



Colorimetric and fluorometric aggregation-based heparin assay by using gold nanoclusters and gold nanoparticles

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Abstract

An optical nanoprobe consisting of gold nanoclusters (AuNCs) and gold nanoparticles (AuNPs) is described for ultrasensitive detection of heparin (Hep). Polyethyleneimine (PEI) induces the aggregation of AuNPs which results in a color change from wine red (peak at 520 nm) to blue (peak at 610 nm). In parallel, the fluorescence of AuNCs (with excitation/emission maxima at 370/610 nm) is weakened. However, in the presence of Hep (which is strongly negatively charged), it will electrostatically bind to positively charged PEI and then will prevent aggregation. Hence, the color changes from blue (aggregated) to red (non-aggregated). In parallel, fluorescence remains unchanged. Hep can be quantified by using the nanoprobe in the range of 4–220 ng·mL⁻¹, with the detection limits as low as 1.6 (colorimetry) and 3.4 ng·mL⁻¹ (fluorometry). The assay was applied to the detection of Hep in (spiked) human serum with satisfactory results.

Keywords Polyethyleneimine · Inner filter effect · Nanoprobe · Surface plasmon resonance · Color change · Fluorescence quenching · Electrostatic interaction · Transmission electron microscopy · Human serum

Introduction

Heparin (Hep) is a highly sulfated glycosaminoglycan with the highest negative charge density among natural biopolymers [1]. It plays an important role in the regulation of diverse physiological processes [2–4] and is extensively used as an anticoagulant or antithrombotic agent in clinical operations [3]. However, overuse can cause unpredictable adverse effects [5]. Traditional clinical laboratory assays for Hep include the activated clotting time assay (ACT), activated partial thromboplastin time assay (APTT), chromogenic antifactor Xa, and electrochemical approaches [6–10]. Although these methods have made great progress in terms of high selectivity and comparative reliability, most of them suffer from such

drawbacks as high costs, sophisticated instruments, and especially requirements of pretreatment and postanalytical variabilities. Therefore, it is very urgent to develop simple, rapid, sensitive, and cost-effective methods to detect Hep.

Considerable efforts have been dedicated to constructing nanomaterial-based optical probe for Hep, including colorimetric [11–15] and fluorimetric [16–28], due to the merit of low cost, high sensitivity, and convenient operation. By taking advantage of high extinction coefficients and localized surface plasmon resonance (LSPR) effect [29, 30], gold nanoparticles (AuNPs) have been exploited as colorimetric probes for the detection of Hep. They are mainly based on distinct color changes associated with the turnover process from the dispersed to aggregated state through electrostatic interaction between the negatively charged Hep and cationic surface modifiers anchored onto AuNPs. However, many environmental variables may cause undesirable aggregation of AuNPs, making the detection results not very accurate. On the other hand, a series of fluorimetric probes based on luminescent NPs including quantum dots, carbon dots (C-dots), silicon NPs, and metal nanoclusters [16–28], have also been well established for Hep assay. For example, our group has reported a fluorescence assay for Hep based on the higher affinity of cetyltrimethyl ammonium bromide (CTAB) with Hep than with gold nanoclusters (AuNCs) [24]. Unfortunately, it acted

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in the on-off mode, which may not be reliable especially in complex biological fluids. Importantly, most of these optical nanoprobe only focus on a single response for the target, which can easily be disturbed by the surroundings and possible falsified signals, eventually affecting the accuracy of the experimental results. To address this issue, multimodal optical detection systems, for example, with colorimetric and fluorometric dual readout, are highly demanded because mutual correction between multiple signals from more than one transduction channel will make the results more convincing [31–33].

In this work, we present a nanoprobe with both colorimetric and fluorometric (“off-on” signal change) readout for selective and sensitive detection of Hep based on AuNCs coupled with AuNPs (Scheme 1). This optical detection platform combines the advantages of the convenience, high sensitivity, and low cost of the fluorometry and colorimetry. More importantly, the simultaneous optical signal changes can enhance the detection accuracy and are very favorable for practical applications, for instance, human serum analysis.

