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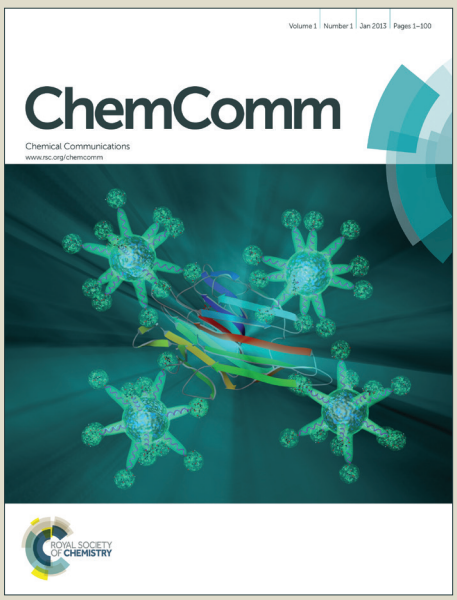

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ARTICLE TYPE

NIR-Responsive and Sugar-Targeted Polypeptide Composite Nanomedicine for Intracellular Cancer Therapy

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The upconversion nanoparticles (UCNPs)-loaded polypeptide composite nanoparticles that present fast NIR-sensitivity and tunable sugar-targeting properties are fabricated, opening a new avenue for on-demand and targeted cancer therapy. The half maximal inhibitory concentration (IC_{50}) of the nanoparticles dropped 4.7-fold or 3.1-fold compared to non-targeted or non-irradiated counterparts.

As the near infrared (NIR, 750~1000 nm) light can penetrate deeply into tissues with less scattering and damage, the NIR-sensitive polymeric nanocarriers might achieve spatiotemporal, pulsatile, and on-demand drug release in the diseased sites and are promising as a noninvasive technology for cancer therapy.[1] To combat cancer, the polymeric nanomedicines have to at least include the four characteristics: (1) extravasation from the vascular wall during long blood circulation, (2) penetration into tumor tissue through the enhanced permeation and retention effect (i.e., EPR effect), (3) tumor cell-uptake via active targeting effect (e.g., ligand-receptor recognition), and (4) quick drug release inside the cells.[2] Both the long-circulating and EPR characteristics can be easily integrated into the poly(ethylene oxide) (PEO)-based micellar nanocarriers (< 200 nm) to enhance accumulation into solid tumors with porous structures. However, most photoresponsive polymeric nanocarriers not only lack of NIR-sensitivity and biodegradability that evoke undesirable drug dosing and some concerns on *in vivo* applications, but also lack of highly active tumor-targeting property to achieve efficient cellular uptake, which is still challenging for polymeric therapeutics and sophisticated drug delivery systems (DDSs).[1-4] To address these problems, herein, we for the first time synthesize a novel class of photoresponsive lactose-terminated PEO-b-poly(*S*-(*o*-nitrobenzyl)-*L*-cysteine) (Lac-PEO-b-PNBC) block copolymers (supporting information, **Scheme S1**, **Table S1**, **Figure S1-3**),[5,6] and fabricate the UCNPs-loaded polypeptide composite nanoparticles (i.e., Lac-PEO-b-PNBC/UCNPs) having fast NIR-sensitivity and tunable sugar-targeting properties that are useful for on-demand and targeted DDSs and cancer therapy (**Figure 1**).

Multivalent carbohydrate ligands (e.g., glycopolymers and sugar-coated nanoparticles) that can specifically recognize and cluster cell-surface receptors (e.g., lectins) throughout the biological world have been increasingly investigated for targeted DDSs and biotechnology.[7] For instance, ricinus communis agglutinin (RCA_{120}) lectin can specifically bind lactose- (Lac) and/or galactose-pendant polymers or nanoparticles, resulting in the lectin-cross-linking aggregates.[7b,8] After the addition of RCA_{120} (0.5 mg/mL), the turbidity of the mixed solution increased with the concentration or the weight percentage of Lac-PEO-b-PNBC (**Figure 2A**, **Figure S4**, **Table S2-3**). This result indicates that (1) the binding between Lac-PEO-b-PNBC or its Lac-coated micelles and RCA_{120} occurred and probably resulted in large cross-linking aggregates, and (2) the binding ability of Lac-coated micelles might be tuned by the Lac density on the surface of micelles.[7b,8] The binding process was further monitored by using online dynamic light scattering (DLS) (**Figure 2B**). The cross-linking aggregates dynamically increased over the incubation time, and then leveled off within ~200 s, demonstrating that the binding between Lac-PEO-b-PNBC or its Lac-coated micelles and RCA_{120} indeed occurred and fast completed.[7b,8a] Note that the cross-linking aggregates became bigger over increasing the concentration or the weight percentage of Lac-PEO-b-PNBC (**Table S4**). In contrast, the addition of nonspecific lectin concanavalin A (Con A) does not result in changing both the optical density and the nanoparticles size of the mixed solution (**Figure 2A**, **B**), which further evidences that Lac-PEO-b-PNBC or its Lac-coated micelles specifically bind with RCA_{120}, but not with Con A.

