A glutathione responsive nanoplatform made of reduced graphene oxide and MnO₂ nanoparticles for photothermal and chemodynamic combined therapy

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Abstract: Based on the specific tumor microenvironment, characterized by an overproduction of H_2O_2 and a high glutathione (GSH) concentration, the cascade reactions of nanomaterials, capable of mediating GSH depletion and reactive oxygen species (ROS) generation, have become a popular strategy to increase cancer therapy efficiency. In this study, we exploited reduced graphene oxide (rGO), one of the most promising graphene-based materials, in combination with manganese dioxide nanoparticles (MnO_2 NPs) to design a multifunctional nanoplatform (rGO@MnO_2) for efficient photothermal/chemodynamic combined therapies. MnO_2 NPs were anchored onto the surface of rGO nanosheets (rGO NSs). MnO_2 NPs oxidize intracellular GSH, and the generated Mn^{2+} ions converted H_2O_2 into $\text{HO}\cdot$ by Fenton reaction. Meanwhile, rGO NSs mediated photothermal therapy (PTT) could further kill cancer cells. High temperature caused by photothermal conversion increased Fenton reaction rate, which enhanced the efficiency of MnO_2 NPs mediated chemodynamic therapy (CDT). Importantly, thanks to the enhanced cell uptake of MnO_2 NPs favored by the delivery properties of rGO, rGO@MnO_2 possessed higher lethality to cancer cells. The decrease in the nanomaterials' effective dose would further improve biosecurity and reduce cost. Therefore, rGO@MnO_2 have great potential in cancer therapy by exploiting the synergistic effect of PTT and photothermal/delivery effect enhanced CDT.

Keywords: GSH depletion, Fenton reaction, Reactive oxygen species, Drug delivery, Cancer therapy

1. Introduction

Recently, the cascade reactions induced by nanoplateforms have become a popular strategy to treat cancer efficiently due to the specific tumour microenvironment. The mechanism is based on GSH depletion and ROS generation [1-5]. Compared to methods that are only based on enhanced ROS generation, the sequential oxidation-reduction reactions could break the intracellular redox homeostasis more easily, therefore leading to cancer cell death more efficiently. For example, copper-cysteine nanoparticles were prepared to treat cancer by Cu^{2+} -caused GSH oxidation and consequent Cu^{+} -mediated Fenton reaction [6]. Although this type of systems resulted very promising, it is necessary to control the dosage due to the relatively high toxicity of metal atom-based nanomaterials [7]. Therefore, manganese-based nanomaterials (e.g. MnO_2) have been explored as a suitable alternative due to the low toxicity of Mn^{2+} ions [8-11]. In physiological environment, Mn^{2+} ions can convert H_2O_2 into hydroxyl radical ($\text{HO}\cdot$) with the assistance of HCO_3^- ions [12-14]. Importantly, Mn^{2+} ions usually come from the reaction between Mn^{4+} ions and intracellular GSH. However, the lethality to cancer cells of MnO_2 -based nanomaterials is not very high due to the relatively high stability [15, 16].

To further promote cancer therapy efficiency, the combination of MnO_2 -mediated chemodynamic therapy (CDT) with chemotherapy, or photodynamic therapy (PDT), or photothermal therapy (PTT) was put forward as an effective strategy. Metal organic framework-coated MnO_2 nanosheets were designed to deliver doxorubicin and self-activate DNzyme for chemo/gene combinatorial treatment of cancer [17]. Alternatively, polydopamine (PDA)-coated $\text{MnO}_2@\text{Ce6}$ nanoparticles can kill cancer cells efficiently by PDT and PTT [18]. Due to the potential drug resistance and the poor tissue penetration of visible light, the chemotherapy and PDT have some limitations in clinical applications. Due to the good tissue penetration of NIR light, PTT should be a better choice for combination with the CDT of MnO_2 [19, 20]. However, when the PTT responsive molecules (such as PDA) are coated onto the surface of MnO_2 , the coating can reduce the lethality of this material towards cancer cells [18, 21].