Experimental

Chemicals and materials

Hydrogen tetrachloroaurate(III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, 99%), glutathione (GSH), polyethyleneimine (PEI), trisodium citrate, and acetonitrile were provided by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China, www.reagent.com). Heparin (Hep, 150 USP units·mg⁻¹) was purchased from Adamas Reagent Co. Ltd. (Shanghai, China, adamas.company.lookchem.cn). Anions including Na_2SO_4 , Na_2CO_3 , $\text{Na}_4\text{P}_2\text{O}_7$, $\text{Na}_2\text{C}_2\text{O}_4$, NaAc, NaCl, NaHCO_3 , and Na_2HPO_4 were obtained from Shanghai Qingxi Technology Co. Ltd. (Shanghai, China, www.qingxihb.com). Biological reagents

including glucose ($\text{C}_6\text{H}_{12}\text{O}_6$), sodium hyaluronate (HA), adenosine triphosphate (ATP), bovine serum albumin (BSA) and all amino acids were bought from Aladdin Reagent Co. Ltd. (Shanghai, China, www.aladdin-e.com). Amino acids used here include tryptophan (Trp), phenylalanine (Phe), lysine (Lys), glycine (Gly), glutamate (Glu), proline (Pro), glutamine (Gln), Aspartic acid (Asp), threonine (Thr), valine (Val), isoleucine (Ile), histidine (His), methionine (Met), cysteine (Cys), serine (Ser), alanine (Ala), tyrosine (Tyr). All chemicals used were at least of analytical grade. Ultrapure water (18.2 M Ω cm⁻¹) was used to prepare all the solution in this study.

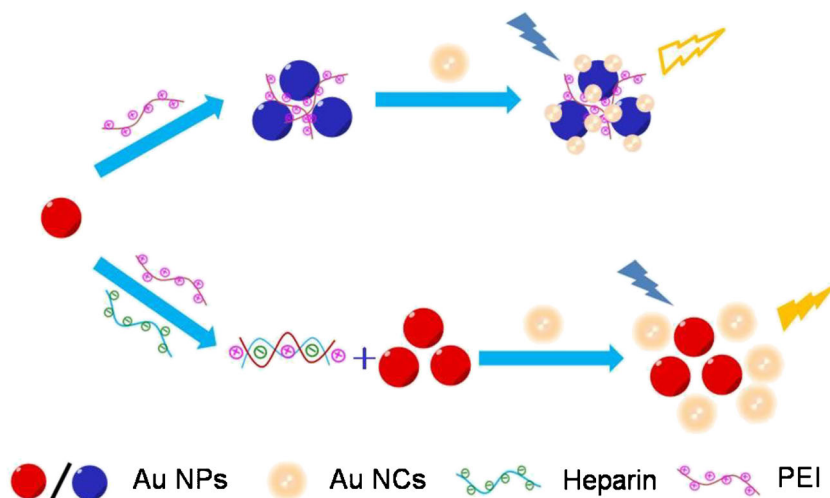
Instruments

Fluorescence spectra were performed on a fluorescence spectrometer (F-4600, Hitachi, Japan, hha.hitachi-hightech.com) equipped with a Xenon lamp source and a 1.0 cm quartz cell. UV-vis absorption spectra were conducted on a UV-2550 spectrophotometer (Shimadzu, Japan, www.shimadzu.com). Fourier transform infrared (FT-IR) spectra were recorded with KBr pellets on a Nicolet 5700 FTIR Spectrometer (Nicolet, USA, www.thermonicolet.co). The data of zeta potential were collected on NPA152 Nanoparticle size analyzer (Microtrac Inc., USA, www.microtrac.com). Transmission electron microscopy (TEM) was carried out with a JEM-2100 transmission electron microscope (JEOL Ltd. Japan, www.jeol.co.jp).

