Overcoming Multidrug Resistance by On-Demand Intracellular Release of Doxorubicin and Verapamil

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Multidrug resistance (MDR) is one of the major obstacles to the successful application of cancer chemotherapy. Herein, we developed light-responsive doxorubicin-and-verapamil-coencapsulated gold liposomes to overcome MDR. Upon ns-pulsed laser irradiation, the highly confined thermal effect increased the permeability of the phospholipid bilayer, triggering the release of doxorubicin and verapamil, leading to high concentrations in cells. Free verapamil efficiently inhibited the membrane multidrug resistance proteins (MRPs), while the high concentration of doxorubicin saturated MRPs, thus overcoming MDR. We showed that nanosecond- (ns-) pulsed laser- (532 nm, 6 ns) induced doxorubicin release from gold liposomes depended on laser fluence and pulse number. More than 58% of the doxorubicin was released with a 10-pulse irradiation (100 mJ/cm²). Furthermore, ns laser pulses also liberated doxorubicin from endocytosed gold liposomes into the cytosol in MDA-MB-231-R cancer cells. The cytotoxicity of doxorubicin coencapsulated with verapamil was significantly enhanced upon laser irradiation. This study suggested that light-triggered on-demand release of chemotherapeutic agents and MRP inhibitors could be used advantageously to overcome multidrug resistance.

1. Introduction

Cancer metastasis is one of the main challenges in cancer therapy, which leads to over 90% of cancer-related deaths [1]. To date, although there are several therapies for cancer metastasis treatment, for example, aggressive surgery, radiotherapy, and chemotherapy, only very limited curative effects in patients have been observed [2]. Chemotherapy is by far the most commonly used cancer treatment. Nanoparticles have successfully been used to carry chemotherapeutic agents and to enable other functions, such as long blood circulation and tumor targeting [3]. The encapsulation of toxic chemotherapeutic agents may reduce their nonspecific toxicity, but meanwhile limits drug release in the tumor sites. Enhancing drug release from endocytosed nanoparticles is of great significance since chemotherapeutic agents have to be released into the cytosol or nucleus to elicit their therapeutic effects. In recent years, controlled drug release has attracted increasing interests. Many controlled drug-release strategies have been reported, especially, the diffusion-based and biologically-activated drug-release system. However, drug release based on these mechanisms are usually slow, cannot be precisely controlled, and do not amplify the selectivity of drug delivery [4]. Strategies that enable fast drug release upon activation is still highly desired to enhance intracellular cancer drug delivery [5].

Liposomes, that is, spherically closed lipid bilayers, have been widely used in the biomedical field [6, 7]. Their hollow spherical structure and high loading capacity make them attractive packaging materials for the encapsulation and delivery of drugs, enzymes, and many other biomolecules [8, 9]. Multifunctional hybrid drug delivery systems, that is, polymer- or nanoparticle-modified liposomes, exhibiting the advantages of two or more delivery systems, have become one of the most popular delivery systems [10, 11]. Recently, gold nanoparticles have been incorporated into liposomes to enable the photosensitivity for photothermal therapy [12]. Gold nanoparticles convert light energy into heat for thermal ablation of cancer cells upon laser irradiation [13, 14]. However, an efficient photothermal therapy requires
continuous light irradiation [15, 16]. Besides, heat-induced damage to the bioactive molecules or normal tissues remains one of the main limitations. Although localized heating can be achieved by a ns-short pulsed laser [17], it requires high laser influence to induce substantial cell apoptosis. Recently, gold nanoparticles also have been conjugated with an antiangiogenic peptide to enhance angiogenesis arrest upon visible laser irradiation in vivo [18]. Another common biological application of gold nanoparticles is to trigger drug release by the photothermal effect, which only requires lower laser fluence compared to thermotherapy.

