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Pedro Gama [1,2], Patricia Juárez [2], Ana G. Rodríguez-Hernández, and Rafael Vazquez-Duhalt[1]*.

[1] Centro de Nanociencias y Nanotecnología, Universidad Nacional Autónoma de México, Km 107 Carretera Tijuana-Ensenada, Ensenada, Baja CA 22860, Mexico.

[2] Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California.

*** Corresponding author**

Prof. Rafael Vazquez-Duhalt

Centro de Nanociencias y Nanotecnología UNAM

Km 107 carretera Tijuana-Ensenada

Ensenada. Baja California

22760 México

Email: *rvd@ens.cnyn.unam.mx*

Keywords: Breast cancer, Brome mosaic virus, Enzymatic nanoreactors, Glucose oxidase, Protein cage, Virus-like particle.

Abbreviations

BMV, Brome Mosaic Virus

CP, coat protein

DLS, dynamic light scattering

GOx, glucose oxidase

HSA human serum albumin

VLP, virus-like nanoparticle

Abstract

Background

Breast cancer is the most common malignant tumor disease and the leading cause of female mortality. The evolution of nanomaterials science opens the opportunity to improve traditional cancer therapies, enhancing therapy efficiency and reducing side effects.

Methods and major results

Herein, protein cages conceived as enzymatic nanoreactors were designed and produced by using virus-like nanoparticles (VLPs) from Brome Mosaic Virus (BMV) and containing the catalytic activity of glucose oxidase enzyme (GOx). The GOx enzyme was encapsulated into the BMV capsid (VLP-GOx), and the resulting enzymatic nanoreactors were coated with human serum albumin (VLP-GOx@HSA) for breast tumor cell targeting. The effect of the synthesized GOx nanoreactors on breast tumor cell lines was studied *in vitro* . Both nanoreactor preparations VLP-GOx and VLP-GOx@HSA showed to be highly cytotoxic for breast tumor cell cultures. Cytotoxicity for human embryonic kidney cells was also found. The monitoring of nanoreactor treatment on triple-negative breast cancer cells showed an evident production of oxygen by the catalase antioxidant enzyme induced by the high production of hydrogen peroxide from GOx activity.

Conclusions and implications

The nanoreactors containing GOx activity are fully suitable for cytotoxicity generation in tumor cells. The HSA functionalization of the VLP-GOx nanoreactors could result in a prevailing strategy to improve selective cancer targeting. The GOx containing enzymatic nanoreactors seems to be an interesting alternative to improve the current cancer therapy. *In vivo* studies are ongoing to reinforce the effectiveness of this treatment strategy.

1. Introduction

Breast cancer is the most common malignant tumor worldwide and the leading cause of female mortality.[1,2] More than half a million deaths caused by breast cancer were recorded in 2020 by the Global Cancer Center at WHO.[3] This histological pathology implies the appearance of adenocarcinoma in mammary glands.[4] Current therapy for breast cancer consists of invasive tumor surgical resection and systemic chemotherapy treatment.[5] However, non-specific chemo-pharmacologic treatments induce several drastic side effects.[6,7] Today, nanomedicine is focusing on designing novel and smart therapies to increase the efficiency of breast cancer treatment.[8-10]

Glucose oxidase (GOx) enzyme activity has been recently proposed for cancer nanomedicine applications. GOx, an intracellular enzyme from*Aspergillus niger* is widely used in glucose determination, fermentation industry, biosensors fabrication, and as a potential antibiotic.[11,12] This enzyme catalyzes glucose oxidation to produce hydrogen peroxide and gluconolactone. In a tumor environment, the GOx activity reduces, both

available glucose and molecular oxygen, essential compounds for cell metabolism. In addition, GOx produces oxidative stress generating hydrogen peroxide, killing tumor cells.[13] Different designs of GOx enzyme systems have been recently developed for cancer therapy, including several mono and multimodal vesicles, polymer dots, and magnetic nanoparticles.[14-17]

