Synthesis of cationic conjugated polymers for use in label-free DNA microarrays

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We describe the synthesis of poly[9,9′-bis(6″-*N*,*N*,*N*-trimethylammonium)hexyl)fluorene-*co*-alt-4,7-(2,1,3-benzothiadiazole) dibromide] (PFBT), a cationic, water-soluble conjugated polymer used in label-free DNA microarrays. This polymer was designed to have a maximum absorbance of close to 488 nm, which meets the excitation wavelength of most commercial microarray readers, and to have efficient emission in the solid state. Starting from commercially available chemicals, five steps are required to synthesize PFBT. The first step involves treatment of 2,7-dibromofluorene in 50% potassium hydroxide solution with excess 1,6-dibromohexene at 75 °C for 25 min to afford 2,7-dibromo-9,9-bis(6′-bromohexyl)fluorene (A). In the second step, a mixture of A, bis(pinacolato)diborane and potassium acetate in dioxane is stirred at 85 °C for 12 h to afford bis[9,9′-bis(6″-bromohexyl)-fluorenyl]-4,4,5,5-tetramethyl-[1.3.2]dioxaborolane (B). The third step involves bromination of 2,1,3-benzothiadiazole using bromine in the presence of hydrogen bromide to afford 4,7-dibromo-2,1,3-benzothiadiazole (C). Suzuki cross-coupling copolymerization of B and C affords the charge-neutral precursor of PFBT. In the final step, quaternization of pendant groups using trimethylamine yields PFBT. Each step takes up to 3 days, including the time required for product purification. The overall protocol requires approximately 3 weeks.

INTRODUCTION

Gene chips and microarrays allow high-throughput screening of hundreds to thousands of genes in a single experiment[1–4]. Nucleic acid detection using such microarrays typically requires labeling of target nucleic acids with fluorophores or other reporter molecules before hybridization, which adds cost, complexity and an element of uncertainty, in particular the efficiency of target labeling[1,4]. Recent studies have shown that cationic conjugated polymers (CCPs) can be incorporated into peptide nucleic acid (PNA)-based microarray assays, removing the requirement for DNA labeling[5]. The overall detection strategy is schematically illustrated in **Figure 1**. One starts with surface containing immobilized PNA (shown in yellow). Hybridization of single-stranded DNA (ssDNA) (shown in blue) to the PNA surface results in a negatively charged surface. Because of electrostatic attraction, the addition of the CCP (shown in orange), followed by washing, results in preferential adsorption onto sites containing complementary ssDNA. After workup, polymer emission indicates that the ssDNA is complementary to the surface-bound PNA. The overall selectivity of this approach relies on the successful removal of CCP from nonhybridized PNA surfaces (**Fig. 1**). Desirable CCP properties that maximize the function of such assays include efficient emission in the solid state and absorption frequencies that are compatible with excitation sources in commercially available microarray readers. One CCP that meets these requirement is PFBT[5]. The key step for the synthesis of PFBT involves the Suzuki cross-coupling co-polymerization of **B** (see **Fig. 2**) and **C**, which yields a neutral precursor material that can be easily tested for purity using standard characterization techniques, such as gel permeation chromatography and NMR spectroscopic techniques. In the following step, treatment of the neutral precursor material with a mixture of trimethylamine and water yields quaternization of the pendant groups and the target cationic structure (**Fig. 3**). This protocol describes the synthesis of PFBT. A similar strategy can be applied to the synthesis of other CCPs[6].

