PEGylated lipid bilayer-wrapped nanographene oxides for synergistic co-delivery of doxorubicin and rapamycin to prevent drug resistance in cancers

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Abstract
Nano-graphene oxide (nGO) is a carbon allotrope studied for its potential as carrier for chemotherapeutic delivery and its photoablation effects. However, interaction of nGO with blood components and the subsequent toxicities warrant a hybrid system for effective cancer drug delivery. Combination chemotherapy aids in effective cancer treatment and prevention of drug resistance. Therefore, in this study, we attempted to prepare polyethylene glycosylated (PEGylated) lipid bilayer-wrapped nGO co-loaded with doxorubicin (DOX) and rapamycin (RAPA), GOLDR, for the prevention and treatment of resistant cancers. Our results revealed a stable GOLDR formulation with appropriate particle size (∼170 nm), polydispersity (∼0.19) and drug loading. Free drug combination (DOX and RAPA) presented synergistic anticancer effects in MDA-MB-231, MCF-7, and BT474 cells. Treatment with GOLDR formulation maintained this synergism in treated cancer cells, which was further enhanced by the near infrared (NIR) laser irradiation-induced photothermal effects of nGO. Higher chromatin condensation and apoptotic body formation, and enhanced protein expression of apoptosis-related markers (Bax, p53, p21, and c-caspase 3) following GOLDR treatment in the presence of NIR laser treatment clearly suggests its superiority in effective chemo-photothermal therapy of resistant cancers. The hybrid nanosystem that we developed provides a basis for the effective use of GOLDR treatment in the prevention and treatment of resistant cancer types.

Supplementary material for this article is available online

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(Some figures may appear in colour only in the online journal)

1. Introduction
Graphene oxides (GO) are one of the rigorously studied nanostructures for drug delivery [16]. Currently, studies involving the dose-dependent toxicity of GO in cells or organisms are of prime interest [16, 20]. Large surface area, high drug loading capacity (LC) mediated by π−π interactions, and near infrared (NIR) induced photothermal effects make GO a potential candidate for cancer therapy [18, 19]. However, interaction of GO with blood components (RBC)
and healthy cell membranes lead to adverse effects [16]. Liposomal formulations are one of the widely studied lipid bilayer formulations, of which several are FDA approved marketed products [4, 8]. They are safe for intravenous delivery and affect blood components minimally, owing to the presence of PEGylated surface [6]. Therefore, engineering these systems as a single hybrid structure would be highly beneficial for effective and safer treatment of cancers.

The strategies for the treatment of cancer include approaches involving chemo-, radio-, photodynamic, and photothermal therapies [21]. Compared to single therapy, a combination treatment offers advantages in preventing possible resistance development, thereby minimizing cancer recurrence [15]. Combinational chemotherapy is employed for effective anticancer activity, and it is mediated by the combination of two or more molecular pathways that lead to the common goal of cancer cell apoptosis [3]. Furthermore, combination of dual chemotherapeutics along with photothermal effects would further enhance the anticancer effects and prevent any possible drug resistance mechanisms.

Doxorubicin (DOX), a potent anticancer agent, intercalates with the DNA double strand and stabilizes topoisomerase II, leading to cancer cell death. However, the ability of cells to activate cell survival pathways such as phosphoinositide-3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) leads to the development of drug resistance [11]. Considering this fact, it seems reasonable to investigate the effect of blocking this pathway on the overall response of DOX to cancer cells. Rapamycin (RAPA), an mTOR inhibitor, leads to the dephosphorylation of downstream targets of mTOR, resulting in the suppression of cell cycle and proliferation [5]. These evidences suggest the relevance of combining RAPA with DOX for improvement of anticancer effects.

In this study, we aimed to engineer a hybrid nanoparticle system comprising nano-GO (nGO) incorporated liposome in which DOX is loaded onto GO and RAPA onto the PEGylated liposomal lipid bilayer (GOLDR). A combination of dual chemotherapeutics along with NIR irradiation, which can induce photothermal effects of GO, were used for the treatment of resistant cancers.

