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A pre-protective strategy for precise tumor targeting and efficient photodynamic therapy with a switchable DNA/upconversion nanocomposite†

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Tumor-specific targeting based on folic acid (FA) is one of the most common and significant approaches in cancer therapy. However, the expression of folate receptors (FRs) in normal tissues will lead to unexpected targeting and unsatisfactory therapeutic effect. To address this issue, we develop a pre-protective strategy for precise tumor targeting and efficient photodynamic therapy (PDT) using a switchable DNA/upconversion nanocomposite, which can be triggered in the acidic tumor microenvironment. The DNA/upconversion nanocomposite is composed of polyacrylic acid (PAA) coated upconversion nanoparticles (UCNPs), the surface of which is modified using FA and chlorin e6 (Ce6) functionalized DNA sequences with different lengths. Initially, FA on the shorter DNA was protected by a longer DNA to prevent the bonding to FRs on normal cells. Once reaching the acidic tumor microenvironment, C base-rich longer DNA forms a C-quadruplex, resulting in the exposure of the FA groups and the bonding of FA and FRs on cancer cell membranes to achieve precise targeting. Simultaneously, the photosensitizer chlorin e6 (Ce6) gets close to the surface of UCNPs, enabling the excitation of Ce6 to generate singlet oxygen ($^1\text{O}_2$) under near infrared light via Förster resonance energy transfer (FRET). *In vivo* experiments indicated that higher tumor targeting efficiency was achieved and the tumor growth was greatly inhibited through the pre-protective strategy.

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Introduction

Cancer has become a major killer that threatens human life and health because of the rapid proliferation and metastasis of cancer cells.^{1,2} With the deeper understanding of cancer in the past decades, targeted cancer therapy has been one of the main choices in clinical trials.^{3–6} Based on the overexpression of the receptors on cancer cell membranes, employing corresponding ligands to improve the targeting efficiency was the most common method.^{7–9} By conjugating a series of targeting groups (*e.g.* FA, TAT peptide, c(RGDyC) peptide, and aptamers) on the nanoparticles, tumor-specific targeting can be realized.^{10–14} Among these approaches, the FA group has been widely used for tumor targeting due to its easy modification and good targeting efficiency.^{15–17} However, there is a key issue that has always been ignored, that is, folate receptors are not only overexpressed at high levels on the membrane of nonmalignant proliferative

cells (*e.g.* bone marrow cells and follicle cells), but also expressed at low levels in most normal tissues, which tremendously reduces the targeting efficiency and therapeutic effect due to the large population of normal cells.^{18–20} Therefore, an effective strategy that can protect the targeting groups and decrease unexpected bonding is urgently needed.

As an important treatment for cancer, PDT possesses superior advantages over other therapeutic methods due to its minimal invasiveness, fast healing process and potential repeatability.^{21–27} A photosensitizer is indispensable for the application of PDT in cancer therapy, however, traditional photosensitizers lack active targeting ability. When the photosensitizer circulates in the body, the retention of agents in the tumor tissues is limited. In this case, the therapeutic effect of PDT without tumor targeting will inevitably be greatly reduced.^{28,29} Besides, the poor penetration of ultraviolet or visible excitation light in conventional PDT also severely limits its clinical application. Improving the penetration of excitation light is also a key factor for enhancing the treatment effects of PDT.^{30–34} Thus, precise tumor targeting accompanied by NIR excitation would be extremely favorable to improve the therapeutic effect of PDT.

Herein, we present a pre-protective strategy for precise tumor targeting and enhanced PDT by constructing a dynamic DNA/UCNP nanocomposite. Two kinds of DNA sequences with

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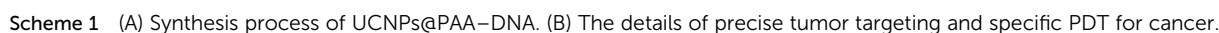
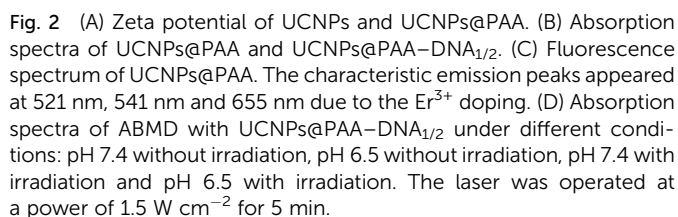


Figure 1 consists of two panels, A and B, showing cryo-electron micrographs of *C. elegans* ommatidia. Panel A shows a single ommatidium, which is a cluster of cells. At the center is a large, dark, circular cone cell. Surrounding it are several smaller, lighter-colored photoreceptor cells. The entire structure is set against a lighter background. Panel B shows a cluster of several ommatidia, each with a central cone cell and surrounding photoreceptor cells. The arrangement is more complex than in panel A. Both panels include a scale bar in the bottom left corner, which is a horizontal line representing 100 nm.

biocompatibility after DNA decoration.⁴⁶ From the absorption spectra in Fig. 2B, obvious absorption peaks of Ce6 at around 400 nm and 655 nm appear after DNA modifications, which provides further evidence for the successful fabrication of UCNPs@PAA-DNA.