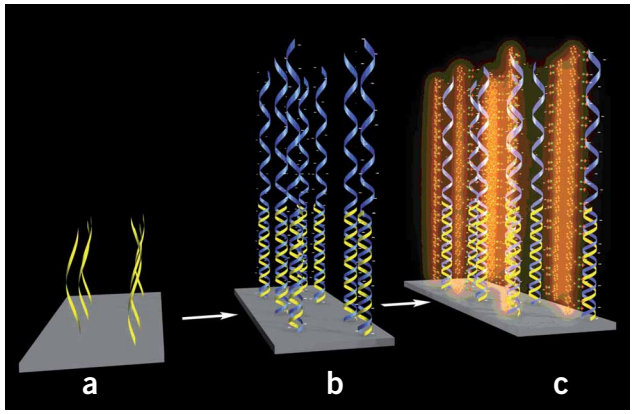

Figure 1 | Schematic illustration of the label-free ssDNA detection using immobilized PNA and CCPs. (**a**) Surface-bound PNA (shown in yellow), (**b**) hybridization with ssDNA (shown in blue) and (**c**) electrostatic adsorption of the CCP onto the PNA/ssDNA surface.

MATERIALS

REAGENTS

- Acetone (ACS reagent, ≥99.5%; Aldrich, cat. no. 154598) ! CAUTION Please refer to the MSD sheets for each chemical for safety information.
- 2,1,3-Benzothiadiazole (98%; Aldrich, cat. no. B10900)
- [1,1-Bis(diphenylphosphino)ferrocene]dichloro palladium (II) (Aldrich, cat. no. 376970)
- Bis(pinacolato)diboron (98%; Aldrich, cat. no. 473294)
- Bromine (≥99.5%; Aldrich, cat. no. 207888)

- Dichloromethane (ACS reagent, ≥99.5%; Aldrich, cat. no. 154792)
- 2,7-Dibromofluorene (97%; Aldrich, cat. no. 342297)
- 1,6-Dibromohexane (96%; Aldrich, cat. no. D41007)
- 1,4-Dioxane (anhydrous, 99.8%; Aldrich, cat. no. 539538)
- Hydrochloric acid (ACS reagent, 37%; Aldrich, cat. no. 3203331), to prepare a 0.1 M solution
- Hydrobromic acid (ACS reagent, 48%; Aldrich, cat. no. 339245)
- Magnesium sulfate (99.99%; Aldrich, cat. no. 203726)
- Methanol (ACS reagent, ≥99.8%; Aldrich, cat. no. 322415)
- Potassium acetate (ACS reagent, ≥99.0%; Aldrich, cat. no. 236497)
- Potassium carbonate (anhydrous, 99.99%; Aldrich, cat. no. 590681)
- Potassium hydroxide (reagent grade, 98%; Aldrich, cat. no. 306568)
- Tetrakis(triphenylphosphine)palladium ($Pd(PPh_3)_4$, 99.5%; Aldrich, cat. no. 216666)
- Tetrahydrofuran (THF; ACS reagent, ≥99%; Aldrich, cat. no. 360589)
- Tetrabutylammonium bromide (ACS reagent, ≥98%; Aldrich, cat. no. 426288)
- Toluene (ACS reagent, ≥99.5%; Aldrich, cat. no. 244511)
- Trimethylamine (anhydrous, 99%; Aldrich, cat. no. 243205)

EQUIPMENT

- Magnetic stirrer with thermal and speed controller (IKA model RCT basic stirring hot plate + IKATRON ETS D4 electronic thermometer; Aldrich)
- Rotary evaporator (Buchi R200)
- Vacuum pump
- Gel permeation chromatography instrument
- Balance
- NMR spectrometer

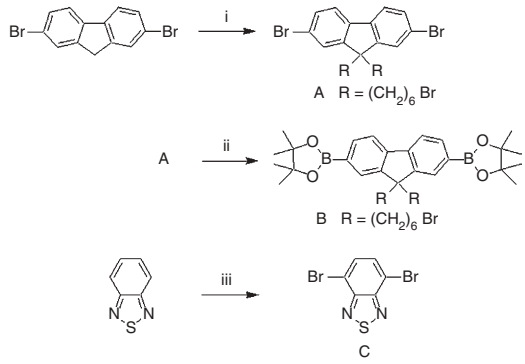

Figure 2 | Synthesis of co-monomers B and C. Reaction conditions: (i) 1,6-dibromohexane, tetrabutylammonium bromide, potassium hydroxide, 75 °C, 25 min; (ii) [1,1-bis(diphenylphosphino)ferrocene]dichloro palladium (II), KOAc, dioxane, 85 °C, 12 h; and (iii) Br_2, 48% HBr, reflux 3 h.

