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A Unique Aliphatic Tertiary Amine Chromophore: Fluorescence, Polymer Structure, and Application in Cell Imaging

Miao Sun, Chun-Yan Hong, and Cai-Yuan Pan*

CAS Key Laboratory of Soft Matter Chemistry, Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei, Anhui, 230026, P. R. China

Supporting Information

ABSTRACT: Although photoluminescence of tertiary aliphatic amines has been extensively studied, the usage of this fundamental chromophore as a fluorescent probe for various applications has unfortunately not been realized because their uncommon fluorescence is easily quenched, and strong fluorescence has been observed only in vapor phase. The objective of this study is how to retain the strong fluorescence of tertiary amines in polymers. Tertiary amines as branching units of the hyperbranched poly(amine-ester) (HypET) display relatively strong fluorescence (Φ = 0.11−0.43). The linear polymers with tertiary amines in the backbone or as the side group are only very weakly fluorescent. The tertiary amine of HypET is easily oxidized under ambient conditions, and red-shifting of fluorescence for the oxidized products has been observed. The galactopyranose-modified HypET exhibits low cytotoxicity and bright cell imaging. Thus, this study opens a new route of synthesizing fluorescent materials for cell imaging, biosensing, and drug delivery.

Fluorescent biomaterials have become a focus of research in biology due to their broad applications in cellular imaging, biosensing, and drug delivery,[1] and the fluorescent materials in the studies include fluorescent organic dyes, fluorescent proteins, and quantum dots.[2] Some aliphatic biodegradable synthetic polymers, which show intriguing photoluminescence phenomena, have developed.[3] However, usage of tertiary amines as a fluorescent probe has never been reported, although the fluorescence of tertiary amine has been extensively studied.[4,5] Tertiary aliphatic amines of different structures display different fluorescence behaviors due to the formation of different excimers, such as α,ω-diaminoalkanes form various intramolecular excimers.[5−7] Very high fluorescence quantum yields (around 98%) of aliphatic tertiary amines including TMA and TEA were obtained only in the vapor phase.[7] However, the fluorescence of primary and secondary amines is not observed even in the vapor phase, owing to easy loss of the hydrogen atom via predissociation,[4] and the quantum yields of tertiary amines measured in various solvents are very low generally due to quenching of the solvents, which is explained by possible energy transfer and electron (or charge) transfer.[8,9] So, the photoluminescence behaviors of tertiary amine chromophores are obviously different from the current fluorescent materials, such as fluorescent organic dyes and quantum dots, and synthesis of the biodegradable, biocompatible, and water-soluble fluorescent polymers containing tertiary amine is relatively easy; with this type of polymer, drawbacks of the current chromophores, such as cytotoxicity and photobleaching,[3] might be avoided. Thus, development of the tertiary amine-based fluorescent polymers is very valuable.

Although the fluorescence nature of tertiary aliphatic amines is continuously investigated for a long time,[10] successful fluorescent labeling with this fundamental chromophore has not been studied. The main problem is how the tertiary amines in the materials can retain their high fluorescence efficiency. Study on the TMA and TEA fluorescent behavior demonstrates that the factors governing the predissociation rate that competes with emission rate are collisional relaxation and quenching.[4] Hyperbranched and dendritic poly(amido amine)s (PAMAMs), which are generally prepared from primary and/or secondary amines, contain different types of amines due to incomplete Michael addition reaction. Their photoluminescence was observed,[11,12] but the chromophore was not investigated,[11] except a speculation based on fluorescence behaviors in our previous report.[12] Plus, the fluorescence of nitrogen vacancy defects in diamond has been extensively studied, and under excited at 514 nm, a strong fluorescence appears at around 700 nm.[13] This encourages us to study the fluorescence properties of hyperbranched polymers produced by linking aliphatic tertiary amine molecules, and this type of polymers is expected to retain high fluorescence efficiency of the starting amine because they are fixed in the polymer network, similar to nitrogen in the diamond. In addition, the microenvironment around the chromophores can be adjusted in order to reduce the fluorescence quenching by solvent molecules.[8] Thus, the hyperbranched poly(amine ester)s, HypET11, HypET15, HypET20, and HypET24, were prepared by Michael addition polymerization of tri(2-mercaptoethyl)amine (TMEA) and ethylene glycol diacrylate (EGDA) for 11, 15, 20 and 24 h, respectively, as shown in Scheme 1.

