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FRET Interaction Between Pyrene-Tagged β-Hydroxy Acid and Perylen-3-ylmethylmethacrylate *co*-*N*-Dodecylmethacrylamide in Film and Solution

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ABSTRACT: Synthesis, characterization, and FRET interaction of pyrene-tagged β-hydroxy acid and perylen-3-ylmethylmethacrylate co-*N*-dodecylmethacrylamide polymer are described. The fluorescent donor, β-hydroxy acid-pyrene boronic ester was synthesized from commercially available alkyl ketene dimer via subsequent hydrolysis, reduction and esterification providing the donor. Conversely, the fluorescent acceptor was synthesized by co-polymerization of N-dodecylmethacryamide and perylen-3-ylmethylmethacrylate, where the latter was prepared from commercially available perylene in three steps. FRET interaction between the donor and acceptor was carried out by titration and monitored using fluorescence spectroscopy. Fluorescence quenching and enhancement in both film and solution formats were observed; furthermore, a more prominent lipophilic-induced interaction was observed in solution. Fluorescence enhancement (at 460 nm) is higher for the acceptor and quenching efficiency (at 420 nm) is higher for donor-acceptor pair, pyrene-tagged β-hydroxy acid and perylen-3-ylmethylmethacrylate co-*N*-dodecylmethacryl-amide polymer, compared to control, pyrene boronic ester. The quenching is due to dynamic quenching between the donor and acceptor. Furthermore, aggregation was not observed from the pyrene-tagged β-hydroxy acid ester donor as the concentration of the solution is increased up to 3000 ppm. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 2865–2872, 2013

KEYWORDS: films; copolymers; dyes/pigments; optical properties; synthesis; processing

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INTRODUCTION

Copolymers containing covalently-linked fluorescent dye are materials with interesting chemical and optical properties. These copolymers can be easily tuned or modified by careful choice of monomers to obtain materials with high photostability and good processability. Consequently, these copolymers have been used in various applications, such as organic or polymeric electroluminescent devices,[1–4] liquid crystalline system,[5] as material for studying interaction,[6] reaction,[7] and aggregation,[8–10] and as material for sensing.[11]

Fluorescent co-polymers are attractive materials for sensing and recognition application, since analyte binding results to change in photophysical response of the material. These systems have been used for the detection of metal ions, anions, nucleic acid, and carbohydrates[12–14] where the binding event results to enhancement or quenching response. Other advantages of using fluorescent co-polymer include processability and ease of structural modification.

Although there are numerous advantages on the use of fluorescent copolymer as indicated by the number of papers published in this area, and the use of fluorescence resonance energy transfer (FRET) as means for quantifying the signal, there are limited studies carried out on recognition under lipophilic conditions. Once such study is the work reported by Yamaguchi.[15]

Herein, we report a FRET recognition between a lipid-containing β-hydroxy acid moiety and a fluorescent copolymer. The FRET pair is composed of pyrene-tagged β-hydroxy acid donor

Additional Supporting Information may be found in the online version of this article.

and perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer as acceptor fluorophore. The use of pyrene–perylene as FRET pair has already been reported in the literature.[16,17] Herein, we report a recognition system where a β-hydroxy acid was tagged with pyrene boronic acid, producing donor fluorophore conjugate **1**. The acceptor is composed of a hydrophobic monomer, *N*-dodecylmethacrylamide **2**, copolymerized with perylen-3-ylmethyl-methacrylate **3**. The lipophilic interaction between the tail of the tagged β-hydroxy acid and the fluorescent lipophilic polymer would allow the partner fluorophores to interact with each other. The resulting FRET interaction between the fluorescence acceptor co-polymer and pyrene-tagged donor was investigated both in film format and in solution.

EXPERIMENTAL

Azobisisobutyronitrile (AIBN) was obtained from Shandong Haiming Chemical and Industrial, China and recrystallized from absolute ethanol prior to use. Unless indicated, all solvents were used straight from the bottle. Hexane (technical grade) was fractionally distilled and stored under sodium sulfate before use. Tetrahydrofuran was distilled over sodium and benzophenone before use. Chloroform, triethylamine and *o*-dichlorobenzene were dried over calcium hydride and distilled prior to use. 3 Å molecular sieves were activated prior to use.

Preparatory TLC plates (1 mm thick) were prepared using Merck silica gel 60 GF254 (0.063–0.200mm). Silica gel was suspended in ethanol (4.0 g silica:12.0 mL) and transferred to a 20 X 20 cm glass plates. The solvent was allowed to evaporate and the plates were placed in an oven for 30 min at 110°C.

