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Cascade enzymatic reaction activatable gemcitabine prodrug with AIE-based intracellular light-up apoptotic probe for *in situ* self-therapeutic monitoring

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A targeted cathepsin B-activatable gemcitabine prodrug with caspase-3 specific light-up tetraphenylene (TPE) as apoptotic probe based on aggregation-induced emission (AIE) properties was designed for *in situ* self-therapeutic monitoring of pancreatic cancer cells.

With a 5-year survival rate of only 5% and a median survival of 6 months, pancreatic cancer is a notoriously lethal cancer worldwide.[1] Owing to a lack of effective diagnostic methods, more than 80% of patients with unresectable locally advanced or metastatic pancreatic cancer are restricted to palliative treatment and gemcitabine (GEM) has been the standard first-line drug for these patients since 1997.[2] However, the clinical application of GEM is severely limited due to the rapid deamination of GEM with an extremely short plasma circulation time.[3] Our group and others have found the use of GEM prodrugs could effectively avoid the deactivation of GEM.[4]

An early assessment of therapeutic efficiency on pancreatic cancer is particularly important in clinic since it can minimize the possible ineffective therapy duration.[5] GEM, a non-fluorescent drug, can be hardly detected in the process of GEM delivery or release.[4a] Recently, some theranostic GEM delivery systems loaded with fluorescent dyes were reported for real-time monitoring of GEM delivery.[6] Furthermore, strategies for real-time monitoring of GEM release *via* GEM release-induced fluorescence intensity change upon tumour-associated stimuli were developed.[7] However, in these works, *in situ* evaluation of therapeutic effect could hardly be realized, which means extra detections are required for therapeutic evaluation. Though magnetic resonance imaging (MRI) is widely used in estimating the effectiveness of pancreatic cancer treatment by measuring the tumour size, it still has the problem in evaluating the early response of cancer to specific treatment as the change in tumour size is not obvious at the early stage of therapy. One of the most promising strategies is to combine an apoptotic probe into the system.[9] When the activated GEM kills pancreatic cancer cells by inducing cell apoptosis, the apoptotic probe can be used to evaluate the therapeutic effect simultaneously. As is known, caspase-3 plays an important role in signal transforming in the procedure of cell apoptosis. It was already reported that caspase-3 could be activated by chemotherapeutic agents including GEM.[10]

Up to date, fluorescent probes with aggregation-induced emission (AIE) characteristics have been widely reported and utilized as chemosensors and fluorescent bioprobes.[11] Tetraphenylethene (TPE), for instance, shows weak or no fluorescence in the molecularly dissolved state but strong blue fluorescence in the aggregated state on account of the restriction of intramolecular rotations (RIR), which can be utilized for cellular imaging and medical imaging.[12] TPE has many advantages in biomedical applications, such as easy synthesis and functionalization, strong and stable fluorescence, and low cytotoxicity. Due to these unique characteristics, it is of particular interest in developing AIE-based stimuli-responsive light-up probe for specific biological processes. As is known, among the endogenous stimuli, such as pH, redox potential and hypoxia in pathological conditions, the dysregulation of certain enzymes is a more specific character for many types of tumours.[13] For instance, lysosomal protease cathepsin B, overexpressed in many types of malignant tumours, was widely exploited for enzyme-responsive drug delivery.[14]

Herein, a targeted theranostic GEM (prodrug **1**, Scheme 1) with cancer cell overexpressed cathepsin B-sensitive GEM release and subsequent caspase-3 triggered AIE-based intracellular light-up was developed as an apoptotic probe for therapeutic monitoring of pancreatic cancer cells. This prodrug consisting of RGD peptide as the targeting moiety is expected

