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Ultrasensitive colorimetric detection of heparin based on self-assembly of gold nanoparticles on graphene oxide†

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A novel colorimetric method was developed for ultrasensitive detection of heparin based on self-assembly of gold nanoparticles (AuNPs) onto the surface of graphene oxide (GO). Polycationic protamine was used as a medium for inducing the self-assembly of citrate-capped AuNPs on GO through electrostatic interaction, resulting in a shift in the surface plasmon resonance (SPR) absorption of AuNPs and exhibiting a blue color. Addition of polyanionic heparin disturbed the self-assemble of AuNPs due to its strong affinity to protamine. With the increase of heparin concentration, the amounts of self-assembly AuNPs decreased and the color changed from blue to red in solution. Therefore, a "blue-to-red" colorimetric sensing strategy based on self-assembly of AuNPs could be established for heparin detection. Compared with the commonly reported aggregation-based methods ("red-to-blue"), the color change from blue to red was more eye-sensitive, especially in low concentration of target. Moreover, stronger interaction between protamine and heparin led to distinguish heparin from its analogues as well as various potentially coexistent physiological species. The strategy was simply achieved by the self-assembly nature of AuNPs and the application of two types of polyionic media, showing it to be label-free, simple, rapid and visual. This method could selectively detect heparin with a detection limit of 3.0 ng mL^{-1} in standard aqueous solution and good linearity was obtained over the range 0.06–0.36 μg mL^{-1} ($R = 0.9936$). It was successfully applied to determination of heparin in fetal bovine serum samples as low as 1.7 ng mL^{-1} with a linear range of 0–0.8 μg mL^{-1}.

Introduction

Heparin, a highly sulfated polysaccharide with an average charge up to ~70, is widely used in medicine as an anticoagulant during clinical procedures such as cardiac/vascular surgery and kidney dialysis.[1] Heparin has a long history of providing therapeutic benefit to patients; meanwhile, close monitoring of heparin levels is of vital importance to avoid adverse effects such as hemorrhages and thrombocytopenia caused by heparin overdose.[2] Since the "heparin incident" in 2008, inspection standards of heparin in the United States, European Union and China have been upgraded.[3] However, quantitative measurement of heparin is generally difficult due to its natural polydispersity, chemical heterogeneity and lack of significant absorbance or fluorescent properties.[4] The conventional methods such as activated clotting time,[5] ion chromatography,[6] and ion pair high-performance liquid chromatography[7] are often not sufficiently sensitive or reliable, or amenable to clinical settings as well as time-consuming. Accordingly, a number of advanced alternatives have recently been increasingly developed to identify and quantitate heparin, such as electrochemistry,[1,8] light scattering,[9] nuclear magnetic resonance,[10] and fluorometry.[11–13] Although each mentioned contributes to an improvement in detection, there are still some limitations such as complicated procedures (synthesis, modification, *etc.*) or instruments, high-cost, or narrow applications. Therefore, the development of simple, rapid, and cost-effective methods with improved analytical performances is urgently required for the detection of heparin.

Colorimetric methods, in particular, are extremely attractive because their detection results can be easily read by the naked eye, as well they possess obvious advantages of simple operation processes, low-cost portable instruments and easy-to-use applications.[14,15] Colorimetry has proven to be effective for the inspection of heparin.[16] Recently, gold nanoparticles (AuNPs) have been widely employed in colorimetric analysis due to their interesting chemical and physical properties, based on their strongly size-dependent surface plasmon resonance (SPR) absorption and extremely high extinction coefficients.[17–20] Generally, aggregation-based AuNPs are utilized to investigate polyions. For example, Jena and Raj developed a colorimetric sensor for detection of protamine and heparin.[21] Cao and Li have

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employed positively charged AuNPs for optical detection of heparin.[22] Such aggregation is commonly accompanied by a "red-to-blue" color change of the AuNPs. Although AuNPs are applicable for accurate on-site and real-time detection, notable challenges associated with these detection systems still exist, such as their relatively low sensitivity.

