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Conjugated Poly(pyridinium salt)s as Fluorescence Light-Up Probes for Heparin Sensing

Lei Wang,[1] Yandong Li,[2] Jingfen Sun,[1] Yan Lu,[1] Yujiao Sun,[1] Dandan Cheng,[1] Chenxi Li[2]

[1]Key Laboratory of Display Materials & Photoelectric Devices, Ministry of Education, School of Materials Science & Engineering, Tianjin University of Technology, Tianjin 300384, China

[2]Key Laboratory of Functional Polymer Materials, Ministry of Education, Institute of Polymer Chemistry, Nankai University, Tianjin 300191, China

Correspondence to: Y. Lu (E-mail: luyan@tjut.edu.cn)

ABSTRACT: Because of its interaction with diverse proteins, heparin plays an important role in the regulation of various normal physiological and pathological processes. However, heparin overdose can induce some complications such as hemorrhages and thrombocytopenia. Therefore, it is critical to detect and quantify heparin accurately and quickly. Recently, conjugated polyelectrolytes (CPEs)-based fluorescent probes for heparin sensing have attracted considerable interests. Unfortunately, only few kinds of CPEs such as polyfluorenes derivatives, polythiophene derivatives, are reported and effective in fluorescent assays of heparin by now. This contribution aims to develop a new kind of polyelectrolytes based on poly(pyridinium salt)s derivatives for heparin detection with a light-up signature. An alternative conjugated poly(pyridinium salt)s (**P1**) containing carbazole segments in the main chain was designed and synthesized, which are weakly emissive in solution but highly luminescent upon binding with heparin. Fluorescence light-up probes for heparin detection based on **P1** were developed with high selectivity. **P1** shows the similar fluorescence response toward heparin both in a broad pH range of about 3–12 and in the presence of various competing anions. Heparin quantification with a practical calibration range (0–14 μ*M*) covering the whole therapeutic dosing levels (0.2–1.2 U mL^{-1}, 1.7–10 μ*M*) is realized based on the polymer probe. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40933.

KEYWORDS: conjugated polymer; fluorescent probe; heparin sensing; light-up; poly(pyridinium salt)s

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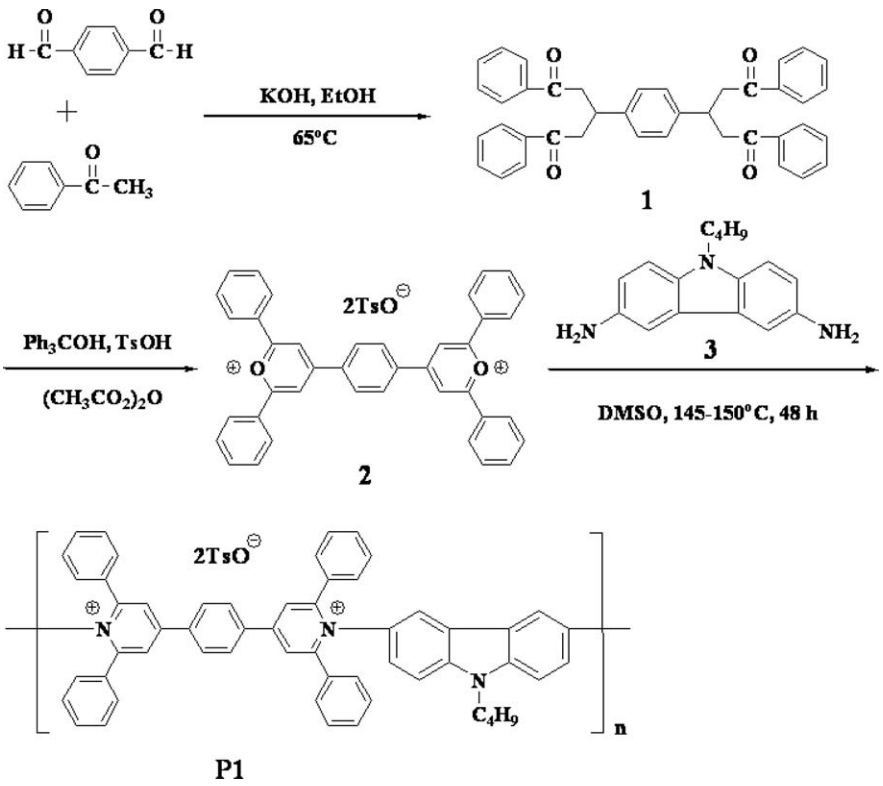
INTRODUCTION

