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Local drug delivery is highly demanded in inoperable cancers and for prevention of local tumor recurrence. Consider that the increment of several months of survival by new chemotherapy drug is significant, the increase of survival of two years using a localized therapeutic implant post-surgery is remarkable.^[1] Localized delivery of chemotherapeutic drugs has shown great promise in enhancing treatment efficacy and reduction of recurrence. However, insufficient or excess dosage due to unknown drug concentration at the tumor site remains a challenge to achieve successful chemotherapy. Real-time monitoring of drug concentration in local drug delivery system (DDS) is of great importance to tune the *in vivo*

drug release rate and optimal treatment concentration, and it is a critical and unsolved challenge. Monitoring of drug concentration by direct tracking of dye-conjugated drug molecules may be challenging since the drug signals may be resulted from unreleased drugs, free/unconjugated dyes (dye may detach due to phagocytic process), drugs being internalized by the cells, and released drugs for intended study and low signal intensity. In this regards, a self-indicating DDS that can reveal the drug concentration via signals from the drug delivery carrier is of great interest. By taking the advantage of a self-indicating system, one can easily detect the depletion of drugs, and re-inject to maintain a dosage in the optimal therapeutic window. In addition, the signals can be amplified without additional dose of drug that may harm patients. Fluorescent molecules are potential candidates to fulfil this aim. At present, there is a few fluorescent DDS designed to monitor chemotherapeutic drugs based on fluorescence resonance energy transfer (FRET) due to interaction between the donor (the matrix) and the acceptor (the drugs).^[2] The concentration of drug in the carrier is reflected based on the change in fluorescent emission of the carrier. However, because of aggregation-caused quenching effect (ACQ), the fluorescence of these molecules is commonly weakened in a poorly dispersed system or complex environments like human body. Since the concept of aggregation-induced emission (AIE) was proposed by Tang and co-workers in 2001,^[3] various new AIE materials such as tetraphenylethene (TPE), triphenylethene and siloles have been developed.^[4] AIE phenomena is observed when the AIE molecules are aggregated in poor solvents such as body fluid or in solid state, it shows strong emission. This phenomenon is totally opposed of the ACQ effect exhibited by typical fluorescent materials such as FITC (fluorescein dye functionalized with an isothiocyanate (ITC) unit, for flow cytometry). This unique property makes them ideal for *in vitro* cell imaging,^[5] chemical sensors,^[6] and monitoring drug delivery mechanisms.^[7]

AIE luminogens are inherently hydrophobic and hindered from direct biological and biomedical applications. To enable water-solubility, AIE molecules were formulated (by

physical blending, co-precipitation) or conjugated (by covalent bonds) with hydrophilic functional moieties. For example, hydroxyl,^[7b] sulfonate^[8] and ammonium (cationic salts)^[5b] were integrated into the AIE luminogens to obtain amphiphilic molecules that could form AIE micelles. These AIE micelles were reported to provide stable self-assembled nano-aggregates thru enhancement of intermolecular interactions (π - π stacking, hydrogen bonding etc.). However, in serum solution or body fluid they may form large aggregates that could result in enhanced inflammation and attract ingestion by macrophage in *in vivo* experiments.^[9] AIE micelles which were prepared by conjugation of TPE with poly(ethylene glycol) (PEG) were used as both nanocarriers to deliver anti-cancer drug, and intracellular imaging probe. However, these AIE micelles flow in the blood stream and could not station in specific location for long-term *in vivo* monitoring purposes. For long-term *in vivo* applications, the luminogens need to be positioned at the specific area, possess leakage-free staining behaviour, and the fluorescence quantum yield should be as high as possible to achieve deep penetration and long-lasting tracking. Conjugation of TPE onto chitosan (a biocompatible polysaccharide derived from shells of crabs and shrimps) can yield AIE bioconjugates that allow long term *in vitro* cellular tracking for as long as 15 passages.^[5c] However, study on *in vivo* drug release monitoring by using AIE gel was not reported.

In this study, we developed a self-indicating DDS, with strong emission for real-time monitoring of drug concentration in local *in vivo* DDS. Current *in vitro* and *in vivo* tracking of drug released from a DDS can be achieved by fluorescence or absorbance spectroscopy, but the fluorescence emission of the drug were limited by ACQ effect and the fate of matrix remains largely unknown. When injected into a tumour, it is usually difficult to detect the drug and fate of the gel by non-destructive methods. We wish to develop a new self-indicating DDS for long-term monitoring purpose, having advantages of: (i) injectable non-invasive system, (ii) able to monitor bulk state of materials, (iii) fluorescent dye does not leach out, and (iv) able to encapsulate drugs and protein under benign conditions. The properties of AIE

micelles and AIE thermogel were characterized by direct critical micellar concentration (CMC) measurement, fluorescent assay and rheology. Biocompatibility studies were conducted to examine the cytotoxicity of the AIE thermogel. *In vitro* cellular tracability and *in vivo* mouse model were studied in detailed.