Acid triggered $^1\text{O}_2$ generation

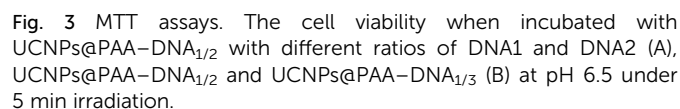
Acid environment triggered C-rich DNA folding was verified. A C-rich DNA sequence modified with Cy3 and BHQ1 was designed to demonstrate the C-quadruplex i-motif structure formation of C-rich DNA at pH 6.5 through fluorescence analysis (DNA4, Table S1[†]). As shown in Fig. S1 in the ESI,[†] the fluorescence intensity of Cy3 decreased obviously when DNA4 was at pH 6.5, which indicated that Cy3 was quenched by BHQ1 and further confirmed the DNA folding. Then we verified whether the nanocomposite could generate $^1\text{O}_2$ in the mild acid environment under NIR irradiation. As shown in Fig. 2C, there are three characteristic emission peaks in the fluorescence spectrum of the UCNP_s NaYF₄:Yb³⁺,Er³⁺ at 521 nm, 541 nm and 655 nm, which corresponded to $^2\text{H}_{11/2}$ - $^4\text{H}_{15/2}$, $^4\text{S}_{3/2}$ - $^4\text{S}_{15/2}$, and $^4\text{F}_{19/2}$ - $^4\text{I}_{15/2}$ transformations of Er³⁺, respectively.^{47,48} Combined with the absorption spectrum of UCNP_s@PAA-DNA_{1/2}, we found that the absorption band of Ce6 around 655 nm overlapped well with the UCNP emission, which meets the needs of FRET. A special probe, 9,10-anthracenediyl-bis(methylene)



Optimization of the DNA ratio

various shorter and longer DNA ratios were prepared. The optimal ratio was based on the viability of cancer cells. We chose human breast cancer cells (MCF-7) as a cell model due to their high FR level (Fig. S4, ESI†). After incubation at pH 6.5 for 12 h, human breast cancer cells (MCF-7) were irradiated with 980 nm NIR for 5 min. Then we conducted MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays to evaluate their ability in inducing cell apoptosis^{49,50} and further made the optimal choice on the DNA ratio. As shown in Fig. 3A, the nanocomposite with the DNA ratio of 2 : 8 treated MCF-7 cells exhibited the minimum value in viability, demonstrating that this DNA ratio was the best. Thus, the nanocomposite with the DNA ratio of 2 : 8 was chosen for further use. What's more, to demonstrate the precise targeting and therapeutic ability of our designed nanocomposite, we constructed UCNP@PAA-DNA_{1/3} as a negative control, in which C base-rich DNA2 was replaced by a scrambled sequence DNA3. As DNA3 could not switch its structure in an acid environment, FA was protected all the time and Ce6 remained far away from the UCNP surface, and both the targeting and therapeutic functions were restricted. After incubation with UCNP@PAA-DNA_{1/3}, most of the cells were still alive and displayed high viability up to about 90%. However, the viability of cancer cells incubated with UCNP@PAA-DNA_{1/2} at pH 6.5 decreased markedly, suggesting excellent therapeutic effects (Fig. 3B).

FRs are generally overexpressed on the membrane of cancer cells and are expressed at low levels on normal cells. The presence of FA on the nanoparticles leads to the feature of specific recognition. The FR mediated uptake of the nanocomposite was investigated by employing a confocal laser scanning microscope (CLSM). As shown in Fig. 4, a bright red fluorescence signal of Ce6 was observed in the MCF-7 cells incubated with the UCNPs@PAA-DNA_{1/2}. However, it was invisible when MCF-7 cells were pre-treated with FA before incubation with the nanocomposite, which was due to the competitive manner of free FA molecules.⁵¹ And a similar trend was observed in the fluorescence quantification analysis (Fig. S5, ESI[†]). Imaging flow cytometry (IFC) experiments were also carried out to confirm the FR mediated cell uptake. As shown by confocal images and statistical data, cells without pre-