Since there are two types of terminal groups, mercapto and acryloyl, in the resultant HypETs, cross-linking reactions occur during storage. All HypETs were treated with mercaptoethanol; both reactions produced the same terminal hydroxyl group. This is confirmed by the ^{1}H NMR spectra (Figure S1) with two methylene group proton signals next to OH group, one at δ = 3.91 ppm, terminal SH reaction with mercaptoethanol forming the linear unit, and the other at δ = 3.75 ppm, acyloyl reaction with mercaptoethanol, indicating existence of linear and terminal

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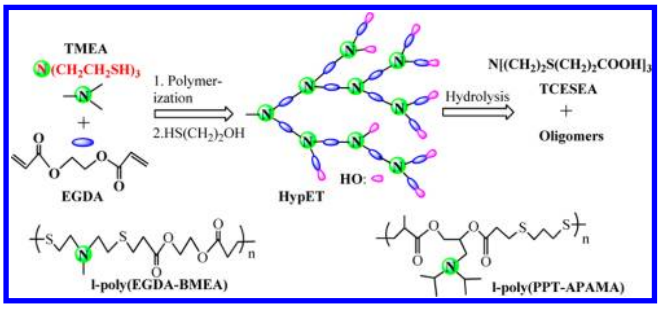
Scheme 1. Synthesis and Hydrolysis of the Hyperbranched Polymer HypET[a]



[a]Structures of the Linear Polymers with Tertiary Amine in the Backbone [L-poly(EGDA-BMEA)] and as Side Group [L-poly(PPT-APAMA)].

units in the HypETs. ^{1}H NMR spectrum in Figure S1 supports the HypETs formation. Based on integral values of signals at 2.5−3.0 and 4.32 ppm, the degree of branching (DBs) was calculated, and the results are shown in Figure 2. The molecular weights and molecular weight distributions were estimated by size exclusion chromatography (SEC) measurements with multi-angle laser light scattering (MALLS) detector, and the results are listed in Table S1 and Figure S2.

Examining hyperbranched structure effects on fluorescence, the fluorescence spectra of starting materials TMEA and EGDA were measured. As shown in Figure 1a,a′, EGDA is non-

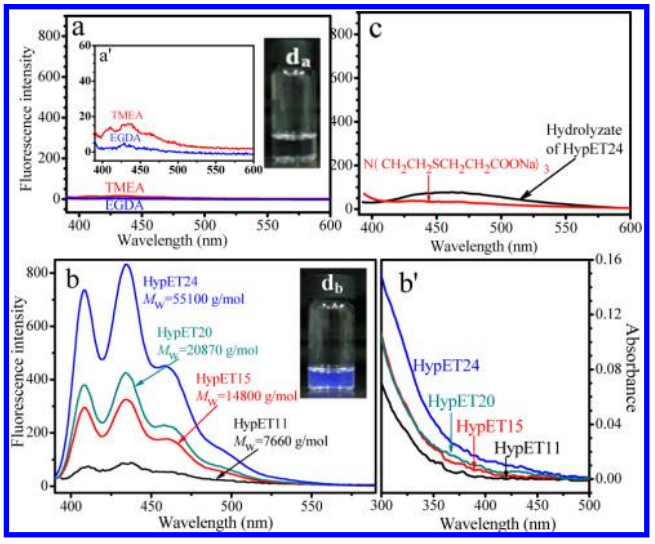


Figure 1. TMEA and EGDA fluorescence used in the preparation of HypETs (a, a′); fluorescence (b) and UV/vis spectra (b′) of the HypET11, HypET15, HypET20, and HypET24 using Michael addition polymerization of TMEA and EGDA with feed molar ratio of 1:2 at 50 °C for 11, 15, 20, and 24 h, respectively; and fluorescence of the hydrolyzed products of HypET24, $N(CH_2CH_2SCH_2CH_2COOH)_3$ (c). Conditions: 1 mg/mL in $CHCl_3$; λ_{ex} = 375 nm at rt. TMEA and EGDA solution (d_a); and HypET24 solution in $CHCl_3$ (d_b).