On the other hand, the aggregation often results from self-assembly of AuNPs. Organizing AuNPs into ordered arrays on a substrate with well-defined location and geometry is crucially important in addressing their properties, and extremely desirable for developing AuNPs-based analytical methods and fabricating integrated AuNPs devices.[23] Zettl *et al.* systematically studied the self-assembly of AuNPs at the surface of amine- and thiol-functionalized boron nitride nanotubes, showing great potential as nanoscale templates for assembly and integration with other nanoscale materials.[24] Wang *et al.* achieved a self-assembly hybrid of AuNPs on graphene modified electrodes for efficient catalytic oxidation and high sensitive electrochemical determination of nicotinamide adenine dinucleotide.[25] Meanwhile, as is well known, graphene oxide (GO) is a rising star on the horizon of nanomaterial science due to its extraordinary electrical, mechanical, chemical, optical and structural proterities.[26] Self-assembly is an ordinary method to fabricate the GO–nanoparticle hybrids in which the loading ratio and the morphology of the nanoparticles are tunable.[27] Wang *et al.* employed GO–AgNPs (silver nanoparticles) hybrids to develop a binary functional substrate for enrichment and ultrasensitive surface-enhanced Raman spectroscopy (SERS) detection of folic acid.[27] The AgNPs were self-assembled onto the GO/PDDA *via* strong electrostatic effect; benefiting from the enrichment from the AgNPs modified GO, the SERS signals of the folic acid were further amplified.[27] Lately, our group has developed a novel label-free colorimetric sensor for ultrasensitive detection of heparin by using the super color quenching capacity of GO.[28] CTAB-stabilized gold nanorods (AuNRs) could easily self-assembly onto the surface of GO through electrostatic interaction, resulting in decrease of SPR absorption and consequent color quenching change of the AuNRs from deep to light.[28] However, to the best of our knowledge, constructing the GO–AuNPs hybrids for colorimetric detection of heparin based on self-assembly of AuNPs through electrostatic interaction has not been reported.

Herein, we established a convenient and easy-handling colorimetric sensor using GO–AuNPs hybrids based on a self-assembly strategy for ultrasensitive and highly selective detection of heparin in aqueous media and fetal bovine serum samples. Polycationic protamine was chosen as the medium for inducing AuNPs self-assembly on GO. The strong affinity of protamine for heparin assured the specificity of this system. Citrate-capped AuNPs were employed for monitoring changes in the SPR absorptions and thereby measuring the concentrations of heparin. Moreover, a "blue-to-red" color change was exhibited, more eye-sensitive.

Experimental

Chemicals and instrumentation

Graphene oxide (GO) was purchased from Nanjing XFNano Materials Technology Company (Nanjing, China). Heparin sodium salt (185 U mg^{-1}) from bovine intestinal mucosa was obtained from Aladdin Chemistry Co. Ltd (Shanghai, China). Protamine sulfate salt was purchased from Sigma-Aldrich (USA). Hydrogen tetrachloroaurate(III), trisodium citrate, NaCl, $NaHCO_3$, Na_2HPO_4, KCl, $CaCl_2$, $MgCl_2$ and Na_2SO_4 were obtained from Sinopharm Chemical Reagent (Shanghai, China). Hyaluronic acid (HA) salt was obtained from *Streptococcus equi* (BioChemika). Chondroitin sulfate (Chs) was purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). DNA, glucose, adenosine triphosphate (ATP), bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were provided by Shanghai Shenggong Co. (Shanghai, China). Fetal bovine serum (FBS) stock solution (HyClone, Thermo Scientific, Australia) was stored at −20 °C and was thawed at room temperature before use. All other solvents and reagents were of analytical grade and used without further purification. HEPES buffer solution was used for all the experiments, adjusted with 1 M NaOH to desired pH values. All solutions were prepared with freshly deionized water (18.2 MΩ cm specific resistance) obtained with a Pall Cascada laboratory water system (USA).

