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Synthesis of a New Conjugated Polymer for DNA Alkylation and Gene Regulation

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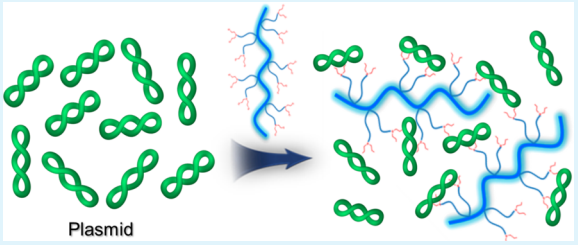
Supporting Information

ABSTRACT: A new polyfluorene derivative containing pendent alkylating chlorambucil (**PFP-Cbl**) was synthesized and characterized. Under direct incubation with DNA in vitro, **PFP-Cbl** could undergo an efficient DNA alkylating reaction and induce DNA cross-linking. In vitro transcription and translation experiment exhibited that the **PFP-Cbl** significantly down-regulated the gene expression of luciferase reporter plasmid. The down-regulation of gene expression was also verified through the transfection experiment of p-EGFP plasmid, which showed decreased green fluorescent protein (GFP) in cells. Meanwhile, the self-luminous property of **PFP-Cbl** could make it able to trace the internalized **PFP-Cbl** and plasmid complexes resulted from cross-linking in cells by fluorescent microscopy. Combining the features of alkylating function, multivalent binding sites, and fluorescent characteristics, **PFP-Cbl** provides a new insight in the area of gene regulation and extends the new applications of conjugated polymers (CPs).



KEYWORDS: *conjugated polymers, DNA alkylation, plasmid, GFP, gene regulation, protein expression*

1. INTRODUCTION

The study of gene regulation has been a major subject in life science. There are several different levels to regulate gene expression, including DNA modification, transcription regulation, post-transcriptional regulation, and translation regulation.[1] Among these gene regulation levels, DNA modification is the most direct and fundamental one, because the accurate readout of DNA sequence is firmly related to the transcription of functional mRNA and, ultimately, the protein expression in cells.[2] Among plenty of chemicals that can result in DNA modification, chlorambucil (**Cbl**) is widely used in current cancer therapy. As a bifunctional alkylating agent, **Cbl** can mediate an SN2 process to attack the nucleophilic site, mainly the N7-position of the guanine residue and induce DNA cross-linking.[3,4] Several approaches have focused on the selectivity investigation by the conjugation of **Cbl** with various functional groups, such as tumor cell targeting,[5−7] specific organelle targeting,[8] and specific DNA sequence targeting.[9,10] Besides, methods for improving the **Cbl** delivery have been explored.[11,12] However, the introduction of the **Cbl** alkylating groups into the side chains of polymers has been less reported.

Due to the unique physiochemical properties, conjugated polymers (CPs) have aroused great interest as a kind of very promising macromolecular material.[13,14] Conjugated backbones offer CPs with excellent photophysical properties such as broad absorption and emission spectra, strong light-harvesting ability, and high quantum yield. They have been used for highly sensitive detections for a wide range of chemical and biological molecules.[15−29] Except for biosensing applications, CPs have also been explored for biomedical applications, such as fluorescence imaging,[30−35] drug delivery and release,[36] gene delivery,[37] drug screening,[38] cell engineering,[39,40] and anti-microorganism and anticancer therapy.[41−46] Recently, the gene regulation ability of CPs has been reported,[47] where CPs can damage DNA and therefore affect gene expression due to their sensitizing of oxygen to produce reactive oxygen species (ROS) under light irradiation. In this paper, a new polyfluorene derivative containing pendent alkylating **Cbl** moieties (**PFP-Cbl**) was synthesized for DNA alkylation and gene regulation. By linking **Cbl** to the side chains, the conjugated polymer **PFP-Cbl** was equipped with numerous alkylating groups. Facilitated by the multivalent enrichment effect of CPs, the cross-linking of DNA by alkylation could be significantly enhanced in comparison with **Cbl** itself. As expected, the down-regulation of gene expression could be effectively achieved. Meanwhile, the self-luminous property of **PFP-Cbl** could make it be able to trace the internalized **PFP-Cbl** and plasmid complexes resulted from cross-linking in cells by fluorescent microscopy. Combining the features of alkylating function, multivalent binding sites, and fluorescent characteristic, **PFP-Cbl** provides a new insight in the area of gene regulation and extends the new applications of CPs.

