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A Direct Crossed Polymerization of Triphenylamines and Cyclohexanones via C=C bond formation: the Method and Its Bioimaging Application

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The effective polymerization process is very desirable in the field of macromolecule science. In this paper, we present the facile synthetic method by Aldol Addition and Condensation (AAC), leading to the formation of florescent linear and branched polymers by cross coupling triphenylamines (TPA) and cyclohexanones (CYC) via C=C bond. The methodology takes advantages of easy operations, mild reaction conditions, and high yield. By the analysis of NMR, FT-IR, GPC, PL, UV, SEM, and theoretical calculation, both of polymers' structure, physical properties, and optical behaviors are well characterized. The great demand to understand cell transplantation, migration, division, fusion, and lysis is a very challenge task. In this work, the linear polymer (LP) exhibits excellent biocompatibility and the low cytotoxicity, which could be readily internalized by living cells with noninvasive manner. The cell iamges of MPC5 cells indicate LP could be a promising emissive fluorescence probe for bioimaging application.

1. Introduction

Research on fluorescent polymers (FPs) has been a classic topic for many decades owing to their diverse molecular structures,¹⁻⁴ fascinating photophysical and optoelectronic properties.⁵⁻⁸ Especially, triphenylamine (TPA)-based FPs have been explored as an important functional florescent materials with photosensitive, redox-active and hole-transporting properties.^{9,10} In 2001, Tang's group observed a peculiar photophysical phenomenon named as aggregation-induced emission (AIE) and aggregation-induced-enhanced emission (AIEE).¹¹ As the terms indicate that an AIE or AIEE molecule is almost nonemissive or very weak emissive in dilute solution but becomes highly fluorescent in the aggregate or total solid state.¹²⁻¹⁴ Up to now, TPA derivatives have been found as the representative AIE or AIEE materials,¹⁵⁻¹⁷ which is employed as cellular tracers to insight into the biological processes of cell transplantation, migration, division, fusion, and lysis. That is due to the fact that when AIE or AIEE molecules enter into cells, enriched concentrations of fluorophores in the cells and the restriction of intramolecular rotation (RIR) are able to increase the emissive capability of fluorophores, therefore,

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The development of effective polymerization route to functional materials is a fundamental important area in macromolecule science. Aldol Addition and Condensation (AAC) reaction has been discovered as a versatile and powerful synthetic tool in the unsaturated carbonyl compounds formation and asymmetric synthesis.²¹⁻²⁴ It takes the advantages of high efficiency, mild reaction conditions, and broad tolerance to functional groups. However, what should be noted that this reaction has been seldom seen in the polymerization process, which is probably due to the presence of water. Water is employed as solvent, very necessary to dissolve inorganic base. And also, one of the products of this reaction is water, which presents an obstacle with regard to the elimination step in the aldol condensation. But, it was convinced that the aldol condensation product is usually insoluble in water, and as it forms, usually precipitate falls out of solution, removing the organic products from the equilibrium. Not only that hydrophobic effect is favourable to accelerate the coupling reaction more effectively.²⁵

In recent years, we have succeeded in exploring a group of novel TPA and tetraphenyl ethylene (TPE)-containing fluorescent molecules.^{15-17,26} These materials possess an diversified molecular structures, and interesting optical property, the further applications were also extended. In this paper, we report the preparation of the linear and branched fluorescent polymers by the facile ACC reaction of TPA derivatives and cyclohexanone (CYC) (Scheme 1). The TPA moieties as electron donor are expected to enhance the electron donating and charge transport ability to CYC moieties

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Scheme 1. The synthetic route to LP and BP.

(electron acceptor) via C=C bond. CYCs are deserved as soft linkers between the more hard TPA segments, which could promote the solubility of the polymers in the common organic solvents. Under very mild reaction conditions, highly emissive polymers with satisfactory molecular weight were obtained. The chemical structures and the optical properties of polymers LP and BP were also characterized. Additionally, by virtues of the biocompatibility and the low cytotoxicity, the linear florescent polymer LP is used as a promising emissive fluorescent biomarker for cell imaging.