Nanoparticle specific-targeting is an emerging field to deliver cytotoxic activity to tumor cells selectively. The specific anticancer-drug delivery increases the treatment effectiveness and reduces the drastic side effects.[18] A diversity of nanosystems, such as polymer, protein, metallic, organic, and inorganic nanoparticles, has been proposed for biomedical applications.[19] Protein cages based on viral capsids, or virus-like particles (VLPs), are interesting nanosystems that have been proposed as nanocarriers for delivering anticancer therapies to specific tumor cells.[20,21] VLPs are widely used in vaccine technology and have recently recently been proposed as nanoplatforms as carriers of drugs for different biomedical therapies.[22-25] VLPs can be derived from native viruses or obtained by recombinant technology. After removing the genetic material, the purified monomeric coat protein is self-assembled under certain conditions forming hollow nanoparticles. Self-assembly property is used to confine or encapsulate several cargo molecules producing well-defined symmetry and homogenous size nanoparticles. The VLPs are highly stable in carrying and delivering cargo molecules, are biocompatible and biodegradable, and show low toxicity.[26,27] The surface of VLPs can be functionalized with a diversity of ligands to be specifically targeted to cells and tissues [28,29] making smarter and more efficient therapies. In addition, the suspension of VLPs is highly stable in biological fluids.

VLPs can contain active enzymes, and the arrangement is called an enzymatic nanoreactor.[22] Improved catalytic properties have been reported for virus-based enzymatic nanoreactors.[30-32] VLP-based enzymatic nanoreactors containing cytochrome P450 activity have been proposed for prodrug activation in breast cancer therapy.[33-35] The protein cage structure protects the catalytic molecule from protease degradation and decreases enzyme recognition by the immune system conferring a better catalytic performance.[36-38] Moreover, the intrinsic porous structure of the VLP-based nanoreactors can allow the substrates and products to flow through the system.[39-41]

This work encapsulated glucose oxidase in VLPs from Brome Mosaic Virus (BMV). The catalytic properties of the enzymatic nanoreactors were analyzed, and their effect on tumor cell lines was determined.

2. Materials and Methods

2.1 Materials

Type VII glucose oxidase from *Aspergillus niger* , human serum albumin (HSA, 96%), dextrose (99.5%), hydrogen peroxide (30 wt%), guaiacol (99%), L-glutathione reduced (98%), and all other general reagents, precursors and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Salts used for buffer solutions were purchased at Fermont (Monterrey, MX). TEM analysis was performed using copper grids (400-mesh) coated with formvar/carbon support film (Ted-Pella, USA). All chemicals were of the highest grade commercially available. Cancer cell lines were acquired at ATTC (USA).

2.2 BMV virus production

The VLP particles were produced and purified from the native virus as previously reported.[42] The BMV native virus was amplified through plant infection to produce VLPs. First, barley plants (*Hordeum vulgare*) were grown under controlled conditions. Then, young barley leaves were inoculated with the BMV virus. Once the leaves showed the infection pattern, they were harvested and stored at -20°C to be later liquefied in a blender (Osterizer) with virus extraction buffer (0.5 M sodium acetate, 80 mM magnesium acetate, pH 4.5). The resulting macerated material was filtered through cheesecloth, then the flowthrough was mixed with chloroform 1:1 v/v. The mixture was centrifuged at 10,000 rpm, 4°C, for 20 min. Since two phases formed in the centrifuge bottle; the upper phase (aqueous phase) was collected and stirred at 4°C overnight. Next, the aqueous extract containing the BMV virions was ultracentrifuged on a 5 mL sucrose cushion in a 32 mL centrifuge tube at 32,000 rpm, 4°C for 2 h. After centrifugation, the supernatant was discarded and a pellet containing the BMV virions was resuspended in a virus suspension buffer (50 mM sodium

acetate, 8 mM magnesium acetate, pH 4.5). Then, the BMV virion suspension was poured into a previously prepared centrifuge tube with a sucrose density gradient (10-40%) and centrifuged at 30,000 rpm, 4°C for 2 h. After centrifugation, the tube was examined under white light to confirm the presence of the blue phase containing the viruses. The blue phase was then recovered and diluted 1:4 v/v in a virus suspension buffer. The dilute blue phase was centrifuged at 32,000 rpm, 4°C for 3 h to concentrate the virions in a pellet. After centrifugation, the supernatant was discarded and the pellet containing the virions was resuspended in virus suspension buffer and stored at -70°C.

