Electronic Supplementary Information (ESI) for Chemical Communications.

Ultrafine fluorene-pyridine oligoelectrolyte nanoparticles for supersensitive fluorescent sensing of heparin and protamine

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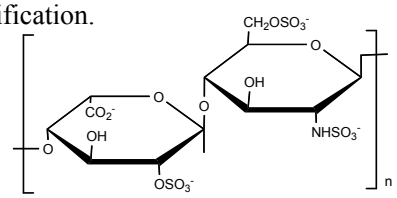
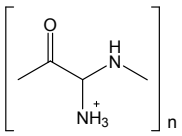
EXPERIMENTAL SECTION

Materials and instruments. Protamine sulfate was purchased from TCI (Shanghai) and heparin sodium salt was obtained from Aladdin (Shanghai, China). Clinical serum samples were provided by Hospital of Hunan Normal University, China. Other materials obtained from commercial suppliers were used without further purification.

Structural formulas of **Hep** and **Pro**:

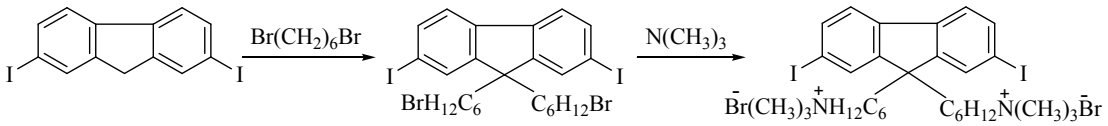


Heparin



Protamine

Fluorescence spectra were recorded on a Hitachi F-7000 fluorometer (Japan). UV-visible absorption studies were carried out on a UV-2450 spectrophotometer (Shimadzu Co., Japan). The ^{1}H NMR spectrum was taken on a Bruker 500 MHz NMR spectrometer. MALDI-TOF mass spectrum was obtained with a Bruker ultrafle Xtreme MALDI-TOF/TOF spectrometer with matrix. Transmission Electron Microscope (TEM) measurement was carried out with a JEM-2010 (JEOL, Japan). Zeta potential of OFPNPs probe was measured on a Zetasizer Nano ZS analyzer (Malvern, UK). Fluorescence images of cells were taken on a confocal laser scanning microscope (Nikon A1, Japan). The LC-MS analysis was performed using LCMS 8050 mass spectrometer (Shimadzu). Fluorescence images of OFPNPs samples were observed by an inverted fluorescence microscope (Eclipse Ti-S).



(1) (2) (3)

Synthesis of Compound (1). Compound **(1)** was prepared according to published literature with minor adjustment.[1] 165 mL of mixed solution (CH_3COOH:H_2O:H_2SO_4 = 50:4:1) was put into a 250 mL three-necked flask, and the mixture was stirred and heated to 80 ℃. Then fluorene (2.54 g, 15 mmol), KIO_3 (1.28 g, 6 mmol) and I_2 (4.1 g, 16.2 mmol) were added. After 10 h, the mixed solution turned purple and the precipitate was formed. After cooling, the precipitate was collected by filtration, and the solid was washed with water and 2 M Na_2CO_3. The crude product was recrystallized from dichloromethane to give a pale yellow solid (4.76 g, 76%). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 3.83 (s, 2H), 7.49 (d, 2H), 7.70 (d, 2H), 7.87 (s, 2H).

Synthesis of Compound (2). 30 mL of 50% aqueous KOH was added to a 250 mL three-necked flask, then stirred and heated to 75 ℃. Then 2,7-diiodo fluorene **(1)** (0.5 g, 1.2 mmol), tetrabutyl ammonium bromide (0.085 g, 0.26 mmol) and 1,6-dibromohexane (3.0 g, 12 mmol) were added. After 15 min, the mixture was rapidly cooled to room temperature. It was extracted three times with dichloromethane, and the organic layer was washed with 1 M HCl, water and brine, and then dried over anhydrous Na_2SO_4. After filtration, the excess 1,6-dibromohexane was distilled under reduced pressure, and the residue was purified by column chromatography (silica gel, hexane/chloroform). Pale yellow solid **(2)** was obtained (0.54 g, 60.5%). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.55-0.61 (m, 4H), 1.06-1.25 (m, 8H), 1.55-1.70 (m, 4H), 1.88-1.92 (m, 4H), 3.30 (t, 4H), 7.41 (d, 2H), 7.64 (d, 2H), 7.67 (s, 2H).

Synthesis of Compound (3): 2,7-diiodo-9,9-bis(6'-bromohexyl) fluorene **(2)** (0.446 g, 0.6 mmol), 10 mL of trimethylamine and 10 mL of THF were added to a 100 mL three-necked flask and then refluxed for three days. The solvent and remaining trimethylamine were distilled under reduced pressure to give a crude product. Then the crude product was washed with diethyl ether to give a white solid **(3)** (0.465 g, 90%). ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 0.54-0.57 (m, 4H), 1.13-1.18 (m, 8H), 1.48-1.60 (m, 4H), 1.85-2.06 (m, 4H), 3.05 (s, 18H), 3.18 (m, 4H), 7.54 (d, 2H), 7.69 (d, 2H), 7.78 (s, 2H).