Preparation of AuNPs

All pieces of the experimental glassware used were cleaned with aqua regia solution (HCl: HNO₃, 3:1) and then rinsed thoroughly with H₂O and finally dried at 100 °C before use. Citrate-capped AuNPs were synthesized based on the Turkevich’ method [34]. The procedures for the preparation

Scheme 1 Schematic illustration for Hep detection based on the nanoprobe with both colorimetric and fluorometric readout



of 13-nm AuNPs was described as follows: 45 mL solution containing 1.0 mM of HAuCl₄ was prepared and heated under reflux to boiling, and then 5 mL of 38.8 mM trisodium citrate was quickly added to the solution under vigorous stirring, and the mixture was allowed to continue for another 30 min. The wine-red solution was cooled to room temperature and stored at 4 °C for further use. The concentration of AuNPs calculated according to Beer's law was 4.8 nM.

Synthesis of GSH-AuNCs

GSH-AuNCs were synthesized as reported previously [24]. Briefly, HAuCl₄ (1 g) was formulated into an aqueous solution (4 mM, 1 L). Then 50 mL of the above solution and the GSH aqueous solution (6 mM, 50 mL) were mixed to form a 100 mL solution. The mixture was immediately transferred to a 250 mL round bottom flask, and heated in an oil bath at 90 °C for 6.5 h. After naturally cooling to room temperature, it was purified by adding acetonitrile, and the precipitate was obtained by centrifugation (12,000 rpm, 10 min). The above centrifugation operation was repeated several times, except that acetonitrile was replaced with a mixed solution of acetonitrile and ultrapure water (v/v, 3:1). Finally, the purified precipitate were dried under vacuum and stored at 4 °C prior to use.

Procedures for the detection of Hep

First, 1700 µL of phosphate buffer (0.01 M, pH 7.0) containing various concentrations of Hep and PEI (50 ng·mL⁻¹) was mixed with 300 µL AuNPs, and the resulting mixture was equilibrated at room temperature. Then 1 mL of 0.3 mg·mL⁻¹ AuNCs was added into 1 mL of the AuNPs. After incubating for 15 min, UV-vis absorption and fluorescence spectra were conducted accordingly. For the UV-vis absorption measurement, the absorbances at 520 and 610 nm were recorded, and their ratio was calculated for the calibration plot. For the fluorescence measurement, the excitation and emission wavelengths were set at 370 and 610 nm, respectively.

Human serum analysis

Human serum was acquired from Nanchang University Hospital from healthy donors. Prior to analysis, a simple pre-treatment was performed. Briefly, acetonitrile (2.0 mL) was added to the serum (0.5 mL). After shaking thoroughly, it was centrifuged (12,000 rpm, 15 min). The supernatant was then aspirated and diluted to 25 mL. Thus, a diluted serum sample from which the protein was removed was obtained.

To evaluate the reliability in human serum analysis, the standard addition method was used to detect Hep. Simply, Hep at known concentrations (62.5, 125, and 188 ng·mL⁻¹) and PEI (50 ng·mL⁻¹) was first mixed separately, and then the

mixture was pipetted into the pretreated serum sample (50 µL). The subsequent procedures were carried out similar to those described above. The recovery results were calculated based on the calibration curves obtained in aqueous solution.

Results and discussion

Choice of materials

In the present detection system, glutathione protected AuNCs (GSH-AuNCs) were selected as the fluorometric reporter. AuNCs are a newly-emerged class of promising fluorophores with intriguing properties such as tunable photoluminescence, high quantum yield, large Stokes shift, and excellent biocompatibility [35]. These features make them very suitable as the fluorometric reporter. Besides, AuNPs functioned as the colorimetric reporter and furthermore as the fluorescence switch for GSH-AuNCs due to the distance-dependent optical properties. With respect to other materials such as C-dots, ZnO, MoS₂ or other oxides, when they act as the fluorometric reporters, their emission wavelengths are often shorter than 500 nm [27], which result in little spectral overlap with the absorption bands of AuNPs either in the dispersed or aggregation state. On the other hand, they do not have high extinction coefficients or localized surface plasmon resonance (LSPR) effect like AuNPs [29, 30], so they are not suitable as the colorimetric reporters.