Synthesis of Donor Fluorophore

Ring Opening of Alkyl Ketene Dimer. The ring opening of alkyl ketene dimer (AKD, 6.11 g, 13.6 mmol) was carried out under NaOH (0.09 g, 2.25 mmol) in methanol (11.0 mL) for 12 h. The resulting β-keto ester (6.17 g, 12.83 mmol) was reduced with sodium borohydride (0.73 g, 19.3 mmol) in 1:1 tetrahydrofuran/methanol (300 mL) for 5 h to provide the β-hydroxy ester **4**. The crude product was purified using flash chromatography (2% EtOAc/Hexane) to obtain a white solid (5.99 g, 81%). Hydrolysis of the crude β-hydroxy ester (5.99 g, 12.40 mmol) with KOH (0.84 g, 15.0 mmol) in ethanol (300 mL) and water (15 mL) yielded β-hydroxy carboxylic acid **5** (2.93 g, 50%) as a white solid. Purification of crude β-hydroxy carboxylic acid using silica gel (Merck silica gel, 40-60 μm mesh) and 8% EtOAc/Hexane with 0.5% acetic acid gave two β-hydroxyl carboxylic acids. β-hydroxyacid **5-1** (R_f = 0.31, 1:5 EtOAc/Hexane): IR (neat) 3327, 3237, 2916, 2849, 1697, 1468 and 719 cm^{-1}. β-hydroxyacid **5-2** (R_f = 0.19, 1:5 EtOAc/Hexane); MP: 69.8-70.2°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72 (m, 1H), 2.46 (m, 1H), 1.15-1.8 (bm, 48 H), 0.88 (t, J = 8.0 Hz, 6H) (Supporting Information Figure S2); IR: (KBr) 3334, 2917, 2850, 1703, 1468, 1218, 1107 cm^{-1} (Supporting Information Figure S3).

Synthesis of Pyrene-Tagged β-Hydroxy Acid. β-hydroxyacid **5-2** (200 mg, 0.43 mmol), pyrene boronic acid (Aldrich, 95 mg, 0.39 mmol), 3 Å MS (Merck, 2.37 g) in $CHCl_3$ (25 mL) were stirred under N_2 for 48 h. The mixture was removed and the molecular sieve was washed with $CHCl_3$. The combined organic layer was dried *in vacuo* to provide a pale yellow solid (0.27 g, 95%). The racemic mixture was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ aromatic 9.06 (d, J = 7.99 Hz, 1 H), 8.59 (d, J = 4.00 Hz, 1 H), 8.05 (m, 9 H), 4.55, 4.38 (m, 1 H), 2.99, 2.76 (m, 1 H) (Supporting Information Figure S4). ^{13}C NMR (60 MHz, $CDCl_3$,) 169.3, 137.0, 134.2, 131.1, 130.5, 129.2, 128.3, 127.4, 125.7, 124.5, 124.1, 47.7, 35.8, 31.9, 29.7, 27.3, 26.9, 25.4, 22.7, 14.1 (Supporting Information Figure S5).

Synthesis of the Acceptor Fluorophore

Synthesis of Perylen-3-ylmethanol. A 250-mL flask was charged with *N*-methylformanilide (Aldrich, 11.0 g, 0.081 mol) and freshly distilled o-dichlorobenzene (60 mL). Phosphorus oxychloride (Merck, 11.0 g, 0.072 mol) was added and stirred for 15 minutes at room temperature under N_2 gas. Perylene (Suzhou Sinosun, 8.7 g, 0.034 mol) was added into the flask and stirred at 85°C for 18 h. Saturated sodium acetate solution (50 mL) was added and stirred for 2 h. The reaction mixture was then extracted with DCM (3 × 650 mL). The extracts were then combined, and washed with distilled water (1 × 1 L), and dried over anhydrous sodium sulfate. The purple crude was purified by column chromatography using EtOAc/hexane (1:9 to 3:1). Light brown crystals (5.0 g, 53%) were obtained. λ_{max}= 477 nm ($CHCl_3$), R_f = 0.74 (20% EtOAc/Hexane).

A 250-mL flask was charged with perylene-3-carbaldehyde (3.0 g, 0.011 mol), sodium borohydride (Hi-Media, 2.2 g, 0.058 mol) and 1:1 methanol/tetrahydrofuran (1:1, 60 mL). The mixture was stirred at 80°C for 4 h under N_2 gas. The reaction was monitored via TLC using EtOAc/Hexane (1:4). After the disappearance of the starting material, 1M HCl (50 mL) was added slowly until no effervescence was observed. The organic solvent was removed *in vacuo* then extracted with DCM (3 x 200 mL). The organic layer was then combined, washed with 5% aqueous sodium bicarbonate (100 mL), and water (100 mL), and dried over anhydrous sodium sulfate. Yellow crystals (1.98 g, 64%) were obtained. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.20 (m, 4 H), 7.97 (d, J = 8.39 Hz, 1 H), 7.70 (d, J = 7.99 Hz, 2 H), 7.54 (m, 4 H), 5.10 (s, 2 H). Hydroxyl proton was not detected as the sample is allowed to undergo D_2O exchange. Rf (20% EtOAc/Hexane) = 0.37. The solubility of perylen-3-ylmethanol is as follows: 10 mg of the alcohol dissolves in 4 mL DCM, 3 mL $CHCl_3$, or 1 mL tetrahydrofuran.

Synthesis of Perylen-3-ylmethylmethacrylate. Methacryloyl chloride (0.10 mL, 1.01 mmol) in tetrahydrofuran (0.9 mL) was added dropwise to a solution of perylen-3-yl methanol (68.4 mg, 0.241 mmol) in tetrahydrofuran (5.00 mL) at 0°C. The resulting solution was stirred for 24 h at room temperature and then filtered to remove triethylammonium chloride salts. The filtrate was neutralized with 1M HCl and washed thrice with 5% $NaHCO_3$ and brine (10 mL). The organic layer was collected and dried using sodium sulfate. The solvent was removed *in vacuo*. The crude product was purified using preparative TLC with DCM in hexane (1:4) as the eluent. Yellow powder (0.0733 g, 0.208 mmol, 86%) was obtained. ^{1}H-NMR (400 MHz, $CDCl_3$) aromatic δ 8.20 (dd, J = 7.6, 0.76 Hz, 1 H), 8.17 (m, 2

