CTAB-CAPPED GOLD NANOPARTICLES AS A NEW PROBE FOR SPECTROPHOTOMETRIC DETERMINATION OF HEPARIN

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A simple, fast, and sensitive spectrophotometric method is reported for the determination of heparin concentration in human plasma samples. The assay is based on enhancing the extinction intensity of CTAB-capped gold nanoparticles in the presence of heparin molecules. The strong electrostatic interaction of the positively-charged gold nanoparticles with the negatively-charged heparin molecules induces a marked increase in size of gold nanoparticles, which results in a dramatic rise in the extinction spectrum of gold nanoparticles. Since the enhanced extinction intensity at 528 nm is proportional to the concentration of heparin, quantitative determination of heparin is possible. Under optimum conditions, the enhanced extinction intensity is linearly correlated with the concentration of heparin in the range 0.18–11.98 U/mL with limit of detection 0.08 U/mL.

Keywords: *determination of heparin, CTAB-capped gold nanoparticles, extinction spectrum.*

НАНОЧАСТИЦЫ ЗОЛОТА, ПОКРЫТЫЕ БРОМИДОМ ЦЕТИЛТРИМЕТИЛАММОНИЯ, КАК НОВЫЙ ЗОНД ДЛЯ СПЕКТРОФОТОМЕТРИЧЕСКОГО ОПРЕДЕЛЕНИЯ ГЕПАРИНА

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Предложен спектрофотометрический метод определения концентрации гепарина в образцах плазмы крови человека, основанный на повышении интенсивности экстинкции наночастицами золота, покрытыми бромидом цетилтриметиламмония (БЦТА), в присутствии молекул гепарина. Сильное электростатическое взаимодействие положительно заряженных наночастиц золота с отрицательно заряженными молекулами гепарина вызывает заметное увеличение размера наночастиц золота, что приводит к резкому росту интенсивности в спектре поглощения наночастиц золота. При повышении концентрации гепарина растет интенсивность при λ = 528 нм, поэтому возможно количественное определение содержания гепарина. При оптимальных условиях интенсивность поглощения линейно зависит от концентрации гепарина в диапазоне 0.18–11.98 ед./мл с пределом обнаружения 0.08 ед./мл.

Ключевые слова: *определение содержания гепарина, покрытые бромидом цетилтриметиламмония наночастицы золота, спектр ослабления излучения.*

Introduction. Heparin, a sulfated polysaccharide belonging to the family of glycosaminoglycans, has numerous important biological manifestations, associated with its interaction with diverse proteins. Heparin has been widely employed as a major clinical anticoagulant drug to prevent thrombosis during surgery and to treat thrombotic diseases [1]. However, heparin overdose can induce some adverse effects, such as hemorrhage and heparin-induced thrombocytopenia [2]. So, there is a consistent demand for reliable analytical methods for quantitative determination of heparin in human plasma samples. To date, various analytical methods

have been reported for the determination of heparin, including electrochemical methods [3–6], colorimetry [7–10], resonance light scattering technique [11], fluorimetry [12–16], and chromatographic methods [17].

Gold nanoparticles have been a subject of intensive research in the recent decades for they have the fascinating property of surface plasmon resonance (SPR) [8, 11, 18]. SPR is the optical response of metallic nanoparticles to incident light in the form of collective oscillations of their free conduction electron, which usually appear as a strong band in the visible and UV regions. The intensity and wavelength of this band depends on the factors affecting the electron charge density on the particle surface, including metal type, size, shape, structure, composition, and the dielectric constant of the surrounding medium of particle [19, 20]. So, a number of methods based on SPR have been reported for determination of chemical and biological compounds [21–23].

In this work, we report the use of CTAB-stabilized gold nanoparticles (CTAB-AuNPs) for the sensitive, rapid, and simple determination of heparin based on the electrostatic interaction of the positively-charged gold nanoparticles and the negatively-charged heparin that result in enhancing the extinction intensity of CTAB-AuNPs. Under optimized conditions, the enhanced intensity of SPR was proportional to the concentrations of heparin. Thus, the CTAB-AuNPs was successfully applied as a new probe for determination of heparin in human plasma samples.

