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Dual Aptamer Modified Dendrigraft Poly-L-lysines Nanoparticle for Overcoming Multi-drug Resistance through Mitochondrial Targeting

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A smart dendrigraft-poly-L-lysines (DGL) nanoparticle for mitochondria-targeted chemotherapy was devised, which aims to achieve enhanced efficacy against drug resistance tumor cells. In this system, doxorubicin (Dox) was intercalated into the DNA duplex containing an ATP aptamer, which subsequently condensed by DGL to form a nanoscaled controlled-release system. A nucleolin-specific binding aptamer, AS1411 and a cytochrome c aptamer were then incorporated into the system to give the nanoparticles (Dox/Mito-DGL) for biological evaluations. This dual modified system has been shown to selectively accumulate in the mitochondria of cancer cells and promptly release the loaded Dox in virtue of the high concentrations of ATP in mitochondria. The mitochondria-specific and spatiotemporally controlled release of Dox led to enhanced therapeutic outcomes both in vitro and in vivo. More significantly, Dox/Mito-DGL was successfully applied to improve the efficacy towards multi-drug resistance cancer cells by altering mitochondrial membrane potential and bypassing the P-glycoprotein-mediated drug efflux. This work presents a paradigm for mitochondria-targeting therapy against mitochondria-associated diseases and provides a potential avenue for overcoming MDR in the treatment of solid tumors.

Introduction

The mitochondrion is a vital cellular organelle that plays essential roles in a variety of critical physiological processes. It is, for example, an integration point in cell metabolism, including the modulation of calcium homeostasis, fatty acid oxidation, amino acid metabolism, redox signaling and so on. On the other hand, mitochondria as a decisive regulator of cell death pathways are also involved in pathophysiological processes. Many human diseases are closely related to mitochondrial dysfunction, especially cancer. It is reported that mitochondrial dysfunction is implicated in the process of carcinogenesis because of its crucial role in DNA alterations, cell proliferation, apoptosis resistance, and several other key events in cancer cells. Thus, mitochondria-targeted treatment could lead to novel and effective therapy for aggressive cancers. More significantly, mitochondrial targeting potentially offers an opportunity to overcome multi-drug resistance (MDR) of cancer cells. In MDR cells, the mitochondrial membrane potentials are normally higher than drug sensitive cells, and energy production rely on glycolysis due to the inhibition of oxidative phosphorylation. These metabolic shifts not only cause apoptosis resistance but also provide potential opportunities for exploring mitochondria as a potential therapeutic target to overcome tumor resistance. For example, several mitochondria-targeted drugs were employed to selectively kill MDR cancer cells by modulating the abnormal cellular metabolism and altering mitochondrial membrane potential. However, to the best of our knowledge, most of the mitochondria-targeted chemotherapy systems for overcoming MDR were based on platinum drugs and chemical modifications on drug molecule. To extend the mitochondria-targeted therapy in MDR tumor to other types of chemo-therapy drugs, a versatile strategy for mitochondria-targeted drug-delivery is highly desired.

For mitochondria-targeting, the primary challenge is the difficulty in traversing its double-membrane structure. Thus, developing new delivery vectors that can penetrate the barriers protecting mitochondria is essential for mitochondria-targeted therapy and bio-imaging. The ideal organelle-specific targeting vector should possess such features as high drug loading dose, small particle size, good biocompatibility and facile access to chemical modification with targeting ligands. Given all this, biocompatible dendritic poly(L-lysines) (DGL) bearing multiple functional groups have emerged as a promising vector for targeted drug/DNA delivery. DGL, which exhibits a highly branched, 3D architecture and comprise an initiator core, several interior layers composed of repeating units, and rich external amino groups, becomes one of the most versatile compositionally and multifunctional nanoscale drug/DNA vectors. Comparing with the traditional nanocarriers,
DGL self-assembling nanoparticles hold great superiority in targeted therapy at subcellular level due to its small size and the presence of numerous surface groups. These groups could be modified with polyethylene glycol and targeting ligand to achieve long circulation and mitochondria-targeting properties. In particular, DGL nanoparticles (DGL NP) possess a greater capability to facilitate the transport of agents across various cell membranes via endocytosis, and then functions as a proton sponge to promote endolysosomal escape of DGL NP by causing osmotic swelling and rupture of the endosome membrane. These characteristics of DGL NP afford to an attractive nanocarrier for constructing mitochondria-targeted drug-delivery systems.

