Light-triggered plasmonic vesicles with enhanced catalytic activity of glucose oxidase for programmable photothermal/starvation therapy

Zhuoting Deng[†], Yaling He[†], Muhammad Rizwan Younis, Shan Lei, Chao Jiang, Yanyan Yuan, Peng Huang and Jing Lin^{*}

ABSTRACT Glucose oxidase (GOx)-based nanotheranostic agents hold great promise in tumor starvation and its synergistic therapy. Self-assembled plasmonic gold vesicles (GVs) with unique optical properties, large hollow cavity, and strong localized surface plasmon resonance, can be used as multi-functional nanocarriers for synergistic therapy. Herein, GOx-loaded GV (GV-GOx) were developed for light-triggered GOx release as well as enhanced catalytic activity of GOx, achieving programmable photothermal/starvation therapy. Under near-infrared laser irradiation, the GV-GOx generated strong localized hyperthermia due to plasmon coupling effect of GV, promoting the release of encapsulated GOx and increasing its catalytic activity, resulting in enhanced tumor starvation effect. In addition, the high photothermal effect improved the cellular uptake of GV-GOx and allowed an efficient monitoring of synergistic tumor treatment *via* photoacoustic/photothermal duplex imaging *in vivo*. Impressively, the synergistic photothermal/starvation therapy demonstrated complete tumor eradication in 4T1 tumor-bearing mice, verifying superior synergistic anti-tumor therapeutic effects than monotherapy with no apparent systemic side effects. Our work demonstrated the development of a light-triggered nanoplatfor for cancer synergistic therapy.

Keywords: light-triggered release, plasmonic vesicles, glucose oxidase, photothermal therapy, starvation therapy

INTRODUCTION

Tumor starvation therapy through chocking off the tumor site energy supply has attracted great interest in recent years [1–5]. Intratumoral glucose is one of the most common and essential energy supplies in tumor, which

can be transformed to gluconic acid and hydrogen peroxide (H_2O_2) through the catalysis of glucose oxidase (GOx) [6–9]. The GOx-mediated catalysis effectively consumes intratumoral glucose, which results in significant glucose depletion, thus triggering the tumor starvation process [10–14]. Although tumor starvation is an ideal tumor treatment approach, the poor reaction kinetics of enzyme-based catalysts and their safe delivery to the tumor site are the prominent bottlenecks, which impede its further applications [15].

Interestingly, it has been demonstrated that heat can accelerate the catalytic reactions, resulting in improved catalytic activity of catalysts [16–19]. However, the controlled generation of hyperthermia at the tumor site remains a hard nut to crack. As we know, photothermal therapy (PTT) involves photothermal agents to generate the localized hyperthermia under laser irradiation, resulting in ablation of tumor cells [20–23]. Integrating PTT with starvation therapy in one platform could realize the controlled generation of hyperthermia, which accelerates the catalytic reaction of GOx, achieving high anti-tumor effects due to the synergistic photothermally enhanced starvation therapy [24–26].

Additionally, the safe and controlled delivery of enzymes to avoid their potent toxicity and degradation remains a formidable challenge. In spite of the development of a wide range of nanocarriers such as liposomes [27], polymer micelles [28], polymersomes [29,30], and mesoporous silica [31], self-assembled plasmonic gold vesicles (GVs) [32–37] with near-infrared (NIR) absorption have aroused great research interest owing to their large hollow cavity and plasmonic coupling effect. These fea-

Marshall Laboratory of Biomedical Engineering, International Cancer Center, Laboratory of Evolutionary Theranostics (LET), School of Biomedical Engineering, Shenzhen University Health Science Center, Shenzhen 518060, China

[†] These authors contributed equally to this work.

^{*} Corresponding author (email: jingl@szu.edu.cn)

resulting solution was centrifuged and resuspended in aqueous solution for further studies.

