Synthesis and functionalization of a highly fluorescent and completely water-soluble poly(*para*-phenyleneethynylene) copolymer for bioconjugation

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Bioconjugation of a highly fluorescent water-soluble poly (*para*-phenyleneethynylene) (PPE) copolymer with ionic and non-ionic side chains is achieved by means of chain-end modification, providing a design principle for biosensor development.

Conjugated polymers are emerging materials for many modern technologies. One of the attractive applications of conjugated polymers is sensor design, because an environmental change at a single site can affect the properties of the collective system, producing large signal amplification.[1,2] In particular, the detection of biological analytes such as DNA, proteins and biological warfare agents has been receiving wide scale attention recently.[3] Receptors can be rationally designed and covalently connected to a conjugated polymer main chain.

A conjugated polymer should be water-soluble, highly fluorescent and have appropriate functional groups for conjugation with biological receptors to be a good molecular biosensor, because most target biological analytes are analyzed in an aqueous environment. However, by their nature, conjugated polymers have a hydrophobic and rigid main chain, which results in poor solubility in water and subsequent fluorescent quenching by micelle formation in an aqueous phase.[4] Even worse, once the polymer dries completely, it is extremely difficult to re-dissolve it in water again due to its strong aggregation. To address this problem, many research groups have been working on developing water-soluble conjugated polymers. Khan *et al.* very recently reported an effective method to suppress the aggregation of poly(*para*-phenyleneethynylene)s in water by introducing branched ethylene oxide units as a side chain.[5]

Here we describe the synthesis and functionalization of a completely water-soluble conjugated polymer, poly{[1,4-bis(1,3-bis(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)propan-2-yloxy) benzene]-*alt*-[2,5-diethynylbenzene-2,4-(bis(3-propoxy-sulfonic acid)) sodium salt]} (**PPE-R$_1$**, Scheme 1), to improve the emissive property even further and give bioconjugation capability. The copolymer, **PPE-R$_1$**, is composed of alternating ionic sulfonate units and bifurcated non-ionic ethylene oxide units on the main chain to provide water solubility and prevent micelle formation. Conventional palladium-catalyzed Sonogashira–Hagihara copolymerization was used. We developed a method to introduce a carboxylic acid group, a versatile functional group for bioconjugation, at the end of the PPE chain. The chemically modified polymer, with a carboxylic acid group at both ends, was subsequently conjugated with a model peptide, pentatyrosine.

Monomer synthesis for the PPE copolymer starts by reacting 1,4-dimethoxybenzene with I_2 (I_2, HIO_3, H_2SO_4, AcOH, 85%). Demethylation (BBr_3, CH_2Cl_2, −78 °C to room temperature, 90%) was then achieved by means of BBr_3. The resulting diiodohydroquinone was reacted with the tosylated bifurcated ethylene oxide molecule,[5,6] followed by the reaction with trimethylsilylacetylene and a subsequent deprotection reaction to give monomer **1**. A diiodo compound, **2**, having sulfonic acid sodium salt units, was prepared according to the literature[7] (Scheme 1). The copolymerization of **1** and **2** was carried out in the presence of a palladium catalyst (tetrakis(triphenylphosphine)palladium, $Pd(PPh_3)_4$) at 50 °C in a water/DMF cosolvent system (50/50 v/v). The synthesized **PPE-R$_1$** showed excellent solubility in water or methanol but poor solubility in common organic solvents such as THF and chloroform. The *in situ* end-capping reaction was undertaken by adding 4-ethynylbenzoic acid with additional palladium catalyst.[8] The crude polymer solution was precipitated in acetone, filtered, and washed with ethyl acetate and THF to remove diacetylene side product. Further purification of the polymer was achieved by dialysis against de-ionized water for 3 days. The *in situ* end-capping reaction of **PPE-R$_1$** with carboxylic groups was investigated by ^{1}H NMR spectroscopy (Fig. 1). Two aromatic proton peaks from the main chain of **PPE-R$_1$** appeared at 7.27 and 7.20 ppm. After the *in situ* end-capping reaction, two new peaks emerged at 7.73 and 7.49 ppm, corresponding to the aromatic protons of the end-capper, confirming that the carboxylic group was chemically attached. The molecular weight of the functionalized PPE (**PPE-R$_1$–COOH**), confirmed by ^{1}H NMR end-group analysis, was 13 000. We also prepared **PPE-R$_2$** as a control which does not have the bifurcated ethylene oxide units.