2.3 Capsid protein (CP) purification

The native BMV virions were disassembled to obtain the CP protein needed to synthesize the VLPs. First, the BMV virions were dialyzed against the disassembly buffer (0.5 M $CaCl_2$, 50 mM Tris-HCl, pH 7.4) for 5 h. Then, the resulting disassembled CP was centrifuged at 50,000 rpm, 4°C for 4 h. After centrifugation, the supernatant containing the CP was collected in 0.5 mL fractions. The CP purity of the fractions was estimated using the A_{280}/A_{260} nm quotient. The best fractions (A_{280}/A_{260}[?] 1.6) were combined and dialyzed against the protein buffer (1 M NaCl, 20 mM Tris-HCl, pH 7.2) for 2 h. Finally, the dialyzed CP was recovered and then quantified by absorbance using the CP extinction coefficient.

2.4 VLP-GOx nanoreactors synthesis

The VLP-GOx nanoreactors were synthesized by combining the disassembled BMV capsid and free-GOx enzyme and using the auto-assembly method. First, the disassembled CP protein was mixed with the free-GOx enzyme (in phosphate buffer pH 7.0) at different molar ratios (1:2, 1:3, 1:6, and 1:23 GOx:CP). Then, the GOx:CP mixtures were dialyzed in two steps. First, initial buffer (50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 50 mM Tris, pH 7.2); and second, auto-assembly buffer (50 mM sodium acetate, 750 mM NaCl, 8 mM magnesium acetate, pH 5.1), 4 h, 4degC. After dialysis, the samples were centrifuged at 40,000 rpm, 4degC for 2 h to obtain the nanoreactors in a pellet. After centrifugation, the supernatant was removed and the pellet containing the VLP-GOx nanoreactors was resuspended in the auto-assembly buffer. The resulting VLP-GOx nanoreactor suspensions were filtered using a 0.2 μm filter for further high-performance liquid chromatography (HPLC) purification. Next, the nanoreactors suspension was injected in a size exclusion column (TSKgel Column, Super SW2000, Supelco-Tosoh Bioscience, Bellefont, PA) for HPLC (Agilent 1100 series) purification of the VLP-GOx nanoreactors. The VLP-GOx nanoreactor fractions were collected and then ultrafiltered through a 30,000 Da membrane (Merck Amicon Ultra-15 Centrifugal Filter Units) to adjust the work concentration. Finally, the resulting VLP-GOx nanoreactors were estimated by Dynamic Light Scattering (DLS) with a Zetasizer Nanoseries device (Nano-ZS, Malvern Instruments). The total amount of protein in all preparations was estimated via the Bio-Rad Bradford Protein Assay (Hercules, CA).

2.5 VLP-GOx surface functionalization

The VLP-GOx nanoreactors were prepared by functionalizing their surface to improve their affinity to breast cancer cells. Hence, the outer surface of the VLP-GOx nanocages was covered with human serum albumin (HSA) protein. First, the HSA was activated with reduced glutathione as reported by Chauhan, et al, 2020. Second, the VLP-GOx nanoreactors (synthesized at 1:3 molar ratio) and reduced HSA were mixed at 1:8 VLP:HSA w/w ratio and stirred overnight at 4°C for the coating reaction. Next, the HSA functionalized nanoreactors were centrifuged at 40,000 rpm, 4°C for 2 h. After centrifugation, the supernatant was discarded and a pellet containing the HSA functionalized VLP-GOx nanoreactors (VLP-GOx@HSA) was resuspended in the assembly buffer. Finally, the VLP-GOx@HSA suspension was purified by HPLC to get rid of free-HAS and aberrant capsids.

2.6 GOx Nanoreactors Characterization

The nanoreactor catalytic activity was characterized by a peroxidase-coupled reaction followed by a colorimetric assay. The last product in this coupled reaction is tetraguaiacol, which absorbs at 470 nm. The increased absorbance is directly related to the glucose transformation rate. A Michaelis-Menten regression was performed by preparing reactions at different glucose concentrations (0, 25, 50, 100, and 150 mM) to

describe the catalytic parameters of the VLP-GOx nanoreactors.

The encapsulation of the GOx enzyme in VLP nanoparticles and the HSA coating was confirmed by SDP-PAGE. Finally, the size and morphology of the nanoreactor were studied with transmission electron microscopy (TEM, JEOL-2010, JEOL) operated at 100 kV.