UV-Visible (UV-Vis) absorption spectra were measured on a Thermo Scientific NanoDrop 2000/2000C spectrophotometer (USA). Transmission electron microscopy (TEM) analyses were performed on a JEM-1230 electron microscope (JEOL, Ltd., Japan) operating at 100 kV. Atomic force microscopy (AFM) images of GO were recorded using a Nanoscope V multimode atomic force microscope (Veeco Instruments, USA) in tapping mode on a freshly cleaved mica surface to simultaneously collect height and phase data.

Synthesis of AuNPs

AuNPs were synthesized by the citrate reduction of $HAuCl_4$ method[29] with necessary modification. All glassware used was thoroughly cleaned in aqua regia (3 parts HCl, 1 parts HNO_3), rinsed in triply distilled H_2O, and oven-dried prior to use. A 100 mL aqueous solution consisting of 1 mM $HAuCl_4$ was brought to boil with vigorous stirring in a round-bottom flask fitted with a reflux condenser, and then 38.8 mM trisodium citrate (10 mL) was added rapidly to the solution. The solution was boiled for another 15 min, during which time its color changed from pale yellow to deep red. The solution was cooled to room temperature with continuous stirring. The size of the nanoparticles determined by TEM image was about 13 nm. The particle concentration of the AuNPs was about 15 nM, which was determined according to the Beer's law by using the extinction coefficient of $\sim 10^8$ M^{-1} cm^{-1} at 520 nm.[30] By adjusting the addition volumes of 38.8 mM trisodium citrate, including 8.3, 6.7, and 5.0 mL, different sizes AuNPs were correspondingly obtained, *i.e.* 18, 22 and 33 nm, respectively. And 4 nm AuNPs were synthesized according to the reported method.[31]

Standard solution analysis

The mixed standard solution of GO–protamine was prepared using 10 mM HEPES (pH 7.4) buffer solution. Then different amounts of heparin (0–180 μL, 0.002 mg mL^{-1}) were added into the above solution for 5 min. Finally, 200 μL of AuNPs were

added to the mixture and incubated for another 5 min, for UV-Vis analysis.

FBS samples analysis

Work solutions of FBS at 0.2‰ (v/v) were prepared by diluting the stock solutions with HEPES buffer (10 mM, pH 7.4). And the work solutions contained the GO–protamine mixture of 3 μg mL^{-1}. Then the same procedure was performed as that of the standard solution analysis mentioned above.

Safety consideration

Aqua regia has strong oxidizing capacity and adverse effects to human health, all experiments involving aqua regia should be performed with protective glasses and gloves.

Results and discussion

Sensing principle for heparin

Fig. 1 illustrates the proposed self-assembly strategy for the sensing of heparin. Protamine is a low molecular weight (*ca.* 4500 Da) protein, rich in basic arginine residues and has ~20 positive charges in physiological condition.[32] GO, which has a lot of oxygen-containing groups, such as hydroxyl, epoxide, and carboxyl, is highly negatively charged and hydrophilic.[33] As illustrated in Fig. 1, since both of the negatively charged GO and citrate-capped AuNPs could bind with positively charged protamine through electrostatic interaction, AuNPs could indirectly self-assembly on GO through protamine, resulting in the appearance of two SPR absorption bands and a blue color, corresponding to "NO target" circumstances. On the contrary, in the presence of polyanionic heparin, the target, the polycationic protamine could firstly be combined due to their strong affinity, and then the negatively charged AuNPs would be prevented from self-assembling onto the surface of GO, displaying an intense SPR absorption of the AuNPs and a red color, as seen in Fig. 1. Hence, the SPR changes of AuNPs are expected to provide a quantitative readout for the detection of heparin.