Characterizations of GV-GOx

The morphologies of GV-GOx were characterized by FEI-APREO-S high-resolution scanning electron microscopy (SEM) and HITACHI HT7700 scanning transmission electron microscopy (TEM). Dynamic light scattering and zeta potential were recorded on Zeta sizer Nano-ZS90. Ultraviolet-visible (UV-Vis)-NIR absorption spectra were collected by Cary 60 UV-Vis spectrophotometer. Fourier transform infrared (FTIR) spectra were measured on a Spectrum Two Perkin Elmer spectrometer.

GOx loading content

The total amount of GOx and the amount of free GOx in the supernatant after dialysis were determined by protein quantification method [38]. GOx loading content was calculated by the formula: loading content (%) = (total GOx – remaining content of GOx)/total GOx × 100%.

Evaluation of the activity of loaded GOx

To estimate the GOx activity, GV-GOx (optical density (OD)_{808 nm} = 0.4) solutions were mixed with different concentrations of glucose solutions: 20, 15, 5, 4, 2, 1, 0.5 mmol L⁻¹, followed by laser irradiation (808 nm, 1 W cm⁻², 10 min). Then H₂O₂ kit was used to detect the activity of GOx, and the pH value was measured by using a pH meter. The solution of GV-GOx without laser irradiation was used as a control. Similarly, time-dependent generation of H₂O₂ and decrease of pH with/without laser irradiation were also monitored at a fixed concentration of glucose (20 and 3.2 mmol L⁻¹, respectively).

In vitro PA imaging

Aqueous suspensions of GV-GOx with different optical densities (OD₈₀₈ = 0.1, 0.2, 0.3, 0.4, 0.5) were prepared and their PA signals were recorded by a Vevo 2100 LAZR system. Ultrapure water was used as a control.

In vitro cellular experiments

4T1 cells were seeded into 96-well plates at a density of 5×10^3 cells/well ($n = 5$) and incubated at 37°C in DMEM media with 10% fetal bovine serum (FBS) (100 µL) under CO₂ atmosphere for 24 h. For *in vitro* studies, all the irradiated groups were exposed to 808 nm laser at a power density of 1 W cm⁻² for 5 min. For cytotoxicity assays, the cells were incubated with different concentrations of GVs or GV-GOx (equiv GOx: 0, 1.875, 3.75, 7.5, 15 and 30 µg mL⁻¹) for 12 h. Cell viability was

quantified by the methyl thiazolyl tetrazolium (MTT) assay.

In cell fluorescence imaging studies, the cells were incubated with GVs (42.3 µg mL⁻¹) or GV-GOx (42.3 µg mL⁻¹) for 4 h, followed by treatment with/without laser irradiation. For live/dead cell observations, calcein acetoxymethyl ester (Calcein AM, live, green) and propidium iodide (PI, dead, red) dyes were added to each well, and then washed with serum-free DMEM. For intracellular reactive oxygen species (ROS) level observations, the cells were stained with 2',7'-dichlorofluorescein diacetate (DCFH-DA) and washed. Finally, the images were acquired by a Nikon Eclipse Ts2 inverted fluorescence microscope. For subcellular localization, the cells were fixed, washed and then stained with DAPI for 10 min, followed by washing again. Finally, images were acquired by a laser scanning confocal microscope.

In vivo PA/photothermal imaging

Female BALB/c mice (4–5 weeks age) were purchased from Guangdong Medicinal Laboratory Animal Center and were used in accordance with the regulations of the Animal Ethical and Welfare Committee of Shenzhen University (AEWC-SZU). To build the subcutaneous xenograft model, 4T1 cells ($\sim 2 \times 10^6$) were subcutaneously injected into the right flank of the mice (18–22 g). When the tumor size reached ~ 60 mm³, 50 µL of phosphate-buffered saline (PBS), GVs (400 µg mL⁻¹), or GV-GOx (400 µg mL⁻¹) was injected into the tumor-bearing mice. Then the tumors were exposed to 808 nm laser (1 W cm⁻², 10 min). Thermal imaging was recorded by a SC300 infrared camera (FLIR). PA imaging and blood oxygen saturation were performed by a Vevo 2100 LAZR system.