To obtain efficient cascade reactions, a challenging design is to anchor MnO_2 nanomaterials onto the surface of a material endowed of PTT properties. Due to the good biocompatibility and high photothermal conversion efficiency, reduced graphene oxide nanosheets (rGO NSs) have been widely used as an efficient medium to treat cancer by PTT [22-24]. rGO possesses high specific surface area,

and is an excellent carrier for drugs or nanoparticles. Therefore, the combination of MnO_2 and rGO might possess the dual functions of CDT and PTT. Interestingly, it is known that high temperatures (e.g. 5 to 20 °C above the physiological temperature) will increase Fenton reaction rate, meaning that heat generated during PTT can enhance the efficiency of CDT [25-27]. Therefore, if the rGO functions as an efficient transporter to increase intracellular content of MnO_2 , the cascade reactions based on MnO_2 with GSH and H_2O_2 will kill more cancer cells by enhanced CDT.

In this work, BSA modified- MnO_2 nanoparticles (MnO_2 NPs) with negative charges were complexed to the surface of polyethyleneimine modified rGO NSs by electrostatic attraction, forming the $\text{rGO}@\text{MnO}_2$ composites. The mechanism of the combined therapy is shown in **Scheme 1**. The MnO_2 NPs efficiently oxidize intracellular GSH, and the generated Mn^{2+} ions subsequently convert H_2O_2 into $\text{HO}^\bullet$ by Fenton reaction. Both GSH depletion and $\text{HO}^\bullet$ generation increase the intracellular ROS level to cause cancer cells death. In addition, rGO NSs mediated PTT, under NIR light irradiation, can further kill cancer cells. Photothermal effect of rGO caused high temperature increases Fenton reaction rate to produce more $\text{HO}^\bullet$, thus improving the efficiency of CDT of MnO_2 NPs. Interestingly, we found that $\text{rGO}@\text{MnO}_2$ possess higher CDT efficiency than MnO_2 NPs at equivalent concentration, which should be attributed to a higher delivery of MnO_2 NPs into cells by rGO NSs. Therefore, $\text{rGO}@\text{MnO}_2$ have a great potential in efficient cancer therapy due to the synergistic effect of PTT and photothermal/delivery effect enhanced CDT.

Scheme 1. $\text{rGO}@\text{MnO}_2$ -mediated PTT and photothermal/delivery effect enhanced CDT for synergistic cancer therapy.

2. Experimental section

2.1. Materials

GO was synthesized following the modified Hummers method and purified by dialysis at least for 10 days. Branched polyethyleneimine (MW~800) (PEI) was purchased from Sigma-Aldrich. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (>99%) was purchased from Honeywell Fluka. Bovine serum albumin (BSA) was purchased from Calbiochem. NaOH (>99.5%) was purchased from Thermal Fisher Scientific. All chemical reagents were of analytical grade and used without any further purification. CellTiter 96® AQueous one solution cell proliferation assay (MTS) Kit and GSH/GSSG-Glo™ Assay Kit were purchased from Promega (France). Reactive oxygen species (ROS) molecular probes™ CM-H2DCFDA was purchased from Thermal Fisher Scientific.

2.2. Preparation of $\text{rGO}@ \text{MnO}_2$

rGO NSs were prepared by a modified method [28]. Briefly, 10 mL of 10 mg/mL PEI was mixed with 10 mL of 1 mg/mL GO dispersion, and the mixture was sonicated for at least 10 min. Then, the mixture was treated in water bath at 80 °C for 20 min to obtain rGO NSs. After cooling down to room temperature, rGO NSs were collected by centrifugation, and washed by Milli-Q water for three times. MnO_2 NPs were synthesized by a modified method according to a previous report. A 1M MnCl_2 aqueous solution (480 μL) was added into 20 mL BSA aqueous solution (20 mg/mL) under stirring. After 10 min, 1200 μL of 1M NaOH was added, and the mixture was stirred for 3h. Then, MnO_2 NPs were dialyzed for 12h, and collected after centrifugation at 13.2k rpm for 45 mins. $\text{rGO}@ \text{MnO}_2$ were prepared based on the electrostatic attraction. Under strong sonication, 500 μL rGO NSs (0.5 mg/mL) with positive charges were added into 500 μL MnO_2 NPs (2.5 mg/mL) with negative charges solution dropwise. The prepared rGO NSs, MnO_2 NPs, and $\text{rGO}@ \text{MnO}_2$ were kept at 4 °C.