As a part of our research interests in searching for smart and targeted delivery of anticancer drugs, herein, we developed a mitochondria-targeted DGL self-assembling NP (Dox/Mito-DGL) for controlled drug release, and for overcoming MDR in tumor cells. Considering mitochondrion is the cellular powerhouse and produce excess of Adenosine-5’-triphosphate (ATP) at the inner mitochondrial membrane, ATP was chosen as the stimulus to trigger drug release in this system. Firstly, Doxorubicin (Dox) as a model anticancer drug was intercalated into the DNA duplex hybridized by the ATP aptamer and its complementary single-stranded DNA to form a stable complex. Secondly, Dox/Duplex complex was condensed by DGL to form a nanoscaled self-assembling delivery system. Cyt c is normally bound to the inner mitochondrial membrane by anionic phospholipid cardiolipin. A DNA aptamer that showed specific binding to Cyt c through systematic evolution of ligands by exponential enrichment (SELEX) was reported recently. Thus, a cytochrome c-specific binding aptamer (Cyt c aptamer) in combination with a nucleolin-specific binding aptamer AS1411 were linked to PEG-modified DGL as active-targeting ligand to achieve tumor and mitochondria dual-targeted properties (Scheme 1a). After injected into the blood vessel, the Dox/Mito-DGL could be selectively taken up by nucleolin-overexpressed tumor cells, and subsequently deliver Dox/Duplex directly into mitochondria by taking the advantages of Cyt c aptamer. As a result, the Dox/Mito-DGL could selectively unload the encapsulated Dox/Duplex and induce dissociation of the DNA duplex upon the high levels of ATP in mitochondria, which thereby causes rapid release of Dox (Scheme 1b). The integrating utilization of dual-targeted aptamer and ATP-controlled drug release led to a significant enhancement of chemotherapy selectivity and efficiency to cancer cells. Moreover, Dox/Mito-DGL which acts directly on mitochondria, can overcome MDR by changing the mitochondrial membrane potential and bypassing abnormal membrane protein trafficking. To the best of our knowledge, this appears to be the first example showing that a general strategy can be designed to achieve not only mitochondria-targeting drug release but also highly efficient chemotherapy against MDR cancer.

Results and discussion

Dox/Duplex and Dox/Mito-DGL Characterization

Dox was intercalated into the double-stranded DNA which hybridized with an ATP aptamer and its complementary DNA to formulate the Dox/Duplex. The fluorescence and cytotoxicity of Dox was quenched when inserted into the duplex, and will become activated in the presence of ATP, leading to a fluorescence increase along with the Dox release. Spectroscopic evaluation of the amount of Dox loaded into the duplex was performed by monitoring the fluorescence of Dox upon incubation with duplex (λ_em=535 nm, λ_ex=595 nm), showing that the fluorescence intensity decreased gradually with the increasing concentration of duplex (Fig. 1a). The Dox/Duplex binding ratio was deduced to be about 4:1 by Job plot (Fig. S1). The responsiveness of Dox/Duplex to ATP was also investigated by fluorescent monitoring. As shown in Fig. 1b, a significant fluorescence recovery was observed upon treatment of Dox/Duplex with increased concentrations of ATP, which suggested the release of Dox triggered by ATP. In contrast, the fluorescence did not change evidently when the ATP aptamer was replaced by a control DNA sequence of equal length, which further proved that the Dox release was induced by specific interaction between ATP and its aptamer (Fig. 1c). To study the selectivity of Dox/Duplex toward ATP, the effect of interference of different concentrations of ATP analogues was detected. Dox/Duplex exhibited a remarkable increase in fluorescence intensity upon reaction with ATP, whereas the response toward other bioanalytes was negligible, suggesting the high specificity to ATP (Fig. 1d).

