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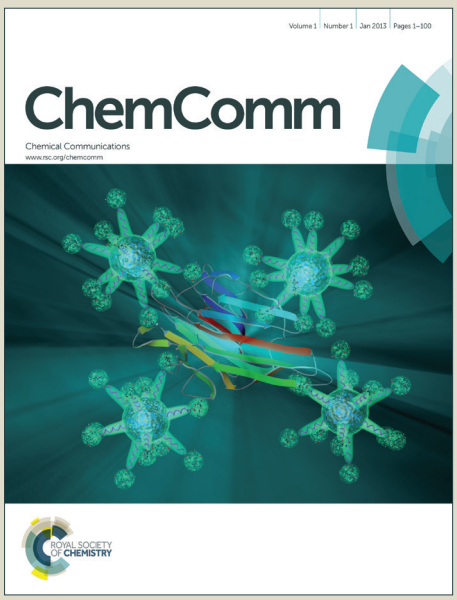

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Biotin-guided anticancer drug delivery with acidity-triggered drug release

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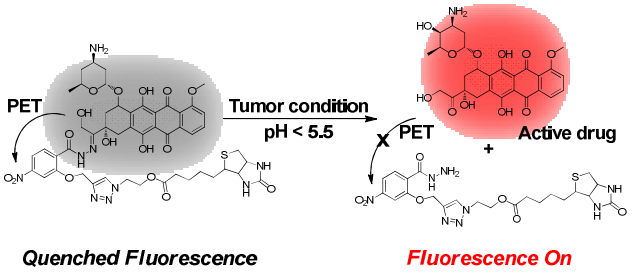
A novel biotin-guided anticancer drug delivery system, prodrug 9, consisting of biotin, nitrobenzene, and doxorubicin, with acid-triggered drug releasing capability was synthesized. Its cellular uptake and anticancer activity are selective to the HepG2 cell line over the WI-38 cell line, as revealed by fluorescence confocal microscopic experiments and MTT assay.

Drug delivery systems (DDS) that are capable of selective targeting and real-time monitoring of drug release have drawn much attention owing to their capability of reporting the drug delivery location and delivered amount *in situ,*[1] allowing determination of the drug delivery kinetics. Moreover, since these drug delivery systems can be modified by specific external or internal stimuli to selectively release their drugs at malignant cancer cells, the cancer cells can be selectively killed without affecting normal cells, minimizing undesirable side effects.

Diverse drug release mechanisms for DDS have been reported that utilize the different environments of tumor cells compared to normal cells, e.g., lower pH,[2] hypoxia,[3] overexpressed enzymes,[4] or higher levels of thiols.[5] Among them, the lower pH environment draws particular interest as an anticancer drug-releasing mechanism because both primary and metastasized tumors have a lower pH than non-malignant tissues.[6] Thereby, a DDS with acid-triggered drug release capability can selectively deliver drugs to tumor tissues. Furthermore, this system may have an additional advantage of minimizing non-selective delivery since the blood stream has a relatively high pH and the DDS would not release drug molecules prior to reaching the target tissues.

Monitoring drug release from DDSs has often been performed through Forster resonance energy transfer (FRET),[7] aggregation-induced emission (AIE),[8] or a quenching system,[9] consisting of a fluorophore, sometimes with a quencher. These ways usually have complicated chemical structures, often resulting in increased hydrophobicity accompanied by decreased bioavailability, as well as poor control of drug release. Therefore, introducing a minimally sized quencher moiety on the fluorescent drug molecule, *e.g.,* doxorubicin (Dox) would be an ideal way to obtain efficient monitoring capabilities in a DDS. Nitrobenzene is a well-known small fluorescent quencher that is able to lower the LUMO level of aromatic molecules, making it an excellent acceptor in photo-induced electron transfer (PET) to induce fluorescence quenching.[10] The PET effect is then diminished by cleavage of the bond between the fluorophore and the prodrug-bearing quencher in acidic conditions enhancing the fluorescence of the drug (Dox in this case).

In this context, we herein present a novel DDS with minimized complexity in size, but still capable of targeting tumor sites and releasing anticancer drugs under the tumor tissue's acidic conditions. The DDS we have designed is composed of three parts: a fluorescence quencher moiety, a tumor-targeting group, and an acid-triggered releasable anticancer drug, as shown in Scheme 1. Nitrobenzene is adopted as the quencher. Biotin, which has often been used as a cancer-targeting moiety, selectively guides the DDS to tumor cells and markedly enhances the water solubility of the prodrug.[11] Dox, linked to the nitrobenzene–biotin scaffold by an acid-cleavable hydrazone bond, is employed as the pH-responsive part. The combination of these parts is expected to provide selective tumor targeting and Dox release with dramatic fluorescence enhancement upon activation by the acidic environment of the tumor cells (**Scheme 1**), allowing for the real-time assessment of its drug release efficiency by imaging the changes in the fluorescence intensity *in situ*.



Scheme 1 Illustration of functioning mechanism of acid-activated tumor-targeted prodrug **9** at the tumor site.