2.2. Synthesis of nGO

nGO were produced using the spark discharge method as explained by Byeon et al [1]. The specifications of the spark discharge configuration were as follows: electrode (C-072561, Nilaco, Japan) length and diameter, 100 and 3 mm, respectively; capacitance, 1.0 nF; resistance, 0.5 MΩ; applied voltage, 3.0 kV; loading current, 2.0 mA; and frequency, 667 Hz. Spark discharge was applied for the production of graphite nanoparticles that were then carried to an impinging device using nitrogen gas (99.9999% purity, 31 min⁻¹). nGO was formed by reacting the graphite particles in the simplified Hummer’s solution (present in the impinging device) with the aid of an ultrasound probe. nGO was synthesized following oxidation of the graphite particles by 40 ml H2SO4 and 1.8 g KMnO4. Ultrasound (250 W cm⁻²) was applied to the graphite particles once they reach the gas (the graphite particle laden flow)-liquid (the simplified Hummer’s solution in the impinging device) interface. The time required to produce nGO in the impinging device was 3.8 min. The prepared nGO was analyzed for particle size and morphologically characterized using transmission electron microscopy (TEM).

2.3. Preparation of doxorubicin-loaded nGO (nGO/DOX)

The prepared nGO dispersion was used for loading DOX. A DOX loading dose of 1:1 w/w ratio for GO:DOX was added to the nGO dispersion and stirred in dark for 24 h. Then, the DOX-loaded nGO sheets were separated by using Millipore Ultracentrifugal device (MWCO 10 000) and dispersed in the desired volume of distilled water to obtain the final nGO/DOX dispersion.

2.4. Preparation of GOLDR formulation

The prepared nGO/DOX dispersion in distilled water was used for the preparation of GOLDR. Lipid phase constituting RAPA, DPPC, CHO and DSPE-PEG was dissolved in a mixture of chloroform:methanol (4:1) and then rotary evaporated at 60°C under vaccum. Thus obtained thin lipid film was subsequently hydrated using nGO/DOX aqueous dispersion. Then, large unilamellar vesicles were extruded using a mini-extruder (Avanti Polar Lipids, Inc, Alabaster, AL, USA) to obtain a monodisperse GOLDR formulation (figure 1(a)).

2.5. Hydrodynamic particle size, polydispersity and zeta potential analysis

The particle size, polydispersity, and zeta potential of GOLDR formulation were determined using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK), which utilized dynamic light scattering technique for measurement of each sample with at least three sets of 13 runs.
2.6. Morphological evaluations

TEM (H7600, Hitachi, Tokyo, Japan) was used for the determination of cross-sectional morphology of GOLDR. Briefly, a drop of GOLDR dispersion (mixed with 2% phosphotungstic acid) was placed on carbon-coated copper grid and dried under IR radiation. This grid was then viewed under the microscope at an accelerating voltage of 100 kV.

2.7. Fourier transform infrared (FTIR) spectroscopy analysis

GOLDR formulations were characterized by the FTIR spectroscopy using our previously described method [17].

2.8. Determination of drug loading

The drug entrapment efficiency (EE) was calculated by separating the unbound drugs from the drug-loaded nanoparticles using an ultrafiltration device (MWCO 10 000 Da). The nanoparticulate dispersion was centrifuged at 5000 rpm for 10 min, and the unbound drugs in the filtrate were analyzed using a high-performance liquid chromatography (HPLC) method. A Hitachi L-2400 equipped with a C18 column (5 μm, 4.6 × 150 mm) was used for the analysis. An isocratic elution with a mobile phase comprising methanol: acetonitrile:acetic acid (1%) at a ratio of 50:49:2 (v/v/v) was used for the determination of DOX content. Similarly, RAPA
content was determined using a mobile phase constituting ammonium acetate (10 mM); acetonitrile (30:70, v/v) at a flow rate of 1 ml min\(^{-1}\).

The drug LC and EE were calculated using the following equations:

\[
\text{LC(\%)} = \frac{W_{\text{drug in NP}}}{W_{\text{NP}}} \times 100, \quad (1)
\]

\[
\text{EE(\%)} = \frac{W_{\text{drug in NP}}}{W_{\text{total drug}}} \times 100, \quad (2)
\]

where, \(W = \) weight and NP = nanoparticles.

