Light-triggered dePEGylation with decreasing the diameter of hydroxyapatite nanocarriers for enhanced cellular uptake and tumor penetration

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Abstract
PEGylation of nano-drug delivery systems (NDDSSs) has been investigated to overcome the side effect of conventional chemotherapy including improving pharmacokinetics by prolonging drug circulation, reducing immune clearance and premature drug leakage. Although, PEGylation of NDDSSs can also disturb the tumor penetration and cellular uptake with the diameter enhancement. Therefore, in this work, we constitute a light-triggered dePEGylation strategy, which results in the decrease of diameter of the pH-responsive hydroxyapatite drug nanocarriers (DOX@HAP-PEG) for enhanced cellular uptake and tumor penetration. Under light irradiation (650 nm), PEG chains can be available separated from the nanocarrier by cleaving the Cy linker. Moreover, the cellular uptake of DOX@HAP-PEG and DOX@HAP-PEG+L (DOX@HAP-PEG under light irradiation) are explored against MCF-7, Hela, and HepG2 cancer cells. The results show that the cellular uptake of DOX@HAP-PEG is lower than that of DOX@HAP-PEG+L. In addition, in 3D tumor model, DOX@HAP-PEG+L can better penetrate into the cell spheroid than DOX@HAP-PEG, which is demonstrated by the accumulated fluorescence signals in the cell spheroid.

KEYWORDS
dePEGylation, hydroxyapatite, light-responsiveness, particle size change, tumor penetration

1 | INTRODUCTION

Hydroxyapatite (HAP, Ca_{10}(PO_{4})_{6}(OH)_{2}), as the component of teeth and bones, has been widely used in tissue engineering in the dental and orthopedic fields.[1–4] Recently, HAP nanoparticle has attracted more attention for NDDSSs, owing to its biocompatible and non-inflammation properties.[3,5–9] Moreover, pH-sensitive HAP nanoparticle can be degraded into calcium and phosphorous elements to ensure the effective release of the doped cargos under the slightly acidic conditions.[5,7,10] HAP as nanocarriers also have achieved targeted drugs delivery to promote the treatment efficiency and reduce side effects to the normal tissue and organs by the surface...
Our group succeeded in synthesizing biodegradable drug-loaded hydroxyapatite nanoparticles modified with folic acid for targeted drug release and ratiometric real-time monitoring of nanodrug based on hydroxyapatite for tumor targeted chemotherapy. Thus, it is necessary to investigate NDDSs based on HAP that are functionalized with active or positive targeting groups.

PEG as a linear polymer, showing negative potential, has been extensively used to modify nanocarriers to improve the biocompatibility, solubility and prolong drug circulation. PEG ligands conjugated to the surface of nanoparticles can effectively reduce the nonspecific protein adsorption and cellular uptake of the reticuloendothelial system (RES) to improve the biodistribution and prolong the blood circulation half-life of the DDSs by the enhanced permeability and retention (EPR) effect. However, due to the increase in the size of the nanoparticles, PEGylation of the NDDSs disturbs the cellular uptake and tumor penetration. Recently, the deshielding strategies were extensively investigated, which effectively triggered PEG chains deshielded from nanocarriers in response to the tumor microenvironment including slightly acidic conditions, reactive oxygen species, and high levels of enzymes. Nevertheless, the differences in tumor microenvironment leads to varied PEG shielding effects and uncontrollable anticancer effect, which limited its clinical application. Thus, it was urgent to investigate alternative strategies with precisely controlled PEG deshielding capability to the desired site.

At present, light has attracted more concern and attention due to the temporal and spatial specific features. Near infrared cyanine dyes were used as the light trigger to constitute light control strategies based on a regioselective photooxidative polyene cleavage resulted from self-sensitized \( ^{1}\text{O}_2 \). In addition, Indocyanine green (ICG) is an Food and Drug Administration (FDA)-approved near-infrared (NIR)-absorbing dye for imaging probes. ICG as a clinical diagnostic agent with a long history of well-tolerated human use, indicating that cyanine dyes had good biocompatibility. Considering the photodegradation of cyanine dyes, in this work, we constituted a light-triggered dePEGylation with decrease of diameter of the pH-responsive hydroxyapatite nanocarriers (DOX@HAP-PEG) for enhanced cellular uptake and tumor penetration. Under light irradiation (650 nm), PEG chains could be available separated from the nanocarrier by cleaving the Cy linker. Due to the diameter change of the nanoparticles, the cytotoxicity of DOX@HAP-PEG+L against cancer cells was higher than DOX@HAP-PEG owing to the enhanced cellular uptake. More importantly, DOX@HAP-PEG+L could better penetrate into the cell spheroid (3D tumor model) than DOX@HAP-PEG, further suggesting that light-triggered dePEGylation of DOX@HAP-PEG resulted in the enhanced tumor penetration. The light-triggered PEGylation/dePEGylation strategy with accurately controlled PEG deshielding at the desired site, providing a remote-controlled drug delivery method for various tissues and tumors.