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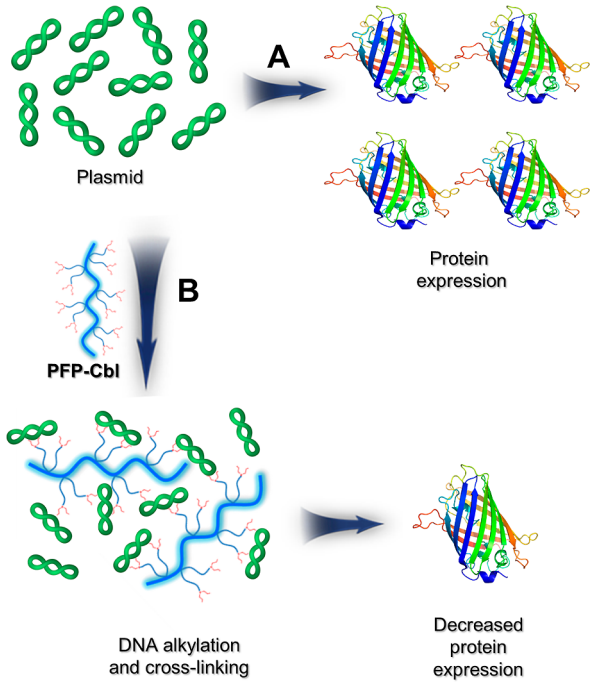
2. RESULTS AND DISCUSSION

The proposed principle of **PFP-Cbl** for DNA alkylation is illustrated in Scheme 1. Polymer **PFP-Cbl** contains a great number of alkylating groups by covalently linking **Cbl** to its side chains. Upon incubation of **PFP-Cbl** with plasmid DNA, the **Cbl** groups alkylate DNA by attacking the nucleophilic center in DNA, which ultimately gives rise to cross-linking DNA. Compared with the plasmid DNA without treatment with **PFP-Cbl** (situation A), the protein expression is greatly inhibited for the plasmid treated with **PFP-Cbl** (situation B).

Scheme 1. Schematic Mechanism of PFP-Cbl for Alkylating and Cross-Linking of DNA and Gene Regulation[a]



[a]Situation A: normal gene expression. Situation B: decreased gene expression affected by DNA alkylation and cross-linking.

Scheme 2 depicts the synthetic route of polymer **PFP-Cbl**. Reaction of *N*-hydroxy succinimide (NHS) with **Cbl** (**1**) gives esterification-activated compound **2**. Polymerization of monomer **3** with phenyldiboronic acid ester via Suzuki coupling affords polymer **PFP-Boc**. The weight average molecular weight (M_w) of **PFP-Boc** is 20 720 with a polydispersity index (PDI) of 1.96 based on GPC analysis. The *N*-Boc groups are then deprotected under hydrochloride atmosphere to yield polymer **PFP-$NH_3^+Cl^-$**. Polymer **PFP-$NH_3^+Cl^-$** is neutralized with triethylamine followed by the reaction of secondary amino groups with compound **2** to give the final conjugated polymer **PFP-Cbl**. The linking ratio of **Cbl** to the polymer side chain is measured to be nearly 100% based on the ^{1}H NMR data (Figure S1). The photophysical properties of the polymer **PFP-Cbl** is investigated in aqueous solution. As shown in Figure 1, **PFP-Cbl** exhibits maximum absorption at 375 nm and maximum fluorescence emission at 425 nm. Using quinine bisulphate as a reference, the fluorescence quantum yield of **PFP-Cbl** is measured to be 68%.