Synthesis of OFP. Monomer 2,6-dialkynyl pyridine was synthesized according to our previous reports.[16a] OFP was prepared as following. Typically, a mixed solution (5 mL water, 7.5 mL DMF, 2.5 mL diisopropylamine) was deoxygenated with nitrogen for ten minutes. 2,7-Diiodo-9,9-bis(6'-(N,N,N-trimethyl ammonium)-hexyl)bromine fluorine **(3)** (0.132 g, 0.15 mmol), 2,6-dialkynyl pyridine (0.0194 g, 0.15 mmol), $Pd(Ph_3P)_4$ (17.40 mg, 15.0 μmol) and CuI (2.90 mg, 15.0 μmol) were added to a 100 mL three-necked flask under N_2 atmosphere. The solvent was added by a syringe. After reacting at 50 ℃ for 24 h, the solution was brownish yellow and bright blue fluorescence could be observed. After cooling to room temperature, the insoluble matter was removed by centrifugation, and the filtrate was concentrated. The resulting solution was slowly dropped into 200 mL acetone/diethyl ether (75/50) mixture, and flocculent precipitate appeared. The supernatant was removed, and the lower layer was centrifuged. After repeated precipitation, the solid was obtained and dried at room temperature in vacuum. ^{1}H NMR (500 MHz, CD_3OD): δ (ppm) 0.60-0.78 (br, 4H), 1.17-1.34 (br, 8H), 1.59-1.75 (br, 4H), 2.10-2.30 (br, 4H), 2.89-3.10 (m,

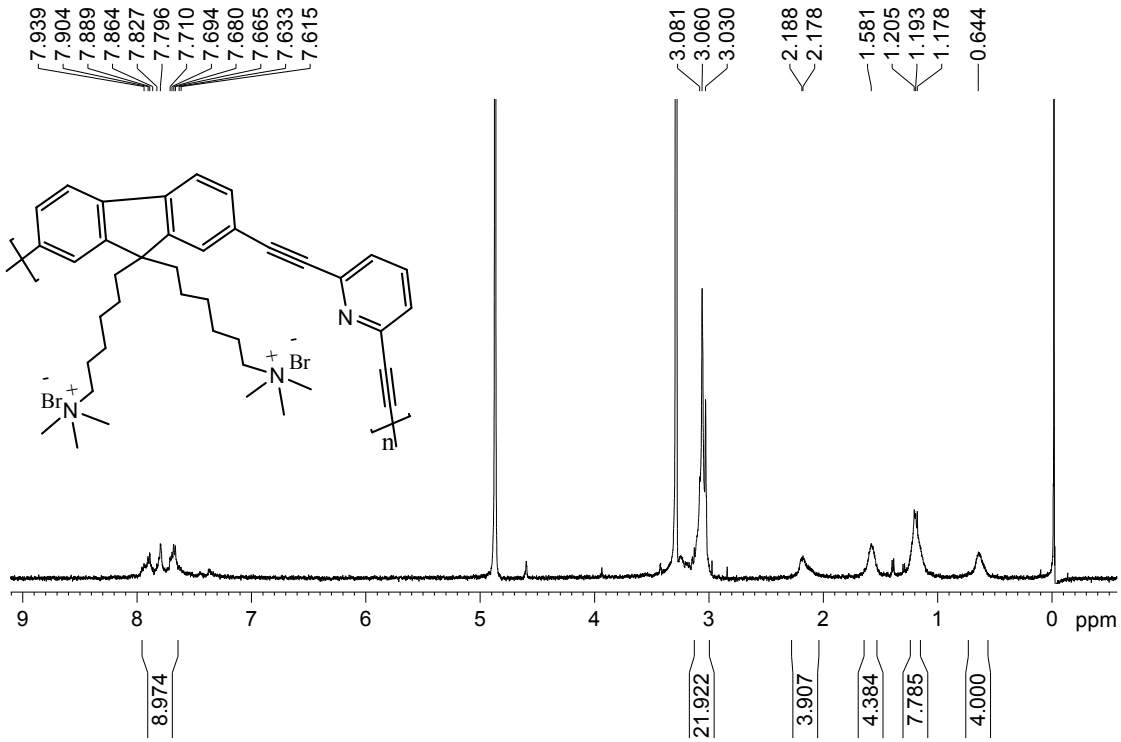
22H), 7.71-8.00 (m, 9H).

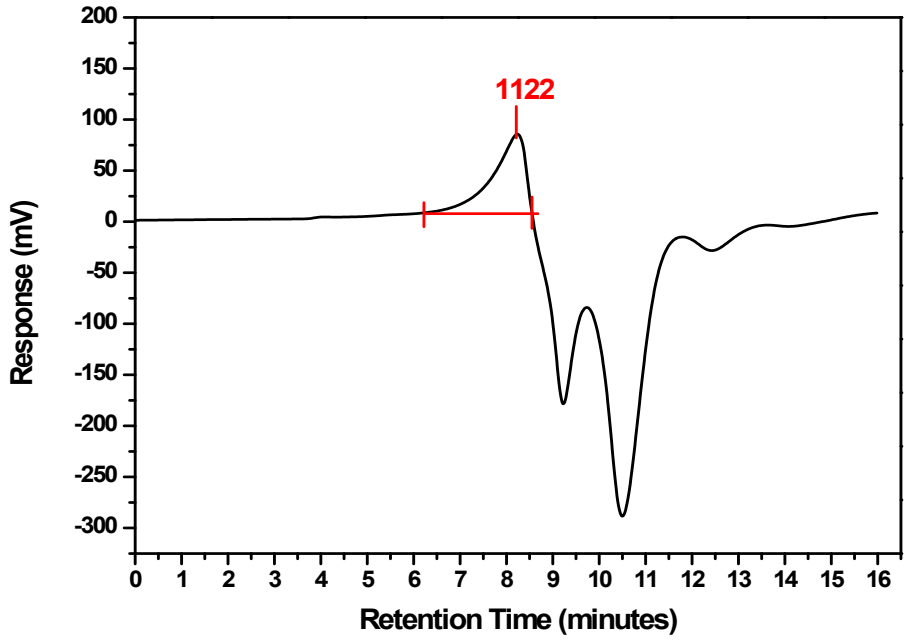
Synthesis of OFPNPs. Ultra-small OFPNPs were produced via a process reported in the literature.[2] Briefly, 0.02 g of PEG-300-COOH was dissolved in 20 mL of H_2O under stirring. Then 8.0 mL of CH_3OH containing OFP was added dropwise. The obtained mixture was sonicated for 0.5 h until the system turned transparent. After the evaporation of CH_3OH, the mixture was centrifuged at 10000 rpm for 10 min. The supernatant was obtained after filtration with a 0.1 μm filter and stored at 4 ℃.