Heparin is a naturally occurring sulfated polysaccharide belonging to the category of glycosaminoglycans.[1] Because of its interaction with diverse proteins, heparin plays an important role in the regulation of various normal physiological and pathological processes, such as cell growth and differentiation, inflammation, immune defense, lipid transport and metabolism, and blood coagulation.[2] Especially, it has been widely used as clinical anticoagulant drug to prevent thrombosis.[3] However, heparin overdose can induce some complications such as hemorrhages and thrombocytopenia.[4] Therefore, it is critical to detect and quantify heparin accurately and quickly. To date, many methods have been established for heparin detection and quantification, including traditional activated clotting time (ACT),[5] activated partial thromboplastin time (aPTT),[6] as well as fluorescent method.[7–14] Compared with the traditional techniques, the fluorescent method provides several unique advantages in terms of rapid response, low cost, and high sensitivity.

In recent years, a variety of fluorescent probes for heparin sensing have been reported, including synthetic cationic chromophores,[15] polycationic calyx[8]arenes,[16] a chromophore-tethered flexible copolymer,[17] conjugated polyelectrolytes (CPEs)[18–20], and so on. Among them, CPE-based probes have attracted considerable interests. Compared with small-molecule fluorophores, CPEs, which combining the optoelectronic properties of conjugated polymers with water-solubility and electrostatic behaviors of electrolytes, exhibit higher sensitivity with lower signal-to-noise ratio and a boarder detecting window. For example, Liu et al. have introduced 2,1,3-benzothiadiazole (BT) units into polyfluorenes (PFs) derivatives to develop several CPE turn-on or ratiometric fluorescent probes for heparin sensing.[8,18,21,22]

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



Scheme 1. Synthesis route for the conjugated poly(pyridinium salt)s (**P1**).

Unfortunately, only few kinds of CPEs such as PFs derivatives, polythiophene (PT) derivatives, are reported and effective in fluorescent assays of heparin by now.

In addition, most of reported assays for heparin rely on fluorescence quenching as the signal output, which is often unfavorable for heparin detection because of large environment effects or low sensitivity. Only a few fluorescence turn-on probes for heparin sensing have been developed and described up to now.[9,18,23,24] Based on the fact that fluorescence turn-on sensors have a significant advantage of reduced false-positive signals as compared to fluorescence turn-off sensors, it is still highly desirable to develop new CPEs-based probes that allow direct fluorescence turn-on heparin sensing.

Recently, we devote ourselves to develop a new kind of polyelectrolytes based on poly(pyridinium salt)s derivatives for biomacromolecules sensing.[25–27] Unlike common *p*-conjugated polyelectrolytes characterized by a backbone with a delocalized electronic structure and the charged functional side chains, poly(pyridinium salt)s, which belong to a class of main-chain cationic polymers, consist of 4,4′-(1,4-phenylene)bis(2,6-diphenylpyridinium) ions along the backbone of the polymer chains. They exhibit a number of interesting properties such as redox behavior, liquid crystalline, light-emitting properties, and so on.[28–30] Considering the good optoelectronic property, the charged nature as well as facile preparation of the kind of polyelectrolytes, we believe that poly(pyridinium salt)s derivatives are expected to become a potential alternate used as fluorescent probes for biomolecules sensing and even complicated biological application.