- Separatory funnel
- Chromatography column
- Magnetic stir bars
- Round-bottom flask
- Condenser
- Low-temperature condensation finger for trimethylamine

PROCEDURE

Synthesis of compound A

1| Mix tetrabutylammonium bromide (300 mg, 0.93 mmol) and aqueous potassium hydroxide (100 ml, 50%) in a 250 ml round-bottom flask with a stir bar.

2| Heat the mixture to 75 °C.

3| Add 1,6-dibromohexane (22.6 g, 92.6 mmol) to the mixture.

4| Add 2,7-dibromofluorene (3.24 g, 10 mmol) to the mixture.

5| Allow the reaction to proceed for 25 min at 75 °C under stirring.

6| Cool down the reaction to room temperature (approximately 25 °C) and extract the mixture with CH_2Cl_2 (100 ml×3).

7| Wash the CH_2Cl_2 layer with water (50 ml×1) and aqueous HCl (0.1 M, 50 ml×1).

8| Dry the CH_2Cl_2 layer with $MgSO_4$ (5–10 g).

9| Filter off $MgSO_4$.

10| Distill off CH_2Cl_2 using a rotary evaporator.

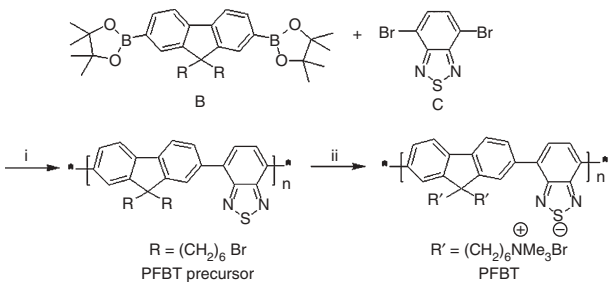

Figure 3 | Synthesis of PFBT. Reaction conditions: (i) $Pd(PPh_3)_4$, 2 M K_2CO_3, toluene/H_2O and (ii) THF/H_2O, NMe_3.

11| Distill off excess 1,6-dibromohexane under reduced pressure (120 °C per 10 mm Hg) using a vacuum pump.

12| Purify the residue using silica gel column chromatography (hexane:$CHCl_3$ = 9:1) to give **A** (5.0 g, 80% yield) as a white solid, R_f = 0.47. The diameter of the column should be approximately 5 cm and the length of packed support should be approximately 20 cm. The overall reaction has been scaled up to a final product yield of approximately 10 g.

■ **PAUSE POINT** Compound **A** can be stored at room temperature for months.

Synthesis of compound B

13| Set up a reaction by mixing the compounds and regents in a 250 ml round-bottom flask with a stir bar using the following table.
! CAUTION Dioxane is a carcinogen. The experiment should be performed in a fume hood.

Compound **A**	6.5 g, 10 mmol
Bis(pinacolato)diboron	6.0 g, 24 mmol
Potassium acetate	7.0 g, 71 mmol
Dioxane	100 ml
[1,1-Bis(diphenylphosphino)ferrocene]dichloro palladium (II)	0.5 g

14| Degas the round-bottom flask equipped with a condenser by applying two freeze–pump–thaw cycles and protect the reaction flask with nitrogen.

15| Allow the reaction to proceed for 12 h at 85 °C under stirring.

16| Cool down the reaction to room temperature.

17| Distill off dioxane at 40 °C using a rotary evaporator.

18| Add water (50 ml) to the reaction flask and extract the mixture with CH_2Cl_2 (100 ml×3).

19| Wash the CH_2Cl_2 layer with water (50 ml×3) using a separatory funnel.