To evaluate the alkylation ability of **PFP-Cbl** to genes, p-EGFP plasmid DNA was used as a model in the alkylation experiment. The p-EGFP samples in 5 μL of TB buffer were respectively mixed with varying amounts of **PFP-Cbl**, **Cbl**, and **PFP-Boc** in 5 μL of DMSO. The p-EGFP sample with DMSO alone was used as the blank control. All the mixtures were incubated at 37 °C for 1 h before adding into the agarose gel. The results of agarose gel assay are displayed in Figure 2. As shown in Figure 2a, as the increase of **PFP-Cbl** concentration, the brightness of p-EGFP plasmid electrophoretic bands gradually decreased. Because conjugated polymer contains a lot of repeated units, **PFP-Cbl** possesses numerous alkylating reactive sites in a single molecule, which greatly enhances the cross-linking ability to gene. Therefore, the cross-linking **PFP-Cbl** /plasmid complexes could not easily migrate through the gel because of large molecular weight. Compared with the small molecule drug **Cbl** that exhibits delay phenomenon of plasmid electrophoretic bands through cross-linking, **PFP-Cbl** shows more cross-linking effect. To confirm that the gel results mainly result from the alkylation ability of **PFP-Cbl**, a control experiment was carried out by using conjugated polymer **PFP-Boc** that contains the same backbone as **PFP-Cbl** but without pendent alkylating groups. As revealed in Figure 2b, the results of agarose gel indicate that the introduction of alkylating group **Cbl** played a key role in the concentration-dependent brightness decrease of p-EGFP plasmid electrophoretic bands.

We conducted dynamic light scattering (DLS) tests to measure the size of cross-linked complex prior to cell transfection. As seen in Supporting Information Figure S2, the complex size is in the range of 200−600 nm and shows a concentration-dependence of **PFP-Cbl**. The gene down-regulation ability of **PFP-Cbl** was first determined by an in vitro transcription and translation system and further verified by cell transfection experiment. To qualitatively measure the gene down-regulation ability of **PFP-Cbl**, luciferase reporter gene plasmid was used in the in vitro transcription and translation studies. The luciferase reporter gene plasmid was respectively treated with **PFP-Cbl** and **PFP-Boc** at 37 °C for 1 h, and then added into the in vitro transcription and translation system according to the provided protocol. The luciferase activity that is determined by the luciferase reporter gene plasmid expression was measured by the luciferase assay reagent. As shown in Figure 3a, compared with the blank control group that was treated under the same conditions without polymer, the **PFP-Cbl** treated plasmid showed dramatically decreased expression. As for the **PFP-Boc** treated plasmid, no obvious inhibition effect was observed. Furthermore, to demonstrate the gene down-regulation ability of **PFP-Cbl** in cell level, the transfection experiment was carried out in HeLa cells. The cell cytotoxicity of **PFP-Cbl** was tested using MTT assay. As shown in Supporting Information Figure S3, no obvious cytotoxicity was observed for **PFP-Cbl** (0−64 μM). The GFP-expressing plasmid p-EGFP was respectively treated with **PFP-Cbl** and **PFP-Boc** at 37 °C for 1 h, and then lipofectamine 2000 reagent was used as the delivery vector of plasmid DNA. The corresponding fluorescence images were taken and given in Figure 3b. Compared with the blank control group, where the plasmid DNA was not treated with polymer, the **PFP-Cbl** treated group showed significant decrease of GFP expression. Meanwhile, the self-luminous property of **PFP-Cbl** could make it be able to trace the internalized **PFP-Cbl** and plasmid complexes resulting from cross-linking in living cells by fluorescent microscopy. Taken together, these results suggest that the alkylating and cross-linking abilities of **PFP-Cbl** could disturb the gene expression process and achieve the gene down-regulation.

