

# pH Induced Self-assembly of AIE Fluorescent Probe for Tumor Imaging

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## Electronic Supplementary Information

**Abstract** This work reported an AIE fluorescent probe for tumor imaging based on the pH induced self-assembly strategy. The fluorescent probe was composed of an acid-responsive soluble copolymer PEG-*b*-PAMA-DMMA with a maleic acid amide group and an anionic soluble aggregation-induced emission fluorogen (AIEgen) TPE-2SO<sub>3</sub><sup>−</sup>. The polymer could be transformed into a protonated amine-containing polymer after the hydrolysis of maleic acid amide in acidic tumor microenvironment, which would be further self-assembled with TPE-2SO<sub>3</sub><sup>−</sup> to form aggregated nanoparticles. The transition of TPE-2SO<sub>3</sub><sup>−</sup> from dispersed state to aggregated state led to an obvious increase in fluorescence intensity due to its AIE characteristics.

**Keywords** pH-responsive; Self-assembly; Fluorescent probe; AIE; Tumor imaging

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## INTRODUCTION

Due to the advantages of quick response, high resolution, low detection limit, and minor damage to organisms, a variety of stimuli-responsive fluorescent probes for tumor imaging have been explored in recent years, which would be very helpful for tumor diagnosis and surgical navigation.<sup>[1–8]</sup> Anyway, the fluorophores often have large conjugate chemical structure, and they usually have a strong tendency to aggregate under the water condition. The aggregation of fluorophores can quench the fluorescence very much. The aggregation-caused quenching (ACQ) effect will hinder the further application of such fluorescent probes for bio-imaging. To overcome this disadvantage, different kinds of fluorescent molecules with aggregation-induced emission (AIE) characteristics have been developed.<sup>[9–19]</sup> Due to the high brightness and biocompatibility, the AIE materials have gained great success in bio-imaging application in recent years.<sup>[20,21]</sup> Up to now, a variety of AIE fluorescent probes have been designed and reported for tumor imaging according to the characteristics of tumor microenvironment.<sup>[22–28]</sup> The main principle of designing such stimuli-responsive AIE fluorescent probes was to link the proper stimuli-responsive moiety with AIE fluorophores together, while the stimuli-responsive moiety also acted as fluorescent quencher. For example, we reported a “turn-on” fluorescence probe of azobenzene-caged tetraphenylethene (TPE) for tumor hypoxia imaging.<sup>[23]</sup> The

azobenzene moiety can quench the fluorescence of the AIE probe efficiently by fluorescence resonance energy transfer (FRET) process. When incubated with tumor cells, the azo double bonds were reduced by the overexpressed azoreductase under hypoxia conditions. Then, the fluorescence of the core AIE fluorogen would be restored. The absorbance spectrum of the azobenzene moiety and the emission spectrum of the AIE moiety should be overlapped. Since the hypoxia-responsive moiety also acted as the fluorescent quencher, it should be very careful to design the chemical structure of the fluorescent probe.

As we know, the unique property of AIE fluorophore is that AIE molecules can show strong fluorescence in the aggregation state, but no fluorescence in the molecular dispersed state. Thus, the variation of solubility of AIE fluorophore in aqueous solution can be used to prepare the stimuli-responsive fluorescent probe for tumor imaging. For example, Tang and coworkers reported a stimuli-responsive AIE probe for tumor imaging with N<sup>+</sup>-O<sup>−</sup> group based on the variation of solubility in water.<sup>[29]</sup> The N-oxide groups could disperse well in aqueous media, leading to the negligible fluorescence of the AIE probe. Under tumor hypoxic condition, the deoxygenation of N-oxide could take place, which would change the groups from hydrophilic groups to hydrophobic groups. Then, strong fluorescence could be observed. In this case, the hypoxia-responsive group N-oxide did not need to act as the quencher group. The FRET process would not be needed to care about anymore. For polymer, it is easy to control the aggregation state by self-assembly.<sup>[30]</sup> Recently, we developed an *in situ* self-assembly method to construct hypoxia-triggered AIE fluorescent probe using water-soluble polymer

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and AIE molecule.<sup>[31]</sup> The polymer was a water-soluble azobenzene containing copolymer. The AIE molecule was TPE with four anionic groups. As the TPE molecule was in the molecular dispersed state in water, it could not show obvious fluorescence due to the AIE characteristics. After reduction reaction of the polymer under hypoxic condition, cationic groups would be formed in the polymer. Then the anionic TPE and cationic polymer would be self-assembled into nanoparticles, leading to the apparent increase of fluorescence.