H), 8.12 (d, $J = 7.6$ Hz, 1 H), 7.83 (dd, $J = 8.5$, 0.72 Hz, 1 H), 7.68 (m, 2 H), 7.53 (m, 2 H), 7.47 (ddd, $J = 7.6$, 7.6, 1.2 Hz, 2 H); 6.16 (dddd, $J = 1.9$, 0.96, 0.96, 0.96 Hz, 1 H), 5.58 (dddd, $J = 1.7$, 1.7, 1.7, 1.7 Hz, 1 H); 5.56 (s, 2 H); methyl 1.98 (dd, $J = 1.5$, 1.0 Hz, 3H) (Supporting Information Figure S6). ^{13}C NMR (60 MHz, $CDCl_3$,) 167.32, 136.18, 134.53, 132.94, 132.05, 131.75, 131.03, 130.98, 130.82, 128.94, 128.39, 128.09, 127.98, 126.98, 126.56, 126.55, 126.00, 123.26, 120.48, 120.47, 120.27, 119.54, 64.90, 18.37 (Supporting Information Figure S7); IR: (KBr) 3045, 2955, 1713, 1640-1527, 1500, 1150, 814, 762 cm^{-1} (Supporting Information Figure S8).

Synthesis of N-dodecylmethacrylamide (N-DODMA). A 1-L flask was charged with n-dodecylamine[18–19] (Aldrich, 27.6 g, 0.15 mol), triethylamine (Merck, 71.1 mL, 0.51 mol) and tetrahydrofuran (300 mL). The solution was stirred at 0°C for one h, and then methacryloyl chloride (16.5 mL, 0.172 mol) in tetrahydrofuran (100 mL) was added dropwise over 30 min. The solution was removed from the ice bath and allowed to stir at rt overnight. The reaction mixture was then filtered, washed with HCl (1*M*, 100 mL), 5% sodium bicarbonate (100 mL), and with brine (50 mL). The organic layer was collected and dried with sodium sulfate. The solvent was removed *in vacuo* to produce a viscous yellow oil. The crude product was recrystallized with hexane. White solid, 28.25 g, 65% was obtained. R_f (EtOAc/Hexane 1:9) = 0.27; ^1H-NMR (400 MHz, $CDCl_3$) δ 5.76 (bs, 1H), 5.66 (s, 1H), 5.31 (s, 1H), 3.30 (dd, $J = 6.3$ Hz, 2H), 1.96 (s, 3H), 1.2-1.6 (bm, 20 H), 0.88 (t, J = 7 Hz, 3 H). IR: ($CHCl_3$) 3410, 2928, 2928, 2857, 1659, 1064 cm^{-1}

Synthesis of Perylen-3-ylmethylmethacrylate co-N-dodecylmethacrylamide Polymer. A test tube was charged with *N*-DODMA (0.200 g, 0.789 mmol) and perylen-3-ylmethylmethacrylate (1.26 mg, 3.6×10^{-3} mmol) in methanol (1.26 mL). The solution was sonicated for 15 min, after which, 3 mol % of AIBN (0.0039 g) was added. The test tube was purged with N_2 for one min and heated at 80°C for 24 h. The resulting polymer was diluted with $CHCl_3$ (1 mL) and precipitated with methanol (5 mL). The polymer was washed with methanol three times and air-dried overnight to obtain a translucent yellow solid with an average yield of 40%. (HNMR, Supporting Information Figure S9); IR: (neat) 3345, 2920, 2850, 1645, 1515, 1465, 1195, 714 cm^{-1}(Supporting Information Figure S10).

Relative molecular weights and molecular distribution were determined using Gel Permeation chromatography (GPC) using Waters (Alliance GPCV 2000) Styragel HR [5E-Linear and 3 (10^3 Å)] columns; polystyrene standards were used to calibrate. The mobile phase used was tetrahydrofuran with a flow rate of 1.00 mL/min at 40°C (Supporting Information Figure S11).

FRET Study

FRET Film. Polymer solution in $CHCl_3$ (10 μL) was deposited on a quartz plate using a micropipette positioned one cm above the slide. The solvent was allowed to evaporate under rt for 15 min. The thickness of the films was obtained using a profilometer (Alpha-Step® 500 Surface Profiler with scan length of 500 μm, scan speed of 50 μm/s, horizontal resolution of 1.00 μm, and stylus force of 2.4 mg). Nine different points of the film were determined.

Figure 1. Fluorescence spectroscopy set-up for the film study. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The schematic diagram of the fluorescence spectroscopy set-up employed in this study is shown in Figure 1. The excitation source is a 25 mW Q-switched Nd:YVO laser (CrystaLaser) with an output wavelength of 355-nm and a repetition rate of 20 kHz. A mirror is used to direct the beam at normal incidence onto the film. Spectral data were collected with a USB2000 spectrometer (Ocean Optics) interfaced to a computer. The beam direction, film long axis and detector orientation are mutually orthogonal. Distance of the probe from the film is maintained at a fixed distance for all runs.

Enhancement Study. Five solutions were prepared containing 0, 100, 200, 300, and 400 ppm pyrene-tagged β-hydroxy acid and constant perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer concentration of 200 ppm in $CHCl_3$.

Quenching Study. Five solutions were prepared containing 0, 18, 37, 56, and 75 mg perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer/mL $CHCl_3$ and a constant pyrene-tagged β-hydroxy acid concentration of 200 ppm.