2. Results and discussion

Following the standard reaction conditions, we carry out the polymerization of PDBD or NTBD with CYC with feed molar ration of 1:1 or 2:3. CYC is determined as representative nucleophilic reagent, which is commercially available and commonly used in the ACC reaction.²⁷ The dual and more active-carbon position could give rise to the polymerization take place. But also, the aliphatic framework of CYC probably favours of better solubility of polymers in organic solvents. The purified LP and BP emit orange and red light under UV illumination respectively. Obviously, this polymerization reaction is conducted with very desirable style. Catalyst-free, inert gas protect-free, solvent dryness-free and moderate temperature, all of them render ACC reaction advantages in the polymerization process.

2.1 Structural characterization

The linear polymer (LP) and the branched polymer (BP) were characterized by spectroscopic methods. Both of the polymers gave satisfactory analytical data that corresponded to their molecular structures. The IR spectra of LP, BP and the related monomers are shown in Figure 1. The absorption band 1690cm⁻¹ in the spectrum is stemmed from the C=O bond stretching vibrations of cyclohexanone. Obviously, 1504 cm⁻¹ in Figure 1 is associated with the strong absorption bands of CH=C bond vibrations. Correspondingly, both these bands could be observed in the spectrum of LP. Therefore, we confirm that LP is obtained desirably according to our original

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expectation. Meanwhile, the branched BP was also produced successfully because of the existence of 1691 cm⁻¹, and it is corresponding to the C=O bond vibrations in cyclohexanone, respectively. And the sharp peaks of 1500 cm⁻¹ is related to the CH=C stretching vibrations (Figure 1).

Their ¹H NMR spectra were also compared in Figure 2. Obviously, in the polymer LP, the typical CH₂ protons of CYC resonances at the blue color-marked region (from 0.5-3.0 ppm), the CH=C (7.71 ppm)and ArH protons of PDBD peaked in the pink color-marked region (from 7.0-8.0 ppm). In sharp contrast, the resonance strength of CH₂ from CYC, which neighbors to C=O group, is decreased greatly in LP. This result confirms the aldol condensation taking place between the two partners of CYC and PDBD. We also found that due to the feed ratio of two monomers is 1:1, the small peak resonances at 10 ppm could be seen, which is due to the non-reacted protons of -CHO. The ¹³C NMR spectrum of LP is given in Figure 2. No unexpected signals are detected and all the peaks could be readily assigned by comparing the spectrum with those of its monomers. The ¹H NMR and ¹³C NMR spectrum of BP is also given in supporting information. Thus, all the ¹H NMR and ¹³C NMR data prove that the polymeric product is indeed LP and BP, with a molecular structure as shown in Scheme 1.



Figure 1.(Left) FT-IR spectra of (A) CYC and (B) PDBD and (C) LP. (Right) FT-IR spectra of (A) CYC and (B) NTBD and (C) BP.



Figure 2. (Left) 1 H NMR spectra comparison of CYC (A), PDBD (B) and LP (C). (Right) 13 C NMR spectra of LP.

2.2 The solvent effect

The steric hindrance of twisted and propeller-shape configuration of TPA in the polymer chains could result in large intermolecular distances and large free volume to accommodate solvent molecules. Therefore, LP has very good solubility in different solvents. On the contrary, BP has lower miscibility to the organic solvents due to the more rigid

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skeleton. Initially, we studied the optical properties in different solvents. LP and BP illustrate a negligible influence on the absorption with the variation in solvent. This indicates that the solvent polarity has little effect on its ground-state electronic transitions.²⁸ However, the emission behaviors vary dramatically according to different solvent. LP and BP fluorescence emission spectra are given in Figure 3. To see whether there is any correlation between the optical properties of the luminophores and the solvent polarity, the solvent polarity parameters (Δ f) are also shown in Figure 3, which are expressed by the following equation:

$$\Delta f = f(\varepsilon) - f(n^2) \simeq \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1}$$

where ε and n are the permittivity (or dielectric constant) and the refractivity (or refractive index) of the solvent, respectively.^{29,30} In the solvent of toluene ($\Delta f \approx 0.014$), LP shows emission peak at 495 nm (Figure 3). When the solvent polarity is increased, the emission band is red-shifted (from 495 to 580 nm) with a concomitant decline in the emission intensity. In the very polar solvent of acetonitrile (ACN, $\Delta f \approx$ 0.306), the emission spectra are so weak. Similarly, the emission of BP is red-shifted by 17 nm and weakened in intensity with an increase in the solvent polarity.