Herein, a new conjugated poly(pyridinium salt)s, poly{(4,4′-(1,4-phenylene)bis-(2,6-diphenylpyridinium))-*co*-N-butyl-3,6-carbazolylene ditosylates} (**P1**) were designed and synthesized for fluorescence light-up heparin detection. The working principle is as follows: Free **P1** are weakly emissive in solution because of intramolecular charge transfer (ICT) from carbazole segments (electron donating part) to bispyridinium salts segments (electron withdrawing part). Upon adding heparin, **P1** exhibits strong fluorescence because of the inhibited ICT effect, which should be attributed to the restriction of the intramolecular rotation of **P1** after the formation of aggregates with negative charged heparin through electrostatic interactions. By taking advantage of the influence of complexation between **P1** and heparin on ICT effect and stronger electrostatic interactions between **P1** and heparin *vs.* its analogies, we demonstrated that poly(pyridinium salt)s **P1** can be used light-up probes for sensitive detection of heparin with high selectivity. Moreover, **P1** exhibited a wide quantification range for heparin (0–14 μ*M*) in aqueous ethanol solution, which may find applications in related research fields requiring heparin quantification.

EXPERIMENTAL

Materials and Characterization

Heparin, chondroitin sulfate (ChS), and hyaluronic acid (HA) were provided by Aladdin Reagent Company (Shanghai, China) for direct use. All solvents and reagents (analytical grade and spectroscopic grade) were obtained commercially and used as received unless otherwise mentioned. The water used throughout all experiments was purified by a Millipore filtration system.

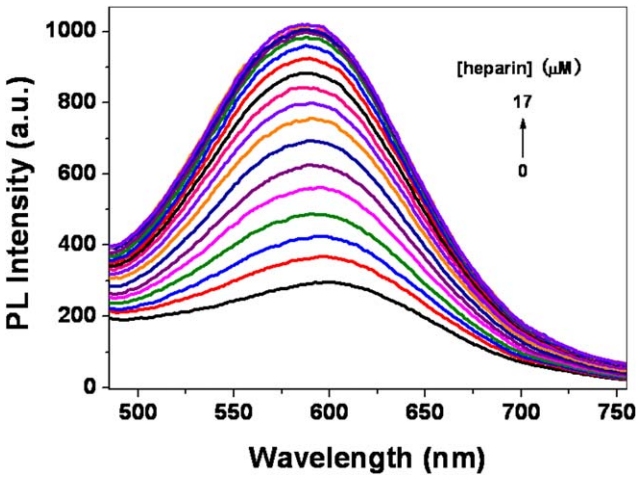


Figure 1. Changes in the PL spectra of **P1** upon addition of different amounts of heparin (excitation at 400 nm). The heparin concentration changes from 0 to 17 μ*M* at intervals of 1 μ*M* upon each addition. Experiments were conducted in ethanol-PBS (2 m*M* pH 7.4) (v/v 1 : 1), [**P1**] = 10 μ*M*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Heparin concentration was calculated using disaccharide as the repeat unit. The stock solutions of heparin (1.0 m*M*) in 2.0 m*M* phosphate buffer solution (PBS) (pH = 7.4) were prepared for spectral measurements. The polymer concentration was also calculated based on the repeat unit.

^{1}H NMR spectra were recorded on a Bruker spectrometer at 400 MHz. Chemical shifts (δ values) were reported in ppm down field from internal Me_4Si. Elemental analyses were performed on a Vanio-EL elemental analyzer (Analysensysteme GmbH, Germany). GPC analysis was conducted with a Waters 2690 liquid chromatography system equipped with Waters 996 photodiode detector and Phenogel GPC columns, using polystyrenes as the standard and DMF as the eluent at a flow rate of 1.0 mL/min at 35°C. A 50 μL of 0.2 wt % of polymer in DMF containing 0.01 *M* LiBr was injected in the columns. The pH measurements were carried out on a Mettler-Toledo Delta 320 pH meter. The absorption spectrum was recorded on a Shimadzu UV-2550 spectrometer. Fluorescence measurements were performed using an F-4600 fluorescence spectrophotometer (Hitachi, Japan) equipped with a quartz cell (1 cm × 1 cm). Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano ZS90 (Malvern Instruments Co., UK) equipped with a He-Ne laser.