fluorescent, and TMEA emits very weak fluorescence. When EGDA and TMEA solution in chloroform was irradiated at λ_{ex} = 375 nm, no fluorescence was observed (Figure 1d_a). But HypET24, from polymerization of TMEA and EGDA for 24 h, displays very strong fluorescence (Figure 1b), and under irradiation at λ_{ex} = 375 nm, a blue solution of the HypET24 in $CHCl_3$ is clearly seen (Figure 1d_b). Distribution of intensity in Figure 1b shows that vibrational relaxation in the excited state occurs prior to emission.[4] Using Williams' method,[14,15] the quantum yield (Φ) of HypET24 in $CHCl_3$ was measured and is 0.43. All these data support that the tertiary amine in the hyperbranched polymers can retain high fluorescence efficiency. Ester linkages of EGDA units are easily hydrolyzed in alkaline solution; theoretically the completely hydrolyzed product of HypETs is tri(5-carboxylate-ethylene-3-sulfo-ethylene)amine (TCESEA). Fluorescence of the hydrolyzed products, obtained by hydrolysis of the HypET24 in NaOH solution of THF/H_2O (1:1, v/v), was measured, and a significant decrease of the fluorescence was observed (Figure 1c). The fluorescence is stronger than the pure TCESEA obtained from reaction of TMEA and methyl acrylate and subsequent hydrolysis (Figure 1c), which is ascribed to small amount of branched oligomers in the hydrolyzate. Results demonstrate again the importance of the hyperbranched structure for retaining high fluorescence efficiency of the tertiary amine.

The emission spectra of different molecular weight HypETs have similar profiles, but the relative fluorescence intensity increases with increasing the molecular weight (Figure 1**b**), which was also observed in the study of PAMAM dendrimers[16] and hyperbranched PAMAM (HPAMAM).[12] Quantum yields of HypETs have the same trend as the fluorescence intensity (Figure 2b) are presumed to be mainly due to decreased interior mobility in the high molecular weight HypETs, because solution NMR studies demonstrated that the interior mobility of the dendrimer decreased with increasing generation.[17] The high molecular weight HypETs also contain a high tertiary amine content in the branching units than the lower molecular weight HypETs, which is supported by the DBs increase of the HypETs with increasing molecular weights (Figure 2b).

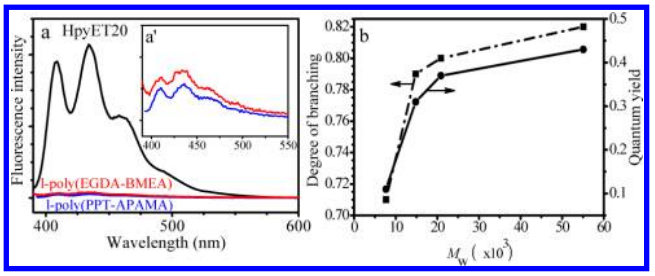


Figure 2. Influence of the polymer structures on fluorescence behaviors. (a) Fluorescence spectra of the HypET20 with M_n = 6000 g/mol and M_w/M_n = 3.50, the linear polymer with the tertiary amine in the backbone (L-poly(EGDA-BMEA), M_n = 6570 g/mol, M_w/M_n = 1.74) and the linear polymer with tertiary amine as side group (L-poly(PPT-APAMA), M_n = 4300 g/mol, M_w/M_n = 1.34). (a′) Fluorescence spectra of linear polymers. Conditions: 1 mg/mL in $CHCl_3$; and λ_{ex} = 375 nm at rt. (b) Relationship of degree of branching and quantum yield with weight-average molecular weight.