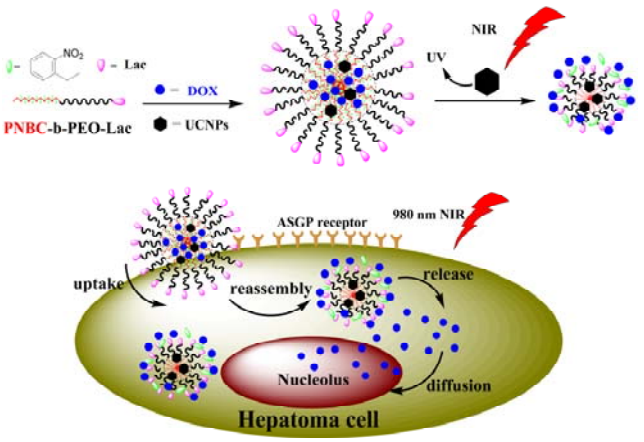

Figure 1. Illustration of the self-assembled NIR-responsive and sugar-targeted polypeptide composite nanomedicine, the cellular uptake, the NIR-triggered reassembly, and the quick DOX release.

Do the Lac-coated micelles have adjustable targeting effect to the mammalian cells? HepG2 cells have overexpressed asialoglycoprotein (ASGP) receptors compared to HeLa cells,[7c] to demonstrate the targeting effect, the cytotoxicity of the anticancer drug doxorubicin (DOX)-loaded and Lac-coated micelles against the different type cells is evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The DOX-loaded and Lac-coated micelles with different Lac densities produce a similar cell viability of about 40 % and half maximal

inhibitory concentration (IC_{50}, as calculated from 6 samples) ranging from 6.82 ~ 7.08 μg DOX equiv/mL when being cultured against HeLa cells for 48 h ((0, 30%, 70%, and 100% denote the weight percentage of Lac-PEO-b-PNBC used for the fabrication of Lac-coated micelles, **Table S2**); **Figure 2C** and **Table S5**). These results demonstrate the Lac ligands dangled on the micelles do not enhance the micelles to be internalized by HeLa cells. However, IC_{50} greatly decreases from 10.07 to 2.14 μg DOX equiv/mL (4.7-fold decrease) when 70% Lac-coated micelles are cultured against HepG2 cells for 48 h (**Figure 2D** and **Table S5**). Compared with 30% and 100% Lac-coated micelles, 70% Lac-coated micelles give the lowest IC_{50} and the highest targeting effect to HepG2 cells. These results convincingly confirm that the Lac-coated micelles with different Lac densities exhibit a tunable active targeting effect to HepG2 cells compared to HeLa cells.[7c,8]

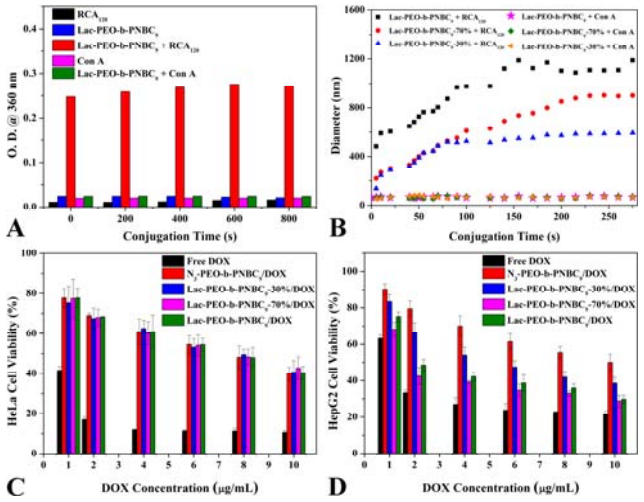

Figure 2. The optical density at 360 nm of Lac-coated micelles solution (30 μg/mL) after addition of RCA_{120} or Con A lectins at different times (A); the size dependence of Lac-coated micelles (30 μg/mL) on the time monitored by on-line DLS (B); the viability of HeLa cells (C) or HepG2 cells (D) being incubated with DOX-loaded and Lac-coated micelles for 48 h.