Working principle of the assay

As depicted in Scheme 1, the positively charged polyethyleneimine (PEI) can induce the aggregation of citrate-capped AuNPs through electrostatic interaction followed by a color change from wine red to blue. The absorbance band of aggregated AuNP overlaps the emission spectrum of AuNCs, and the adsorption of PEI on AuNP surface also facilitates the contact with negatively charged AuNCs. Therefore, an efficient inner filter effect (IFE) occurs with AuNCs acting as the donors and AuNP acting as the acceptors, leading to the fluorescence quenching. However, in the presence of PEI and the target Hep simultaneously, Hep deactivates the aggregation agent PEI because they have a higher affinity than that between PEI and AuNPs. AuNPs are still well dispersed in solution with wine red color. When AuNCs are added, a sufficient distance is maintained with AuNPs owing to strong electrostatic repulsion and the emission spectrum does not overlap with the absorption peak of dispersed AuNPs; hence, IFE does not occur. As a result, the fluorescence of AuNCs changes little.

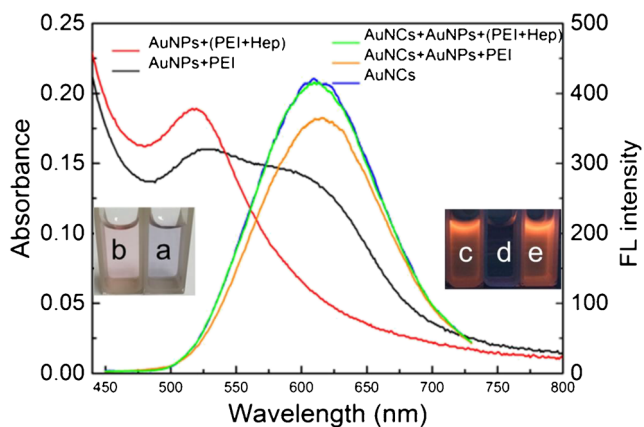


Fig. 1 UV-vis absorption spectra of AuNPs in the presence of PEI (red line) and PEI with Hep (black line). The inset shows the corresponding photographs: *a* AuNPs+PEI, *b* AuNPs+(PEI + Hep). Fluorescence spectra of AuNCs mixing with AuNPs upon addition of PEI or PEI with Hep. The inset shows the corresponding photographs: *c* AuNCs, *d* AuNCs+AuNPs+PEI, *e* AuNCs+AuNPs +(PEI + Hep)

Mechanistic investigation of the assay

As illustrated in Scheme 1, PEI served as a medium to tune the inter-particle distance among AuNPs regarding the color change and to modulate the occurrence of IFE between AuNPs and AuNCs leading to the fluorescence quenching or recovery of AuNCs. Citrate-stabilized AuNPs are negatively charged ($\zeta = -15.6$ mV), while PEI is positively charged ($\zeta = +13.9$ mV). As shown in Fig. 1, PEI can easily induce the aggregation of citrate-capped AuNPs due to electrostatic interaction, which results in a clear color change from red to blue. Concurrently, the characteristic SPR peak at 520 nm of AuNPs decreases and a new peak at 610 nm emerges (black line). Because the maximum emission peak of the GSH-AuNCs locates at about 610 nm (blue line), the absorption spectrum of aggregated AuNPs largely overlaps with the emission spectrum of AuNCs, indicating the potential of an efficient IFE process. In addition, since the GSH-AuNCs also bear negative charges ($\zeta = -9.18$ mV) due to the presence of carboxyl groups, polycationic PEI attached onto AuNPs makes AuNPs and AuNCs close to each other and thus ensures the IFE process to occur. These results virtually