Detection of heparin and protamine. Newly fabricated OFPNPs and buffer solution were added to a quartz cell. After the introduction of heparin, this solution was incubated for ~ 16 min before recording the spectra by a fluorescence spectrophotometer excited at 365 nm. Protamine may be added and incubated for 10 min before collecting the spectra data.

Detection of human serum samples. Human serum samples were used for heparin or protamine analysis to further confirm the reliability of the method. Human serum was collected from the Hospital of Hunan Normal University. Then, the obtained samples were centrifuged at 10,000 rpm for 10 min to remove possible proteins and subjected to a 10-fold dilution. The content of heparin or protamine was determined by the standard addition method.

Cytotoxicity and fluorescence imaging. MCF-7 cells (human breast carcinoma cell line) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cytotoxicity of OFPNPs was evaluated by MTT assay using various concentrations of OFPNPs (0, 5.0, 10.0, 15.0, 25.0, 35.0 and 40.0 μg/mL). The absorbance at 490 nm was monitored in a spectrophotometer. Before fluorescence imaging, MCF-7 cells were incubated with OFPNPs in RPMI-1640 (3 mL) for 0.5 h at 37 ℃, and the extracellular OFPNPs were eliminated by washing thrice with D-Hank's. Afterward, the cells were imaged by a confocal microscopy.





Broad Unknown Relative Peak Table

Distribution Name	Mp (Daltons)	Mn (Daltons)	Mw (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Mv (Daltons)	Polydispersity	Mz/Mw	Mz+1/Mw
1	1122	1662	3577	10172	18936	3042	2.15223	2.843723	5.293821

Fig. S1 ^{1}H NMR of OFP in CD_3OD and molecular weights of OFP indicated by GPC.

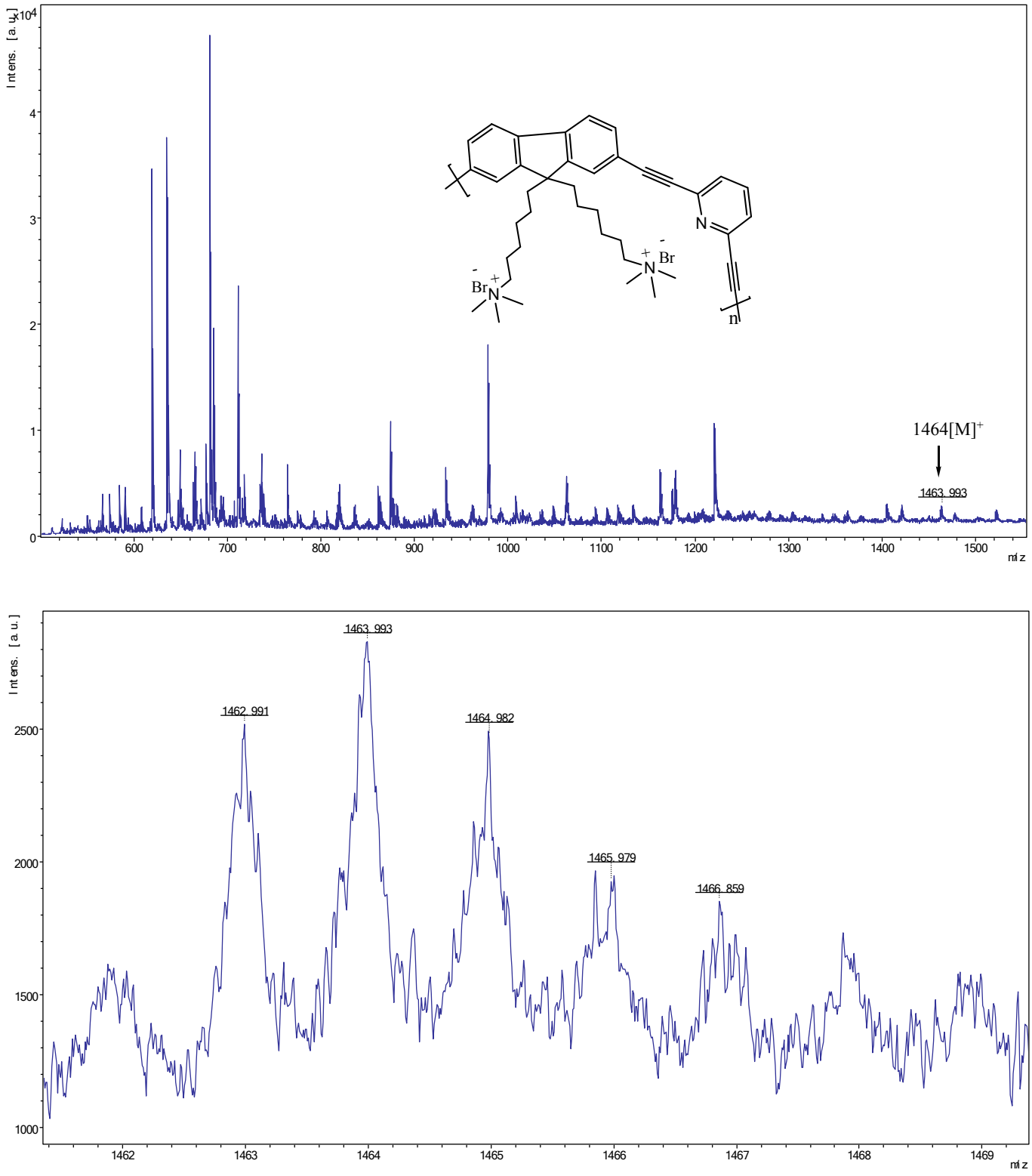
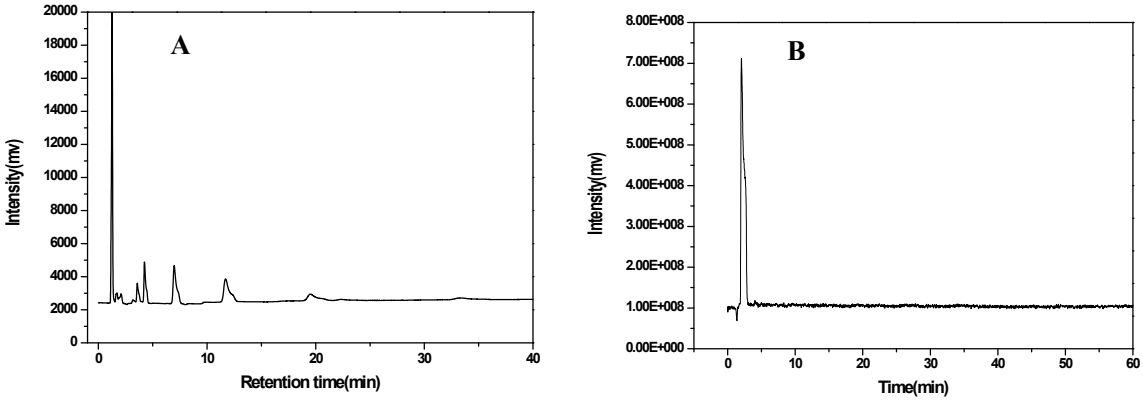
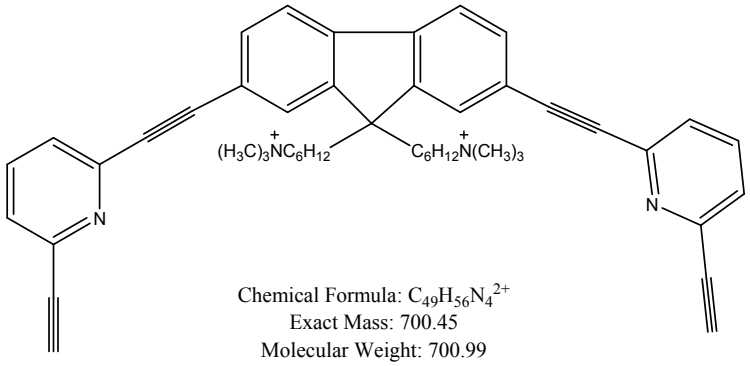