Multidrug resistance (MDR) is one of the major unsolved problems for successful cancer chemotherapy. A combination of chemotherapeutic agents with multidrug resistance protein (MRP) inhibitors, such as verapamil, has been shown to have an in vivo anticancer synergistic mechanism [19]. However, to effectively inhibit MRPs, a high concentration of MRP inhibitors are required, especially for small molecule inhibitors, which may induce unwanted toxicity to normal tissues. To overcome the current limitations, we coencapsulate doxorubicin and verapamil into gold liposomes and use a ns-pulsed laser to trigger fast intracellular drug release. High intracellular concentrations of doxorubicin and verapamil were achieved upon light irradiation, leading to the increased cytotoxicity of doxorubicin. Gold-nanoparticle-decorated liposomes, that is, gold liposomes were developed, and the light-triggered drug release was tested in vitro and in cells. Under light irradiation, gold nanoparticles generate a highly localized heat owing to the surface plasmon resonance [20], and the cargo was released due to the increased permeability of liposomes (Schematics 1). We demonstrated that the ns laser pulse (532 nm, 6 ns) liberated doxorubicin from gold liposomes into the cytosol in drug resistant MDA-MB-231-R cancer cells. Moreover, the cytotoxicity of doxorubicin coencapsulated with verapamil was significantly enhanced upon laser irradiation. This study showed that the photothermal-triggerable liposome is a promising strategy to overcome multidrug resistance and enhance therapeutic effects by on-demand release of chemotherapeutic agents and MRP inhibitors.

2. Results and Discussion


Gold liposomes were developed by the reduction of gold chloride onto the 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipid surface following a previously reported method [21]. As shown in Table 1, gold liposomes showed a hydrodynamic size around 103 nm, and uncoated liposomes were around 92 nm. Transmission electron microscopy (TEM) observation showed that gold nanoparticles were distributed on the surface (Figure 1(a)). The structure of gold liposomes we observed was similar to the previously reported gold liposomes prepared by the same method [22]. UV-Vis spectra of gold liposomes exhibited an absorption peak at around 650 nm, while uncoated liposomes did not show any resonance peak in the wavelength range of 400–800 nm (Figure 2(b)). The results suggested that the gold nanoparticles have been successfully decorated onto the liposome surface. The gold liposomes that we developed showed high absorbance in the wavelength range of 500–750 nm, suggesting that these gold liposomes could be activated by light in the range of 500–750 nm.

2.2. Thermal Effect and Nanosecond Laser Pulses Induced Doxorubicin Release.

As shown in Figure 2(a), for uncoated liposomes, when the bulk temperature was lower than 35°C, no obvious leakage was observed. Doxorubicin release sharply increased from 10% to 41% when the temperature reached 38°C. After 38°C, doxorubicin release became slowly increased. The thermal-effect-triggered release is due to the gel-to-liquid crystalline phase transition of the phospholipid bilayer. The pretransition temperature and major transition temperature of DPPC liposomes are 35°C and 41°C, respectively [23]. There was no significant difference between uncoated liposomes and gold liposomes, suggesting that the gold decoration did not significantly alter the phase transition temperature of the DPPC phospholipid bilayer.

Nanosecond-pulsed laser-triggered doxorubicin release was tested with different laser pulse numbers and influence (Figure 2(b)). As expected, doxorubicin release from gold liposomes was dependent on the laser pulse number and influence. With the same pulse influence (100 mJ/cm²), the doxorubicin release increased from 20.8% to 58.3% by increasing the pulse number from 1 to 10. A single pulse with a higher influence in the range of 50–200 mJ/cm² also led to a higher doxorubicin release. No temperature increase was observed under current experiment conditions, suggesting that a ns-pulsed laser-induced thermal effect was highly localized and no bulk heating was involved in our system. Nanosecond laser irradiation did not trigger any significant doxorubicin release from uncoated liposomes, suggesting that gold nanoparticle decoration was required for ns-pulsed laser activation.

