Drug Design

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Near-Infrared Light-Mediated Photoactivation of a Platinum Antitumor Prodrug and Simultaneous Cellular Apoptosis Imaging by Upconversion-Luminescent Nanoparticles**

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Abstract: *Platinum-based drugs are among the most active antitumor reagents in clinical practice; their application is limited by side effects and drug resistance. A novel and personalized near-infrared (NIR) light-activated nanoplatform is obtained by combining a photoactivatable platinum(IV) prodrug and a caspase imaging peptide conjugated with silica-coated upconversion-luminescent nanoparticles (UCNPs) for the remote control of antitumor platinum prodrug activation, and simultaneously for real-time imaging of apoptosis induced by activated cytotoxicity. Upon NIR light illumination, the Pt^{IV} prodrug complex is activated at the surface of the nanoparticle and active components are selectively released which display cytotoxicity against human ovarian carcinoma A2780 cells and its cisplatin-resistant variant A2780cis cells. More importantly, the caspases enzymes triggered by cytotoxicity would effectively cleave the probe peptide, thereby allowing the direct imaging of apoptosis in living cells.*

Platinum(II) compounds including cisplatin, carboplatin, and oxaliplatin are metal-based drug molecules that have been successfully used as chemotherapeutic reagents in clinics for the treatment of a broad spectrum of solid tumors.[1] Unfortunately, the lack of tumor specificity, severe toxic side-effects, and acquired or intrinsic tumor resistance of platinum(II) drugs remain the serious problems in clinical practice.[2] Therefore, systematic studies aimed at designing and preparing alternative platinum analogs that are potent and have minimal side-effects are currently the topics of considerable activities.[3] One promising strategy to address the shortcomings of cisplatin and its variant molecules is to rely on nonclassical platinum(IV) antitumor complexes with octahedral structures that have been found to exhibit effective antitumor activities in several preliminary clinical investigations, mainly attributed to their chemical inertness and redox properties. To achieve effective antitumor treatment, the bioactive Pt^{II} counterparts need to be activated first in the tumor cells through reduction of Pt^{IV} by several extra- or intracellular reducing agents such as glutathione, metallothionein, cysteine, and ascorbic acid.[4] In spite of their initial success, some Pt^{IV} prodrugs displayed inert kinetics in redox reactions. Furthermore, pervasive reductants in both healthy and tumor tissues may potentially compromise tumor specificity during the process of chemotherapy. An interesting alternative design based on photoactivatable antitumor Pt^{IV} complexes has been proposed recently to meet the challenging requirements of ensuring both tumor specificity and localized distribution of platinum drug moieties within tumors. In this case, a beam of light with high spatiotemporal resolution is needed to trigger the activation process which leads to the controlled release of cytotoxic Pt^{II} components into target tumor cells.[5] However, until now, most of the photoactive Pt^{IV} prodrug molecules respond mainly to relatively short wavelengths of light irradiations (e.g. UV, blue or green light). The unexpected photodamage to tissues, nucleic acids, proteins, and inefficient light/tissue penetration could potentially hamper the use of short-wavelength light-activated Pt^{IV} complexes for wide clinical applications.[6] Although two-photon photolysis using long-wavelength excitation has been used to investigate the possibility of ligand substitution in square-planar Pt^{II} complex,[7] the probability of two-photon absorption is typically small and large cross-sections of selected ligands are required for the system to be susceptible to longer-wavelength light irradiation. Given the clinical significance of photoactive Pt^{IV} prodrugs, the design of a simple and specific strategy that offers precise control of long-wavelength light activation of Pt^{IV} prodrug complexes specifically to tumor cells with minimum biological damage remains a big challenge. Therefore, further studies on this pertinent subject are urgently needed.