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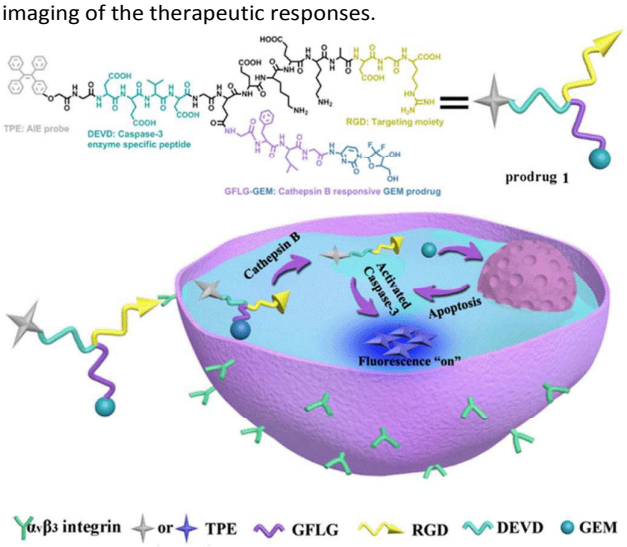
to be accumulated preferentially in pancreatic cancer cells with overexpressed $\alpha_v\beta_3$ integrin.[15] Following cellular internalization, the GFLG peptide in GEM prodrug is hydrolysed in the presence of overexpressed cathepsin B,[4b, 14a, 16] followed by the release of the active drug (GEM) and the apoptotic probe (TPE-DEVD-RGD) (Scheme S2). GEM-induced cell apoptosis will be initiated by the released GEM, and then DEVD peptide in TPE-DEVD-RGD will be cleaved by the apoptosis activated caspase-3.[5,9,17] Therefore, strong blue fluorescence will be observed because of the hydrophobic TPE residues with AIE characteristics. As a proof-of-concept, such cascade enzymatic reaction activatable GEM prodrug system can be used as theranostic platform for killing pancreatic cancer cells and simultaneously for real-time and noninvasive imaging of the therapeutic responses.



Scheme 1 Schematic illustration of cascade enzymatic reaction activatable gemcitabine prodrug **1** with AIE-based intracellular light-up apoptotic probe for *in situ* self-therapeutic monitoring of pancreatic cancer cells.

Carboxylated TPE (TPE-COOH, Scheme S1) was first synthesized and confirmed by ^{1}H NMR and electrospray ionization-mass spectrum (ESI-MS) (Fig. S1, S2). Prodrug **1** (Scheme 1) was then custom-synthesized by ChinaPeptides Co., Ltd with more than 95% purity and confirmed by High Performance Liquid Chromatography (HPLC) and ESI-MS spectrum (Fig. S3, S4). Meanwhile, caspase-3-responsive GEM prodrug **2** (Scheme S3, Fig. S5, S6) but without cathepsin B-responsiveness, and cathepsin B-responsive GEM prodrug **3** (Scheme S4, Fig. S7, S8) but without caspase-3-responsiveness were also custom-synthesized as controls.

For the prodrug delivery system, it is crucially important to release original active drug for restoring its therapeutic ability.[5a,18] Since cathepsin B is overexpressed in pancreatic cancer cells,[19] the release behaviour of pharmaceutically active GEM from prodrug **1** incubated with 0.5 UN mL^{-1} cathepsin B was investigated by HPLC[12b] (Fig. 1a). Approximately 60% of GEM was released in 6 h and close to 80% of GEM was released after 24 h since GEM was conjugated to prodrug **1** by cathepsin B-cleavable GFLG peptide sequence. Meanwhile, we found that the release behaviour of GEM from prodrug **1** is cathepsin B-concentration dependent. However, there is almost no GEM release from prodrug **1** in the absence of cathepsin B. In addition, only few GEM was released from prodrug **2** with stable GAAG peptide sequence in the presence of cathepsin B. These results suggested that prodrug **1** could generate pharmaceutically active GEM trigged by cathepsin B.