FRET Solution

Enhancement Study. Stock solutions of donor fluorophore (pyrene boronic acid and pyrene-tagged β-hydroxy acid), and acceptor fluorophore (perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer) in $CHCl_3$ were prepared. Pyrene boronic acid and pyrene-tagged β-hydroxy acid served as the donor.

To check for fluorescence enhancement, acceptor fluorophore (perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer) concentration was kept constant at 1000 ppm, whereas the donor fluorophore (pyrene boronic acid or pyrene-tagged β-hydroxy acid) concentrations were varied from 0 ppm to 1000 ppm, in 100 ppm increments. Samples of 1 mL solution containing both the donor and acceptor fluorophore in $CHCl_3$ were prepared and were analyzed using a Grenier F-bottom sample plates in a BMG FluoStar spectrofluorometer. Gain adjustment was set at 850 for all runs. The excitation and emission wavelength were set at 355 nm and 460 nm.

For dilute runs, the concentration of pyrene-tagged β-hydroxy acid concentration was varied—0, 50, 100, 150, 200, 250, and 300 ppm. For concentrated samples, the pyrene-tagged β-hydroxy acid concentration was varied from 0 ppm to 3000 ppm, in 500 ppm increments, while the concentration of the acceptor fluorophore perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer concentration was kept constant at 1000 ppm.

Quenching Study. To check for fluorescence quenching, donor fluorophore—pyrene boronic acid or pyrene-tagged β-hydroxy

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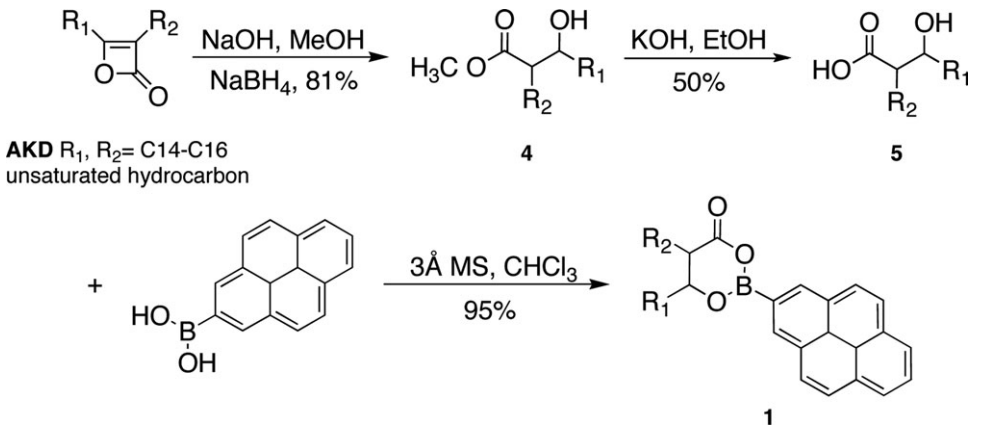


Scheme 1. Preparation of donor fluorophore, pyrene-tagged β-hydroxy acid **1**.

acid—concentration was kept constant 1000 ppm for all 1 mL solutions, whereas the acceptor fluorophore—perylen-3-ylmethylmethacrylate *co*-*N*-dodecylmethacrylamide polymer—concentrations were varied from 0 ppm to 1000 ppm, in 100 ppm increments. The sample solutions with a total volume of 1 mL were prepared containing both the donor and acceptor fluorophore in $CHCl_3$. The excitation and emission wavelength were set at 355 and 420 nm.

RESULTS AND DISCUSSION

Preparation of Donor and Acceptor Fluorophore

The donor, a compound containing β-hydroxy acid moiety with shorter alkyl side chains, was synthesized starting from commercially available alkyl ketene dimer (AKD) (Scheme 1). The preparation of β-keto esters from AKD is derived from an industrial process developed by Boese for the preparation of hydrophobic cellulose.[20] AKD undergoes rapid ring opening with methanol to form β-keto esters under basic conditions.[21] The intermediate was transformed into β-hydroxy esters by subsequent reduction with sodium borohydride to obtain **4** in 81% yield. The β-hydroxy ester was hydrolyzed to provide the hydroxy acid **5** as a white powder. Facile condensation of the acid **5** with pyrene boronic acid gave the donor ester **1**.