2.3. Characterization

A Varian Cary winUV 50 Bio-spectrophotometer (USA) was used to obtain absorbance spectra. Raman spectra were measured by a Renishaw inVia microscope equipped with 532 nm laser. A Perkin Elmer Spectrum One ATR-FT-IR spectrometer was used to perform FT-IR spectra measurement. Morphology of MnO_2 NPs and $\text{rGO}@ \text{MnO}_2$ was observed by transmission electron microscope (TEM, Hitachi 7500, Hitachi High Technologies Corporation, Tokyo, Japan). XPS spectra were obtained by a Thermo Scientific K-Alpha X-ray photoelectron spectrometer with a basic chamber pressure of 10^{-8}

to 10^{-9} bar with an anode using Al K α radiation ($h\nu = 1486.6$ eV). The Zeta potentials of rGO NSs, MnO₂ NPs, and rGO@MnO₂ were measured by a Beckman Coulter “Delsa Nano Submicron Particle Size and Zeta Potential” instrument. Fluorescence images were obtained by fluorescence microscope (ZEISS, Axiovert 200M).

2.4. Cell Viability

HeLa (human cervical adenocarcinoma) cells were cultured in high-glucose Dulbecco’s modified Eagle medium (H-DMEM) with 10 μ g/mL gentamycin (Lonza BioWhittaker), 10 mM N-(2-hydroxyethyl)-piperazine-N’-ethanesulfonic acid (Lonza BioWhittaker), 0.05 mM β -mercaptoethanol (Lonza BioWhittaker) and 10% fetal bovine serum (FBS). HeLa cells were maintained under a humidified atmosphere of 5% CO₂ at 37 °C, and the culture medium was changed every 2 days. Cell viability was measured by MTS Assay Kit, and live/dead cells staining were performed by live/dead staining Kit.

2.5. Photothermal Effect of rGO@MnO₂

To measure the photothermal effects of rGO@MnO₂, 1 mL rGO@MnO₂ aqueous solution was put into glass tubes with different concentrations (50, 100, and 200 μ g/mL) by irradiation by NIR laser (808 nm, 2 W/cm²) for 10 min. An infrared thermal imager was used to read the temperature of the solution.

2.6. Generation of HO $\cdot$ by Fenton Reaction

To confirm Mn²⁺ ions mediated Fenton reaction, the degradation of methylene blue (MB) was measured. Ten μ L of 10 mg/mL MB solution was added into 1 mL of 25 mM NaHCO₃ solution containing Mn²⁺ ions at different concentration (50 μ M, 100 μ M, and 200 μ M), and then 10 μ L of 1M H₂O₂ was added. Under different temperature, the degradation rate of MB was calculated by absorbance value in UV-vis spectra. The Fenton reactions of MnO₂ NPs and rGO@MnO₂ after reaction with GSH were detected following the same procedures.

2.7. TEM Measurement of HeLa Cells

HeLa cells were cultured in 24-well plates at a density of $\sim 2 \times 10^4$ cells per well. After incubation for 12 h, MnO₂ NPs and rGO@MnO₂ were added at 125 μ g/mL and 150 μ g/mL, respectively. After another 12 h, cells were washed with PBS twice, and then fixed in 2.5% glutaraldehyde in PBS at 4 °C overnight. Then, HeLa cells were rinsed thrice with cacodylate buffer alone, and were post-fixed with 0.5% osmium tetroxide for 30 min at RT. After washed thrice with MilliQ water, gradient alcohol (25%,

50%, 70%, 95%, and 100%) dehydration was performed. After dehydration, cells were soaked in 1:1 ratio of 100% ethanol and Epon overnight at 4 °C. After rinsed twice with Epon for 8 h, the final inclusion of Epon into the cells was done by polymerizing Epon at 40 °C for 30 min then incubating at 60 °C for 48 h. Afterward, the polymerized blocks were removed and sliced into ultrathin sections using a diamond knife attached to a ultramicrotome cutter (Leica). The ultrathin sections were then collected on copper grids coated with Butvar B-98 and stained with 1% uranyl acetate for 30 min followed with lead citrate staining for 2 min. Grids were then examined by TEM.