2.7 Cytotoxic effects of nanoreactors

Breast tumor cell cytotoxic effects of GOx nanoreactors were studied by means of an *in vitro* assay. Two cell lines were exposed to different nanoreactor amounts. First, human embryonic kidney (293T) as a human healthy cell model; second 4T1 (murine breast cancer tumor). The nanoreactor-exposed cells were analyzed by an MTT colorimetric assay (TOX-1, Sigma-Aldrich, St. Louis, MO) to estimate the cytotoxic effect. Firstly, the cells were cultured in 96 well-plates; for 293T, 10,000 cells/well; for 4T1, 4,000 cells/well at 37°C and 5% CO_2. After 24 h, the cells were treated with different nanoreactor amounts. Finally, 48 h later starting the cultures, the MTT reagent (methyl-134-thiazolyl-tetrazolium) was added to the cells, and the absorbance (570 nm) showed in the well-plate was measured with a Multiskan GO spectrophotometer (Thermo Scientific). The resulting absorbance from negative control (cell media) was established as 100% of cell survival. Then, experimental groups were compared to estimate a survival rate.

2.8 Fluorescence microscopy

Cell culture of $5x10^5$ of MDA-MB231/eGPF (Cell Biolabs Inc, Ref. AKR-201) was seeded onto cell culture-treated Petri dishes (3 cm of diameter) and incubated in DMEM cultute medium supplement with 10% foetal bovine serum (Biowest, France) and 1% of antibiotic/antimycotic (GIBGO) overnight at 37°C under 5% CO_2 atmosphere. Afterward, cells were washed with phosphate buffer saline (PBS) 1X. Then, 100 µg of VLP-GOx resuspended in DMEM were added to the cell culture and incubated. Time-lapse images of cells were obtained by LS720 Microscope Lumascope (Etaluma Inc., San Diego, CA) every 2 minutes for 4 hours. Phase contrast and green fluorescent (green filter 473-491/502-561 excitation/emission) images were obtained.

2.9 Statistical analysis

Differences between data were tested using a two-way repeated measures (RM) ANOVA with Sidak or Tukey post hoc tests as appropriate. Statistically significant differences were accepted as P-values of < 0.001 and Excel statistics (Microsoft, Redmond, WA) was used for all analysis.

3. Results

3.1 Enzymatic nanoreactor synthesis and functionalization

The complementary electrostatic charge between the negatively charged GOx enzyme surface and the positively charged inner face of the BMV capsid drive the enzyme encapsulation (Fig. 1a). The isoelectric point of GOx was determined by electrophoretic light scattering using the Z potential at different pH and showed to be 4.6 (SI 1). The self-assembly process was modulated by ionic strength (0.75 M) at pH 5.1. The GOx enzyme was successfully encapsulated inside VLPs from BMV by the self-assembly synthesis method (Fig. 1b).

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Figure 1. a) Surface electric charge distribution on the GOx enzyme and BMV capsomer. The GOx surface mostly exhibits negative electric charges (red color), and the inner surface of BMV capsomers shows a more positive (blue color) electric charge distribution. The difference in electric charge distribution between GOx and the inner BMV surface results in electric charge complementarity. *In silico*results by Maestro Schrodinger®. b) Schematic representation of VLP-GOx nanoreactor self-assembly encapsulation and HSA functionalization.

To optimize the enzyme encapsulation, four different enzyme-coat protein molar ratios (GOx:CP) were assayed (Table 1). Nanoreactors containing enzymatic activity (VLP-GOx) were obtained in all mixtures tested. All nanoreactor preparations were purified by size exclusion chromatography (SEC) with an HPLC (Fig. 2). The purified preparation showing the highest enzymatic activity (1:3 molar ratio) was then functionalized with human serum albumin (HSA) (Fig. 1b). HSA shows binding affinity for specific receptors on the surface of different cells in diseased organs that permits the active targeting and the specific recognition of the albumin-based formulations. *In vitro* and *in vivo*experiments HSA conjugated preparations showed higher cellular uptake efficiency, longer half-life, higher cytotoxicity, and accumulation in tumors to a much greater extent than the free drug preparations.[43, 44] The functionalization was carried out through previously reduced cysteine groups of HSA protein, producing di-thiol cross-linking with the cysteine (Cys108) of CP-BMV (SI 2).