Figure 3. (Left) The emission spectra of LP in different solvents and the photographs of LP in different solvents taken under UV illumination at 365 nm. (Right) The emission spectra of BP in different solvents and the photographs of BP in different solvents taken under UV illumination at 365 nm.

As reports declared that polymers containing both electron donor and acceptor moieties within a repeating unit contribute to electronic transition between the ground and excited states, which could be manipulated by the induced charge transfer (CT) from donor (D) to acceptor (A).^{31,32} The intramolecular charge transfer (ICT) has been well explained and is responsible to the solvatochromism.³³ Then, theoretical calculation using DFT//B3LYP/6-31G level in the Gaussian 09program is carried out to characterize the three-dimensional structures and the frontier molecular orbital energy levels of the repeating unite (DBC). As illustrated in Figure 4, DBC shows the three-dimensional propeller structure with a narrow energy gap of 0.12743 eV. The HOMO orbital is mostly developed in the TPA core, and LUMO orbital extends across

to the CYC segment, which indicates that DBC has obvious ICT tendency. Therefore, LP and BP are able to display the solvent-dependent chromic effects.^{34,35} This structural pattern renders materials more emissive and sensitive to the micro-environment changes. Especially, among of research context, TPA-based D-A polymers especially attracted significant attentions.³⁶

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Figure 4. (A) The chemical structure of DBC. (B) and the optimized molecular conformation. (C) orbital amplitude plots of HOMO and LUMO energy levels of DBC.

2.3 AIEE properties

Irradiating LP and BP by UV light affords emissive green, and red colors in THF, with emission peaks at 528, and 583 nm, respectively. Their solids emit maximally at 575, and 615 nm, which are red-shifted with 47 and 32 nm respectively when compare with those in THF (Figure 5). Fortunately, thanks for the structurally soft cyclohexanone and the propeller-shaped TPA, the resulted polymers LP and BP demonstrate excellent gelating ability in the solvent of CH_2Cl_2 and THF respectively. The critical gelation concentration (CGC) value is 2.9 and 0.45 wt%, and the phase transition temperature (T_g) could be maintained at 8 and 35 °C respectively. Correspondingly, what could be noted that less emissive of the sol, but more emissive of the gel (Figure 6). These results indicate the typical AIE performance of both materials and the great potential applications in the filed of soft&smart materials.

Accordingly, we investigate this physical property in THF/water



Figure 5. (A) The emission spectra of LP in THF (conc.: $6x10^{-2} \text{ g/L}$) and solid state. ($\lambda_{ex} = 435 \text{ nm}$). (B) The emission spectra of BP in THF (conc.: $1x10^{-1} \text{ g/L}$) and solid state.($\lambda_{ex} = 480 \text{ nm}$). Insets shown the images of solid LP and BP under UV light.

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Figure 6. (A, B) Gel and sol states of LP (in CH₂Cl₂) and (C, D) BP (in THF) under room light and UV lamp.

mixtures following the documented method.³⁷ Since both of LP and BP are insoluble in water, they will aggregate in the aqueous mixtures with high water fractions. As shown in Figure 7, the emission intensity of LP is suppressed further and further when water is charged from 0 to 40% in volume, and the emission band become broader and broader. The enhancement of the ICT effect by increasing the solvent polarity upon water addition in LP may be responsible for the emission quenching.³⁸ Along with the increasing water fractions, the florescence intensity elevates gradually, and the maximum emission band narrows into a more sharp peak. Notably, whole spectrum shifts from 545 nm into 561 nm when charging water from 40% to 90%. Meanwhile, the emission peak intensity increases about 3-folds during this