2.9. In vitro drug release study

The drug release profiles from GOLDR formulation were determined using the dialysis method. Release media simulating physiological pH (phosphate buffered saline (PBS), pH 7.4) and tumor pH (acetate buffered saline (ABS), pH 5.0), supplemented with 1.0% Tween 80 to maintain sink conditions were used. Briefly, 1 ml GOLDR formulation was sealed in Spectra/Por\textregistered dialysis membrane (MWCO 3500) and placed in the release media at 37 °C with stirring rate of 100 rpm. At predetermined time intervals, the samples were withdrawn and replaced with equal amounts of fresh media. The amounts of DOX and RAPA released were determined using the HPLC method as described previously.

2.10. In vitro cell viability assay

The in vitro cytotoxicity of free drugs and GOLDR was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, USA). Briefly, 1 \times 10^4 cells were seeded into 96-well plates. These cells were then incubated for 24 h, treated with different concentrations of DOX, RAPA, DOX + RAPA (DR), and GOLDR with or without NIR (808 nm, 3.0 W cm\(^{-2}\) for 5 min) irradiation, and further incubated for 24 h at 37 °C. Finally, the cells were treated with MTS solution. Untreated cells served as the control, and the absorbance was measured at 493 nm using an automated microplate reader to determine the cell viability.

2.11. Cellular uptake study

The qualitative cellular uptake of GOLDR in MDA-MB-231, MCF-7, and BT474 cell lines was studied using a confocal laser scanning microscope (CLSM, Leica Microsystems, Wetzlar, Germany). Initially, the cells were seeded separately on coverslips placed in 12-well plates and incubated for 24 h. Then, GOLDR was added to each cell line, incubated for 30 min, followed by the addition of LysoTracker green, and further incubated for 10 min. The media was then removed. The cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, washed with PBS, and stained with Hoechst 33342 for 30 min. Finally, the cells were rinsed with PBS and observed under an inverted fluorescence microscope (Nikon Eclipse Ti).

2.12. Cell cycle and apoptosis

Cell cycle analysis of untreated and treated cells was performed using the Cell Clock\textsuperscript{TM} Assay kit (Biocolor Ltd, UK), which can be used for live-cell detection and analysis of the four major phases (G1, S, G2, M) of mammalian cell cycle during in vitro culture. Live cancer cells in 12-well plates were treated with redox dye (Cell-Clock Dye Reagent) for 1 h at 37 °C, and images were taken using the DMIL LED microscope. The images of cell cycle were used for the qualitative evaluation of apoptosis in cells treated with free drugs or formulations.

Furthermore, cellular apoptosis was determined by using AnnexinV/7-aminoactinomycin D (7AAD) kit. Briefly, the breast cancer cells (MDA-MB-231, MCF-7, and BT474) were treated with free drugs, combination, and GOLDR formulation. Then, the cells were harvested, washed with PBS twice, stained with Annexin V and 7AAD for 15 min, and examined using FACS.

2.13. Determination of morphological changes in the nucleus

Cancer cells were seeded into 6-well plates and incubated for 24 h. Then, the cells were treated with free drugs, combination, and GOLDR with or without NIR (808 nm, 3.0 W cm\(^{-2}\) for 5 min) and incubated further for 24 h. The cells were then washed twice with PBS, fixed in 4% paraformaldehyde, washed with PBS, and stained with Hoechst 33342 for 10 min. Finally, the cells were rinsed with PBS and observed under an inverted fluorescence microscope (Nikon Eclipse Ti).

2.14. Western blot analysis

The expression of apoptotic markers following treatment with free drugs or GOLDR was determined by using western blot analysis. The cells grown in 6-well plates were treated with free drugs or GOLDR formulation, washed with PBS, and harvested. Then, the cells were lysed using sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). The proteins were extracted, and then separated using SDS-PAGE gel. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bellerica, MA). The membrane was blocked with 5% dry milk in Tris-buffered saline with 1% Tween-20 (TBST) and incubated with a specific primary antibody. The membrane was then washed three times with TBST and incubated with the secondary antibody. Finally, protein expression was detected using chemiluminescence detection reagents via imaging in Kodak imaging film (Kodak, USA).
liposomes were spherical in shape with an approximate particle size of 120 nm (figure 2(b)). The particle size increased slightly with the incorporation of nGO into the liposomes (figure 2(c)). Furthermore, the addition of RAPA and DOX in the system further increased the particle size of GOLDR formulation (figure 2(d)).