Table 1. Hydrodynamic diameter determined by Dynamic Light Scattering (DLS) of proteins and catalytic contants of enzymatic nanoreactors assembled at different GOx:CP molar ratios.

Preparation	**Preparation**	**Hydrodymanic diameter (nm)**	**V_{max} (U/g protein)**	**K_M (mM)**
Free GOx	Free GOx	12.2 ±4.1	237,985	27.8
Coat protein (CP)	Coat protein (CP)	6.8 ±1.2	–	–
Native BMV	Native BMV	27.6 ±6.7	–	–
GOx:CP molar ratio	**VLP-GOx nanoreactors**	**VLP-GOx nanoreactors**	**VLP-GOx nanoreactors**	**VLP-GOx nanoreactors**
1:2	1:2	25.8 ±8.5	507	47.7
1:3	1:3	28.8 ±7.9	1,208	34.6
1:6	1:6	33.4 ±14.2	872	40.0
1:23	1:23	26.1 ±8.9	137	49.0
1:3 VLP-GOx@HSA	1:3 VLP-GOx@HSA	29.7 ±8.9	106	70.4

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Figure 2. Purification of VLP-GOx nanoreactors by SEC HPLC chromatography using differences in retention time **A)**Overlapped chromatograms show the retention time for native BMV=5.7 min, free GOx=7.5 min and disassembled CP=8.4 min. **B)** VLP-GOx nanoreactors retention time=5.7 min, similar to native virions of BMV. The VLP-GOx nanoreactors were purified by collecting the fractions from 5.5 to 6.5 min.

3.2 Nanoreactors characterization

The nanoreactor's hydrodynamic size was estimated by Dynamic Light Scattering (DLS). No significant changes in hydrodynamic diameters were found. All the assayed ratios produced VLP-GOx particles with a similar hydrodynamic diameter to the native virus ([?]28 nm). The unfunctionalized VLP-GOx showed a hydrodynamic diameter of 28.8 ±7.9 nm, while the functionalized VLP-GOx@HSA showed a value of 29.7 ±13.3 nm (Table 1).

The structure of the VLP-GOx nanoreactors was also characterized by transmission electron microscopy (TEM) (Fig. 3). The obtained micrographs of the VLP-GOx nanoreactor show a quasi-spherical shape with similar symmetry to the native BMV virus (28 nm). In addition, functionalized VLP-GOx@HSA nanoreactors show an ornamented outer surface displaying HSA protein molecules on the nanoreactor surface.

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Figure 3. Transmission electron microscopy (TEM) of nanoreactors. A) Quasi-spherical unmodified VLP-GOx nanoreactors, and B) VLP-GOx@HSA functionalized nanoreactors.

3.3 Catalytic characterization

The protein proportions in the nanoreactors were estimated by densitometry on an SDS-PAGE gel. According to the band's intensity, the GOx enzyme in the unfunctionalized VLP-GOx corresponds to 3.58% of the total protein, while in the HSA functionalized preparations (VLP-GOx@HSA) the GOx represented only 0.82%, coat protein (CP) 22.8% and the cover shell of HSA 76.38% of the total protein in the nanoreactor.

The catalytic constants for GOx activity of nanoreactors were estimated using a Michaelis-Menten nonlinear regression. The assembly from 1:3 GOx:CP molar ratio showed the highest specific activity (1,208 U/g) and a Michaelis constant (K_M) of 34.6 mM (Table 1). Thus, this molar ratio was used for further studies and for HSA functionalization. Considering the GOx content in the nanoreactor preparations, the V_{max} of VLP-GOx, in enzyme activity basis, is estimated to be 33,743 U/g of GOx, representing 14% of the transformation rate of free enzyme. On the other hand, the V_{max} of functionalized VLP-GOx@HSA en enzyme basis is 12,990 U/g of GOx, 5.5% of the value of the free enzyme. Using the amount of enzyme contained in the nanoreactors, the transformation rate constant (k_{cat}) could be obtained. The free enzyme showed a k_{cat} value of 634 s^{-1}, while the preparations VLP-GOx and VLP-GOx@HSA showed values of 90 s^{-1} and 34 s^{-1}, respectively. In addition, it is possible to estimate the number of GOx molecules (active dimer of 160,000 Da) per capsid or nanoreactor (180 monomers of 20,385 Da). The number of GOx molecules inside each VLP was estimated as an average of 0.85 molecules of GOx per VPL nanoreactor.