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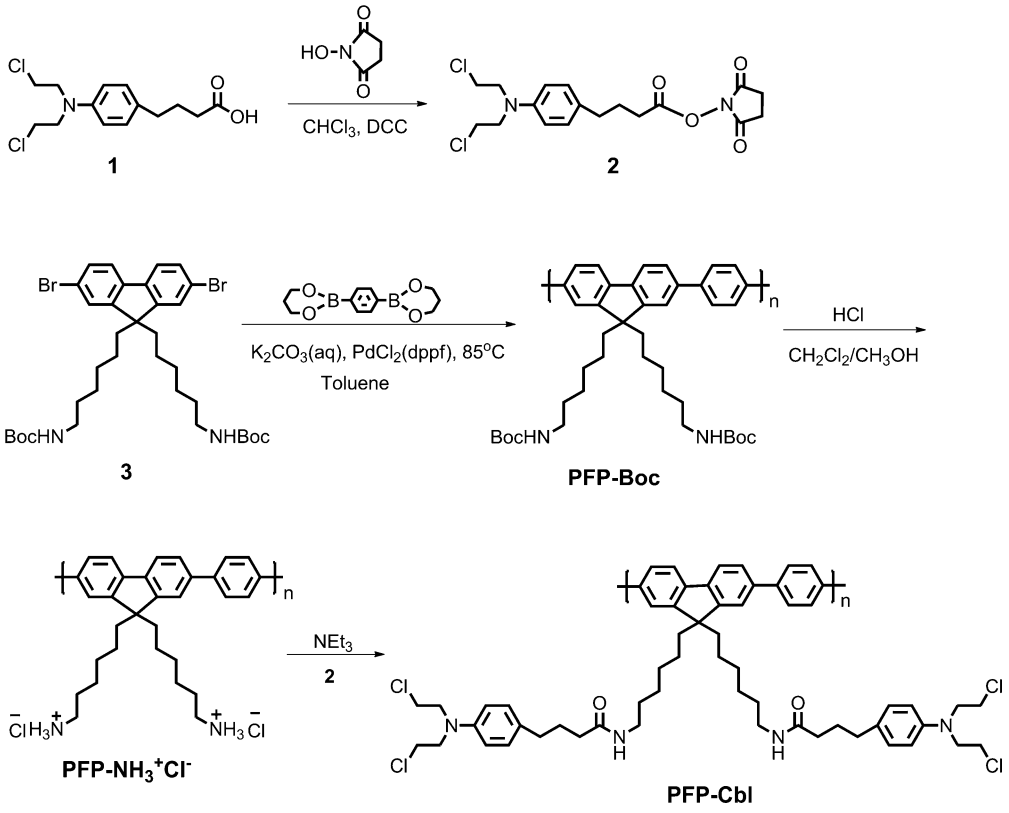
Scheme 2. Synthetic Route of the Polymers PFP-Boc and PFP-Cbl



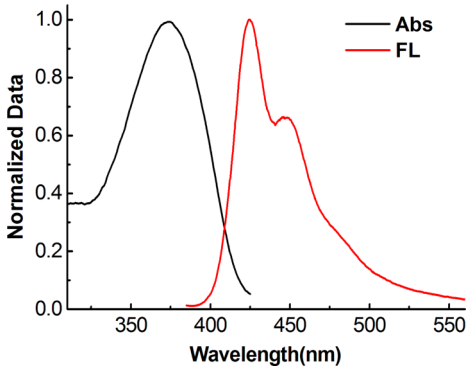


Figure 1. Normalized absorption and fluorescence spectra of **PFP-Cbl** in aqueous solution.

3. CONCLUSION

A new polyfluorene derivative containing pendent alkylating chlorambucil (**PFP-Cbl**) was synthesized and characterized. Under direct incubation with DNA in vitro, **PFP-Cbl** could undergo efficient DNA alkylating reaction and induce DNA cross-linking. In vitro transcription and translation experiments of luciferase reporter plasmid revealed that **PFP-Cbl** could efficiently down-regulate gene expression. The down-regulation of gene expression was also verified through the transfection experiment of p-EGFP plasmid, which exhibited the decreased GFP expression in cells. Meanwhile, the self-luminous property of **PFP-Cbl** could make it be able to trace the internalized **PFP-Cbl** and plasmid complexes resulting from cross-linking in cells by fluorescent microscopy. Combining the features of alkylating function, multivalent binding sites, and fluorescent characteristics, **PFP-Cbl** provides a new insight in the area of gene regulation and extends the new applications of CPs.

4. EXPERIMENTAL SECTION

Materials. All the reagents and solvents used were commercially available. Benzen-1,4-bis(boronic acid)-propane-1,3-diol diester and chlorambucil was purchased from Sigma Chemical Co. Compounds **2** and **3** were synthesized according to the procedures in the literatures.[48,49] Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials (Hangzhou, China). Ethidium bromide (EB) was purchased from Beijing SBS Genetech Co. Ltd. *E. coli* TOP10 (CB104-02) and pure plasmid extraction kit (DP107-02) were purchased from TIANGEN Biotech (Bejing) Co., Ltd. A T7 coupled reticulocyte lysate system (L4610) and luciferase assay system (E1500) were purchased from Promega Corporation. Nuclease-free water was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. Lipofectamine 2000 (Cat. No. 11668-019) was purchased from Invitrogen (Beijing). HeLa (human cervical cancer) cell was purchased from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 4500 mg/L glucose, and 4.0 mM glutamine. Plasmid expressing GFP (p-EGFP) was amplified in the *E. coli* strain DH5α and purified according to the manufacturer's protocol (TIANGEN Biotech). The water was purified using a Millipore filtration system.