Polymer Synthesis

The bis(pyrylium salt)s (**2**) was polymerized with 3,6-diamino-*N*-butylcarbazole (**3**) by ring-transmutation polymerization reaction[25–28] to yield polymer **P1** that was carried out on heating in DMSO as shown in Scheme 1. A solution of the monomer **2** (40 mmol) and the another monomer **3** (40 mmol) in 20 mL of DMSO was vigorously stirred and heated at 110°C under nitrogen for 2 h. Toluene (10 mL) was then added so that the water generated by the transformation of the pyrylium ring to the pyridinium ring could be removed as a toluene/water azeotrope. The bath temperature was increased to 150°C and the azeotrope and the excess toluene were gradually distilled from the reaction mixture using a Dean-Stark trap over 4–5 h. The bath temperature was maintained at that temperature of 145–150°C for 48 h. The resulting viscous solution was slowly poured into a large excess (25 times in volume) of rapidly stirred ethyl acetate. The yellow poly(pyridinium salt)s that precipitated was collected by filtration, purified by precipitation from DMSO with water and dried under reduced pressure at 120°C overnight. ^{1}H NMR (400 MHz, d_6-DMSO, ppm): 8.85–7.08 (m, 42H, Ar-H), 4.17 (t, 2H, -N-CH_2-), 2.25 (s, 6H), 1.79 (m, 2H), 1.41 (m, 2H), 0.93 (t, 3H, R-CH_3). Anal. calcd for $C_{70}H_{57}N_3O_6S_2$: C, 76.41; H, 5.22; N, 3.82. Found: C, 76.52; H, 5.23; N, 3.85.

Heparin Detection in Aqueous Ethanol Solution

The stock solution of **P1** in ethanol (10 μL, 5.0 m*M*), 2.5 mL of ethanol, and 2.5 mL of PBS (2.0 m*M*, pH 7.4) were mixed together to yield testing used solution (10 μ*M* of **P1**, ethanol-PBS, 1 : 1, v/v). Then heparin stock solution (1.0 m*M*) in 2.0 m*M* PBS (pH = 7.4) was added dropwise into 2 mL of the above mixture solution for spectral analysis. Upon each addition, the mixture was gently shaken before fluorescence analysis. The fluorescence spectra were collected in the range of 450–800 nm with an excitation wavelength of 400 nm.

RESULTS AND DISCUSSION

Synthesis and Characterization of P1

Scheme 1 depicts the synthetic route towards the carbazole derivative-containing alternating copolymer, poly[(4,4′-(1,4-phenylene)bis(2,6-diphenylpyridinium))-*alt*-(*N*-butyl-3,6-carbazolylene) ditosylate] (**P1**). The starting materials, 3,3′-(1,4-phenyl-ene)bis(1,5-diphenyl-1,5-pentadione) (**1**) and 4,4′-(1,4-phenylene)bis(2,6-diphenyl-pyrylium) ditosylate (**2**), were synthesized according to our previous report.[25–27] The diamino monomer, 3,6-diamino-9H-*N*-butylcarbazole (**3**), was synthesized in an 63% yield according to the reported synthetic procedure.[31] The correct chemical structures of **1**, **2**, and **3** were confirmed using NMR spectroscopy, elemental analysis. The ring-transmutation polymerization reactions of **2** with **3** afford a new conjugated poly(pyridinium salt)s (**P1**) in yields of over 85% shown in Scheme 1. In this step, the increasing reaction time and the removal of the water generated by the transformation of the pyrylium rings to the pyridinium rings from the polymerization medium by using

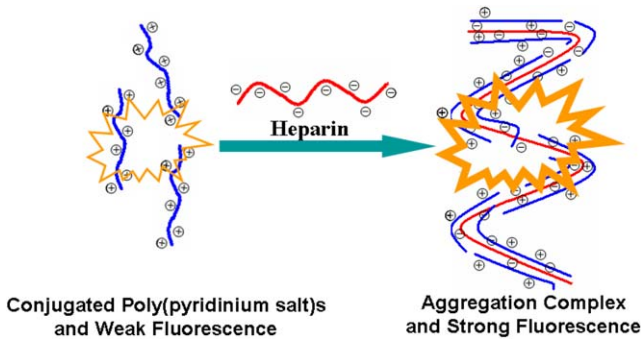


Figure 2. Schematic illustration of heparin detection. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

