Nanoarchitectonics of the Aggregation-Induced emission Luminescent Molecule Tetraphenylethylene-COOH (TPE-COOH): Fluorescence Imaging of Targeted Cancer Microsphere Cells

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Abstract: Cancer microspheres are an ideal cellular model for studying cancer stem cells. However, the lack of effective and rapid fluorescent imaging reagents for cancer microspheres has severely hampered cytological studies associated with cancer microspheres. Here, we have identified an aggregation-induced emission (AIE) probe, **TPE-COOH**, to bind to a targeting cyclic heptapeptide molecule to form a new **TPE-Peptide** probe. This probe has been employed for fluorescence imaging of normal, cancer and cancer microsphere cells. A novel analogue of **TPAPy-1** was synthesized to verify the cell-specific targeting. For the first time, the fluorescence imaging of cancer microspheres was achieved and microspheres were imaged due to changes in viscosity, thereby providing a means to visualize and identify cancer microspheres.

1. Introduction

Cancer stem cells (CSCs) are stem cells with self-renewal ability, and can readily achieve tumour heterogeneity and metastasis, which is one of the reasons for the failure of radiotherapy and chemotherapy [1–3]. Therefore, the ability to precisely discriminate cancer stem cells and eliminate them is of high importance, and such targeting offers new possibilities for improving the treatment of cancer. The main prerequisites for studying CSCs is to isolate them *in vitro* and have the ability to culture them from the corresponding 'daughter' tumour cells. The main methods commonly used for CSC isolation are the sorting method and suspension culture method [4,5]. In 1992, Reynolds *et al* [6] introduced suspension culture to the study of stem cells for the first time, and the results of the study showed that only a very small number of cells in neuronal cells survived and aggregated to form spheres under serum-free and low-adsorption suspension culture conditions, verifying that the spheres had stem cell properties. The advantage of such methods is that they provide CSCs with a growth environment similar to the "niche" (niche) of the tumour stem cells, in which non-tumour stem cells cannot survive. Subsequently, sphere forming assays have been widely used in CSC enrichment cultures, and researchers have established corresponding CSC suspension culture systems for breast, gastric, colon, lung, glioma, liver cancers and many other tumour cells; the obtained microspheres (spheres) were verified to have tumour stem cell properties [7–11]. Therefore, the use of a suspension culture for cancer cells with a suspension medium to obtain microspheres is currently an effective and commonly used method to study cancer stem cells (CSCs) *in vitro*. Indeed, cancer microspheres are an

ideal cell model to study CSCs.

Cancer microspheres are made by culturing cancer cells in suspension in a specific medium for 5 to 8 days, gradually transforming from an adherent state to a suspended state and eventually forming three-dimensional (3D) spheres *in vitro*. However, the lack of effective and rapid methods for the identification and characterization of cancer microspheres has severely hampered cytological studies related to cancer microspheres. It would greatly facilitate cancer microsphere research if useful strategies could be provided to assess and identify cancer microsphere cells residing in tumor or cancer cell lines, and to screen for drugs specifically targeting CSCs. Our group has previously performed differential screening using phage display technology and has identified a cyclic heptapeptide molecule that binds to hepatocellular carcinoma microspheres in a highly specific manner. Therefore, it is hypothesized that the cyclic heptapeptide could be used as a bio-targeting molecule to specifically target hepatocellular carcinoma cells [12].

Bioimaging has become an integral part of modern biology, offering advantages such as high sensitivity, simple operation and biocompatibility for molecular-level detection, visualization and dynamic tracking, and *in vivo* imaging [13]. However, conventional fluorophores with π-planar structures usually suffer from aggregation quenching (ACQ), poor photostability and small Stokes shifts. Since the discovery of aggregation-induced luminescence by Tang, aggregation-induced emission (AIE) probes have been widely used in chemical sensors and bioimaging given their high emission efficiency, high signal-to-noise ratio, strong photostability, and large Stokes

shifts in the aggregated state [14–16]. To our knowledge, there are few reports involving specific fluorescence imaging using cancer microspheres.