To prepare Dox/Mito-DGL, DGL-PEG-aptamer was diluted to 600 mg/mL in phosphate buffer (pH 7.4). Dox/Duplex complex solution (100 mg/mL, 50 mM sodium sulfate solution) was added at mass ratio of 6:1 (DGL to DNA) and immediately vortexed for 30 s at room temperature. Dox/Mito-DGL was characterized after purifying by ultra filtration using a 30 kDa molecular weight cut off membrane. The morphology of Dox/Mito-DGL was analyzed by...
transmission electron microscopy (TEM). As shown in Fig. 2a, Dox/Mito-DGL NPs were well dispersed with spherical morphology. The dynamic light scattering (DLS) showed that the average hydrodynamic diameter of Dox/Mito-DGL was 38 nm (Fig. 2b). The size was small enough for mitochondria-targeted drug delivery systems as the diameter of mitochondria is about 0.5-10 μm. To evaluate the stability of Dox/Mito-DGL in physiological environments, the NPs were suspended in two commonly used biological media: Dulbecco's Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 10% fetal bovine serum (FBS). Nanoparticle size was measured by DLS. No significant size change was observed after incubation of the NPs in these media for at least 6 days (Fig. S2), suggesting the Dox/Mito-DGL is integrate before they go across the membrane.

The ζ potential results showed that DGL was positively charged with a surface charge of 3 mV, due to the protonated amino group at the surface of DGL, suggesting that DGL was suitable for encapsulating DNA duplex to neutralize its negative charges. After modification with the AS1411 aptamer and Cyt c aptamer, the ζ potential of DGL changed from 3 to 2.5 mV, confirming the presence of aptamers at the surface. The positive charge favors the binding with cell membrane, which could facilitate the adsorptive-mediated endocytosis. Furthermore, when DGL NPs derivatives were complexed with Dox/Duplex to form Dox/Mito-DGL, the ζ potential was changed to 1.5 mV. The positive charge may make it easier to penetrate into cell membrane, which facilitate the cellular internalization of Dox/Mito-DGL.

The Cellular Selectivity and Localization of Dox/Mito-DGL

In order to track Dox/Mito-DGL in living cancer cells, the Cyt c aptamer on Dox/Mito-DGL surface was labeled by Cy 5.5, a near-infrared fluorescent dye, which was also suitable for in vivo imaging in this system due to the deep penetration depth of its wavelength. Nucleolin-overexpressed HeLa cells were used to investigate the cellular internalization of Dox/Mito-DGL, as shown in Fig. 3, remarkable red fluorescence was observed in the cytoplasm after incubation with 50 μg mL⁻¹ Dox/Mito-DGL for 3 h. The result indicated that the Dox/Mito-DGL can be easily internalized by HeLa cells. To demonstrate that the cellular uptake of Dox/Mito-DGL was attributed to the specific binding of AS1411 aptamer to nucleolin on tumor cells, the cellular internalization of Dox/Mito-DGL without AS1411 was also investigated by confocal imaging. After incubated with 50 μg mL⁻¹ Dox/Mito-DGL (no AS1411) for 3 h, the cells showed a very weak fluorescence signal, confirming the essential role of the aptamer in the recognition of nucleolin-rich cancer cells. To study the selectivity of Dox/Mito-DGL to distinguish tumor cells from normal cells, HaCaT cells as model were also used for the bioimaging experiment of Dox/Mito-DGL. Compared to HeLa cells, the fluorescence in cytoplasm was again negligible, suggesting that Dox/Mito-DGL selectively internalized into the nucleolin-overexpressed tumor cells due to the specific binding to the nucleolin transmembrane protein.
Furthermore, the subcellular localization of Dox/Mito-DGL was investigated by fluorescence imaging on HeLa cells. As shown in Fig. 4, cells incubated with 50 μg mL⁻¹ Dox/Mito-DGL for 3h showed remarkable fluorescence in discrete subcellular locations as revealed by confocal microscopy. Co-staining experiments with LysoTracker Green, GolgiTracker Green, Hoechst 33342, and MitoTracker Green (commercially available organelle green-fluorescent probe) has been carried out to determine the distribution of Dox/Mito-DGL. The overlay images showed that Dox/Mito-DGL was retained in the mitochondria of living tumor cells (colocalization efficient=90.6%), whereas negligible portion of Dox/Mito-DGL was distributed in lysosome (colocalization efficient=5.2%), Golgi apparatus (colocalization efficient=4.1%) or nucleus (colocalization efficient=0.8%). After an additional 3h of incubation, the mitochondrial fluorescence gradually decreased, and the cytoplasm fluorescence became brighter (Fig. S3), suggesting that the DGL transported to the cytosol after Dox/Mito-DGL were disassembled and released Dox. Similar results were observed from the same experiment on PC3 cells (Fig. S4). Thus, this mitochondria-targeting capability of Dox/Mito-DGL provides a unique drug delivery system employing mitochondria as therapeutic target.

