Mesoporous Polydopamine Nanobowls Toward Combined Chemo- and Photothermal Cancer Therapy

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To enhance therapeutic efficacy and reduce side effects in cancer treatment, multimodal therapies are increasingly desired. In particular, combined chemo- and photothermal therapy has been developed as an approach with significantly higher therapeutic efficacy. However, long-term cytotoxicity arising particularly from poor biodegradability of the nanoparticles typically used for such treatment remains a challenge. In the present in vitro study, a new approach targeted toward cancer treatment that combines chemo- and photothermal therapy using bio-derived polydopamine (PDA) bowl-shaped mesoporous nanoparticles with exceptional biocompatibility is indicated. The potential of PDA mesoporous nanobowls as a new chemo- and photothermal agent was explored by loading the anti-cancer drug doxorubicin (DOX) into nanobowls and investigating their photothermal performance upon near-infrared (NIR) illumination. Strikingly, DOX loaded nanobowls show significantly higher pharmaceutical cytotoxicity to HeLa cells in vitro in comparison with free DOX due to their preferential uptake into cells. Following this with photothermal treatment resulted in nearly 100% cell death from the combined treatment of DOX loaded nanobowls and NIR illumination. This first step highlights the potential of PDA mesoporous nanobowls as a scaffold for combined chemo- and photothermal therapy for cancer treatment that offers new opportunities for combined pharmacological therapies to treat advanced disease states.

1. Introduction

Nanoparticles have been extensively used to transport chemotherapeutic drugs to malignant tissues/cells for cancer treatment, as they can be tailored to cross biological barriers, enable selective targeting of malignant cells, and offer strategies for sustained release of drugs to the targeted site.[1] A wide range of nanoparticles have been fabricated to use as drug delivery systems to target cancer cells including carbon, silica, gold, and various polymers, yet the long-term toxicity of these nanocarriers remain a significant challenge.[2] Besides, cellular uptake efficiency of the nanocarriers and delivering an effective dose of drug to targeted tumor sites before particles are cleared remain big barriers to their ultimate success as drug nanocarriers for cancer treatment.[3] Moreover, traditional cancer treatment is insufficient to completely kill cancer cells due to multiple drug resistance of malignant tissue/cells increasing their tolerance to toxic drugs.[3,4]

Recently, extensive research has been carried out on developing combined treatment of near infrared (NIR) induced photothermal therapy and chemotherapy to augment the cytotoxicity of chemotherapeutic agents.[3] Researchers have devoted efforts to the development of photothermal therapy agents that include carbon-based nanomaterials, noble metal nanostructures, semiconductors, black phosphorus, and their composites, comprehensively utilizing their intense absorption characterized by a large molar extinction coefficient and relatively high photothermal conversion efficiency.[6] Nevertheless, not only the fabrication process of these particular nanomaterials is elaborate and challenging, but also the majority of these substances show long-term toxicity, which remains problematic.[5,6,7]

There is an urgent demand for effective cancer therapies that possess a unique combination of photothermal and chemotherapeutic benefits, and which can eliminate large solid tumors as well as disseminated, metastatic nodules, while simultaneously preventing tumor recurrence without any residual toxicity. By selecting drug nanocarriers with long-term biocompatibility, free-radical-scavenging activity, cellular internalization efficiency, and by loading a sufficient dosage of drug in combination with the photothermal response, it may be possible to treat local tumors with improved therapeutic outcomes in malignant cancers.
In recent years, the melanin-like, naturally inspired biopolymer, polydopamine (PDA), has been widely used in various biomedical applications including drug delivery due to its excellent biocompatibility, colloidal stability, and distinctive drug loading facility.[10] Moreover, due to the adhesive nature of catechols and amines, similar to those in mussel adhesive proteins, PDA tends to show strong adhesion to all types of surfaces.[16] Finally, PDA has high photothermal conversion efficiency, making it ideal for photothermal transduction in the IR region of the spectrum. For instance, Lu and co-workers have demonstrated 40% photothermal energy conversion efficiency of PDA, indicating promising applications in photothermal-based cancer therapeutics, which is emerging as a powerful technique in cancer therapy due to the potential for localized treatment and minimal invasiveness.[8b,10] Additionally, due to its excellent flexibility through a high degree of control in shape and size of nanostructures that can be formed, and with high thermal stability (when compared to many nanostructured soft matter delivery systems), PDA offers opportunities for synthesis of various shapes and sizes of nanoparticles including spherical, cup-/bowl-shaped, and walnut-shaped.[11] For instance, in a previous study, we successfully synthesized various sizes of PDA mesoporous nanobowls by tuning reaction parameters.[12]