Lanthanide-doped upconversion-luminescent nanoparticles (UCNPs) serve as promising vectors to achieve remote control of light irradiation mostly because of their unique photoluminescent properties. In particular, UCNPs can absorb near-infrared (NIR) light and convert it into multiplexed emissions that span over a broad range (i.e., from UV to NIR region).[8] Such novel and amazing photoconversion allows UCNPs to function as a powerful NIR-induced mediator for their extensive in vitro and in vivo biomedical applications including remotely regulated photodynamic tumor inactivation,[9] photothermal therapy,[10] biosensing and imaging,[11] tuning of biological activities,[12] and site-specific delivery of gene or drug molecules to the targeted cells or living subjects.[13]

Herein, we demonstrated the functionality of a novel and personalized NIR light-mediated antitumor drug activation

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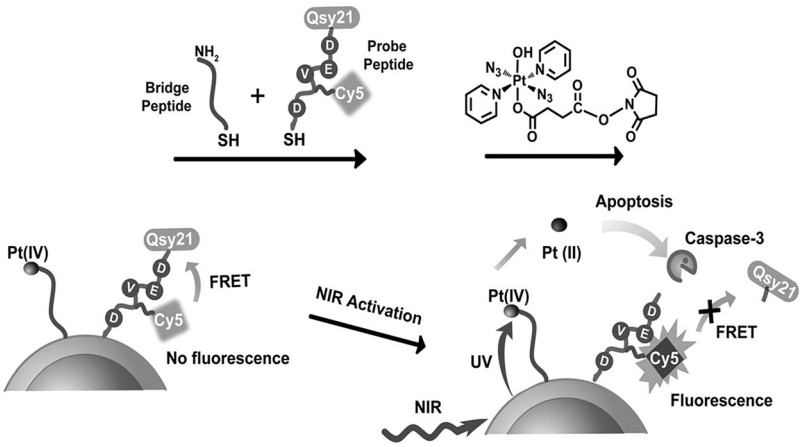
system compromising of a specific photoactive Pt^{IV} prodrug and an apoptosis sensing peptide incorporated onto the silica-coated UCNPs surface. Such NIR light-responsive nanoconjugate selectively triggers the localized activation of potent Pt^{II} complex at targeted tumors, thus maximally enhancing light penetration and avoiding nonspecific toxicity to healthy tissues. More importantly, the proposed nanoparticle conjugate is also capable of allowing simultaneous real-time imaging of the remotely controlled drug release process and evaluation of the corresponding antitumor activities in targeted tumor cell lines.

Scheme 1 illustrates the rational design for the NIR-mediated activation of the antitumor Pt^{IV} prodrug on core–shell silica-coated UCNPs and simultaneous apoptosis imaging. Typically, silica-coated upconversion-luminescent nanoparticles (UCNPs@SiO_2, $NaYF_4$: 30 mol% Yb, 0.5% mol Tm) with reactive amino terminal groups were first prepared according to the reported procedures.[11] TEM images showed that the average size of silica-modified UCNPs@SiO_2 was around 35 nm (see Figure S1 in the Supporting Information), and importantly, both the solutions of UCNPs and modified UCNPs@SiO_2 demonstrated similar photoluminescent properties that are suitable for light activation of Pt^{IV} prodrugs found on the surface of nanoparticles (Figure S1).



Scheme 1. Schematic illustration of NIR light activation of platinum(IV) prodrug and intracellular apoptosis imaging through upconversion-luminescent nanoparticles.

To ensure a high loading efficiency of the antitumor prodrug on the particle surface, a photoactivatable Pt^{IV} complex *trans,trans,trans*-$[Pt(N_3)_2(OH)(O_2CCH_2CH_2CO_2H)(py)_2]$ with one carboxyl group at axial position modified with *N*-hydroxysuccinimide (NHS) was chosen to conjugate with UCNPs@SiO_2,[5b,c] which were first functionalized with a bridge peptide sequence (KKKKKC) through an oligo(ethyl glycol) ($dPEG_6$) linker that contained a maleimide group to react with thiols. Furthermore, to conduct real-time apoptosis imaging, a short peptide probe with a flanking activatable fluorescence resonance energy transfer (FRET) pair consisting of a far-red fluorescence donor, Cy5, and a NIR quencher, Qsy21 (Cy5-acp-CGDEVDAK-Qsy21), for specific recognition of caspase-3, one of the key executioner caspase enzymes in apoptosis,[14] was also designed to couple with the maleimide-$dPEG_6$ UCNPs@SiO_2 to afford the final product of Pt^{IV} probe UCNPs@SiO_2 (see Scheme 1 and the Supporting Information). The optimal amounts of Cy5 probe and Pt content on the nanoparticle surface after cross-coupling reactions were determined to be 2.0 $\mu M\,mg^{-1}$ and 3.5%, as confirmed by UV/Vis absorption spectroscopy and inductively coupled plasma spectrometry (ICP) analysis (Figure S2).