Evaluation of the In Vitro Drug Release and Anti-cancer Efficiency of Dox/Mito-DGL

To evaluate the effectiveness of Dox/Mito-DGL for ATP-triggered drug-release applications, the in vitro Dox release kinetics of Dox/Mito-DGL with and without ATP was determined by a fluorescence spectrometer. When Dox/Mito-DGL were suspended in HEPES buffer solution with 5 mM ATP, the amount of released Dox increased with the incubation time, reaching a plateau after 16h incubation. After incubated with ATP for 24h, more than 50% of Dox originally encapsulated within Dox/Mito-DGL was released into the medium. In contrast, the amount of Dox released from Dox/Mito-DGL in the absence of ATP was much lower after 24h (Fig. 5a), suggesting that the drug release was specifically induced by ATP. To further prove that the significantly higher drug release was caused by specific binding of ATP and its aptamer, the release behavior of Dox/Mito-cDGL which prepared by replacing the duplex in Dox/Mito-DGL to the non-ATP-responsive duplex, was also investigated by the same method. After incubation with 5 mM ATP for 24h, a much smaller amount of Dox was released from Dox/Mito-cDGL compared to Dox/Mito-DGL, which suggest that the ATP aptamer played an essential role in drug-controlled release.

High antitumor efficacy is the necessary property of a drug-delivery system. Confocal fluorescence imaging was used to monitor the release of Dox in HeLa cells. As shown in Fig. S5, red fluorescence was observed in the mitochondria after incubation for 4h, suggesting that Dox were released from Dox/Mito-cDGL triggered by ATP in mitochondria. Furthermore, to compare the in vitro cytotoxicity of Dox/Mito-DGL and free Dox, cell viability was assessed by the MTT assay in HeLa cells. As shown in Fig. 5b, the IC50 value of Dox/Mito-DGL was relatively lower than that of free Dox. Since Mito-DGL showed negligible cytotoxicity, it can be concluded that integrating Dox into a subcellular therapeutic system can improve the antitumor activity of Dox. On the other hand, non-ATP-sensitive Dox/Mito-cDGL was basically non-cytotoxic at the same concentration with Dox/Mito-DGL. Similar results were observed from the same experiment on PC3 cells (Fig. S6). These results indicated that the enhanced cytotoxicity of Dox/Mito-DGL was attributed to the prompt drug release specifically triggered by the high level of ATP in mitochondria.

The Application of Dox/Mito-DGL for Overcoming Multi-drug Resistance in Cancer Cells

The development of MDR in many cancers poses a significant obstacle to many forms of chemotherapy. It is reported that many clinically observed MDR phenotypes, especially cancer resistant to Dox, are proposed to arise from drug mediated by P-glycoprotein (P-gp) pumps. Thus, mitochondrial targeting of anticancer drug delivery may be a new avenue to evade the acquired tumor...
resistance through sequestration of the drug into mitochondria. Herein, a lipophilic cationic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine-iodide (JC-1) \(^2\), which is widely used to determine the early stage of apoptosis was adopted to evaluate the ability of Dox/Mito-DGL to promote apoptosis in cancer cells by monitoring the changes of mitochondrial transmembrane potential. In healthy cells with high mitochondrial membrane potential, JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. On the other hand, in apoptotic or unhealthy cells with low mitochondrial membrane potential, JC-1 remains in the monomeric form, which shows only green fluorescence. As shown in Fig. 6, the mitochondrial membrane potential of cells dramatically decreased after 24-h incubation with Dox/Mito-DGL or free Dox, suggesting that Dox/Mito-DGL as well as free Dox had high antitumor activity on the drug-sensitive MCF-7 cells. But there was no significant effect for free Dox on the drug-resistant MCF-7/ADR cells under the same condition. Notably, prominent apoptosis was observed in MCF-7/ADR cells after treatment with Dox/Mito-DGL for 24 h, indicating the capability of Dox/Mito-DGL for overcoming MDR in cancer cells. The results were further confirmed by MTT assay (Fig. 57 and Fig. 58), which validate that the antitumor activity of Dox against MDR tumor cells was significantly improved after incorporating into a mitochondria-targeting smart nano-system. Similar results were observed from the same experiment on HeLa cells and the Dox-resistant HeLa/ADR cells (Fig. 59 and Fig. 60). The enhanced therapeutic efficacy in MDR cells may be attributed to evasion of P-gp resistance mechanism that induced by mitochondrial sequestration of Dox. Moreover, as mitochondria play a major role in cell apoptosis, the ability of Dox/Mito-DGL to overcome MDR effect can be also attributed to the direct action on the cell death machinery.