To demonstrate the strategy, the ability of GO as a platform for self-assembly of AuNPs was firstly investigated. GO was characterized by AFM and TEM to confirm the single layer formation through exfoliation. As shown in Fig. 2A, the thickness of GO was attained about 1.3 nm by AFM, which was reasonable for single layer GO. The thin nanoplate motif of GO was examined by TEM, which exhibited a typical wrinkle morphology of GO, as seen in Fig. 2B. The negatively charged AuNPs were synthesized by trisodium citrate reduction of hydrogen tetrachloroaurate(III) in the liquid phase.[29] Initially, the citrate-capped AuNPs were well dispersed in solution with an intense SPR peak at 520 nm and exhibited red (Fig. S1†).[34] In the presence of GO (Fig. S2d†) or GO and heparin (Fig. S2e†), the AuNPs showed no obvious difference and still displayed strong SPR absorption at 520 nm. Interestingly, as expected from the original design, the polycationic protamine induced self-assembly of AuNPs onto the surface of GO. After adding into GO–protamine solution, the citrate-capped AuNPs showed a color change from red-to-blue and displayed two SPR peaks (Fig. 3A(a)): one was at 520 nm, corresponding to the dispersed AuNPs; the other was at 655 nm, corresponding to the self-assembly AuNPs. However, in the presence of 0.36 μg mL^{-1} polyanionic heparin, the AuNPs were almost completely prevented from being absorbed onto the surface of GO due to stronger interaction between protamine and heparin, remaining as native color and displaying one intense SPR peak at 520 nm (Fig. 3A(b)). This phenomenon was further evidenced by TEM images that self-assembly of AuNPs occurred in the absence of heparin while significant mono-dispersion was observed in the presence of heparin (Fig. 3B). To further prove the phenomena, different sizes of AuNPs were tested, showing consistent observations. All these results indicated successful self-assembly of AuNPs onto the surface of GO by virtue of polycationic protamine.

Parameters optimization

Sensitivity of the self-assembly colorimetric strategy strongly depended on the relevant experimental parameters including

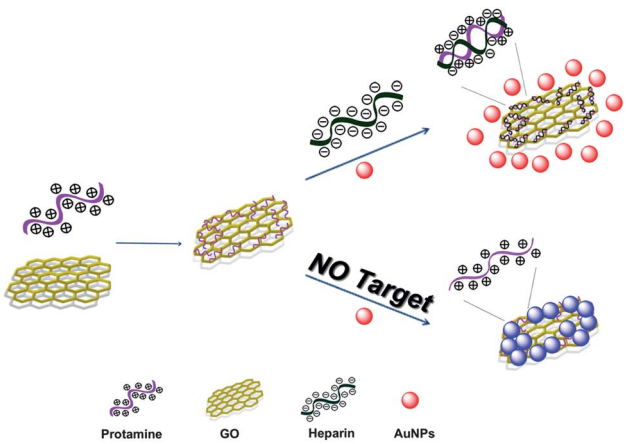

Fig. 1 Schematic illustration of colorimetric sensing heparin based on self-assembly of AuNPs onto the surface of GO.