Besides hypoxia, extracellular acidity is also one of the main characteristics of the tumor microenvironment, which can be due to the enhanced glycolytic rates (known as Warburg effects) and the changes in the expression of plasma membrane ion pumps and transporters that facilitate proton efflux.<sup>[32–34]</sup> Liu and co-workers successfully designed and synthesized a pH-responsive light-up nanoparticle probe with AIE features for targeted imaging and selective suppression of cancer cells.<sup>[35]</sup> They also reported a pH-responsive fluorescent light-up probe for tumor acidic extracellular microenvironment imaging based on a hybrid of gold nanoparticle and conjugated poly electrolytes with high solubility or dispersibility in aqueous media.<sup>[36]</sup> However, AIE probe through controlling the molecular dispersed state based on the pH induced self-assembly strategy was rarely reported. In this work, we further used the *in situ* self-assembly strategy to construct the pH-responsive AIE fluorescent probe for tumor imaging. The fluorescent probe was composed of an acid-responsive soluble copolymer PEG-*b*-PAMA-DMMA with a maleic acid amide group and an anionic soluble AIE fluorogen TPE-2SO<sub>3</sub><sup>−</sup>. The polymer could be transformed into a protonated amine-containing polymer after the hydrolysis of maleic acid amide in acidic tumor microenvironment, which would be further self-assembled with TPE-2SO<sub>3</sub><sup>−</sup> to form aggregated nanoparticles. The transition of TPE-2SO<sub>3</sub><sup>−</sup> from dispersed state to aggregated state led to an obvious increase in fluorescence intensity due to its AIE characteristics.

## EXPERIMENTAL

### Materials and Methods

Ethanolamine hydrochloride was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. 2,2-Azobisisobutyronitrile (AIBN), 2,3-dimethylmaleic anhydride (DMMA) and succinic anhydride (SA) were purchased from J&K Chemical. Methacryloyl chloride was purchased from HEOWNS Technologies, LLC, Tianjin. Poly(ethylene glycol) 4-cyano-4-pentanoate (PEG-CTA,  $M_n = 2000$ ) was purchased from Sigma-Aldrich. The phosphate buffers of different pH were obtained from Beijing Solarbio Co., Ltd. HeLa cells line were supported by the Chinese National Infrastructure of Cell Line Resource and cultured in DMEM (Dulbecco's Modified Eagle's medium, Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin at 37°C and 5% CO<sub>2</sub>. AIBN was recrystallized in anhydrous ethanol before use. Other reagents and solvents were purchased commercially if not mentioned specifically and used without further purification. Ultrapure water (resistivity > 18 MΩ·cm) was obtained using Milli-Q water purification system.

<sup>1</sup>H-NMR spectra were measured by a JEOL JNM-ECA 600 NMR spectrometer. The molecular weights and polydis-

persity index (PDI) were measured by a SHIMADZU LC-20A gel permeation chromatography system using pure tetrahydrofuran (THF) as the eluent. The fluorescence spectra were measured on a HITACHI F-7000 fluorescence spectrophotometer. TEM images were captured with a HITACHI H-7650B TEM at 80 kV, and the nanoparticle size measurements were performed on ALV/DLS/SLS-5022F Laser Light Scattering System with a solid-state 17 mW He-Ne laser ( $\lambda = 633$  nm). Cellular imaging was carried out by Nikon ECLIPSE Ti2 confocal laser scanning microscope.

### Synthesis of 2-Aminoethyl Methacrylate Hydrochloride (AMA)

AMA was commercially available but its technical grade purity was 90% as well as containing polymerization inhibitor phenothiazine, making it unsuitable for current study. AMA used in the experiment was prepared as follows.<sup>[37]</sup> Ethanolamine hydrochloride (6.50 g, 0.067 mol), methacryloyl chloride (10.00 mL, 0.096 mol), and hydroquinone (0.05 g) were mixed in a three-necked round-bottomed flask with a condenser. The mixture was then heated to 93–95 °C under a nitrogen atmosphere. The heterogeneous mixture of the molten salt and methacryloyl chloride was stirred vigorously for 1 h under this temperature, and the evolved hydrogen chloride was absorbed by a dilute sodium hydroxide solution. Next, the obtained solution was stirred for a further 2 h at 70–75 °C. The mixture was then cooled to 40 °C, and THF (30 mL) was added. This solution was then slowly dropped to cold *n*-pentane (80 mL), and a white precipitate was collected by filtration. After drying under vacuum, the crude product was recrystallized using a mixture of ethyl acetate/2-propanol (7/3, V/V) to give the pure product in its hydrochloride salt form. Yield: 72%. <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O,  $\delta$ , ppm): 1.93 (s, 3H), 3.38 (t, 2H), 4.43 (t, 2H), 5.77 (s, 1H), 6.19 (s, 1H).