3.4 Antitumoral effect

The antitumoral effect of nanoreactors was assayed in 4T1 breast tumor cells, a breast cancer cell line derived from the mammary gland tissue of a mouse. In addition, the cytotoxic effect of nanoreactors was also assayed on cultures of 293T healthy cell line from the human embryonic kidney. Cytotoxicity was directly estimated as the cell viability as a metabolic activity using the MTT colorimetric assay.

Control experiments were carried out with VLP deprovided of GOx (Fig. 4). Interestingly, high concentrations of empty VLPs and VLPs@HSA (>25 μg/mL) significantly reduced the cell viability of 4T1 cancer cells. In contrast, VLPs treatment of 293T epithelial cells did not reduce cell viability at the highest concentration tested of 100 μg/mL, except for unfunctionalized VLPs.

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Figure 4 . Cytotoxicity activity of empty unmodified and modified VLPs on 4T1 breast tumor mouse cell line model, and 293T healthy embryonic kidney human cells. Two-way RM ANOVA ($p<0.001$). *** Significative difference between the two preparations at the same concentration. ++ Significative difference with the control.

The same cytotoxicity experiments were performed with GOx-containing nanoreactors (Figs. 5 and 6). The results were expressed based on both GOx content and nanoreactor GOx activity. These two forms to express the treatment doses are due because the protein content of the two preparations is significantly different, and if the GOx content is similar in capsid base the enzymatic activity changes when the nanoreactor is covered with HAS (Table 1). On the 4T1 breast tumor cells, the LD_{50} values were 0.04 μg GOx/mL for VLP-GOx and around 0.1 μg GOx/mL for VLP-GOx@HSA, and no metabolic activity was detected at 1 μg GOx/mL

for both preparations (Fig. 5a). The 4T1 tumor cells showed an LD_{50} at around 1 mU of GOx activity per mL of cell culture, and no metabolic activity at around 10 mU/mL in both preparations (Fig. 5b).

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Figure 5 . Cytotoxicity of unfunctionalized VLP-GOx and functionalized VLP-GOx@HSA nanoreactors on 4T1 breast tumor mouse cell line.

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Figure 6 . Cytotoxicity of unfunctionalized VLP-GOx and functionalized VLP-GOx@HSA nanoreactors on 293T kidney cell line.

The cytotoxicity was also assayed in 293T healthy embryonic kidney human cells (Fig. 6). The results were expressed as both GOx protein content and nanoreactor GOx activity. The LD_{50} values were 0.45 μg GOx/mL for VLP-GOx and around 0.79 μg GOx/mL for VLP-GOx@HSA, and no metabolic activity was detected at 1 μg GOx/mL for VLP-GOx preparation (Fig. 6a). The VLP-GOx nanoreactors on 293T kidney cells showed an LD_{50} at around 15 mU of GOx activity per mL of cell culture, and no metabolic activity at around 30 mU/mL (Fig. 6b). On the other hand, the VLP-GOx@HSA nanoreactors showed an LD_{50}around 8 mU/mL (Fig. 6b).

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Figure 7. Fluorescence microscopy images of the triple-negative breast cancer cell line MDA-MB-231 expressing GFP. The images were taken at different times after treatment with VLP-GOx@HSA. The bubble-like formations are shown with yellow arrows. See the videos in the Supplementary information.

The effect of the treatment with VLP-GOx@HSA on a triple-negative breast cancer cell line (MDA-MB-231) expressing the green fluorescent protein (GFP) was monitored by fluorescence microscopy (Fig. 7 and videos SI). After 1-hour treatment in the presence of nanoreactors, the morphology of cells changed drastically, maybe due to oxidative stress. After 2 hours, bubbles are produced inside the cells suggesting a high production of hydrogen peroxide, and the fluorescence starts to diminish. The bubble production is increasing, and the fluorescence continues to diminish after 3 hours. Finally, after 4 hours the cells are very damaged, and the fluorescence has completely disappeared.