Experimental. All reagents were of analytical-reagent grade, and doubly distilled de-ionized water (obtained from Ghazi Co., Tabriz, Iran) was used for the preparation of all solutions. Heparin sodium salt from porcine intestinal mucosa (180 U/mg) was obtained from Sigma-Aldrich. Trisodium citrate (Merck), $HAuCl_4.3H_2O$ (Merck), ascorbic acid (Merck), $NaBH_4$ (Merck), and cetyltrimethylammonium bromide (CTAB) (Fluka) were used for preparation of gold nanoparticles. Sodium acetate buffer (1.0 mol/L) was prepared by dissolving the appropriate amount of sodium acetate in water and adjusting the pH value.

The UV-Vis absorption spectra were measured using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) with 1.0 cm quartz cells. The pH value was measured using a pH meter (HANA 211, Romania). Transmission electron microscopy (TEM) images of the AuNPs were obtained by a Leo 906 transmission electron microscope (Germany). Zeta potentials were measured by a Nanotrac zeta potential analyzer (Microtrac Inc., USA).

Procedure for preparation of gold nanoparticles. The CTAB-AuNPs were synthesized by applying the seeding-growth approach according to [24] with minor changes. At first, gold seeds were prepared by mixing 10 mL aqueous solution containing 2.5×10^{-4} M $HAuCl_4$, 10 mL aqueous solution containing 2.5×10^{-4} M trisodium citrate, and 0.3 mL ice-cold freshly prepared 2.5×10^{-3} M $NaBH_4$ solution. The particles in this solution were used as seeds within 2 h after preparation. Then, for preparation of growth solution, a mixture of 3 g solid cetyltrimethylammonium bromide (0.08 M final concentration) and 100 mL aqueous solution of 2.5×10^{-4} M $HAuCl_4$ was heated to about 45ºC until the solution turned to a clear amber color. This solution after cooling to room temperature was used as a stock growth solution. Finally, the gold nanoparticles were prepared as follows: 7.5 mL of growth solution was mixed with 0.05 mL of freshly prepared 0.1 M ascorbic acid solution. Next, 5 mL of seed solution was added while stirring. Stirring was continued for 10 min after the solution turned wine red.

General procedure. Into a 5 mL volumetric flask, 1.25 mL GNPs, 100 μL acetate buffer solution (1.0 M, pH 4.0), and appropriate amounts of heparin were added in turn. After addition of each reagent, the resulting solution was mixed. The mixture was finally diluted to the mark with water. After shaking and waiting for 5 min, the extinction intensity of the reaction product (I) and of the reagent blank (I_0) were measured at 528 nm, and the measurement parameter was calculated as $\Delta I = I - I_0$.

Procedure for plasma sample. An aliquot of 0.5 mL human plasma, spiked with different amounts of heparin, was transferred to a centrifuge tube. Then 1.0 mL of 5% HNO_3 (Merck) was added to precipitate proteins and the tube was centrifuged for 45 min. The clear supernatant solution was transferred to a 5 mL volumetric flask and diluted to the mark with water. A suitable aliquot of this solution was used for the determination of heparin according to the general procedure. The corresponding blank was also prepared by the same procedure using drug-free plasma.

Results and discussion. The extinction spectrum is the SPR of metallic nanoparticles including both scattering and absorption components. The cross-sections of the two components in extinction spectrum can be substantially different depending on the composition, shape, aggregation state, and size of nanoparticles [25, 26]. The size of nanoparticles has the most important effect on the relative percentage of scatter or absorption components in SPR. The smaller nanoparticles will have a higher percentage of extinction due to absorption, while the ratio of scattering to absorption increases dramatically for larger size of particles [19, 27].