We then examined the feasibility of photoinduced Pt^{IV} prodrug activation through the use of Pt^{IV} probe UCNPs@SiO_2.

The photoactive $[Pt(N_3)_2(OH)(O_2CCH_2CH_2CO_2H)(py)_2]$ complex was used as a control (Scheme S1). The precise control of drug release from the complex was demonstrated by measuring the absorbance change and the concentration of released Pt^{II} component from nanoparticle was determined by ICP analysis. Prior to NIR light illumination, platinum release was not observed from both the free Pt^{IV} complex and the Pt^{IV} probe UCNPs@SiO_2, indicating the stability of the Pt^{IV} prodrug and the Pt^{IV} probe UCNPs@SiO_2 under dark conditions. Upon 980 nm laser excitation, the effective release of the Pt component could be detected from the solution of Pt^{IV} probe UCNPs@SiO_2 and the release of Pt was found to increase with prolonged light irradiation (Figure 1). By contrast, similar NIR illumination of the free the Pt^{IV} prodrug did not lead to obvious Pt release and the effective Pt content could only be identified when the Pt^{IV} complex was exposed to UV light (Figure S3), clearly demonstrating that the Pt^{IV} nanoparticle prodrug conjugate could be photo-

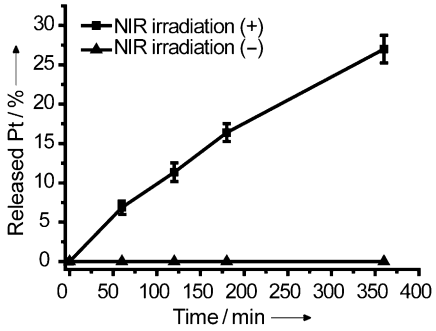


Figure 1. NIR light-responsive photoactivation of the Pt^{IV} probe UCNPs@SiO_2 in phosphate-buffered saline (PBS) at increasing irradiation times. The sample without NIR laser treatment is used as control.

activated by 980 nm laser irradiation, and such NIR light activation allowed the remote control of the Pt^{II} complex release for specific tumor treatment.

The controlled photoactivation of the Pt^{IV} prodrug was further performed in target tumor cells. In this study, a well-characterized tumor cell line pair; cisplatin susceptible human ovarian carcinoma A2780 cell and its derivative cisplatin-resistant subline A2780cis cell were chosen to incubate with the Pt^{IV} probe UCNPs@SiO_2. After 1 h of NIR irradiation and followed by another 24 h of incubation, the photo-activated cytotoxicity was measured by a standard MTT assay.[15] Similar cellular incubation but no NIR light illumination was used as a parallel control. To assess the potential phototoxicity, the NIR irradiation measurements were also conducted on tumor cells and the cells were incubated with free Pt^{IV} complexes and the peptide (KKKKKC/DEVD-probe) UCNPs@SiO_2 conjugate without the Pt^{IV} prodrug on the particle surface (e.g. NH_2-UCNPs@SiO_2). As shown in Figure 2 A and B, NIR light treatment of 10 μM Pt^{IV} probe UCNPs@SiO_2-incubated A2780 cells for 1 h resulted in