Fig. S2 MALDI-TOF mass spectra of OFP.





Chemical Formula: $C_{49}H_{56}N_4^{2+}$
Exact Mass: 700.45
Molecular Weight: 700.99
m/z: 350.22 (100.0%), 350.73 (53.0%), 351.23 (13.8%), 351.73 (2.3%), 350.72 (1.5%)
Elemental Analysis: C, 83.96; H, 8.05; N, 7.99

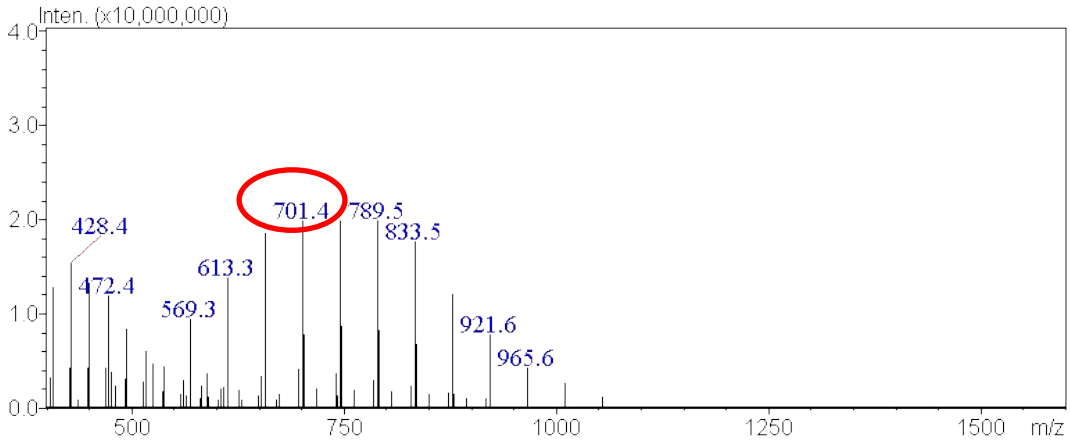


Fig. S3 Analysis of conjugated oligoelectrolyte OFP by LC-MS.

S1: Calculation of Quantum Yield

Calculation of quantum yield: The quantum yield (QY) of OFP or OFPNPs was calculated using quinine sulfate (QY = 0.55) in sulfuric acid (0.1 M, $\eta = 1.33$) as the standard and 360 nm as the excitation wavelength as reference. For calculation of quantum yield, six concentrations of each compound were made, all of which had absorbance less than 0.1. The OFP or OFPNPs sample was dissolved in water ($\eta = 1.33$). Their fluorescence spectra were recorded at excitation of 365 nm. Then by comparing the integrated fluorescence intensities (excited at 365 nm) and the absorbency values (at 361 nm) of the OFP or OFPNPs sample with the reference of quinine sulfate, QY of the OFP or OFPNPs sample was determined. The quantum yield was estimated with the equation:

$$\Phi_x = \Phi_{std}(F_x A_{std} \eta_x)/(F_{std} A_x \eta_{std})$$

Where Φ, F, A, and η are the quantum yield of the standard sample, integrated fluorescence intensity, absorbance, and refractive index, respectively. The subscript "std" refers to the standard fluorophore of known quantum yield, for an example, quinine sulfate used in present work.