The optical properties of TPE-COOH and prodrug **1** were then studied. It is well known that AIE fluorogen shows weak fluorescence in good solvents but strong fluorescence in the aggregated state. As is expected, TPE-COOH emitted strong blue fluorescence in DMSO/PBS mixture (1/199, v/v), while prodrug **1** was almost non-fluorescent in the same medium on account of its excellent solubility (Fig. 1b). The aggregation of hydrophobic TPE-COOH in DMSO/PBS mixture (1/199, v/v) was confirmed by dynamic light scattering (DLS) with an intensity-average hydrodynamic diameter of 825 nm (Fig. S9). This cellular aggregation of TPE might be beneficial for enhanced intracellular retention of the probe and thus for improving tumor imaging *in vivo*.[20] In view of these interesting optical properties, prodrug **1** can be a potential specific light-up apoptotic probe for therapeutic monitoring with minimal background signals.

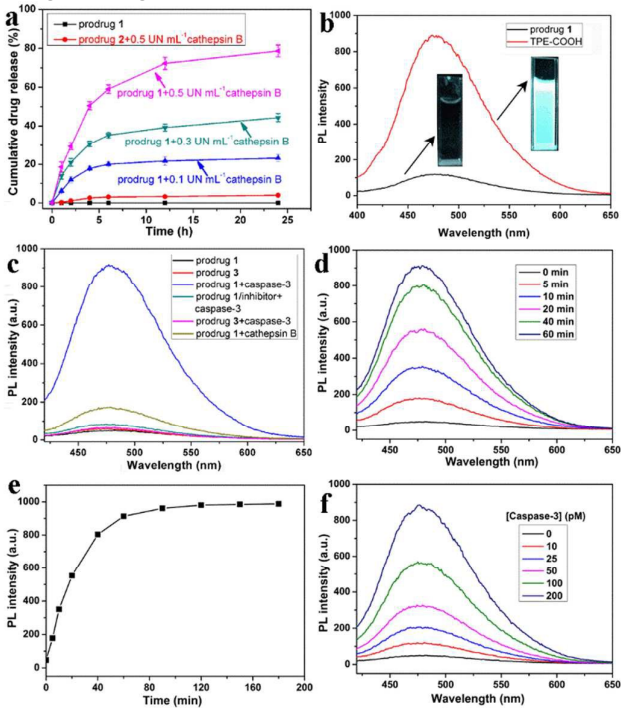


Fig.1 (a) *In vitro* GEM release from prodrug **1** incubated with various concentration of cathepsin B (0, 0.1, 0.3, 0.5 UN mL^{-1}) and prodrug **2** inbucated with cathepsin B (0.5 UN mL^{-1}). (b) Fluorescent emission spectra of prodrug **1** and TPE-COOH in DMSO/PBS mixture (1/199, v/v). Inset: Corresponding digital photos taken under irradiation by a UV lamp at 365 nm. (c) Fluorescent emission spectra of prodrug **1** (10μM) or prodrug **3** (10μM) upon treatment with caspase-3 (200 pM) in the presence or absence of Z-DEVD-FMK (20μM, caspase-3 inhibitor) and cathepsin B (0.5 UN mL^{-1}). (d) Time-dependent fluorescent emission spectra of prodrug **1** (10μM) in the presence of caspase-3 (200 pM) in DMSO/PBS mixture (1/199, v/v). (e) PL intensity of prodrug **1** at 480 nm upon addition of caspase-3 (200 pM) from 0 to 180 min. (f) PL intensity of prodrug **1** (10 μM) treated with various concentration of caspase-3 (0, 10, 25, 50, 100, and 200 pM) in DMSO/PBS mixture (1/199, v/v) for 60 min.