contribute to the decrement of the fluorescence of AuNCs (orange line). However, Hep, a highly negatively charged macromolecule ($\zeta = -27.7$ mV), preferentially interacted with PEI due to stronger electrostatic interaction than that between AuNPs and PEI as well as hydrogen bonding between amino groups of PEI and hydroxyl groups of Hep [23]. In this case, AuNPs are still in a dispersed state, retaining wine red, only showing the absorption band at 520 nm (red line). In addition, the distance between AuNPs and AuNCs enlarges due to strong electrostatic repulsion. Therefore, IFE is invalidated and the fluorescence of AuNCs slightly changed (green line). It should be noted that in the presence of AuNPs, PEI, or Hep alone, the fluorescence of AuNCs keeps almost constant (Fig. S1). The phenomenon is further confirmed by TEM (Fig. 2). Upon addition of PEI into the mixture of AuNPs and AuNCs, AuNPs are highly aggregated (Fig. 2A). When PEI and Hep are added into the detection system at the same time, the solution is retained in a dispersed state owing to the interaction between PEI and Hep (Fig. 2B). These results strongly support our principle of detection of Hep that PEI may lead to the aggregation of AuNPs accompanied with the corresponding color change and fluorescence quenching of AuNCs while Hep can reverse the color change of AuNPs and restore the fluorescence of AuNCs by altering the inter-particle distance.

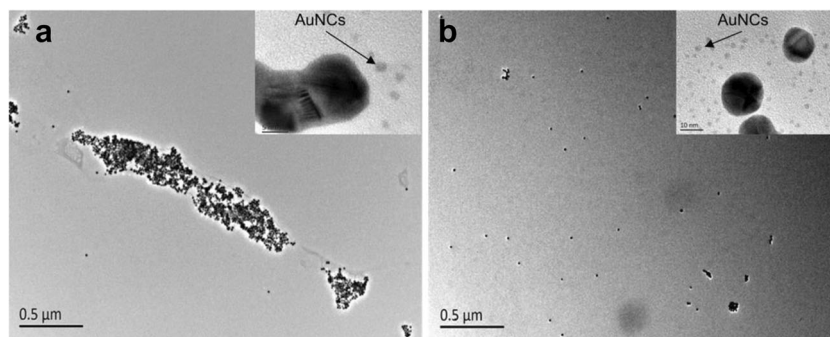
Optimization of method

The following parameters were optimized: (a) Concentration of AuNPs; (b) concentration of PEI; (c) pH value; (d) incubation time. Respective data and Figures are given in the Electronic Supporting Material. The following experimental conditions were found to give best results: (a) Best concentration of AuNPs: 0.36 nM; (b) Best concentration of PEI: 50 ng·mL⁻¹; (c) Optimal pH: 7.0; (d) Optimal incubation time: 15 min.

Colorimetric and fluorimetric detection of Hep

Under the optimal conditions, AuNPs and AuNCs were incubated with different concentrations of Hep and 50 ng·mL⁻¹ PEI, stirring and standing 15 min. As shown in Fig. 3a, as the concentration of Hep increases, the absorption peak of AuNPs at 610 nm ascribed to the aggregation state drops gradually,

Fig. 2 TEM images of AuNPs **a** in the presence of PEI and AuNCs; **b** and in the presence of PEI, Hep, and AuNCs. The insets are the corresponding HRTEM images, respectively