To verify the contribution of branching structure to tertiary amine fluorescence, we synthesized a linear polymer with tertiary amine in its backbone by Michael addition polymerization of EGDA and bis(2-mercaptoethyl)methylamine with equal molar ratio (Figure S3). The resultant L-poly(EGDA-BMEA), M_n(GPC) = 6750 and M_w/M_n = 1.74 (Figure S4), emits weak fluorescence (Φ = 0.025), as shown in Figure 2**a**. Since collisional relaxation is very important factor influencing the fluorescence efficiency,[4] the tertiary amine in the linear polymer backbone has relatively high mobility in comparison with the tertiary amine in the branching units of HypEts, decreasing fluorescence via

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collisional relaxation. If this is correct, then a polymer with a tertiary amine side group should display more weak fluorescence because the side groups have higher mobility than the groups in the polymer backbone. Thus a polymer with a tertiary amine side group was prepared by polymerization of 1,3-propanedithiol and 1-acryloyl-1-di(isopropyl)amineethyl-2-methacryloyl-glycol (Figure S5) and the resulting L-poly(PPT-APAMA) with M_n(GPC) = 4300, M_w/M_n = 1.34 (Figure S6) display weaker fluorescence (Φ = 0.006) than L-poly(EGDA-BMEA) (Figure 2a). However, their fluorescence spectra are almost the same with HypET in both appearance and position (Figure 2a′). We reasonably infer that the hyperbranched structure with the tertiary amine as branching unit is a key for reserving fluorescence efficiency of the tertiary amine. Based on the this discussion, terminal and linear units emit very weak fluorescence, and branching units situated in the interior of HypETs have the biggest contribution to the fluorescence, which is consistent with the previous fluorescence-quenching study of the PAMAM dendrimers where the fluorescence originates from the interior region, and the fluorescence of surface region was not observed.[16] To further understand the fluorescence, a similar method reported[16] was applied to measure the lifetimes of fluorescence for HypETs, and the results are listed in Table S1. Different from the results of PAMAM dendrimers that have two lifetimes,[16] only one lifetime (around 2.5 ns) for the HypETs was obtained. This suggests that only a single fluorescent moiety, TMEA, exists in the HypETs.

HypETs fluorescence is centered at 440 nm, which is much lower than the fluorescence of nitrogen-vacancy defects in diamond.[13] To explore the red-shifting possibility of the fluorescence, the HypET24 dissolved in THF was stirred under ambient conditions at ~35 °C for 7 and 20 days, respectively, and oxidized products, HypET24-O7 and HypET24-O20, were obtained after removal of THF and subsequently dried. UV/vis and fluorescence spectra were measured in chloroform. Figure 3a shows the UV/vis spectra of HypET24 and its oxidation products. HypET24 has

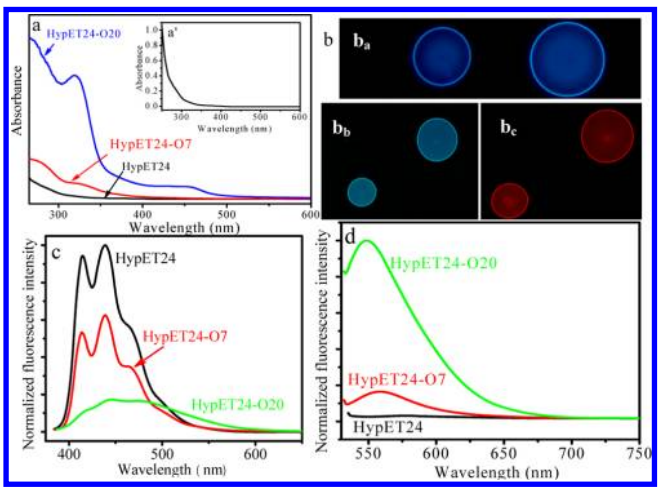

Figure 3. UV/vis absorption (a) and fluorescence spectra (c,d) of HypET24, HypET24-O7, and HpyET24-O20 solutions in $CHCl_3$ (1 mg/mL) measured at rt; excited wavelength for (c) λ_{ex} = 365 and (d) λ_{ex} = 510 nm. (b) Optical fluorescence microscope photos of the ring-shape films prepared, respectively, by evaporating a drop of HypET24 (b_a) and HypET24-O20 solutions in $CHCl_3$ on the clean glass slides under excited wavelength of 330−385 nm (WU) filter (b_b) and 510−550 nm (WG) filters (b_c).