The light-up capability of prodrug **1** triggered by caspase-3 was subsequently investigated. As shown in Fig. 1c, prodrug **1** was also non-fluorescent in DMSO/PBS mixture (1/199, v/v), but strong fluorescence signals were recorded upon incubation with 200 pM caspase-3. However, when pretreated with

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caspase-3 inhibitor Z-DEVD-FMK, almost no fluorescence could be observed in the presence of caspase-3, indicating the specific cleavage of DEVD peptide sequence in prodrug **1** was severely inhibited. It is worth noting that, upon incubation with 0.5 UN mL^{-1} cathepsin B, the fluorescence of prodrug **1** increased little, indicating hardly no AIE effect happened since hydrophilcity of the prodrug changes little with an almost unchanged basic hydrophilic segment of the prodrug. In addition, prodrug **3** was almost non-fluorescent in DMSO/PBS mixture (1/199, v/v) and the fluorescence spectrum remained no change in the presence of caspase-3 since the DAAD peptide sequence was stable and TPE kept in the molecularly dissolved state under this condition.

The fluorescence change of prodrug **1** upon treatment with caspase-3 was also recorded over time in DMSO/PBS mixture (1/199, v/v). As shown in Fig. 2d, after incubation with caspase-3, the fluorescence of prodrug **1** increased dramatically. The fluorescence reached a plateau in 60 min which was 22-fold higher than the intrinsic emission of prodrug **1** (Fig. 2e). The effect of caspase-3 concentration on the fluorescent emission of prodrug **1** was further investigated. As shown in Fig. 2f, with increasing concentrations of caspase-3 from 0 to 200 pM, the fluorescence of prodrug **1** increased gradually, which might be ascribed to the increased amount of hydrophobic TPE aggregates. These results confirmed that the caspase-3 triggered release of TPE might be used as a specific indicator of caspase-3 activity in the cells.

In order to evaluate the therapeutic effect of prodrug **1**, the *in vitro* cell proliferation inhibition against human pancreatic cancer cells BxPC-3 cells compared to free GEM, prodrug **2**, and prodrug **3** was studied using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Fig. 2a). After incubation for 72h, the cell viabilities upon treatment with prodrug **1** and prodrug **3** were almost the same but much lower than that with prodrug **2**. This is because GFLG peptide sequence of prodrug could be specifically cleaved by cathepsin-B and active GEM would be thus released in BxPC-3 cells, which kills the cells, while GAAG could not. Interestingly, high dose of prodrug **1** showed superior cell proliferation inhibition compared to the same concentration of GEM, probably owing to efficient cellular internalization of prodrug **1** and successful release of GEM from the prodrug.

GEM kills BxPC-3 cells by inducing cell apoptosis with activated caspase-3,[20] and caspase-3 plays an important role in signal transforming of cell apoptosis.[10] In order to verify whether the caspase-3 could be activated by prodrug **1** in BxPC-3 cells, the expression of caspase-3 was investigated using Ac-DEVD-pNA, a fluorogenic caspase-3 substrate (Fig. 2b). Compared to the control, BxPC-3 cells incubated with prodrug **2** only had a slight increase of the expression of caspase-3 because GEM cannot be effectively released. However, when incubated with prodrug **1** or prodrug **3**, the expression of caspase-3 increased dramatically owing to the effective cathepsin B triggered GEM release, which was consistent with the previous MTT results. These results suggested that BxPC-3 cells could generate caspase-3 stimulated by prodrug **1** and the generated caspase-3 could be used as specific initiator for intracellular light-up apoptotic probe for therapeutic monitoring of BxPC-3 cells.

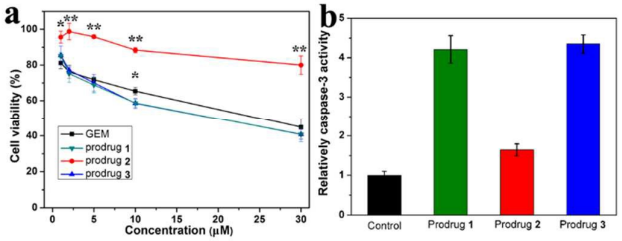

Fig. 2 (a) Cell viability of BxPC-3 cells incubated with various concentrations of GEM, prodrug **1**, prodrug **2**, and prodrug **3** for 72 h. *P<0.05, **P<0.01, compared to prodrug **1**. (b) Caspase-3 expression in BxPC-3 cells incubated with prodrug **1**, prodrug **2**, and prodrug **3** at the concentration of 20 μM for 72 h.

