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Partially polymerized liposomes: stable against leakeage yet capable of instantaneous release for remote controlled drug delivery

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Abstract

A critical issue for current liposomal carriers in clinical applications is their leakage of the encapsulated drugs that are cytotoxic to non-target tissues. We have developed partially polymerized liposomes composed of polydiacetylene lipids and saturated lipids. Cross-linking of the diacetylene lipids prevents the drug leakage even at 40 °C for days. These inactivated drug carriers are non-cytotoxic. Significantly, >70% of the encapsulated drug can be instantaneously released by a laser that matches the plasmon resonance of the tethered gold nanoparticles on the liposomes, and the therapeutic effect was observed in cancer cells. The remote activation feature of this novel drug delivery system allows for precise temporal and spatial control of drug release.

1. Introduction

Liposomes as carriers for drug delivery have attracted tremendous interest due to its great tunability in physical and chemical properties for different therapeutic applications [1–14]. To improve the therapeutic index and minimize the cytotoxicity of the drug to normal tissues, the encapsulated drug should be released precisely at the targeted tissues at the desired time and duration. However, this precise control is hindered by the limited stability of conventional liposomes at physiologic conditions against drug leaking at non-target sites. For example, the only heat sensitive liposome (Thermodox) currently under clinical trial released ~5% of doxorubicin in only 2 min at 37 °C and >40% at 40 °C, leading to potential toxicity to non-target tissue [15]. Crosslinking the lipids in liposomes has been shown to greatly reduce breakage [16–18] and phospholipid exchange or fusion with other liposomes or cell membranes [19, 20]. However, it also becomes exceedingly difficult to release the encapsulated contents instantaneously at the desired location. Therefore, it remains a great challenge to construct liposomal drug delivery carriers that are stable while capable of rapid release of the drug on demand.

In this work, we developed a new liposomal system to achieve stability as well as precisely controlled release. Our system is based on stable, partially polymerized liposomes (PPLs) with gold nanoparticles (GNPs) attached on the outer surface. We demonstrated that this system retained the encapsulated content at 40 °C for 3 days while instantaneously releasing over 70% of the contents upon triggering with a laser matching the surface plasmon

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resonance of the GNPs. Furthermore, doxorubicin was encapsulated using our GNP attached PPLs (PPL/GNP), and its therapeutic effect was tested on breast cancer cell line MDA-MB-231. We showed a significant killing of the cancer cells only upon laser irradiation of the doxorubicin-loaded PPL/GNP complexes.

2. Experimental details

2.1 Materials

Lipids 1,2-di-(10Z,12Z-tricosadiynoyl)-sn-glycero-3-phosphoethanolamine (DiynePE), 1,2-di-(10Z,12Z-tricosadiynoyl)-sn-glycero-3-phosphocholine (**2**), 1-myristoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (**3**), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchase from Avanti Polar Lipids. Inc. (Alabaster, AL) and used without further purification. ^{99m}Tc chelate was a generous gift from Nuclear Medicine Department at the Methodist Hospital. N-Hydroxysuccinimide (HOSu), calcein, and *N,N'*-Dicyclohexylcarbodiimide (DCC) were purchased from Sigma Aldrich (St. Louis, MO), and used without further purification. Thioctic acid was purchased from Acros Organics (Belgium) and used without further purification. Gold nanoparticles were purchased from Nanopartz (Loveland, CO).

2.2 Synthesis of lipid 1

To the solution of thioctic acid (21 mg, 0.10 mmol) in 1 mL dichloromethane, DMSO (0.1 mL), HOSu (12 mg, 0.10 mmol), DCC (21 mg, 0.10 mmol) and lipid diynePE (100 mg, 0.11 mmol) were added. The reaction mixture was stirred at room temperature overnight, the purified product was obtained through a semi preparative reversed- phase high-performance liquid chromatography (HPLC) system (Agilent 1200 series). $C_{59}H_{98}NO_9PS_2$: m/z 1058.4 (100, $[M-H]^-$. calcd 1059.6). ^{1}H NMR (300 MHz, $CDCl_3$): 4.12 (m, 1H), 3.87 (m, 4H), 3.42 (m, 2H), 3.14 (m, 2H), 2.64 (m, 4H), 2.48 (m, 1H), 2.25(m, 14H), 1.53(m, 16H), 1.27(m, 46H), 0.898(m, 6H) (See figure S1 in supporting information)