Thermogelling copolymer, poly(PEG/PPG/TPE urethane) (EPT) consisted of PEG, poly(propylene glycol) (PPG) and TPE was prepared based on polymerization route provided in **(Figure 1(a))**, with molecular weight of ~10 kDa, TPE of 1.5 wt. % of the copolymer, as determined by GPC, ^1H NMR. (Table S1 and Figure S1). EPT was soluble and light-emitting in water. The amphiphilic thermogelling copolymer self-assembled into EPT micelles in water, and they exhibit strong emission with the TPE molecules aggregated in the core of the micelles. The AIE behavior of EPT was characterized by fluorescence assay. In Figure S2(a), fluorescence intensity of EPT aqueous solutions increased as the concentration was increased, with a peak at 479 nm, at 25 °C. The polymer appeared as white solid flakes under room light, and it emitted bright blue fluorescence under UV illumination, as presented in Figure S2(c and d). Photograph of these solutions under UV lamp was displayed in Figure S2(b).

Water is a poor solvent for TPE segments. In dilute solution, unimers self-assemble into micelles at CMC (Figure S3 and Table S2), with hydrophobic PPG and TPE in the core, and hydrophilic PEG at the corona.^[10] In dilute solution above CMC, micelles aggregation is driven by hydrophobic interaction and dehydration of PEG at elevated temperature.^[11] Above sol-gel transition temperature, a higher degree of aggregation occurs. These EPT micelles transform into a closed pack three-dimensional network structure. This is a physical self-assembly method that may achieve higher AIE intensity than their micellar solution. Further increase temperature leads to excessive hydrophobicity that destroys the micellar network, and results in macroscopic precipitation.^[12] Temperature-controlled rheometer was used to examine the thermogelling properties of EPT thermogel. As temperature increased from 10-70 °C, sol-gel transition was observed at 21 °C as indicated by the modulus crossover point of

storage modulus (G') and loss modulus (G'') in Figure S4 (a). Sol-gel transition temperature is tunable via hydrophilic-hydrophobic balance in the copolymer and polymer concentration in aqueous solution.^[10c] At low temperature, AIE polymer solution was a white flowable liquid, as indicated by G'' above G' and in the inserted photograph. At temperature above gelation temperature, the AIE polymer solution became a white non-flowable physical gel. This time, the G' was above G'' . At 37 °C, the value of G' was raised to 550 Pa, which was a soft physical gel with modulus similar with brain, neuron and epithelial cells.^[13] Oscillation frequency sweep experiment was carried out to determine the dynamic gel properties under physiological temperature. At 37 °C, G' is above G'' indicates a physical gel was obtained, as shown in Figure S4(b).

The effect of thermogelation on AIE effect was compared in Figure 1(b). Fluorescence emission was concentration and temperature dependent. At free/unimer state, the emission was considered negligible. The phenyl rings of TPE underwent active intramolecular rotations that caused non-radiative energy decay and resulted in the non-emissive state. At micelle state, unimers self-assembled into micelles, and the solution started to fluoresce. Intramolecular rotations of phenyl rings of TPE were restricted in the micelles. This restricted intramolecular rotation (RIR) of the TPE rotors blocked the non-radiative decay pathways and collected radiative transitions, leading to a highly fluorescent micellar solution.^[4, 14] At gel state, micelles aggregated into a close-packed three dimensional physical network structure. This further enhanced the RIR process, and thus, an exceptionally highly emissive fluorescent gel was produced. The fluorescence intensity of the thermogelling EPT solution (at unimer, micelle and gel states) was examined at a range of temperatures (15 – 45 °C). There was a fluorescent decay as temperature increased. Photoluminescence (PL) intensity was relatively lower at 15 °C before sol-gel transition occurred at 21 °C. At 25 °C, at gel state, it exhibited the strongest emission. At 35 - 45 °C, these AIE systems may gain thermal energy, activating

intra- or inter-molecular rotations and encouraging the relaxation of the excitation energy in non-radiative decay. Temperature effect on dilute aqueous solution was shown in Figure S5.

Doxorubicin (Dox) is an anthracycline antibiotic, which is used as a chemotherapy drug for a wide range of cancers including leukemia, and various tumours. However, because the drug is administered via injection into blood stream, patients with high dose may experience severe side effects such as cardiotoxicity. Local DDS which consisted of Dox in AIE micelles and nanoparticles provide DDS to encapsulate the drug, with good biocompatibility.^[7] The photoluminescence signal was substantially reduced in the presence of 10-50 $\mu\text{g/mL}$ doxorubicin in a dose-dependent manner (Figure 1(c, d)). The blue shift of the emission peaks suggested that the presence of Dox reduced the aggregation of TPE at the core of micelles.^[4, 15] There was a transfer of emission from TPE to Dox, and caused fluorescence intensity to decrease.^[7a] As Dox concentration in EPT thermogel was increased, Dox's emission was quenched due to ACQ effect.^[7a, 16]