### Synthesis of PEG-*b*-PAMA

PEG-*b*-PAMA was synthesized by RAFT polymerization. PEG-CTA (100 mg, 0.05 mmol), AMA (165.53 mg, 1 mmol), AIBN 0.82 mg, 0.01 mmol) and DMF (1.8 mL) were added to a Schlenk flask. The solution was degassed by freeze-pump-thaw cycle for three times, and then put into an oil bath at 70 °C. The reaction was carried out for 24 h, and quenched by liquid nitrogen. The solution was slowly dropped into cold ether, and the product was obtained after centrifugation. <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O,  $\delta$ , ppm): 0.94–1.14 (−CH<sub>2</sub>C(CH<sub>3</sub>)−), 2.04 (−CH<sub>2</sub>C(CH<sub>3</sub>)−), 3.37 (−CO(O)CH<sub>2</sub>CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>Cl<sup>−</sup>), 3.69 (−CH<sub>2</sub>CH<sub>2</sub>O−), 4.29 (−CO(O)CH<sub>2</sub>CH<sub>2</sub>NH<sub>3</sub><sup>+</sup>Cl<sup>−</sup>).  $M_n$  (GPC)=6.84×10<sup>3</sup> and PDI=1.07.

### Synthesis of PEG-*b*-PAMA-DMMA and PEG-*b*-PAMA-SA

PEG-*b*-PAMA (58.7 mg, 0.3 mmol −NH<sub>3</sub><sup>+</sup>) was dissolved in NaHCO<sub>3</sub>-Na<sub>2</sub>CO<sub>3</sub> buffer at pH=9.0 and stirred for 1 h. 2,3-Dimethylmaleic anhydride (DMMA, 189 mg, 1.5 mmol) was added into the solution while the solution pH was kept at 8.5–9.0 by adding sodium hydroxide solution. After the pH value remained constant, the reaction was carried out overnight. The solution was ultrafiltered using Amicon YM-30 centrifugal filter devices (Millipore, MWCO=3000 Da) and the product was obtained after freeze-dried. <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O,  $\delta$ , ppm): 0.94–1.14 (−CH<sub>2</sub>C(CH<sub>3</sub>)−), 1.84 (−NHC(CH<sub>3</sub>)=C(CH<sub>3</sub>)COOH), 1.99 (−CH<sub>2</sub>C(CH<sub>3</sub>)−), 3.47 (−CO(O)CH<sub>2</sub>CH<sub>2</sub>NH−),

3.67 (—CH<sub>2</sub>CH<sub>2</sub>O—), 4.06 (—CO(O)CH<sub>2</sub>CH<sub>2</sub>NH—).

PEG-*b*-PAMA-SA was synthesized through a similar method and only DMMA was replaced by succinic anhydride (SA). <sup>1</sup>H-NMR (600 MHz, D<sub>2</sub>O, δ, ppm): 0.94–1.14 (—CH<sub>2</sub>C(CH<sub>3</sub>)—), 1.89 (—CH<sub>2</sub>C(CH<sub>3</sub>)—), 2.44 (—NHCH<sub>2</sub>CH<sub>2</sub>COOH), 3.45 (—CO(O)CH<sub>2</sub>CH<sub>2</sub>NH—), 3.67 (—CH<sub>2</sub>CH<sub>2</sub>O—), 4.04 (—CO(O)CH<sub>2</sub>CH<sub>2</sub>NH—).

### Synthesis of TPE-2SO<sub>3</sub><sup>−</sup>

2-((4-(2,2-Bis(4-hydroxyphenyl)-1-phenylvinyl)phenyl)-(phenyl)methylene)malononitrile (TPE-2OH) was synthesized following previous reported procedure.<sup>[38]</sup> Sodium ethoxide (0.4 g, 5.88 mmol) was dissolved in 2 mL of ethanol and added slowly into the ethanol solution of TPE-2OH (0.5 g, 1.0 mmol). The mixture was stirred for 1 h, and 1,3-propanesultone (0.33 g, 2.7 mmol) was added and the reaction was carried out for 8 h at room temperature. After filtration and drying, the final product was obtained as red solid with a yield of 35%. <sup>1</sup>H-NMR (600 MHz, DMSO-*d*<sub>6</sub>, δ, ppm): 1.96 (m, 4H), 2.52 (m, 4H), 3.97 (m, 4H), 6.66–7.64 (m, 22H). HRMS(ESI) *m/z*: [C<sub>42</sub>H<sub>34</sub>N<sub>2</sub>O<sub>8</sub>S<sub>2</sub>]<sup>2−</sup>: Calcd.: 379.0884, Found: 379.0877.