2.8. Intracellular Mn Content Measurement

HeLa cells were treated by MnO₂ NPs and rGO@MnO₂ according to the similar procedures to prepare TEM sample, but without fixation and resin inclusion. After treatment with the nanomaterials, HeLa cells were trypsinized and collected. Cells containing materials were sonicated, and freeze-dried. Then, Mn content was measured by ICP-MS (7700x, Agilent Technologies) and XPS.

2.9. Intracellular GSH/GSSG Ratio Measurement

After treatment with MnO₂ NPs and rGO@MnO₂ for 12 h, the cells cultured in 96-well plates were washed by PBS twice. Then, 50 µL of Total GSH Lysis Reagent or GSSG Lysis Reagent were added per well. After shaking at RT for 5 min, the cell lysis solution was centrifuged at 4 °C for 20 min, and the supernatant was transferred into opaque 96-well plate. Then, 50 µL per well of Luciferin Generation Reagent was added to all wells. The plate was incubated at RT for 30 min after shaking briefly. Finally, 100 µL per well of Luciferin Detection Reagent was added. After 15 min, the luminescence was measured.

2.10. ROS Content Measurement

After treated with MnO₂ NPs and rGO@MnO₂ for 12 h, HeLa cells was stained with 10 µM CM-H₂DCFDA work solution for 30 min at 37 °C. After washed twice by PBS, the fluorescence intensity was measured by Varioskan™ LUX multimode microplate reader at Ex485 nm/Em525 nm.

2.11. NPs Delivery Enhanced CDT

HeLa cells were cultured in 96-well plate at a concentration of 2000 cells per well. After 12 h, the old medium was replaced by the new medium with rGO NSs, MnO₂ NPs, and rGO@MnO₂ at different concentration. Then, cell viability was measured after 12, 24, 36, and 48 h, respectively. GSH/GSSG ratio and ROS content measurements were performed at the time point after 24 h. Live/dead cell staining was performed at the time point after 48 h.

2.12. Synergistic Effect of PTT and Enhanced CDT

After treatment with rGO@MnO₂ for 12 h at different concentration, the old medium was discarded, and new medium without materials was added. Then, cells were irradiated by 808 NIR light for 10 min. After cultured for additional 24 h, cell viability and live/dead cell staining measurements were performed. GSH/GSSG ratio and ROS content measurements were performed after irradiation for 4 h.

2.13. Statistical Analysis

The statistical significance of the differences was determined by one-way ANOVA. Values with $P < 0.05$ were considered statistically significant (* $p < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

3. Results and discussion

3.1. Preparation and characterization of rGO@MnO₂

MnO₂ NPs with negative charges were prepared by a modified method according to the previous reports [29, 30]. MnO₂ NPs had a good dispersibility, and the size was about 20 nm (Fig. 1a and b). PEI was used to reduce and modify GO to form rGO NSs with positive charges [28]. After reduction, the color of rGO NSs changed into black from light brownish GO (inset in Fig. S1a), and the enhanced absorbance at 800 nm guarantees a good photothermal conversion capability of rGO NSs under NIR (Fig. S1a). The prepared rGO NSs had a size of ~ 500 nm, and the surface was relatively smooth (Fig. S1b). Additional characterization using Raman evidenced an increased value of I_D/I_G ratio, meaning that the number of smaller graphitic domains increased after the reduction of GO by PEI (Fig. S2), similar to the results reported previously [31, 32]. Further, XPS results demonstrated that a lot of oxygen-containing functional groups were removed by reduction with PEI, causing O content to decrease to 15.1% (rGO) from 29.4% (GO). Meanwhile, the content of N increased to 15.0% (rGO) from 0.4% (GO), which means that rGO surface was modified by PEI successfully (Fig. S3).