We performed a comprehensive cytocompatibility assessment of AIE thermogel preparation using a multimodal platform comprising of four different human cell types - primary dermal fibroblasts, lung epithelial cells A549, liver hepatocellular carcinoma cells HepG2 and embryonic kidney cells HEK293. The results showed that EPT thermogel is biocompatible (Figure S6). We examined if the AIE thermogel preparation was self-traceable in human epithelial cells. The “blue” fluorescence of thermogel was clearly visible in the cytoplasm of the cells treated for 3h or 6h (Figure S7). There was no or limited “blue” fluorescence in the nucleus (Figure S7) suggesting that thermogel did not enter into the nucleus. Further analysis of xz- and yz-sections of confocal images confirmed cellular uptake of the AIE thermogel (Figure 2). Next, we investigated whether the cellular uptake of the thermogel was mediated by an energy-dependent mechanism. For this purpose, A549 cells were incubated with AIE thermogel with concentration as above at low temperature (4°C) for 3h. The amount of thermogel uptake by A549 cells incubated at 4°C was found to be

significantly reduced (94.4 % reduction) in comparison to the equivalent incubation at 37°C (Figure 2). Moreover, blocking of the active transport mechanism of A549 cells by an ATP synthesis inhibitor sodium azide significantly impeded AIE thermogel uptake (93.6 % reduction in comparison untreated 37°C control) (Figure 2). These data suggest that thermogel is taken up into A549 epithelial cells *via* an energy-dependent endocytosis.

EPT thermogel exhibits exceptionally high emission from agglomeration of AIE micelles. Tumour bearing nude mice was taken as models to study its real-time monitoring of chemotherapeutic drug delivery ability. In Figure 3(a), the injection of Dox in saline was followed by fast diffusion spreading the whole solid tumour after 3h and quick metabolism or disappearance of red fluorescent Dox after 24h. Significantly different from Dox in saline, Dox in AIE thermogel (Figure 3(b)) experienced much slow spreading of Dox and maintained full cover and high concentration of Dox through the whole tumour even after 24h, indicating the sustained release of Dox for much longer time than Dox alone to effectively prevent the fast metabolism of Dox.

The AIE thermogelling solutions were injectable at room temperature and formed gel depots containing the chemotherapeutic drugs at the injection site, as evidenced by fluorescent signals from aggregation state of AIE thermogel in Figure 3(c). The photoluminescence signal of EPT thermogel was increasing in a dose-dependent manner, because the aggregation of TPE was recovered as Dox was released overtime. The self-indicating EPT thermogel is advantageous because the signals from matrix revealed the release of Dox precisely based on the change of Dox concentration. Moreover, the fluorescent signal intensity of EPT matrix was significantly higher than Dox signals, indicating a higher imaging quality based on matrix signals. The signals of the EPT matrix reached a plateau after 48h.

Significant shrinkage of tumour size in mice treated with EPT based thermogel with Dox was observed in Figure 3(d) and (e), in comparison with mice treated with Dox only. Also, body weight of mice bearing tumour was plotted in Figure S8. These results revealed

that inhibition of tumour growth by using chemotherapeutics might benefit from the slow release of drug encapsulated micelles from AIE thermogel. In addition, slow release results in less toxicity to mice.

By tracking the spatiotemporal change of fluorescent signals from Dox and self-indicating AIE thermogel, Dox transportation pathway was summarized. After being internalized by the tumour cells and transported into lysosome, Dox could detach from AIE micellar aggregates, due to acidic conditions.^[7a] The free Dox escaped and entered nucleus.^[7a] The AIE micellar aggregates stayed in the cytoplasm, as shown in previous *in vitro* section. Fluorescent signal from Dox in saline showed a diminishing trend over time. This is similar with some reports on commercial cell trackers and AIE nanoparticles.^[17] These small molecules leaked out easily from the cells due to change in concentration gradient. In contrast, Dox released from AIE thermogel exhibited continuously high emission because the thermogel and Dox aggregates were large in size (100-200 nm based on dynamic light scattering) that could hardly leak out once internalized.^[5c] This is an advantage over TPE nanoparticles that showed reduction in size from 70 to 8 nm after adding Dox.^[7a] After free Dox reached the nucleus, ACQ effect was reduced, thus a boosted emission from AIE thermogel was detected.

In vivo study further demonstrated the success of constantly monitoring of AIE thermogel depot in the tumour up to 18 days (Figure S9). Decrease of fluorescent signal of gel matrix at 18 days was attributed to gel erosion or swelling that leads to reduction in gel aggregation. As compared with commercial Qtracker[®] 655 that showed severe fluorescent decay in less than 7 days,^[17] current AIE thermogel tracing system is much more superior. It not only serves as sustained release depot, but also a long-term self-indicating gel for tracing concentration of drug in the matrix.

Conclusions

An AIE thermogel based on PEG, PPG and TPE was developed, with tuneable aggregation-induced emission dependent on Dox concentration and temperature. AIE thermogel exhibited advantages over typical DDS as the thermogel matrix is capable of revealing drug release status and the erosion condition of the matrix. It is superior to current cell tracking systems as the AIE thermogel showed exceptionally higher emission than AIE micelles because of enhanced RIR process in the close-packed gel. The EPT thermogel was non-toxic and did not enter into the nucleus. Slow and localized *in vivo* drug release from EPT thermogel depot in the tumour resulted in high efficiency at reducing tumour size. The fluorescent signal from EPT gel depot was detectable up to 18 days, thanks to the thermogelation behaviour which immobilized the AIE solution at the tumour and emission enhancement at the gel state. These observations suggested that a new self-indicating, biocompatible formulation based on AIE thermogel is an excellent candidate for *in vivo* detection of drug concentration and localized delivery of chemotherapeutic drugs.