### Hydrolysis Process of PEG-*b*-PAMA-DMMA Studied by <sup>1</sup>H-NMR Spectroscopy

The commercially available DCI (35 wt%) was diluted with D<sub>2</sub>O to a pH of 6.8. PEG-*b*-PAMA-DMMA was added to the solution and the <sup>1</sup>H-NMR spectrum was monitored at regular intervals.

### Determination of the Optimal Ratio

Four groups of different ratios were studied where the molar ratio of positive and negative charges was 0.5:1, 1:1, 2:1, 3:1, respectively. That is, to the solution of TPE-2SO<sub>3</sub><sup>−</sup> of 0.01 mg/mL, PEG-*b*-PAMA-DMMA was added to the concentration of 0.004, 0.009, 0.018, 0.026 mg/mL, respectively, and fluorescence emission spectra were measured every 5 min. The measurement was carried out in a buffer with a pH of 6.8 and the excitation wavelength was 420 nm.

### pH Sensitivity of the Fluorescent Probe

PEG-*b*-PAMA-DMMA (0.009 mg/mL) and TPE-2SO<sub>3</sub><sup>−</sup> (0.01 mg/mL) were added into PBS buffers with pH of 6.0, 6.4, 6.8, 7.4, respectively, and fluorescence emission spectra of the solution were measured every 5 min with an excitation wavelength of 420 nm.

### Formation and Imaging of 3D Multicellular Spheroids (MCTS)

HeLa cells lines were chosen as cell models to form MCTS. Agarose (1.5 g) was evenly dispersed in 100 mL of DMEM. Then, appropriate amounts of the resulting solution were added into 96-well microplates. Cell suspension (75 μL, 1.0×10<sup>4</sup> cells/mL) was plated onto the agarose-coated 96-well microplates. The plates were centrifuged at 1500 rcf for 15 min, and the cells were allowed to solidify without agitation for 24 h to form single spheroids.

The fluorescent probe solution was added to the 3D multicellular spheroids to achieve a concentration of 0.01 mg/mL (PEG-*b*-PAMA-DMMA of 0.009 mg/mL and TPE-2SO<sub>3</sub><sup>−</sup> of 0.01 mg/mL). The fluorescence images were captured by confocal laser scanning microscopy (CLSM).

### Cytotoxicity Assessment

Cytotoxicity of the fluorescent probe was assessed using

thiazolyl blue tetrazolium bromide (MTT) assay. HeLa cell suspension was seeded in 96-well microplates at 1.0×10<sup>4</sup> cells/well and pre-incubated for 24 h. The fluorescent probe solution was added at the concentration ranging from 0.001 mg/mL to 0.04 mg/mL, and HeLa cells was incubated for another 24 h. The medium was replaced with MTT (20 μL, 5 mg/mL), and after 3 h the supernatant was replaced with DMSO (150 μL). The absorbance was measured at 570 nm using an Infinite M200 microplate reader. All experiments were repeated for four times.