In vivo antitumor efficacy

The mice were randomly divided into seven groups ($n = 5$ per group), including (1) PBS (50 µL), (2) PBS-Laser (50 µL), (3) GOx (283.7 µg mL⁻¹, 50 µL), (4) GVs (400 µg mL⁻¹, 50 µL), (5) GVs-Laser (400 µg mL⁻¹, 50 µL), (6) GV-GOx (400 µg mL⁻¹, 50 µL) and (7) GV-GOx-Laser (GV-GOx-L, 400 µg mL⁻¹, 50 µL). For irradiation groups, the tumors were exposed to 808 nm laser at a power density of 0.5 W cm⁻² for 10 min. The spot of laser beam was adjusted to cover the entire region of tumor. All the mice received once intratumoral injection on the first day. The size of the tumor was measured by a caliper every other day, and the tumor volume was calculated according to the formula: volume = (length × width²)/2. Besides, the body weights were also recorded

during the treatment. All the mice were sacrificed after 15 days of treatment, and then the tumors and major organs were collected.

Statistical analysis

All data were expressed as means $\pm$ standard deviation (SD). All figures shown in this article were obtained from at least three independent experiments with similar results. Statistical analyses between multiple groups were performed with the one-way ANOVA followed by Tukey's post-test or two-way ANOVA analysis followed by Bonferroni post-test (GraphPad Prism 6.0). Statistical significance is denoted by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS AND DISCUSSION

PEG-*b*-PCL copolymer was synthesized according to the

reported protocol (Fig. S1a, b) [38]. GV-GOx was prepared by the improved selective solvent approach to maintain the catalytic activity of GOx. Briefly, the polymer-modified GNPs were slowly dripped into GOx aqueous solution to mix evenly, and then self-assembled by dialysis. The as-prepared GV-GOx exhibited the spherical morphology with high dispersity (Fig. 1a, b). The hydrodynamic size of GV-GOx is ~ 245 nm with a polydispersity index of 0.19 (Fig. S2a). The surface zeta potential changes from -18 to -29 mV due to the loading of an anionic GOx into the cavity of GVs (Fig. S2b). The GV-GOx was homogeneously dispersed in different physiological media including deionized water, PBS buffer, saline and cell culture medium containing 10% FBS for over 24 h (Fig. S3). As shown in Fig. 1c, the UV-Vis-NIR absorption spectrum of GV-GOx showed a prominent red-shifted broad band from visible to NIR region after

Figure 1 (a) SEM and (b) TEM images of GV-GOx self-assembled from GNPs. (c) UV-Vis-NIR spectra of GNPs and GV-GOx. (d) Temperature increase profile of varying concentrations of GV-GOx under laser irradiation. (e) GOx loading efficiency in GV-GOx as a function of GOx concentration. (f) GOx release profile at different times under laser irradiation.

self-assembly, suggesting that GV-GOx could convert optical energy into heat. To investigate the light-to-heat conversion efficiency, varying concentrations of GV-GOx ($OD_{808} = 0.1, 0.2, 0.3, 0.4$, and 0.5) underwent 808 nm laser irradiation at a power density of 1.0 W cm^{-2} for 5 min (Fig. 1d). An obvious concentration-dependent temperature increase was observed, whereas, by adjusting the content of GV-GOx, the temperature was precisely controlled from 25 to 63°C , suggesting that GV-GOx possessed the capability of rapid and efficient conversion of NIR laser to generate strong localized hyperthermia. Then, the loading and light-regulated release of GOx in GV-GOx was determined. The GOx loading efficiency can reach to $\sim 12\text{ wt\%}$ (Fig. 1e). To track the light-controlled GOx release of GV-GOx, GOx was conjugated with fluorescein isothiocyanate isomer (FITC) to afford GOx-FITC, verified by characteristic fluorescence peak (Fig. S4a) and FTIR spectra (Fig. S4b). The GOx-FITC-loaded GVs have no fluorescence attributed to the fluorescence quenching by plasmonic GVs. Whereas, under laser irradiation (808 nm , 1.0 W cm^{-2}), the enhanced fluorescence intensity of FITC was observed over time (Fig. S4c, d). The localized hyperthermia-triggered release of GOx reached nearly 55% within 40 min (Fig. 1f). Meanwhile, morphological changes of GV-GOx further revealed that GV-GOx gradually disintegrated under irradiation (Fig. S5), facilitating the release of GOx. Meanwhile, *in vitro* PA imaging of GV-GOx was examined. As shown in Fig. S6a, GV-GOx showed strong PA signal, which was linearly correlated within a certain concentration range (Fig. S6b), allowing an effective treatment monitoring *in vivo* by PA imaging.