Through electrostatic attraction, MnO₂ NPs were uniformly anchored onto the surface of rGO NSs to form rGO@MnO₂ (Fig. 1c and d). rGO@MnO₂ had a good dispersibility and the size was around 500 nm. We measured then zeta-potential, obtaining a positive value of 36.5 eV for rGO, while the value of MnO₂ NPs was about -25.9 eV. Zeta-potential value of rGO@MnO₂ was also negative (-21.4 eV), meaning that MnO₂ NPs were successfully complexed to the surface of rGO NSs (Fig. 1e). In addition, the appearance of typical peaks (such as COOH/~1643cm⁻¹ and amide II/~1524 cm⁻¹) of BSA appeared both in the FT-IR spectra of MnO₂ NPs and rGO@MnO₂ [33], confirming that MnO₂ NPs

were covering well the surface of rGO NSs (Fig. 1f). Furthermore, XPS spectra provided similar evidence of a good combination between MnO₂ NPs and rGO NSs (Fig. 1g). The valence state of manganese in MnO₂ NPs are mainly Mn⁴⁺ (~53.9%) and Mn³⁺ (~38.7%) according to the high resolution Mn 2p_{3/2} XPS spectra (Fig. 1h and Fig. S4).[34, 35] Mn ions with high valence state provide the basis for the depletion of intracellular GSH.[36] According to UV-Vis absorbance spectra, MnO₂ NPs with yellow color almost had no absorbance at 800 nm (Fig. 1i). However, the black rGO@MnO₂ dispersion had strong absorbance at 800 nm, meaning that rGO@MnO₂ certainly possess good photothermal effect. The content of Mn in MnO₂ NPs was then measured by ICP-MS and corresponded to 32.0% (w/w). Taking into account the valence contribution of Mn to MnO₂, the amount of BSA embedded into the nanoparticles was 51.5% (w/w). This percentage of BSA endows MnO₂ NPs with good water dispersibility.

Fig. 1. Characterization of rGO@MnO₂. (a) and (b) TEM images of MnO₂ NPs under different magnification. (c) and (d) TEM images of rGO@MnO₂ under different magnification. Zeta-potential (e), FT-IR spectra showing the change of the surface functional groups in each material (f), and XPS survey spectra (g) of MnO₂ NPs, rGO NSs, and rGO@MnO₂ confirming the type of element present in each material. (h) Mn high resolution XPS spectra of MnO₂ NPs and rGO@MnO₂ to confirm Mn

valence state. (i) UV-Vis absorbance spectra of MnO_2 NPs and rGO@MnO_2 .

3.2. Enhanced Fenton reaction rate by photothermal effect

A faster Fenton reaction rate can generate more hydroxyl radicals, causing stronger lethality to cancer cells. We observed that MnO_2 NPs reacted with GSH in a few minutes, and the brown solution gradually faded with the increased concentration of GSH (**Fig. 2a**), due to GSH oxidation and Mn^{2+} ion generation [2]. Under room temperature, it is clear that the colour of methylene blue (MB) changed from blue to colourless after 1 hour only in the presence of Mn^{2+} ions and H_2O_2 , meaning that the degradation of MB is occurring through Mn^{2+} -mediated Fenton reaction (**Fig. 2b**). To investigate the influence of the temperature on Fenton reaction, the degradation rate of MB was measured at different temperatures. Interestingly, the rate of this reaction speeded up with increasing the temperature of the dispersion (**Fig. 2c and d**). At 50°C , the reaction rate at $50\ \mu\text{M}$ Mn^{2+} ion concentration was similar to the reaction rate using a concentration of $100\ \mu\text{M}$ at 37°C . Even, the reaction rate using $100\ \mu\text{M}$ Mn^{2+} at 50°C surpassed the reaction rate at 37°C with $200\ \mu\text{M}$ of Mn^{2+} (**Fig. 2e**). Therefore, the increase in temperature clearly accelerates Mn^{2+} -mediated Fenton reaction.

Fig. 2. Enhanced Fenton reaction by photothermal effect of rGO@MnO_2 . (a) Reaction between GSH and MnO_2 NPs. (b) Mn^{2+} ion-mediated Fenton reaction. (c) and (d) Fenton reaction rate changes at

different temperature. (e) MB degradation rate at different conditions. (f) Absorbance spectra of rGO@MnO₂ at different concentration. (g) Temperature curves at different concentration under NIR irradiation. (h) GSH and H₂O₂ triggered cascade reactions of rGO@MnO₂. (i) Photothermal effect enhanced Fenton reaction.