Measurements. The ^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. The GPC measurement was performed on a Waters-410 system against polystyrene with THF as eluent. The fluorescence spectrum was measured on a Hitachi F-4500 fluorometer with a Xenon lamp as excitation source. UV-vis absorption spectrum was taken on a Jasco V-550 spectrometer. Phase contrast bright-field and fluorescence images were taken by fluorescence microscope (Olympus IX71) with a mercury lamp (100 W) as light source. Gels and chemiluminescence images were visualized and taken by Bio-Rad Molecular Imager ChemiDoc XRS system.

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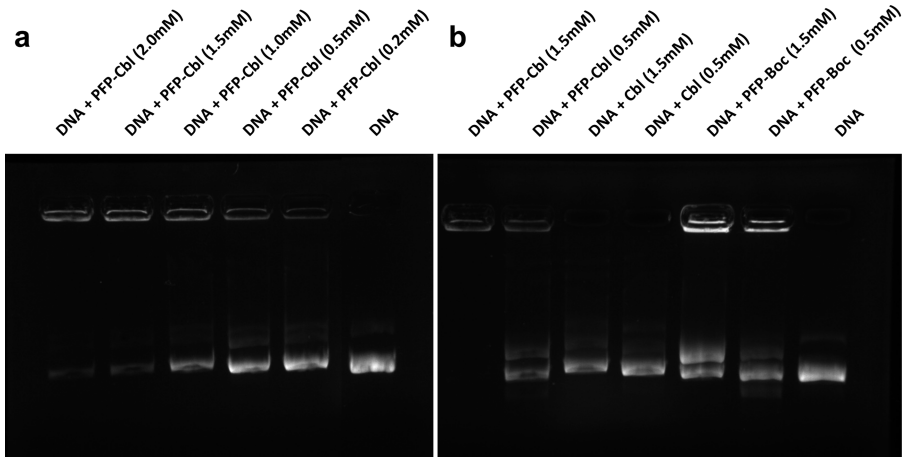

Figure 2. Alkylating and cross-linking activity of **PFP-Cbl** to p-EGFP plasmid DNA by agarose gel assay. (a) p-EGFP plasmid (0.75 μg) in 5 μL of TB buffer was mixed with different amounts of **PFP-Cbl** in 5 μL of DMSO. (b) p-EGFP plasmid (0.75 μg) in 5 μL of TB buffer was respectively mixed with different amounts of **PFP-Cbl**, **Cbl**, and **PFP-Boc** in 5 μL of DMSO. The mixture was incubated at 37 °C for 1 h and loaded onto a 0.8 % agarose gel.