4. Discussion

Thanks to the complementarity of charges between the negative charge of the glucose oxidase protein and the positively charged internal side of the coat protein of BMV, it was possible to encapsulate the GOx inside the BMV capsid. An average of 0.85 GOx molecules per capsid was obtained. Considering the hydrodynamic diameter of the dimeric enzyme ([?]12.2 nm) and the internal diameter of the BMV capsid of 17.8 nm,[45] it seems not possible the encapsulation of more than one GOx dimeric molecule per capsid. GOx was previously encapsidated in cowpea chlorotic mottle virus (CCMV) for cascade reaction study with the so-called DNAzyme, a peroxidase mimic formed in situ by a specific sequence of ssDNA in the

presence of hemin, and gluconokinase.[46] However, no catalytic information of nanoreactors containing only GOx was reported.

As expected, the nanoreactors showed a higher K_M than the free enzyme, especially in the HSA-functionalized nanoreactors. This could be due to the substrate mass transfer limitations when the enzyme is inside a protein

cage. This mass transfer limitation seems to be also the origin of the decrease of transformation rate (k_{cat}) of nanoreactors VLP-GOx and VLP-GOx@HSA to 14% and 5.5% of the value of free enzyme, respectively. Nevertheless, both preparations showed high GOx activity.

Unexpectedly, we found a slight cytotoxic effect of unloaded VLPs, especially on 4T1 cell line (Fig. 4). These results should be taken cautiously due to the high protein concentration. It is known that *in vivo* virus and virus-like nanoparticles are able to enhance specific anti-cancer immune reactions when delivered directly to the tumor.[47-49] VLPs from Cowpea mosaic virus (CPMV) have shown potential as safe anti-cancer immunotherapy (Beatty and Lewis, 2019).[50] Systemically administered CPMV can stimulate an additive therapeutic immune response following treatment of the tumor with a conventional agent such as radiation.[51] However, scarce information is available on *in vitro* cytotoxicity of empty and unfunctionalized VPLs. Unfunctionalized P22 VLPs showed a cell survival rate of 90% of at concentrations as high as 200 μg/mL, indicating that VLP had almost no toxicity to bone marrow-derived dendritic cells.[52] On the other hand, unloaded Human hepatitis B virus-derived VLPs also showed no cytotoxicity on 293 kidney cell line.[53]

The LD_{50} in both cell lines is similar for VLP-GOx and VLP-GOx@HSA when evaluated in GOx content or GOx activity (Fig. 4 and 5). Significant damage in cancer cells was found in the triple-negative breast cancer cell line MDA-MB-231/eGFP (Fig. 6 and videos SI). MDA-MB-231/eGFP stable cells express a green fluorescent protein (GFP) as cytoplasmatic proteins. This mammalian cell is transfected with a cDNA chimera composed of a fusion of the genes encoding the fluorescent protein and β-actin. After 1-hour incubation in the presence of GOx enzymatic nanoreactors, a drastic change in the cell morphology is noted. A plasmatic membrane protrusion after 2 hours of incubation was clearly observed in phase contrast images (videos SI). This seems to be due to the formation of gas bubbles inside the cells produced by the metabolic oxygen production by the antioxidant system (e.g. catalase) induced by te excess of hydrogen peroxide produced by the GOx activity. As shown in the videos (SI), when the cell "explodes" the fluorescence immediately disappears due to the cell membrane destruction and the subsequent dilution of the fluorophore in the medium. Glucose oxidase has been reported as a cytotoxic agent for tumor cells and its capacity to reduce the tumor tissue *in vivo*experiments.[54] The cytotoxic effect of GOx is originated from the production of hydrogen peroxide, causing oxidative damage in the cells, hypoxia as oxygen is consumed, and glucose consumption that starves the cells.[55-57] The advantages of VLPs and nanocarriers of enzymatic activity are the protection against the proteases,[38] the reduction of immunogenic response, especially when the nanoreactors are covered with compatible proteins or non-immunogenic polymers such as polyethylene glycol.[58]

