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Photosensitizer-conjugated polymeric nanoparticles for redox-responsive fluorescence imaging and photodynamic therapy†

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Photosensitizers conjugated with hyaluronic acid *via* disulfide linkers were developed for biologically activatable near-infrared fluorescence imaging and photodynamic therapy. The nanoparticles prepared from the conjugate are nonfluorescent and nonphototoxic in their native state, but become highly fluorescent and phototoxic in response to reductive agents inside cancer cells.

Photodynamic therapy (PDT) using combinations of chemical photosensitizers, light, and molecular oxygen has long been used successfully to treat cancers and other nonmalignant conditions.[1] Compared with conventional chemotherapy, photosensitizers become cytotoxic only in regions that receive the appropriate wavelength of light. However, problems with water-insolubility, limited tumor selectivity, and poor pharmacokinetics of PDT agents have been the main drawbacks to their clinical application. In particular, nonspecific activation of singlet oxygen generation in normal tissues causes prolonged skin photosensitivity, thereby limiting their utility as theranostic agents.[2] Various types of activatable photosensitizing agents have been tested to try and control their optical properties,[3] and development of a biocompatible and biologically tunable PDT agent is an ongoing challenge.

Here, we propose photosensitizer-conjugated polymeric nanoparticles as a redox-responsive theranostic agent for selective near-infrared (NIR) fluorescence imaging and photodynamic therapy of cancers (Fig. 1a). Chlorin e6 (Ce6), a second-generation photosensitizer with NIR fluorescence emission and high singlet oxygen quantum yield (0.75),[4] was conjugated to a hyaluronic acid (HA) backbone *via* redox-responsive cleavable disulfide linkers (Fig. 1b). HA is a component of the extracellular matrix and is thus a highly biocompatible, water-soluble biopolymer.[5] We expected nanoparticles prepared from the amphiphilic HA–Ce6 conjugate would be self-quenched; therefore, fluorescence and singlet oxygen generation (SOG) are inhibited in the native state (Fig. 1a). After the nanoparticles accumulate in tumor tissues through the enhanced permeability and retention (EPR) effect,[6] they enter the cancer cells *via* endocytosis, and then intracellular redox agents (in particular, glutathione) degrade the nanoparticles by cleaving the disulfide linker between Ce6 and the HA backbone. This will result in photosensitizer release from the nanoparticles, and fluorescence emission and singlet oxygen generation are restored. The large glutathione (GSH) concentration gradient between extracellular (2 μM) and intracellular (2–10 mM) compartments has long been utilized for triggered drug release inside cancer cells.[7]

To synthesize the HA–Ce6 conjugates, HA backbone carboxylic acids were first modified with cystamine dihydrochloride using EDC/sulfo-NHS (ethyl-(dimethyl aminopropyl) carbodiimide/sulfo-*N*-hydroxysuccinimide) chemistry, as shown in Fig. S1 (ESI†).

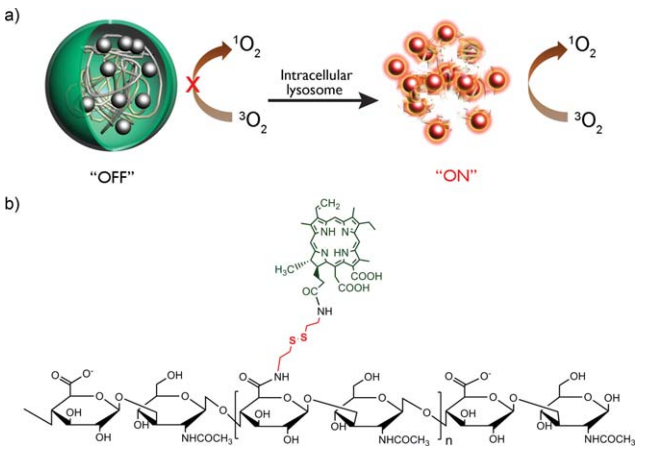

Fig. 1 (a) Schematic drawing of the redox-responsive nano-PDT agent. Fluorescence and singlet oxygen generation (SOG) are quenched in the native state. When the nanoparticles enter cancer cells *via* endocytosis, disulfide bonds are cleaved by intracellular reductive agents, resulting in dequenching of fluorescence and SOG. (b) Chemical structure of the synthesized HA–Ce6 conjugate.