Figure 1 shows the extinction spectrum of CTAB-capped AuNPs in the presence of different concentrations of heparin. The CTAB-capped AuNPs have an absorption maximum at 528 nm as a result of surface plasmon resonance. Upon addition of heparin molecules, a significant increase in extinction spectrum of AuNPs is observed. Interestingly, with addition of different concentrations of heparin on AuNPs, only negligible shifts in maximum wavelength appeared and no color changes were observed. Thus, CTAB-capped AuNPs attached to heparin molecules are dispersed in solution and not aggregated. The TEM images (Fig. 3) shows that CTAB-capped AuNPs are spherical and have an average size 4 and 29 nm before and after addition of heparin molecules respectively. In order to confirm the principle of the assay, the *z*-potential of the system was also measured. Both CTAB-capped AuNPs and heparin attached CTAB-capped AuNPs (11.98 U/mL of heparin) have +0.7 mV zeta potential, i.e., after addition of heparin, no change in zeta potential was observed. So, the dramatic enhancement of extinction intensity of gold nanoparticles in the presence of heparin molecules could be assigned to electrostatic attraction of highly negative charged heparin with the CTAB-capped AuNPs [28] and assembling of excessive amount of CTAB molecules in gold nanoparticles solution on them that causes steric and electrostatic restabilization and marked enlarging of AuNPs (Fig. 2).

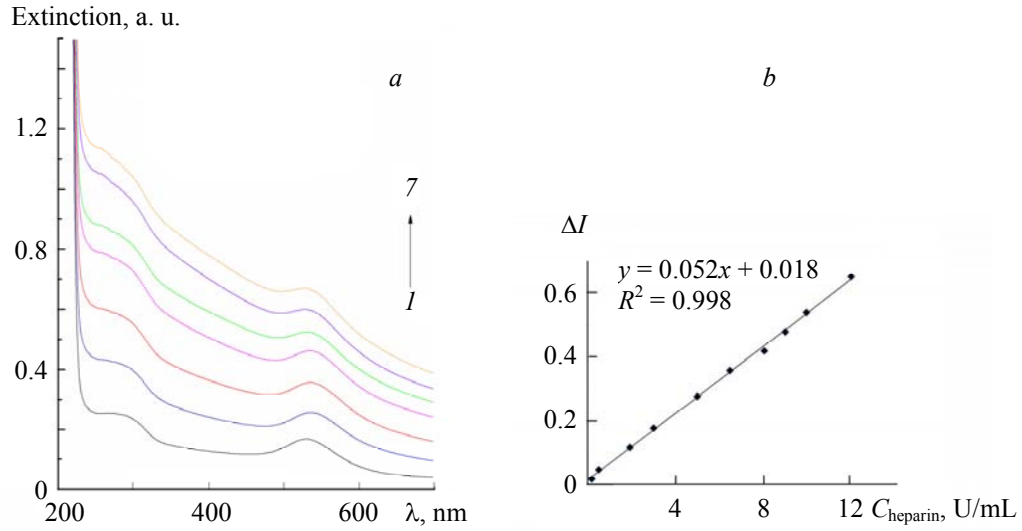

Fig. 1. Extinction spectra of AuNPs in the absence (1) and in the presence of 2 (2), 3.9 (3), 5.3 (4), 6.8 (5), 7.9 (6), 9.8 U/mL (7) of heparin. pH 4.0, AuNPs volume is 1.25 mL (a) and the calibration graph at 528 nm (b).

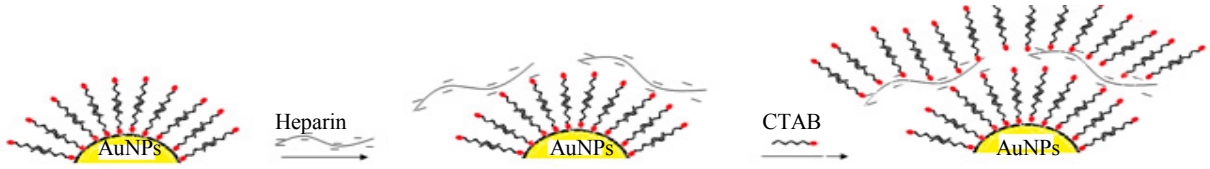

Fig. 2. Schematic diagram of proposed assay for the determination of heparin.