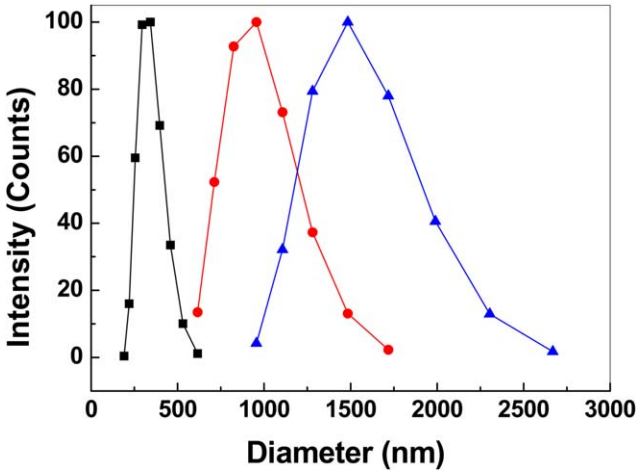


Figure 3. Hydrodynamic diameter of **P1** (10 μM) in the presence of heparin in EtOH-PBS (2 m*M* pH 7.4) (v/v 1:1) obtained from DLS. **P1** (square); **P1** + 5 μM of heparin (circle); **P1** + 15 μM of heparin (triangle). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

toluene as an azeotrope agent were crucial for the high yield. The number, average molecular weight, and polydispersity of **P1** were 13, 760, and 1.83, respectively, determined by gel-permeation chromatography (GPC) using DMF containing 0.01*M* LiBr as the solvent, and polystyrene standards. In addition, the elemental analysis results for **P1** coincide well with theoretical calculations, reflecting the high purity of the polymer. **P1** exhibits a maximal absorption at 334 nm in ethanol-PBS (2 m*M*, pH 7.4) (v/v 1 : 1) ($\varepsilon = 7.8 \times 10^4\ M^{-1}\ cm^{-1}$) in the UV-vis spectrum, which is assigned to π–π^* transition in the polymer backbone (Supporting Information Figure S1).

Complexation-Enhanced Fluorescence

To investigate the effect of polymer-heparin complexation on the fluorescence of **P1**, titration experiments were conducted by adding heparin into the polymer solution gradually in ethanol-PBS (2 m*M*, pH 7.4) (v/v 1 : 1) mixture solution (Figure 1 and Supporting Information Figure S2). As can be seen from Figure 1 and Supporting Information Figure S2, the PL intensity of polymer increased gradually with the increase of heparin concentrations at various polymer concentrations, such as 10, 20, and 50 μM. The optimized concentration of polymer can be easily selected as **P1** exhibits the weakest background fluorescence at [**P1**] = 10 μM.

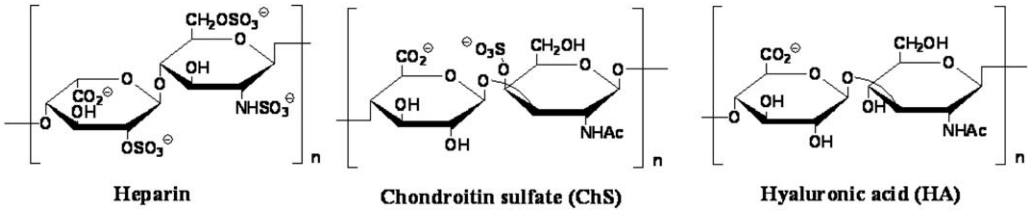
Figure 1 shows the changes in the PL spectra of **P1** upon addition of heparin ranging from 0 to 17 μM. Free **P1** are weakly emissive in solution in the absence of heparin because of ICT from carbazole segments (electron donating part) to bispyridinium salts segments (electron withdrawing part). With the increment of heparin concentration, the fluorescence intensity increased gradually to about 3.5 times its individual intensity in the absence of heparin, concomitant with a gradual blue-shift of the emission maximum from 604 to 586 nm and then tended to saturation when the heparin concentration reached 15 μM, indicating the analyte-receptor saturation. At the saturation point, the negative charges (60 μM) from heparin (15 μM) are about 3 times more than that for the positive charges (20 μM) from **P1**, which indicates that the aggregation of polymer occurs around heparin as depicted as follows (Figure 2).