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Amino-terminated HA linked with a disulfide bond was sequentially conjugated with Ce6–NHS ester. The degree of substitution of Ce6 molecules per unit (2 glucose rings) of HA was analyzed by ^{1}H-NMR and calculated to be 0.27 (Fig. S2, see ESI†). HA–Ce6 conjugates showed self-organizing behavior in aqueous solution due to aggregation of the hydrophobic Ce6 molecules, thereby forming nanoparticles. The nanoparticles prepared from the HA–Ce6 conjugate (*i.e.*, HA–Ce6 NPs) were round, as observed by scanning electron microscopy (SEM, Fig. 2a). The hydrodynamic size and zeta potential of HA–Ce6 NPs in aqueous solution were 245.85 ± 51.46 nm and −40.23 ± 5.99 mV, respectively. Comparison of the UV/Vis absorption spectra of HA–Ce6 NPs and free Ce6 in phosphate buffered saline (PBS) solution confirmed aggregation of the conjugated photosensitizers within the nanoparticles (Fig. 2b). Significant broadening of the Soret band region of the HA–Ce6 NP UV/Vis spectrum is the hallmark of photosensitizer aggregation for effective self-quenching.[8] As expected, fluorescence of the aggregated Ce6 in the nanoparticles was quenched (inset image of Fig. 2b), and SOG of HA–Ce6 NPs was significantly inhibited in comparison with free Ce6 (Fig. 2c).

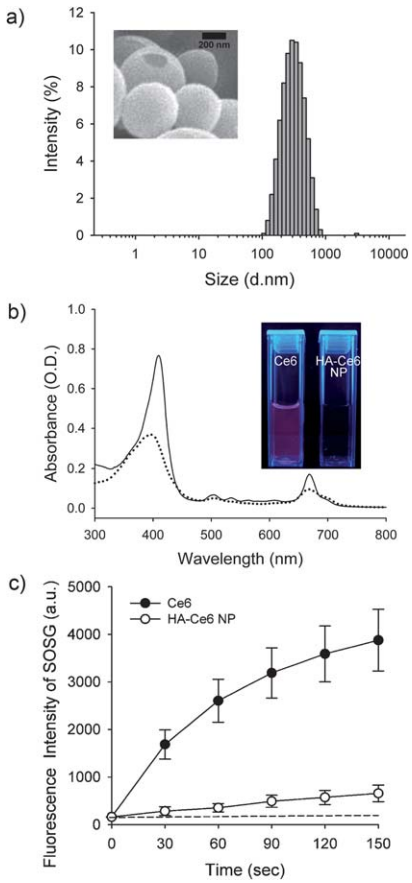

Fig. 2 (a) Hydrodynamic size distribution and SEM images of HA–Ce6 NPs. (b) UV/Vis absorption spectra of free Ce6 (solid line) and HA–Ce6 NPs (dotted line) at 5 μM Ce6 equiv. Inset image: fluorescence image of free Ce6 and HA–Ce6 NPs in aqueous solution at 5 μM under 380 nm UV light. (C) Time-dependent singlet oxygen generation of free Ce6 and HA–Ce6 NPs during light irradiation (650 nm diode laser, light dose rate: 38 mW cm^{-2}). The dashed line represents baseline SOSG fluorescence.

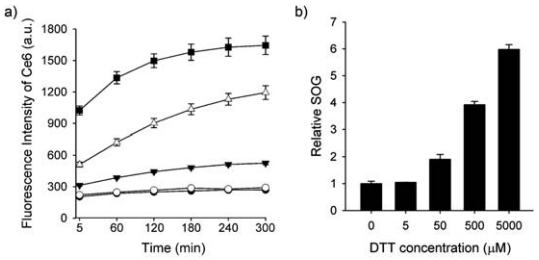

Fig. 3 (a) Recovery of NIR fluorescence by HA–Ce6 NPs over time upon addition of various concentrations of DTT (●: 0 μM, ○: 5 μM, ▼: 50 μM, △: 500 μM, ■: 5 mM). (b) Relative SOG and DTT concentration. SOG was measured after treating HA–Ce6 NPs with DTT for 5 h.

Given that the disulfide linker in the nanoparticles is key for quenching and dequenching of fluorescence and SOG, we evaluated NIR fluorescence recovery of HA–Ce6 NPs in the presence of a reductive agent, dithiothreitol (DTT). The degree and rate of fluorescence recovery were dependent on DTT concentration, as shown in Fig. 3a and S3 (ESI†). Notably, an 8 fold increase in fluorescence was obtained by treating the nanoparticles with 5 mM DTT for 5 h, whereas no enhancement in fluorescence was observed in samples treated with 5 μM DTT. Fluorescence signal recovery was particularly fast when the sample was treated with 5 mM DTT (*i.e.*, about 5 fold increase in 5 min). SOG from HA–Ce6 NPs was measured in the absence and presence of DTT, using singlet oxygen sensor green (SOSG) as a singlet-oxygen-detecting reagent (Fig. 3b). As in the fluorescence experiment, addition of DTT triggered DTT dose-dependent recovery of SOG. About 6.2 fold increase in SOG was obtained by addition of 5 mM DTT; no enhancement was observed with 5 μM DTT.