Figure 7. The emission spectra of LP (Top)&BP (Bottom) in THF and in the mixture of THF&water and the plot of ratiometric fluorescence intensity and emission wavelengths versus the water fractions. Inset shown the images of LP&BP in different water fractions under UV light.

process. This fluorescence changes probably indicates the presence of the twisted intramolecular charge transfer (TICT) when solvent polarity is enhanced by adding water.³⁹⁻⁴¹ Thereby, the above results exhibit a potential AIEE effect in the case of LP. In contrast to LP, the branched BP has a lower solubility and more emissive intensity in THF, which is originated from the fact that its intramolecular rotations are highly restricted because of the much-stiffer molecular skeleton and the crowded configuration. Further fluorescence

measurement in THF/water mixtures reveals that the emission intensity of BP decreases gradually with the increasing water fractions (Figure 7). Which consistent with the emission intensity of BP in THF is strong than the solid.

From the SEM images of LP and BP in different mixture of water&THF (Figure 8), we can see that nanocale objects could not be observed when dissolving LP and BP in pure THF. In contrast, nanospheres with uniform size of 100 nm are formed and scattered on the silicon wafer clearly if water fraction rises to 40%. This property confirms the aggregation ability of the polymers LP and BP over the hydrophobic environment.



Figure 8. (A) SEM images of LP in the mixture of THF&water when $f_w = 90\%$ (B) BP in the mixture of THF&water when $f_w = 40\%$, (C) $f_w = 90\%$.

2.4 Thermosensitivity

As can be seen from the temperature-dependent florescence spectrum, LP exhibits obvious temperature sensitive property within a broad window from 0 to 60 °C (Figure 9A). For instance, the fluorescence intensity reduces as much as 3 folds, and the emission band is blue-shifted as high as 25 nm during the temperature increasing process. Comparely, BP is more sluggish than LP towards the temperature change. A small blue-shift and a little intensity reduce could be observed within the same temperature change windows (Figure 9B). This results verify the different freedom of the rotation of TPA groups in the different polymer matrix. Furthermore, LP could be possibly used as thermo-powered high-throughput visual iluminescence sensor. This work is undergoing in our research group.



Figure 9. (A) The emission spectra of LP in THF under different temperatures. (conc.: $6x10^{-2}$ g/L, $\lambda_{ex} = 435$ nm). (B) The normalized emission spectra of BP in THF under different temperatures. (conc.: $1x10^{-1}$ g/L, $\lambda_{ex} = 480$ nm). Inset shown image of LP and BP in THF taken at 0 and 60 °C under UV light.

2.5 Cell cytotoxicity assessment and cellular imaging applications

The novel AIEE effect and bio-compatibility of LP encourage us to utilize it as a bioprobe for cellular imaging applications.^{42,43} With concentration of 50 μ g/mL, DLS measurement presents a hydrodynamic diameter of 126 nm and a polydispersity index

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(PDI) value of 0.583 (Figure S5). The cell viability is up to 92% at the concentration of 50 µg/mL (Figure 10). Conventionally, fluorophores can hardly be retained inside live cells for a long period of time.^{44,45} To examine if LP will diffuse from the intracellular compartments to the extracellular media, living MPC5 cells stained by LP are studied. From Figure 10, we can see that the bright orange-red fluorescence is collected from the cytoplasm, which suggests that LP molecules enter into the cells and aggregate in the cytoplasm successfully. In other words, the cytoplasm is successfully labeled by the AIEE materials. These results also confirm that the AIEE-active molecules show low cytotoxicity and can be efficiently internalized into MPC5 cells. Notably, LP molecules behaves remarkable leakage-free staining properties which are difficult to extrude out once they are internalized into the cells, but also the fluorescence intensity of the stained cells does not change much. Therefore, we can conclude that LP is of great potential to be employed as a promising bioprobe for cell imaging.



Figure 10. Cell viabilities of MPC5 cells which treated with different concentrations of LP for 96 h by MTT assay.