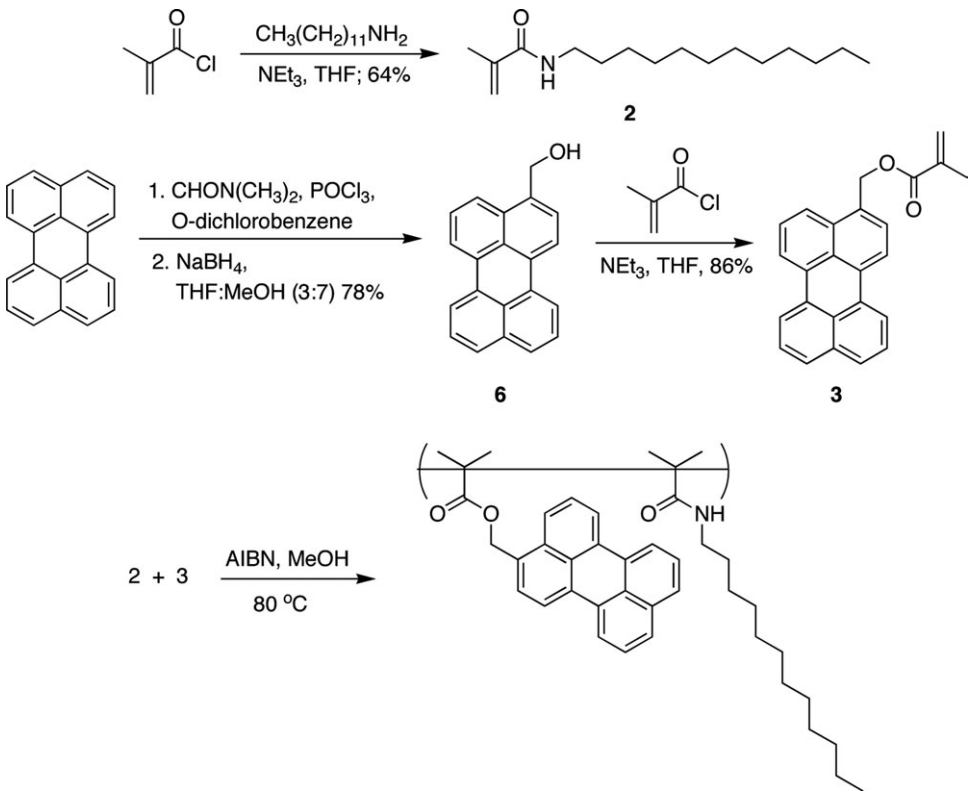
The acceptor copolymer was synthesized from *N*-dodecylmethacrylamide **2** (*N*-DODMA) and perylen-3-ylmethylmethacrylate **3** (Scheme 2). Compound **2** was synthesized starting from *n*-dodecylamine and methacryloyl chloride. Perylen-3-ylmethylmethacrylate **3** was obtained from commercially available perylene. The carbonyl moiety was installed under Vilsmeier–Haack condition,[16,22] reduction of the aldehyde provided alcohol **6** in 78 % yield over two steps. Esterification provided monomer **3** in 86% yield.

Poly(*N*-dodecylmethacrylamide) has been prepared previously.[18,19] In our hands, thermal-initiated polymerization of *N*-dodecylmethacrylamide (*N*-DODMA) in various solvents (toluene, methanol, tetrahydrofuran, acetonitrile, chloroform, dichloromethane) gave a clear polymer, while polymerization in the presence of ethylene glycol dimethacrylate (EDGMA) at 50 mol % total monomer concentration gave a translucent polymer both in toluene and methanol. In this study, the acceptor fluorophore was prepared under thermal polymerization in the presence of *N*-DODMA **2** with fluorescent monomer perylen-3-ylmethylmethacrylate **3** (219: 1 mole ratio) and AIBN in methanol with no stirring of the reaction mixture (Scheme 2). The product, perylen-3-ylmethylmethacrylate *co*-*N*-dodecylmethacrylamide polymer, is soluble in most organic solvents, such as ethyl acetate, chloroform, methylene chloride, hexane, toluene, and partially soluble in isopropanol, and insoluble in acetonitrile, dimethylformamide and dimethylsulfoxide. GPC chromatogram showed three overlapping peaks (Supporting Information Figure S11). The M_w, M_n, PDI of the polymer were summarized: Peak 1- M_n 90,541 (Da), M_w 103,581 (Da), PDI 1.14; Peak 2- M_n 25,281 (Da), M_w 29,404 (Da), PDI 1.16; Peak 3- M_n 8,326 (Da), M_w 9,171 (Da), PDI 1.10. Although the PDI is around 1.1, peaks 2 and 3 are not as well resolved. Therefore, the polymer is polydispersed. Furthermore, ^{1}H NMR shows that there are 1:449 perylene to dodecyl groups (Supporting Information Figure S12).

FRET Interaction in Film Format and in Solution

Forster resonance energy transfer, or FRET, is a distance-dependent interaction involving the transfer of energy from an excited donor fluorophore to a nearby acceptor fluorophore. The transferred energy is then released by the acceptor fluorophore at a longer wavelength. The excitation and emission of the donor fluorophore pyrene-tagged β-hydroxy acid ester is at 242–393 and 360–428 nm in 200 ppm in $CHCl_3$, respectively; for the acceptor fluorophore perylen-3-ylmethylmethacrylate *co*-*N*-dodecylmeth-acrylamide polymer (prepared with no stirring) the absorbance is at 382–465 and emission at 433–570 nm in 25 ppm $CHCl_3$, which shows FRET overlap is possible (Figure 2).

FRET film study was carried out by applying known concentration of donor (pyrene-tagged β-hydroxy acid) on a thin film of acceptor (perylen-3-ylmethylmethacrylate *co*-*N*-dodecylmethacrylamide polymer). The unexpanded laser beam was incident only on one point, since it was previously shown that aggregation of chromophore molecules in low molecular weight fatty film is possible[23,24] and the aggregation is more prominent in the condensed phase. From the emission, FRET interaction was evident. As more donor was added on the quartz slide, an increase in the emission of the acceptor was observed. However,



Scheme 2. Preparation of acceptor fluorophore perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer (~449:1 dodecyl:perylene ratio by ^{1}H-NMR, Supporting Information Figure S13) from *N*-dodecylmethacrylamide (*N*-DODMA) **2** and perylen-3-ylmethylmethacrylate **3**.

since the acceptor is not covalently linked to the quartz surface, inconsistent emission of the acceptor confirms the displacement as the donor is added. Hence, fluorescence enhancement of the acceptor molecule was investigated by mixing different concentration of the donor (0, 100, 200, 300, and 400 ppm pyrene-tagged β-hydroxy acid) with constant perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer concentration of 200 ppm (Figure 3). These solutions (10 μL) were drop-casted on a quartz slide. Since the thickness of the film is not easy to control using drop-casting method, different representative films were subjected to thickness study using a profilometer. These films were determined to have a thickness ranging from 1.111 to 5.870 μm. Figure 3 shows the fluorescence intensity of the acceptor molecule was enhanced by the addition of the donor fluorophore.