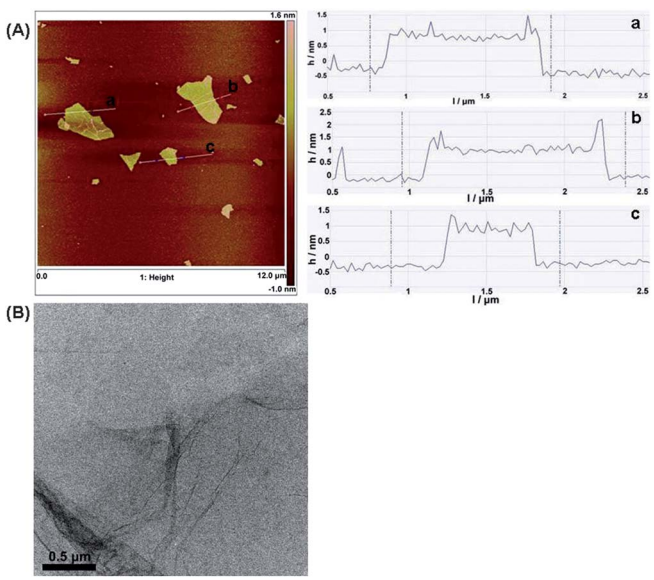

Fig. 2 GO characterization: (A) AFM image (left) and height profile (taken along the white line in the AFM image; right) and (B) TEM image.

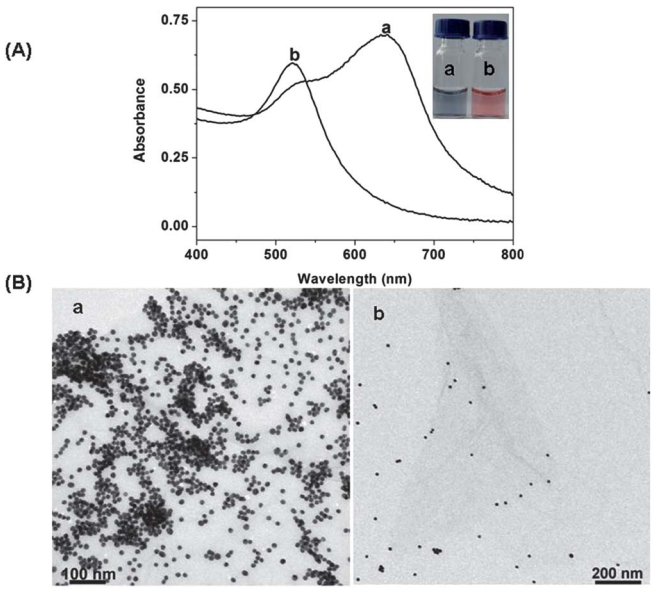

Fig. 3 (A) UV-Vis absorption spectra (inset images were the corresponding colorimetric responses) and (B) TEM images of AuNPs in the GO–protamine mixed solution in the absence (a) and presence (b) of heparin (0.36 μg mL^{-1}).

concentrations of GO and protamine, pH values of solution, and sizes of AuNPs. Their effects for SPR absorption were systematically investigated as follows.

Since protamine was firstly bound with GO through electrostatic interaction and then for the following procedures, as illustrated in Fig. 1, the concentrations of the mixed GO–protamine were chosen, not separate GO or protamine, to evaluate for the minimum detectable concentration of the colorimetric strategy. The SPR absorption values of AuNPs at 520 and 655 nm corresponded to the quantities of dispersed and self-assembled AuNPs, respectively. Thus the molar ratio of dispersion to self-assembly of AuNPs can be expressed by the ratio of the absorption value at 520 nm to 655 nm, *i.e.* A_{520}/A_{655}. As shown in Fig. S3†, the lower the concentration of GO–protamine was, the more sensitive of the strategy was to heparin. Thus, 1.0 μg mL^{-1} of GO–protamine was selected.

The pH values of the solution would affect the interaction of protamine and heparin as well as protamine and AuNPs. Therefore, it was necessary to investigate the effect of pH values for the detection of heparin. Fig. S4† shows the ratio of A_{520}/A_{655} of AuNPs in various pH solutions. It could be seen that the ratio values gradually increased and thereby sensitivity gradually increased in the range of 6.0–7.4, which might be caused by the enhanced affinity of heparin to protamine as well as AuNPs to protamine. At pH > 7.4, the minimum detectable concentration of heparin increased to a certain degree. Therefore, pH 7.4 was chosen in this work.