Proposed Mechanism of Light-up Fluorescence

To further study the mechanism of enhanced fluorescence, the complexation behavior between **P1** and heparin was probed by DLS experiments in aqueous ethanol solution. DLS data obtained for a fixed concentration of **P1** (10 μM) with varying concentrations of heparin (0–15 μM) showed changes in the hydrodynamic diameter depending on the progress of titration (Figure 3). **P1** forms small aggregates with a mean diameter of 340 nm, which is ascribe to its poor water-solubility. When heparin was added to the **P1** solution, larger aggregates form with a mean diameter of 950 nm at [Heparin] = 5 μM. Near the endpoint of the titration, **P1**-heparin complexes exist in the highly aggregated state, whereas further addition of heparin causes electrostatic repulsion between heparins and thus the heparin-**P1** complexes are dispersed. The results indicate that polymer aggregation around heparin lead to enhanced fluorescence through the inhibited ICT effect, which should be attributed to the restriction of intramolecular rotaion of **P1**.

Selectivity

To investigate the selectivity of probe **P1** and the influence of electrostatic interaction on the optical properties of heparin-polymer complexes, heparin derivatives, such as chondroitin 4-sulfate (ChS) and HA have also been added to **P1** solution. As shown in Scheme 2, all three molecules have a similar main-chain structure. However, on the basis of sugar dimer as the repeated units, ChS and HA have two and one negative charges on the side chains, respectively. Under the same experimental conditions used for Figure 4, addition of 20 μM ChS or HA into the **P1** solution did not lead to significant fluorescence increase (Figure 4). The good selectivity for heparin is associated with much stronger electrostatic interactions between heparin and **P1**, as compared to that for ChS/**P1** or HA/**P1**, which consequently induces more compact polymer aggregation in the presence of heparin to give enhanced fluorescent emission. This selective optical signature not only allows distinguishing heparin from its analogues but also highlights the importance of



Scheme 2. Chemical structures of Heparin, ChS, and HA.

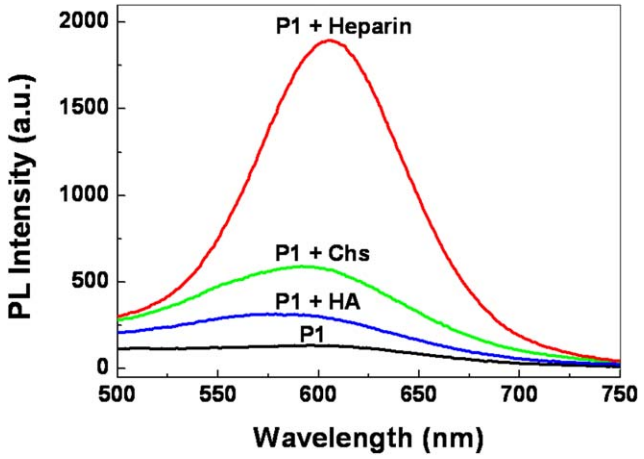


Figure 4. PL spectra of **P1**, **P1**/heparin, **P1**/ChS, and **P1**/HA in ethanol-PBS (2 m*M* pH 7.4) (v/v 1 : 1). [**P1**] = 10 μM, [heparin] = [ChS] = [HA] = 20 μM, (excitationat 400 nm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

electrostatic attraction between the polymer and the analyte in polymer aggregation process.