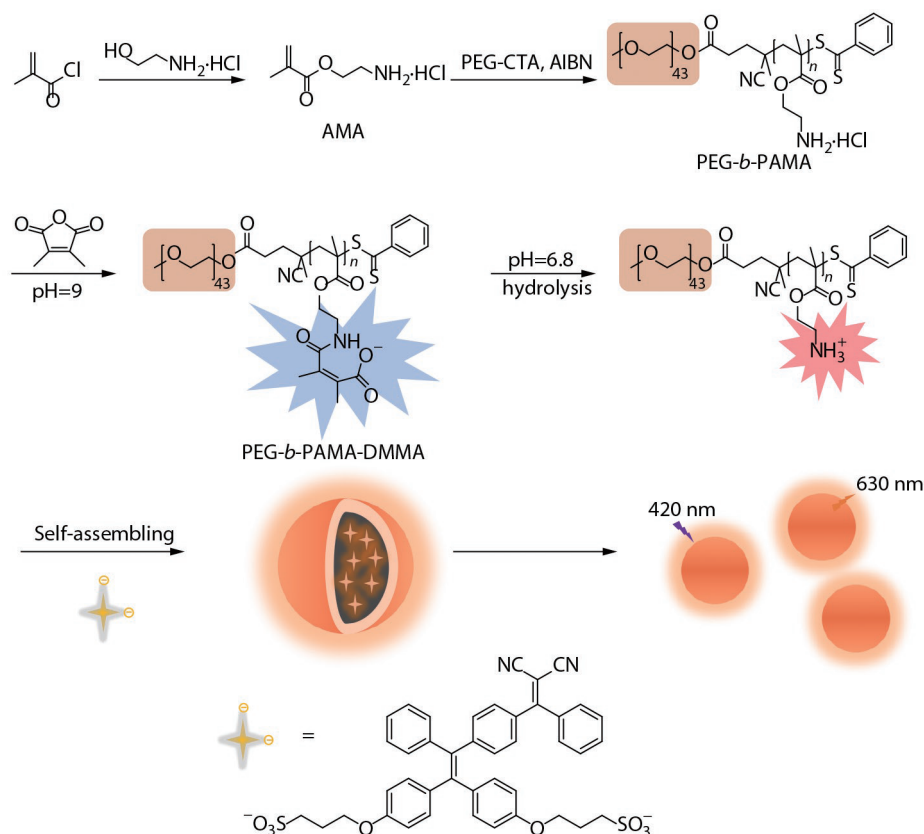
## RESULTS AND DISCUSSION

As shown in Scheme 1, the soluble block copolymer PEG-*b*-PAMA was synthesized via RAFT polymerization, then the pH-sensitive maleic acid amide was prepared by the reaction between PEG-*b*-PAMA and DMMA. The chemical structure was confirmed by <sup>1</sup>H-NMR (Figs. S1–S4 in the electronic supplementary information, ESI). As shown in Fig. 1(a), the characteristic signals of PEG (δ=3.69), PAMA (δ=4.29 and 3.37) could be recognized clearly, and the degree of polymerization was calculated to be 30 by comparing the integration ratio of these two peaks. The GPC trace of PEG-*b*-PAMA in Fig. S5 (in ESI) exhibited a unimodal peak with a narrow polydispersity index of 1.07, which confirmed the well-controlled nature of RAFT polymerization. The characteristic peak of PAMA had a significant shift from δ=4.29 and 3.37 to δ=4.06 and 3.47 respectively, after PEG-*b*-PAMA was treated with DMMA, as shown in Fig. 1(b). Also, a doublet peak corresponding to hydrogen in the methyl of DMMA was observed, confirming the reaction was successfully performed.

TPE-2SO<sub>3</sub><sup>−</sup> was synthesized following previous report and the synthesis route is shown in Fig. S6 (in ESI).<sup>[39]</sup> Its chemical structure was confirmed by <sup>1</sup>H-NMR, <sup>13</sup>C-NMR and ESI-MS spectra, as shown in Fig. S7, Fig. S8 and Fig. S9 (in ESI), respectively. Furthermore, with the addition of a large number of poor solvent of THF into the aqueous solution of TPE-2SO<sub>3</sub><sup>−</sup>, its emission increased significantly, indicating its AIE feature (Figs. S10a and S10b, in ESI). The AIE characteristic was also studied by adding positively charged polymer PEG-*b*-PAMA to the solution of TPE-2SO<sub>3</sub><sup>−</sup>, and FL spectra and the images (Fig. S11 in ESI) indicated a significant increase in fluorescence as the AIEgen changed from dispersed state to aggregated state via electrostatic interaction, confirming the AIE characteristic of TPE-2SO<sub>3</sub><sup>−</sup>.

Previous studies showed that the hydrolysis process of maleic acid amide went through an anhydride intermediate, because amide bond hydrolyzed faster than anhydride, as illustrated in Fig. 1(e).<sup>[40,41]</sup> Comparing Fig. 1(b) with Fig. 1(c), the characteristic signal of PAMA shifts back after hydrolysis for 30 min, which indicated the hydrolysis of the amide bond. The doublet peak disappears and is replaced by a new singlet peak at δ=1.97, confirming the existence of the anhydride intermediate. After 24 h, the chemical shift of the characteristic peak moves from 1.97 to 1.77, as shown in Fig. 1(d), indicating that the anhydride was hydrolyzed. The <sup>1</sup>H-NMR spectra of neat 2,3-dimethylmaleic anhydride before and after hydrolysis further confirmed this process (Figs. S12a and S12b in ESI).

The fluorescent probe was composed of acidic tumor mi-



**Scheme 1** Synthesis route to PEG-*b*-PAMA-DMMA and schematic illustration of the *in situ* self-assembly process of the fluorescent probe.