Quenching experiment was also performed to verify the FRET interaction between the donor and acceptor fluorophore. Quenching is a process where a decrease in the emission intensity of the sample occurs in the presence of molecular contact between the fluorophore and the quencher. Different molecular interactions can result to quenching, some examples are: excited-state reactions, molecular rearrangements, energy transfer, and ground-state complex formation.[25]

Energy transfer from the donor fluorophore to the acceptor fluorophore would naturally entail the quenching of the fluorescence emission of the donor fluorophore, as more acceptor

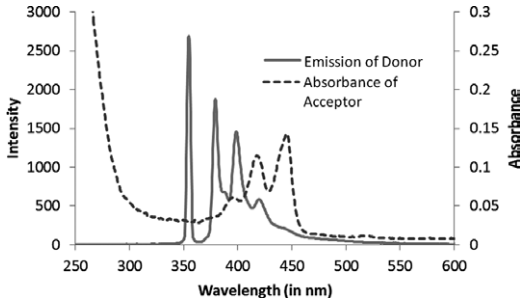


Figure 2. Emission spectrum of donor, pyrene-tagged β-hydroxy acid using 25 mW Q-switched Nd:YVO laser (CrystaLaser) with an output wavelength of 355-nm (200ppm in $CHCl_3$); Absorbance spectrum of acceptor, perylen-3-ylmethylmethacrylate *co-N*-DODMA (25 ppm in $CHCl_3$).

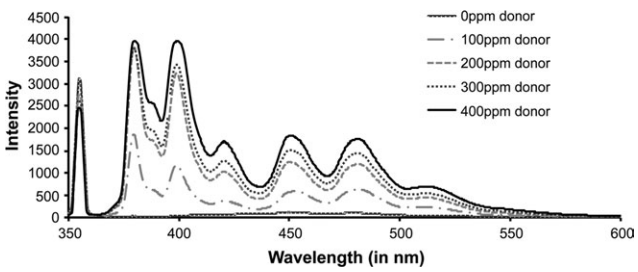


Figure 3. Fluorescence spectra of five films each containing different concentration of pyrene-tagged β-hydroxy acid while the concentration of perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer was kept constant at 200 ppm with laser excitation at 355 nm.

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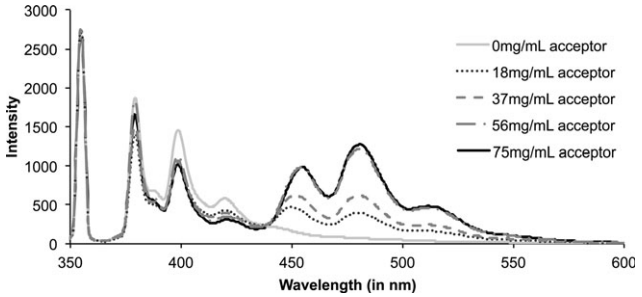

Figure 4. Fluorescence spectra of five films each containing different concentration of perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer while the concentration of pyrene-tagged β-hydroxy acid was kept constant at 200 ppm with laser excitation at 355 nm.

fluorophore is available for energy transfer. The donor concentration was kept constant while acceptor concentration was varied. Five solutions containing perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer (0, 18, 37, 56, and 75 mg/mL chloroform) and a constant pyrene-tagged β-hydroxy acid concentration of 200 ppm were used. Figure 4 shows a decreasing trend in the fluorescence intensity of the donor molecule as the concentration of the acceptor increases.

Emission profile or FRET interaction between the donor and acceptor fluorophore can differ in the polymer versus solution state due to interpolymer chain aggregation and difference in energy migration.[24] The emission profiles of pyrene-tagged β-hydroxy acid and β-hydroxy acid-free pyrene donor with the acceptor are similar. This suggests that in order for optimum interaction of the fluorophores to take place, aggregation must be minimized. Hence, fluorescence enhancement and quenching were investigated in solution.

Figure 5 plots the normalized relative fluorescence intensity (RFI) at 460 nm versus donor concentration (ppm) in chloroform. Excitation wavelength is 355 nm. As the concentration of the pyrene-tagged β-hydroxy acid donor increased, there is a corresponding increase in the fluorescence emission of the acceptor. Conversely, no significant increase in the fluorescence emission was observed using pyrene boronic acid as the donor.

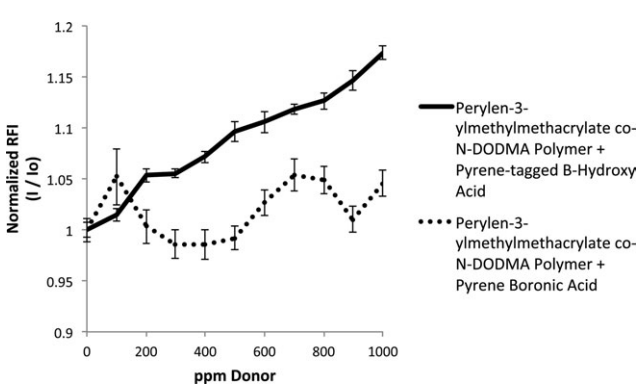

Figure 5. Fluorescence enhancement of perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer via FRET with Increasing Concentrations of pyrene-tagged β-hydroxy acid (solid) versus that of pyrene boronic acid (dotted) in chloroform.