To test whether there was a triggering effect in time, we investigated the kinetics of doxorubicin release upon ns-pulsed laser activation. The results showed that doxorubicin release reached the maximum within 1.0 min (Figure 2(c)), confirming that there was a triggering effect

Schematics 1: (a) Schematics for the formation of gold liposomes. (b) Nanosecond-pulsed laser-triggered on-demand intracellular release of verapamil and doxorubicin for overcoming multidrug resistance. AA, ascorbic acid; Dox, doxorubicin.
in cargo release upon light activation. The permeability of gold liposomes was characterized by carboxyfluorescein (CF) leakage studies following a previously reported method [24]. We observed acute CF leakage upon ns-pulsed laser activation and the CF leakage only lasted for 1 min, which was in the same time scale as doxorubicin release (Figure 2(d)). The enhanced membrane permeability and cargo release may happen in an even shorter time scale, unfortunately, limited by current experimental conditions, we cannot do real-time observation.

2.3. Intracellular Doxorubicin Release. The ns-pulsed laser-triggered intracellular doxorubicin release was observed by confocal scanning laser microscopy. The fluorescence of doxorubicin was quenched due to its self-association when loaded by the ammonium sulfate method [25]. The release of doxorubicin from liposomes and dilution by the surrounding medium dequench doxorubicin and increase its fluorescence intensity. As shown in Figure 3, ns laser pulses (532 nm, 5 pulses, and 100 mJ/cm²) triggered doxorubicin release from the gold liposomes into the cytosol, dramatically increasing the cytosol and nuclear fluorescence intensity. No significant difference was observed for cells treated with uncoated liposomes before and after laser pulse irradiation. The results again confirmed that gold nanoparticles decorated on the liposome surfaces were required for light-induced release. Many nanocarriers were trapped in endolysosomes after endocytosis, and cannot efficiently liberate encapsulated active compounds, which significantly limits their therapeutic effects. This technique enables intracellular release of trapped therapeutic agents and is promising for enhancing the therapeutic effects. In a previous report, picosecond-pulsed laser-triggered cargo release from gold liposomes has been used to manipulate cell signaling [22]. We believe that our technique may also find many other biological applications where intracellular cargo release is required.

2.4. Cytotoxicity of Doxorubicin-Loaded Liposomal Carriers. To test the capability of on-demand release to enhance therapeutic effects, the cytotoxicity of doxorubicin coencapsulated with verapamil in liposomes was measured by MTT. The results showed that laser irradiation dramatically increased the cytotoxicity of doxorubicin loaded in gold liposomes with or without verapamil (Figure 4). The IC₅₀ of doxorubicin decreased from 29.5 μM to 2.7 μM after incorporation into gold liposomes combined with laser activation. Based on in vitro doxorubicin-release studies, around 36% doxorubicin was liberated, which makes the concentration of free doxorubicin in cells treated with gold liposomes and

<table>
<thead>
<tr>
<th>Samples</th>
<th>Diameter (nm)</th>
<th>Polydispersity index (PDI)</th>
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<tbody>
<tr>
<td>Uncoated liposomes</td>
<td>92.5 ± 5.3</td>
<td>0.034 ± 0.005</td>
</tr>
<tr>
<td>Gold liposomes</td>
<td>103.8 ± 10.8</td>
<td>0.128 ± 0.039</td>
</tr>
<tr>
<td>Gold liposomes with laser irradiation</td>
<td>101.5 ± 13.7</td>
<td>0.157 ± 0.051</td>
</tr>
<tr>
<td>Dox/Ver loaded liposomes</td>
<td>101.1 ± 7.1</td>
<td>0.052 ± 0.013</td>
</tr>
<tr>
<td>Dox/Ver loaded gold liposomes</td>
<td>115.2 ± 12.5</td>
<td>0.171 ± 0.045</td>
</tr>
</tbody>
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**Figure 1:** Characterization of gold liposomes. (a) Typical TEM image of gold liposomes; (b) UV-Vis spectra of uncoated and gold liposomes.
lasers much lower than those treated with free doxorubicin. However, the cytotoxicity of doxorubicin was enhanced more than 10-fold, which suggested that the triggering effect significantly contributed to enhanced cytotoxicity. Incorporation of verapamil into doxorubicin liposomes significantly increased cytotoxicity of doxorubicin. More importantly, the cytotoxicity of doxorubicin was further enhanced by laser irradiation and the IC_{50} decreased to 1.5 μM. Under the current condition, laser irradiation alone did not induce substantial dead cells (Figure 5), suggesting that the enhanced cytotoxicity of doxorubicin by light irradiation was not because of the laser ablation of cancer cells. There are probably two main mechanisms contributing to the enhanced cytotoxicity. First, laser irradiation triggered doxorubicin release into the cytosol, which provides more free doxorubicin to elicit its pharmacological activity. Second, instant high concentration of verapamil was achieved upon laser activation, which resulted in inhibition of MRPs and reducing doxorubicin efflux mediated by MRPs. However, for doxorubicin encapsulated in uncoated liposomes, no significant difference was observed after laser irradiation.