absorption between 250 and 400 nm (Figure 3a′), but for the HypET24-O7, a new absorption band at 320 nm is clearly seen and is strengthened significantly in the case of HypET24-O20. Fluorescence behaviors of the HypET24 and its oxidized products are quite different (Figure 3c,d); HypET24 emits fluorescence at 440 nm, altering the excited wavelength from 305 to 390 nm does not change the fluorescence both in appearance and in position. For the ring films formed by evaporation of the HypET24 solution in $CHCl_3$, only blue rings were observed by optical fluorescence microscope under excitation at 330−385 nm (Figure $3b_a$), and the color was not changed with altering excited wavelengths, which is different from the HPAMAMs that exhibit color change from blue to green to red with increasing the excitation wavelength.[12,15] However, the oxidized products of HypET24 show two fluorescence peaks centered at 440 and ~560 nm, respectively (Figure 3c,d), thus, blue and red ring films of the HypET24-O20 were observed by optical fluorescence microscope under excitation of 330−385 and 510−550 nm, respectively (Figure $3b_b$,b_c). Compared to the fluorescence of HypET24, the fluorescence intensities of the oxidized HypET24s at 440 nm decrease greatly, indicating continuous decrease of the unoxidized tertiary amine content with increasing oxidization time from 7 to 20 days (Figure 3c), which is contrary to the oxidation of PAMAM dendrimers.[18] But the fluorescence at 560 nm is strengthened significantly with increasing oxidization time (Figure 3d) due to the content increase of a new chromophore. We tried to completely oxidize the tertiary amine in the HypETs using a strong oxidation agent, such as H_2O_2, to further understand the influence of oxidation on fluorescence but was unsuccessful, owing to HypET degradation during the oxidation reaction.

What is the new chromophore formed during the oxidation? Imae assumed a chromophore of oxygen-doped tertiary amine formed in oxidation of the PAMAM dendrimers.[18] Oxygen-amine "contact" donor−acceptor complex formed in the oxidation of aliphatic tertiary amine was presumed as a chromophore.[8] We suggest that the ≡N→O may be formed based on IR (Figure S8) and ^{1}H NMR spectra (Figure S7) of the HypET24 and its oxidized products. The ^{1}H NMR spectrum of HypET24-O20 in Figure S7 reveals that all proton signals remain unchanged, except the proton signals (d and e) of three nitrogen-attached methylene groups at 2.54−2.72 ppm are shifted to downfield, and the unshifted tail of d and e signals represents unoxidized tertiary amine. With increasing oxidization time, tail strength decreases gradually, but weak proton signals still remain, demonstrating the oxidation reaction is incomplete. To explore the resulting oxidized product, we measured the IR spectra of HypET24 and its oxidized products (Figure S8). Although the IR spectrum of HypET-O7 has no big difference with that of the HypET24, the spectrum of HypET24-O20 clearly shows characteristic N→O absorption bands at υ = 1184, 1362, and 1460 cm^{-1} (dimer),[19] but the N═O absorption band in the region of 1585−1539 cm^{-1}[19] was not found. The characteristic absorption bands for the unconjugated C−N of tertiary amine in the region of 1250−1100 cm^{-1}[19] decreased significantly but did not disappear completely, which supports the result of ^{1}H NMR analysis. The N→O formed may be responsible for the fluorescence.