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Supporting Information

Supporting Information is available online from the Wiley Online Library or from the author.

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Figure 1. (a) Synthesis procedure of EPT thermogelling copolymer, and schematic drawing of unimers, AIE micelles, micelles aggregation and in-situ gelling DDS; (b) Photoluminescence intensities of EPT solutions at free/unimer state (0.086 g/L), micelle state (2.75 g/L) and gel state (200 g/L) were plotted at different temperatures (15 – 45 °C) (λ_{ex} = 340 nm); (c) Photoluminescence intensities changed when different concentration of Dox (10 – 50 $\mu\text{g/mL}$) were added into EPT solution at gel state (λ_{ex} = 340 nm); (d) Photoluminescence intensities at 479 nm were fitted into an exponential function, $R^2=0.999$.

Figure 2. Energy-dependent cellular uptake of AIE thermogel by human epithelial cells. (A) A549 cells growing on cover slips were incubated with thermogel [20% thermogel (w/v) further diluted with medium to gel volume ratio of 2:1 (v/v), blue] for 3 h at 37 °C and fixed. Cells were counterstained with rhodamine-phalloidin to visualize cytoplasm (red) and DRAQ5 to visualize nuclei (purple). Intracellular accumulation of the thermogel was detected by confocal microscopy and a representative image-set is shown. The maximum projection of XY, XZ and YZ-sections of the confocal images indicate cytoplasmic distribution of thermogel. (B) A549 cells growing on cover slips were incubated without or with the above diluted thermogel (blue) for 3 h at 37 °C, 4 °C or with sodium azide (pre-treated cells) and fixed. Cells were counterstained with rhodamine-phalloidin (red) and DRAQ5 (purple). Intracellular accumulation of the thermogel was detected by confocal microscopy and representative images are shown. Scale bar 10 μ m (C) Cellular uptake of thermogel was quantified using INCell Investigator software and cellular intensity of thermogel fluorescence is presented. Data is mean values $\pm$ SEM of three independent experiments in triplicates from five randomly selected fields per sample. * $p < 0.05$ compared to untreated control.

Figure 3. *In vivo* mouse model. (a-c) *In vivo* non-invasive fluorescence imaging of nude mice bearing HepG2 tumour with post intravenous injection of 50 μL DOX in saline or DOX loaded thermogel for indicated time points. The black arrow indicated the location of tumour. (d-e) Inhibition of tumour volume by intratumoural injection of saline, DOX or DOX loaded EPT thermogels. Dorsal subcutaneous implantation of HepG2 cancer cells into mice was followed by administration of each solution after tumours had reached a volume of $\sim 150 \text{ mm}^3$. The excised tumour removed after 14 days was taken for evaluation.

Monitoring of drug release *in vivo* is an important requirement in the development of localized drug delivery system (DDS). However, current technologies and materials do not effectively support *in vivo* non-invasive detection of actual drug release profile. Here, we report a new aggregation-induced emission (AIE) thermoresponsive hydrogel, which is capable of showing fluorescent signals with emission peak at 479 nm when excited at 340 nm, indicative of the concentration of drug in the gel matrix. The photoluminescence signal is substantially reduced in the presence of 10-50 $\mu\text{g/mL}$ doxorubicin in a dose-dependent manner. AIE molecule – tetraphenylethene (TPE) is incorporated into a thermogelling copolymer in a condensation reaction and the resultant thermogel is biocompatible, which shows cellular internalization via an active cell uptake mechanism. This injectable formulation exhibits a critical sol-gel transition temperature that allows the formation a drug delivery gel depot in situ at body temperature. *In vivo* experiments demonstrate the potential of AIE thermogel as a self-indicating formulation for monitoring drug release and fate of matrix.

Keyword (see list)

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Supporting Information

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Table S1. Molecular characterization of TPE diol and multiblock polyurethane copolymers.

Sample ID	M _n	M _w	PDI	Composition ¹ H NMR (wt. %)		
				PEG	PPG	TPE
TPE diol	1200	1300	1.09	-	-	100
EPT	13800	18000	1.31	70.0	30.0	-
EP	10100	15400	1.53	60.5	38.0	1.5

(B)

(C)

(D)

(E)

Figure S1. ^1H NMR spectra of (A) tetraphenylethene (TPE) diol and (B) poly(PEG/PPG/TPE urethane) (EPT) in CDCl_3 and in (C) D_2O , at 25 °C, (D) ^{13}C NMR of TPE diol in CDCl_3 and (E) HRMS MALDI-TOF analysis of TPE diol.

Figure S2. (a) EPT aqueous solution with various concentrations at 25 °C, $\lambda_{\text{ex}} = 340$ nm, $\lambda_{\text{max}} = 479$ nm; (b) Photograph taken under UV illumination ($\lambda = 365$ nm) at room temperature (sample 1-9 corresponding to concentration 2.75-0.011 g/L); (c and d) Solid state EPT flakes under room light (RL) and UV illumination ($\lambda = 365$ nm).