20| Dry the CH_2Cl_2 layer with $MgSO_4$ (5–10 g).

21| Filter off $MgSO_4$.

22| Distill off CH_2Cl_2 using a rotary evaporator.

23| Purify the residue using silica gel column chromatography (hexane:dichloromethane = 2:1) to give **B** (4.2 g, 59% yield) as a white solid.
■ **PAUSE POINT** Compound **B** can be stored at room temperature for months.

Synthesis of compound C

24| Mix 2,1,3-benzothiadiazole (6.8 g, 50 mmol) and hydrobromic acid (10 ml) in a 100 ml round-bottom flask equipped with a condenser and a stir bar.

25| Heat the mixture to 110 °C.

26| Add bromine (8.3 ml, 150 mmol) dropwise to the mixture.
! CAUTION Bromine is volatile and poisonous. The experiment should be performed in the fumehood.

27| Add another portion of hydrobromic acid (10 ml) to the mixture.

28| React under reflux at 110 °C for 3 h under stirring.

29| Cool down the reaction to room temperature and filter the mixture.

30| Wash the solid residue with water (50 ml×3), and dry the solid at 50 °C under vacuum (10 mm Hg) for 4 h.

31| Recrystalize the solid using chloroform to yield **C** as a white solid (12 g, 82%).
■ **PAUSE POINT** Compound **C** can be stored at room temperature for several months.

Synthesis of poly[9,9′-bis(6″-bromohexyl)fluorene-*co*-alt-4,7-(2,1,3-benzothiadiazole)] (PFBT precursor)

32| Set up reaction by mixing the compounds and regents in a 25 ml round bottle flask using the following table.

Compound **B**	186 mg, 0.25 mmol
Compound **C**	73.5 mg, 0.25 mmol
$Pd(PPh_3)_4$	5 mg
Potassium carbonate	830 mg, 6 mmol
Toluene	5 ml
Water	3 ml

33| Degas the round-bottom flask by applying two freeze–pump–thaw cycles, wrap the flask in aluminum foil to protect from light and maintain the reaction mixture under nitrogen.
▲ **CRITICAL STEP** If degassing is not performed and the reaction is not under the protection of nitrogen, the catalyst color can darken and lose catalytic activity quickly. As a consequence, one will obtain polymers with low molecular weight. The reaction should be protected against light, using aluminum foil, to avoid photochemical decomposition of the catalyst.

34| Allow the reaction to proceed at 85 °C for 24 h under vigorous stirring.

35| Cool down the reaction mixture to room temperature.

36| Pour the reaction mixture into 200 ml methanol.

37| Collect the polymer by filtration.

38| Wash the polymer with hot (70 °C) methanol (50 ml×1) and acetone (50 ml×1).

39| Dry the polymer at 40 °C in a vacuum oven for 24 h to yield PFBT precursor polymer (105 mg, 65%).
■ **PAUSE POINT** The isolated polymer can be stored at room temperature under the protection of nitrogen for months.

Synthesis of PFBT

40| Dissolve PFBT precursor (70 mg) by stirring in 10 ml THF using a 100 ml round-bottom flask.

41| Cool the reaction solution to −78 °C using a dry ice-acetone bath.

42| Degas the round-bottom flask.

43| Add trimethylamine (1 ml) by condensing on a cold finge runder vacuum and react at room temperature for 24 h.

44| Add water (10 ml) and cool the reaction solution to −78 °C using a dry ice-acetone bath.

45| Degas the round-bottom flask.

46| Add trimethylamine (1 ml) and react at room temperature for 12 h.

47| Distill off the solvents at 30 °C using the vacuum pump (20 mm Hg).
▲ **CRITICAL STEP** Distillation should be carried out in a fumehood using traps filled with liquid nitrogen to minimize the smell of trimethylamine.

48| Add acetone to the residue in the flask to induce precipitation.

49| Collect the precipitate by centrifugation.