Figure 11. Images of MPC5 cells stained with LP (50 µg/mL).(A) Bright field image , (B) confocal microscopy images of MPC5 cells incubated with LP (50 mg/mL) for 0.5 h, (C) overlay of (A) and (B).

3. Experimental section

3.1 Materials and instrumentation

Triphenylamine (TPA, 99%), phosphorus oxychloride (POCl₃), cyclohexanone (99.5%), sodium hydroxide (NaOH, 96.0%), were purchased from Aladdin Co. Other commercial reagents were purchased from Aladdin Co. and Energy Chemical Co. and used without further purification unless otherwise stated. Room temperature ¹H NMR (600 MHz) and ¹³C NMR (150 MHz) spectra were recorded on a MERCURY spectrometer with tetramethylsilane (TMS) as the internal reference. Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet AVATAR 360 FT-IR spectrophotometer using KBr tablets. UV- visible absorption spectra (UV) were recorded on a TU-1901 spectrometer from 190 to 1100 nm. Fluorescence spectra were measured using a PE LS-55 Luminescence/Fluorescence Spectrophotometer. The morphology of the compound was observed by scanning electron microscopy (SEM, Zeiss Ultra Plus. The number-average molecular mass (Mn), weight-average molecular mass (Mw), and polydispersity (Mw/Mn) of polymer were determined by a gel permeation chromatography (GPC). THF was employed as the eluent at a flow rate of 1.0 mL/min at 35 °C. Particle size measurements process were performed on a Zetasizer Nano ZS dynamic light scattering (DLS) system.

3.2 Synthesis

3.2.1 Compound 4,4'-(phenylazanediyl)dibenzaldehyde (PDBD)⁴⁶

Triphenylamine (4.91 g, 20 mmol) was dissolved in DMF (16 mL) and placed in a 100 mL flask. Phosphorous oxychloride (16 mL) was added dropwise in ice bath, and the reaction mixture was stirred for 10 min at 0 °C. And then the mixture was refluxed at 80 °C for 10 h under N₂ atmosphere. Then, reaction was quenched with cold water (100 mL) and yellow solid was precipitated. The crude product was purified over a silica gel column with mixture (ethyl acetate /petroleum ether, 1:40) as eluent to give PDBD as a yellow solid (yield: 79%). ¹H NMR (600 MHz, CDCl₃), δ (TMS, ppm): 9.90 (s, 2H), 7.78 (d, J = 8.0 Hz, 4H), 7.40 (t, J = 7.6 Hz, 2H), 7.29-7.24 (t, 1H), 7.21-7.17 (d, J = 8.0 Hz, 4H), 7.18-7.15 (d, J = 7.2 Hz, 2H). ¹³C NMR (150 MHz, CDCl₃), δ (TMS, ppm): 190.38, 151.99, 131.27, 130.13, 126.96, 126.23, 122.63. IR (KBr), v (cm⁻¹): 3058, 2921, 2852, 1589, 1280, 835, 751, 697.

3.2.2 Compound 4,4',4"-nitrilotribenzaldehyde (NTBD)⁴⁷

PDBD (3.01 g, 10 mmol) was dissolved in DMF (8 mL) and placed in a 100 mL flask. Phosphorous oxychloride (8 mL) was added dropwise in ice bath, and the reaction mixture is stirred for 10 min at 0 °C. And then the mixture was refluxed at 100 °C for 8 h under N₂ atmosphere. The reaction solution was purified over a silica gel column with mixture (ethyl acetate /petroleum ether, 1:20) as eluent to give NTBD as a dark yellow solid (yield: 63%). ¹H NMR (600 MHz, CDCl₃), δ (TMS, ppm): 9.93 (s, 3H), 7.83 (d, J = 3.6 Hz, 6H), 7.23 (d, J = 6.6 Hz, 6H). ¹³C NMR (150 MHz, CDCl₃), δ (TMS, ppm): 190.42, 151.14, 132.58, 131.41, 124.51. IR (KBr), v (cm⁻¹): 3052, 2924, 2852, 1600, 1275, 818.