Figure S3. CMC determination of EPT by direct fluorescent method, the intersection of the tangents to the two slopes of the graph where the log concentration was plotted against fluorescent intensity at 479 nm, at 25 °C. ($\lambda_{\text{ex}} = 340 \text{ nm}$)

Table S2. CMC of EPT and EP dilute solutions at various testing temperatures.

Sample ID	T (°C)	CMC ($\times 10^{-5}$) g. mL ⁻¹
EPT	15	40.6
	25	43.2
	35	24.8
	45	22.9
EP	25	41.7

Figure S4. (a) Modulus of EPT aqueous solution of 20 wt. % over 10-70 °C, with heating rate set at 5 °C/min, and 1 Hz and 0.1% strain. Dotted line indicates the modulus crossover point at 21 °C; (b) Oscillation frequency sweep at 0.1% strain at 37 °C

Figure S5. Fluorescence spectra of EPT aqueous solution with various concentrations at different temperatures (a) 15 °C, (b) 25 °C, (c) 35 °C, and (d) 45 °C. ($\lambda_{\text{ex}} = 340 \text{ nm}$)

The EPT thermogel is biocompatible

We next performed a comprehensive cytocompatibility assessment of AIE thermogel preparation using a multimodal platform comprising of four different human cell types - primary dermal fibroblasts, lung epithelial cells A549, liver hepatocellular carcinoma cells HepG2 and embryonic kidney cells HEK293. Cells were treated without or with various concentrations of thermogel (5, 25, 50 or 100 μg of AIE thermogel/ml cell culture medium, w/v) for 6 h, 24 h, 48 h or 72 h. After treatment periods, cells were fixed and fluorescently stained for cytoskeletal proteins tubulin and actin (as indicators to visualize cellular morphologies) and nucleus. Automated microscopy showed no detectable change in the gross structure of the cytoskeletal proteins tubulin and actin or the overall morphology of cells exposed to as high as 100 μg AIE thermogel/ml medium, w/v (Figure S6A). Multiple parameters representing cellular health such as shapes and sizes of cells and nuclei including number of adhered viable cells, average cell area, cell gyration, cell 1/form factor, and nuclear area were within the normal variation range (Figure S6B). There were no signs of significant cellular or nuclear abnormalities due to AIE thermogel treatment in comparison to that observed in untreated cells (Figure S6B). In contrast, cells treated with nocodazole (used as a cytotoxicity control) showed significant cell detachment from the culture plate, denatured cytoskeleton and reduced cell spreading (Figure S6A and S6B).

To further assess the cytotoxic effect of thermogel over time, all the four cell types were exposed to a high concentration of EPT thermogel preparation (100 μg AIE thermogel/ml medium, w/v) and were monitored in real-time using a whole cell-based electrical impedance sensing technique. Cells were allowed to grow on the electrode surface (E-Plate 16®) to confluence over a period of 20 h and then treated with thermogel and incubated for further 48 h. Changes in resistance of the electrode, depending on various morphological parameters of cells that may be affected by thermogel treatments, were quantified automatically and plotted as an arbitrary unit “Cell Index”. As illustrated in Figure S6C, there was no detectable cytotoxicity of AIE thermogel preparation in all the four cell types tested.

In parallel, we performed another independent assay to measure the effect of AIE thermogel preparation on cell viability via quantifying the MTS conversion to formazan. No significant reduction in the viability of any of the cell types were observed when they were treated with

100 $\mu\text{g/mL}$ thermogel for 24 h (Figure S6D) further confirming the biocompatibility of the thermogel preparation.

Figure S6. Multimodal biocompatibility analysis of AIE thermogel. Cultured human dermal fibroblasts, A549, HEK293 and HepG2 cells growing on 96-well tissue culture plates were incubated with increasing concentrations of AIE thermogel (5, 25, 50 or 100 μg of AIE thermogel/ml cell culture medium, w/v) for 6 h, 24 h, 48 h or 72 h and fixed. Cells were treated with a cytotoxic drug nocodazole as control. Cells were counterstained with anti- α -tubulin-FITC (green), phalloidin-rhodamine (red) and Hoechst (blue) and imaged using an automated microscope IN Cell Analyzer 2200. **(A)** Representative images of cells treated with the highest concentration of thermogel (100 μg AIE thermogel/ml medium, w/v). **(B)** Multiple

cellular and nuclear morphological parameters to determine the effect of thermogel on cell health were automatically quantified by IN Cell Investigator software. Normalized morphological parameters 9 randomly selected fields/well containing at least 300 cells were converted into heatmaps and presented. (C) Cells (2×10^3 cells/well) growing on 16-well E-Plate16 for 20 h were treated with the 100 μg AIE thermogel/ml medium (w/v) thermogel in triplicates and incubated for additional 48 h. Cell growth curves were automatically recorded (Cell Index $\pm$ SEM) in real time using xCELLigance electrical impedance sensing platform. (D) Percentage (%) cell viability following treatment with 100 μg AIE thermogel/ml medium (w/v) thermogel for 24 h was determined using MTS-based assay and plotted. Data represent mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared to untreated control. NT, no treatment, Noc, Nocodazole.