while the intensity of characteristic SPR peak centered at 520 nm increases a little. Figure 3b shows a good linear relationship between the ratio of $A_{520\text{nm}}$ to $A_{610\text{nm}}$ ($A_{520\text{nm}}/A_{610\text{nm}}$) and the concentration of Hep from 4 to 220 $\text{ng}\cdot\text{mL}^{-1}$ ($A_{520\text{nm}}/A_{610\text{nm}} = 0.017 C_{\text{Hep}}/(\text{ng}\cdot\text{mL}^{-1}) + 1.01$, $R^2 = 0.995$). The limit of detection (LOD) was calculated to be 1.6 $\text{ng}\cdot\text{mL}^{-1}$. At the same time, the fluorescence recovery of AuNCs due to competitive interaction of Hep with PEI can also be demonstrated by the turn-on fluorescence response. As described in Fig. 3c, the fluorescence intensity increases gradually with increasing the concentration of Hep. And the fluorescence recovery correlates well to a concentration range of 4 to 220 $\text{ng}\cdot\text{mL}^{-1}$ ($F = 0.699 C_{\text{Hep}}/(\text{ng}\cdot\text{mL}^{-1}) + 108$, $R^2 = 0.994$) with a LOD of 3.4 $\text{ng}\cdot\text{mL}^{-1}$ (Fig. 3d). These results suggest that the nanoprobe with two signals can be used for sensitive detection of Hep. Compared with many reported optical nanoprobe for Hep (Table S1), the nanoprobe shows better or comparable detection sensitivity. This assay is also rapid and complex synthetic or modification procedures are not involved [11, 12, 24]. With colorimetric and fluorimetric read-out, this assay is highly reliable for the quantification of Hep levels by eliminating possible false response in a single signal system. It should be noted that the linear range of this assay is not wide enough relative to some other assays.

Selectivity of Hep detection

To evaluate the selectivity of the presented nanoprobe for Hep, we investigated the influences of the potentially

interfering substances under the same detection conditions by UV-vis absorption and fluorescence spectra. The main relevant species include common anions (CO_3^{2-} , HCO_3^- , Cl^- , HPO_4^{2-} , SO_4^{2-} , $\text{P}_2\text{O}_7^{4-}$, Ac^- , $\text{C}_2\text{O}_4^{2-}$, citrate) and biomolecules (BSA, ATP, HA, glucose, Trp, Phe, Lys, Gly, Glu, Pro, Gln, Arg, Asp, Thr, Val, Ile, His, Met, Cys, Ser, Ala, Tyr). As shown in Fig. 4a and B, the value of $A_{520\text{nm}}/A_{610\text{nm}}$ is the biggest only when adding Hep, indicating that the addition of interferents does not lead aggregated AuNPs induced by PEI to dispersed state. Similarly, the results obtained from fluorescence response also reveal that only the presence of Hep caused a remarkable fluorescence increase (Fig. 4c and d). In addition, the tolerance experiments show that the coexistence of these species with Hep poses negligible effects on the colorimetric and fluorimetric responses toward Hep (Fig. S3). We reason that these species cannot form a stronger binding than that between Hep and PEI as Hep has a much higher negative charge density [1]. All the results clearly demonstrate that the detection system has excellent selectivity and reliability toward Hep detection.

Detection of Hep in human serum samples

In order to verify the capability of this assay in complex biological environments, the human serum samples were analyzed colorimetrically and fluorometrically with the nanoprobe by spiking Hep at known concentrations. As listed in Table 1, the recoveries of the spiked samples are

Fig. 3 **a** UV-vis absorption spectra of the AuNP-based detection system containing AuNCs and PEI ($50 \text{ ng}\cdot\text{mL}^{-1}$) incubated with various concentrations of Hep. **b** Linear plot of $A_{520\text{nm}}/A_{610\text{nm}}$ versus Hep concentration in the range of 4–220 $\text{ng}\cdot\text{mL}^{-1}$. **c** Fluorescence spectra of the AuNC-based detection system containing AuNPs and PEI ($50 \text{ ng}\cdot\text{mL}^{-1}$) incubated with various concentrations of Hep. **d** Linear plot of the fluorescence intensity at 610 nm of AuNCs versus Hep concentration in the range of 4–220 $\text{ng}\cdot\text{mL}^{-1}$