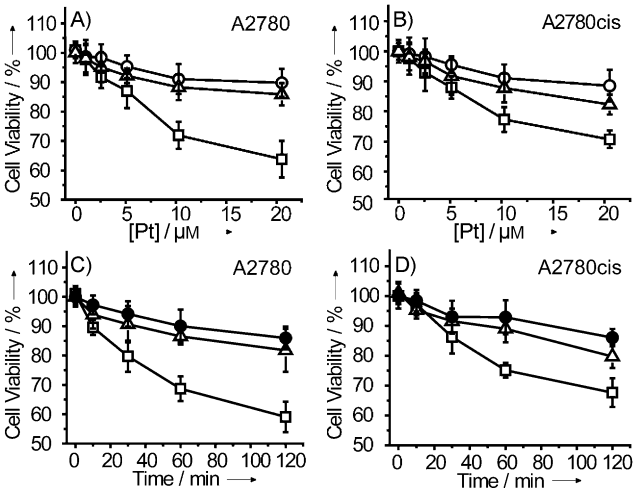


Figure 2. The cytotoxicity assays of the Pt^{IV} probe UCNPs@SiO_2 (□) and NH_2-UCNPs@SiO_2 (△) in A2780 and cisplatin-resistant A2780cis cells. A and B) NIR irradiation of incubated cells with different concentrations at 1 h of NIR irradiation (1.5 $W cm^{-2}$). The cells treated with the Pt^{IV} probe UCNPs@SiO_2 but no light illumination were used as controls (○). C and D) NIR irradiation of 10 μM sample-incubated cells under increasing exposure time. The cells treated with NIR light illumination alone but no prodrug incubation were used as controls (●).

obvious cytotoxicity with about 72% cell viability; a similar cell viability (77%) was also found in the cisplatin-resistant A2780cis cells incubated with the Pt^{IV} probe UCNPs@SiO_2 (10 μM). Both A2780 and A2780cis cells displayed increasing NIR responsive cytotoxicity (e.g., 64% cell viability for A2780 cells, and 70% for A2780cis cells) when higher concentrations of Pt^{IV} probe nanoconjugates (e.g. 20 μM) were used (Figure 2 A and B). Moreover, the NIR-responsive Pt^{IV} probe UCNPs@SiO_2 was found to indicate the higher antitumor activities in A2780 and A2780cis cells when compared to the free Pt^{IV} complex, which was activated by UV light to control the release of a similar amount of Pt^{II} component (see Figures S4A and S4B). Although more potent cytotoxicity of Pt^{IV} complex could be easily achieved using longer time UV irradiation, excessive exposure to UV light could lead to drastic photodamage to the cells which should be best avoided (Figure S4C and S4D). We further compared the cytotoxicity of the Pt^{IV} probe UCNPs@SiO_2 with that of standard cisplatin in tumor cells. Considering the effective Pt release from nanoparticles, the NIR-mediated Pt^{IV} probe UCNPs@SiO_2 conjugate exhibited slightly higher drug activity in A2780 cells when compared to cisplatin, however, a more significant antitumor activity could be observed in cisplatin-resistant A2780cis cells when the Pt^{IV} probe UCNPs@SiO_2 was irradiated with NIR light (Figure S5). Furthermore, the photoactivatable tumor cytotoxicity of Pt^{IV} probe UCNPs@SiO_2 was also found to be dependent on the duration of NIR irradiation. As shown in Figure 2 C and D, greater cell lethality in A2780 (59%) and A2780cis cells (68%) could be observed when longer times of light exposure (120 minutes) were applied. Obvious cytotoxic effects were not detected for both A2780 and A2780cis cells when treated with the Pt^{IV} probe UCNPs@SiO_2 without light illumination. Moreover, NIR irradiation of tumor cells alone and the cells treated with peptide NH_2-UCNPs@SiO_2 conjugate did not give rise to significant cytotoxicity (Figure 2), unequivocally suggesting that the Pt^{IV} probe UCNPs@SiO_2 could serve as a reliable prodrug conjugate that enables NIR light irradiation to remotely control the effective release of drugs to inactivate targeted tumor cells, including those with cisplatin resistance.