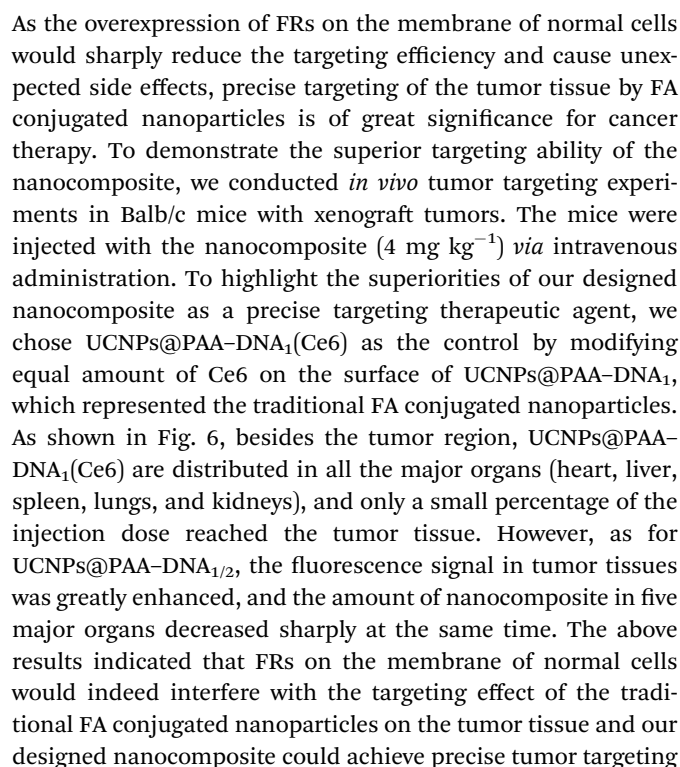


Fig. 6 *In vivo* precise targeting. *In vivo* imaging of five major organs harvested from a mouse at 8 h post-injection with UCNP@PAA-DNA₁(Ce6) (left) or UCNP@PAA-DNA_{1/2} (right).

and reduced side effects on normal tissues. The targeting effect of UCNP@PAA-DNA_{1/2} on tumors was quantified to be $12.1 \pm 0.7\%$ ID per g using inductively coupled plasma-atomic emission spectrometry (ICP-AES), which is higher than that of UCNP@PAA-DNA₁(Ce6) ($4.1 \pm 0.4\%$ ID per g) (Fig. S9, ESI†). The metabolism kinetic study of UCNP@PAA-DNA_{1/2} was also carried out by collecting the feces and urine. As shown in

Fig. S10 in the ESI,[†] the retention content of nanoparticles in the body decreased to less than 5% at 72 h post injection. And ¹O₂ generation of UCNPs@PAA-DNA_{1/2} at pH 6.5 with human serum albumin (HSA, 10 mg mL⁻¹) was detected by ABMD. As shown in Fig. S11 in the ESI,[†] the absorption of ABMD decreased obviously and was similar to that without HSA, which indicated that the protein has a negligible influence on our designed nanoparticles.

Tumor growth inhibition *in vivo*

Encouraged by the outstanding therapeutic effect *in vitro* and good targeting ability *in vivo*, we then evaluated the efficacy in tumor growth inhibition. Nude mice were subcutaneously injected with MCF-7 cells to form a xenograft tumor. When the solid tumor reached about 150 mm³, the mice received different treatments: PBS, NIR irradiation only, UCNP@PAA-DNA_{1/3} with NIR irradiation, and UCNP@PAA-DNA_{1/2} with NIR irradiation. The nanoparticles were injected *via* intravenous administration only once and NIR irradiation was conducted for 5 min (1.5 W cm⁻²) at 8 h post injection. The tumor volume was monitored every other day in a period of 14 days without extra irradiation. As shown in Fig. 7, the volume of tumors in mice treated with PBS or NIR only increased greatly and was

