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A novel fluorescence assay for the detection of hemoglobin based on the G-quadruplex/hemin complex

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ABSTRACT

A novel sensing strategy for specific recognition of hemoglobin has been designed. In the presence of trypsin, hemoglobin was cleaved to release heme which could effectively bind to a FITC-labeled guanine (G)-rich oligonucleotide sequence to form the G-quadruplex/hemin complex. This binding process would lead to fluorescence quenching of FITC via photoinduced electron transfer. Therefore, a fluorescence assay for the determination of hemoglobin could be established. Under the optimized conditions, there was a linear relationship between the fluorescence intensity of probe DNA and the hemoglobin concentration in the range of 5–200 nmol/L, with the limit of detection (LOD) as low as 2 nmol/L.

1. Introduction

Hemoglobin (Hb) is a metalloprotein existing in red blood cells. It transports molecular oxygen physiologically in mammalian blood from the lungs to tissues, and specifically interacts with three other gases, CO_2, CO and NO, which have important biological roles [1,2]. One hemoglobin molecule consists of four globular protein subunits. Each subunit comprises a polypeptide chain and a non-protein heme group, in which an iron atom is coordinated by protoporphyrin IX and an imidazole nitrogen atom of a histidine residue [3,4]. Significant variations in the hemoglobin concentration may have relationship with several diseases, such as anemia, erythrocytosis [2], leukemia, heart diseases, etc. [5]. Several methods have been applied for the determination of hemoglobin, including spectrophotometry [6,7], fluorimetry [8], electrochemistry [9–14], chemiluminescence [15], high performance liquid chromatography [16,17] and mass spectrometry [18]. Chen and co-workers have presented a gold nanodots-based luminescent assay for the detection of hemoglobin in biological samples, based on hemoglobin-induced photoluminescence quenching [1]. Prasad and co-workers have synthesized a molecularly imprinted polymer (MIP)-based electrochemical sensing system for human hemoglobin (Hb) at the surface of quantum dots–multi-walled carbon nanotubes (MWCNTs) nanoconjugate [19]. Ramanavicius et al. designed a selective immunosensor for hematoporphyrines assays based on fluorescence quenching matrix of the conducting polymer polypyrrole [20,21].

Trypsin is a serine protease. Its enzymatic mechanism involves two steps. First, the target amino acid is recognized in a substrate binding pocket; then, the C-terminal amide bond is cleaved by a mechanism involving a serine residue on the protease. Trypsin preferably cleaves the C-terminal side of arginine and lysine residues, for its deep narrow binding pocket has a negatively charged aspartate at the bottom and binds basic amino acids via an ionic interaction. The target amino acid for cleavage should be positively charged and have long side chains. And the only two amino acids which fulfill these criteria are arginine and lysine [22].

The chains of hemoglobin can be cleaved into peptide fragments by trypsin [23], after that, heme groups are released. As reported, the free heme group oxidizes immediately (less than 1 s) in air at room temperature, making the ferrous iron transform to its ferric state [24]. The formed hemin (Fe(III)–heme) is the active cofactor for numerous enzymes, including catalases, peroxidases and monooxygenases (including the cytochrome P450 family) [25]. Hemin is able to bind to a G-rich oligonucleotide sequence that can form G-quadruplex structure in the presence of Na^+ or K^+ [26]. The resulting complex is a horseradish peroxidase (HRP)-mimicking DNAzyme [27,28], and has been used as a catalytic amplifier for several biorecognition events [29,30].

In this paper, we built a new assay platform for the determination of hemoglobin. Trypsin was adopted to cleave hemoglobin into peptide fragments, and the released heme oxidized immediately. The formed hemin could bind to a fluorescent dye-labeled G-rich oligonucleotide sequence to form the G-quadruplex/hemin complex. Such complex could act as an electron acceptor, and photo-induced electron transfer (PET) from the fluorescent dye (FITC) to the G-quadruplex/hemin complex would lead to quenching of the

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fluorescence of the dye [31]. Thus we were able to detect hemoglobin quickly, sensitively and selectively.

2. Experimental

2.1. Materials

DNA oligonucleotides were synthesized and purified by TAKARA Biotechnology (Dalian, China). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Sinopharm Chemical Reagent Co. Ltd. (China). TE buffer (pH 8.0), trypsin (1:250) and dimethyl sulfoxide (DMSO) were purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China). Hemoglobin (bovine erythrocytes, BR) was obtained from Shanghai Kayon Biological Technology Co. Ltd. Hemin (BioXtra, from Porcine, HPLC grade), pepsin, urease, lactate dehydrogenase (LDH), glucose oxidase, bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Sigma-Aldrich. All chemicals used were at least of analytical reagent grade and used without further purification. The water used in all experiments had a resistivity higher than 18 MΩ cm.

Dye-labeled G-rich oligonucleotide sequence (P):

5′-FITC-TTTGGGTAGGGCGGGTTGGG-3′

The oligonucleotide sequences were dissolved in TE buffer to prepare 250 nM stock solution. Hemin stock solution was prepared by dissolving hemin in DMSO, with a final concentration of 1 mM, and stored in the dark at −20 °C. All the protein solutions were stored at 4 °C before use.

2.2. Instrument

All fluorescence measurements and spectra were obtained on a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Co., Kyoto, Japan). In this experiment, we used a 1 cm path length quartz cuvette to measure the fluorescence spectrum. The pH value of the buffer solution used was measured with a PHS-3C pH meter (Tuopu Co., Hangzhou, China). The absorption spectra were performed using a UV-1700 spectrophotometer (Shimadzu Co., Japan).

2.3. Fluorescent DNA assays

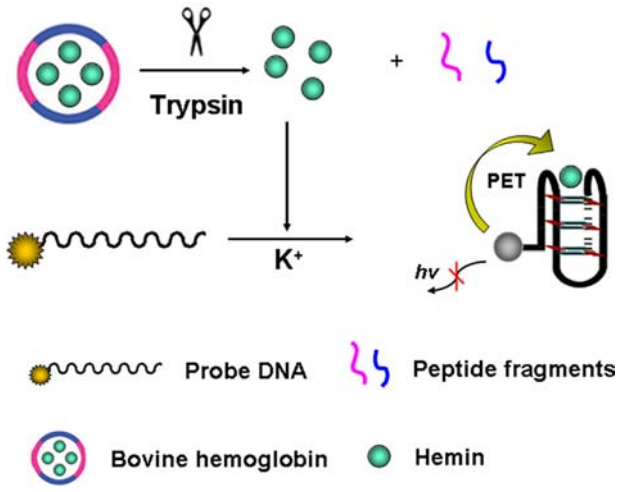
An aliquot of 2 mL dye-labeled G-rich oligonucleotide sequence (P) in Tris–HCl buffer (25 mM, pH 8.1, containing 10 μM KCl) was prepared to measure the initial fluorescence intensity in the quartz cuvette. Then another DNA solution was mixed with hemin solution and incubated for 2 h at room temperature to record the quenched fluorescence intensity.

For the detection of hemoglobin, different amounts of hemoglobin were mixed with trypsin and incubated for 90 min at 37 °C. Then the solution was mixed with DNA after being cooled down to room temperature [32]. The fluorescence intensity was detected when the mixture has been incubated for 2 h at room temperature. The fluorescence intensity was obtained from the emission spectrum at the wavelength of 520 nm, the excitation wavelength was set at 480 nm. The slit widths for excitation and emission were both set at 10 nm. The experiments to optimize for sensing conditions were carried out under identical conditions.

3. Result and discussion

3.1. Design of the sensing strategy

A new strategy for the detection of hemoglobin has been developed by using a fluorescent dye-labeled G-rich oligonucleotide sequence (P) as the probe. Scheme 1 shows the assay strategy.



Scheme 1. Schematic representation of the fluorescent sensor for the determination of hemoglobin.

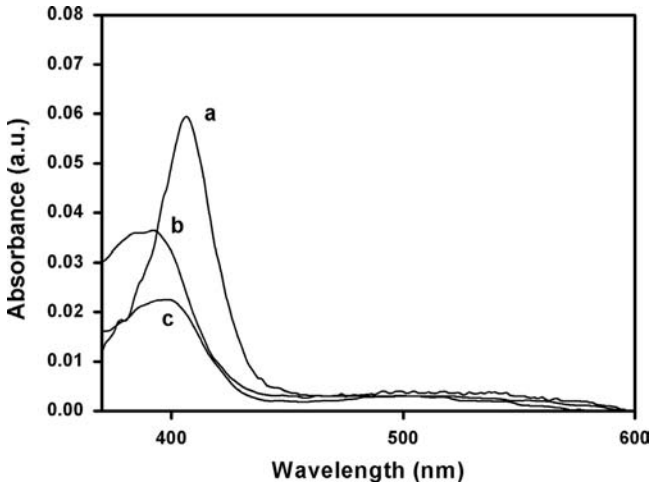


Fig. 1. UV–vis absorption spectra of (a) hemoglobin (200 nmol/L), (b) hemin (800 nmol/L) and (c) hemoglobin (200 nmol/L) in the presence of trypsin (70 mU/mL). Conditions: Tris–HCl buffer (25 mM, pH 8.1, containing 10 μM KCl), 90 min for protein digestion at 37 °C.

In this method, hemoglobin was digested by trypsin and released heme molecules. The free heme oxidized immediately and formed hemin. The probe DNA sequence could be folded properly and bind hemin to form a G-quadruplex/hemin complex. Such G-quadruplex bound hemin so tightly that the electron could transfer from FITC to the hemin Fe^{III} center more easily, and gave rise to the fluorescence quenching of P [31].

UV–vis absorption spectrum (Fig. 1) shows that hemoglobin had a strong absorption peak at 406 nm (curve a) which corresponded to the R state [33], and the free hemin had a soret absorption band centered at about 392 nm (curve b) [34]. When hemoglobin was incubated with trypsin, the absorption peak shifted to 398 nm (curve c), coinciding with that of hemin. The results proved that hemoglobin was cleaved into peptide fragments in the presence of trypsin, and the released heme was oxidized to form hemin immediately. The absorption of the complex did not overlap with the emission spectrum of P, in which the maximum intensity was obtained at the wavelength of 520 nm. Thus, the possibility for fluorescence resonance energy transfer (FRET) from FITC to the complex could be eliminated. Based on previous reports, the fluorescence quenching processes proceed via photoinduced electron transfer (PET) mechanism [31,35].

3.2. Detection of hemin

In this study, the kinetic behavior of FITC-labeled G-rich oligonucleotide sequences in the presence of hemin (400 nmol/L) was studied by monitoring fluorescence intensity as a function of time. Fig. 2 shows that in the presence of K^+, hemin could bind to a fluorescent dye-labeled G-rich oligonucleotide sequence to form the G-quadruplex/hemin complex through external π-stacking [26], promoting the electron transfer from FITC to hemin. As a result, the fluorescence of P was quenched. Such quenching process reached equilibrium when P had been incubated with hemin for 2 h, proving that all the hemin had bound to P to form the G-quadruplex/hemin complex. We also investigated the quenching effect of hemin on the fluorescence intensity of P (Fig. 3A). Fig. 3B shows the fluorescence spectra changes of P with the increasing concentration of hemin from 0 to 2500 nmol/L. The fluorescence intensity decreased linearly upon increasing the concentration of hemin from 10 nmol/L to 500 nmol/L (Fig. 3A inset). The limit of detection (LOD) corresponding to the fluorescence intensity of 3 times standard deviation obtained from 11 fluorescence measurements in the absence of analytes was 5 nmol/L. The linear regression equation was

$$F_0/F = 0.97719 + 0.00124[\text{hemin}],\ \text{nmol/L}$$

(F and F_0 were the fluorescence intensity of P in the presence and absence of hemin, respectively), with $R^2 = 0.992$. The relative standard deviation for determination ($n = 3$) of 50 nmol/L hemin was 1.0%.

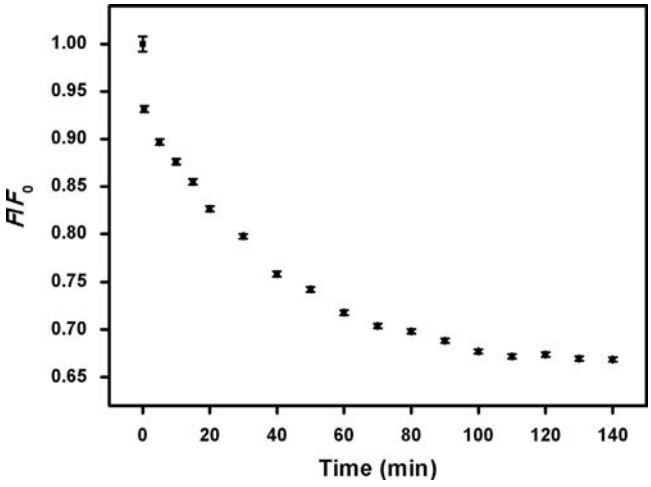


Fig. 2. Fluorescence intensity changes of P in the presence of hemin (400 nmol/L) with different incubation times. Conditions: Tris–HCl buffer (25 mM, pH 8.1, containing 10 μmol/L KCl), 3.75 nmol/L P. F and F_0 were the fluorescence intensity of P in the presence and absence of hemin, respectively. Error bars represent standard deviations from three measurements.

3.3. The effect of pH

The effect of pH on the enzymatic digestion reaction of trypsin has been investigated over the range of pH 7.5–8.7. Firstly, as shown in Fig. 4, the fluorescence quenching efficiency increased with the increase of pH. Then the maximum fluorescence quenching efficiency appeared at pH 8.1. When pH was further increased,

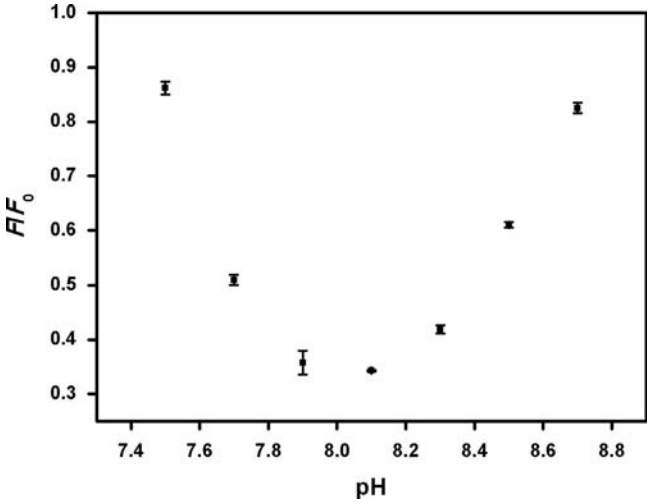


Fig. 4. The effect of pH on the fluorescence intensity ratio F/F_0 of P in the presence of hemoglobin and trypsin. Conditions: Tris–HCl buffer (25 mM, containing 10 μmol/L KCl), 3.75 nmol/L P, 100 nmol/L hemoglobin and 70 mU/mL trypsin, 90 min for protein digestion at 37 °C and 2 h for fluorescence quenching. F and F_0 were the fluorescence intensity of P in the presence and absence of hemoglobin, respectively. Error bars represent standard deviations from three measurements.

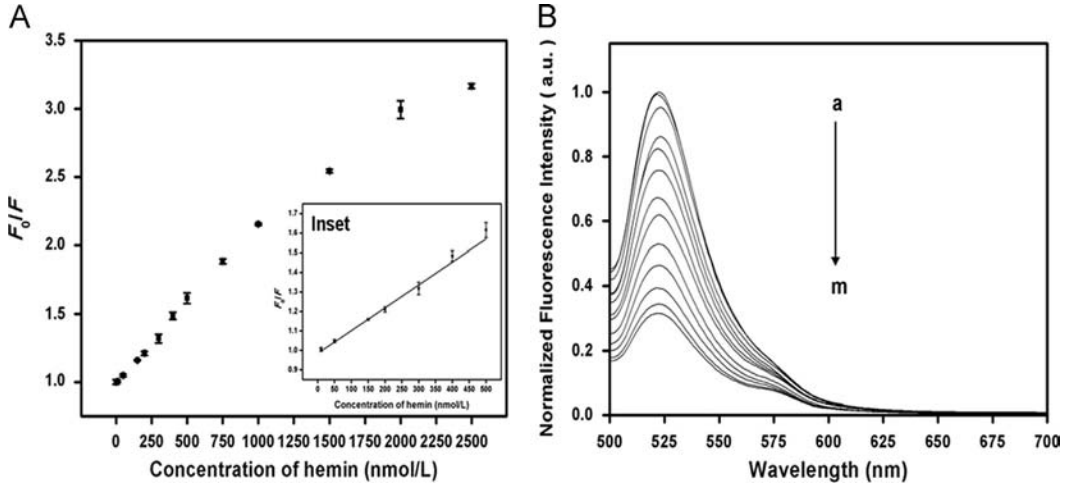


Fig. 3. (A) The corresponding relationship between F_0/F and the concentration of hemin. The inset shows the Stern–Volmer linear plot between F_0/F and the concentration of hemin in the range of 10–500 nmol/L. (B) The fluorescence spectra of P in the presence of different concentrations of hemin: (a) 0, (b) 10 nmol/L, (c) 50 nmol/L, (d) 150 nmol/L, (e) 200 nmol/L, (f) 300 nmol/L, (g) 400 nmol/L, (h) 500 nmol/L, (i) 750 nmol/L, (j) 1000 nmol/L, (k) 1500 nmol/L, (l) 2000 nmol/L, and (m) 2500 nmol/L. Conditions: Tris–HCl buffer (25 mM, pH 8.1, containing 10 μmol/L KCl), 3.75 nmol/L P, 2 h for fluorescence quenching. F and F_0 were the fluorescence intensity of P in the presence and absence of hemin, respectively. Error bars represent standard deviations from three measurements.

the quenching efficiency decreased quickly over the range of pH 8.1–8.7. According to previous report, trypsin was most active under slightly alkaline conditions [36]. The maximum fluorescence quenching efficiency at pH 8.1 shows that the activity of trypsin was the highest at pH 8.1, and the result was consistent with a previous report [37]. At pH 8.1, more chains of hemoglobin were cleaved into peptide fragments and more heme groups were released to form the G-quadruplex/hemin complex. Thus the fluorescence of P was quenched more efficiently. So we chose pH 8.1 buffer in the further study.

3.4. Effect of the concentration of trypsin

As shown in Fig. 5, when the concentration of hemoglobin was fixed (100 nmol/L), the fluorescence intensity ratio F/F_0 (F and F_0 were the fluorescence intensity of P in the presence and absence of hemoglobin, respectively) of P decreased with the increasing trypsin concentration. Then the fluorescence intensity ratio was nearly unchanged when the concentration of trypsin reached 70 mU/mL, indicating that hemoglobin was cleaved completely. Therefore, 70 mU/mL trypsin was used in the further experiments.

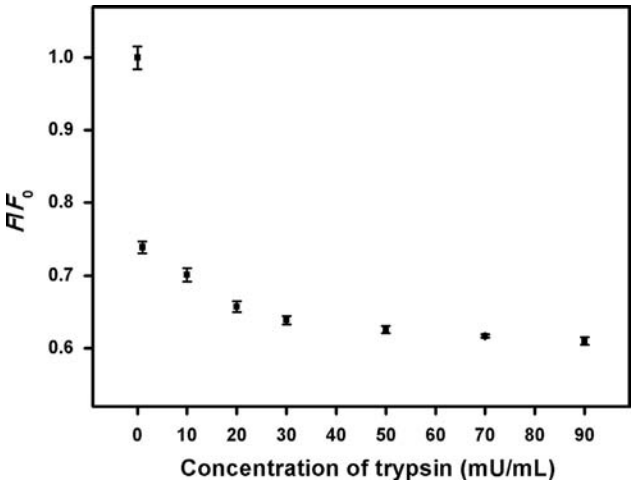


Fig. 5. The effect of trypsin concentration on the fluorescence intensity ratio F/F_0 of P. Conditions: Tris–HCl buffer (25 mM, pH=8.1, containing 10 μmol/L KCl), 3.75 nmol/L P, 100 nmol/L hemoglobin, 90 min for protein digestion at 37 °C and 2 h for fluorescence quenching. F and F_0 were the fluorescence intensity of P in the presence and absence of hemoglobin, respectively. Error bars represent standard deviations from three measurements.

3.5. Detection of hemoglobin

Under the optimum conditions, we studied the relationship between the fluorescence intensity of P and the concentration of hemoglobin. As shown in Fig. 6, the fluorescence intensity of P decreased with the increase of hemoglobin concentration. Then, a plateau was reached when the concentration of hemoglobin was 750 nmol/L. A linear quenching equation was obtained of

$$F_0/F = 0.96962 + 0.00605[\text{hemoglobin}],\ \text{nmol/L}$$

in a concentration range of 5–200 nmol/L, with the correlation coefficient (R^2) of 0.992, as shown in Fig. 6A inset. The limit of detection (LOD) corresponding to the fluorescence intensity of 3 times standard deviation obtained from 11 fluorescence measurements in the absence of analytes was 2 nmol/L. Compared with other methods (Table 1) [1,13,16,38–40], our method had the wider linear range and the similar LOD. The relative standard deviation for determination ($n=3$) of 10 nmol/L hemin was 2.1%.

3.6. Selectivity

Here, we also investigated the selectivity of the designed fluorescent sensing method. As shown in Fig. 7, five different enzymes (70 mU/mL trypsin, pepsin, urease, LDH and glucose oxidase) were adopted to digest hemoglobin (100 nmol/L) under the same condition. 38.3% quenching was observed for P in the presence of hemoglobin and trypsin, while in the presence of hemoglobin and other enzymes, none of these enzymes could produce noticeable P fluorescence intensity changes. These results clearly demonstrate that the assay highly depends on trypsin. We also tested the effect of other proteins, 100 nmol/L bovine serum albumin (BSA) and 100 nmol/L human serum albumin (HSA), on the fluorescence of P in the presence of 70 mU/mL trypsin. The results show that they did not significantly interfere with our assay.

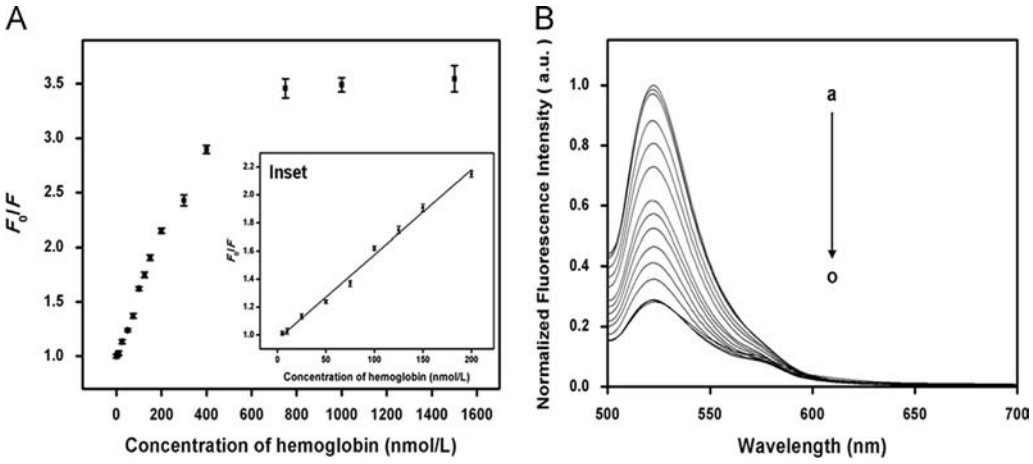


Fig. 6. (A) The corresponding relationship between F_0/F and the concentration of hemoglobin. The inset shows the Stern–Volmer linear plot between F_0/F and the concentration of hemoglobin in the range of 5–200 nmol/L. (B) The fluorescence spectra of P in the presence of different concentrations of hemoglobin: (a) 0, (b) 5 nmol/L, (c) 10 nmol/L, (d) 25 nmol/L, (e) 50 nmol/L, (f) 75 nmol/L, (g) 100 nmol/L, (h) 125 nmol/L, (i) 150 nmol/L, (j) 200 nmol/L, (k) 300 nmol/L, (l) 400 nmol/L, (m) 750 nmol/L, (n) 1000 nmol/L, and (o) 1500 nmol/L. Conditions: Tris–HCl buffer (25 mM, pH 8.1, containing 10 μmol/L KCl), 3.75 nmol/L P, 70 mU/mL trypsin, 90 min for protein digestion at 37 °C and 2 h for fluorescence quenching. F and F_0 were the fluorescence intensity of P in the presence and absence of hemoglobin, respectively. Error bars represent standard deviations from three measurements.

Table 1
Comparison of different methods for the determination of hemoglobin.

Technique	Linear range (nmol L^{-1})	Detection limit (nmol L^{-1})	Ref.
Electrochemistry	50.0–300.0	10.0	[13]
	10.0–20,000.0	5.0	[38]
	100–1000	–	[39]
	10–500	48	[40]
High performance liquid chromatography	2.5–50	–	[16]
Fluorimetric method	1.0–10	0.5	[1]
	5–200	2	This work

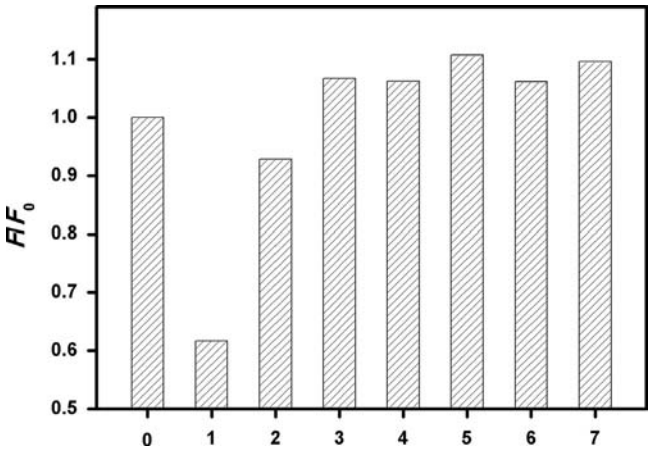


Fig. 7. Study on the effect of trypsin, pepsin, urease, LDH, glucose oxidase, BSA and HSA on the detection process. (0: DNA, 1: hemoglobin+trypsin, 2: hemoglobin+pepsin, 3: hemoglobin+urease, 4: hemoglobin+LDH, 5: hemoglobin+glucose oxidase, 6: BSA+trypsin, and 7: HSA+trypsin) Conditions: Tris–HCl buffer (25 mM, pH 8.1, containing 10 μmol/L KCl), 3.75 nmol/L P, 90 min for protein digestion at 37 °C and 2 h for fluorescence quenching. F and F_0 were the fluorescence intensity of P in the presence and absence of hemoglobin, respectively.

4. Conclusion

In this paper, we developed a sensitive and selective fluorescent sensing method for the detection of hemoglobin by using a fluorescent dye-labeled G-rich oligonucleotide sequence as the probe. One hemoglobin molecule contained four non-protein heme groups that would be released and oxidized when hemoglobin was cleaved by trypsin under proper conditions. The formed hemin was able to bind to the FITC-labeled G-rich oligonucleotide sequence and the G-quadruplex/hemin complex could accept the photoinduced electron from FITC, resulting in the fluorescence quenching of FITC. By measuring the fluorescence intensity, we were able to detect hemoglobin quantificationally. The low detection limit and high selectivity have made our approach a potential method for recognizing hemoglobin efficiently.

Acknowledgments

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References

[1] L.Y. Chen, C.C. Huang, W.Y. Chen, H.J. Lin, H.T. Chang, Biosens. Bioelectron. 43 (2013) 38–44.
[2] A.N. Schechter, Blood 112 (2008) 3927–3938.
[3] M.F. Perutz, Annu. Rev. Physiol. 52 (1990) 1–25.
[4] J.M. Friedman, Science 228 (1985) 1273–1280.
[5] X.F. Yang, X.Q. Guo, H. Li, Talanta 61 (2003) 439–445.
[6] K. Zhang, R. Cai, D. Chen, L. Mao, Anal. Chim. Acta 413 (2000) 109–113.
[7] K. Takahata, S. Igarashi, Clin. Chim. Acta 283 (1999) 129–138.
[8] Q.L. Wang, Z.H. Liu, H. Yuan, R.X. Cai, Chin. J. Anal. Chem. 4 (2001) 421–424.
[9] J.F. Song, X.F. Kang, W. Guo, Fresenius' Journal of Analytical Chemistry 357 (1997) 127–129.
[10] X. Bao, Z. Zhu, N.Q. Li, J. Chen, Talanta 54 (2001) 591–596.
[11] H.X. Ju, H.B. Sun, H.Y. Chen, Anal. Chim. Acta 327 (1996) 125–132.
[12] G.X. Li, Y.T. Long, H.Y. Chen, D.X. Zhu, Fresenius' Journal of Analytical Chemistry 356 (1996) 359–360.
[13] Z.W. Zhu, N.Q. Li, Electroanalysis 10 (1998) 643–646.
[14] C.M.A. Brett, G. Inzelt, V. Kertesz, Anal. Chim. Acta 385 (1999) 119–123.
[15] T.I. Quickenden, P.D. Cooper, Luminescence 16 (2001) 251–253.
[16] M.R. van BommelA.P.J.M. de JongU.R. TjadenH. IrthJ. van der Greef, J. Chromatogr. A 886 (2000) 19–29.
[17] T.H.J. Huisman, Anal. Chim. Acta 352 (1997) 187–200.
[18] P. Kleinert, M. Schmid, K. Zurbriggen, O. Speer, M. Schmugge, B. Roschitzki, S.S. Durka, U. Leopold, T. Kuster, C.W. Heizmann, H. Frischknecht, H. Troxler, Clin. Chem. 54 (2008) 69–76.
[19] B.B. Prasad, A. Prasad, M.P. Tiwari, Talanta 109 (2013) 52–60.
[20] A. Ramanavicius, N. Ryskevic, Y. Oztekin, A. Kausaite-Minkstimiene, S. Jursenas, J. Baniukevic, J. Kirlyte, U. Bubniene, A. Ramanaviciene, Anal. Bioanal. Chem. 398 (2010) 3105–3113.
[21] A. Ramanavicius, N. Kurilcik, S. Jursenas, A. Finkelsteinas, A. Ramanaviciene, Biosens. Bioelectron. 23 (2007) 499–505.
[22] J.V. Olsen, S. Ong, M. Mann, Mol. Cell. Proteomics 3 (2004) 608–614.
[23] M. Bergmann, J.S. Fruton, F.F. Nord, Advances in Enzymology, Interscience Publishers, Inc., New York (1941) 1941; 63.
[24] B.A. Springer, K.D. Egeberg, S.G. Sligar, R.J. Rohlfs, A.J. Mathews, J.S. Olson, J. Biol. Chem. 264 (1989) 3057–3060.
[25] R.A. Sheldon, Metal-Catalyzed Oxidations of Organic Compounds, Academic Press, New York (1981) 1981; 216–268.
[26] Y. Shi, W.T. Huang, H.Q. Luo, N.B. Li, Chem. Commun. 47 (2011) 4676–4678.
[27] P. Travascio, P.K. WittingA.G. MaukD. Sen, J. Am. Chem. Soc. 123 (2001) 1337–1348.
[28] D.J.F. Chinnapen, D. Sen, Biochemistry 41 (2002) 5202–5212.
[29] Y. Xiao, V. Pavlov, R. Gill, T. Bourenko, I. Willner, ChemBioChem 5 (2004) 374–379.
[30] D. Li, B. Shlyahovsky, J. Elbaz, I. Willner, J. Am. Chem. Soc. 129 (2007) 5804–5805.
[31] L.B. Zhang, J.B. Zhu, S.J. Guo, T. Li, J. Li, E.K. Wang, J. Am. Chem. Soc. 135 (2013) 2403–2406.
[32] W.Y. Li, J. Chen, H.P. Jiao, Q.F. Zhang, H.P. Zhou, C. Yu, Chem. Commun. 48 (2012) 10123–10125.
[33] K.M. Faulkner, C. Bonaventura, A.L. Crumbliss, Inorg. Chim. Acta 226 (1994) 187–194.
[34] S.M. Jia, X.F. Liu, P. Li, D.M. Kong, H.X. Shen, Biosens. Bioelectron. 27 (2011) 148–152.
[35] E. Sharon, R. Freeman, I. Willner, Anal. Chem. 82 (2010) 7073–7077.
[36] G.H. Jeohn, S. Serizawa, A. Iwamatsu, K. Takahashi, J. Biol. Chem. 270 (1995) 14748–14755.
[37] B.A. Zaccheo, R.M. Crooks, Anal. Chem. 83 (2011) 1185–1188.
[38] M.C. Liu, G.Y. Shi, L. