Fluorescent metallacycle-cored polymers via covalent linkage and their use as contrast agents for cell imaging

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The covalent linkage of supramolecular monomers provides a powerful strategy for constructing dynamic polymeric materials whose properties can be readily tuned either by the selection of monomers or the choice of functional linkers. In this strategy, the stabilities of the supramolecular monomers and the reactions used to link the monomers are crucial because such monomers are normally dynamic and can disassemble during the linking process, leading to mixture of products. Therefore, although noncovalent interactions have been widely introduced into metallacycle structures to prepare metallosupramolecular polymers, metallacycle-cored polymers linked by covalent bonds have been rarely reported. Herein, we used the mild, highly efficient amimation reaction between alkylamine and N-hydroxysuccinimide-activated carboxylic acid to link the pendant amino functional groups of a rhomboidal metallacycle 10 to give metallacycle-cored polymers P1 and P2, which further yielded nanoparticles at low concentration and transformed into network structures as the concentration increased. Moreover, these polymers exhibited enhanced emission and showed better quantum yields than metallacycle 10 in methanol and methanol/water (1/5, vol/vol) due to the aggregation-induced emission properties of a tetraphenylethene-based pyridyl donor, which serves as a precursor for metallacycle 10. The fluorescence properties of these polymers were further used in cell imaging, and they showed a significant enrichment in lung cells after i.v. injection. Considering the anticancer activity of rhomboidal Pt(II) metallacycles, this type of fluorescent metallacycle-cored polymers can have potential applications toward lung cancer treatment.

Fluorescent polymers play an important role in bioimaging due to their improved brightness, inertness to microenvironment, and good biocompatibility. In this article, we used tetraphenylethene (TPE) derivatives that give strong fluorescence emission in an aggregated state as fluorophores and synthesized fluorescent polymers via the covalent linkage of TPE-based rhomboidal Pt(II) metallacycles. Due to the integration of covalent linkage-induced aggregation of the monomers, the aggregation-induced emission character of TPE derivatives together with Pt(II)-based metal–ligand interactions, these polymers exhibit enhanced emission compared with their corresponding precursors, making them applicable as excellent cell imaging agents. Considering the potential anticancer activity of rhomboidal Pt(II) metallacycles, these polymers may serve as theranostic agents for both bioimaging and cancer therapy.

Significance

Fluorescent polymers play an important role in bioimaging due to their improved brightness, inertness to microenvironment, and good biocompatibility. In this article, we used tetraphenylethene (TPE) derivatives that give strong fluorescence emission in an aggregated state as fluorophores and synthesized fluorescent polymers via the covalent linkage of TPE-based rhomboidal Pt(II) metallacycles. Due to the integration of covalent linkage-induced aggregation of the monomers, the aggregation-induced emission character of TPE derivatives together with Pt(II)-based metal–ligand interactions, these polymers exhibit enhanced emission compared with their corresponding precursors, making them applicable as excellent cell imaging agents. Considering the potential anticancer activity of rhomboidal Pt(II) metallacycles, these polymers may serve as theranostic agents for both bioimaging and cancer therapy.

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(ii) the introduced functional groups should not interfere with the metal–ligand bonds; (iii) the reaction used to link the metallacycles should be mild and highly efficient; and (iv) any reagents used to promote the reaction should be easy to remove. Herein, by examining the structures of a number of metallacycles and chemical reactions, we chose rhomboidal Pt(II) metallacycles and the amidation reaction to overcome the above-mentioned challenges. Thus, polymers 1 and 2 (P1 and P2) were synthesized by linking a tetraamino-functionalized rhomboidal Pt(II) metallacycle 10 using N-hydroxsuccinimide-activated carboxylic acid-based linkers 11 or 12. Both P1 and P2 consist of a tetraphenylenethene (TPE) derivative which is a well-known AIE fluorophore (7–11). The aggregation of the monomers by polymerization inhibits the rotations of the aromatic rings of TPE, making P1 and P2 more emissive than their metallacycle precursor 10. At higher concentrations, the resulting polymers further aggregate into network structures, thereby even further enhancing their fluorescence, and hence may serve as potentially useful cell imaging agents. By investigating the distribution of P2 in mice 6 h after i.v. injection, we found that P2 showed significant enrichment in the lung. Based on the potential anticancer activity of rhomboidal Pt(II) metallacycles (42), these metallacycle-cored polymers may show potential applications as theranostic agents for both cell imaging and tumor therapy.

Results and Discussion

The synthetic procedures for P1 and P2 are shown in Fig. 1. A TPE-derivative 8, having two pyridyl groups for metal coordination and two amino groups for polymerization, was synthesized in a four-step pathway starting from commercially available benzophenone derivatives. The key intermediate TPE-derivative 4 was prepared by a classical McMurry coupling reaction and isolated in 45% yield. After nucelophilic substitution at the phenolic hydroxyl site of 4, a palladium-catalyzed Suzuki coupling reaction was carried out to obtain the 120° dipyridyl ligand 7, which was further reduced in the presence of hydrazine to yield ligand 8. The rhomboidal Pt(II) metallacycle 10 was prepared in quantitative yield by heating the 120° dipyridyl donor 8 and 60° platinum acceptor 9 at 50 °C in methanol for 24 h. Simple stirring of a mixture of 10 with 11 or 12 (1:2 molar ratio) in methanol solution at room temperature for a day, followed by dialysis with methanol, gave P1 and P2 in 83% and 86% yields, respectively.

The formation of metallacycle 10 was confirmed by multinuclear NMR (31P and 1H) analysis and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). The 31P(1H) NMR spectrum of 10 exhibits a sharp singlet (13.92 ppm) with concomitant 195Pt satellites corresponding to a single phosphorus environment (Fig. 2G), indicating the formation of a discrete, highly symmetric metallacycle. In the 1H NMR spectrum of metallacycle 10, the expected downfield chemical shifts were observed for the α-pyridyl protons Hα (from 8.54 ppm to 8.87 ppm) and β-pyridyl protons Hβ (from 7.68 ppm to 7.99 ppm) and both of them split into two set of signals (Fig. 2A and B), in a similar fashion to what was observed in analogous reaction systems (43). ESI-TOF-MS provided further evidence for the stoichiometry of formation of 10. Peaks at m/z = 877.6365, 1,220.1737, and 1,904.7172 were found (SI Appendix, Fig. S18), corresponding to [10 – 4OTf]1+, [10 – 3OTf]2+, and [10 – 2OTf]3+, respectively.

Fig. 1. Synthetic routes and cartoon representations of P1 and P2 and model compound 3.
The $^{31}$P($^1$H) NMR spectra of P1 and P2 exhibit broader singlets but with chemical shifts similar to that of rhomboid 10 (Fig. 2 G–I), indicating that the metallacycle structures were maintained in P1 and P2. In the $^1$H NMR spectra of P1 and P2, the aminomethylene protons $H_8$ shifted from 2.75 ppm to 3.18 ppm (Fig. 2 B–D) because the amidation reaction changes the chemical enviroment of $H_8$. No chemical shift changes were observed for the pyridyl protons $H_{2c}$, $H_{6c}$ and the aromatic protons $H_{1c}$, $H_{6a}$, $H_{6c}$, and $H_{6e}$ indicating that the amidation reaction does not perturb the rhomboidal metallacycle 10. To prove the efficiency of the amidation reaction, model compound 3 was also synthesized by stirring n-butyamine and linker 12 (2:1 molar ratio) overnight. Fig. 2E shows the $^1$H NMR spectrum of the reaction mixture of 3 after 8 h. As seen, almost all of the reactants were consumed and a new peak for N-hydroxysuccinimide appeared, indicating the efficiency of the amidation reaction between alkyamine and N-hydroxysuccinimide-activated carboxylic acid. Moreover, protons $H_8$, $H_{10m}$, and $H_{10e}$ of P2 and compound 3 appear at the same location and all of them exhibit triplet signals, indicating that the amidation reaction was also highly efficient during the covalent linking process.

The morphology of P1 and P2 was characterized by scanning electron microscopy (SEM) (Fig. 3 and SI Appendix, Figs. S27–S32). The samples were prepared by dropping their methanol solution onto a silica wafer followed by evaporation. At lower concentrations (0.1 mg/mL), well-dispersed nanoparticles were observed (Fig. 3 A and D) for both P1 and P2. However, when the concentration increased to 1.0 mg/mL, network structures emerged (Fig. 3 B and E) for both species, due to further aggregation of nanoparticles. We also found the coexistence of both dispersed nanoparticles and network structures at the edge of the silica wafer (SI Appendix, Figs. S29 and S32), which provides evidence for the concentration-dependent transformation of their morphology. The size of P1 and P2 was determined by dynamic light scattering (DLS). At a concentration of 0.1 mg/mL, P1 and P2 showed average hydrodynamic diameter ($D_h$) values of 296 and 283 nm (Fig. 3 C and F), respectively, consistent with the size of the particles observed by SEM (~250 to 310 nm).

The UV and visible (UV-Vis) absorption and fluorescence emission spectra of ligand 8, rhomboid 10, P1, and P2 in methanol and methanol/water (1/9, vol/vol) are shown in Fig. 4. Ligand 8 displays two broad absorption bands centered at 262 and 336 nm with molar absorption coefficients ($\epsilon$) of $3.30 \times 10^4$ and $2.03 \times 10^4$ M$^{-1}$·cm$^{-1}$, respectively (Fig. 4 A and SI Appendix, Table S1). Upon the formation of rhomboidal metallacycle 10, the lowest energy band is moderately red-shifted (ca. 26 nm). Rhomboid 10 exhibits four absorption bands centered at 257, 266, 290, and 362 nm with $\epsilon$ = $1.46 \times 10^4$, $1.48 \times 10^4$, $1.06 \times 10^4$, and $5.78 \times 10^3$ M$^{-1}$·cm$^{-1}$, respectively (Fig. 4 A and SI Appendix, Table S1). The absorption spectra of P1 and P2 are quite similar to that of rhomboid 10, providing further evidence for the retention of the rhomboidal metallacycle structures in P1 and P2.

Ligand 8 is weakly emissive (Fig. 4B) in methanol because of the nonradiative decay via intramolecular rotations of the pyridyl and phenyl rings (7–11). Upon formation of rhomboid 10, the pyridyl rings are partially rigidified, which limits their rotation, giving a moderate emission band centered at 522 nm. After the formation of P1 and P2, the TPE derivatives further aggregate, making P1 and P2 even more emissive than their metallacycle precursor 10 (Fig. 4B). The absorption and emission spectra of the four species in methanol/water (1/9, vol/vol) are quite similar to that in methanol, except for the fluorescence intensity increase due to the AIE effects of TPE-type compounds in poor solvents (Fig. 4 C and D). The changes in quantum yields ($\Phi_F$) in methanol and methanol/water (1/9, vol/vol) are in good agreement with the emission enhancement. In methanol, a very low $\Phi_F$ value (less than 0.05%) was observed for ligand 8. For rhomboid 10, the value rises to 0.237%. For P1 and P2, the values further increase to 0.329 and 0.337%, respectively. While in methanol/water (1/9, vol/vol), the $\Phi_F$ value of ligand 8 increases to 1.22% due to the AIE effect. Correspondingly, the $\Phi_F$ values of rhomboid 10, P1, and P2 increase to 2.13, 2.77, and 2.89%, respectively (SI Appendix, Table S1).

The fluorescent properties of P1 and P2 inspired us to explore their applications as bioimaging agents. Confocal laser scanning microscopy (CLSM) was used to evaluate the cellular uptake efficiency and intracellular localization of P2 in single cells. Based on the CLSM data, a bright fluorescence derived from P2 was observed in the cytoplasm of the cells after 6 h of incubation (Fig. 5 A–H), suggesting that the polymers can be applied for cell imaging. Moreover, the emission spectrum of P2 by CLSM (Fig. 5I) is consistent with their fluorescence spectra described above (Fig. 4 B and D), with maximum emission at 521 nm. This result suggests that the metallacycle structure remains intact during the imaging process. The fluorescence of P1 and P2 at different concentrations (40 and 200 µg/mL) was also collected by flow cytometric analysis (Fig. 5 J and K), indicating that P1
and P2 serve as contrast agents for cell imaging in the concentration range of 40 μg/mL to 200 μg/mL.

In vivo experiments were performed to evaluate the efficiency and distribution of P2 as contrast agent. Aqueous suspensions of P2 at various concentrations (~7.8 to 500 μg/mL) were imaged using an in vitro phantom study. A linear dependence of the fluorescence intensity on concentration was observed in the tested range (Fig. 6A), revealing the potential of using P2 for real-time imaging and quantitative analysis. To verify this, 20 μL of P2 (10 mg/mL) was intratumorally injected into a mouse bearing an MDA-MB-231 (a human breast adenocarcinoma cell line) tumor. A significant fluorescence of the tumor was observed even 24 h after injection (Fig. 6B), indicating that P2 is both chemostable and photostable in vivo, which is an essential criterion for bioimaging agents. The same mouse was killed 24 h after injection, and the tumor, major organs, and lymph nodes were imaged (Fig. 6C). A significant transfer of P2 from the tumor to the liver and lung was observed. In addition, there was an accumulation of P2 in lymph nodes, which is associated with tumor metastasis and early diagnostics. We next explored the in vivo distribution of P2 in tumor-bearing mouse following systematic administration. By the investigation of the images and fluorescence counts of different organs 6 h after i.v. injection, we found that P2 showed a significant enrichment in the lung over the other organs (Fig. 6D and E). Hence, given the known anticancer activity of rhomboidal Pt(II) metallacycles (42), the possible use of these polymers toward lung cancer therapy could be explored.

**Conclusion**

In summary, by linking the rhomboidal metallacycles via amidation reaction between N-hydroxysuccinimide-activated carboxylic acid and alkylamine, two polymers, P1 and P2, were successfully prepared and characterized by multinuclear NMR (1H and 31P) and SEM. The structure of the metallacycles was maintained in the polymers due to the mild, highly efficient and catalysis-free amidation reaction, providing a method to polymerize metallacycles to give functional polymers and an alternative approach for postfunctionalization of metallacycles. The metal coordination limits the free rotation of the aromatic rings of TPE, and the formation and further aggregation of polymers match well with the AIE properties of TPE derivatives, thereby providing these polymers with enhanced fluorescence emission properties useful as bioimaging agents. Moreover, this covalent linking approach to aggregate AIE-type compounds also provides a good method to further enhance the AIE effects. The use of these fluorescent polymers as bioimaging agents was
explored, and their biodistribution after intratumoral and i.v. injection was also studied. A significant enrichment of the polymers in the lung was observed after i.v. injection. Other studies could explore further tuning the emission of the polymers by changing the linkers and the metallacycles, as well as their applications in bioimaging, drug delivery, and cancer therapy (42, 44–47).

Materials and Methods

All reagents were commercially available and used as supplied without further purification. Deuterated solvents were purchased from Cambridge Isotope Laboratory. Compounds 5 (48), 9 (49), 11 (39), and 12 (39) were prepared according to the literature procedures. NMR spectra were recorded on a Varian Unity 300-MHz or 400-MHz spectrometer. $^1$H and $^{13}$C NMR chemical shifts are reported relative to residual solvent signals, and $^{31}$P($^1$H), NMR chemical shifts are referenced to an external unlocked sample of 85% H$_3$PO$_4$ (δ = 0.0). Mass spectra were recorded on a Micromass Quattro II triple-quadrupole mass spectrometer using electrospray ionization with a MassLynx operating system. The melting points were collected on an SHPSC CR–22 automatic melting point apparatus. The UV-Vis experiments were conducted on a Hitachi U-4100 absorption spectrophotometer. The fluorescent experiments were conducted on a Hitachi F-7000 fluorescence spectrophotometer. Quantum yields were determined using quinine sulfate at 365 nm (Φ$_{FL}$ = 56%). TEM was performed on a FEI Quanta 650 FEG (field emission gun). CLSM was performed with a Zeiss LSM 710 Confocal Microscope using a 63x objective. Flow cytometry was performed with a Fluorescence Activated Cell Sorter Calibur Flow Cytometer (BD Biosciences). The size of polymers was measured using a Malvern ZS90 DLS instrument with an He–Ne laser (633 nm) and 90° collecting optics. The data were analyzed using Malvern Dispersion Technology Software 5.10. The mice were obtained from Beijing HFK Bioscience Co., Ltd.