Effect of sizes of AuNPs was further investigated to improve the detection sensitivity toward heparin. Five different diameters of AuNPs were used including 4, 13, 18, 22 and 33 nm. As shown from Fig. S5†, the SPR absorption was proportional to the amount of heparin. The smaller the diameter of AuNPs, the higher the sensitivity of the colorimetric strategy was for detection of heparin. When the diameter of AuNPs was 13 nm, the strategy displayed the highest sensitivity. It should be noted that the smaller size AuNPs of 4 nm could not bind with protamine, and consequently the colorimetric strategy would not work. This was most probably due to the fact that, for 4 nm AuNPs, there was too much citrate so that the AuNPs had no chance to bind with protamine, that is, excess citrate in the solution bonds with protamine and thereby disturbs the interaction between AuNPs and protamine. So, the optimal diameter of AuNPs was chosen to be 13 nm.

Sensitivity and selectivity for the detection of heparin

Under the above optimized conditions, the UV-Vis absorption spectra of AuNPs in the presence of different concentrations of heparin were recorded. The process of protamine-induced self-assembly of AuNPs was followed by monitoring the shift in the band position and change in the absorbance. The degree of self-assembly of AuNPs was dependent on the concentration of heparin. As shown in Fig. 4A, upon addition of increasing concentrations of heparin, there was an increase in the plasmon absorption bands at 520 nm region along with a concomitant decrease at 655 nm, showing blue-to-red color change as seen in Fig. 4B. These two wavelengths were employed to represent the relative amounts of mono-dispersed and self-assembled AuNPs, respectively. For quantitative analysis of heparin, the ratio of absorption at 520 nm and 655 nm was used. The absorption ratio values (A_{520}/A_{655}) exhibited a good linear relationship to the concentrations of heparin in the range of 0.06–0.36 μg mL^{-1} (R = 0.9936) (inset of Fig. 4A). Based on $3\sigma/s$ (σ is the standard deviation of the blank measurements and s is the sensitivity of the calibration graph), a relative low detection limit for heparin was determined to be 3.0 ng mL^{-1}. The colorimetric strategy was demonstrated to be ultrasensitive and highly reliable for the detection of heparin levels.

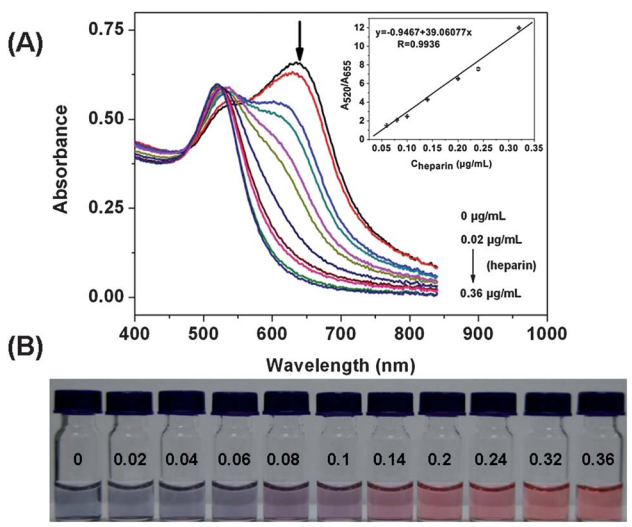

Fig. 4 (A) UV-Vis absorption spectra of AuNPs in the GO–protamine mixed solution upon addition of different concentrations of heparin (0–0.36 μg mL^{-1}) and (B) photographic images of the corresponding colorimetric responses (from left to right: 0–0.36 μg mL^{-1}). Inset of (A): plot of A_{520}/A_{655} values *versus* the concentrations of heparin in 10 mM HEPES (pH 7.4) buffer solution; the error bars denote standard deviations from three parallel experiments.