As the photolabile moiety *o*-nitrobenzyl (NB) has a large absorption coefficient,[1f] the Lac-PEO-b-PNBC micelles present a fast UV-sensitivity upon 365 nm UV irradiation and the photocleavage reaction finished within 20 min (**Figure S5**). However, the Lac-PEO-b-PNBC micelles do not exhibit a NIR-sensitivity even after 4 h irradiation using a high power-density femtosecond pulsed laser (**Figure S6**). This is because the simultaneous two-photon process of NB derivatives is inefficient and slow due to the lower two-photon absorption cross-section (0.01~0.03 GM).[1f,1g] Inspired by the core-shell lanthanide-doped UCNPs that can efficiently convert continuous-wave NIR light (e.g., a cheap 980 nm diode laser) into UV or visible light,[9] we have fabricated the UCNPs-loaded polypeptide composite nanoparticles by the film rehydration method.[10] Characterized by DLS and transmission electron microscopy (TEM), the composite nanoparticles have a hydrodynamic diameter (D_h) of 116 ± 6 nm and a spherical morphology (**Figure 3A** and **3C**). The clear contrast between the composite nanoparticles and the blank ones shows that these composite nanoparticles have a dark UCNPs-encapsulated core. After 980 nm NIR irradiation on the composite nanoparticles solution, the broad absorption peaks ranging from 300 ~ 400 nm increased over irradiation time, and then leveled off after 30 min (**Figure 3B**). Monitored by on-line DLS, the composite nanoparticles progressively reduced from 116 ± 6 nm to 43 ± 4 nm during the NIR irradiation process. These results demonstrate that the pendant NB groups can be gradually photocleaved from the PNBC core and the composite nanoparticles present a fast NIR-sensitivity in aqueous solution.[1f,1g,6e] This conclusion can be explained by the followng facts: (1) UCNPs absorb 980 nm NIR light and convert it to high-energy photons in UV and visible regions (**Figure S7**);[9] and (2) the emitted UV photons that are absorbed by NB groups activate the photocleavage reaction, resulting in both the disassembly of the composite nanoparticles and the reassembly of decaged Lac-PEO-b-poly(L-cysteine) (PLC) into smaller micelles (**Figure 1** and **Scheme S2**). TEM further confirmed most of the composite nanoparticles disassembled and reassembled into smaller micelles after 980 nm NIR irradiation for 40 min (**Figure 3D**), which is consistent with the DLS analysis.

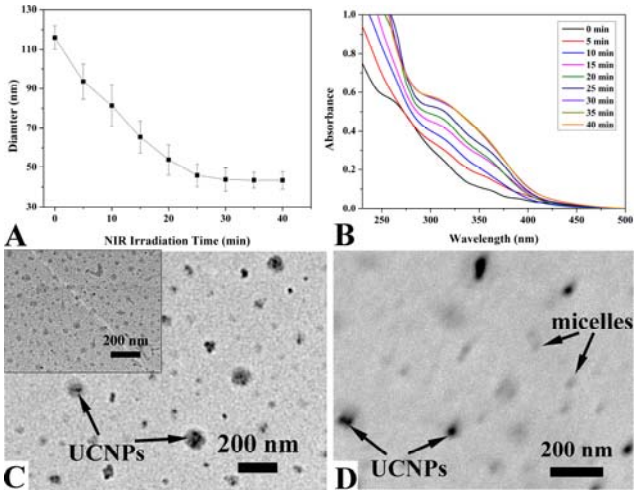

Figure 3. The dependence of the average diameter of Lac-PEO-b-PNBC/UCNPs composite nanoparticles on NIR irradiation time (A); UV-vis time-resolved spectra of the composite nanoparticles upon NIR irradiation (B); TEM photographs of the composite nanoparticles before (C; inset is Lac-PEO-b-PNBC micelles without UCNPs) and after 40 min NIR irradiation (D).

To demonstrate the concept of NIR-triggered drug release, the DOX-loaded composite nanoparticles with a D_h of (132 ± 10) nm and a drug-loading capacity of 10.9 wt% were fabricated by the film rehydration method (**Figure S8**). The accumulative DOX release from the composite nanoparticles linearly increases with NIR irradiation time, demonstrating that DOX can be released in a controllable manner (**Figure 4A**). The released DOX attained 73 % within 12 h and the apparent drug-release rate increased about 2.2-fold after the composite nanoparticles were irradiated by periodic NIR light (i.e., 6 × 5 min irradiation). As the photocleavage reaction of NB groups induced the disassembly of the nanoparticles, the instantaneous drug release profile can be switched in a pulsatile or "on-off" mode given the effect of the off-state drug leakage from nanoparticles.[6b]

Do the DOX-loaded composite nanoparticles possess the NIR-triggered cytotoxicity against HeLa cells? The cell viability remained > 90 % after 48 h culture and both the blank composite nanoparticles and the NIR irradiation demonstrated a lower cytotoxicity (**Figure S9**). After 5 min or 10 min NIR irradiation upon the DOX-loaded composite nanoparticles that were incubated with HeLa cells (**Figure 4B**), however, the cell

viability decreased obviously and IC_{50} dropped from 7.20 μg DOX equiv/mL to 4.08 μg DOX equiv/mL (1.7-fold decrease), and then to 2.32 μg DOX equiv/mL (3.1-fold decrease) compared to the non-irradiated sample. These results convincingly demonstrate that the DOX-loaded composite nanoparticles exhibit a tunable NIR-triggered cytotoxicity.