To evaluate the in vivo tumor-targeting efficiency of Dox/Mito-DGL, non-invasive near-infrared optical imaging was performed at different time points in subcutaneous HeLa tumor-bearing BALB/c nude mice (Fig. 7a). After administration with Dox/Mito-DGL via tail vein, the fluorescence at tumor site increased gradually with time and could be distinguished clearly from the surrounding normal tissue at 4 h post injection. A distinct near-infrared fluorescence signal was displayed at the tumor region while negligible fluorescence was observed at other regions of the mouse body after injection for 24 h, demonstrating the high targeting efficiency of Dox/Mito-DGL in cancer tissue. In contrast, Cy5.5 labeled Dox/Duplex without DGL encapsulation (Dox/Duplex-Cy5.5) presented a very weaker fluorescence signal in cancer tissue within 24 h post injection, indicating the much lower cancer specificity compared with Dox/Mito-DGL. Moreover, to explore the role of AS1411 aptamer in tumor targeting, Dox/Mito-DGL (no AS1411)-based bioimaging was also carried out. During 24 h post injection with Dox/Mito-DGL (no AS1411), the fluorescence signal at tumor site was still much lower compared with Dox/Mito-DGL, which further proved that the high tumor-targeting capability of Dox/Mito-DGL was attributed to the selective recognition of AS1411 aptamer to nucleolin on surface of cancer cells.

Fig. 6 Confocal fluorescence images of apoptosis by the JC-1 assay in a) drug-sensitive MCF-7 and b) drug resistance MCF-7/ADR cells treated with free Dox or Dox/Mito-DGL for 24 h. Scale bars: 20 μm.

In Vivo Targeted Imaging and Therapy on Subcutaneous Tumor-Bearing Mice

![Fig. 6](image_url)
fluorescence biodistribution imaging was also adopted to investigate the targeting behavior of Dox/Mito-DGL. As shown in Fig. 7b, the excised tissues displayed the strongest fluorescence occurred in the cancer tissue, which further validated the high tumor selectivity of Dox/Mito-DGL.

The in vivo targeted therapy efficacy of Dox/Mito-DGL to tumor was investigated in HeLa tumor-bearing mice by monitoring the changes of relative tumor volumes (Fig. 8a). After intravenous administration with Dox/Mito-DGL into the mice, the growth of the tumor was significantly inhibited, as only a small flat tumor mass under the skin of the mice was apparent after 12-day treatment. In contrast, the therapeutic efficiency of free Dox was much lower than that of Dox/Mito-DGL which may be explained by lack of cancer targeting capability and the MDR effect. Notably, no significant anticancer activity on inhibiting tumor growth of the mice treated with ATP-insensitive Dox/Mito-cDGL, validating that the controlled intracellular release mediated by ATP played an essential role in improving the therapeutic efficacy in vivo.

The effectiveness of the treatment in terms of tumor cell death were also evaluated by performing TUNEL staining on tissue sections after different treatments (Fig. 8b). A much greater population of apoptotic cells were observed in histological sections treated with Dox/Mito-DGL, which suggested that Dox/Mito-DGL was highly capable of inducing tumor cell apoptosis in vivo. On the other hand, tissue sections from the mice treated by free Dox or Dox/Mito-cDGL exhibited smaller extent of cell death, which was attributed to the low cancer selectivity. The results revealed that Dox/Mito-DGL could achieve highly efficient in vivo cancer therapy taking advantages of the tumor-specific nanocarrier and spatiotemporally controlled drug release.

**Experimental section**

**Materials and Reagents**

Dendrigraftpoly-L-lysines (DGL) generation 3 with 123 lysine groups were purchased from Colcom, France. α-Malemidyli-u-N-hydroxysuccinimidyl polyethylene glycol (NHS-PEG-MAL, MW 500) was obtained from Jenkem Technology (Beijing, China). ATP aptamer, single-stranded cDNA of ATP aptamer, random DNA, Cy5.5-labelled mitochondria targeting aptamer and AS1411 aptamer were purchased from Sangon Biotech Ltd. Co (Shanghai, China). ATP aptamer sequence is: 5’-ACC TGG GGG AGT  ATT GCG GAG GAA GGT-3′. The Cy5.5-labelled mitochondria targeting aptamer sequence is designed as follows: 5′-Cy5.5-CCG TGT CTG GGG CCG ACC GGC GTA TGT AC CAG TTG TG TTT TT TTT-thiol-3′. Doxorubicin hydrochloride and TUNEL Assay Kit were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342, LysoTracker Green, MitoTracker Green, GolgiTracker Green were all obtained from Invitrogen (Carlsbad, CA, USA). Ultrapure water was prepared using a Millipore Simplicity System (Millipore, Bedford, USA).