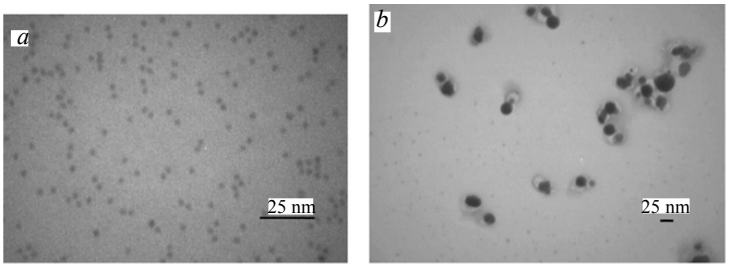

Fig. 3. Transmission electron microscope images of CTAB-stabilized AuNPs (a) and heparin conjugated CTAB-stabilized AuNPs (b). [heparin] = 2.0 U/mL.

Optimization of experimental conditions. The effect of important variables (pH, kind of buffer, the amount of AuNPs, and incubation time) on the measurement parameter was studied by the one-at-a-time method, and ΔI was used as an analytical signal. The pH of the solution plays an important role in the interaction between heparin and gold nanoparticles. As seen in Fig. 4a, the extinction intensity increases to a maximum at pH 4 and then drops. The low intensity in the cases of lower and higher pH is probably related to poor dissociation of carboxyl groups in the heparin structure ($pK_a = 3.1$) [29] and competition of hydroxyl ions with heparin for interaction with CTAB molecules on gold nanoparticles respectively. So, the pH of solutions was adjusted to 4 by acetate buffer. Then the volume of acetate buffer was optimized. According to Fig. 4b, at concentrations above 0.03 M, the extinction intensity decreases since acetate ions can compete with heparin to bind on surface of CTAB-stabilized gold nanoparticles. Hence, 100 μL of 1.0 M acetate buffer was selected as the optimum amount for adjusting pH. The effect of concentration of AuNPs solution on extinction intensity was also investigated. As seen in Fig. 4c, 1.25 mL of AuNPs obtains maximum intensity for 1.92 U/mL heparin. At low concentration of AuNPs, the concentration of the binding products is less, which makes the extinction intensity lower; on the contrary, excessive AuNPs will bind with heparin competitively, so the number of the binding drug for every AuNP will decrease, which results in decreasing the intensity. Finally, the influence of incubation time on extinction intensity was studied. According to the obtained results, for both blank and sample solutions, the extinction intensity remains nearly constant for a long time (at least 2 h). So, all measurements were done 2 min after preparation of solutions.

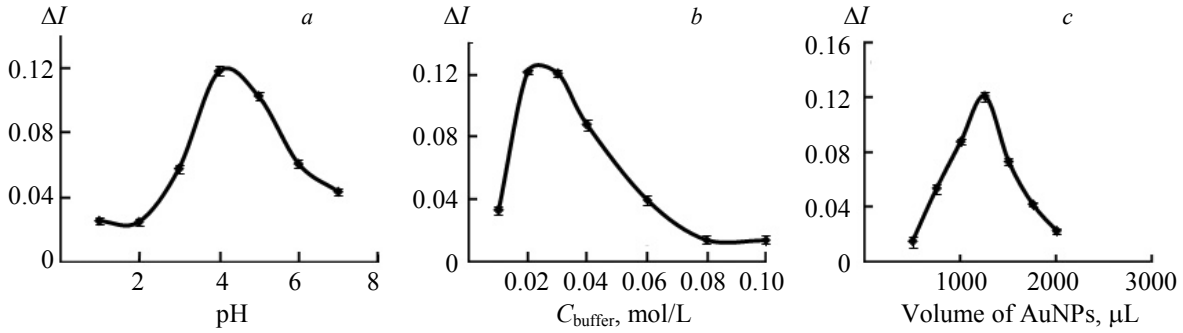

Fig. 4. (a) Effect of pH on extinction intensity; AuNPs volume =1.25 mL, [heparin] = 2.0 U/mL. (b) Effect of buffer concentration; pH 4, AuNPs volume =1.25 mL, [heparin] = 2.0 U/mL. (c) Effect of AuNPs volume; pH 4, [buffer]=0.02 mol/L, AuNPs volume =1.25 mL, [heparin]= 2.0 U/mL.