Fig. 2 illustrates the absorption and emission spectra of the prepared PPEs. The absorption spectra of **PPE-R$_2$**, unlike that of **PPE-R$_1$** and **PPE-R$_1$–COOH**, shows a pronounced shoulder in the longer wavelength region, typical of an aggregation band. **PPE-R$_2$** also shows a broad emission spectrum with the suppressed 0–0 band at λ_{max} = 460 nm and a long tail, a characteristic shape of excimer/aggregation-like emission caused by polymer aggregation, as expected.[9] On the contrary, the emission spectra of **PPE-R$_1$** and **PPE-R$_1$–COOH** are narrow with a well-defined 0–0 band at λ_{max} = 460 nm. We achieved a high quantum yield of PPE in

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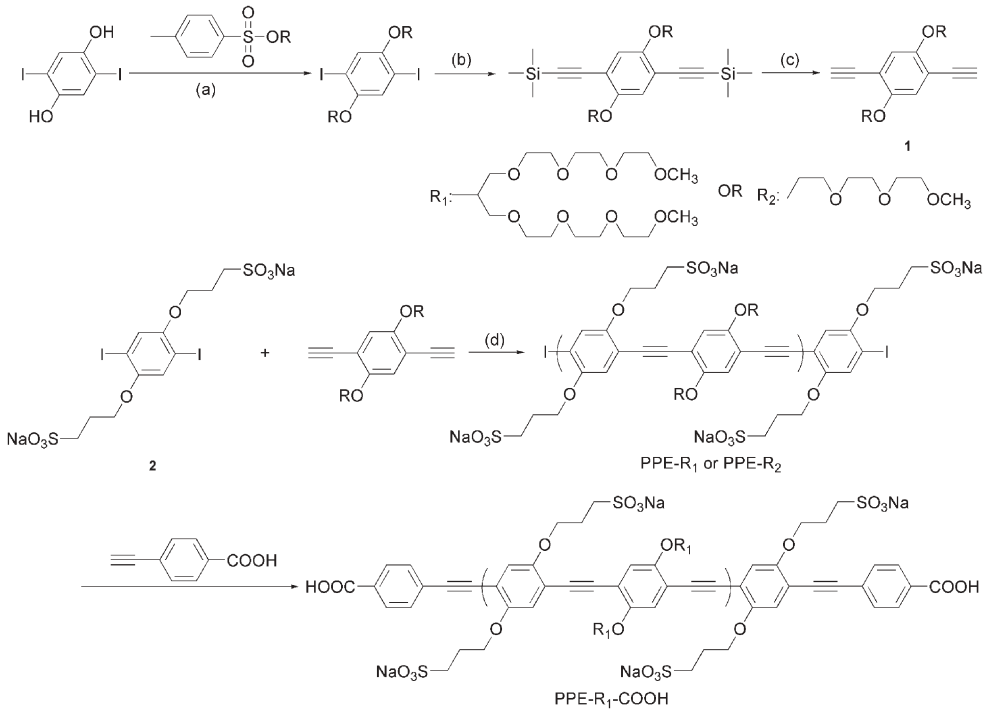


Scheme 1 Polymer synthesis: (a) DMF, K_2CO_3, 75 °C, 72 h; (b) THF, $Pd(PPh_3)_4$, DIPA (diisopropylamine), CuI; (c) KOH, water/methanol.

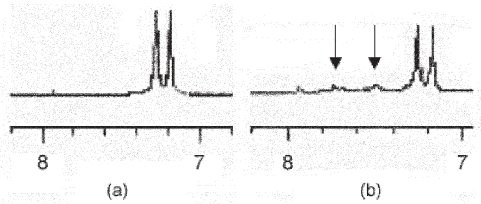


Fig. 1 ^1H NMR spectra of **PPE-R$_1$** in D_2O (a) before and (b) after end-capping.

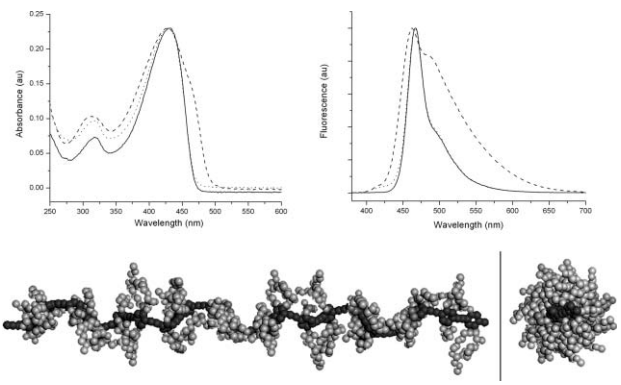
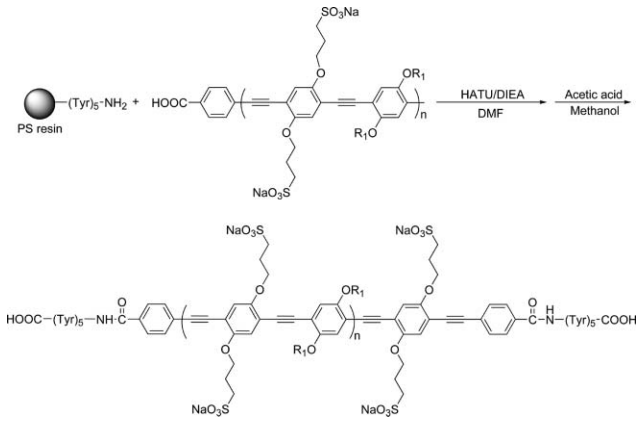