It has been reported that HSA functionalization increases tumor cell affinity.[43] The HSA provides an interaction between the nanocage surface and secreted protein acidic and rich in cysteine (SPARC). HSA-SPARC interaction allowed breast tumor cell internalization.[44] The role of SPARC in cancer development is still controversial. Higher levels of SPARC expression have been reported in glioblastomas,[59]pancreatic cancer,[60] gastrointestinal cancer,[61] cervical cancer,[62] bone cancer,[63]and breast cancer.[64-66] In contrast, lower levels of SPARC expression have been found in pancreatic cancers,[67] ovarian,[68]and colorectal.[69,70] HSA-based drugs can bind to SPARC.[71,72] With this in mind, VLP-GOx nanoreactors were functionalized with HSA. The HSA covered nanoparticles (Fig. 3B) significantly increased the protein content of nanoparticles. However, no advantage in cytotoxicity in cell cultures was found when the cytotoxicity results were based on GOx content or GOx activity (Fig. 5 and 6). The effect of HSA functionalization *in vivo* experiments is currently being explored.

5. Conclusions

Self-assembly is a highly efficient procedure for active enzyme encapsulation. The VLP-encapsulated GOx enzyme showed significant catalytic activity. The nanoreactors containing GOx activity are entirely suitable for cytotoxicity generation in tumor cells. The HSA functionalization of the VLP-GOx nanoreactors could result in a prevailing strategy to improve selective cancer targeting. The GOx containing enzymatic nanoreactors seems to be an efficient alternative to improve the current cancer therapy. *In vivo* studies are envisaged to reinforce the effectiveness of this treatment strategy.

Author contributions

Pedro Gama: Formal analysis, investigation, methodology, writing – original draft, writing – review & editing. Patricia Juarez: Formal analysis, investigation, methodology, writing – review & editing. Ana G. Rodríguez-Hernández: Investigation, methodology. Rafael Vazquez-Duhalt: Conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing – review & editing.

Acknowledgments

This work has been funded by UNAM (grant PAPIIT IN209722). We thank Dr. Oscar González Davis and Itandehui Betanzo for their technical assistance, and Francisco Ruiz Medina for TEM analysis.

Conflict of interest statement

The authors declare no competing interests.

Data availability statement

Additional data that support the findings of this study are available upon reasonable request from the corresponding authors.

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Figure leyends

Figure 1. a) Surface electric charge distribution on the GOx enzyme and BMV capsomer. The GOx surface mostly exhibits negative electric charges (red color), and the inner surface of BMV capsomers shows a more positive (blue color) electric charge distribution. The difference in electric charge distribution between GOx and the inner BMV surface results in electric charge complementarity. In silico results by Maestro Schrodinger(r). b) Schematic representation of VLP-GOx nanoreactor self-assembly encapsulation and HSA functionalization

Figure 2. Purification of VLP-GOx nanoreactors by SEC HPLC chromatography using differences in retention time A) Overlapped chromatograms show the retention time for native BMV=5.7 min, free GOx=7.5 min and disassembled CP=8.4 min. B) VLP-GOx nanoreactors retention time=5.7 min, similar to native virions of BMV. The VLP-GOx nanoreactors were purified by collecting the fractions from 5.5 to 6.5 min.

Figure 3. Transmission electron microscopy (TEM) of nanoreactors. A) Quasi-spherical unmodified VLP-GOx nanoreactors, and B) VLP-GOx@HSA functionalized nanoreactors.

Figure 4. Cytotoxicity activity of empty unmodified and modified VLPs on cell cultures of 4T1 breast tumor mouse cell line model, and 293T healthy embryonic kidney human cells. Two-way RM ANOVA ($p<0.001$). *** Significative difference between the two preparations at the same concentration. ++ Significative difference with the control.

Figure 5. Cytotoxicity of unfuctionalized VLP-GOx and functionalized VLP-GOx@HSA nanoreactors on 4T1 breast tumor mouse cell line. Mean and standard deviation were obtained from three independent experiments.

Figure 6. Cytotoxicity of unfuctionalized VLP-GOx and functionalized VLP-GOx@HSA nanoreactors on 293T kidney cell line. Mean and standard deviation were obtained from three independent experiments.

Figure 7. Flourescence microscopy images of the triple negative breast cancer cell line MDA-MB-231 expressing GFP. The images were taken at different times after treatement with VLP-GOx@HSA. The bubble-like formations are shown with yellow arrows. See videos on the Supplementary information.