Anion Interference Experiments

As electrostatic attraction was the main driven force for the **P1**-heparin complex, other negative ions maybe compete with heparin to bind with **P1**. Herein, ion interference experiments were carried out in aqueous ethanol solution (EtOH-H_2O, 1 : 1, v/v) for evaluation of the sensing ability of **P1** to heparin in the presence of three equivalents of a series of background anions. Sufficient fluorescence enhancement was observed for **P1** after the addition of heparin in the presence of various anions (Figure 5), suggesting that the **P1** can be used as a selective fluorescent probe for heparin in the presence of a wide range of the environmentally relevant anions.

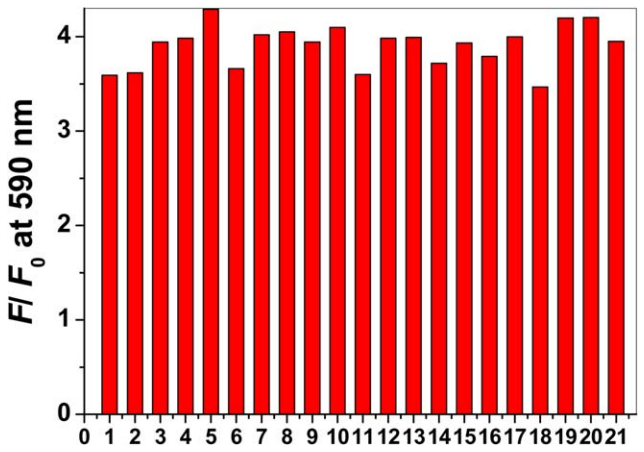


Figure 5. Ratio (F/ F_0) of fluorescence intensity at 590 nm of **P1** (10 μM) in EtOH-H_2O (v/v 1 : 1) upon the addition of 3 equiv heparin in the presence of 3 equiv competing anions. Interfering species: (1) none; (2) $CH_3CO_2^-$; (3) Br^-; (4) ClO_4^-; (5) F^-; (6) HPO_4^{2-}; (7) HSO_4^-; (8) I_3^-; (9) BF_4^-; (10) PF_6^-; (11) I^-; (12) NO_3^-; (13) CO_3^{2-}; (14) Cl^-; (15) HCO_3^-; (16) OH^-; (17) NO_2^-; (18) $P_2O_7^{4-}$; (19) PO_4^{3-}; (20) S^{2-}; (21) SO_4^{2-}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

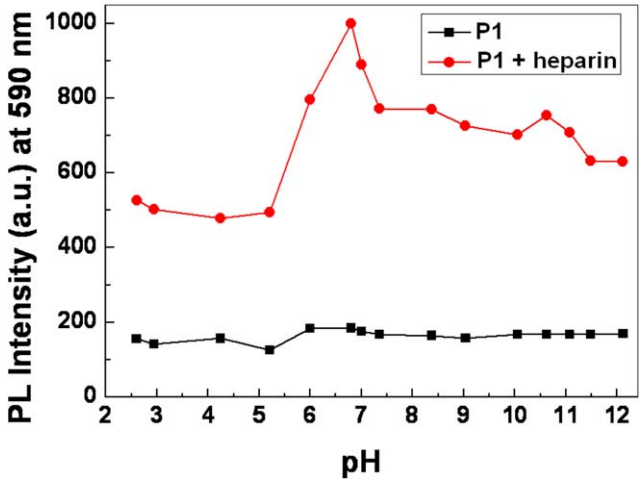


Figure 6. Effect of pH on the fluorescence intensity of **P1** (10 μM) (black squares) and **P1**/Heparin (30 μM) mixtures (red dots) at room temperature (excitation at 400 nm, fluorescent intensity at 590 nm) in EtOH-H_2O (v/v 1 : 1). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effect of pH on Performance of Probe