2 | RESULTS AND DISCUSSION

2.1 | Characterization

As shown in Scheme 1, DOX@HAP was prepared via the coprecipitation and hydrothermal method, modified with Cy-\( \text{N}_3 \) by coupling reaction of APTES, further functionalized with PEG-alkyne by copper(I)-catalyzed alkyne–azide cycloaddition reaction. DOX@HAP were firstly characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Figure 1A and 1B). The results revealed that DOX@HAP nanoparticles were uniform nanorods with the average length, diameter and height (170, 55, and 32 nm). In Figure S9, PEG ligands conjugated to the surface of DOX@HAP could improve its dispersion with the PDI value of the hydration particle size decreased from 0.42 to 0.06. According to previous
reports, DOX@HAP-PEG nanorods obtained better tumor-targeting property than spherical nanoparticles because nanorods flowed in the blood vessels with different motion characteristics such as rolling and tumbling to facilitate the EPR effect.\(^5\) In addition, the hydration particle size of DOX@HAP-PEG reduced from 414 to 178 nm with irradiation (Figure 1E), demonstrating light-triggered dePEGylation of DOX@HAP-PEG by the photobleaching of Cy resulting in the decrease of the particle size. The BET surface areas of DOX@HAP measured by N\(_2\) adsorption–desorption isotherm was 58.37 m\(^2\) g\(^{-1}\) (Figure 1C). After functionalized, the BET surface of DOX@HAP-PEG gradually decreased to 50.51 m\(^2\) g\(^{-1}\) (Figure 1D), respectively, suggesting that DOX@HAP nanoparticles were successfully modified with PEG chains. Fourier transform infrared (FT-IR) spectra of HAP, DOX@HAP, DOX@HAP-PEG and DOX@HAP-PEG+L were shown in Figure S8. The main characteristic peaks of HAP located at 1039, 603, and 567 cm\(^{-1}\) were, respectively, assigned to the stretching vibration and the bending vibration of the phosphate
(PO₄³⁻) group. A new peak at 1585 cm⁻¹ (C = C(Ar) stretching vibration) emerged after loaded DOX, which suggested that DOX@HAP nanoparticles were successfully synthesized. The peaks of DOX@HAP-PEG located at 2930, 1645, and 1270 cm⁻¹ were ascribed to the C-H stretching vibration, N-H bending vibrations and C-O-C stretching vibration, indicating that PEG chains were successfully grafted to DOX@HAP by click reaction. After irradiated, the peak of DOX@HAP-PEG+L located at 1270 cm⁻¹ reduced, indicating that PEG chains could be effectively deshielded from nanocarriers by cleaving the Cy linker. XPS measurement confirmed that the ratio of Ca/P of the DOX@HAP was 1.63, which is consistent with the reported range of HAP⁵,3₄ (1.33–1.67) (Figure S7 and Table S1). In Figure S6, the characteristics peaks of DOX@HAP measured by the X-ray diffraction located at 25.9°, 31.9°, 33°, 34°, and 40° matched with the standard reference of HAP (PDF#09-0432⁵), indicating that there is no significant phase change of HAP after doped with DOX. Moreover, the absorption and fluorescence spectra of Cy were measured. In Figure S11, the excitation and emission peak of Cy mainly were located at 625 and 750 nm, respectively. In Figure 1F, the characteristic absorption and fluorescence spectra of DOX@HAP-PEG were located at 640 and 760 nm, respectively, indicating that DOX@HAP nanoparticles were further functionalized by Cy.

2.2 Drug release profile and PEG deshielding from DOX@HAP-PEG

By measuring the released DOX in the acidic condition (pH 3.0) through UV–vis absorption spectra, the drug-doping capability of DOX@HAP-PEG was ~114 mg g⁻¹ (Figure S4 and S5). Furthermore, in vitro, at different pH values, the drug release behavior of DOX@HAP-PEG was studied (Figure 2A). After treatment for 24 hours at pH 7.4, the DOX release amount was only 16.3 %, demonstrating

FIGURE 2 A, The release curve of DOX from DOX@HAP-PEG in PBS buffer with different pH values. B, TEM images of DOX@HAP-PEG for 2, 6, 12, and 24 hours (pH 6.5; Scale bar: 1 µm). C, Evolution of the fluorescence spectra of DOX@HAP-PEG in DMSO with irradiation every 5 minutes. D, The color of the DOX@HAP-PEG solution without (I) and with (II) irradiation
that DOX@HAP-PEG possessed high stability to avoid the drug burst release from the nanocarriers and reduce the toxic side effects to healthy organs and tissues. Meanwhile, the total DOX release from DOX@HAP-PEG was dramatically increased to 88.9 % (pH 5.0) and 53.4 % (pH 6.5) for 24 hours, respectively, revealing that DOX could be effectively release from DOX@HAP-PEG under the slightly acidic conditions. The results verified that the nanostructure of HAP was gradually degraded in acid conditions. In Figure 2B, TEM results showed that the nanorods of DOX@HAP-PEG gradually collapsed with different time at pH 6.5, further demonstrating that the pH-sensitive HAP had the potential as nanocarriers to deliver drugs. In addition, the evolution of the fluorescence spectra of DOX@HAP-PEG in DMSO with irradiation every 5 minutes was measured to prove the light-triggered dePEGylation. In Figure 2C, with irradiation at 650 nm (0.2 W cm$^{-2}$), the fluorescence spectra of DOX@HAP-PEG (1.0 mg mL$^{-1}$, 5 mL) about 750 nm gradually decreased, providing a proof that light-triggered dePEGylation of DOX@HAP-PEG via the photobleaching of Cy. The intensity of fluorescence spectrum was almost not changed after 20 minutes. The color of the DOX@HAP-PEG solution transformed from blue to pink after irradiated (Figure 2D).
2.3 Cellular internalization studies on cancer cells