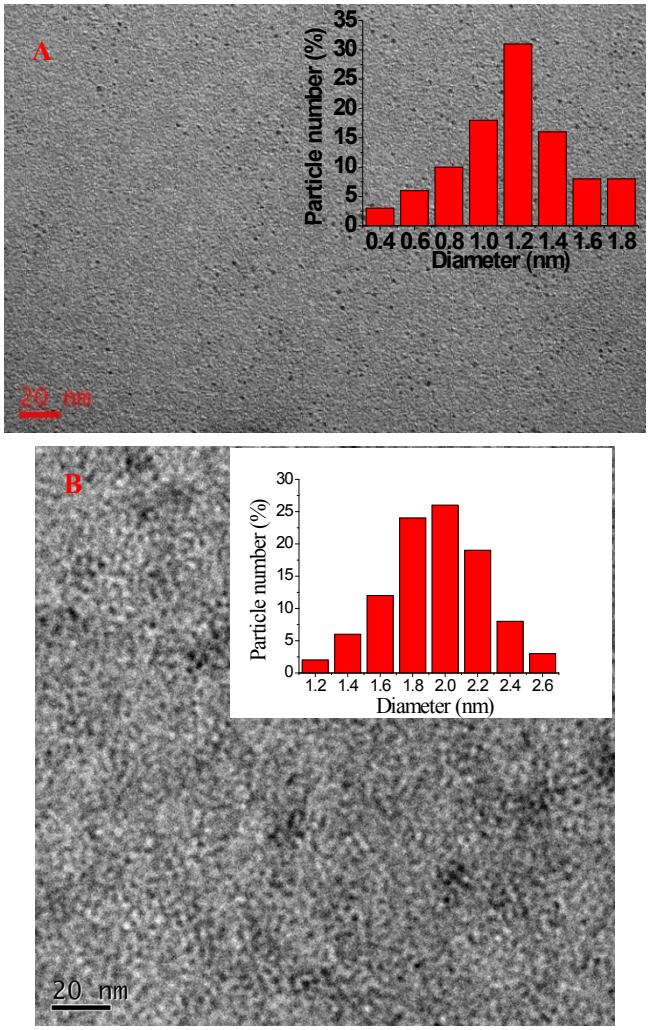


Fig. S4 TEM images of OFPNPs prepared using low (A) or higher (B) concentrations of OFP.

Table S1

OFPNPs prepared via a modified reprecipitation method using different concentrations of **OFP** under ultrasonication.

OFPNPs	**OFP (s)**	**CH_3OH solvent**	**PEG**	**H_2O**	**OFPNPs diameter**
a	0.8 mg	8.0 mL	0.0210 g	20 mL	~ 1.2 nm (0.4-1.8 nm)
b	0.8 mg	1.0 mL	0.0219 g	20 mL	~ 2.0 nm (1.2-2.6 nm)

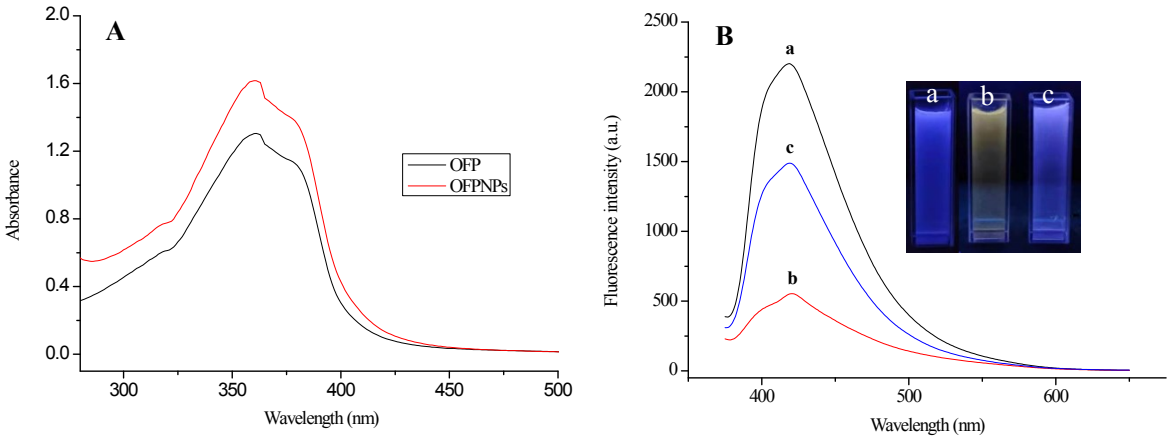


Fig. S5 (A) UV-vis absorption spectra of OFP and OFPNPs. (B) Fluorescence emission spectra (λ_{ex} = 365 nm) of OFPNPs (a), OFPNPs/Hep (b) and OFPNPs/Hep/Pro (c) in pH = 5.0 buffer solution (HAc-NaAc). Inset: the corresponding photos of OFPNPs, OFPNPs /Hep and OFPNPs /Hep /Pro under 365 nm UV light.