3.2.3 Polymers

The polymerization was carried out according to the standard ACC reaction conditions.⁴⁸ PDBD (330 mg, 1 mmol) and cyclohexanone (97 mg, 1 mmol) were charged into the solvent of EtOH (20 mL) in the presence of 20% NaOH aqueous (5 mL). The mixture was heated to 60 °C and vigorously stirred for 14 h. EtOH was evaporated and the resulted precipitates were washed with distilled water several times to judge pH = 7.0. Crude product was extracted by mixture of ethyl acetate and petroleum ether (1:10 by volume) at 85 °C for 48 h by Soxhlet extraction. Then the powders were dried under vacuum at 70 °C for 5 h to give light orange solids LP. Mw=29800; Mw/Mn =

1.45 (GPC, polystyrene calibration). ¹H NMR (600 MHz, CDCl₃), δ (TMS, ppm): 9.85 (CHO), 7.75 and 7.05-7.49 (ArH), 7.71 (CH=C), 1.0-2.6 (CH₂). ¹³C NMR (150 MHz, CDCl₃), δ (TMS, ppm): 212.19, 190.70, 152.76, 145.83, 131.88, 131.27, 129.88, 129.60, 125.56, 124.57, 41.95, 26.99, 24.97. IR (KBr), v (cm⁻¹): 3062, 3036, 2924, 2854, 1690, 1602, 1589, 1504, 1272, 832, 752, 695. The same process could be applied for the synthesis of BP. BP was afforded as red powders. Mw = 15820; Mw/Mn = 1.43. ¹H NMR (600 MHz, d₆-DMSO), δ (TMS, ppm): 9.92 (CHO), 7.89 and 7.26 (ArH), 7.79 (CH=C), 2.24, 1.8-1.7 and 1.68-1.57 (CH₂). ¹³C NMR (150 MHz, d₆-DMSO), δ (TMS, ppm): 210.85, 191.93, 151.25, 132.66, 131.89, 124.89, 41.77, 26.86, 24.77. IR (KBr), v (cm⁻¹): 3064, 3032, 2923, 2852, 1691, 1601, 1500, 1273, 830, 751, 697.

3.3 Cell culture

The cytotoxicity test was performed with MPC5 cells. LP was sterilized with ultraviolet light and dissolved in DMSO. Then the solution was diluted with PBS buffer (pH = 7.4) to different concentrations (30, 40, 50, 60, 70 and 80 μ g/mL). To obtain complete cell culture medium, 10% FBS, 100 units mL⁻¹ penicillin, and 100 units mL⁻¹ streptomycin were added in the mixture. MPC5 cells (purchased from Gansu Provincial Cancer Hospital, Gansu, China) were cultured in the conditioned medium at 37 °C in a humidified environment of 95% O₂ and 5% CO₂. After 96 h of incubation, cell viability was determined bythe3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

3.4 Cell imaging

Living MPC5 cells $(1.0 \times 10^5 \text{ per well})$ were cultured overnight and then stained in a Petri dish at 50% confluence with a LP solution (50 µg/mL). An image was taken after 0.5 h incubation.

4. Conclusions

In this paper, we present the facile synthetic method by Aldol Addition and Condensation (AAC), leading to the formation of florescent linear (LP) and branched polymers (BP) by cross coupling triphenylamines (TPA) and cyclohexanones (CYC) via C=C bond. That methodology takes advantages of easy operations, mild reaction conditions (*Inert gas protect-free, Solvent dryness-free, Moderate temperature*). The further optical analysis reveals that LP and/or BP demonstrate solventdependent chromic effect, AIEE property, organogel formation ability, thermosensitivity, and better bio-compatibility. Notably, LP is readily internalized by MPC5 cells, and it behaves good biocompatibility and high resolution. Therefore, the readily available materials are of the potential versatile applications in the fields of bioimaging. Further research is underway in our lab.

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Text: Fluorescent polymers synthesized by ACC reactions with interesting optical performances and the potential cell imaging applications.