Increasing the concentration of the composite, its absorbance intensity around 800 nm becomes also stronger (Fig. 2f). Consequently, the temperature increases under NIR irradiation raising about 20 °C at 200 µg/mL concentration after 10 min, demonstrating that rGO@MnO₂ possess a good photothermal response (Fig. 2g). rGO@MnO₂ could degrade MB in the presence of both GSH and H₂O₂ (Fig. 2h), confirming that MnO₂ NPs on rGO NSs surface were reduced into Mn²⁺ that efficiently converts H₂O₂ into HO•. In this context, we verified that rGO NSs did not interfere with the cascade reactions of MnO₂ involving GSH/H₂O₂. Under NIR irradiation, rGO@MnO₂ cause an increase in the temperature of the dispersion by photothermal effect, then accelerating the Fenton reaction (Fig. 2i). Overall, rGO@MnO₂ can deplete GSH, and the reaction product consisting on Mn²⁺ ions converts H₂O₂ into HO• by Fenton reaction. Meanwhile, rGO@MnO₂ have a good photothermal effect, and the increased temperature further accelerates the redox reaction. This photothermal effect enhanced Fenton reaction could enhance the lethality of MnO₂ to cancer cells, leading to a better cancer therapy efficiency.

3.3. rGO NSs Delivery Effect Enhanced CDT

The enhancement of cellular uptake of NPs could make CDT more efficient [37]. Therefore, to confirm the delivery effect of our materials, cell viability was measured after incubation with MnO₂ NPs, rGO NSs and rGO@MnO₂ at equivalent concentration (Fig. 3a-b, the corresponding statistic analysis shown in Fig. S5-8), respectively. After treated with rGO NSs, the cells maintain high viability at the different concentrations even after 48 h, suggesting a good cytocompatibility. After 12 h, MnO₂ NPs and rGO@MnO₂ had similar mortality effects on HeLa at the different concentrations tested (Fig. 3a and S5). Interestingly, cell viability decreased to ~55.5% after MnO₂ NP treatment for 24 h at the highest concentration of 250 µg/mL (Fig. 3b and S6), while the viability was only ~23.1% after rGO@MnO₂ treatment at 300 µg/mL concentration (corresponding to a concentration of 250 µg/mL of MnO₂ NPs plus 50 µg/mL rGO NSs). After 36 h, cell viability slightly decreased to 52.5% in MnO₂ NP group at 250 µg/mL concentration, while it decreased to 16.9% in rGO@MnO₂ treatment group at

300 $\mu\text{g/mL}$ concentration (Fig. 3c and S7). After 48 h, almost all cells (93.6%) were dead in rGO@MnO₂ group at 300 $\mu\text{g/mL}$ concentration, whereas there were still 43.2% alive cells in MnO₂ NP group at 250 $\mu\text{g/mL}$ (Fig. 3d and S8). Interestingly, even at the lowest concentration of 75 $\mu\text{g/mL}$ (equal to 62.5 $\mu\text{g/mL}$ MnO₂ NPs plus 12.5 $\mu\text{g/mL}$ rGO NSs), rGO@MnO₂ had a stronger cytotoxic effect to HeLa cells than MnO₂ NPs at 250 $\mu\text{g/mL}$ concentration after 24 h. Meanwhile, the corresponding bright field images after treatment for 24 h also evidenced very little amount of remaining living cells in the case of rGO@MnO₂ treatment (Fig. S9). The low concentration of the effective dose increases the biosecurity of this nanomaterial. Therefore, rGO@MnO₂ possess greater potential than MnO₂ NPs in cancer therapy.

In addition, the live/dead staining results also proved that rGO NSs possess high cytocompatibility, and MnO₂ NPs have only low lethality to HeLa cells, as there are still many live cells (Fig. 3e). However, after the treatment by rGO@MnO₂, a lot of cells were dead, and only some cells remained alive, which is consistent with cell viability measurement results. The enhanced lethality to cancer cells of rGO@MnO₂ at equivalent concentration of MnO₂ NPs is likely due to rGO NSs that can deliver more MnO₂ NPs into cells, leading to a stronger CDT effect.