**Fabrication and Characterization of Dox/Mito-DGL**

DGL were reacted with NHS-PEG-MAL (MW 500) at a ratio of 1:10 (mol/mol) in phosphate buffer (pH 8.0) for 2 h at room temperature. The primary amino groups on the surface of DGL were specifically reacted with the terminal NHS groups of the PEG derivative. The resulting conjugate, DGL-PEG, was purified by Amicon Ultra centrifugal filter units, and dissolved in phosphate buffer (pH 7.0). Then DGL-PEG was reacted with Cy5.5-labelled mitochondria targeting aptamer and AS1411 aptamer (5% weight compared with DGL concentration) in phosphate buffer (pH 7.0) for 24 h at room temperature. The MAL groups of DGL-PEG were specifically reacted with the thiol groups of the two aptamers, yielding the DGL-PEG-aptamer vectors. From the fluorescence intensity of the supernatant and the standard curve of Cy5.5, the number of Cy5 aptamer on the surface of each DGL NP was estimated. The amount of Cy5 aptamer on each DGL NP was determined to be 10^5 with the same method.

The Dox-Duplex complex was prepared by incubating Dox with a hybridized duplex of ATP aptamer and its cDNA at a molar ratio of 1:1 for 15 min. DGL-PEG-aptamer was diluted to 600 mg/mL in phosphate buffer (pH 7.4). Dox-Duplex complex solution (100 mg/mL, 50 mM sodium sulfate solution) was added at mass ratio of 6:1 (DGL to DNA) and immediately vortexed for 30 s at room temperature. To separate DGL-PEG-aptamer from Dox/Mito-DGL after self-assembly process, Dox/Mito-DGL was purified by ultra...
filtration using a 30 kDa molecular weight cut off membrane. The similar procedures were used to prepare Dox/Mito-cDGL.

The transmission electron microscopy (TEM, JEM-2100) measurement of Dox/Mito-DGL was prepared by dropping the solution onto a carbon-coated copper grid following negative staining with 2.0% (w/v) phosphotungstic acid. The particle size and size distribution of Dox/Mito-DGL were measured by dynamic light scattering (DLS) (a Mastersizer 2000 particle size analyzer) with a fixed scattering angle of 90°. Zeta potential measurement was performed at 25 °C on a Malvern Zeta sizer-Nano Z instrument.

In Vitro Release of Payload from Dox/Mito-DGL and Dox/Mito-cDGL

The profiles for in vitro release of Dox were established by dialysis of the Dox/Mito-DGL suspensions in HEPES buffer solution. Briefly, 3 mL Dox/Mito-DGL suspensions (50 μg mL⁻¹) were dialyzed against 15 mL HEPES buffer (molecular-weight cut off: 12000) and gently shaken in a thermostatic rotary shaker at 100 rpm and 37 °C. Samples were removed at different intervals, and an equal amount of the same medium was added to maintain a constant volume. The amount of Dox released from Dox/Mito-DGL was analyzed by using a fluorescence spectrometer.

Cell Culture and Confocal Fluorescence Imaging

Human cervical carcinoma cell line (HeLa cells) and human epidermal cell line (HaCaT cells) were maintained following protocols provided by the American type Tissue Culture Collection. Cells were seeded at a density of 1×10⁶ cells/mL in RPMI 1640 supplemented with 10% FBS, NaHCO₃ (2 g/L) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). The cells were maintained in a humidified incubator at 37 °C, in 5% CO₂/95% air. One day before imaging, cells were passed and plated on 10-mm glass bottom dishes. Cell imaging was carried out after washing cells with PBS for three times. Confocal fluorescence imaging studies were performed with a ZEISS Laser Scanning Microscope (Zeiss LSM 710).