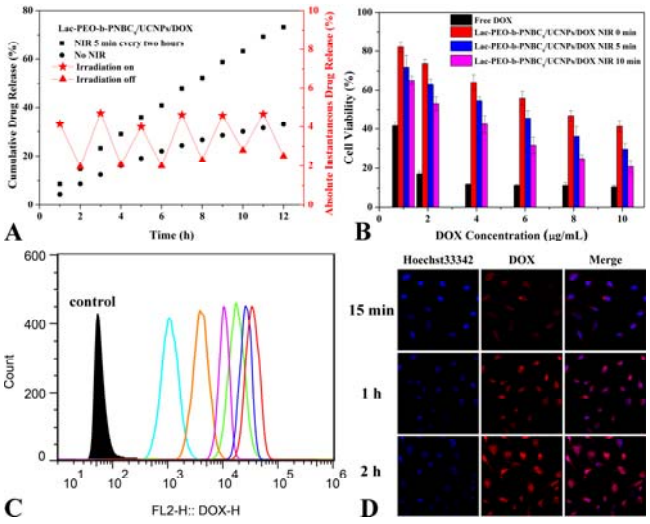

Figure 4. NIR-triggered DOX release from the composite nanomedicine (A); NIR-triggered cytotoxicity against HeLa cells (B); flow cytometry histogram profiles (C) of HeLa cells incubated with free DOX and the composite nanomedicine (cyan line (15 min), orange line (1 h), and green line (2 h) for DOX; violet line (15 min), blue line (1 h), and red line (2 h) for the composite nanomedicine); CLSM images (D) of HeLa cells incubated with the composite nanomedicine.

Whether the DOX-loaded composite nanoparticles can enter into cells and then release DOX inside cells is a key step because DOX kills cells via its intercalation with DNA backbone.[2] **Figure 4C** shows the histograms of HeLa cells incubated with free DOX and DOX-loaded composite nanoparticles at the predetermined time intervals of 15 min, 1 h and 2 h. Based on the mean fluorescence intensity at 2 h, 39% and 73% DOX-loaded composite nanoparticles entered into cells while only 11% and 30% free DOX did at 15 min and 1 h, respectively. This result indicates that the DOX-loaded composite nanoparticles can be quickly internalized by HeLa cells, as further confirmed by confocal laser scanning microscopy (CLSM) (**Figure 4D)**. The cells incubated with the DOX-loaded composite nanoparticles show weak red fluorescence in cytoplasm after 15 min, strong red fluorescence both in cytoplasm and nuclei after 1 h, and then stronger red fluorescence in nuclei after 2 h. Therefore, these results convincingly confirm that the DOX-loaded composite nanoparticles quickly enter into the mammalian cells, and then release DOX inside the cells.

In summary, we synthesize a novel class of sugar-terminated photoresponsive polypeptide block copolymers, and fabricate the UCNPs-loaded polypeptide composite nanoparticles having fast NIR-sensitivity and tunable sugar-targeting properties. The Lac-coated micelles with different Lac densities produce a tunable active targeting effect to HepG2 cells and 70% Lac-coated micelles give a 4.7-fold decreased IC_{50} compared with non-Lac-coated micelles. The polypeptide composite nanoparticles can release DOX in a controllable or on-off mode and present a tunable NIR-triggered cytotoxicity (e.g., 3.1-fold decreased IC_{50} upon 10 min NIR irradiation). The DOX-loaded composite nanoparticles can quickly enter into the mammalian cells and then release DOX inside the cells. This work opens a new avenue for on-demand and targeted DDSs and cancer therapy.

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NIR-Responsive and Sugar-Targeted Polypeptide Composite Nanomedicine for Intracellular Cancer Therapy

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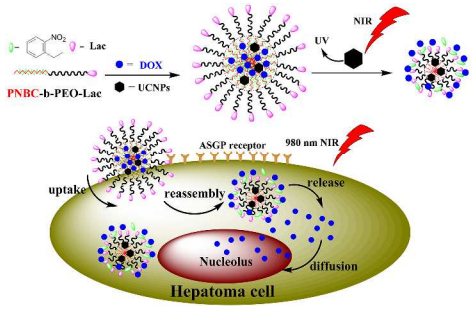

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