Fig. 2 Top: Normalized absorption and emission spectra of the polymers: **PPE-R$_1$** (solid); **PPE-R$_1$–COOH** (dotted); **PPE-R$_2$** (dashed). Bottom: molecular modelling of **PPE-R$_1$** simulated by Materials Studio 3.0 (Accelrys®). The **dark black** chain indicates the polymer backbone (left: side view, right: edge view).

water by adding ionic and bulky non-ionic side chains. The absolute quantum yield of **PPE-R$_1$** in water (1 mg L^{-1}) was 53%.[10] Conversely, the absolute quantum yield of **PPE-R$_2$** in water (1 mg L^{-1}) was only 19%, suggesting that the ionic side chain, sulfonic acid sodium salts, provide additional water-solubility but that the bulky non-ionic side group is required to prevent aggregation. Molecular modelling of **PPE-R$_1$**, presented in Fig. 2, shows that the hydrophobic PPE backbone is sheathed by the bulky ethylene oxide side chain, effectively preventing aggregation.[11]

Chemical modification of **PPE-R$_1$** was done by an *in situ* end-capping reaction at the end of the copolymerization. We selected 4-ethynylbenzoic acid as an end-capper because a carboxylic group is of practical use for bioconjugation.[12] The absolute quantum yield of the resulting **PPE-R$_1$–COOH** was 45%, lower than that of **PPE-R$_1$**. This drop in the quantum yield is believed to be due to the carboxylic acid group being directly connected to the conjugated backbone. We have made various water-soluble PPE copolymers with carboxylic acid side chains in every other repeating unit. We consistently observed that the fluorescent quantum yields of PPEs with directly connected carboxylic acid side groups are always substantially lower than those of PPEs with carboxylic acid side chains connected to the conjugated backbone through a non-conjugated linker group.[13] The reason why **PPE-R$_1$–COOH** has only a slightly smaller quantum yield than **PPE-R$_1$** is likely to be because there are only two carboxylic acid groups at the ends of the conjugated backbone.

We carried out the peptide conjugation reaction on the carboxylic acid groups of **PPE-R$_1$–COOH** by using 4-chloro-trityl resin bound with pentatyrosine as a model peptide (Scheme 2). We chose 4-chloro-trityl polystyrene (PS) resin because the cleavage reaction can be undertaken using mild conditions, meaning that the PPE backbone is not damaged. After cleavage of the pentatyrosine from the resin, the quantitative coupling reaction of **PPE-R$_1$–COOH** with the pentatyrosine was



Scheme 2 Peptide–PPE coupling reaction.

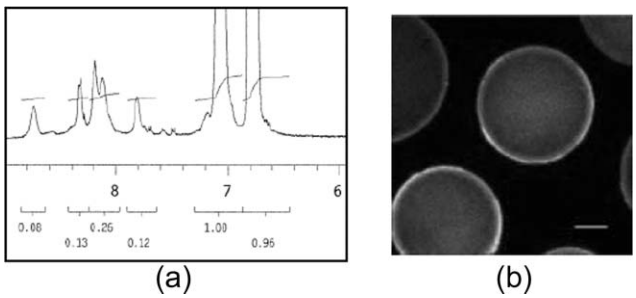


Fig. 3 (a) ^{1}H NMR spectrum of pentatyrosine–PPE in DMSO (b) A confocal image of pentatyrosine–PPE (scale bar: 20 μm).

confirmed by NMR. New aromatic proton peaks at 7.8–8.8 ppm, corresponding to pentatyrosine, are shown in Fig. 3 (a). It was confirmed that pentatyrosine units were coupled at both ends of the **PPE-R$_1$–COOH** by end-group analysis.[14] Fig. 3 (b) shows a confocal microscope image of photoluminescent 4-chloro-trityl resin reacted with **PPE-R$_1$–COOH**. The image was taken after 3 stringent rinses of the resin with methanol, DMF, water and dichloromethane to remove any unreacted copolymers. The filtrate of the washing step to remove unbound polymers hardly showed any fluorescence, confirming that almost every polymer chain end has a carboxyl group that had reacted with the PS resin. After cleaving the pentatyrosine from the resin, the resulting peptide-conjugated PPE does not have any carboxylic acid directly bound to the conjugated backbone .[15]

In conclusion, we have established a simple and practical approach for the bioconjugation of a conjugated polyelectrolyte and a pentatyrosine, a model biological molecule. We designed and synthesized completely water-soluble and highly fluorescent sulfonated PPE with bifurcated ethylene oxide side chains. End-functionalized PPE, prepared by *in situ* chemical modification during polymerization, was successfully attached to a model peptide, pentatyrosine on a 4-chloro-trityl PS resin. This study provides a design principle for the preparation of functionalized, water-soluble, fluorescent, conjugated polymers for bioconjugation. Bio/synthetic hybrid conjugated polymers have a large potential as molecular biosensors to detect biological analytes quickly and selectively.

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