3.2. Characterization of photothermal, spectroscopic and drug release behaviors

The UV–vis–NIR absorption of GO and GOLDR are shown in supplementary figure S1 available online at stacks.iop.org/NANO/28/295101/mmedia for the same mass concentration (0.25 mg ml\(^{-1}\)). A typical absorption peak at 230 nm with broad absorption spectrum in the NIR range was evident for nGO, which could contribute to NIR laser-induced photothermal effects. GOLDR also presented similar UV–vis–NIR spectrum with slight reduction in absorbance. Initially, different concentrations of nGO were irradiated with NIR for defined time points, and the results suggested a concentration- and time-dependent temperature elevation of the system (figure 3(a)). When nGO was incorporated within the lipid bilayer, not much change in the pattern of thermal heat induction for temperature elevation was observed upon NIR irradiation, suggesting the possibility of using GOLDR for exerting photothermal effects (figure 3(b)). FTIR spectroscopy measurements of GOLDR formulation presented characteristic peaks of liposomes with no interference peaks of nGO, DOX and RAPA suggesting their successful incorporation within the system (figure 3(c)). In vitro drug release studies were performed to evaluate the drug release patterns of DOX and RAPA in different pH conditions (figure 3(d)). The drug release profiles for both drugs were significantly higher at pH 5.0 compared to those at pH 7.4.

3.3. Cellular cytotoxicity and uptake studies

Free DOX and RAPA presented concentration-dependent cell cytotoxicity (figures 4(a)–(c)). Differences in cell cytotoxicities observed among the cell lines were in the order of MDA-MB-231 > BT474 > MCF-7. Combination of DOX and RAPA enhanced cell cytotoxicity in all the three cell lines similar to that of the free drug. The determination of combination indices suggested that synergistic effects were observed in all three cell lines, with highest degree of synergism in MDA-MB-231 and least in MCF-7 cells. Treatment of all cell lines with GOLDR formulation led to the maintenance of synergistic effects as presented by their respective combination indices. Furthermore, NIR irradiation improved cellular cytotoxicities in all three cell lines, suggesting the beneficial effect of combining chemo- and photothermal therapy in treating resistant cancers.

Confocal images clearly demonstrated the uptake of GOLDR formulation in the lysosomes of cancer cells (figure 4(d)). Furthermore, the evaluation of quantitative cellular uptake performed with FACS clearly demonstrated a concentration- and time-dependent uptake of GOLDR formulation in all three cell lines (supplementary figure S2).
3.4. Evaluation of apoptosis in cancer cells

The optical images of different cancer cells stained with Cell Clock™ are shown in figure 5(a). The control for all three cancer cell lines presented cells with different colors, suggesting their distribution in G1, S, G2 and M phases. Treatment of cells with DOX led to cell death as presented in the images for all three cell lines. Combination of free DOX and RAPA increased cellular apoptosis further, as presented by the remaining number of live cells and their morphologies. GOLDR formulation was almost equipotent to free drug combination in inducing cellular apoptosis. NIR irradiation significantly reduced the number of live cells, and the morphology of the remaining cells was highly distorted, suggesting the beneficial effects of using the combination chemo- and photothermal therapy.

FACS analysis of the treated cells for determination of cellular apoptosis is presented in figure 5(b). As expected, DOX treatment led to an increase in the number of apoptotic cells in all three cell lines and the results were consistent with those obtained by MTS assay. RAPA treatment led to a relatively smaller number of cells in the apoptotic region. Combination of DOX and RAPA led to a significant increase in the number of apoptotic cells in early and late apoptosis phase. Finally, NIR irradiation of cells treated with GOLDR formulation enhanced the number of apoptotic cells, and in particular, improved the number of cells in the late apoptosis phase. These results support the rationale of using a combination of chemotherapeutics and photothermal therapy for the effective treatment of resistant cancers.