This suggests that the energy transfer between the lipophilic pyrene-tagged β-hydroxy acid ester donor is more pronounced than the more hydrophilic pyrene boronic acid. Dilute and concentrated fluorescence enhancement runs were also performed using pyrene-tagged β-hydroxy acid ester donor. Under a more dilute condition (<100 ppm), the fluorescence enhancement trend is not evident (see Supporting Information Figure S14). The concentration of donor was increased up to 3000 ppm to determine if there is pyrene excimer formation at higher concentration. From the results, aggregation was not observed from the pyrene-tagged β-hydroxy acid ester donor as evident in the controlled enhancement trend up to 3000 ppm (Supporting Information Figure S15). In the event of pyrene aggregation, a red shift on the emission profile is expected due to the pi-pi stacking.[23] FRET interaction may no longer take place since the emission of the donor may not match the absorption of the acceptor fluorophore.

When the concentration of the donor fluorophore—pyrene boronic acid or pyrene-tagged β-hydroxy acid—concentration was kept constant 1000 ppm, and the amount of acceptor fluorophore—perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer—concentration was varied from 0 ppm to 1000 ppm (in 100 ppm increments), an observed decrease in the fluorescence emission intensity of the donor was observed (Figure 6).

The Stern–Volmer plot $I_o/I = 1 + K_{sv}[Q]$, allows the investigation of the quenching event between the donor and the acceptor. The ratio I_o/I is the fluorescence intensity in the absence (I_o) and presence (I) of the quencher; this is related to the molar concentration of the quencher [Q], and the quenching constant K_{sv}.[25] The Stern-Volmer plot of pyrene-tagged β-hydroxy acid and pyrene boronic acid with increasing amount of acceptor or quencher, perylen-3-ylmethylmethacrylate *co-N*-DODMA polymer with a slope of K_{sv} is shown in Figure 7.

Since the quenching process requires the formation of tight molecular interaction between pyrene-tagged β-hydroxy acid and the acceptor fluorophore quencher, perylen-3-

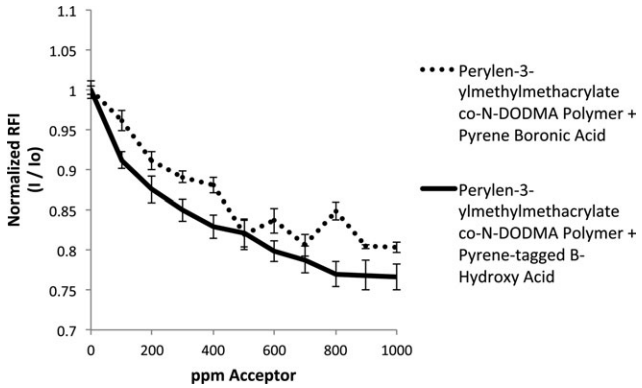

Figure 6. Fluorescence quenching of pyrene boronic acid (dotted) versus pyrene-tagged β-hydroxy acid (solid) via FRET with Increasing Concentrations of perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer in chloroform.

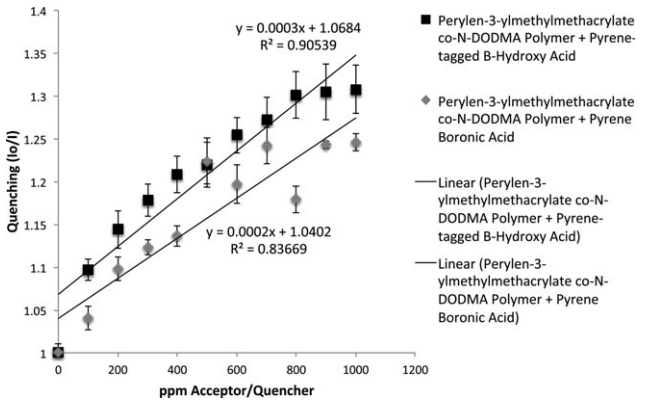

Figure 7. Stern–Volmer plot of Pyrene-tagged β-Hydroxy Acid I_o/I versus concentration of Perylen-3-ylmethylmethacrylate *co-N*-DODMA Polymer. The linear approximation gives $I_o/I = 0.0003x + 1.0684$, $R = 0.9054$. Stern–Volmer plot of Pyrene Boronic Acid I_o/I versus concentration of Perylen-3-ylmethylmethacrylate *co-N*-DODMA polymer. The linear approximation gives $I_o/I = 0.0002x + 1.0402$, $R = 0.8367$.

ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer, the transfer due to the dynamic quenching or static (complex formation) quenching is not easily distinguished in Figure 7. In dynamic quenching, the quencher must interact with an excited fluorophore, the fluorophore returns to ground state without emission of photon upon the presence of the quencher. This quenching event occurs without permanent change in the molecule. Conversely, static quenching occurs when the fluorophores form a stable complex with another molecule and this complex is not fluorescent.[25]

Since dynamic quenching always occurs, a modified Stern-Volmer plot was utilized to determine if static quenching is involved. The modified equation ($F_o/F = 1 + K_{app}[Q]$) was utilized, and the apparent quenching constant, K_{app}, is calculated at each quencher concentration. A plot of K_{app} or $(F_o/F - 1)/[Q]$ vs. $[Q]$ should yield a straight line with intercept of $K_D + K_S$ and slope of $K_D K_S$. In order to confirm the presence of both quenching mechanisms, the combined plot must show positive linearity. However, the plots obtained did not show positive linearity for both FRET pairs (Perylen-3-ylmethylmethacrylate *co-N*-DODMA Polymer + Pyrene-tagged β-Hydroxy Acid and Perylen-3-ylmethylmethacrylate *co-N*-DODMA Polymer + Pyrene Boronic Acid), suggesting that the only quenching mechanism for pyrene in this study is through dynamic quenching (Supporting Information Figure S16).