Tetraphenylethylene (TPE) is the 'star molecule' of aggregation-induced luminescence. In solution, TPE exhibits weak or no fluorescence because the four benzene rings rotate around the carbon-carbon single bond, releasing energy, whereas in the aggregated state, rotation within the molecule is restricted, resulting in intense blue fluorescence. TPE has also used for the identification of Fe^{3+}, Al^{3+} and other ions [17-20], bioinformatics detection [21, 22], and the ablation of bacteria and cancer cells [23-25] etc. Therefore, the modification of **TPE-COOH** with AIE properties onto the targeting peptide molecule in order to synthesize a new **TPE-Peptide** has been investigated. On the other hand, triphenylamine (TPA) is also an important basic group for the construction of fluorescent compounds, consisting of a single nitrogen atom linked to three benzene rings, and has similar fluorescent properties to TPE with superior electron-giving and hole-transport properties [26,27]. In our previous work, we have synthesized and analyzed triphenylamine-based fluorescent probes [28,29], and so we have used triphenylamine as a backbone to synthesize a new fluorescent probe, **TPAPy-1**, in order to demonstrate the targeting properties of **TPE-COOH**. Through spectroscopic analysis, cytotoxicity, fluorescence imaging and viscosity studies, we found for the first time that **TPE-COOH** and the **TPE-Peptide** were capable of the fluorescent imaging of cancer microspheres cells and could effectively identify and characterize cancer microspheres.

2. Experimental

2.1. Materials and instrumentation

All chemical materials used in this study were obtained from commercial suppliers and were not further purified. Silica gel (Guiyang Chaoyuan Zhi-Cheng Biotechnology Co.) was used for column chromatography. ^{1}H NMR and ^{13}C NMR were measured on a Bruker Avance III, and chemical shifts are reported in ppm (in d_6-DMSO, TMS is the internal standard). Absorption spectra were recorded on a UV-2600 spectrophotometer. Fluorescence emission spectroscopy was performed on a Cary eclipse fluorescence spectrometer from Varian, USA. Fluorescence imaging was observed under an Olympus SpinSR10 confocal fluorescence microscope. HepG2 (Procell CL-0103) was provided by Rolcell Life Science &Technology Co. The remaining cells were obtained from the Guizhou Medical University Cell Bank (Guizhou, China), and the cells were cultured at 37°C and 5% CO_2 supplemented complete medium.

2.2 Synthesis of TPE-COOH, TPE-Peptide and TPAPy-1

Benzophenone (18.2 g, 0.1 mol), 4-hydroxybenzophenone (19 g, 0.1 mol), zinc powder (16 g, 0.24 mmol) and 400 mL of tetrahydrofuran (THF) were added to a three-necked flask at 0 °C, followed by the slow addition of titanium tetrachloride ($TiCl_4$) (13 mL, 0.12 mmol). The system was refluxed overnight under nitrogen. After the reaction was complete, the mixture was cooled to room temperature, and the solvent was removed by distillation under reduced pressure. The resulting solid was dissolved in dichloromethane, and 50 mL of dilute hydrochloric acid (1 mmol L^{-1}). Following extraction, the organic phase was dried with anhydrous $MgSO_4$ and left to stand

overnight. Following purification by silica gel column chromatography, a white solid (**TPE-COOH** intermediate 1) was obtained. Intermediate 1 (3.5 g, 10 mmol), *tert*-butyl bromoacetate (2 g, 15 mmol), K_2CO_3 (2 g, 15 mmol) and 50 ml acetonitrile were added in a round bottom flask and refluxed overnight. After the reaction, the mixture was separated by filtration and purified again by silica gel column chromatography to extract **TPE-COOH** intermediate 2. The product was added to a solvent mixture of DCM: TFA = 1:1 and stirred for 3 h. After the reaction, the mixture was poured into water and extracted with dichloromethane (DCM) three times, and the white solid precipitated from the organic phase was **TPE-COOH**.

^{1}H NMR (400 MHz, d_6-DMSO) δ 12.95 (s, 1H), 7.15-7.08 (m, 9H), 6.99-6.94 (m, 6H), 6.87-6.85 (d, J = 8.0 Hz, 2H), 6.68-6.66 (d, J = 8.0 Hz, 2H), 4.58 (s, 2H). ^{13}C NMR (100 MHz, d_6-DMSO) δ 172.8, 159.0, 146.1, 142.8, 142.5, 138.6, 134.6, 133.4, 130.6, 130.5, 129.2, 129.1, 67.1. MS (HR-ESI): calcd for $C_{28}H_{22}O_3$+Na^+: 429.1569 ; found:429.14565. **TPE-Peptide** was synthesized by the Shanghai Top-Peptide Biotechnology Co. Ltd. MS(HR-ESI) calcd for $C_{68}H_{79}N_9O_{18}S_2$+H^+: 1360.4658; found:1360.810.