Similar to current fluorescent materials studied, this fluorescent polymer may have various applications. We applied this polymer in the cell imaging, which requires high water solubility of the HypET. Considering special interaction of HepG2 cells with galactose,[20] a water-soluble galactopyranose-

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modified HypET (HypET-AlpGP) was prepared by hydrolysis of 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose-grafted HypET with quantum yield = 0.10 in acidic solution (pH = 3) of THF/H_2O (Figures S9 and S10). First we tested cytotoxicity of the HypET-AlpGP by MTT assay because toxicity is very important for biological applications. Branched PEI with molecular weight of 25k (PEI25k) was used as the control. In Figure 4a, the PEI25k exhibits high cytotoxicity to HepG2 cells

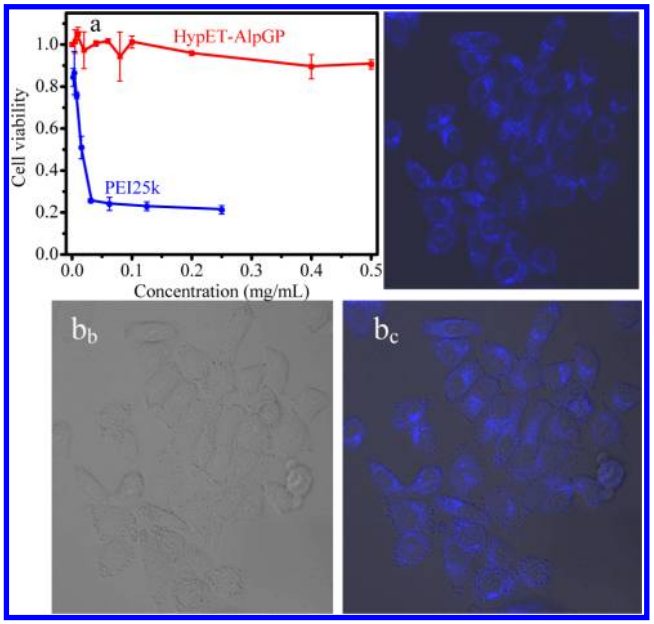

Figure 4. Cytotoxicity and cell imaging of the HypET-AlpGP. (a) Cytotoxicity of the HypET-AlpGP to HepG2 cells. (b) Laser confocal scanning microscopic images of the HepG2 cells after 9 h incubation with a serum-free DMEM solution of HypET-AlpGP (2 mg/mL) under excitation at λ_{ex} = 375 nm (b_a) and bright field (b_b); (b_c) is a merged picture of (b_a) and (b_b).

with 50% cell viability at the concentration of 15 μg/mL. In contrast, only slight decrease (less than 10%) of the cell viability was observed for the HypET-AlpGP at a dose up to 500 μg/mL.

Liver carcinoma HepG2 cells can recognize galactose residues through an asialoglycoprotein receptor on the surface,[20] so modified HypET (2 mg/mL) was incubated with HepG2 cells at 37 °C for 9 h, and then the cells were washed with phosphate-buffered saline for removal of the HypET-AlpGP that was not internalized. The obtained cells were observed under laser confocal scanning microscopy. When excited at 375 nm (Figure 4b), blue HepG2 cells are clearly observed, indicating potential application of HypETs in cell imaging. These preliminary results demonstrate that the HypET-AlpGP is a suitable candidate for cell imaging, biosensing, and drug delivery.

In summary, we demonstrated that the tertiary aliphatic amine in branching units of hyperbranched polymers is key in retaining high fluorescence efficiency of the tertiary amine. Linear polymers with a tertiary amine in the backbone or as a side group emit weak fluorescence. Molecular weight of hyperbranched polymers is an important factor, and the fluorescence efficiency increases with increasing molecular weights. Aliphatic tertiary amine-based hyperbranched polymers are easily oxidized, and the oxidized products exhibit different fluorescence properties from the precursors. HypET-AlpGP has low cytotoxicity and displays bright cell imaging. Thus we provide a new route of designing fluorescent materials for various applications including cell imaging, biosensing, and drug delivery.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

pcy@ustc.edu.cn

Notes

The authors declare no competing financial interest.

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