To determine the quantitative distribution of effective antitumor Pt in cellular nucleus upon NIR light irradiation of 10 μM Pt^{IV} probe UCNPs@SiO_2-incubated tumor cells, the nucleus DNA was extracted based on the standard protocol from Promega (Figure S6).[15] The ICP measurement confirmed the effective Pt content in A2780 (0.51 ± 0.03 ng Pt per microgram DNA) and cisplatin-resistant A2780cis (0.31 ± 0.01 ng Pt per microgram DNA) cell nucleus. As control, similar Pt^{IV} probe UCNPs@SiO_2 incubation but no 980 nm laser irradiation demonstrated no obvious Pt distribution in the nucleus. The enhanced platination of DNA observed in the Pt^{IV} probe UCNPs@SiO_2 pre-treated cells further indicated the possibility of employing UCNPs as effective platforms to specifically activate the Pt^{IV} prodrugs at targeted tumor areas. The higher DNA platination distribution of the NIR-responsive Pt^{IV} probe UCNPs@SiO_2 when compared to cells treated with either free Pt^{IV} prodrug complexes or cisplatin molecules suggests that UCNPs@SiO_2 is effectively delivered to the cell structures and is consistent with results obtained in antitumor cytotoxicity studies for A2780 and A2780cis cells (Figure S6).

A cytotoxic mechanism study was also performed to investigate the feasibility of the photoactivated Pt^{IV} nanoparticle prodrug responsible for executing programmed cell death. Typically, the Pt^{IV} probe UCNPs@SiO_2 (0.2 μM) was incubated with recombinant human active caspase-3/CCP32 in caspase-3 working buffer at 37°C and a 10-fold enhancement in the fluorescent intensity of Cy5 at 665 nm was observed (Figure 3). Similar enzyme hydrolysis upon treat-

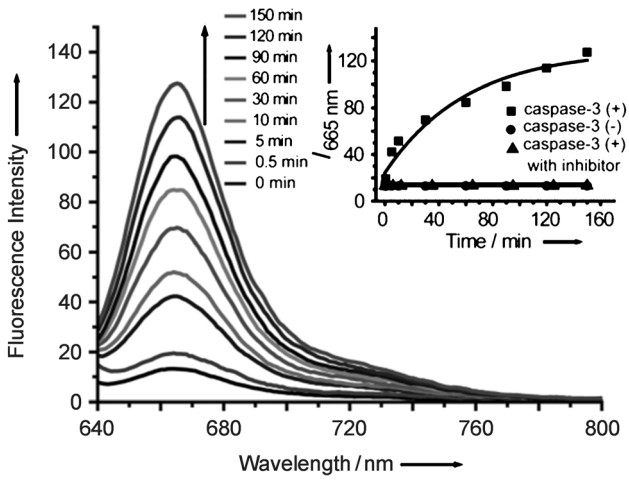


Figure 3. Time course of fluorescence emission spectra of the Pt^{IV} probe UCNPs@SiO_2 incubated with recombinant human active caspase-3 (2 Unit) at 37 °C. Excitation wavelength: 620 nm.

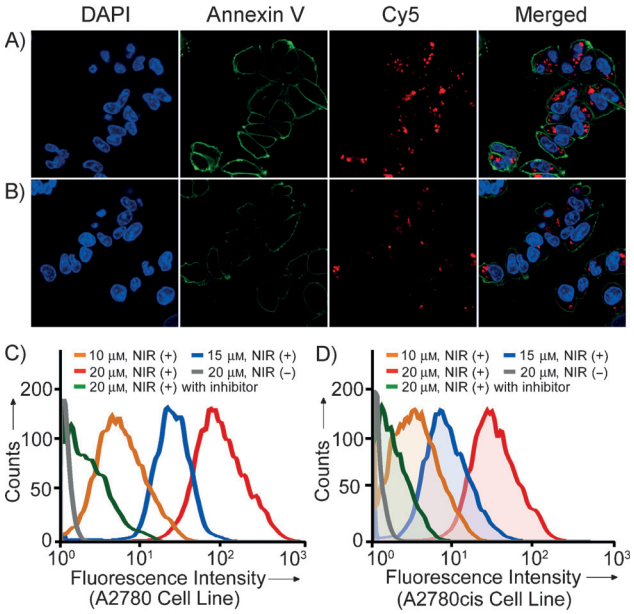


Figure 4. Live-cell apoptosis imaging for NIR irradiation (1 h) of 15 μM Pt^{IV} probe UCNPs@SiO_2-incubated cells: A) A2780 cells, and B) Cisplatin-resistant A2780cis cells. (blue: DAPI; green: Annexin V; red: Cy5.) Quantitative flow cytometric analysis of C) A2780 and D) A2780cis cells treated with different concentrations of the Pt^{IV} probe UCNPs@SiO_2 (10, 15, and 20 μM, respectively) and 1 h of NIR irradiation. Cells treated with Ac-DEVD-CHO (20 μM) inhibitor and NIR irradiation of cells without Pt^{IV} probe UCNPs incubation were used as controls.