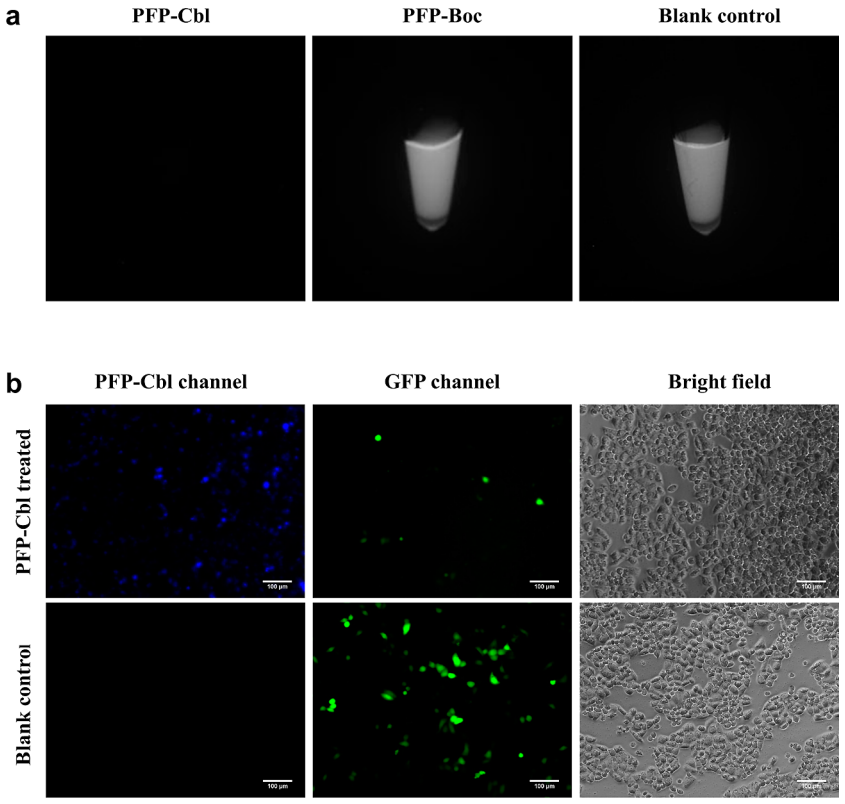

Figure 3. (a) Down-regulation of luciferase expression by **PFP-Cbl** compared with a control polymer **PFP-Boc**. The blank control group was treated under the same condition without polymers. Luciferase expression was measured by the luciferase assay reagent and recorded by Bio-Rad Molecular Imager ChemiDoc XRS system. (b) Down-regulation of GFP expression by **PFP-Cbl**. The blank control group was treated under the same conditions only without polymer. Fluorescence images of **PFP-Cbl** and GFP were recorded using the 380/30 and 455/70 nm excitation filter, respectively.

Synthesis of Polymer PFP-Boc. A mixture of compound **3** (108 mg, 0.15 mmol), benzen-1,4-bis(boronic acid)-propane-1,3-diol diester (37 mg, 0.15 mmol), and 2 mL of 2.0 M K_2CO_3 aqueous solution in 4 mL of toluene was degassed, and 15 mg of $PdCl_2$(dppf) (dppf = 1,1′-bis(diphenyl-phosphine)-ferrocene) was added under a nitrogen atmosphere. The resulting mixture was stirred at 85 °C for 48 h under nitrogen. After cooling down to room temperature, 30 mL of distilled water was added, and the mixture was extracted with chloroform. After the organic solvent was removed, the residue was precipitated in methanol. The crude polymer was purified by precipitation from chloroform into methanol twice and dried under vacuum to give 38 mg of product as a solid (yield 40%). ^{1}H NMR (400 MHz, $CDCl_3$, ppm): 7.82 (br, 6H), 7.78−7.25 (br, 4H), 4.41 (br, 2H), 2.99 (br, 4H), 2.08 (br, 4H), 1.40 (br, 16H), 1.30 (br, 4H), 1.12 (br, 8H), 0.77 (br, 4H). M_n = 10 560, M_w = 20 720, and PDI = 1.96 based on GPC analysis.

Synthesis of Polymer PFP-Cbl. Hydrochloride was bubbled into a solution of **PFP-Boc** (12 mg, 0.02 mmol) in 5 mL of

dichloromethane for 3 h. After the solid was precipitated, 10 mL of methanol was added to dissolve the precipitation and hydrochloride was bubbled into the solution for another 3 h. Excessive hydrochloride and the solvent were removed under vacuum to afford **PFP-$NH_3^+Cl^-$**. The solid was then dissolved in 3 mL of methanol, after 500 μL of triethylamine was added, the resulting mixture was stirred for 30 min at room temperature. The solution of compound **2** (20 mg, 0.05 mmol) in 15 mL of dichloromethane was then added in the mixture, and the new result mixture was stirred overnight at 45 °C. After cooling down to room temperature, the organic solvent was removed and the residue was precipitated into methanol. The crude polymer was purified by precipitation from chloroform into methanol twice and dried under vacuum to give 10 mg of product as a solid (yield 50%). ^{1}H NMR (400 MHz, CD_3OD, ppm): 7.82 (br, 6H) 7.73−7.36 (br, 4H), 7.01 (br, 4H), 6.58 (br, 4H), 5.35 (br, 2H), 3.66 (br, 8H), 3.59 (br, 8H), 3.10 (br, 4H), 2.49 (br, 4H), 2.07 (br, 8H), 1.84 (br, 4H), 1.28 (br, 4H), 1.11 (br, 8H), 0.77 (br, 4H).