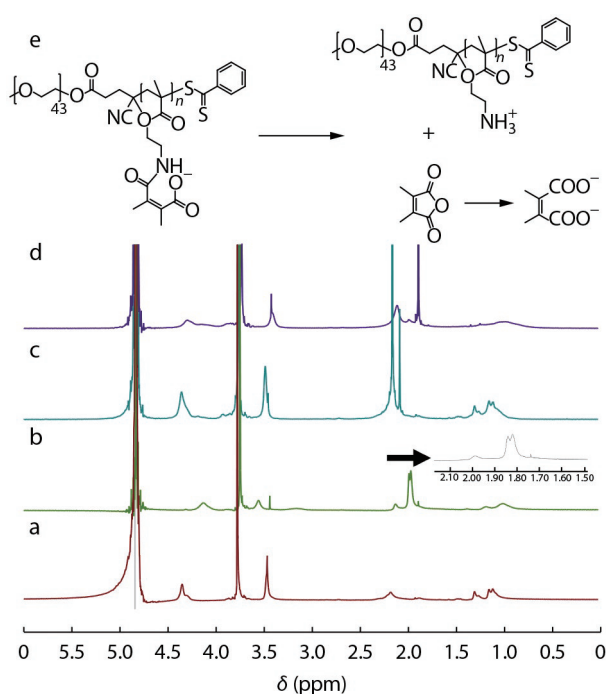


**Fig. 1** <sup>1</sup>H-NMR spectra (600 MHz, D<sub>2</sub>O) of (a) PEG-*b*-PAMA, (b) PEG-*b*-PAMA-DMMA and PEG-*b*-PAMA-DMMA after hydrolysis for (c) 30 min and (d) 24 h; (e) Hydrolysis process of PEG-*b*-PAMA-DMMA.

environment responsive polymer PEG-*b*-PAMA-DMMA and AIEgen TPE-2SO<sub>3</sub><sup>-</sup>, thus the optimal ratio of these two components was important for getting the best fluorescence imaging effect. According to previous study,<sup>[42]</sup> when the amount of positive and negative charges was equal, the most stable state of the polyion complex nanoparticles was obtained, which was also proved as shown in Fig. S13 (in ESI) that the group at a ratio of 1:1 between positive charges and negative charges shows the strongest fluorescence emission, and the optimal ratio was used in subsequent experiments.

Then, the pH sensitivity of the fluorescent probe was studied as shown in Fig. 2(a). The fluorescence intensity almost did not change at a pH of 7.4, but increased significantly under acidic conditions, and the “off-on” fluorescence ratio was about 4 at a pH of 6.8 after 30 min, indicating the good responsiveness of the fluorescent probe to the weakly acidic condition. The emission peak of the fluorescence spectra (Fig. 2b) was observed at 630 nm which was corresponding to the emission peak of TPE-2SO<sub>3</sub><sup>-</sup>.

The pH sensitivity of maleic acid amide was further studied by replacing PEG-*b*-PAMA-DMMA with PEG-*b*-PAMA-SA (Fig. 3a), in which the amide bond would not undergo hydrolysis in weakly acidic environment with a pH above 6.0.<sup>[43]</sup> No fluorescence emission can be observed after adding PEG-*b*-PAMA-SA, which indicated that there was no interaction between PEG-*b*-PAMA segment and TPE-2SO<sub>3</sub><sup>-</sup>, and it was the protonated amino group generated by the hydrolysis of maleic acid amide that assembled with TPE-2SO<sub>3</sub><sup>-</sup> to form nanoparticles aggregation, which led to the increase of fluor-

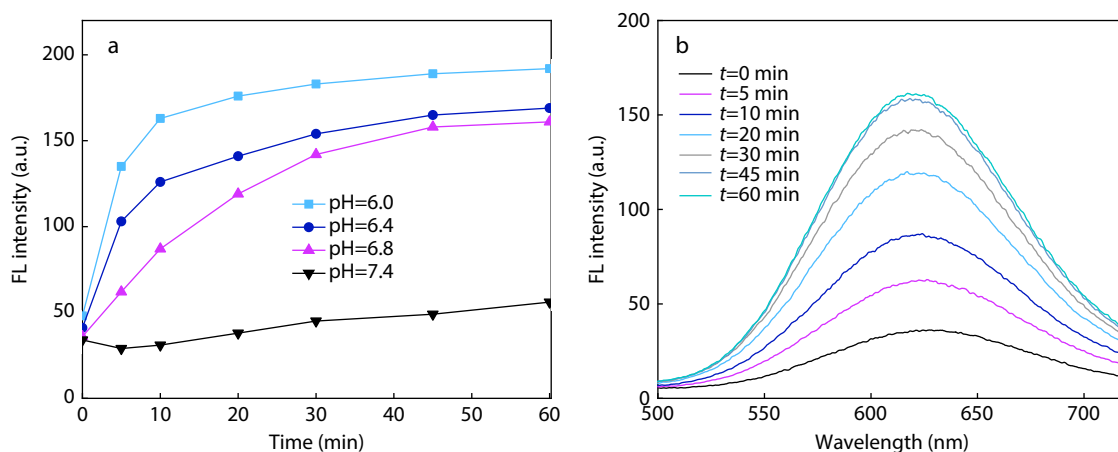


**Fig. 2** (a) FL spectra of the mixture of PEG-*b*-PAMA-DMMA (0.009 mg/mL) and TPE-2SO<sub>3</sub><sup>−</sup> (0.01 mg/mL) under different pH conditions over time; (b) FL spectra of mixture of PEG-*b*-PAMA-DMMA (0.009 mg/mL) and TPE-2SO<sub>3</sub><sup>−</sup> (0.01 mg/mL) under pH of 6.8 ( $\lambda_{\text{ex}}$ =420 nm).