Cellular internalization of the AIE thermogel

We examined if the AIE thermogel preparation was self-traceable in human epithelial cells. A549 cells were treated with AIE thermogel [20% thermogel (w/v) further diluted with medium to gel volume ratio of 2:1 (v/v)] for 3 h or 6 h and its cellular internalization was visualized by confocal laser scanning microscopy. The “blue” fluorescence of thermogel was clearly visible in the cytoplasm of the cells treated for 3 h or 6 h (Figure S4). There was no or limited “blue” fluorescence in the nucleus (Figure S7) suggesting that thermogel did not enter into the nucleus.

Figure S7. Cellular uptake of EPT thermogel by human epithelial cells. A549 cells growing on 8-well chamber slides were incubated with EPT thermogel [20% thermogel (w/v) further diluted with medium to gel volume ratio of 2:1 (v/v), blue] for 3 h or 6 h and fixed. Cells were counterstained with phalloidin-rhodamine (blue) and nucleotracker (purple). Intracellular accumulation of the thermogel was detected by confocal microscopy and representative images are shown. At least 20 different fields were imaged in three independent experiments and representative images are shown.

Figure S8. Body weight of mice bearing tumor, with intratumoural injection of saline, DOX or DOX loaded EPT thermogels.

Figure S9. In vivo non-invasive fluorescence imaging of nude mice bearing HepG2 tumor with post intravenous injection of 50 μ L DOX loaded thermogel for indicated time points, indicating the degradation or metabolism of polymeric structures after 18 days.

Materials and Methods

Poly(ethylene glycol) (PEG) with M_n 2050 $\text{g}\cdot\text{mol}^{-1}$ and poly(propylene glycol)(PPG) with M_n 2000 $\text{g}\cdot\text{mol}^{-1}$, dibutyltin dilaurate (DBT) (95%), 1,6-hexamethylene diisocyanate (HDI) (98%), 1,6-diphenyl-1,3,5-hexatriene)(DPH), anhydrous toluene, and diethyl ether were purchased from Sigma-Aldrich. All the reactants were used as received.

Molecular characterization

NMR spectra were measured at room temperature using Bruker AV-400 NMR spectrometers in CDCl_3 solvent. Gel permeation chromatography (GPC) analysis was carried out with a Viscotek GPC max module equipped with two phenogel columns (10^3 and $10^5 \text{ \AA}$) (size: $300 \times 7.80 \text{ mm}$) in series and a Viscotek TDA 305 triple detector. Tetrahydrofuran was used as eluent at a flow rate of 1.0 mL min^{-1} at column temperature of 40°C . Mono-dispersed polystyrene standards were used to obtain calibration curve.

Synthesis of TPE diol

4-(6-Hydroxyhexyloxy)benzophenone was first prepared according to previous literature.^[1] Into a 250 mL two-necked round bottom flask equipped with a condenser were added zinc powder (2.19 g, 33.5 mmol) and 4-(6-hydroxyhexyloxy)benzophenone (5.0 g, 16.8 mmol) in 60 mL freshly distilled THF under nitrogen and dry-ice bath. Titanium(IV) chloride (3.82 g, 20.1 mmol) was added in dropwise and the mixture was warmed up to room temperature. The mixture was heated to reflux for 8 h. After cooling to room temperature, the mixture was

extracted with dichloromethane (DCM) for three times and washed with water twice. The organic phase was collected and dried over anhydrous sodium sulfate. After solvent evaporation, the crude product was purified by silica-gel column chromatography using hexane/ethyl acetate (v/v = 9:1 to 3:7) as eluent to furnish a yellow oil as product. Yield: 80%. ^1H NMR (400 MHz, CDCl_3) δ (ppm):^[2] 7.13–7.06 (m, 6H, ArH), 7.05–7.02 (m, 4H, ArH), 6.94–6.88 (m, 4H, ArH), 6.65–6.59 (m, 4H, ArH), 4.66 (s, 2H, OH), 3.89–3.63 (t, 8H, $\text{CH}_2\text{-O}$), 1.71–1.41 (t, 16H, CH_2). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 156.3, 143.4, 135.0, 131.8, 130.8, 128.7, 127.8, 127.5, 127.4, 127.0, 126.9, 125.5, 125.0, 113.5, 113.4, 113.0, 112.0, 67.0, 62.3, 55.0, 32.0, 28.6, 25.3, 25.2, 24.9. HRMS (MALDI-TOF): m/z 564.3228 (M^+ , calcd. 564.3240). NMR spectra were displayed in supplementary information (Figure S1). $M_n \sim 1222 \text{ g mol}^{-1}$, $M_w \sim 1329 \text{ g mol}^{-1}$ and PDI ~ 1.09 obtained by GPC.