Interactions with Plasmid DNA. A solution containing 75 ng/μL of p-EGFP DNA in TB buffer was incubated for 1 h at 37 °C in the presence of **PFP-Cbl** at different concentrations (2.0, 1.5, 1.0, 0.5, and 0.2 mM) and **PFP-Boc** (1.5 and 0.5 mM), respectively. A sample treated under the same condition without polymers was used as blank control. The resulting complexes were loaded onto a 0.8% agarose gel containing EB DNA dye. Further electrophoresis was conducted at 135 mA for 15 min, and the gels were visualized in Chemi-Doc (Bio-Rad, USA).

In vitro Cytotoxicity Using MTT Assay. The in vitro cytotoxicity of **PFP-Cbl** was measured by MTT assay. The stock solution of **PFP-Cbl** was prepared in DMSO ([**PFP-Cbl**] = 10 mM). The final concentration of DMSO in the culture medium is below 0.25%, which scarcely induces cytotoxicity. In all the experiments, cells were seeded in 96-well plates at a density of 4×10^3 cells/well. After 12 h, cells were incubated with various concentrations of **PFP-Cbl** (0−64 μM) in fresh medium. After treatment for 48 h, MTT (5 mg mL^{-1} in water, 10 μL/well) was added to the wells by incubation at 37 °C for 4 h. The supernatant was removed and 100 μL DMSO per well was added to dissolve the produced formazan. After shaking the plates for 5 min, absorbance values of the wells were recorded on a microplate reader at 570 nm.

In vitro Transcription and Translation. A 3 μL of luciferase reporter gene plasmid (0.3 μg/μL) was incubated with 2 μL of **PFP-Cbl** (10 mM), **PFP-Boc** (10 mM), and DMSO, respectively, for 1 h at 37 °C. Cell-free transcription and translation of luciferase reporter gene plasmid was carried out using the rabbit reticulocyte lysate-based T7 transcription-translation coupled system by conforming to the provided protocol. Firstly, 12.5 μL of TNT rabbit reticulocyte lysate, 0.25 μL of amino acid mixture minus methionine, 0.25 μL of amino acid mixture minus leucine, 1 μL of TNT reaction buffer, 0.5 μL of T7 RNA polymerase, and 0.5 μL of RNase inhibitor were mixed together in each tube of experiment groups, 0.5 μL of each treated plasmids sample was then separately added, and finally, nuclease-free water was added in a total reaction volume of 25 μL for each tube. The reaction mixture was incubated at 30 °C for 100 min and stored at −20 °C. To measure luciferase activity, 5 μL of reaction mixture was gently mixed with 40 μL of luciferase assay reagent by repeatedly pipetting. After a time-delay of 30 s, the assay solution was placed in a dark chamber to measure the chemiluminescence for 500 s and the resulting images were taken by the Bio-Rad Molecular Imager ChemiDoc XRS system.

Transfection Experiment for Gene Regulation. HeLa cells were cultured in DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS). All of cell lines were discarded after 3 months and new lines obtained from frozen stocks. In the transfection experiment, HeLa cells were first seeded onto 24-well plates (1×10^5 cells per well, in 0.5 mL growth medium with 10% FBS). After 24 h culture prior to the experiment, 1 μg of plasmid DNA encoding GFP in 3 μL of TB buffer was incubated with 3 μL of **PFP-Cbl** in DMSO ([**PFP-Cbl**] = 10 mM) at 37 °C for 1 h, and the control group was treated under the same condition without any polymer. After the treatment, 3 μL of treated sample was diluted each with 50 μL RPMI-1640 medium without FB, and then mixed gently. A 1.5 μL portion of Lipofectamine 2000 was diluted in 50 μL of RPMI-1640 medium without FBS and incubated for 5 min at room temperature. Then, the diluted 50 μL Lipofectamine 2000 solution was added to the 50 μL diluted DNA−polymer solution. The mixture incubated for 20 min at room temperature. The 100 μL of complex was then added to a well containing cells and 400 μL serum-free RPMI-1640 medium, mixing gently by rocking the plate back and forth. The final concentration of polymers was 30 μM in the culture medium. After transfection for 6 h, the medium was replaced with fresh supplemented DMEM (0.5 mL). After initial transfection for 24 h, the medium was removed and the cells were washed with phosphate buffered saline (PBS, pH 7.4) three times for imaging the transfected cells. The fluorescence images of **PFP-Cbl** and GFP were recorded using the 480/30 and 455/70 nm excitation filter, respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectra of **PFP-Cbl**, dynamic light scattering, and cell viability data. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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