The detection specificity of the self-assembly-based strategy toward heparin was then investigated relative to other potentially competing substances, including structural analogues and commonly coexistent physiological level species. As shown in Fig. 5, only heparin caused a significant increase in relative absorption of AuNPs accompanied with a remarkable red color, while other species had no evident effect on the SPR band showing a light blue color. The results demonstrated that physiological levels of K^+, Ca^{2+}, Mg^{2+}, HCO_3^-, HPO_4^{2-}, Cl^-, SO_4^{2-}, and glucose did not interfere with the detection. In addition, 100 mM of Na^+, 0.16 μM of ATP and 0.8 μg mL^{-1} of BSA did not affect heparin detection. DNA was used as a model to investigate the effect of other polyanionic substances and the results showed that plasma DNA normal level at 50 ng mL^{-1} could not interfere with the detection. To study the response of self-assembly AuNPs toward other polysaccharides, HA (0.28 μg mL^{-1}) and Chs (0.28 μg mL^{-1}), analogues of heparin, were also investigated. As shown in Fig. 5, neither caused interferences. This was simply due to the fact that both HA and Chs bear low charge density per disaccharide unit: one is formed by a 1 → 3 linked *N*-acetylglucosamine and glucuronic acid, which has only one carboxyl group; the other is formed by 1 → 3 linked *N*-acetylgalactosamine and glucuronic acid and modified by sulfation in position 4, which possesses one sulfate and one carboxylate moieties.[11] In contrast, interestingly, heparin is composed of a 1 → 4 linked iduronic acid and glucosamine per repeating disaccharide unit, which carries three sulfate groups and one carboxylate per repeat unit.[11] Therefore, the electrostatic attraction between HA or Chs and protamine is significantly weaker than that between heparin and protamine.[11] All these results indicated the developed sensor program possessed excellent selectivity and reliability toward heparin.

Application to FBS samples

To demonstrate the applicability of the developed self-assembly colorimetric strategy with high sensitivity and selectivity for practical sample analysis, heparin was measured in FBS samples. As shown in Fig. 6, remarkable absorption peaks and their changes occurred for the spiked FBS with heparin. The values of A_{520}/A_{655} were found to be dependent on the heparin concentrations, *i.e.* increased linearly with increasing the spiked concentration of heparin in FBS over the range of 0–0.8 μg mL^{-1} (inset of Fig. 6). The lowest detectable concentration of heparin was estimated to be 1.7 ng mL^{-1} based on $3\sigma/s$. Herein, 3.0 μg mL^{-1} of GO–protamine was selected for eliminating the possible matrix interference, different from the optimal 1.0 μg mL^{-1} in standard solution. It is noted that the detection limit value was slightly lower than that of 3.0 ng mL^{-1} in the standard solution of HEPES. In a certain sense, the results from FBS samples also suggested that the developed self-assembly-based colorimetric detection had wide applicability without significant matrix interference to heparin.

Method performance comparison

The performance of the developed self-assembly colorimetric strategy toward heparin was compared with some reported colorimetric, fluorescent, light scattering and electrochemistry methods for heparin determination. As can be seen from Table S1†, the reported colorimetric methods present good selectivity for heparin, and exhibit a wide linear range and simple operation processes. However, they are also involved in lower sensitivity,[15,21,22,28] rigorous testing media,[15,22] or restricted applicability in real samples.[21,28] The fluorometry and light scattering methods possess a wide quantitation span and good selectivity, but they are also associated with some problems such as complicated synthesis and purification procedures, poor water-solubility and low practicability.[9,11–13,35,36] The electrochemistry methods have problems of lower sensitivity or complicated modification.[1,8] Our newly developed self-assembly colorimetric strategy presents a number of attractive analytical features such as high sensitivity, wide linear range, good reproducibility, high selectivity, simple operation process, short analysis time,