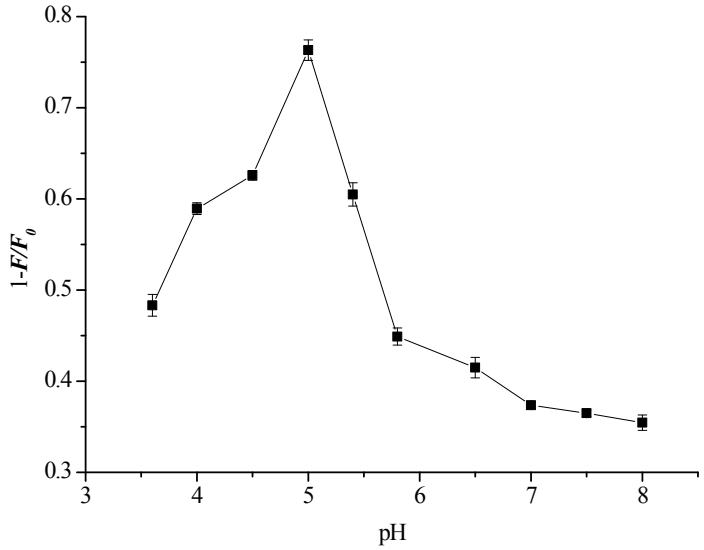


Fig. S6 Effect of pH on the detection of heparin. [OFPNPs] = 5.0×10^{-7} g/mL, [heparin] = 2.0×10^{-7} g/mL, λ_{ex} = 365 nm. Buffer solutions: 5.0 mM HAc-NaAc (pH 3.6-5.8), 5.0 mM NaH_2PO_4-Na_2HPO_4 (pH 6.5-8.0).

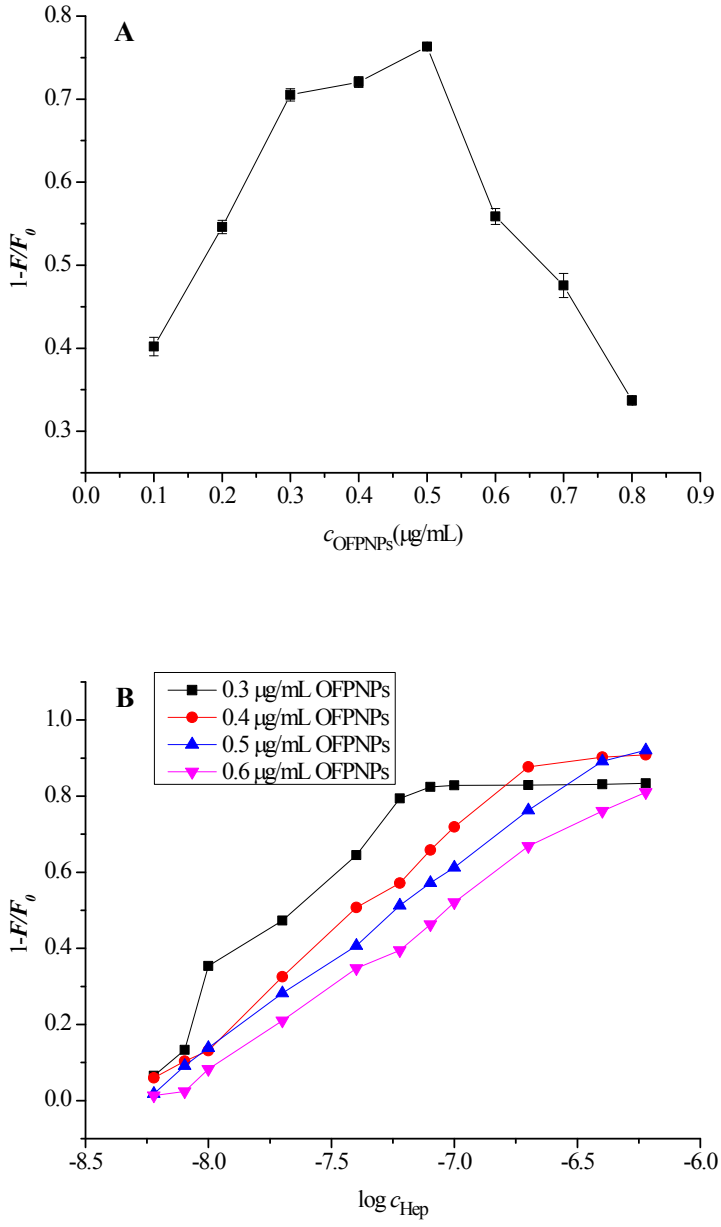


Fig. S7 (A) Effect of OFPNPs concentration on the fluorescent sensing of heparin (λ_{ex} = 365 nm). [heparin] = 2.0 × 10^{-7} g/mL, pH = 5.0 (HAc-NaAc). (B) Fluorenscence response of different concentrations of OFPNPs to heparin in pH 5.0 buffer solution. [heparin]: 6.0 × 10^{-9}, 8.0 × 10^{-9}, 1.0 × 10^{-8}, 2.0 × 10^{-8}, 4.0 × 10^{-8}, 6.0 × 10^{-8}, 8.0 × 10^{-8}, 1.0 × 10^{-7}, 2.0 × 10^{-7}, 4.0 × 10^{-7}, 6.0 × 10^{-7} g/mL.

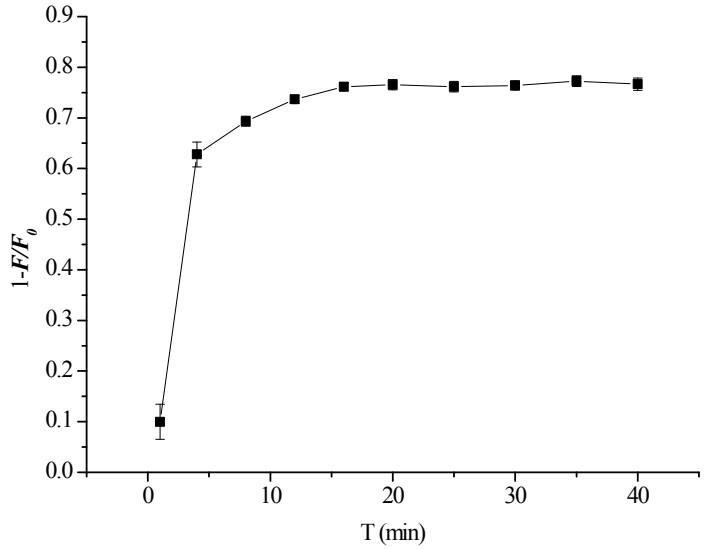


Fig. S8 Effect of time on the detection of heparin, [OFPNPs] = 5.0×10^{-7} g/mL, [heparin] = 2.0×10^{-7} g/mL, pH = 5.0 (HAc-NaAc). The excitation wavelength was 365 nm.