50| Dry the precipitate at 40 °C in a vacuum oven for 24 h to yield PFBT (72 mg, 89%). Store the product in nitrogen at room temperature.

● **TIMING**
For the synthesis of **A**:
Steps 1–5, 1 h; Step 6, 2 h; Step 7, 30 min; Step 8, 4 h; Steps 9–10, 1 h; Step 11, 2 h; Step 12, 6–10 h
For the synthesis of **B**:
Steps 13–14, 2 h; Step 15, 12 h; Step 16, 3 h; Step 17, 30 min; Steps 18–19, 1 h; Step 20, 4 h; Steps 21–22, 1 h; Step 23, 6–10 h
For the synthesis of **C**:
Steps 24–25, 1 h; Steps 26–27, 1 h; Step 28, 3 h; Step 29, 3 h; Step 30, 4 h; Step 31, 12 h
For the synthesis of PFBT precursor:
Step 32, 30 min; Step 33, 1 h; Step 34, 24 h; Step 35, 3 h; Steps 36–37, 30 min; Step 38, 1 h; Step 39, 24 h
For the synthesis of PFBT:
Step 40, 30 min; Step 41, 30 min; Step 42, 1 h; Step 43, 10 min followed by 24 h reaction; Step 44, 1 h; Step 45, 1 h; Step 46, 10 min followed by 12 h reaction; Step 47, 1 h; Steps 48–49, 30 min; Step 50, 24 h

ANTICIPATED RESULTS

The overall yield of the procedure is approximately 25%.
NMR spectroscopy data of **A**, **B**, **C**, the precursor polymer and PFBT are given below.

Compound **A**: ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.2–7.4 (m, 6H), 3.12 (t, 4H), 1.75 (t, 4H), 1.5 (m, 4H), 1.0 (m, 8H), 0.4 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 152.3, 139.2, 130.5, 126.2, 121.7, 121.4, 55.7, 40.2, 34.1, 32.8, 29.1, 27.9, 23.6.
Compound **B**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.83–7.72 (m, 6H), 3.27–3.24 (t, $J = 6.8$ Hz, 4H), 2.03–1.99 (q, $J = 4.0$ Hz, 4H), 1.64–1.57 (q, $J = 7.2$ Hz, 4H), 1.39 (s, 24 H), 1.17–1.13 (q, 4H), 1.06–1.02 (q, 4H), 0.55 (m, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 150.27, 144.09, 133.99, 128.93, 119.67, 83.97, 55.24, 40.12, 34.19, 32.84, 29.15, 27.93, 23.55.
Compound **C**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.73 (s, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 153.3, 132.7, 114.3. MS(EI) *m/z*: 744.
PFBT precursor: ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.07–7.91 (m, 6H), 7.87–7.72 (m, 2H), 3.36–3.29 (t, 6H, $J = 6.6$ Hz), 2.19 (m, 4H), 1.7 (m, 4H), 1.3–1.2 (m, 8H), 0.9 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 154.5, 151.6, 141.2, 136.8, 133.8, 128.5, 124.3, 120.6, 55.6, 40.4, 34.3, 32.9, 29.3, 27.9, 23.9. Gel permeation chromatography analysis using THF against crosslinked polyethylene standards: $M_w \sim 20$ K, $M_n \sim 12$ K. Elemental analysis: calculated, C, 59.43; H, 5.47; N, 4.47; found, 60.13; H, 5.29; N, 3.35.
PFBT: ^{1}H NMR (200 MHz, CD_3OD): δ 8.30–7.80 (m, 8H), 3.3–3.2 (t, 4H), 3.1 (s, 18H), 2.3 (br, 4H), 1.6 (br, 4H), 1.3 (br, 8H), 0.9 (br, 4H). ^{13}C NMR (50 MHz, CD_3OD): δ 155.7, 152.7, 142.7, 138.2, 134.6, 129.9, 125.3, 121.5, 67.8, 56.9, 52.5, 41.4, 30.5, 27.1, 25.2, 23.9.

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COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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