To further explore the practical applicability of the probe, the effects of pH on the fluorescent response to heparin of the new sensor **P1** were also investigated. Experimental results show that the intensities of free **P1** have no significant changes in a wide pH range from 1 to 12 (Figure 6). However, upon addition of heparin, **P1** shows different intensity enhancements. Under acidic conditions (pH < 6), the carboxylate and sulfate groups of heparin may be partially protonated, which decreases the electrostatic interactions of **P1** and heparin. At pH over 7, quaternary ammonium cations (Nc+) of **P1** may form an NcOH complex, which can weaken the electrostatic interactions of **P1** and heparin. **P1** shows the highest fluorescence response toward

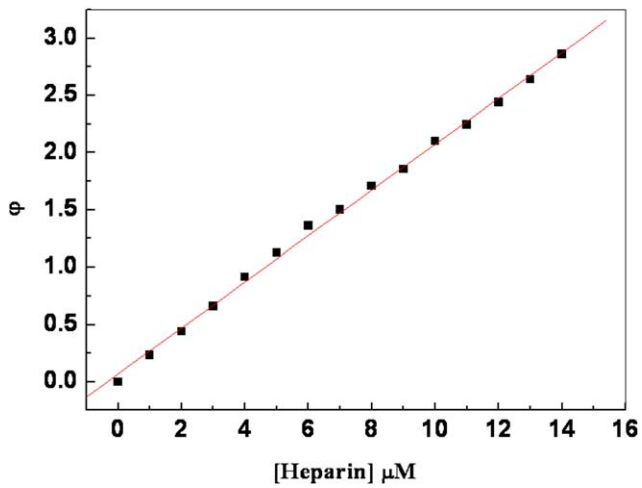


Figure 7. φ as a function of [heparin] at [**P1**] = 10 μM in ethanol-PBS (2 m*M* pH 7.4) (v/v 1 : 1). The data are based on the average value of the three independent experiments at exicitation wavelengthe of 400 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

heparin in the pH range of 6.0–8.0. Therefore, the probe **P1** can be used effectively at pH values near physiological conditions.

Heparin Quantification

To demonstrate heparin quantification, changes in the PL spectra of **P1** in Figure 1 are correlated to heparin concentration. We define φ as: $\varphi = (F - F_0)/F_0$, where F and F_0 are the intensities at 590 nm in the presence and absence of heparin, respectively. Figure 7 shows φ as a function of heparin concentration. The linearity in the concentration range of 0–14 μM indicates that it is feasible to use **P1** as a heparin probe for heparin quantification. As the heparin concentration at the therapeutic dosing level is 0.2–1.2 $U{\cdot}mL^{-1}$ (1.8–10.8 μM) in postoperative and long-term care, the obtained heparin calibration curve with [**P1**] = 10 μM can be used for detecting heparin required for clinical applications. At [**P1**] = 10 μM, the slope of the curve is 0.201 μM^{-1} and the correlation coefficient is 0.998.

The limit of detection for heparin in ethanol-PBS (2 mM pH 7.4) (v/v 1 : 1) mixture is about 7.6×10^{-8} M, based on $3 \times S_0/S$, where S_0 is the standard deviation of background and S is the sensitivity (detailed information see also ESI).

CONCLUSIONS

In summary, different from the reported fluorescence turn-on probe for heparin, which sensing mechanism mainly takes advantage of the distance-dependent fluorescence resonance energy transfer or aggregation-induced emission, we have successfully developed a fluorescence light-up macromolecular probe for detection and quantification of heparin by taking advantage of inhibited ICT effect because of the restriction of intramolecular rotaion of **P1** through complexation between **P1** and heparin, which increases the polymer fluorescence in aqueous ethanol solution. The novel sensing strategy will open new routes to simple synthesis of macromolecular probes for heparin sensing with high sensitivity and selectivity. Electrostatic interaction between the probe and heparin are used to regulate the supramolecular aggregation process. A good linear response for heparin was found in the range of 0–14 μM (approximately 1.6 U mL^{-1}) in ethanol-PBS solution. Furthermore, **P1** exhibits good selectivity to distinguish heparin from its analogue ChS and HA. The facile preparation and the robust aqueous detection condition make conjugated poly(pyridinium salt)s become a good candidate for the applicable heparin sensors and even complicated biological application.

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