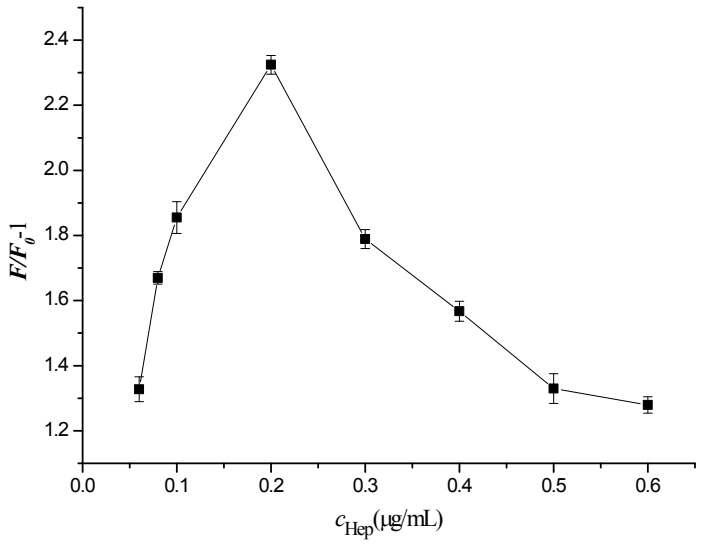


Fig. S9 Effect of heparin concentration on the fluorescent sensing of protamine (λ_{ex} = 365 nm). [OFPNPs] = 5.0×10^{-7} g/mL, [protamine] = 5.0×10^{-7} g/mL, pH = 5.0 (HAc-NaAc).

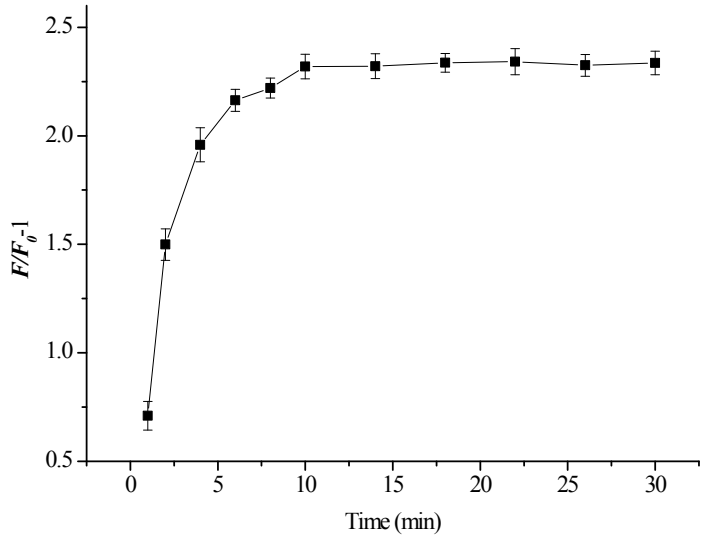


Fig. S10 Effect of time on the detection of protamine. [OFPNPs] = 5.0×10^{-7} g/mL, [heparin] = 2.0×10^{-7} g/mL, [protamine] = 5.0×10^{-7} g/mL, pH = 5.0 (HAc-NaAc).