2.3 Synthesis of partially polymerized liposomes (PPLs) Loaded with imaging agent or drug

Lipids **1** (2.6 mg, 2.5 μmol), **2** (65.3 mg, 71.4 μmol), and **3** (3.5 mg, 8.2 μmol) were dissolved in chloroform/ methanol (9:1 v/v). The solvent was evaporated with a rotary evaporator under reduced pressure, and the residue was dried under vacuum while shielded from light. After the addition of 8 mL phosphate buffered saline solution of Tc-99m Mebrofenin, calcein (0.16 M), or doxorubicin (0.04 M), the solution was stirred for 3 h, and then transferred to a 10 mL Lipex extruder (Northern Lipids Inc. Canada) equipped with two stacked polycarbonate filter membranes with 100 nm pore size. The emulsion was then extruded at 45 °C for 11 times, resulting in an opalescent solution. This solution was transferred onto a petri dish sitting on ice, and irradiated under 254 nm UV for 1 h, yielding partially polymerized liposomes. The liposomes were then filtered using a 220 nm filter and heated at 45 °C for 15 min followed by purification with Sephadex G50 columns (GE healthcare).

2.4 Synthesis of Non-polymerized liposomes (NPLs)

It follows the same procedure as above. After extrusion, the generated non-polymeried liposomes were cooled down and filtered using a 220 nm filter followed by purification with Sephadex G50 columns.

2.5 Synthesis of liposomes with DPPC (NPL-DPPC)

Lipids DPPC (30.0 mg, 40.9 μmol) were dissolved in chloroform, then the solvent was evaporated and the residue was dried under vacuum. After the addition of calcein in phosphate buffered saline (4 mL, 0.64 mmol), the solution was stirred for 3h, and then extruded at 45 °C for 11 times as above. The liposomes were then filtered using a 220 nm filter followed by purification with Sephadex G50 columns.

2.6 Synthesis of gold nanoparticle tagged Liposomes

The above liposome solution (~10^{12} particles/mL) was mixed with a solution of gold particles (50 nm in diameter, 1.2×10^{12} particles/mL, Nanopartz) (1:3 v/v), and incubated at room temperature with shaking for 1 h. The mixture was then passed through a 450 nm filter.

2.7 Calculation of the Releasing percentage

The amount of radioactive Tc mebrofenin encapsulated in the liposomes was quantified using Agilent HPLC system equipped with a HiTrap Sephadex G25 column (GE healthcare) and a radiodetector. The radioactivity of the liposome fraction was measured as the integrated peak area. The releasing percentage was calculated as below:

$$\text{Releasing}\ (\%)=(1-\text{Radioactivity inside liposomes after laser treatement}/\text{Radioactivity inside liposome before laser treatment})\times 100$$

The amount of calcein leaked from liposomes was quantified using a FLUOstar OPTIMA plate reader (BMG LABTECH Inc., Germany.) The total encapsulated contents were released using 8M urea with 5% SDS. The releasing percentage was calculated as below:

$$\text{Releasing}\ (\%)=[(\text{Fluorescence after heating}-\text{Background Fluorescence})/(\text{Fluorescence from total release}-\text{Background Fluorescence})]\times 100$$

2.8 Electron Cryo-Microscopy (Cryo-EM)

Particles were suspended across a thin layer of vitreous ice, which was prepared under controlled temperature and humidity conditions within a Gatan Cp3 cryo-plunger (Gatan Inc., Pleasanton, CA.) then vitrified by rapid plunging into liquid ethane. Low dose cryo-EM imaging was performed on a JEOL 2100 electron microscope operating at 200 kV with a Gatan 60 degree liquid nitrogen specimen cryo-holder.

2.9 Cell viability study

PPL/GNP complexes encapsulating doxorubicin as anticancer drug were prepared as described above. Breast cancer cell line MDA-MB-231 was used to test the stability of liposomes and release of doxorubicin from liposomes. Specifically, cells were harvested and seeded into 8-well chambers with a density of 2×10^4 cells/well, and incubated at 37 °C with 5% CO_2 for overnight. Then cells were washed with warm PBS twice and added fresh media with PPLs and treated with 10 pulses of laser irradiation. GNPs and PPL/GNP complexes were pretreated using the same laser condition and added into cell culture. After incubation for 12 h, cell viability was determined by trypan blue exclusion staining.

3. Results and discussion

The stable PPLs were prepared from two types of lipids: the polymerizable lipids with diacetylene moieties (**1** and **2**, figure 1) and the non-polymerizable lipids (**3**, figure 1). To

achieve a strong linkage of PPL and GNP, the polymerizable lipid **1** containing a thioctic acid was used to form two Au–S bonds with GNP [21]. For quantitative analysis of the encapsulation and release of the PPL/GNP carrier, Tc-99m mebrofenin, a clinically used single photon emission computed tomography imaging agent, was encapsulated in the PPL as an indicator. The PPL was prepared using similar procedures described in our previous paper [22]. Briefly, a suspension of the lipids **1**, **2**, and **3** in a solution of Tc-99m mebrofenin in PBS was stirred for 3 h to form a milky emulsion followed by extrusion through a 100 nm membrane at 45 °C. Polymerization was carried out by 254 nm UV irradiation [19, 23, 22] to provide the PPL. The occurrence of polymerization of the diacetylene moieties in the lipid was indicated by the color change of the solution from semi-transparent to bright orange [24] and the appearance of an absorption band at 443 nm (figure 1(c)). Free Tc-99m mebrofenin remained in solution was removed using a size exclusive column (Sephadex G25). The Tc-99m concentration was calculated as 1 uCi/mL. Figure 1b showed a typical cryo-EM image of a liposome with a diameter of ~100 nm, similar to the size measured by dynamic light scattering (figure 1(d)).

The stability of the liposomes was tested at 37 °C and 40 °C. There was no release of encapsulated Tc-99m mebrofenin from the liposomes at both temperatures for 1 hour. Since Tc-99m has a short half-life of 6 hours, for testing the long-term stability of the liposomes against leakage, fluorescence dye calcein was used as the indicator. When the encapsulated, highly concentrated calcein was released from the liposomes, the fluorescence intensity was increased due to dequenching, which can be used to quantify the release. Remarkably, it was found that nearly no content was released from the PPLs after incubating at 40 °C for 3 days (figure 2). The stability of the non-polymerized liposomes (NPLs) with the same formulation as the PPLs, and the liposomes made of DPPC (NPL-DPPC) were further evaluated. DPPC is commonly used as the matrix in the heat-sensitive liposomes [25–28]. In the reported studies, the liposomes prepared solely from DPPC exhibited the highest stability among the other heat-sensitive liposomes. Therefore, we selected DPPC liposomes as the control. In the stability study, NPL-DPPC released ~80% of the encapsulated content in one hour at 40 °C. The NPLs with the same formulation of PPLs but not subjected to UV irradiation exhibited a higher stability than NPL-DPPC, releasing ~20% of encapsulated content in three days. Significantly, no release of encapsulated content in PPLs could be detected for three days at 40 °C. These results demonstrated that PPLs are remarkably stable at the upper-limit of physiologic body temperature long enough for most *in vivo* targeted drug delivery schemes.

To demonstrate the controllability of releasing encapsulated content from the stable PPLs, we utilized laser to excite the gold nanopraticles tethered to PPLs through surface plasmon resonance. The release of encapsulated content was induced by the laser energy absorbed by gold nanoparticles [29–32], and Wu *et al*. has demonstrated that the gold nanoparticles tethered on the outer surface of liposomes released the most of its contents [33]. Therefore, we incubated PPLs with a suspension of GNPs (50 nm) at room temperature for 1 h. The attachment of GNP to PPL is shown in figure 3. The gold nanoparticles were chosen to have a nominal diameter of 50 nm and a maximum absorption at 532 nm (figure 3(b)) that matches the wavelength (λ_0 = 532 nm) of a Nd:YAG laser (Brilliant B, Quantel, Inc.). Furthermore, the absorption spectrum of PPL/GNP complexes also showed strong absorption at 532 nm, in consistant with the laser wavelength (figure S2, Supporting Information). We irradiated the sample with 10 × 6-ns pulses of the laser (10 Hz frequency, intensity up to 400 mJ/pulse, corresponding to a mean power density of 3.3 W/cm^2). Upon laser irradiation, the remaining radioactivity inside the liposomes could be precisely quantified by using a Bioscan radio detector incorporated to a liquid chromatography (LC) system. The results showed that ~70% of the content was released from the gold nanoparticles tagged PPLs (PPL/GNP) by the laser irradiation (figure 4). In contrast, less

than 3% of the encapsulated content was released from the control PPLs without gold nanoparticles after laser irradiation, demonstrating that the gold nanoparticles present on the liposomes are necessary for laser trigged release.

To further demonstrate that the release of liposomal content was indeed induced by the gold nanoparticles upon absorption of laser energy corresponding to the peak absorption wavelength of the gold nanoparticles, the experiment was repeated using a laser with a wavelength of 1064 nm (6-ns duration, 10 Hz frequency, intensity up to 800 mJ/pulse, corresponding to a mean power density of 6.6 W/cm^2). Our data showed that both PPL/GNPs and PPLs released less than 5% of encapsulated content upon irradiation with 10 pulses of the laser with a wavelength far from the peak absorption wavelength of the gold nanoparticles (figure 4).

The morphological change of PPLs and PPL/GNPs induced by pulsed laser irradiation was also investigated by Cryo-EM. No morphological changes was observed for the unlabeled liposomes after laser irradiation. In contrast, for PPL/GNP carriers, the 50 nm GNPs were fragmented into smaller particles with a diameter of less than 10 nm (figure 3(d)). This data suggested that the rapid absorption of laser energy caused the fragmentation of the gold nanoparticles [34] leading to the breakage of PPLs and release of the encapsulated content. Although this process resulted in the breakage of the PPLs, the effect is highly localized and should not cause significant surrounding damage in bulk tissue. In fact, studies have shown that even though GNPs can be heated above their melting point by pulsed laser, the temperature around GNPs drops to the bulk temperature within a few nanometers [35].

For therapeutic studies, doxorubicin was encapsulated to treat breast cancer cell line MDA-MB-231. After incubation with doxorubicin encapsulated PPL/GNP complexes (Dox-PPL/GNP) with a doxorubicin concentration of 10 ug/mL for 12 h, cells were washed with PBS twice and stained with DAPI. Since doxorubicin is fluorescent, the localization of Dox-PPL/GNP in cells can be visualized by confocal fluorescence imaging (Olympus Fluoview 1000 laser scanning confocal microscope with a 20× objective). The images showed that while Dox-PPL/GNPs accumulated in the cytoplasm of the cells (figure 5(c)), free doxorubicin accumulated in the nuclei (figure 5(f)). This result showed that doxorubicin encapsulated in PPL/GNP complexes could enter into cells and the intracellular distribution was different from free doxorubicin. The absence of doxorubicin in the nuclei in figure 5(c) also proved that our liposomal system is very stable and able to prevent the undesired release of doxorubicin causing side effects. Since doxorubicin needs to reach the nuclei to have therapeutic effect, it was hypothesized that doxorubicin encapsulated in PPL/GNPs should have minimum cytotoxicity until triggered release by laser irradiation.

The therapeutic effect of the Dox-PPL/GNPs with and without laser irradiation (λ_0 = 532 nm, 6-ns duration, 10 Hz frequency, intensity up to 400 mJ/pulse) was then evaluated (figure 6). For control experiments, cells were irradiated with the laser at the same condition and cell viability test showed that laser itself did not kill the cells under this condition. After incubating with Dox-PPLs, the cells were treated with or without laser. Our results showed that more than 80% of cells were viable in both cases. To demonstrate that any changes in cell viability was due to the release of doxorubicin from PPL/GNP complexes, the solution containing Dox-PPL/GNP complexes was irradiated and then added into cell culture to eliminate the thermal ablation effect of the GNPs upon laser treatment [36–38]. It was found that only ~20% of cells were viable after incubation with pre-treated Dox-PPL/GNPs compared to more than 80% viability after incubation with Dox-PPL/GNPs without laser treatment. Furthermore, GNPs themselves did not exhibit toxicity to cells with or without laser irradiation as shown by more than 90% cell viability. Therefore, the significant

viability decrease for cells incubated with Dox-PPL/GNPs was due to the release of doxorubicin from the complexes upon laser irradiation.

4. Conclusion

In summary, partially polymerized liposomes are stable at the upper physiologic temperature (40 °C) for days, but can instantaneously release the encapsulated contents upon excitation of the tethered gold nanoparticles with pulsed laser irradiation. The stability of the liposomes at physiologic temperature for prolonged periods of time is important especially for targeted drug delivery which may require recirculation of the liposomes for hours to days to maximize accumulation at target sites. In addition to the stability of the liposomes under physiologic conditions, this approach also provides precise spatial and temporal control of content release which should increase significantly the therapeutic index of encapsulated drugs and decrease the side effects in non-target tissues. We showed that the resonance matching of energy absorber and source is essential for the triggered release. Furthermore, we have demonstrated that the PPL/GNP complexes could enter into cells and had significant therapeutic effect upon triggered release of encapsulated therapeutic drugs. Currently, we are developing partially polymerized liposomes tethered with hollow gold nanoshells with a surface plasmon peak in the near infrared range for potential in vivo pre-clinical and clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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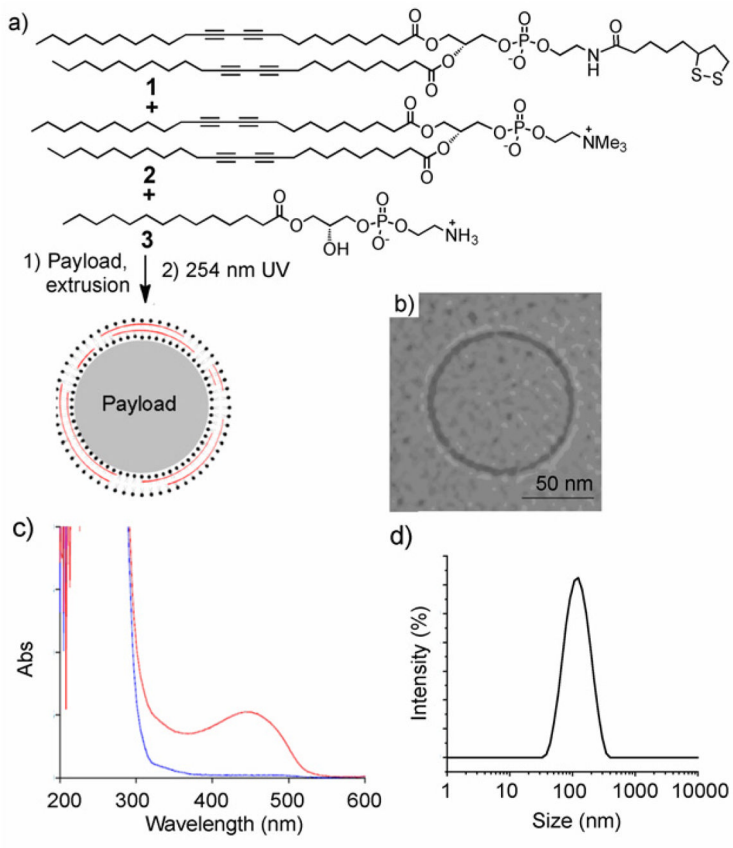

Figure 1.

Preparation and characterization of partially polymerized liposomes (PPLs). (a) Preparation of drug-loaded PPLs (red curves in PPL illustrate the regions containing polydiacetylenes), (b) a typical cryo-EM image showing a PPL, (c) UV-vis absorption spectrum of PPLs (red curve) and non polymerized liposomes (blue curve), and (d) size distribution of PPLs from dynamic light scattering measurement.

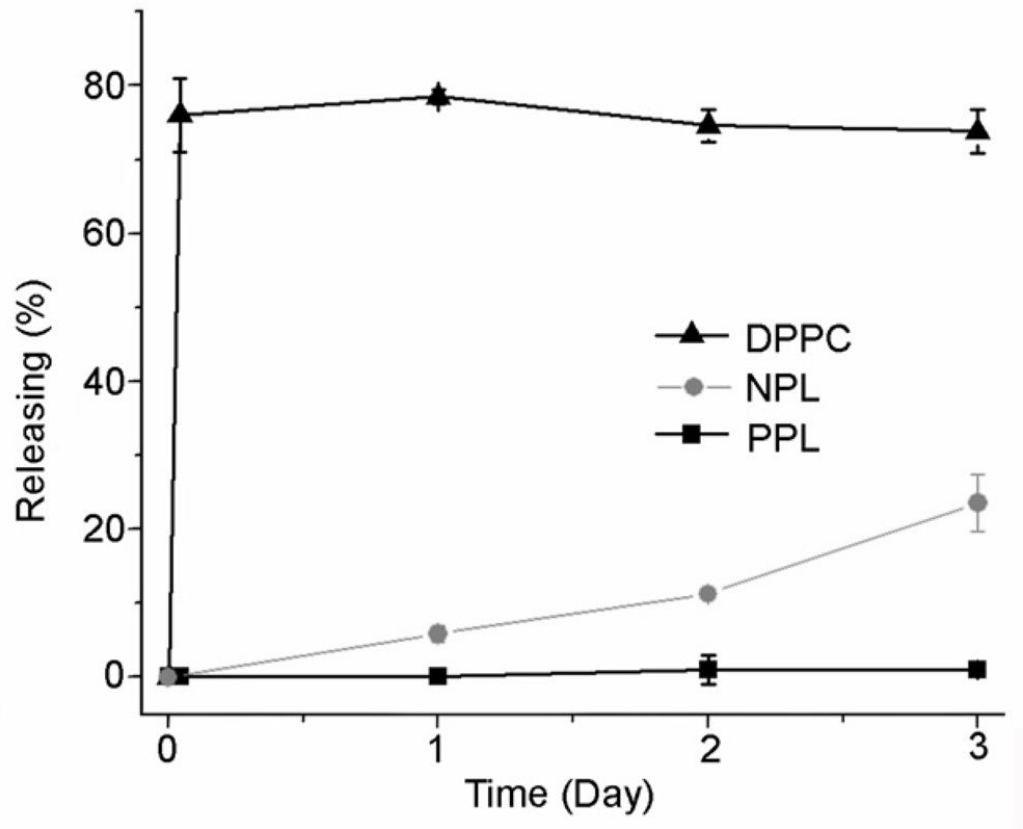

Figure 2.
(a) Stability of PPLs (■), NPLs (●), and NPL-DPPC (▲) at 40 °C over a period of 3 days. (n ≥ 3)

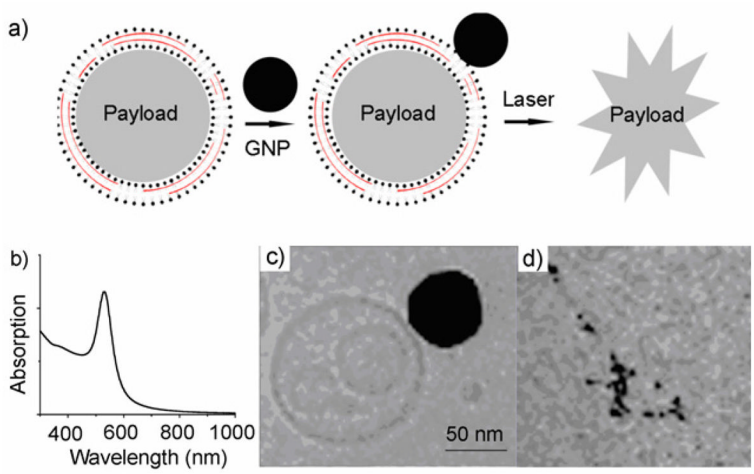

Figure 3.

a) Illustration of the releasing process; b) UV-vis absorption spectrum of gold nanoparticles (GNPs); c) a typical cryo-EM image of a PPL tethering a GNP; and d) a typical cryo-EM image showing PPL/GNP complexes after laser irradiation. The scale bar in c) also applys to d).

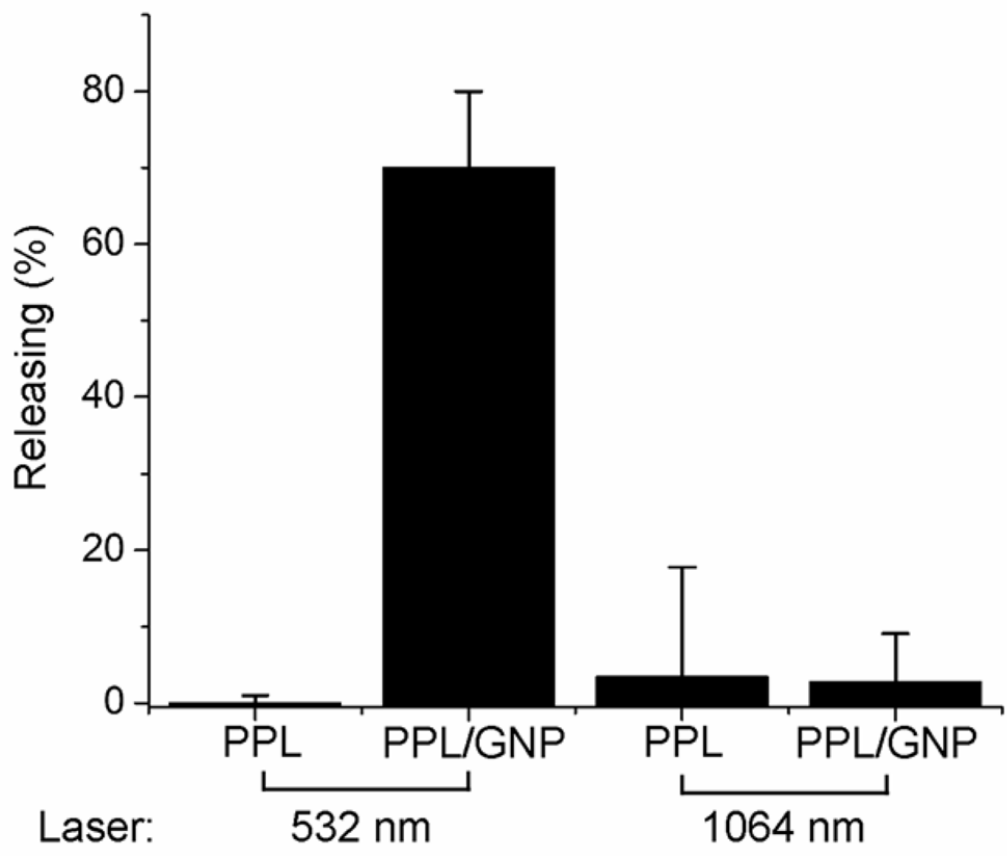

Figure 4.
Content release from PPLs and PPL/GNP complexes upon laser irradiation with a wavelength of 532 nm or 1064 nm for 10 pulses. ($n \geq 3$)

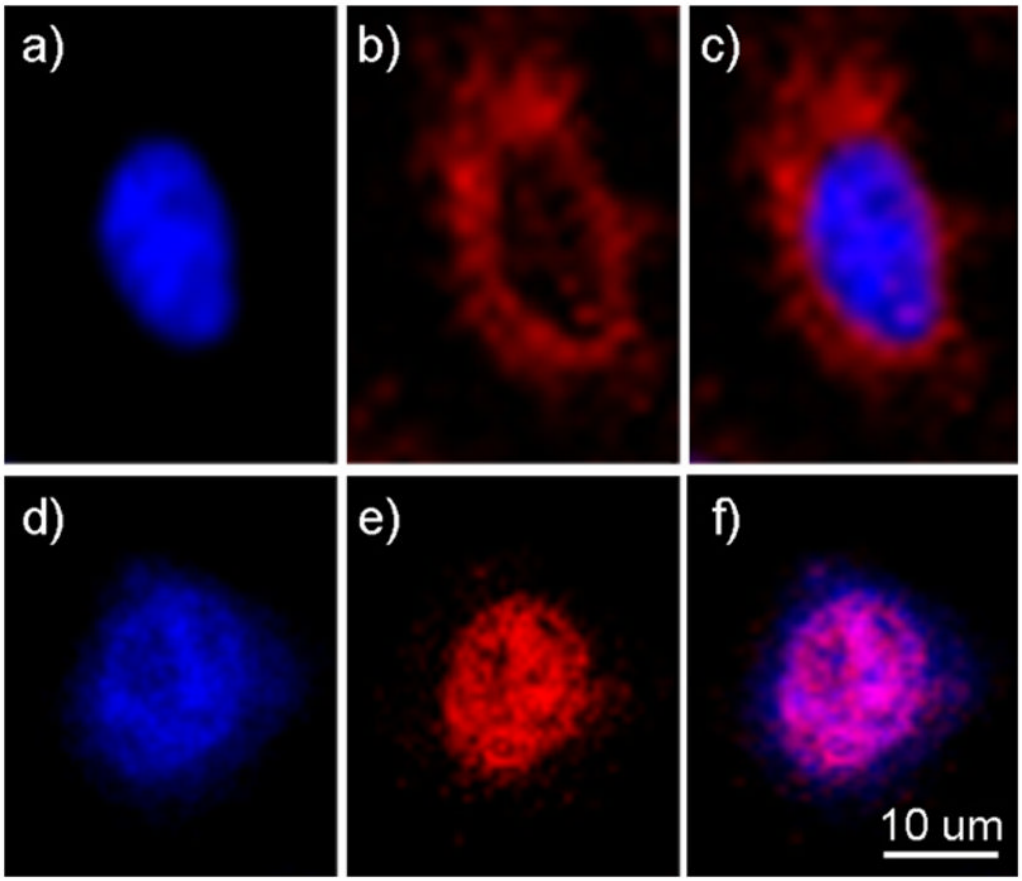

Figure 5.
Confocal fluorescence images of MDA-MB-231 cells incubated with Dox-PPL/GNP complexes shown in a) DAPI stain, b) doxorubicin, and c) the overlay of a) and b); and with free doxorubicin shown in d) DAPI stain, e) doxorubicin, and f) the overlay of d) and e). The scale bar in (f) applies to (a–e).

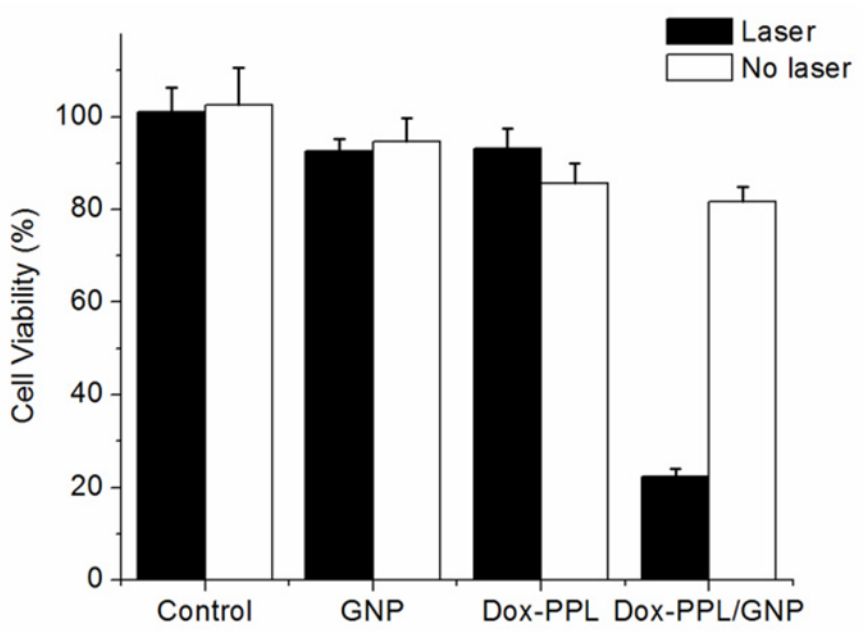

Figure 6.
Viability study of MDA-MB-231 cells incubated with GNPs, Dox-PPLs, and Dox-PPL/GNP with or without laser treatment (532 nm, 10 × 6 ns pulses).