ment of caspase-3 inhibitor (e.g. Ac-DEVD-CHO, 20 μM) further revealed very weak fluorescence, clearly showing that peptide probe, Cy5-acp-CGDEVDAK-Qsy21, found on the UCNPs@SiO_2 surface could be cleaved by caspase-3 which led to a disruption of the FRET process by moving the quencher Qsy21 away from the fluorescent donor Cy5, and thus significantly turned on the emission from Cy5 (Scheme 1). Inspired by the promising fluorescence enhancement from standard enzyme hydrolysis, we systematically examined the ability of the Pt^{IV} probe UCNPs@SiO_2 to image caspase-3 and to evaluate in real time the photoactivated cytotoxicity arising from triggered apoptosis during the localized drug release process. As shown in Figure 4 A and B, NIR light irradiation of Pt^{IV} probe UCNPs@SiO_2-incubated A2780 and A2780cis cells could lead to a remarkable Cy5 fluorescence in both cells. On the other hand, obvious fluorescence was not observed in the control experiments for cells that were either treated with caspase-3 inhibitors or not exposed to laser irradiation (Figure S7 and S8). The cellular imaging clearly suggested the occurrence of apoptosis where the activated caspase-3 cleaved the sensing peptide and deactivated FRET by releasing the Qsy21 quencher from the nanoparticle surface. Furthermore, the induced apoptotic pathway was also confirmed when the Pt^{IV} probe UCNPs@SiO_2 or standard cisplatin-treated tumor cells were incubated with FITC-tagged Annexin V (Figure 4 and Figures S7 and S8).[16] Although the exact nature of the induced programmed cell death remains unclear, both our Pt^{IV} probe UCNPs@SiO_2 conjugate and the standard detection assay provided the clear evidence that the observed programmed cell death most probably involved the photoactivated cytotoxicity of the Pt^{IV} prodrug UCNPs@SiO_2 conjugate. Similar caspase-dependent fluorescence detection was also obtained based on quantitative flow cytometric (FCM) analysis (Figure 4 C and D), which further confirmed the signs of apoptosis and the high specificity of the Pt^{IV} probe UCNPs@SiO_2 for activated caspase. Moreover, FCM studies also revealed that the fluorescence intensities of photoactivated Pt^{IV} probe UCNPs@SiO_2 in A2780 and A2780cis cells were enhanced when the higher concentration of nanoconjugate was used. The activated apoptosis fluorescence increasing trend was in accordance with the results observed in cytotoxicity studies, indicating that NIR light activation of the Pt^{IV} probe UCNPs@SiO_2 initiated apoptosis, and significantly, the induced apoptosis offers the opportunity to perform a real-time evaluation of the antitumor cytotoxicity by light-controlled drug release.

In summary, we have presented a novel and personalized strategy for the remote control of platinum prodrug activation and real-time imaging of apoptosis by integrating photoactivatable Pt^{IV} prodrugs and caspase imaging probes into silica-coated UCNPs. Upon NIR light irradiation, the converted emission from UCNPs@SiO_2 could locally activate the Pt^{IV} prodrug and thus efficiently induced potent antitumor cytotoxcity in both cisplatin-sensitive and resistant tumor cells. Moreover, such NIR light-controlled tumor inactivation triggers the cellular apoptosis and the highly activated caspase could cleave the NIR imaging peptide probe from the nanoparticle surface, thus greatly turning on the quenched NIR fluorescence of Cy5. These systematic studies indicated that the Pt^{IV} probe UCNPs@SiO_2 could not only provide a promising strategy to remotely control the localized activation of the Pt^{IV} prodrug at the target tumor sites, but more importantly, the developed Pt^{IV} probe UCNPs@SiO_2 conjugates may also serve as personalized tumor markers to image in real time and evaluate the antitumor therapy at the cellular level.

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