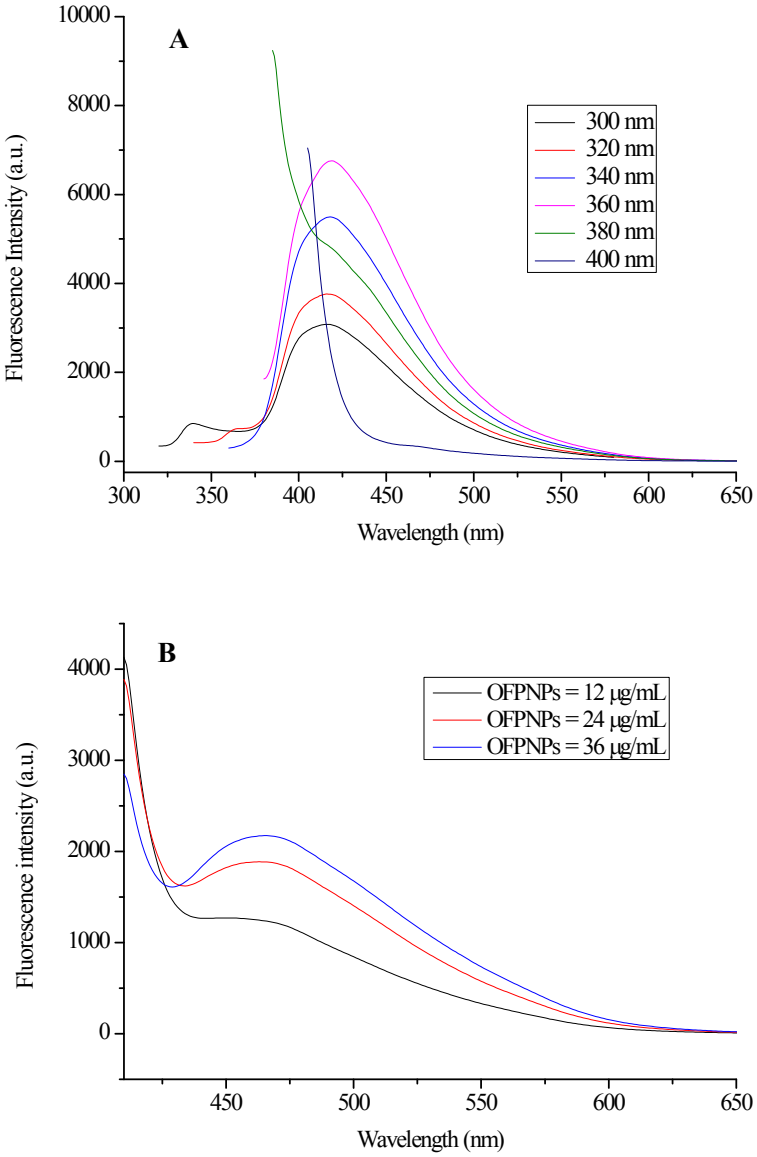


Fig. S11 (A) Fluorescence emission spectra at different excitation wavelengths ranging from 300 nm to 400 nm, OFPNPs = 1.2 μg/mL. (B) Fluorenscence response (λ_{ex} = 405 nm) of the solution containing various concentrations of OFPNPs.

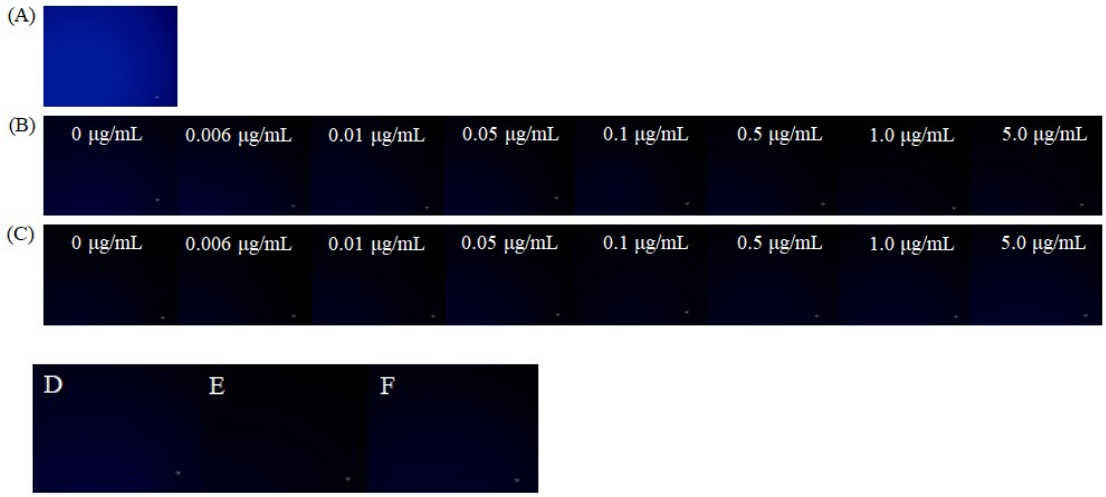


Fig. S12 Fluorescence images of OFPNPs (40.0 μg/mL) (**A**), OFPNPs (0.5 μg/mL) after adding heparin (0-5.0 μg/mL) (**B**), and OFPNPs (0.5 μg/mL)/heparin (0.2 μg/mL) upon adding protamine(0-5.0 μg/mL) (**C**), which were measured by fluorescence inverted microscopy. Exposure time: 600 ms. Selected photographys acquired by fluorescence inverted microscopy: (**D**) 0.5 μg mL^{-1} OFPNPs, (**E**) 0.5 μg mL^{-1} OFPNPs/ 5.0 μg mL^{-1} Hep and (**F**) 0.5 μg mL^{-1} OFPNPs/ 0.2 μg mL^{-1} Hep/ 5.0 μg mL^{-1} Pro. Exposure time: 600 ms.

Table S2

Representative approaches for heparin analysis.

Method	Material	Linear range (ng/mL)	LOD (ng/mL)	Reference
Fluorescence	Perylene derivative	0-49	2.4	[2b]
AIEgen	TPE derivative	0-4000	4	[3]
Colorimetry	AuNRs-GO	20-280	5	[5b]
AIEgen	Tetraphenylbenzene derivative	0-500	5.04	[7a]
IFE	BSA-capped CdS QDs	10-300	2.2	[7d]
Phosphorescent	Cationic polyfluorene-Ir(III)	0-44000	50	[11a]
Fluorescence	Ultrafine OFPNPs	6-600	1.2	This paper

Table S3

Some important probes for protamine sensing.

Method	Material	Linear range (ng/mL)	LOD (ng/mL)	Reference
Fluorescence	CdTe-QDs	2.0-200	1.0	[4]
Colorimetry	HAuNPs	10-70	5	[5c]
AIEgen	Tetraphenylbenzene derivative	0-6000	4.78	[7a]
FRET	UCNPs-STNPs	10-500	3.1	[7c]
Fluorescence	Tb-MOF	3-5000	1.21	[7e]
Fluorescence	Ultrafine OFPNPs	6-750	0.5	This paper

Table S4

Detection of heparin in 1% serum samples. [OFPNPs] = 5.0×10^{-7} g/mL. All measurements were performed in pH = 5.0 buffer solution (HAc-NaAc).

Sample	Spiked (ng/mL)	Detected	Recovery (%)	RSD (%, n = 3)
1	20	18.8	94	2.79
2	60	64.7	108	3.78
3	100	110.1	110	1.45

Table S5

Detection of protamine in 1% serum samples. [OFPNPs] = 5.0×10^{-7} g/mL, [heparin] = 2.0×10^{-7} g/mL. All measurements were performed in pH = 5.0 buffer solution (HAc-NaAc).

Sample	Spiked (ng/mL)	Detected	Recovery (%)	RSD (%, n = 3)
1	50	46.3	93	3.18
2	120	127.2	106	3.49
3	200	192.5	96	3.07

References

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