The theranostic potential of the redox-responsive HA–Ce6 NPs was evaluated *in vitro* with the U-87MG human glioblastoma cell line. U-87MG cells were chosen because they do not express hyaluronidase (HAse), eliminating the possibility of HA–Ce6 NP degradation by HAse.[9] U-87MG cells were treated with free Ce6 and HA–Ce6 NPs for 24 h over a concentration range of 0–20 μM Ce6 equiv. to determine the dark toxicity of these agents. Cells treated with free Ce6 or HA–Ce6 NPs showed no cytotoxicity even at the highest Ce6 concentration. In contrast, dose-dependent phototoxicity was induced by treatment with free Ce6 and HA–Ce6 NPs followed by illumination with a 670 nm CW diode laser. Even

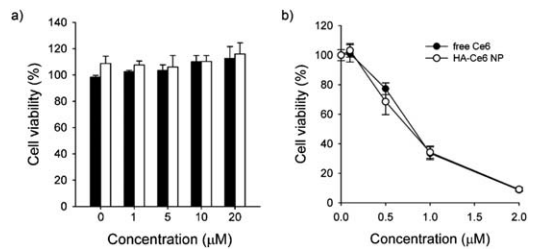

Fig. 4 (a) U-87MG cell viability after treatment with free Ce6 and HA–Ce6 NPs at various concentrations of Ce6 equiv. for 24 h (closed bars: free Ce6, open bars: HA–Ce6 NPs). (b) *In vitro* phototoxicity test. U-87MG cells were treated with free Ce6 and HA–Ce6 NPs for 18 h at 0, 0.1, 0.5, 1, and 2 μM Ce6 equiv., and then irradiated with a 650 nm CW laser (50 mW cm^{-2}, 5 J cm^{-2}).

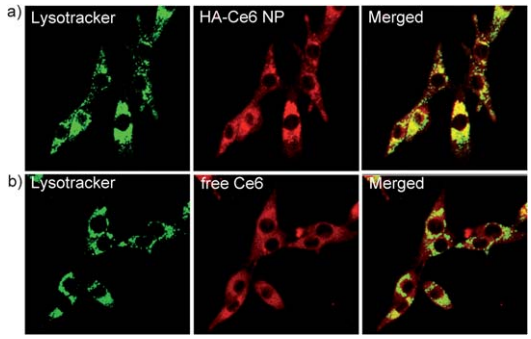

Fig. 5 Confocal fluorescence microscopy of U-87MG cells after treatment with Ce6 and HA–Ce6 NPs for 18 h at 2 μM Ce6 equiv. Yellow regions in the merged images indicate localization of Ce6 molecules in the lysosomes.

though HA–Ce6 NPs were quenched, similar phototoxic effects were observed in this *in vitro* PDT test, indicating that SOG by HA–Ce6 NPs was recovered by intracellular reductive agents after entering the cancer cells. In fact, fluorescence-activated cell sorting (FACS) supports intracellular uptake of HA–Ce6 NPs and subsequent dequenching inside cells (Fig. S4, see ESI†).

To observe the interaction of HA–Ce6 NPs with U-87MG cells and visualize their location in lysosomes, cells were treated with free Ce6 and HA–Ce6 NPs at 2 μM Ce6 equiv. for 24 h, and then the lysosomes were stained with LysoTracker. In the confocal fluorescence microscopy images in Fig. 5a, the bright yellow color in the merged fluorescence images of the photosensitizers and LysoTracker indicates intracellular uptake of HA–Ce6 NPs through endocytosis and preferential localization of HA–Ce6 NPs in lysosomal compartments, where the concentration of glutathione is high (2–10 mM). Since U-87MG cells have no HAse activity, strong fluorescence in the HA–Ce6 NP-treated cells is due to dequenching of Ce6 fluorescence inside the lysosomes. No apparent co-localization was observed in cells treated with free Ce6 (Fig. 5b).

In conclusion, a redox-responsive and biocompatible nano-PDT agent was prepared by conjugating photosensitizers with biocompatible hyaluronic acid *via* disulfide linkers; the agent showed great potential in selective NIR fluorescence imaging and subsequent PDT of cancers. The HA–Ce6 NPs were nonfluorescent and non-phototoxic in the native state, but became highly fluorescent and phototoxic upon entering cancer cells. This activatable PDT agent may have utility for target-cell-specific imaging and therapy by introducing specific targeting-ligands on its surface.

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