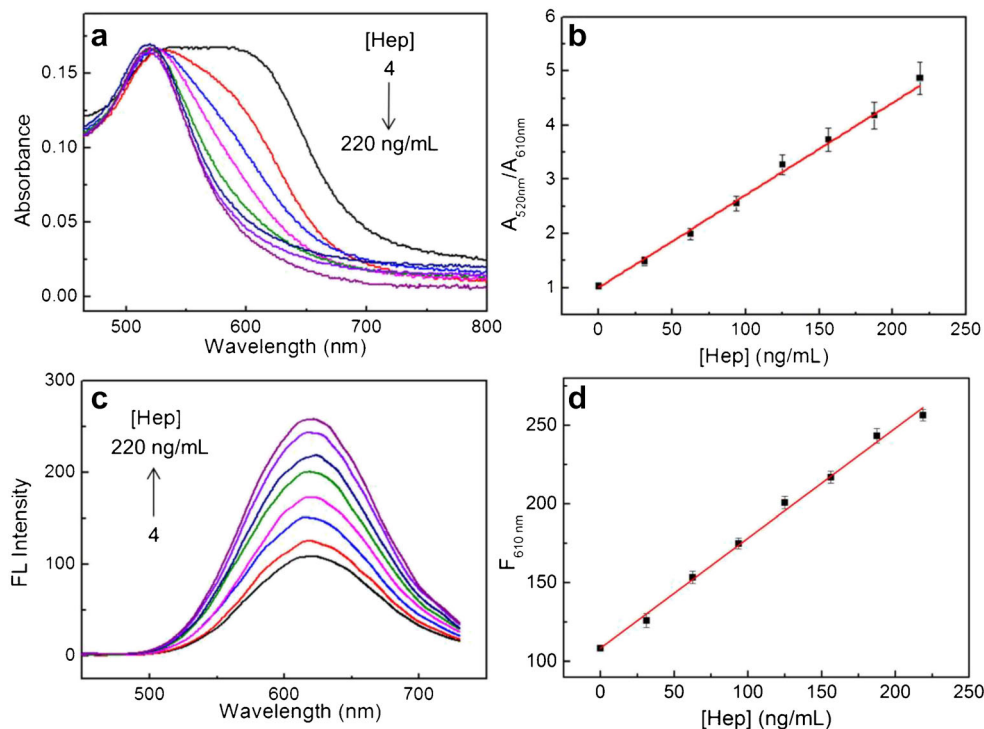
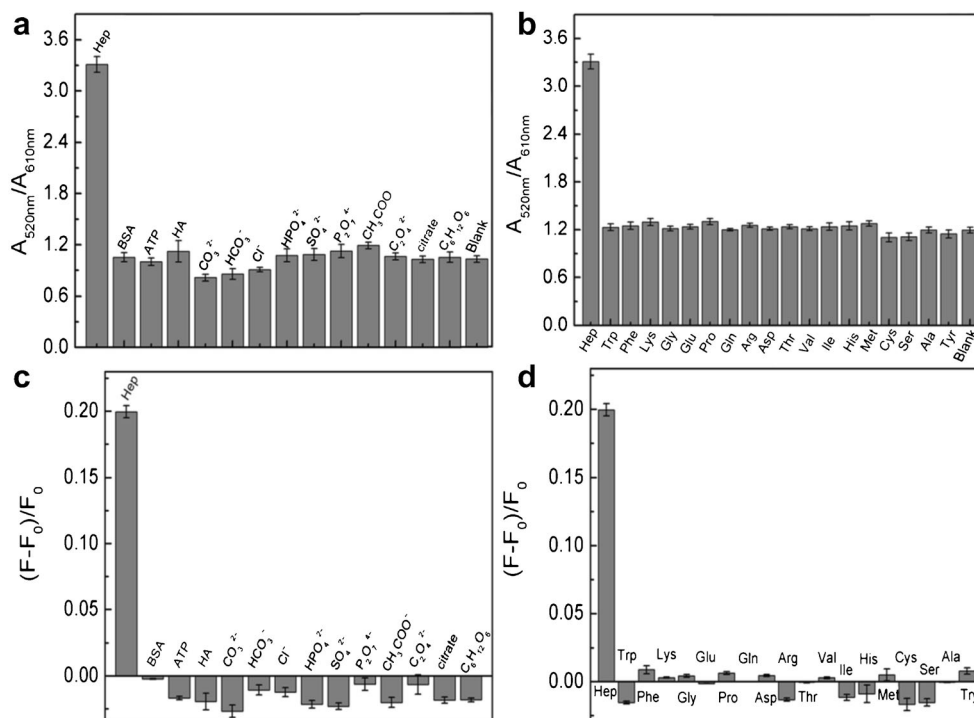