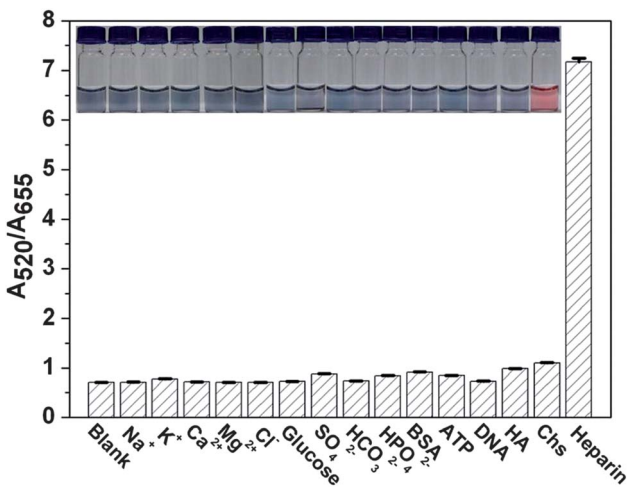

Fig. 5 Relative absorption responses of AuNPs in 10 mM HEPES solutions (pH 7.4) upon the addition of heparin and various potentially coexisting species: heparin, 0.28 μg mL^{-1}; Na^+, 100 mM; K^+, 5 mM; Ca^{2+}, 2.5 mM; Mg^{2+}, 1.5 mM; Cl^-, 103 mM; glucose, 3.9 mM; SO_4^{2-}, 0.5 mM; HCO_3^{2-}, 27 mM; HPO_4^{2-}, 1.0 mM; BSA, 0.8 μg mL^{-1}; ATP, 0.16 μM; DNA, 50 ng mL^{-1}; HA, 0.28 μg mL^{-1}; Chs, 0.28 μg mL^{-1}.

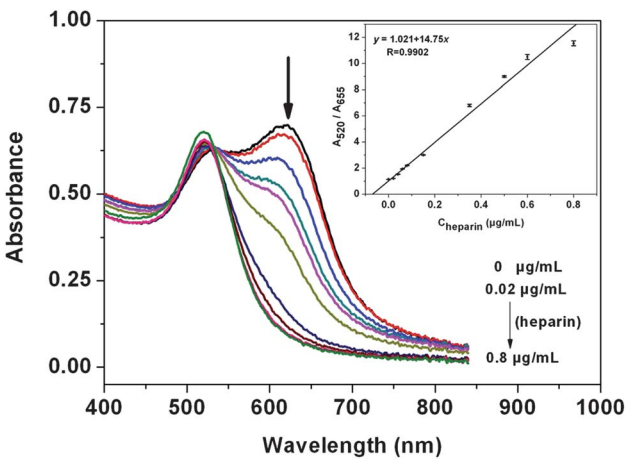

Fig. 6 UV-Vis absorption spectra of AuNPs upon the addition of various concentrations of heparin (0–0.8 μg mL^{-1}) in spiked FBS sample. Inset shows the plot of A_{520}/A_{655} values *versus* the concentrations of heparin and the error bars represent standard deviations from three parallel experiments.

sensitive visualization and good practical applicability. The colorimetric method based on self-assembly of AuNPs on GO is easy to prepare with low cost and can be used for routine analysis of ultratrace levels of heparin in biological samples.

Conclusions

In summary, a new colorimetric method was developed that exhibited high sensitivity and selectivity for detection of heparin in aqueous solution and FBS samples based on self-assembly of AuNPs onto the surface of GO. The color change from blue to red is different from that of usual analyte-induced aggregation methods. As eyes are more sensitive to red than blue, the developed method allows us to detect heparin conveniently by the naked eye. The ultrasensitive detection of heparin was successfully attained as low as 1.7 ng mL^{-1} by taking advantage of the unique absorption change resulting from dispersion or self-assembly of AuNPs as well as high binding affinity of protamine with heparin. Moreover, the sensor showed a remarkable absorption response and color change upon detecting heparin compared to other biologically relevant species. It is anticipated that the self-assembly-based colorimetric method predicts potential applications in biological and environmental samples, by virtue of the extraordinary properties of GO together with the versatility of nanomaterials.

Acknowledgements

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