Prodrug **9** was prepared following the synthetic route shown in Scheme 2. First, the biotin derivative **2** was synthesized in two steps, and nitrobenzene moiety **6** was prepared in four steps from 2-hydroxy-4-nitrobenzoic acid. Compounds **2** and **6** were then coupled by a click reaction in DMF to produce compound **7**. Finally, prodrug **9** was synthesized by attaching Dox to the biotin-conjugated

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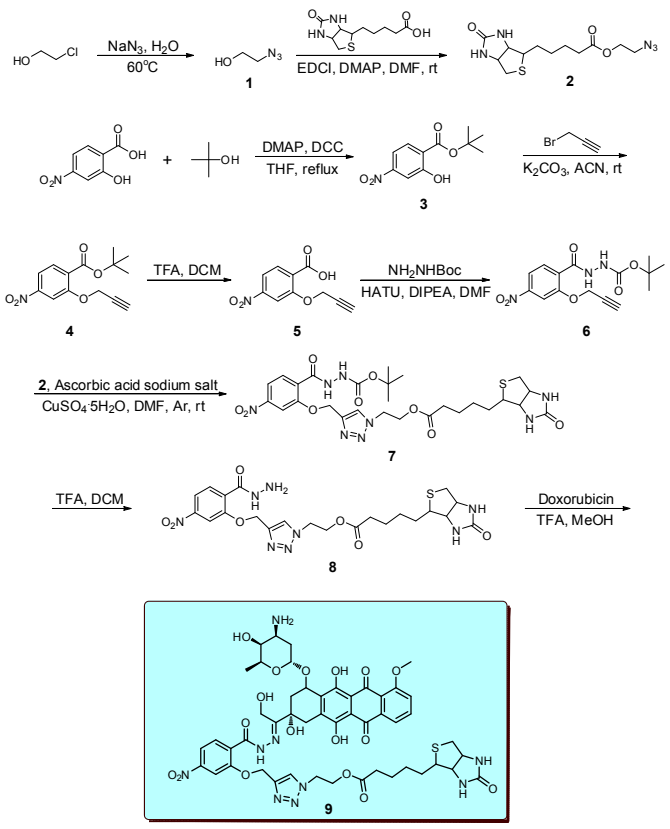
nitrobenzene moiety **7** in two steps. The structures of all the synthesized compounds were confirmed by ^{1}H, ^{13}C NMR, and ESI-mass spectrometry (see Fig. S1–S21).



Scheme 2 Synthetic route of the acid-activated tumor-targeted prodrug **9**.

To examine whether the hydrazone bond between the biotin–nitrobenzene conjugate and Dox of prodrug **9** is cleavable in acidic environments, the fluorescence properties of prodrug **9** were measured in different pH conditions. In a neutral solution (pH 7.4), compound **9** exhibited much weaker emission peak at 558 nm than did free Dox at the same concentration as shown in Fig. 1. The decreased fluorescence intensity of the Dox unit in **9** is presumably quenched by the nitrobenzene moiety through PET mechanism. As predicted, prodrug **9** shows no change in its fluorescence spectrum for at least 7 h under normal physiological pH conditions (pH 7.4), whereas the fluorescence intensity gradually increased in low pH conditions (pH 5.0) in a time-dependent manner (Fig. 1b and c). The fluorescence intensity at 558 nm clearly depends on the acidity of the solution (Fig. 1d). These results can envisage that the hydrazone bond of prodrug **9** could remain stable during blood circulation, then be cleaved and release Dox upon arrival at tumor cells which in general are in an acidic environment. Such a tendency demonstrates that the cleavage of the hydrazone bond will release the active drug, Dox, intracellularly and simultaneously enable us to monitor the distribution of the drug through the fluorescence enhancement of Dox.

In order to confirm the release of Dox from **9** in acidic conditions, compound **9**-containing solution incubated at pH 5.0 for 3 days was analyzed by mass spectrometry, and the data are shown in Fig. S22. We observed molecular ion peaks of *m/z* 566.35 and *m/z* 587.25, which correspond to the molecular ion peaks of Dox ($M + Na^{+}$) and

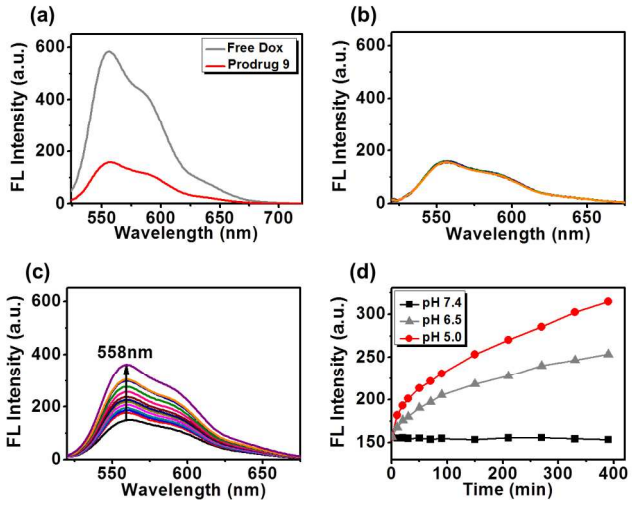


Fig. 1 (a) Fluorescence emission spectra of free doxorubicin and prodrug **9** (5 μM); λ_{ex} = 501 nm. (b) Fluorescence changes of prodrug **9** (5 μM) at various times (0–400 min) at pH 7.4 and (c) at pH 5.0 (0–400 min, overnight); λ_{ex} = 501 nm. (d) Fluorescence intensity changes using prodrug **9** (5 μM) at 558 nm at various times (0–400 min) at pH 7.4, pH 6.5, and pH 5.0. All spectra were recorded in PBS buffer at 37°C. FL: fluorescence.