Heptabromoheptanoic acid (4.18 g, 20 mol) and 4-methylpyridine (1.95 ml, 20 mmol) were dissolved in 40 ml of acetone and the system was refluxed overnight. After the reaction, the mixture was cooled to room temperature and a white solid was precipitated, filtered, washed with acetone and dried under vacuum, to obtain the intermediate. The intermediate (0.45 g, 1.5 mmol) and 5-(4-

(diphenylamino)phenyl)thiophene-2-carboxaldehyde (0.53 g, 1.5 mmol) were dissolved in 40 ml anhydrous ethanol, and 4 drops of piperidine was added and the system was refluxed overnight. After the reaction was cooled to room temperature, a large amount of ether was added, which precipitated a red solid. The solid was filtered and washed with ether, dried under vacuum, to afford red **TPAPy-1**, molecular mass 639.7g/mol.

^{1}H NMR (600 MHz, d_6-DMSO) δ 8.94 (d, J = 6.6 Hz, 2H), 8.26 (d, J = 16.2 Hz, 1H), 8.21 (d, J = 6.6 Hz, 2H), 7.63 (d, J = 8.4 Hz, 2H), 7.52 (m, 2H), 7.36 (t, J = 7.8 Hz, 4H), 7.16-7.12 (m, 3H), 7.09 (d, J = 7.8 Hz, 4H), 6.98 (d, J = 9.0 Hz, 2H), 4.51 (t, J = 7.8 Hz, 2H), 2.19 (t, J = 7.2 Hz, 2H), 1.90 (m, 2H), 1.48 (m, 2H), 1.29-1.26 (m, 4H). ^{13}C NMR (400 MHz, d_6-DMSO) δ174.5, 152.6, 147.8, 147.3, 146.5, 143.9, 138.7, 134.0, 133.8, 129.7, 126.8, 126.3, 124.7, 124.2, 123.9, 123.2, 122.2, 121.2, 59.4, 33.6, 30.4, 27.9, 25.2, 24.3. MS (HR-ESI): calcd for $C_{36}H_{35}N_2O_2S^+$[M-Br]$^+$: 559.24138; found: 559.24116.

2.3. General procedure for analysis

Dimethyl sulfoxide (DMSO)-phosphate buffer solution (PBS) or a methanol-phosphate buffer solution (PBS) was selected as the test system. The probes were configured into the master solution at a concentration of 1×10^{-3} mol·L^{-1} or 1×10^{-2} mol·L^{-1}, and the test solution was prepared by diluting 200 μl of the master solution into 10 ml. All spectra were obtained in a quartz cuvette (path length = 1 cm).

The propylene glycol (99% glycerol)-ethanol system was chosen as the test system.

Firstly, the concentration of the probe was configured as 1×10^{-3} mol·L^{-1} of the mother liquor concentration, and then different ratios of glycerol-ethanol solutions were configured. Finally, 100 μl of the probe was added, sonicated with an ultrasonic instrument for 10 min to remove air bubbles, mixed thoroughly and left for 1 h. The fluorescence spectra were measured.

2.4. Cell culture

Normal and cancer cell culture systems: 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin), 10% FBS and 90% DMEM or 1640 medium. 50 ml microsphere cells culture system were utilized as follows: 48.2 ml for DMEM-F12 medium, 1 ml for B27, 0.5 ml for heparin, hEGF 0.05 ml (final concentration: 100 μg/ml), and bFGF 0.25 ml (final concentration: 50 μg/ml). The cells were digested and centrifuged, resuspended in culture flasks with the desired medium, and the microspheres were incubated in suspension in ultra-low adsorption culture flasks at 37°C in an incubator with 5% CO_2.

cancel the cell suspension (density of about 6×10^4/ml) after centrifugation, was added to the well plate, put in the incubator, and was incubated for 24 h until the cell density was about 70%-90%. The suspended cells need to be incubated in advance with the culture bottle to complete, take the corresponding amount of cells per well and add to the well plate; wash 2 times with pre-warmed PBS, add a certain concentration of probe 500 μl, put in the incubator 37°C. 500 μl of 4% paraformaldehyde was added (at room temperature), fix the walled cells for 15 min to 20 min, fix the microspheres for

1 h, then wash the cells with PBS for 3 times, 5 min each time. Anti-fluorescence quencher was added dropwise, and the binding status of probe and cells was detected under laser confocal microscope. The **TPE-COOH** and **TPE-peptide** probes were excited at 405 nm, and the **TPAPy-1** probe was excited at 561 nm.