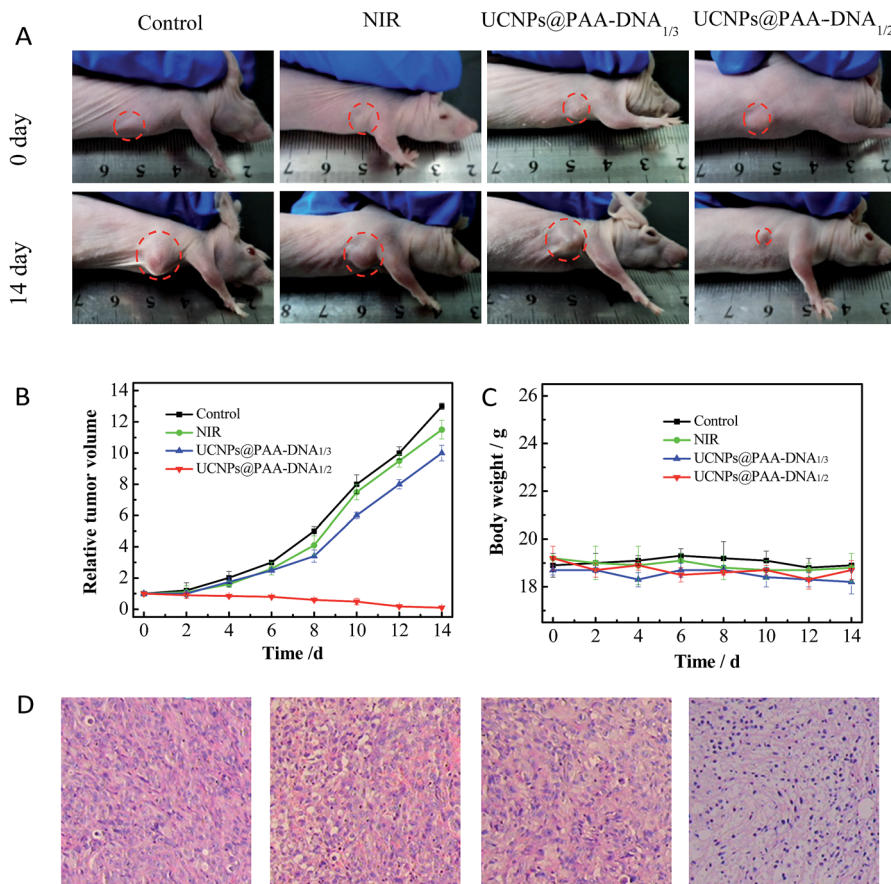


Fig. 7 *In vivo* therapeutic effects. (A) Photographs of mice with different treatments on day 0 (top) and day 14 (bottom). The laser was operated at a power of 1.5 W cm^{-2} for 5 min. Tumor growth curves (B) and mice body weight curves (C) of different groups of tumor-bearing mice. (D) H&E staining of tumors in mice that received different treatments.

about 12 fold larger than its initial volume. As DNA3 could not change its structure in an acid environment, FA groups could be protected and Ce6 could not get close to the surface of UCNPs, resulting in low targeting efficiency and little $^1\text{O}_2$ generation. Similarly, the tumor volume was found to increase nearly 10-fold over this period. Remarkably, the tumor was almost eliminated after being treated with our designed nanocomposite, due to the acid microenvironment triggered precise targeting, which demonstrated its excellent therapeutic effect (Fig. 7A and B). In addition, H&E staining on tumor sections provided further evidence for the treatment efficacy of inducing tumor cell apoptosis. As shown in H&E staining images, UCNPs@PAA-DNA_{1/2} treated tumors exhibited extensive apoptotic cells, which was reflected in a large amount of nuclear shrinkage and fragmentation (Fig. 7D). Meanwhile, no obvious body weight change was observed during the treatments, suggesting that the nanocomposite would not induce negative system toxicity (Fig. 7C). H&E staining on five major organs was also conducted at the end of the treatment period and no histopathological abnormalities appeared in all organs of mice with different treatments (Fig. S12, ESI†).

Conclusions

In summary, a pre-protective strategy was introduced for precise tumor targeting and efficient PDT with a switchable DNA/upconversion nanocomposite. The nanocomposite consists of PAA coated UCNPs that are conjugated with FA and Ce6 functionalized DNA sequences. In normal tissues, the FA group of the shorter DNA sequence was protected by the longer DNA sequence to prevent the conjugation to FRs. Once reaching the tumor region, C base-rich longer DNA would form C-quadruplex and FA groups will be exposed to bond FRs on the membrane of cancer cells, enabling precise tumor targeting. Simultaneously, the photosensitizer Ce6 on the longer DNA moved exactly close to the surface of UCNPs and could be activated to produce $^1\text{O}_2$ under NIR *via* FRET. *In vivo* experiments demonstrated that nanocomposites accumulated in the tumor region and the tumor growth was severely inhibited, suggesting that the targeting efficiency and therapeutic effect of PDT were greatly improved. We anticipate that this pre-protective strategy could provide new insights for precise targeting and highly efficient cancer therapy.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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