Further detailing of the apoptosis induction was observed using Hoechst 33324 staining of the nuclei following treatments with free drugs, combination, or GOLDR formulation. Homogenous staining of the nuclei and cytoplasm were observed in the control cells, whereas the treated cells exhibited condensation of chromatin and apoptotic body formation (figure 6(a)). Compared to free drugs, their combination and GOLDR formulation presented higher number of cells with chromatin condensation and formation of apoptotic bodies, suggesting synergistic anticancer effects. NIR irradiation reduced the number of live cells as observed in the images; however, the morphology of apoptotic cells was still maintained, proving the synergistic effects of chemo- and photothermal therapy combination further.

3.5. Expression of pro- and anti-apoptotic proteins in cancer cells

A further investigation of different proteins expressed in cancer cells was carried out using western blot analyses (figure 6(b)). The expression of pro-apoptotic proteins (Bax, p53, p21, c-caspase 3) and anti-apoptotic protein (BCl2) [18]
was evaluated in cells treated with either free drugs, combination, or GOLDR formulation. The levels of all pro-apoptotic proteins were increased following treatment with combination and GOLDR formulation. In addition, the expression of anti-apoptotic protein was reduced.

4. Discussion

In this study, we aimed to develop a combination of chemotherapeutics and photothermal therapy for the treatment of resistant cancers. nGO of the appropriate size and concentration, suitable to be incorporated within the lipid bilayer system, was synthesized using the spark discharge method. Simultaneously, optimization of liposomes, GOL, and GOLDR formulations were carried out.

The slight increase in particle size of GOL compared to that of blank liposomes could be attributed to the successful incorporation of nGO into the liposomes. Following the optimization of GOL formulation, dual drug-loaded nanoparticles were prepared. A negligible increase in particle size was observed with the addition of DOX in the system. DOX can interact with GO through $\pi-\pi$ interactions leading to high drug loading onto the GO surface [14, 22]. Therefore, no much interference was developed by DOX in the aqueous core of liposomes thus maintaining the nano size of the system. Incorporation of RAPA further increased the particle size of GOLDR formulation. RAPA is lipophilic in nature [10] and can accommodate itself in the lipid bilayer system thus increasing the particle size of GOLDR formulation.

TEM imaging was performed for the morphological evaluation of GOLDR formulation that suggested successful preparation of the system. UV–vis–NIR spectra for nGO presented a peak at 230 nm, contributed by the $\pi$-plasmon of carbon [16]. A broad spectrum absorbance in the NIR range for nGO indicates promising laser-induced photothermal effects. The UV–vis–NIR spectrum of GOLDR exhibited similar pattern with nGO suggesting its potential for the induction of photothermal effects. NIR irradiation (3.0 W cm$^{-2}$) of nGO and GOLDR formulations was performed to determine the photothermal effects of the system. Even after the nGO incorporation within GOLDR formulation, there was no significant reduction in the thermal heat induction by the nGO following NIR irradiation. nGO possesses optical absorption properties that can convert NIR irradiation to heat which can be used for thermal ablation of the treated cancer cells [18]. FTIR analysis was used for further characterization of GOLDR formulation. Blank liposomes and nGO presented their characteristic peaks. After the incorporation of nGO within the lipid bilayer system, the characteristic peaks of nGO were not observed, suggesting their successful accommodation within the core of the system.

Figure 4. In vitro cytotoxicity of various formulations with/without NIR irradiation (808 nm, 3.0 W cm$^{-2}$ for 5 min in (a) MDA-MB-231; (b) MCF-7; and (c) BT474 cells. A 1:1 w/w ratio of DOX and RAPA was used for combination chemotherapeutics treatment (CI = combination index was calculated using Compusyn software). (d) Confocal imaging for the determination of cellular uptake of GOLDR in MDA-MB-231, MCF-7, and BT474 cells (scale bar: 20 $\mu$m). Red color refers to DOX and green refers to LysoTracker.
the successful loading of both the drugs within the formulation. In vitro drug release profiles presented significantly higher DOX and RAPA release in acidic pH conditions. Tumor microenvironment possess acidic pH [17] and our obtained results suggest the suitability of our system for targeted drug release within the cancer cells. 