3. Results and discussion

3.1 Design and synthesis of TPE-COOH, TPE-Peptide and TPAPy-1

Insert Scheme 1 in here

TPE-COOH was synthesized using the reported method in the literature [30] and its synthetic route is shown in (Scheme 1, A). Its structure was characterized by ^{1}H and ^{13}C NMR spectroscopy and HRMS (Figs. S1, S3 and S5). The structure of **TPE-peptide** is shown in Scheme 1 (B), which was synthesized by the Shanghai Tao-pu Biotechnology Co. The synthetic route of **TPAPy-1** is shown in Scheme 1 (C), and the synthesis method is shown in the supporting material. The structure was characterized by ^{1}H and ^{13}C NMR spectroscopy and HRMS (Figs. S2, S4 and S7).

3.2. Properties of TPE-COOH, TPE-Peptide, and TPAPy-1

Insert Fig1 in here

We first investigated the AIE properties of three compounds, namely **TPE-COOH**, **TPE-Peptide** and **TPAPy-1**. The fluorescent probes were dissolved in DMSO, and the fluorescence of **TPE-COOH** and **TPE-Peptide** gradually increased with increasing water content. The fluorescence was strongest when the water content reached 95%, and the maximum emission wavelengths at 480 nm and 476 nm were 216 and 113 times

higher than those of the pure DMSO solution, respectively (Fig1, A and B). Trends in water content of TPE-COOH and TPE-Peptide and fluorescence profiles under UV light in (Fig1, D and E). The addition of unwanted solvent gradually aggregates the molecules, restricting intramolecular rotation and enhancing fluorescence. [31]. The fluorescence intensity of **TPAPy-1** also gradually increased with the increase of water content, and when the water content reached 70%, the fluorescence intensity at the maximum emission wavelength of 634 nm was 13 times higher than that of the pure methanol solution, and gradually decreased after 70% of water content (Fig1, C). Trend diagram of its water content and fluorescence profile under UV light in (Fig1, F). This estimation was due to precipitation, indicating that **TPAPy-1** also had an AIE effect.

Additionally, TPE-COOH and TPE-Peptide, which bear the same tetraphenylene fluorescent group, exhibited similar solid fluorescence. Stronger fluorescence intensity was observed in the solid state and almost no fluorescence was evident in the solution state. At λ_{em} = 468 nm, the solid fluorescence of TPE-COOH was 24 times greater than the solution fluorescence, while at λ_{em} = 480 nm, the solid fluorescence of TPE-Peptide was 120 times greater than the liquid fluorescence (Fig. S8, A). Moreover, the triphenylamine structure of TPAPy-1 emits at a wavelength in the red fluorescence region, where its solution fluorescence is stronger (five times that of the solid fluorescence, Fig. S8, B). The fluorescence plots of the three probes under UV irradiation are shown in Fig. S8 (C). In addition, the fluorescence quantum yields are given in Table S1, and are all higher for the solids *versus* the solution state (Φ).

3.3. Biological applications

Before performing cell imaging, cancer microsphere cells were constructed, and cancer cells were cultured in a microsphere medium and their growth status was observed for eight consecutive days (Fig. S9). From the observations, it can be seen that the cancer microsphere cells showed obvious aggregation into clusters on the fifth day. At this time, the nucleus and cytoplasm of the cells had no clear boundary, and the overall appearance was that of translucent round spheres, and the cells gradually formed a large sac-like structure. By the eighth day, the cells had formed a 3D spherical shape and were completely suspended, indicating that the *in vitro* model of cancer microspheres had been formed.

Insert Fig. 2 in here

Next, we needed to test the cytotoxicity of the three drugs, and three types of cells; normal hepatocytes (LO2), hepatoma cells (HepG2) and hepatoma microsphere cells (HepG2 microsphere) were selected as test subjects. The experimental results of the cytotoxicity assay showed that the cells exhibited almost no toxic changes when **TPE-COOH** and **TPE-peptide** were incubated with the cells at 0-8μM for 48h (Fig. 2, A and B). The cells also showed negligible cytotoxicity after being treated with the **TPAPy-1** probe at 0-15μM for 48h (Fig.2, C), indicating that the three probes can be used for cell imaging with biocompatibility. We selected 5μM as the concentration for fluorescence imaging, and after incubating both walled normal and cancer cells in the incubator for 24 h, the probes were added and co-incubated for 1 h. The probe solution

was washed off with PBS and bright-field and fluorescence images were taken using a laser confocal. Cancer microspheres were pre-cultured for one week and then incubated directly with the probe solution for 1 h, washed with PBS to remove the residual probe solution, fixed and photographed in a bright field and the fluorescence examined under a laser confocal.