Selectivity study. In order to study the selectivity of the proposed method, the effects of physiological levels of some foreign substances, including Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, CO_3^{2-}, PO_4^{3-}, glucose, cysteine, glycine, and tryptophan were examined, and the results showed that none of the tested species interfered with the detection of heparin in human plasma samples. Possible interferences from other anionic biopolymers including hyaluronic acid and chondroitin 4-sulfate were also tested. These polymer solutions caused almost no change in signal intensity of a model solution (1.92 U/mL heparin). The effect of different concentrations of chondroitin 4-sulfate on extinction spectrum of CTAB-stabilized AuNPs was also studied. Even high concentrations of chondroitin 4-sulfate led to small changes in extinction intensity of CTAB-stabilized AuNPs. The results could be assigned to lower negative charge density of these polymers compared to that of heparin [30]. It demonstrated that the developed assay provided attractive specificity toward heparin.

Analytical figures of Merit. By using the optimum conditions, a method was developed for the determination of heparin. The linear equation of the calibration graph obtained by the enhanced extinction intensities (ΔI) of a series of standard solutions of heparin in the range 0.18–11.98 U/mL is $\Delta I = 0.052$ C (U/mL) + + 0.018, with a correlation coefficient of 0.9989. When the amount of heparin goes beyond 11.98 U/mL in the system, the enhancement continues, but the calibration graph becomes nonlinear. The detection limit based on three times the standard deviations of the blank ($3s$) was found to be 0.08 U/mL. The precision of the method expressed as the relative standard deviation (RSD), obtained by five replicate determinations of a model solution (1.92 U/mL heparin), was 1.52%.

Analytical applications. In order to confirm the applicability of the proposed method, it was applied to the determination of heparin in spiked human plasma samples. The results given in Table 1 indicate that the proposed method has sufficient precision and accuracy for the determination of heparin in human plasma.

TABLE 1. Results Obtained for Heparin Determination in Spiked Human Plasma Samples

Sample	Added [a]	Found [b]	Recovery, %
Plasma 1	2.0	1.96±0.09	98±3
	4.94	4.95±0.03	100.2±5
	7.08	6.94±0.27	98±3
Plasma 2	2.0	2.05±0.06	102.5±2
	4.94	4.88±0.23	98.8±3
	7.08	6.8±0.36	96±5

[a] The added and found values are in U/mL unit in initial plasma samples.
[b] Averages of three determinations ± standard deviation.

TABLE 2. Comparison of Analytical Characteristics of the Proposed Method with Those of Some Previously Published Methods in Pure Solutions

Proposed probe	Detection Method	LDR, U/mL	DL, U/mL	Reference
Chitosan-capped AuNPs	Resonance light scattering	1.7–7.2	0.09	[4]
Cysteamine-stabilized AuNPs	Colorimetric	0.045–1.6	0.015	[2]
AuNPs-graphen oxide	Colorimetric	0.03–0.18	0.0015	[8]
Polydiacetylene liposome	Colorimetric	0.30–5.35	–	[5]
Cetyltrimethylammonium bromide	Resonance light scattering	0–0.8	0.6	[21]
Polythiophene	Colorimetric	0–6.7	0.01	[30]
CTAB-capped AuNPs	Spectrophotometric	0.18–11.98	0.08	This work

N o t e. LDR is linear dynamic range, DL is detection limit.

Conclusion. A new, simple, and less time-consuming spectrophotometric method was introduced for determination of heparin. This method is based on increasing the extinction intensity of positively charged CTAB-stabilized gold nanoparticles in the presence of the highly negative charged macromolecule. The proposed method is convenient and inexpensive and can be used for the determination of heparin concentration of human plasma samples in the whole therapeutic dosing concentration range in post-operation and long-term therapy (0.2–1.2 U/mL) and cardiovascular surgery (2–8 U/mL). Comparison of analytical features of this method with those of some previously reported methods in pure solutions (Table 2) indicates that the linear range and LOD of the developed method are better than or comparable with most of the other methods.