![Fig. 5](image-url) CLSM images and flow cytometric analysis of A549R cells after incubation with P1 and P2. (A and E) Images of cells treated with P2. (B and F) Images of cells stained with Alexa Fluor 568. (C and G) Images of cells stained with DAPI. (D) Merged image of A549R cells from A, B, and C. (H) Merged image of A549R cells from E, F, and G. (I) Fluorescence spectrum of P2 in A549R cells taken by CLSM. (J and K) Flow cytometric analysis of P1 and P2 in A549R cells after 6 h of incubation.

![Fig. 6](image-url) Bioluminescence and fluorescence images of P2 in mice after intratumoral injection and i.v. injection. (A) Optical images of aqueous suspensions of different concentrations of P2 in a 96-well plate, showing plot and fitting of the fluorescence intensity of P2 versus their concentration. (B) Optical and fluorescence image of a mouse after intratumoral injection of 200 µg P2. The image was taken 24 h post injection. (C) Bioluminescence intensity of P2 24 h after intratumoral injection. The sequence of the images of the organs is the same as that of the fluorescence counts. (D and E) Optical and fluorescence images of a mouse after i.v. injection of 600 µg P2. (D) Images of different organs and (E) the fluorescence counts of different organs 6 h after i.v. injection. The sequence of the images of the organs is the same as that of the fluorescence counts. ROI, region of interest.
Rhomboid 10 was synthesized by heating 8 with 9 in a 1:1 molar ratio in a 2-dram vial. After cooling, the solvent was removed to give rhomboid 10 as a yellow solid. The formation of polymers P1 and P2 was achieved by stirring rhomboid 10 and linker 11 or 12 (1:2 molar ratio) in methanol (0.25 mmol for 10) for 24 h. After that, the solvent was removed to give a crude product, which was dialyzed with methanol for another 24 h to give polymers that were then collected and dried under reduced pressure for future use.

Rhomboid 10: H NMR (400 MHz, CDCl3, 295 K): 8.87 (m, 8H), 8.46–8.75 (m, 4H), 7.99 (m, 8H), 7.45–7.85 (m, 20H), 7.28 (d, J = 8.2 Hz, 8H), 7.02 (d, J = 8.8 Hz, 8H), 6.74 (d, J = 8.8 Hz, 8H), 4.00 (t, J = 5.6 Hz, 8H), 7.13 (d, J = 7.0 Hz, 8H), 0.97 (t, J = 7.8 Hz, 18H). 13C NMR (121.4 MHz, CDCl3, 295 K) δ (ppm): 13.27 ppm (168 ppm satellites, J13C = 2698.1 Hz).

P1: H NMR (400 MHz, CDCl3, 295 K): 8.87 (m, 8H), 8.46–8.75 (m, 4H), 7.99 (m, 8H), 7.45–7.85 (m, 20H), 7.28 (d, J = 8.2 Hz, 8H), 7.02 (d, J = 8.8 Hz, 8H), 6.74 (d, J = 8.8 Hz, 8H), 4.01 (t, J = 5.6 Hz, 8H), 7.31 (t, J = 7.0 Hz, 8H), 2.91 (t, J = 7.2 Hz, 8H), 2.55 (t, J = 7.2 Hz, 8H), 1.78 (t, J = 6.6 Hz, 8H), 0.90–1.60 (m, 152H). 13C NMR (121.4 MHz, CDCl3, 295 K) δ (ppm): 13.33 ppm (169 ppm satellites, J13C = 2698.1 Hz).

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