Synthesis of EPT

EPT was prepared from PEG, PPG and TPE diol, using HDI as coupling agent. The weight ratio of PEG to PPG was kept at 2:1, and 1 wt. % of TPE diol was used. The amount of HDI added in the reaction was set at $[\text{OH}]:[\text{NCO}]$ 1:1. Typically, 1.33 g of PEG and 0.65 g of PPG were weighed into a 100 mL round bottom flask. TPE diol of 0.02 g was added into the flask, followed by the addition of 10 mL of anhydrous toluene. The reaction mixture was sonicated for 15 min. Trace amount of water in the reaction mixture was removed through an azeotropic distillation by rotary evaporation until viscous solution was being left in the flask. This process was repeated twice. 20 mL of anhydrous toluene was added into the flask, before it was heated to 110 °C. 0.16 mL (9.9×10^{-4} mol) of HDI and two drops of dibutyltin dilaurate ($\sim 8 \times 10^{-3}$ g) were added sequentially. The reaction mixture was stirred at 110 °C under nitrogen atmosphere for 24 h. The resultant copolymer was precipitated from diethyl ether. Yield: 1.6 g, 80 %. ^1H NMR (400 MHz, CDCl_3) δ (ppm): 7.12–6.62 (m, ArH), 4.20 (s, NH), 3.64 (t, CH_2O), 3.55 (t, CH_2O), 3.41 (m, CH), 3.14 (t, CH_2NHCOO), 1.49–1.33 (t, overlap of CH_2 from TPE and HDI), 1.14 (d, CH_3). ^1H NMR spectra with sample in CDCl_3 and D_2O were displayed in supplementary information (Figure S1). Weight ratio (%) based on ^1H NMR peak integration of characteristic peaks (δ (ppm): 3.64, 1.14, and 6.62) EG:PG:TPE 60.5:38.0:1.5; molar ratio EG:PG:TPE 1120:534:1. $M_n \sim 10100 \text{ g mol}^{-1}$, $M_w \sim 15400 \text{ g mol}^{-1}$ and PDI ~ 1.53 obtained by GPC.

Poly(PEG/PPG urethane) (EP) was prepared using the similar protocol as a reference material for comparison. Yield: 1.7 g, 85 %. ^1H NMR (400 MHz, CDCl_3) δ (ppm): 4.21 (s, NH), 3.64 (t, CH_2O), 3.54 (t, CH_2O), 3.40 (m, CH), 3.14 (t, CH_2NHCOO), 1.48 (t, $\text{CH}_2\text{CH}_2\text{NHCOO}$), 1.32 (t, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCOO}$), 1.13 (m, CH_3). Weight ratio (%) based on ^1H NMR peak integration of characteristic peaks (δ (ppm): 3.64 and 1.14) EG:PG 70:30; molar ratio EG:PG 3:1. $M_n \sim 13800 \text{ g mol}^{-1}$, $M_w \sim 18000 \text{ g mol}^{-1}$ and PDI ~ 1.31 obtained by GPC.

Determination of the Critical Micelle Formation Concentration (CMC)

With incorporation of TPE segment in the thermogelling copolymer, the self-assembly of micelles can be observed in solution, and the CMC can be determined by examining the fluorescence of EPT solution. Stock solution was prepared by dissolving small amount of EPT thermogelling copolymer in DI water, equilibrate overnight in fridge. Concentrations ranging from 0.011 to 2.75 g/L were studied, at different temperatures of 15, 25, 35 and 45 °C. The fluorescence intensity at wavelength of 350 – 600 nm were measured (excited at 340 nm), using a fluorescent spectrophotometer (Shimadzu RF5301) with temperature controlled water/IPA bath attached. Slit width of 3 was used for excitation and emission. The CMC was observed as the intersection of the tangents to the two slopes of the graph where the concentration was plotted against fluorescence intensity.

Rheological measurement

Thermosensitive properties were studied using a TA instruments Discovery Hybrid Rheometer Series (DHR-3, Research Instruments, Inc., U.S.A.) fitted with 20 mm parallel plate geometry, and a temperature controlled peltier base plate system. Oscillation amplitude

sweep and oscillation frequency sweep were run at both 25 and 37 °C. Oscillation amplitude sweep was carried out at 0.01-100 % strain, with frequency fixed at 1 Hz. Oscillation frequency sweep was studied at 1-100 Hz, with strain fixed at 0.1 % (which is within linear viscoelastic region). Temperature sweep was run from 10-70 °C, with strain fixed at 0.1 %, frequency fixed at 1 Hz, and heating rate was set to 5 °C.min⁻¹.