8 ($M + K^{+}$), respectively (graph = cationic measurement). This provided further strong evidence that prodrug **9** releases strongly fluorescent Dox through cleavage of the hydrazone bond of prodrug **9** in acidic conditions.

Next, we investigated the antitumor efficacy of prodrug **9** towards cancer cells. To evaluate the cytotoxicity of prodrug **9**, MTT assay was carried out in two different cell lines, biotin receptor-positive HepG2 cells and negative WI-38 cells as shown in Fig. 2. Upon treatment of free Dox alone, both cell lines were highly susceptible to the drug treatment (Fig. 2(a)). In the case of prodrug **9** treatment, the cytotoxicity was reduced against both cell lines compared to the cytotoxicity of Dox alone, but the HepG2 cells were more susceptible to the prodrug than the WI-38 cells (Fig. 2(b)). This result demonstrates that prodrug **9** can exhibit antitumor activity in biotin receptor-positive cells, the HepG2 cell line in this study, obviously due to selective uptake by the biotin receptor.

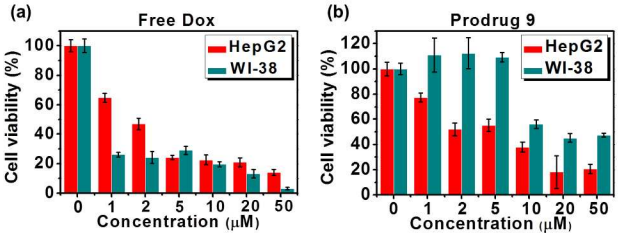


Fig. 2 Cytotoxicity of (a) free doxorubicin and (b) prodrug **9** in HepG2 cells and WI-38 cells after 48-h incubation.

To get insight into the selective uptake of prodrug **9** by biotin receptor-positive cells over biotin receptor-negative cells, confocal microscopy was carried out for both Dox and prodrug **9** using HepG2 and WI-38 cells. As shown in Fig. 3, after 1 h incubation with free Dox, the fluorescence of Dox was observed in both HepG2 cells and WI-38 cells, with little bit more intense fluorescence in the WI-38 cells. Conversely, in the presence of prodrug **9**, the fluorescence of Dox released from prodrug **9** was much stronger in the HepG2 cells than that in WI-38 cells. These confocal microscopic images provide evidence of prodrug **9** being selectively

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taken up by biotin-positive cancer cells. Taken together, these results show that prodrug **9** preferentially targets biotin-positive cancer cells, where the prodrug is accumulated and is activated by acidic conditions to deliver the drug (Dox) to the cancer cells with fluorescence enhancement. The delivery of the Dox may result in apoptosis and eventually inhibition of tumor growth in tumor models. Such theranostic strategies based on acidity-triggered drug release with markedly fluorescence changes could be simple and efficient ways for the early diagnosis and precise treatment of cancer.

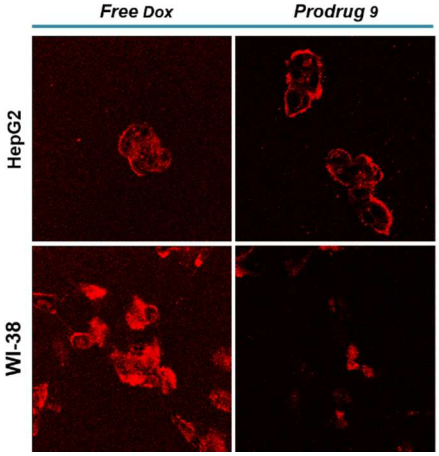

Fig. 3 Confocal microscopy images of HepG2 and WI-38 cells treated with free Dox and prodrug **9** at 5 μM for 1 h. Image was acquired at an excitation wavelength of 488 nm. Dox: doxorubicin.

In conclusion, a novel biotin-guided anticancer drug delivery system, prodrug **9**, consisting of biotin, nitrobenzene, and Dox, was synthesized. The drug release was mediated by acid-triggered hydrazone bond cleavage and accompanied by the enhanced fluorescence intensity of Dox which was initially quenched by a nitrobenzene moiety. In the cytotoxicity experiment, biotin receptor-positive HepG2 cells were found to be more sensitive to prodrug **9** than biotin receptor-negative WI-38 cells. Hence, prodrug **9** can be a promising candidate as a selective tumor-targeted drug delivery system with applications in anticancer therapy and imaging as a theranostic tool.

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<Graphical Abstract>