The viabilities of MDA-MB-231, MCF-7, and BT474 cells were carried out using MTS assay. Differences in the degree of synergism between MDA-MB-231, MCF-7 and BT474 cells could be attributable to the differential regulation of PI3K/Akt-SKP2-Cip/Kips pathway [7] following treatments with DOX and RAPA. Cellular uptake studies were carried out using confocal imaging and FACS. A concentration- and time-dependent lysosomal uptake of GOLDR formulation was evident, which is highly beneficial for targeted-drug release within the cancer cells, where acidic pH governs the tumor microenvironment [13, 15].

Visual inspection of apoptosis following treatments with free drugs, combinations, or GOLDR formulation was performed using Cell Clock™ Assay followed by imaging under the optical microscope. Cell Clock™ Assay is mainly used for cell cycle analysis based on color differences in the appearance of treated cells, presenting their distribution in G1, S, G2 and M phases. RAPA is an mTOR inhibitor that mainly acts by inhibiting the process of cell cycle [2]. Therefore, in all three cell lines there was an increase in the number of cells in the G1 phase resulting from cell cycle arrest mediated by mTOR inhibition. DOX treatment led to an increase in the number of apoptotic cells in all three cell lines and RAPA addition further led to a significant increase in the number of apoptotic cells in early and late apoptosis. A combination of two different pathways, stabilization of topoisomerase II complex and mTOR inhibition, led to beneficial anticancer effects [12]. However, to eliminate the possibilities of drug resistance mediated by different drug efflux proteins, such as Pgp and breast cancer receptor protein, we prepared a GOLDR formulation that could release DOX and RAPA in a controlled manner for the efficient induction of cellular apoptosis even in resistant cancer cells. GOLDR presented apoptotic level similar to free drug combination in the treated

Figure 5. (a) Microscopic images of MDA-MB-231, MCF-7, and BT474 cells stained with Cell Clock™ Assay (scale bar: 100 μm) and (b) FACS analyses for the determination of cellular apoptosis following treatment with different formulations (DOX, RAPA, DR, and GOLDR with or without NIR: 808 nm, 3.0 W cm⁻² for 5 min).
cells. Considering the fact that DOX and RAPA can be released in a controlled manner inside the cells, we can expect even higher cellular apoptosis if tested for longer treatment times. Chromatin condensation and apoptotic body formation are typical characteristics of drug-induced apoptosis [9]. The cells treated with free drugs, combination, or GOLDR formulation presented these characteristics, which suggest synergistic anticancer effects. In addition, NIR irradiation reduced the number of live cells, but the morphology of apoptotic cells was maintained. Cancer cells express different types of pro-apoptotic and anti-apoptotic proteins. Augmentation in the levels of pro-apoptotic proteins clearly suggests the efficient anticancer effects of GOLDR formulation in sensitive and resistant cancer cells. Furthermore, the reduction in the expression of anti-apoptotic protein supports the rationale of using drug combination along with phototherapy. A schematic representation for the potential anticancer effects of GOLDR formulation, in the treatment of resistant cancers, is presented in supplementary figure S3.

5. Conclusions

Our first attempt in the preparation of nGO-incorporated liposomes, which can be employed for chemo- and photothermal combination therapies of resistant cancers, was successful. GOLDR formulation presented appropriate particle size, high drug loading, and pH-responsive drug release characteristics that favored cancer treatment. Compared to monotherapy, DOX and RAPA combination therapy presented synergistic anticancer effects in sensitive and resistant cancer cells as evidenced by the reduction in the number of viable cells. GOLDR formulation maintained this synergistic anticancer effect, which was further enhanced by the photothermal effect developed upon NIR laser irradiation of the incorporated nGO. Chromatin condensation and apoptotic body formation were promoted in GOLDR-treated breast cancer cells with better activity upon NIR laser irradiation, when compared to that in free drug combinations. Notably, upregulation of apoptotic proteins (Bax, p53, p21, and c-caspase 3) and downregulation...
of anti-apoptotic protein (BCl2) upon treatment with GOLD-R + NIR suggest the possible applicability of the developed GOLDR formulation for effective treatment of cancers, by overcoming drug resistance through a combination of chemo-photothermal therapy.

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