3. Materials and Method

3.1. Materials. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol were purchased from Avanti Polar Lipids. Gold (III) chloride trihydrate (HAuCl₄·3H₂O) and doxorubicin hydrochloride was purchased from Sigma-Aldrich. Ascorbic acid was from Fisher Scientific. DMEM
medium and fetal bovine serum were purchased from GIBCO. 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Thermo Fisher Scientific. Doxorubicin sodium salt was purchased from Alfa Aesar. All other reagents were analytical grade and used without further purification.

3.2. Preparation and Characterization of Gold Liposomes. Liposomes were prepared with DPPC and cholesterol at a molar ratio of 70:30. Briefly, lipid powders were dissolved in chloroform and dried under a nitrogen stream, and then placed in a vacuum overnight to completely remove chloroform. The lipid film was hydrated with 300 mM ammonium sulfate (pH = 7.5) with or without 50 μM verapamil, followed by extrusion for 21 times through 100 nm polycarbonate membrane using an Avanti mini extruder. Empty liposomes were passed through the S1000 column preequilibrated with isotonic N-(2-hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid) (HEPES) buffer (140 mM NaCl, 10 mM HEPES, pH = 7.4) to replace the extra ammonium sulfate solution. Subsequently, doxorubicin hydrochloride was added to the liposome suspension to achieve a drug to lipid ratio of 1/3 (mol/mol). The loading process was carried out at 37°C for 2 h. The free doxorubicin was removed by size exclusion chromatography (SEC) eluted with HEPES buffer. Gold was decorated onto the liposome surface following a previously reported method with minor modification [12]. Briefly, liposomes were diluted to 1 mM using the HEPES buffer. Gold chloride solution at a concentration of 20 mM was added and mixed with liposomes, followed by the addition of ascorbic acid solution (40 mM). Following reduction, gold liposomes were dialyzed against the HEPES buffer for 24 h under 4°C to remove unreacted gold chloride and ascorbic acid. The resulting liposomal samples were stored at 4°C until further use. Transmission electron microscopy (TEM) operating at the voltage of 200 kV was used to observe the morphology of gold liposomes. Gold liposomes were diluted with distilled water and dropped onto a carbon-film-coated copper grid. The samples were air-dried for 2 h at room temperature and then imaged. The hydrodynamic radius and polydispersity of liposomal vesicles were determined using dynamic laser light scattering (DLS). Each sample was measure at least 5 times. The UV-Vis spectra of gold liposomes in the range of 400 nm–800 nm was recorded using a spectrometer.

3.3. Thermal- and Laser-Triggered Doxorubicin Release. The thermal effect-induced doxorubicin release from gold liposomes were investigated under program-increasing temperature conditions following a previously reported method [23]. The measurements were performed on an F7000 fluorescence spectrometer equipped with a RES refrigerated circulating bath. The liposome suspensions were heated from 30 to 50°C in intervals of 1°C. The solution was allowed to

![Figure 3: Nanosecond-pulsed laser- (532 nm, 5 pulses, and 100 mJ/cm²) triggered intracellular doxorubicin release from gold liposomes in MDA-MB-231-R cells. Blue represents nucleus; red represents doxorubicin; scale bar 20 μm.](image-url)

![Figure 4: Cytotoxicity of doxorubicin encapsulated in different liposomal formulations with or without laser irradiation in MDA-MB-231-R cells. 532 nm, 5 pulses, and 100 mJ/cm².](image-url)
leakage upon laser irradiation (10 pulses, 100 mJ/cm²) was monitored at 0, 1, 1.5, 2, 2.5, 3, 4, 6, 9, and 24 h, respectively.