In Vitro Cytotoxicity Assay

MTT assay was carried out to investigate the cytotoxicity of Dox/Mito-DGL, free Dox, Dox/Mito-cDGL and Mito-DGL. HeLa cells were first seeded to two 96-well plates at a seeding density of 1×10⁴ cells well in 200 μL complete medium, which was incubated at 37 °C for 24 h. After rinsing with PBS, HeLa cells were incubated with 200 μL culture media containing serial concentrations of Dox/Mito-DGL, free Dox, Dox/Mito-cDGL or Mito-DGL for 48 h.

Then, 20 μL of 5 mg mL⁻¹ MTT solution in pH 7.4 PBS was added to each well. After 4-h incubation, the medium containing unreacted MTT was removed carefully, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. After 1 h the absorbance (Abs.) was measured at 490 nm in a TRITURUS microplate reader. The cell viability was then determined by the following equation: Cell viability (%) = (mean of Abs. value of treatment group/mean Abs. value of control) × 100%. Calculation of the half lethal dose (IC50) values was done according to Huber and Koella. ²⁸

Animal and Tumor Model

Specific pathogen-free (SPF) female BALB/c mice, 5–6 weeks of age, were purchased from Shanghai Laboratory Animal Center and bred in an axenic environment. All animal operations were in accord with institutional animal use and care regulations approved by the Model Animal Research Center of Nanjing University (MARC). HeLa cells (1×10⁶) were implanted into the nude mice by using a stereotactic fixation device with mouse adaptor. During treatment, mice were anesthetized with 2.5% isoflurane in oxygen delivered at a flow rate of 1.5 L min⁻¹. Tumors were then allowed to grow to 5–8 mm in diameter. To determine tumor volume, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) from each tumor were determined using a vernier caliper. Each tumor’s volume, based on the caliper measurements, was calculated using the following formula: tumor volume = length × width² × 0.5.

In Vivo Imaging Study

The HeLa tumor model was established by implanting the HeLa cells (1×10⁶) into the selected position of nude mice. Dox/Mito-DGL, Dox/Duplex-Cy5.5 and Dox/Mito-DGL (no AS1411) were injected through the tail vein of tumor-bearing nude mice at DOX dosage of 1 mg/kg. At 1, 4, 12, and 24 h, the mice were anesthetized and imaging by a Cambridge Research & Instrumentation (CRI) in vivo imaging system (CRI, MA, USA). After the 24 h scanning, the mice were euthanized to obtain the principal organs (including heart, kidney, liver, lung, and spleen) and tumor tissues for ex vivo imaging.

Targeted Treatment on Subcutaneous Tumor Model and Evaluation of Therapeutic Effect

In vivo targeted treatment was performed using HeLa tumor-bearing mice. The mice were randomly divided into four groups and subjected to the following treatments: group 1, untreated; groups 2–4, Dox/Mito-cDGL, free Dox or Dox/Mito-DGL at Dox dosage of 1 mg kg⁻¹ in PBS. From Day 0, the tumor-bearing nude mice were intravenously injected every three days for 12 days, and meanwhile the tumour size was measured. The therapeutic effects were evaluated by monitoring the changes of relative tumor volumes, TUNEL apoptosis staining.

Conclusions

In conclusion, we have successfully engineered a dual-targeted, ATP-responsive DGL self-assembling NP for precise mitochondrial drug delivery and highly efficient therapy in MDR tumor cells. The dual-modification by AS1411 and Cyt c aptamers render Dox/Mito-DGL a high selectivity for targeted cancer therapy at the subcellular level. Such a mitochondria-specific nanocarrier could deliver Dox into the highly impermeable organelle of tumor cells, and subsequently release the loaded drug in virtue of the high level of ATP within this intracelluar compartment. The controllable antitumor activity and the targeting feature led to high therapeutic outcomes both in vitro and in vivo. More importantly, the smart nano-system was successfully applied to eliminate chemotherapy-refractory by altering the high mitochondrial membrane potential of MDR cancer cells and bypassing the P-gp efflux pumps in plasma membrane. Overall, by exploiting the unique biochemical properties of mitochondria to trigger more precise drug release, this organelle-specific delivery system provides an excellent opportunity to combat cancer or other mitochondria-associated diseases.
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Notes and references

Dox/Mito-DGL could selectively unload the encapsulated Dox/Duplex and induce dissociation of the DNA duplex upon the high levels of ATP in mitochondria, which thereby causes rapid release of Dox. The integrating utilization of dual-targeted aptamer and ATP-controlled drug release led to a significant enhancement of chemotherapeutic selectivity and efficiency to cancer cells.