To confirm that more MnO₂ NPs were taken up by HeLa cells due to rGO NS delivery, we performed TEM, ICP-MS, and XPS measurements. Due to the interference of background, it is difficult to clearly distinguish MnO₂ NPs in the TEM images, although there seemed to be more nanoparticles in vesicles (Fig. 4a and b). Interestingly, we could observe instead many rGO@MnO₂ inside the cells (Fig. 4c, red arrows), meaning that this complex can be easily internalized by HeLa cells. To further quantify the content of manganese into the cells, HeLa treated with MnO₂ NPs and rGO@MnO₂ were analyzed by ICP-MS and XPS. After rGO@MnO₂ treatment and extensive washing, a black residue was obtained, meaning that a lot of rGO@MnO₂ were taken up by HeLa cells (Fig. S10). According to the results of ICP-MS, the Mn content in control group was less than 0.016 pg/cell (Fig. 4d). After the treatment with MnO₂ NPs, the amount of Mn increased to ~ 0.48 pg/cell. Interestingly, the Mn content in cells treated by rGO@MnO₂ reached up ~ 1.75 pg/cell, which was over 3 folds as much as that of MnO₂ NP group. We also prepared a sample for XPS analysis by recovering the cells after MnO₂ NPs and rGO@MnO₂ treatment, then sonicating and lyophilizing the cell mixtures. Although the signal is noisy, the peak of Mn at ~ 642 eV in rGO@MnO₂ treated cells showed a higher intensity than that of MnO₂ NPs treated cells. The calculation of the areas under these peaks afforded a content of Mn

corresponding to ~1.7% and ~1.1% in rGO@MnO₂ treated cells and MnO₂ NP treated cells, respectively (Fig. S11). These two analyses confirmed a higher content of manganese in cells after culturing with rGO@MnO₂, meaning that more MnO₂ NPs were delivered into the cells by rGO NSs.

Fig. 3. Enhanced lethality of rGO@MnO₂ following intracellular delivery. (a-d) Viability of cells treated with rGO NSs, MnO₂ NPs, and rGO@MnO₂, respectively, at different equivalent concentration after 12, 24, 36, and 48 h. (e) Live/dead staining of HeLa cells treated with rGO NSs (25 μg/mL), MnO₂ NPs (125 μg/mL), and rGO@MnO₂ (150 μg/mL) after 48 h.

After cell internalization, MnO₂ NPs will oxidize GSH, and the generated Mn²⁺ ions will be able to convert H₂O₂ into HO• by Fenton reaction. To verify this hypothesis, the intracellular GSH/GSSG ratio was measured firstly to confirm that GSH was depleted. The GSH/GSSG ratio in control cells was ~13.6 (Fig. 4e), which is similar to previous reports [38, 39]. This value decreased to ~11.0 after the treatment with 125 μg/mL of MnO₂ NPs, meaning that part of GSH was depleted by these NPs. After incubation with rGO@MnO₂ (150 μg/mL), the GSH/GSSG ratio decreased dramatically to ~6.9, proving that more GSH was oxidized. In addition, we also measured the relative ROS level change in cells after MnO₂ NPs and rGO@MnO₂ treatment. There was no significant difference between the

control group and rGO NS group, suggesting that rGO NSs almost do not cause an increase of ROS (Fig. 4f). Using MnO₂ NPs, the ROS level was 1.8 folds as much as the control group. Surprisingly, the ROS level in rGO@MnO₂ group augmented by 8.6 folds and 4.8 folds as much as the control group and MnO₂ NP group, respectively. As a consequence, a higher GSH depletion and ROS generation accelerate cancer cell death, which is consistent with cell viability results. The mechanisms involved in the process are illustrated in Fig. 4g and h. During the treatment, only a small quantity of MnO₂ NPs was internalized by cancer cells, triggering a little GSH reduction and a weak Fenton reaction. However, the delivery action of rGO NSs is able to transport many MnO₂ NPs into the cells, causing a large of GSH depletion and a strong Fenton reaction. Large consumption of GSH and more HO• generation lead to cancer cells rapid death.