The catalytic activity of released GOx from the disintegrated GV-GOx was tested by determining the catalytic products (H_2O_2 and gluconic acid) of GOx reaction (Fig. 2a). The generated H_2O_2 concentration of free GOx-mediated catalytic decomposition of glucose was tested at different temperatures ($30, 40, 50$ and 60°C). As shown in Fig. S7, the catalytic activity of GOx can be maintained at $30\text{--}60^\circ\text{C}$. Interestingly, the catalytic activity of GOx can be improved at high temperatures (50 and 60°C), which suggested mild hyperthermia could accelerate the catalytic reaction of GOx. Compared with GV-GOx, laser-irradiated GV-GOx (808 nm , 1.0 W cm^{-2}) generated significantly higher amount of H_2O_2 , which is directly proportional to the glucose concentration (Fig. 2b) and time (Fig. 2c). The catalytic reaction of GOx and the produced gluconic acid lead to lower pH. As shown in Fig. 2d and e, a rapid decrease in the pH value was recorded with glucose concentration and time upon laser irradiation. These

results strongly dictate the light-triggered release as well as enhanced catalytic activity of GOx.

Next, *in vitro* cytotoxicity of GV-GOx was evaluated by MTT assay on HEK293T and 4T1 cells, respectively. As shown in Fig. 3a, the cell viabilities were over 80% at all tested concentrations ($0\text{--}30\text{ }\mu\text{g mL}^{-1}$), which suggests GV-GOx is safe under these concentrations. The viability of free GOx-treated 4T1 cells was less than 20% when GOx concentration was over $1.6\text{ }\mu\text{g mL}^{-1}$ (Fig. S8), which suggests the GV carrier can dramatically reduce the toxicity of GOx. Subsequently, the *in vitro* programmable photothermal/starvation therapy was evaluated on 4T1 cells. Neither the GVs nor GV-GOx decreased the cellular viability in dark. In contrast, under laser irradiation (1.0 W cm^{-2} , 5 min), GV-GOx exhibited more significant cellular destruction of 4T1 cells than all other groups, which was attributed to the synergistic effect of photothermal/starvation therapy (Fig. 3b).

In addition, live/dead assay using Calcein AM/PI co-staining images further endorsed the higher synergistic therapeutic effects than single therapy (Fig. S9). To ensure that the higher killing effect is caused by the synergistic effects, we verified the catalytic activity of GOx at the cellular level using DCFH-DA, an ROS fluorescence probe. Compared with other groups, a markedly higher fluorescence intensity of DCFH-DA was observed in the cells treated with GV-GOx under laser irradiation (Fig. 3c), which confirmed the generation of large amount of intracellular ROS (H_2O_2) by GV-GOx owing to the photothermally enhanced catalytic activity of GOx. Irradiation-enhanced cellular uptake of GV-GOx was also investigated. As shown in Fig. 3d, a higher fluorescence intensity was observed from NIR laser-treated cells incubated with FITC-labeled GV-GOx, suggesting light-triggered cellular uptake enhancement.

Because of high NIR absorption and good photothermal effect *in vitro*, GV-GOx was anticipated to be a high-performance photothermal/PA imaging agent. As shown in Fig. 4a and b, both the GVs and GV-GOx increased the local tumor temperature up to 47°C under laser irradiation (808 nm , 0.5 W cm^{-2} , 10 min), which implies that the GV-GOx can act as a photothermal agent to mediate efficient photothermal destruction of tumor cells *in vivo*. In addition, PA imaging was also conducted to evaluate the PA properties of GV-GOx. Strong PA signals were observed in the tumor region (Fig. 4c), which was approximately 10-fold stronger than pre-injection (Fig. 4d). Three-dimensional (3D) PA imaging of tumor tissue also showed a strong contrast before and after GV-GOx injection (Fig. 4e). Given the fact that the GOx-