The transfer efficiency (E_{FRET}) was also evaluated. E_{FRET} is the fraction of photon energy absorbed by the donor and is transferred to an acceptor. Transfer efficiency can be measured as the relative fluorescence of the donor in presence (F) and absence (F_o) of the acceptor, $E = 1 - F/F_o$.[25,26] Since E_{FRET} is a measure of energy transfer where the end-result is quenching, it is also a measure of quenching efficiency. It can be observed based on the plot of FRET efficiency vs. Acceptor/Quencher concentration, that quenching is much more efficient in pyrene-tagged β-hydroxy acid compared to the pyrene boronic acid (Figure 8).

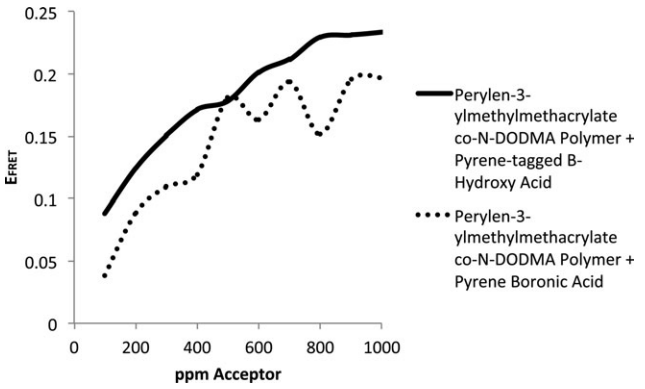

Figure 8. Quenching efficiency of the donor pyrene-tagged β-hydroxy acid as acceptor perylen-3-ylmethylmethacrylate *co-N*-DODMA polymer concentration increases.

Since the E_{FRET} is dependent on the distance between donor-acceptor fluorophore, the donor to acceptor separation distance (r) was calculated for pyrene-tagged β-Hydroxy Acid and pyrene boronic acid. The equation $E = 1/(1+r/R_o)^6$ was utilized, where R_o is the Forster distance of the donor and acceptor pair where the transfer efficiency is 50%, and E is the FRET efficiency at 22 Å. The plot shows that the intermolecular distance between the donor and acceptor is smaller in the pyrene-tagged β-hydroxy acid compared with the pyrene boronic acid, suggesting that the β-hydroxy acid tail plays a role in bringing the FRET pairs together through van der Waals interaction (Figure 9).

From the results, it can be surmised that there is better FRET interaction between the pyrene-tagged β-hydroxy acid donor and the synthesized fluorescent co-polymer perylen-3-ylmethylmethacrylate *co-N*-dodecylmethacrylamide polymer acceptor. The lipophilic tail of the pyrene-tagged β-hydroxy acid ester allowed for better FRET interaction and better quenching efficiency with the acceptor fluorophore compared to pyrene boronic acid. The limit for detection of pyrene-tagged β-hydroxy acid ester using the FRET system *via* fluorescence enhancement is 216.9 ppm (313.1 μM pyrene-tagged β-hydroxy acid).

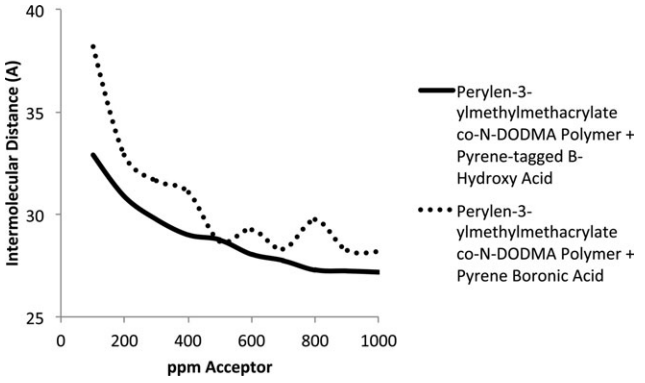

Figure 9. Intermolecular distance (based from FRET calculation) as the function of acceptor concentration.

SUMMARY

The FRET donor was prepared starting from commercially available alkyl ketene dimer (AKD) and pyrene boronic acid. The acceptor was obtained from co-polymerization of *N*-dodecylmethacrylamide (*N*-DODMA) and perylen-3-ylmethylmethacrylate. FRET interaction between pyrene-tagged β-hydroxy acid ester donor and perylen-3-ylmethylmethacrylate *co*-*N*-dodecylmethacrylamide polymer acceptor was observed given the fluorescence enhancement and quenching both in film and solution. Comparing the FRET interaction between the film and solution, FRET results from the films are due to both lipophilic interactions and the aggregation of fluorophore as a result of the condensed solid phase of the film. In solution, the partner fluorophores are free to interact showing more prominent FRET interaction between the pyrene-tagged β-hydroxy acid and perylen-3-ylmethylmethacrylate *co*-*N*-dodecylmethacrylamide polymer. Quenching studies reveal that dynamic quenching, and no static quenching is taking place.

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