Fig. 4. rGO@MnO₂ mediated lethality to cancer cells by delivery effect. (a-c) TEM images of HeLa treated with MnO₂ NPs (125 μg/mL) and rGO@MnO₂ (150 μg/mL), respectively. Red arrows show the location of rGO@MnO₂. Scale bar: 500 nm. (d) Intracellular Mn content change after MnO₂ NPs and rGO@MnO₂ treatment analyzed by ICP-MS. (e) Intracellular GSH/GSSG ratio change after MnO₂ NPs (125 μg/mL) and rGO@MnO₂ treatment (150 μg/mL). (f) Intracellular ROS level change after

MnO₂ NPs and rGO@MnO₂ treatment. (f) Scheme of rGO NS-mediated delivery effect.

3.4. Synergistic effect of PTT and photothermal-enhanced CDT

To further confirm the synergistic effect of PTT and enhanced CDT, the cell viability was measured after NIR irradiation. The cell culture medium containing the nanomaterials was first discarded to avoid their interference and replaced by new medium. Then, the cells treated with rGO@MnO₂ were irradiated by 808 nm NIR light for 10 min. Equally, the medium in rGO NS group and MnO₂ NP group was also replaced by the new medium without materials. As shown in **Fig. 5a** and **b**, the cell viability in rGO NSs and MnO₂ NPs groups recovered to a certain degree after discarding the old medium containing nanomaterials, which we could attribute to the cellular redox self-regulation ability [40, 41]. On the contrary, rGO@MnO₂ still possess a high lethality to cancer cells, and the cell viability further decreased after NIR irradiation (Fig. 5c).

Fig. 5. Synergistic effect of PTT and photothermal-enhanced CDT. (a) Cell viability after rGO NS treatment. (b) Cell viability after MnO₂ NP treatment. (c) Cell viability after rGO@MnO₂ treatment with or without NIR irradiation. (d) Live/dead cell staining images. (e) GSH/GSSG ratio and ROS content after rGO@MnO₂ treatment with or without NIR irradiation.

Moreover, there were a lot of live cells and almost no dead cells after NIR irradiation in the absence of the nanomaterials (Fig. 5d). Even after replacing the medium, the number of live cells decreased remarkably and many dead cells appeared in rGO@MnO₂ group. Following NIR irradiation, more cells were ablated. Therefore, we can conclude that rGO@MnO₂ are able to mediate the combination of a photothermal/delivery effect enhanced CDT and PTT to kill cancer cells efficiently. Moreover, we measured significant changes in ROS level and GSH/GSSG ratio after NIR irradiation in rGO@MnO₂ group (Fig. 5e). These results demonstrate that rGO@MnO₂ caused photothermal-promoted ROS generation and GSH depletion. The photothermal effect enhances the Fenton reaction of Mn²⁺ ions, and the combination of the photothermal/delivery effect-enhanced CDT and PTT has higher synergistic efficiency to kill cancer cells.

4. Conclusion

In summary, highly dispersed rGO@MnO₂ were prepared by a simple electrostatic interaction method between MnO₂ NPs with negative charges and positively charged rGO NSs. MnO₂ NPs are able to oxidize intracellular GSH efficiently, and subsequently, the generated Mn²⁺ ions convert H₂O₂ into HO· by Fenton reaction. Due to the delivery effect of rGO NSs, we found that rGO@MnO₂ possess higher lethality to cancer cells than MnO₂ NPs alone at equivalent concentration by the efficient internalization. Furthermore, under NIR irradiation, rGO NS-mediated PTT could further kill cancer cells, and the increased temperature caused by photothermal conversion accelerated the Fenton reaction rate, enhancing the efficiency of MnO₂ NPs mediated CDT. Finally, the decreased concentration of the nanomaterials' effective dose would further improve biosecurity and reduce preparation costs. Therefore, rGO@MnO₂ have great potential in cancer combined therapy by the synergistic effect of PTT and photothermal/delivery effect-enhanced CDT.

CRedit authorship contribution statement

Baojin Ma: Performed Experiments, analyzed results analysis, and wrote the manuscript. **Yuta Nishina:** Provided GO, review and comments the results and manuscript. **Alberto Bianco:** Supervised the project, discussed the results and reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing for financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/>

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