Insert Fig. 3, Fig. 4, Fig. 5 in here

From the fluorescence imaging results of **TPE-COOH** (Fig.3) and **TPE-Peptide** (Fig.4). The system does not exhibit fluorescence for normal liver cells: LO2 and cancer cells: HepG2, SMMC-7721, Hela. However, we observed bright blue fluorescence for the cancer microspheres: QGY-7701 microsphere, HepG2 microsphere, SMMC-7721 microsphere, Hela microsphere. This indicates that the fluorescent probes **TPE-COOH** and **TPE-Peptide** are able to specifically, via fluorescence, image the cancer microspheres. To verify the targeting of the compounds, we synthesized **TPAPy-1** for comparative experiments. **TPAPy-1** also has an AIE effect and has a similar structure to **TPE-COOH**, which also has a carboxyl group. The fluorescence imaging results of **TPAPy-1** are shown in Fig. 5, and from the experimental results, it was evident that **TPAPy-1** can be used for normal cells: LO2, cancer cells: HepG2, SMMC-7721, Hela and cancer microspheres: QGY-7701 microsphere, HepG2 microsphere, SMMC-7721 microsphere, Hela microsphere all emitted bright red fluorescence. This further indicates that **TPE-COOH** and **TPE-Peptide** are able to target cancer microsphere cells.

3.4. Viscosity determination

Insert Fig. 6 in here

It has been well documented that cancer cells are more viscous than normal cells [32]. Therefore, the difference in viscosity has been used to design and synthesize many fluorescent probes with a viscosity response to specifically identify cancer cells [33]. When constructing cancer microspheres, under the microscope, we observed significant clustering of cells after forming cancer microspheres, and we speculate that the ability of **TPE-COOH** and **TPE-Peptide** to selectively image was caused by the greater viscosity of the cancer microspheres *versus* the cancer cells. To confirm our speculation, we examined the response of the fluorescent probes **TPE-COOH**, **TPE-Peptide** and **TPAPy-1** to viscosity, and the viscosity results (Fig.6) revealed that the fluorescence intensity of the probes increased with an increase in viscosity. However, **TPE-COOH** showed the greatest fluorescence enhancement with increasing viscosity. There is a good linearity between fluorescence intensity and glycerol ratio for **TPE-COOH** and **TPE-Peptide** in the range of 0-60% and 70%-95% (Figs. S10 and S11). However, TPAPy-1 also exhibited good linearity in the range of 0-60% and a poor linear correlation in the range of 70%-95% (Fig. S12).

Insert Fig. 7 in here

Nystatin has been reported to induce changes in cell structure or cell swelling leading to changes in cell viscosity causing dysfunction, and can be used as a detection agent for viscosity changes [34,35]. Therefore, we treated HepG2 and Hela cells with nystatin for 30 min. to increase their viscosity, and then incubated these cells with **TPE-**

COOH and **TPE-Peptide** for 1 h to obtain laser confocal fluorescence images (Fig.7). These images showed that cancer cells not treated with nystatin did not show fluorescence, while HepG2 and Hela cells treated with nystatin showed a distinct blue fluorescence. It was further demonstrated that the ability of **TPE-COOH** and **TPE-Peptide** to specifically recognize cancer microspheres is caused by the relatively high viscosity of the cancer microspheres, which means that in the intracellular environment, the intracellular viscosity has to be large enough to limit the free rotation of TPE to a certain value, so that its rotation is restricted and fluorescence is triggered.

4. Conclusions

In summary, we found that both **TPE-COOH** and **TPE-Peptide** were effective in identifying only cancer microspheres cells from normal cells, cancer cells and cancer microspheres, and in identifying and characterizing cancer microspheres by fluorescence imaging. In the solubilised state, the probes **TPE-COOH** and **TPE-Peptide** exhibit weak fluorescence, with an increase in fluorescence intensity containing water, exhibiting AIE properties caused by restriction of the intramolecular rotation (RIR) effect. On the other hand, both fluorescent probes showed a gradual increase in fluorescence intensity with increasing viscosity and **TPE-COOH** was the most viscosity sensitive of these three probes. Through analysis and imaging, we found that the molecule with the true recognition functional group was tetraphenylethylene, independent of its side chain. This in turn confirms that the viscosity of the cancer microspheres is greater than that of the cancer cells. Here, for the first time, we have

found that the tetraphenylethylene motif can be successfully applied to the fluorescence imaging of cancer microspheres, effectively identifies cancer microspheres and providing a visual means for the identification of cancer microsphere cells.

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Supplementary data

Electronic Supplementary Information (ESI) available: Details of the NMR and MS spectrum，AIE properties of the probe，Cell Cytotoxicity and the Probe viscosity correlation.

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