The fluorescence of each sample was immediately measured, and the release of doxorubicin was calculated using (1):

\[R\% = \frac{F_x - F_0}{F_t - F_0} \times 100\%\]

where \(F_x\) is the fluorescence of samples at each measurement, \(F_0\) is the initial fluorescence of samples before any heating, and \(F_t\) is the fluorescence of 100% release induced by 1% Triton X-100 treatment.

For the light-induced release measurements, uncoated and gold encapsulated liposome suspensions were placed in transparent 96-well plates. A ns laser at 532 nm with a beam diameter of 5 mm was used to induce release. The samples were exposed to the laser beam at room temperature with different pulses (1, 5, and 10 pulses) and different laser influences (50, 100, 150, and 250 mJ/cm²). After laser treatment, the fluorescence of each sample was immediately measured, and the release percentage was calculated using (1). To understand the kinetics of doxorubicin release, the fluorescence intensity of laser-treated samples was monitored at 0, 1, 1.5, 2, 2.5, 3, 4, and 5 min after light irradiation. Carboxyfluorescein (CF) at a concentration of 50 mM was encapsulated into gold liposomes to study the permeability of gold liposomes. CF leakage upon laser irradiation (10 pulses, 100 mJ/cm²) was measured using the same protocol as doxorubicin release.

3.4. Intracellular Doxorubicin Release. To study the intracellular doxorubicin release triggered by laser irradiation. MDA-MB-231-R cells were cultured in 35 mm glass bottom dishes in DMEM medium for 24 h. The cells were washed with PBS and replaced with fresh medium that contains doxorubicin-loaded gold liposomes. After 3 h of incubation, the cell nucleus was stained with 5 μg/mL DAPI for 5 min. Cells were then washed with PBS, and supplied with fresh cell media prior to laser irradiation. Nanosecond pulses (5 pulses, 100 mJ/cm²) was tested. To study the intracellular distribution of gold liposomes, cells with or without laser irradiation were immediately observed by a confocal laser microscope (Olympus FV 1000).

3.5. Cytotoxicity Study. Cytotoxicity studies were done using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. MDA-MB-231-R cells were cultured overnight in 96-well plates at a density of 1 × 10⁴/well. The control group was free doxorubicin-treated cells, and the treatment groups are doxorubicin-encapsulated uncoated liposomes with or without laser irradiation, doxorubicin-encapsulated gold liposomes with or without laser irradiation, and doxorubicin/verapamil-coencapsulated gold liposomes with or without laser irradiation. The cells were washed with PBS and replaced with fresh medium that contains doxorubicin encapsulated in different gold liposomes. After 3 h of incubation, the cells were washed with PBS for 3 times, and the laser treatment groups were irradiated with a ns laser pulse (532 nm, 5 pulses, and 100 mJ/cm²), followed by supplementation with fresh DMEM medium and placed in the incubator for the next 24 h. Subsequently, the cell viability was determined by the MTT assay.

4. Conclusion

In conclusion, we developed doxorubicin-and-verapamil-coencapsulated gold liposomes. Mild ns-pulsed laser exposure induced cargo release from gold liposomes without bulk heating. Ns laser pulses (532 nm, 6 ns) induced doxorubicin release from gold liposomes in a laser pulse- and influence-dependent pattern. In MDA-MB-231-R cancer cells, ns laser pulses (100 mJ/cm², 5 pulses) also liberated doxorubicin from endocytosed gold liposomes into the cytosol. Furthermore, laser irradiation significantly increased the cytotoxicity of doxorubicin-and-verapamil-coencapsulated gold liposomes in MDA-MB-231-R cells. The results suggested that ns-pulsed laser-triggered on-demand release of chemotherapeutic agents and MRP inhibitors is promising to overcome multidrug resistance and enhance therapeutic efficiency.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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