Fig. 4 **a, b** Absorbance ratio $A_{520\text{nm}}/A_{610\text{nm}}$ of the solution containing AuNPs, PEI, AuNCs, and various interferents. **c, d** Fluorescence recovery ratio $(F-F_0)/F_0$ of the solution containing AuNPs, PEI, AuNCs, and various interferents, where F_0 and F represent the fluorescence intensities at 610 nm of the mixture of AuNPs, PEI, and AuNCs without and with various interferents, respectively. Concentrations: BSA, ATP and HA, 62.5 ng mL^{-1} ; CO_3^{2-} , HCO_3^- , Cl^- , HPO_4^{2-} , SO_4^{2-} , $\text{C}_2\text{O}_4^{2-}$, $\text{P}_2\text{O}_7^{4-}$, CH_3COO^- , citrate, and $\text{C}_6\text{H}_{12}\text{O}_6$, $50 \text{ } \mu\text{M}$; amino acids, $5 \text{ } \mu\text{M}$; Hep, 125 ng mL^{-1}



96.8% ~ 103% and 96.8% ~ 102%, respectively. The *t*-test was utilized to analyze the statistical differences, and the results suggest that there is no significant difference between colorimetric and fluorometric methods, indicating that this assay is feasible and reliable for Hep detection in practical samples.

Generally, optical probes that work in the UV are often susceptible to interference from biomatters that always display strong background UV absorption and fluorescence. Fortunately, by virtue of the very high sensitivity of the assay, the interference from biomatters in human serum can be effectively eliminated by diluting the sample. In addition, the maximum emission peak of AuNCs locates at about 610 nm, which is almost in the near infrared region, so the biomatters with background UV

absorption cannot screen off the fluorescence of AuNCs. Also, the emitted UV fluorescence of the biomatters cannot affect the fluorescence of AuNCs due to a big difference between their emission wavelengths.

Conclusions

In summary, we have successfully constructed an economical nanoprobe for Hep detection with both colorimetric and fluorometric readout based on the regulation of the electrostatic interaction of AuNPs and AuNCs by PEI. Such colorimetric and fluorometric feature endows this assay with ultrahigh sensitivity and excellent reliability. It is capable of the detection of Hep in human serum matrix by largely offsetting the interference from environmental fluctuations. It is also expected that this nanoprobe by means of AuNCs and AuNPs has great potential for monitoring Hep in other complex samples considering its simplicity and flexibility. More efforts will be made to broaden the linear range of this assay.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Table 1 Recovery results of the detection of Hep in human serum samples with the presented nanoprobe

Added / ng mL ⁻¹	Found / ng mL ⁻¹	Recovery / %	RSD (% , n = 3)
0	not detected ^a	—	—
	not detected ^b	—	—
62.5	64.4 ^a	103	5.3
	61.8 ^b	98.9	4.8
125	128 ^a	102	5.3
	127 ^b	102	5.2
188	182 ^a	96.8	4.7
	182 ^b	96.8	4.7

^a Colorimetry, ^b Fluorescence

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