The cellular uptake of DOX@HAP-PEG and DOX@HAP-PEG+L against MCF 7, Hela and HepG2 cells were evaluated by confocal laser scanning microscopy (CLSM) and flow cytometry via monitoring the DOX fluorescence. As shown in Figure 3A, MCF 7, Hela, and HepG2 cells incubated with DOX@HAP-PEG and DOX@HAP-PEG+L (100 μg mL⁻¹) for different time, fluorescence intensity of DOX in cancer cell gradually increased over incubation time, indicating that DOX@HAP-PEG and DOX@HAP-PEG+L could be internalized into the cells. Furthermore, the fluorescence intensities in cancer cells incubated with DOX@HAP-PEG were lower than that of DOX@HAP-PEG+L, because DOX@HAP-PEG+L nanorods were preferentially internalized into cancer cell than DOX@HAP-PEG. In addition, the difference in the intake behavior of DOX@HAP-PEG and DOX@HAP-PEG+L were evaluated by flow cytometry (Figure 3B, 3C, 3D, and 3E). The time-dependent average fluorescence intensity of DOX@HAP-PEG was lower than that of DOX@HAP-PEG+L, which was consistent with CLSM images (Figure 3A), further demonstrating that DOX@HAP-PEG+L with smaller hydration particle size were preferentially internalized into cancer cell than DOX@HAP-PEG.

2.4 Cytotoxicity assay

The cytotoxicity of DOX@HAP-PEG and DOX@HAP-PEG+L nanoparticles against MCF 7, Hela and HepG2 cells were investigated by MTT assay. The viability of cancer cells incubated with different concentrations of HAP-PEG was about 100% (Figure 4A), showing that HAP nanorods as nanocarriers were nontoxicity. MCF 7, Hela, and HepG2 cancer cells were incubated with DOX@HAP-PEG and DOX@HAP-PEG+L with the increasing concentrations (0.5, 1, 25, 50 and 100 μg mL⁻¹) for 24 hours. As shown in Figure 4B, 4C, and 4D, the cell viability of group DOX@HAP-PEG+L gradually decreased to 46.1%, 38.0%, and 37.7% lower than group DOX@HAP-PEG (64.4%, 60.2%, and 45.7%), respectively. The results clearly certified that the increased cytotoxicity of DOX@HAP-PEG and DOX@HAP-PEG+L were concentration-dependent.
FIGURE 5 The Z-stack images of 4T1 cells incubated with DOX@HAP-PEG and DOX@HAP-PEG+L for 4 hours in 3D tumor spheres every 10 µm.

The cytotoxicity of DOX@HAP-PEG+L against cancer cells was higher than DOX@HAP-PEG in accordance with the results of the cellular uptake assays, which demonstrated that DOX@HAP-PEG+L nanoparticles were more easily taken up by cancer cells than DOX@HAP-PEG nanoparticles.

2.5 | Tumor penetration capability

To examine the tumor penetration capability of DOX@HAP-PEG and DOX@HAP-PEG+L, avascular 4T1 cells three-dimensional spheroid models were established to simulate solid tumors, and respectively incubated with DOX@HAP-PEG and DOX@HAP-PEG+L (100 µg mL⁻¹) for 4 hours. As shown in Figure 5, the fluorescent images of DOX obtained from the top to the bottom of the spheroids every 10 µm by CLSM. The fluorescence signals of DOX@HAP-PEG+L mainly accumulated in the cell spheroid, but that of DOX@HAP-PEG appeared at the outer layers of the cell spheroid, which indicated that DOX@HAP-PEG+L could better penetrate into the cell spheroid than DOX@HAP-PEG (Figure 5). This might be ascribed to the smaller hydration particle size of DOX@HAP-PEG+L than DOX@HAP-PEG, resulting in the better tumor penetration.

3 | CONCLUSION

In this work, we have successfully synthesized a light-triggered dePEGylation with decrease of diameter of the pH-responsive hydroxyapatite nanocarriers (DOX@HAP-PEG) for enhanced cellular uptake and tumor penetration. Under light irradiation (650 nm), PEG chains could be availablely deshielded from nanocarriers by cleaving the Cy linker. Moreover, the cellular uptake and tumor penetration of DOX@HAP-PEG was lower than that of DOX@HAP-PEG+L in the 2D cancer cells and 3D tumor model due to the smaller hydration particle size of DOX@HAP-PEG+L than DOX@HAP-PEG. Thus, this light control strategy with PEG deshielding provides an accurate and remote-controlled drug delivery method for various tissues and tumors.

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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.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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