Finally, taking advantage of the intracellular cascade enzymatic reaction, fluorescence microscopy was carried out to study the capability of GEM-induced apoptosis in BxPC-3 cells by recording the intracellular fluorescence upon incubation with prodrug **1**, prodrug **2,** or prodrug **3**. As shown in Fig. 3, upon incubation with 10 μM prodrug **1,** strong blue fluorescence was observed in BxPC-3 cells because of the intracellular cascade enzymatic reaction generated hydrophobic TPE residues with AIE characteristics. However, if incubated with prodrug **2**, only weak fluorescence was observed in BxPC-3 cells, since GEM could be hardly released from the stable GAAG peptide sequence, leading to few cell apoptosis, which was in accordance with the results in Fig. 2b. Meanwhile, almost no fluorescence of prodrug **3** could be monitored in cells due to the molecularly dissolved state of TPE in TPE-DAAD-RGD probe that could not be specifically cleaved by capase-3, which was in good agreement with FL results in Fig. 1c. These results strongly proved that prodrug **1** was not only promising in targeted GEM delivery but also potential as intracellular light-up apoptotic probe for *in situ* self-therapeutic monitoring of pancreatic cancer cells.

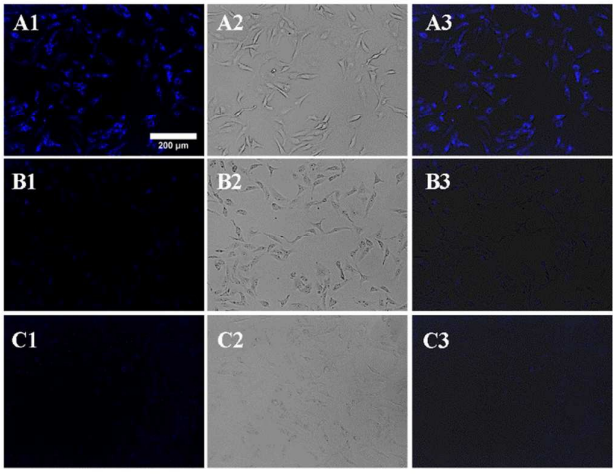

Fig. 3 Fluorescence microscopy images of the prodrug **1** (A), prodrug **2** (B), and prodrug **3** (C) for 3 h. A1, B1, C1: blue fluorescence images of TPE; A2,B2,C2: bright field images; A3,B3,C3: the merge images.

In summary, a theranostic GEM prodrug for targeted drug delivery and evaluation of *in situ* therapeutic effect was developed in this work. After cellular internalization of prodrug **1**, active GEM and the cell apoptotic probe (TPE-DEVD-RGD)

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could be released simultaneously from the prodrug due to the specific cleavage of GFLG peptide sequence in prodrug by the intracellular over-expressed cathepsin B. The pharmaceutically active GEM could induce the apoptosis and activate the caspase-3 in BxPC-3 cells. The DEVD peptide sequence in TPE-DEVD-RGD could be cleaved by the activated caspase-3 and the AIE effect of TPE residues was thus triggered for therapeutic effect evaluation. This cascade enzymatic reaction activatable GEM prodrug system could be used as a theranostic platform for killing pancreatic cancer cells and real-time and noninvasive imaging of the therapeutic responses. On the other hand, the fluorescence of TPE is in the blue spectrum, which strongly restricts the *in vivo* applications. In order to transfer this concept to further *in vivo* applications, we are trying to prepare AIE bioprobes with red fluorescence that can be used for monitoring the drug release and subsequent assessing the therapeutic efficiency. The relevant research is ongoing.

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