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REFRENCES

1. A. L. Lehninger, D. L. Nelson, M. M. Cox, W. H. Freeman, *Lehninger Principles of Biochemistry*, 4th edn., New York (2005).
2. M. D. Freedman, *J. Clin. Pharmacol.*, **32**, 584–596 (1992).
3. K. L. Gemene, M. E. Meyerhoff, *Anal. Chem.*, **82**, 1612–1615 (2010).
4. J. Lei, J. Ding, Y. Chen, W. Qin, *Anal. Chim. Acta*, **858**, 60–65 (2015).
5. L. Li, Y. Liang, Y. Liu, *Anal. Biochem.*, **434**, 242–246 (2013).
6. L. Tan, S. Yao, Q. Xie, *Talanta*, **71**, 827–832 (2007).
7. T. Bříza, Z. Kejík, I. Císařová, J. Králová, P. Martásek, V. Král, *Chem. Commun.*, **28**, 1901–1903 (2008).
8. R. Cao, B. Li, *Chem. Commun.*, **47**, 2865–2867 (2011).
9. Y.-S. Cho, K. H. Ahn, *J. Mater. Chem. B*, **1**, 1182–1189 (2013).
10. X. Fu, L. Chen, J. Li, *Analyst*, **137**, 3653–3658 (2012).
11. Z. Chen, Z. Wang, X. Chen, H. Xu, J. Liu, *J. Nanopart. Res.*, **15**, 1930–1939 (2013).

12. Z. Liu, Q. Ma, X. Wang, Z. Lin, H. Zhang, L. Liu, X. Su, *Biosens. Bioelectron.,* **54**, 617–622 (2014).
13. H. Liu, P. Song, R. Wei, K. Li, A. Tong, *Talanta*, **118**, 348–352 (2014).
14. J. Liu, G. Liu, W. Liu, Y. Wang, *Biosens. Bioelectron.*, **64**, 300–305 (2015).
15. Y. Cao, S. Shi, L. Wang, J. Yao, T. Yao, *Biosens. Bioelectron.*, **55**, 174–179 (2014).
16. X. Peng, Q. Long, H. Li, Y. Zhang, S. Yao, *Sens. Actuators. B*, **213**, 131–138 (2015).
17. R. P. Patel, C. Narkowicz, G. A. Jacobson, *Anal. Biochem.*, **387**, 113–121 (2009).
18. N. Nath, A. Chilkoti, *Anal. Chem.*, **74**, 504–509 (2002).
19. X. Huang, M. A. El-Sayed, *J. Adv. Res.*, **1**, 13–28 (2010).
20. C. J. Murphy, A. M. Gole, S. E. Hunyadi, J. W. Stone, P. N. Sisco, A. Alkilany, B. E. Kinard, P. Hankins, *Chem. Commun.*, 544–557 (2008).
21. A. Reza, A. S. M. Noor, M. Maarof, In *Plasmonics – Principles and Applications*, Ed. K. Y. Kim, InTech (2012).
22. J. Homola, S. S. Yee, G. Gauglitz, *Sens. Actuators. B*, **54**, 3–15 (1999).
23. J. Homola, *Anal. Bioanal. Chem.*, **377**, 528–539 (2003).
24. R. Fenger, E. Fertitta, H. Kirmse, A. F. Thünemann, K. Rademann, *Phys. Chem. Chem. Phys.*, **14**, 9343–9349 (2012).
25. S. K. Ghosh, T. Pal, *Chem. Rev.*, **107**, 4797–4862 (2007).
26. M. Hu, J. Chen, Z.-Y. Li, L. Au, G. V. Hartland, X. Li, M. Marquez, Y. Xia, *Chem. Soc. Rev.*, **35**, 1084–1094 (2006).
27. X. Liu, M. Atwater, J. Wang, Q. Huo, *Colloids Surf. B*, **58**, 3–7 (2007).
28. S. P. Liu, H. Q. Luo, H. Xu, N. B. Li, *Spectrochim. Acta, A*, **61**, 861–867 (2005).
29. D. A. Lane, I. Björk, U. Lindahl, *Heparin and Related Polysaccharides*, Springer US (2013).
30. R. Zhan, Z. Fang, B. Liu, *Anal. Chem.*, **82**, 1326–1333 (2010).