In order to select a drug nanocarrier, shape and size are important factors to consider, as these features determine their efficiency at passing through biological barriers, their cellular uptake efficiency and biodistribution, and ultimately their intracellular fate.[31] For example, Gao and co-workers have demonstrated a faster cellular uptake of bowl-like particles relative to spherical ones.[16] More recently, in our previous work, we found faster cellular internalization of PDA bowls compared to their spherical counterpart, as bowls tend to use their curved side to attach onto the cell membrane and thus are enwrapped faster by the cell membrane leading to faster uptake.[13b] Considering this, PDA bowl-shaped mesoporous nanoparticles (PDA mesoporous nanobowls) can be an ideal candidate as they show significantly faster cellular uptake relative to spherical nanoparticles with the same chemistry[13c] and can load a sufficient quantity of drugs in their cavity and mesopores.

With an aim to explore whether bowl-shaped particles could be utilized in the development of new chemo/photothermal technologies, we developed a simple and versatile strategy to efficiently kill cancer cells in vitro with a combined treatment of doxorubicin (DOX) loaded PDA mesoporous nanobowls, activated by NIR photothermal transduction (shown in Figure 1). Considering the size-dependent endocytosis pathways of PDA mesoporous nanobowls in HeLa cells (human cervical carcinoma epithelial cells) and the photothermal responsive properties of such particles shown in our previous studies, we selected nanobowls of around 180 nm diameter in this work.[12,13] We demonstrate a significant decrease of in vitro cell viability upon incubation with DOX loaded nanobowls followed by NIR illumination on HeLa cells. To our knowledge, this is the first demonstration indicating combined chemo- and photothermal therapeutic potential for cancer treatment in vitro using particles of this type, indicating such systems as a promising candidates for further study in cancer treatment.

Figure 1. Schematic diagram showing chemo- and photothermal properties of PDA mesoporous nanobowls a,b) Healthy adherent HeLa cells, after incubating with DOX loaded PDA mesoporous nanobowls showing morphological changes, and a few of them are dying, followed by near-infrared illumination, almost all cells are dead, causing their detachment from the surface.

2. Results and Discussion

2.1. Synthesis of PDA Mesoporous Nanobowls

PDA mesoporous nanobowls were synthesized following an established emulsion-induced interface anisotropic assembly method.[11c] Briefly, the formation of the nanobowl is based on the simultaneous formation of Pluronic F127/trimethylbenzene (TMB)/polydopamine (PDA) composite micelles, followed by nucleation of PDA and subsequent anisotropic growth of PDA on the surface of emulsion droplet templates. Morphology of the final product was confirmed by scanning electron microscopy (SEM) imaging (Figure 2a), which revealed mesoporous nanobowls of ≈180 nm diameter with ≈80 nm cavity and ≈7 nm mesopores. Transmission electron microscopy (TEM) imaging in Figure 2b further confirms the morphology of the nanoparticiles and reveals that the mesopores form connecting channels known as mesochannels that are arranged radially from the center to the surface of the nanobowls.[11b]

The size distribution and zeta potential of the nanobowls were analyzed using dynamic light scattering (DLS) and phase analysis light scattering (PALS) respectively (Figure 2c,d). These nanobowls have appropriate sizes for cell internalization and a remarkably narrow size distribution, as has been noted previously. Figure 2d shows negative zeta potentials of the nanobowls with moderate magnitudes in both water and cell culture medium, suggesting colloidal stability of the nanobowls in dispersion due to their negative surface charge. Chemical characterization of the nanobowls was accomplished using Fourier transform infrared spectroscopy (FTIR) in our previous study.[13c]

2.2. Photothermal Effect of PDA Mesoporous Nanobowls

Photothermal conversion efficiency of PDA mesoporous nanobowls was investigated in order to determine their potential...
efficacy for photothermal therapy. Experimental results in Figure 2e and f demonstrate that PDA mesoporous nanobowls suspended in PBS absorb light under NIR illumination, which is converted into thermal energy, thus increasing the temperature of the surrounding medium. After 500 s of NIR illumination, temperature of the surrounding medium increased significantly with increasing concentration of nanobowls (0, 25, 50, 100, and 200 µg mL⁻¹ particle loadings gave final temperatures of 37, 49, 53, 58, and 64 °C respectively). This translates to a photothermal conversion efficiency of 17.0%, comparable with the 23% calculated by Yang et al. for spherical polydopamine nanoparticles of similar size. [6e] Duration of NIR exposure was also an important factor in the extent of heat generated photothermal transduction, suggesting that photothermal conversion efficiency is strongly dependent on the absorption spectrum of the PDA mesoporous nanobowls, whereas total heat generation depends also on particle concentration and NIR exposure time.

2.3. Loading DOX into PDA Mesoporous Nanobowls and their Drug Release Profile In Vitro

PDA mesoporous nanobowls are functionalized with aromatic rings, which provide favorable π–π stacking interactions when loading the anti-cancer drug doxorubicin (DOX, Figure 3a). [10b,16] Obtained results in Figure 3b shows a remarkably higher loading efficiency of drugs with increasing concentration. The distinctive DOX peak at around 590 nm confirms adsorption of DOX into the nanobowls (Figure S1, Supporting Information). [10b] A standard curve of DOX was used to calculate loading efficiency of drugs into the nanobowls (Figure S2, Supporting Information). Previously it has been demonstrated that in comparison to non-porous PDA nanoparticles, mesoporous PDA nanoparticles have a higher capacity for loading DOX. [10b,17] In addition to π–π stacking, electrostatic attractions also contribute to loading DOX into the mesopores and cavity of PDA mesoporous nanobowls due to their negative surface charge. [10b,13a,18] It is assumed that their mesoporous structure also facilitates greater drug adsorption due to increased surface area to mass ratio.

DOX release kinetics were investigated after adsorbing DOX into the nanobowls. It has been previously reported that acidic environments are generally ideal to mimic tumor cell extracellular spaces, and considering this, drug release profiles were characterized here in acidic environments (pH = 5.5). [19] Obtained results in Figure 3c demonstrate that the cumulative release of DOX reached over 90% in 24 h at pH 5.5. Previous studies have suggested that this type of acidic environment is favorable for the disruption of π–π stacking between PDA mesoporous nanobowls and DOX due to protonation of the amine group of PDA. [20]

2.4. Cytotoxicity Test of HeLa Cells in Response to Various Treatments

With the aim of investigating the efficiency of PDA mesoporous nanobowls as nanocarriers, and the impact of combined treatments of DOX loaded PDA mesoporous nanobowls and NIR on HeLa cells (Figure 4a), propidium iodide (PI) testing was conducted using flow cytometry analysis. To undertake this investigation, first HeLa cells were incubated with PDA
mesoporous nanobowls, free DOX, or DOX loaded PDA mesoporous nanobowls for 4 and 24 h, and untreated HeLa cells were used as a control. Obtained results in Figure 4b demonstrate a large number of dead HeLa cells in the population that was incubated with DOX loaded PDA mesoporous nanobowls (≈75% and ≈86% at 4 and 24 h respectively). In contrast, negligible cytotoxicity was recorded for cells incubated with free DOX (DOX alone, ≈10% and ≈20% at 4 and 24 h respectively, Figure 4b). These findings not only indicate a faster internalization of DOX loaded PDA mesoporous nanobowls when compared to free DOX, but also suggest the wide distribution of DOX within the cells when carried by nanobowls, causing a greater incidence of cell death. These results suggest that PDA mesoporous nanobowls have successfully played a role as an efficient nanocarrier by carrying DOX inside the cells and evenly distributing it within the cells, while free DOX was not internalized as quickly or efficiently as PDA mesoporous nanobowls. This result can only be indicative of the cellular internalization mechanism of PDA mesoporous nanobowls. In our previous study, we have observed that PDA nanobowls use their curved edge to attach to the cell membrane, which is favorable for easier enwrapping by the cell membrane leading to faster internalization into HeLa cells.\textsuperscript{[13a]} We also demonstrated the location of the nanobowls within the cells, where they were found assembled and widely distributed in the intracellular environment.\textsuperscript{[12,13]} However, chemotherapeutic drug resistance was observed as ≈14% viable cells were found in the cell population after 24 h of incubation with DOX loaded nanobowls, indicating that the combined energy of chemo- and photothermal therapy is needed to completely destroy the cancer cells.

Considering the above findings, the cell samples (untreated cells, cells treated with PDA mesoporous nanobowls, free DOX, and DOX loaded PDA mesoporous nanobowls) were exposed to NIR irradiation (as shown schematically in Figure 4a). As observed in Figure 4c, NIR alone was insufficient to cause noticeable changes in cell viability. In contrast, combined treatment of DOX loaded nanobowls and NIR radiation resulted in significant cytotoxicity to the cells, where ≈98.2% dead cells were observed in the cell population. On the other hand, in comparison to the free DOX sample, nanobowl-incubated cells indicated greater cell death due to the presence of PDA, which efficiently absorbs light and converts it to thermal energy.\textsuperscript{[10a]}

Taken together, the above results suggest a greater therapeutic efficacy of PDA mesoporous nanobowls for combined chemo- and photothermal treatments on HeLa cells, when compared to any component of therapy on its own. Although these in vitro results are initially promising, in vivo studies would be an essential next step to determining the specificity and overall function of these materials.

2.5. MTS Cell Proliferation Assay of HeLa Cells Upon Various Treatments

To further confirm the above flow cytometry analysis results, MTS assay testing was conducted with the same experimental design at 24 h of incubation time. Figure 5a shows that cell viability was ≈95%, ≈72%, and ≈20% after treatment with PDA mesoporous nanobowls, free DOX, and DOX loaded PDA mesoporous nanobowls respectively. These results demonstrate...
that a significantly higher reduction of cell viability was caused by DOX loaded PDA mesoporous nanobowls as they were internalized faster and broadly distributed the drugs inside cells, which supports the data obtained by PI testing (Figures 5a and 4b). After 24 h of incubation with DOX loaded PDA mesoporous nanobowls followed by NIR irradiation, cell viability decreased by 98%, in contrast to negligible changes in cell viability observed with free DOX and NIR treatment (Figure 5b). Additionally, around 70% cell viability was found upon treatment of PDA mesoporous nanobowls and NIR, which indicates the impact of photothermal conversion of PDA on cells (Figure 5b). These findings further confirm that DOX alone (that is, free DOX) is incapable of rapid, efficient internalization into cells compared to DOX loaded into PDA mesoporous nanobowls, revealing the particles’ valuable role in facilitating drug internalization.
2.6. Confocal Imaging of HeLa Cells Upon Combined Chemo- and Photothermal Treatments

Confocal laser scanning microscopy (CLSM) analysis was undertaken to further confirm the above findings, and visually understand the impact of combined treatments of DOX loaded PDA mesoporous nanobowls and NIR irradiation on HeLa cells. Herein, cell samples were prepared using the same experimental design as flow cytometry analysis and MTS assay testing above. In Figure 6, we first focus on confocal images showing a red channel fluorescence signal indicating the presence of DOX. Note that DOX molecules that were loosely attached to the cell membrane were removed during multiple washing steps, whereas cells that were treated with DOX loaded PDA mesoporous nanobowls showed a significant red fluorescence signal relative to similarly concentrated

| Treatment                      | Bright | DAPI | DOX | Merge (DAPI & DOX) |
|-------------------------------|--------|------|-----|--------------------|
| Only Cells                    | (a1)   | (a2) | (a3) | (a4)               |
| Nanobowls 24 h                | (b1)   | (b2) | (b3) | (b4)               |
| Free DOX 24 h                 | (c1)   | (c2) | (c3) | (c4)               |
| DOX loaded nanobowls (4 h)    | (d1)   | (d2) | (d3) | (d4)               |
| DOX loaded nanobowls (24 h)   | (e1)   | (e2) | (e3) | (e4)               |
| DOX loaded nanobowls (24 h) + NIR | (f1) | (f2) | (f3) | (f4)               |

Figure 6. Confocal fluorescence images of HeLa cells after various treatments. NIR illumination for 5 min, 1 W cm⁻¹. Scale bars represent 20 μm.
free DOX, indicating significantly higher intracellular DOX accumulation when carried by PDA mesoporous nanobowls. In our previous study, we demonstrated the intracellular location of PDA mesoporous nanobowls by transmission electron microscopy (TEM) and TEM CLSM imaging analysis, where they were found broadly distributed inside the cells without any aggregation.[13a]

Consequently, these data provide further evidence that DOX loaded PDA mesoporous nanobowls were internalized quickly and evenly distributed the drugs inside the cells, which resulted in significant cell death within 4 h of incubation (Figure 6d1–d4), and could kill around 80% of the cells in 24 h of incubation (Figure 6e1–e4). In contrast, negligible cytotoxicity was observed in the cells after 24 h of incubation with free DOX (Figure 6c1–c4).

Moreover, cells were exposed to photothermal treatment (NIR illumination for 5 min) after 24 h of incubation with DOX loaded PDA mesoporous nanobowls. This process destroyed up to 98% cells (Figure 6f1–f4). Additionally, cells incubated with PDA mesoporous nanobowls did not show any obvious toxicity (Figure 6b1–b4). This outcome aligns with the findings of flow cytometry analysis and MTS assay testing.

3. Conclusions

In summary, we have introduced PDA mesoporous nanobowls as a highly functional drug nanocarrier, with capacity demonstrated in vitro for both chemo- and photothermal therapy. In comparison to free DOX, PDA mesoporous nanobowls show significantly faster cellular uptake, highlighting them as an efficient vector to locate drug molecules within cells. PDA nanobowls also showed excellent photothermal conversion efficiency under NIR illumination. These chemo- and photothermal agents were tested in vitro on HeLa cells, where cytotoxicity and viability of the cells were evaluated via PI and MTS assay testing respectively, along with confocal imaging analysis. Obtained results demonstrated a significant cytotoxic effect of combined treatments of DOX loaded PDA mesoporous nanobowls and NIR illumination on HeLa cells in comparison to free DOX, which suggests that PDA mesoporous nanobowls may offer a promising candidate to be explored for development of synergistic chemo- and photothermal therapy. Further work towards a therapeutic application would require testing the release of drugs from these particles in different pH conditions to determine release efficiency in nontumour environments (which would determine the likely extent of side effects). In vivo studies would then be essential to determine factors such as the biodistribution, degradation, and fate of these particles.

4. Experimental Section

Materials: Trimethylbenzene (TMB, 98%), dopamine hydrochloride, Pluronic F-127, phosphate-buffered saline (PBS), fetal bovine serum (FBS, Gibco), paraformaldehyde (PFA), MTS colorimetric cell proliferation assay kit (where MTS is 3-(4,5-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), antibiotic-antimycotic solution (100X), and 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich. Ammonia solution was from Ajax Finechem Pty., and 4´,6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific. Ethanol 96% was from Univa. Propidium Iodide (PI) was purchased from Merck. Other materials were Dulbecco’s Modified Eagle’s Medium (DMEM, Life Science) and trypsin (0.25%, Merck). All reagents were used as received, without further purification.

Characterization of PDA Mesoporous Nanobowls: Hydrodynamic diameters and zeta potential of particles were determined using dynamic light scattering (DLS) and phase analysis light scattering (PALS) respectively, using a Brookhaven NanoBrook Omni particle sizer and zeta potential analyzer. To examine the size and morphology of the particles, transmission electron microscopy (TEM, FEI Tecnai G2 T20 electron microscope under 200 kV, using LaB6 emitter) and field-emission scanning electron microscopy (FEI Magellan 400 FEG SEM) analysis techniques were undertaken. Fluorescence absorption and emission spectra of samples were recorded using a Varian Cary Eclipse Fluorescence spectrophotometer in 1 cm path-length quartz cuvettes. Chemical characterization of PDA particles was undertaken using a Cary 630 FTIR (Fourier Transform Infrared) spectrometer in the previous study.[13b]

Synthesis of PDA Mesoporous Nanobowls: PDA mesoporous nanobowls were synthesized following an established method known as an emulsion-induced interfacial anisotropic assembly.[20] Briefly, 1.5% (v/v) dopamine hydrochloride (DA) and 1.0% (v/v) Pluronic F127 (block copolymer) were dissolved in 1:1 water: ethanol mixture (total volume 10 mL) followed by addition of 2.0% (v/v) TMB under stirring. An emulsion was formed by ultra-sonication (2 min). In order to achieve an alkaline environment (pH 12.1), ammonium solution (3.75% (v/v), 28%) was added dropwise into the emulsion system. Synthesized nanoparticles were centrifuged with water and ethanol three to four times after 2 h of polymerization time. Eventually, particles were re-dispersed in 10 mL of (1:1) water ethanol mixture for hydrothermal treatment in a sealed Teflon-lined autoclave at 100 °C for 24 h. The final product was washed with water and freeze-dried for characterization and future use.

Loading Doxorubicin (DOX) to PDA Mesoporous Nanobowls: To evaluate the drug delivery properties of PDA mesoporous nanobowls, the anti-cancer drug doxorubicin (DOX) was loaded into the particles. A 1 mg mL⁻¹ nanobowls (total volume 5 mL) suspension was prepared for loading DOX. Various quantities of DOX (0.5, 1.5, and 3 mg) were dispersed in the above nanobowls suspension and this mixture was stirred for 24 h at room temperature to allow binding to the nanobowls. Afterward, DOX loaded nanobowls were washed by centrifugation with ultrapure water three to four times to remove free DOX, followed by freeze-drying for future use. All supernatants were collected to estimate the drug loading capacity. Loading efficiency of DOX in nanobowls was determined by fluorescence spectrophotometry at an excitation wavelength of 480 nm and an emission wavelength of 590 nm by subtracting the mass of the DOX in the supernatant from the total mass of the DOX in the initial solution divided by the total amount of DOX and expressing it as a percentage.[21]

Release Profile of PDA Mesoporous Nanobowls/DOX: To evaluate drug release kinetics, 1 mL PDA mesoporous nanobowls/DOX particle dispersion was loaded into a dialysis bag (molecular weight cutoff 3.5 kDa) with 25 mL 1x PBS buffer solution (pH 5.5). At different time intervals, 1 mL of PBS solution was collected to quantify the amount of released DOX by fluorescence spectrophotometry at wavelength at 590 nm. Note that 1 mL of fresh PBS (pH 5.5) was added into the solution at each sampling time to keep the total volume constant.[20]

Photothermal Performance of PDA Mesoporous Nanobowls: Measurement of photothermal performance of the nanobowls was done by monitoring temperature under NIR illumination (from an OSLON 9 PowerCluster IR, an array of nine OSRAM IR OSLON Black Series LEDs, wavelength = 850 nm, mounted on a heatsink and connected to a power source, providing 1 W cm⁻² radiant power at the sample). A thermocouple probe was used to record the temperature of the dispersions every 30 s. In this experiment, 300 µL of dispersions containing nanobowls of various concentrations including 25, 50, 100, and 200 µg mL⁻¹ were deposited into wells of a 96 well plate.

Herein, all measurements were done in triplicate and NIR illumination duration was 500 s.
Cell Culture: Human cervical cancer cells (HeLa cells) were cultured at 37 °C in a humidified incubator with 5% CO₂ in a complete medium (DMEM medium supplemented with 10% FBS and 1% P/S).

Cell Cytotoxicity Analysis with Flow Cytometry: HeLa cells were seeded at a density of 4 x 10⁶ cells per mL in individual 25 mL culture flasks and left to adhere overnight at 37 °C and 5% CO₂. Before any treatments, the original medium was replaced with a fresh medium to remove any dead cells. Afterward, cells were treated with DOX loaded mesoporous PDA nanobowls, or free DOX at an equivalent drug concentration of 6.8 µg mL⁻¹, or unloaded PDA mesoporous nanobowls, for different time periods (4 and 24 h). Herein, particle concentration was kept fixed at 100 µg mL⁻¹. After the desired incubation time, cells were treated with NIR operating at 1 W cm⁻¹ for 5 min followed by trypsinization and washing steps (centrifugation/re-dispersion cycles 3 times, 1200g, for 5 min with 1x PBS buffer solution). Finally, the supernatant was removed and 340 µL of 1x PBS solution was added to aliquots for flow cytometry analysis using flow core analysis technique (Fortessa X20c Flow Cytometry analyzer with B710 detector). Herein, propidium iodide (PI) was used as a red fluorescent nuclear and chromosome counterstain, which penetrates cells with damaged membrane and is excited at 488 nm. Cell samples were stained with PI dye (0.5 µg mL⁻¹) and kept in the dark for 15 min before flow cytometry analysis. Note that before the washing steps, supernatants of the cell samples were collected and stained with PI for flow cytometry analysis to determine the total number of dead cells in the cell population.

To calculate cell percentages, 20,000 events per sample were read.

In Vitro Cytotoxicity Assay of HeLa Cells: The MTS (3-(4,5-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell viability assay was conducted to investigate in vitro cytotoxicity of HeLa cells after various treatments. For this experiment, cells were seeded at a density of 2 x 10⁵ cells per mL in a 96-well plate with a volume of 50 µL in each well and allowed to adhere overnight at 37 °C and 5% CO₂ before being exposed to any treatments. After the desired treatments, absorbance at 490 nm was recorded using a CLARIOstar microplate reader (BMG Labtech, VIC, Australia). Data was analyzed by setting the absorbance value of the control (untreated cells) to 100%, and cell proliferation was expressed as a percentage of this control. The MTS assay was conducted in triplicate.

Confocal Imaging of HeLa Cells after Various Treatments: For imaging analysis, HeLa cells were seeded (2 x 10⁶ cells per mL) on coverslips into 6-well plates and allowed to adhere overnight at 37 °C and 5% CO₂ prior to undergoing treatments. Under the same experimental conditions as flow cytometry analysis, the cells were treated with PDA mesoporous nanobowls, free DOX, DOX loaded PDA mesoporous nanobowls, and/or NIR illumination. Eventually, samples were treated with DAPI dye (0.5 µg mL⁻¹) and kept in the dark for 15 min before being exposed to any treatments. Herein, particle concentration was kept fixed at 100 µg mL⁻¹ for different time periods (4 and 24 h). After the desired incubation time, cells were treated with NIR operating at 1 W cm⁻¹ for 5 min followed by trypsinization and washing steps (centrifugation/re-dispersion cycles 3 times, 1200g, for 5 min with 1x PBS buffer solution). Finally, the supernatant was removed and 340 µL of 1x PBS solution was added to aliquots for flow cytometry analysis using flow core analysis technique (Fortessa X20c Flow Cytometry analyzer with B710 detector). Herein, propidium iodide (PI) was used as a red fluorescent nuclear and chromosome counterstain, which penetrates cells with damaged membrane and is excited at 488 nm. Cell samples were stained with PI dye (0.5 µg mL⁻¹) and kept in the dark for 15 min before flow cytometry analysis. Note that before the washing steps, supernatants of the cell samples were collected and stained with PI for flow cytometry analysis to determine the total number of dead cells in the cell population.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords
efficient drug nanocarrier, mesoporous nanobowls, photothermal treatments, polydopamine

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