Multimodal biocompatibility testing of AIE thermogel

The biocompatibility of EPT thermogel preparation was determined using a multimodal platform comprising of four different human cell types - primary dermal fibroblasts, A549, HepG2 and HEK293 (American Type Culture Collection, ATCC). All cell lines were cultured in Gibco[®] DMEM medium supplemented with 10% (v/v) fetal bovine serum and antibiotics Pen-Strep (Life Technologies, Singapore) in a humidified incubator at 37 °C in 5% CO₂. For experimentation, cells were seeded into Nunc[®] 96-well tissue-culture plates at a density of 2×10^3 cells/well. After overnight incubation in order to allow cells to attach on the surfaces, they were treated with various concentrations of thermogel preparation (ranging from 5 to 100 µg of AIE thermogel/ml cell culture medium, w/v) for multiple time-points as indicated in the figure legends and High Content Analysis (HCA) was performed as per our previously described protocols 32-34.^[3] Cells were washed with phosphate buffered saline (PBS) at the end of the treatment periods, fixed in 3% paraformaldehyde and permeabilized. Cells were stained with fluorescently labelled cytoplasmic dyes [phalloidin-rhodamine (Molecular Probes[®]) and anti- α -tubulin-FITC (Sigma-Aldrich)] to visualize the cellular morphologies and cytoskeletal structures and Hoechst (Sigma-Aldrich) to visualize the nuclei. The 96-well plates were scanned (9 randomly selected fields/well) by an automated microscope IN Cell Analyzer 2200 (GE Healthcare) using $\times 10$ or $\times 20$ objectives. Quantitative estimations of the acquired images were performed with IN Cell Investigator software (GE Healthcare, version 3.7.3), and the normalized data were converted into heat-maps using Spotfire[®] (Spotfire Decision Site[®] 9.1.1, TIBCO Software Inc., Somerville, MA). For real-time monitoring of cell health based on electrical impedance measurements (which depends on cell number, degree of adhesion, spreading and proliferation of the cells), we used xCELLigence system as per manufacturer's instructions (ACEA Biosciences) and described.^[3b] Briefly, cells were seeded at a density of 2×10^3 cells/well into 200 µl of media in the E-Plate16[®] and allowed to attach onto the electrode surface over time for 20 h. The electrical impedance was recorded every 15 min. At the 20 h time point, cells were treated with 100 µg/mL thermogel preparation (final concentration diluted in cell culture media, w/v) in triplicates and monitored for additional 48 h. The cell impedance, expressed in arbitrary units called the 'Cell Index', were automatically recorded on the xCELLigence system and converted into growth curves. To measure cell viability, we performed a MTS-based assay using CellTier 96[®] Aqueous One solution cell proliferation assay kit according to manufacturer's instructions (Promega Corporation, Madison, WI, USA) and as described previously.^[3b] Briefly, at the end of the treatment period, cells were incubated with 20 µl of MTS tetrazolium solution for 2 h at 37 °C. Subsequently, the absorbance was measured at 490 nm using a microplate reader (BioTek) and then relative cell viability was calculated. Each treatment was performed in triplicate.

Determination of AIE thermogel cellular uptake using confocal microscopy

The cellular uptake of thermogel was determined in A549 cells by fluorescent confocal microscopy. Cells cultured on cover slips were treated with 20% thermogel preparation (w/v), which was further diluted with medium to gel volume ratio of 2:1 (v/v) for 3 or 6 h and then fixed in 3 % paraformaldehyde. To determine the mechanism of cellular uptake of thermogel, treated A549 cells were incubated at 37 °C and 4 °C for 3 h, to monitor active and passive diffusion, respectively. In order to observe the impact of passive transport on uptake, cells were pre-treated with sodium azide for 1 h (0.1 %, 0.015 M). Cells were then washed in PBS and fluorescently labeled with rhodamine-phalloidin (Molecular Probes[®]) to

visualize the cytoplasmic area and DRAQ5 (eBiosciences) to visualize the nuclei. Coverslips were mounted on glass slides using FluoromountTM Aqueous mounting medium (Sigma-Aldrich). High resolution intracellular accumulation of AIE thermogel was visualized by confocal laser scanning microscope (Zeiss LSM800 Airyscan, Carl Zeiss Microimaging Inc., NY, USA) using a Plan-Apochromat 63×/1.4 Oil DIC M27 objective lens. Three channel qualitative imaging was carried out by acquiring a series of Z-stack images using excitation wavelengths 405 nm, 561 nm and 640 nm. Cellular uptake of the gel was further quantified using an IN Cell Investigator software (GE Healthcare, version 3.7.3). At least 20 different microscopic fields were analyzed for each sample.

Statistical analysis

The data was expressed as mean ± standard error of mean (SEM). For comparison of two groups, p-values were calculated by two-tailed unpaired student's t-test. In all cases p-values ≥ 0.05 was considered to be statistically significant.

Preparation of Dox loaded thermogel

The EPT was added in normal saline (n.s.) at a concentration of 20 wt. % at 4 °C for 24 h to form thermogels. Dox in DI water was mixed directly into the thermogelling solution. 50 µg/mL of drug was added into the 1 mL of thermogelling solution (20 wt. %) to form an injectable solution at 4 °C. The prepared drug delivery system was kept at 4 °C before use.

Development of HepG2 hepatocellular carcinoma tumour model in nude mice and in-vivo imaging

All animal experiments were carried out in accordance with guidelines approved by the Animal Care and Ethics Committee of Xiamen University. In order to develop the solid tumour model, HepG2 hepatocellular carcinoma cells (1×10^6 tumour cells suspended in 200 µL PBS) were inoculated into subcutaneous of BALB/c nude mice with body weight of ~20 g. When the tumour volume reached ~150 mm³ (~2 weeks), 50 µL of drug loaded thermogel (Dox in EPT thermogel) was injected at 4 °C into the tumour site of nude mice, with control of normal saline and Dox only. In Vivo Imaging System IVIS ® Lumina II (Caliper Life Sciences, Hopkinton, MA) was applied to collect the fluorescent images, which were performed using a 695-770 nm emission filter for Dox and 510 nm emission filter for EPT. Spectral unmixing (Living Image Software, Caliper Life Sciences, Hopkinton, MA) was utilized to remove the auto-fluorescence.

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