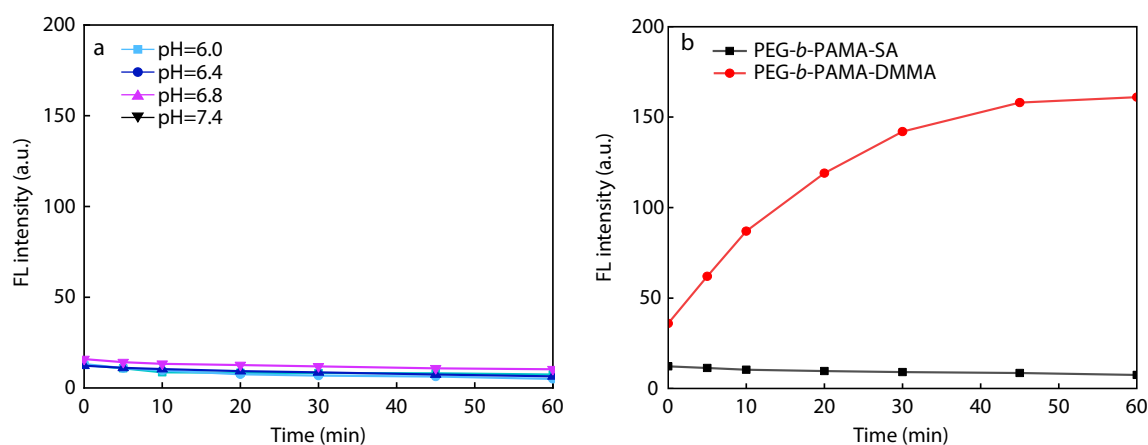


**Fig. 3** (a) FL spectra of the mixture of PEG-*b*-PAMA-SA (0.009 mg/mL) and TPE-2SO<sub>3</sub><sup>−</sup> (0.01 mg/mL) under different pH conditions over time; (b) FL spectra of PEG-*b*-PAMA-SA and PEG-*b*-PAMA-DMMA under pH of 6.8 ( $\lambda_{\text{ex}}$ =420 nm).

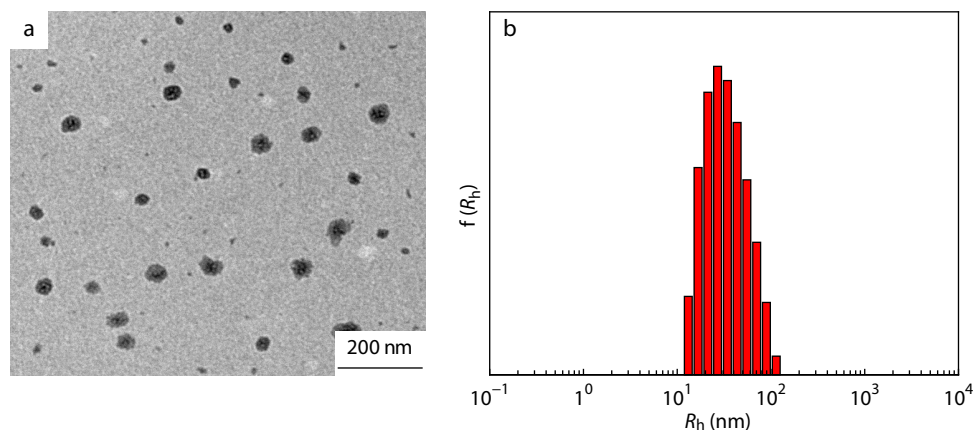


**Fig. 4** (a) Typical TEM image of the assembled nanoparticles of the fluorescent probes; (b) Distribution curve of the hydrodynamic radius ( $R_h$ ) of the self-assembly aggregates in the aqueous dispersion.

escence (Fig. 3b).

TEM and DLS were applied to confirm the *in situ* self-assembling process. Both blocks of PEG-*b*-PAMA-DMMA were water-soluble, thus the polymer could be well dispersed in water, and no aggregates can be observed through DLS or TEM. However, after putting into a weakly acidic environment

(pH=6.8) for 30 min, nanoaggregates could be clearly observed from TEM (Fig. 4a) with an average diameter of  $64 \pm 8$  nm (estimated statistically from TEM images), which was consistent with the result measured by DLS ( $R_h = 34 \pm 5$  nm) (Fig. 4b).

All of the above results indicated that PEG-*b*-PAMA-DMMA

can transform to a hydrophilic cationic polymer with protonated amino groups under weakly acidic condition *via* the hydrolysis of maleic acid amide. This structural transition led to the charge reversal from an anion state to a cationic state and could activate the fluorescence of molecularly-dispersed TPE-2SO<sub>3</sub><sup>−</sup> through an electrostatic interaction induced aggregation process to form polyion complexes nanoparticles *in situ*. Meanwhile, the results demonstrated that PEG-*b*-PAMA-DMMA and TPE-2SO<sub>3</sub><sup>−</sup> could be used as “turn-on” *in situ* fluorescent probes under acidic condition as well.

In order to verify whether the fluorescent probe can be used for tumor imaging, a 3D multicellular tumor spheroid (MCTS) model was used to mimic *in vivo* tumor growth. The MCTSs were incubated with PBS buffer and the fluorescent probe respectively for 48 h to confirm whether the fluorescent probe could be used for tumor imaging *in vitro*. The confocal laser scanning microscopy (CLSM) images of the MCTSs are shown in Fig. 5. It could be observed clearly that there was almost no fluorescence signal when only PBS buffer was added, but strong fluorescence could be seen after adding PEG-*b*-PAMA-DMMA and TPE-2SO<sub>3</sub><sup>−</sup>. Meanwhile, weak fluorescence scattered inside the MCTSs could be observed after incubated for 1 h, indicating the good permeability of the fluorescent probe in tumor. Cytotoxicity was assessed *via* MTT assay, and HeLa cells showed high viability (>80%) in solutions of PEG-*b*-PAMA-DMMA only, TPE-2SO<sub>3</sub><sup>−</sup> only, and the mixture of both with concentrations up to 0.4 mg/mL (Fig. S14 in ESI). The CLSM images and the cytotoxicity results also proved the tumor imaging capabilities of the fluorescent probes.

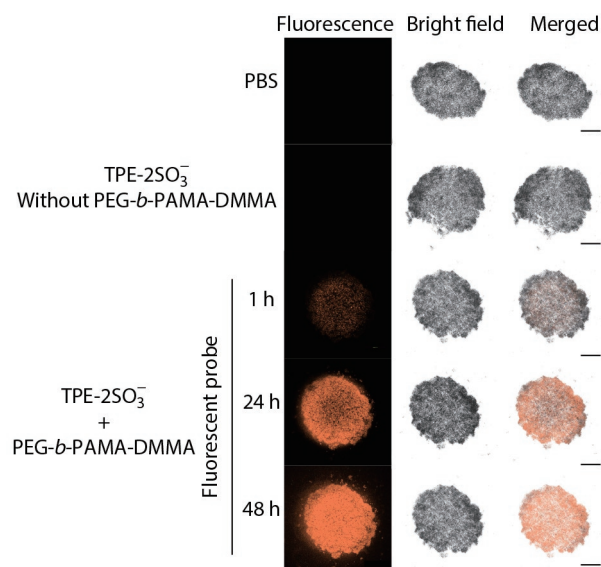


**Fig. 5** CLSM images of HeLa 3D multicellular tumor spheroids after incubating with PBS buffer or the fluorescent probe solution (PEG-*b*-PAMA-DMMA and TPE-2SO<sub>3</sub><sup>−</sup>). Scale bars=200 μm. λ<sub>ex</sub>=420 nm, λ<sub>em</sub>=600–640 nm.

## CONCLUSIONS

In summary, we reported an acidic tumor microenvironment responsive fluorescent probe for tumor imaging based on the *in situ* self-assembly strategy. The fluorescent probe was composed of a weakly acid-responsive water-soluble polymer

PEG-*b*-PAMA-DMMA and an anion AIEgen TPE-2SO<sub>3</sub><sup>−</sup>. The *in situ* self-assembling process could be triggered by the weak acidity of tumor microenvironment, and the maleic acid amide structure of the polymer underwent hydrolysis to transform to a cationic polymer containing protonated amino groups, which further assembled with TPE-2SO<sub>3</sub><sup>−</sup> *via* electrostatic interaction. Thus, the transition of AIEgen TPE-2SO<sub>3</sub><sup>−</sup> from molecular-dispersed state to aggregation state led to an obvious increase of fluorescence and it was also proved by *in vitro* experiment using HeLa MCTSs as models.

## Conflict of Interests

The authors declare no interest conflict.

## Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-023-3000-9>.

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