Figure 2 (a) Illustration of light-triggered GOx catalysis reaction. (b) Glucose-concentration-dependent and (c) time-dependent H₂O₂ generation of GV-GOx with/without laser irradiation. (d) Glucose-concentration-dependent and (e) time-dependent decrease in pH value of GV-GOx with/without laser irradiation.

mediated catalytic decomposition of glucose consumed a large amount of endogenous oxygen (O₂), the intratumoral blood oxygen saturation (sO₂) concentration significantly dropped to 10% after laser irradiation of GV-GOx for 10 min, while the mice in the GV group maintained their original O₂ levels (Fig. 4f and Fig. S10) in accordance with 3D images of sO₂ (Fig. S11). These results suggest the light-triggered release and enhanced catalytic activity of GOx in the tumor tissues lead to the O₂ consumption during the catalytic reaction of GOx.

Finally, the *in vivo* antitumor efficacy of GV-GOx was evaluated on 4T1 tumor-bearing mice. As shown in Fig. 5a, the complete tumor elimination in GV-GOx-L group was observed after 15 day of treatment, whereas, negligible tumor growth inhibition was seen in other control groups, suggesting the prominent antitumor ef-

fect of GV-GOx-L. Meanwhile, no body weight loss was observed during the treatment (Fig. 5b), indicating that GV-GOx has good biocompatibility. To quantitatively assess the antitumor efficacy, the tumor weights were measured and recorded (Fig. 5c). In the absence or presence of laser irradiation, the PBS group had more than 12-fold increase of tumor weight, indicating that laser irradiation alone is unable to trigger the anticancer effect. Without laser irradiation, the tumors in GV-treated mice grew to the same size as those in PBS or GOx-treated mice, while GV-L exhibited much slower tumor growth than control groups, suggesting a certain level of anticancer effects due to the photothermal effect. Impressively, GV-GOx-L demonstrated almost complete tumor growth inhibition throughout the entire experimental period with a tumor inhibition rate of nearly 100%. The photographs

Figure 3 (a) Dark cytotoxicity of GV-GOx in 4T1 and HEK 293T cells. (b) *In vitro* therapeutic efficacy after incubated with varying concentrations of GVs or GV-GOx with/without laser irradiation. *** $p < 0.001$. (c) Fluorescence images of DCFH-DA (scale bar = 500 μm) and (d) confocal fluorescence images (scale bar = 50 μm) in 4T1 cells incubated with GVs or GV-GOx with/without laser irradiation.

of 4T1 tumor-bearing mice at the 15 day further confirmed that the tumors in the GV-GOx-L group were completely ablated (Fig. 5d). H&E stained images further showed that GV-GOx-L significantly damaged the tumor cells (Fig. 5e), while caused negligible damage/inflammation to other organs (Fig. S12). All results indicate that the light simultaneously triggered GOx release and enhanced GOx activity, achieving synergetic photothermal/starvation therapy and leading to nearly

complete tumor destruction without any tumor recurrence.

The delivery of GOx by GVs shows the following advantages: (1) high GOx loading efficiency (~12 wt%) due to the hollow interior of GVs; (2) the controllable release of GOx by laser irradiation; (3) enhanced catalytic activity of GOx by laser irradiation; (4) PA/photothermal duplex imaging-guided synergistic photothermal/starvation therapy with improved treatment efficacy. In addition,

Figure 4 (a) Thermal images in the tumor region treated with PBS, GV and GV-GOx under laser irradiation. (b) The corresponding temperatures at different time points of (a). (c) *In vivo* PA images (scale bar = 1 mm), (d) average PA intensity and (e) 3D PA images of tumor tissues before and after injection of GV-GOx. (f) *In vivo* oxygen saturation (sO_2) images of tumor tissues before/after injection and after laser irradiation (scale bar = 1 mm).

the multifunctional nanoplatform has high solubility and stability in aqueous solution and excellent biocompatibility, thus promising for biomedical applications.

CONCLUSIONS

In summary, GOx-loaded GVs have been explored as a nanotheranostic agent for duplex imaging-guided sy-

Figure 5 (a) Time-dependent tumor growth curves, (b) relative body weight and (c) tumor weight from 4T1-tumor bearing mice with different treatments ($n = 5$, mean $\pm$ SD). (d) Photographs and (e) H&E stained tumor sections at the Day 15 after different treatments (scale bar = 100 μm).

nergistic photothermal/starvation cancer therapy. Due to the plasmon coupling effect, the GV-GOx exhibited strong photothermal effect, generating strong localized hyperthermia to trigger the release of encapsulated GOx as well as enhance its catalytic activity, resulting in photothermally augmented tumor starvation to realize complete 4T1 tumor eradication *in vivo*. Our study demonstrated GVs, as a multifunctional cancer nanotheranostic platform with high antitumor efficacy and biological safety, hold biomedical potential for cancer management.

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Author contributions Lin J conceived the idea, supervised the study and wrote the manuscript; Deng Z participated in the fabrication and characterization of GV-GOx; Jiang C and Yuan Y participated in the photoacoustic imaging; Deng Z and He Y completed the subsequent experiments, drew all figures and wrote this manuscript; Lin J, Huang P, Younis MR and Lei S helped editing this manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Supporting data are available in the online version of the paper.

Zhuoting Deng received her BSc degree in pharmaceutical engineering from the Southeast University in 2017. Currently, she is studying for her MSc degree in the Laboratory of Evolutionary Theranostics (LET), School of Biomedical Engineering, Shenzhen University. Her current research interests mainly focus on the development of multifunctional nanotherapeutic agent based on gold nanovesicles and their applications in cancer therapy.

Yaling He received her BSc degree from Anhui Normal University in 2018. Currently, she is studying for her MSc degree in the LET, School of Biomedical Engineering, Shenzhen University. Her current research interests mainly focus on the development of pH-sensitive gold vesicles for biomedical applications.

Jing Lin is a Distinguished Professor at the School of Biomedical Engineering, Shenzhen University Health Science Center, China. She received her PhD degree in organic chemistry from Donghua University and Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences in 2010. Then she joined the PharmaResources (Shanghai) Co., Ltd. as a group leader. After two years, she moved to the United States of America and spent four years as a postdoc at the University of Maryland and the National

Institutes of Health (NIH). She joined the faculty of Shenzhen University in 2016 and was promoted as a Distinguished Professor in 2018. Her research focuses on molecular imaging, nanomedicine and theranostics.

光触发增强葡萄糖氧化酶催化活性的等离子体囊泡用于程序化光热-饥饿疗法

邓卓婷[†], 何亚玲[†], Muhammad Rizwan Younis, 雷珊, 蒋超, 袁燕燕, 黄鹏, 林静^{*}

摘要 在肿瘤的饥饿治疗及协同治疗中, 基于葡萄糖氧化酶(GOx)的纳米诊疗剂展现出巨大的应用前景. 自组装等离子体金囊泡(GV), 由于具有独特的光学性能、巨大空腔和强局域表面等离子体共振等特性, 可作为协同治疗的多功能纳米载体. 本文中, 我们开发了一种装载GOx的GV(GV-GOx)用于光触发释放GOx, 同时增强GOx的催化活性, 从而实现程序化光热-饥饿治疗. 在近红外激光照射下, 由于GV具有等离子体耦合效应, GV-GOx可以产生很强的局部高热, 引起封装的GOx释放, 同时高热可提高GOx催化活性, 从而增强肿瘤的饥饿效应. 此外, 高光热效应可促进细胞对GV-GOx的摄取, 并可通过活体光声/光热双模态成像对协同治疗进行有效监测. 令人印象深刻的是, 协同光热/饥饿疗法能完全消融4T1荷瘤小鼠的肿瘤, 抗肿瘤效果明显优于单一疗法, 且没有明显的系统毒性. 本工作展示了一种光触发的纳米平台, 可用于癌症协同治疗.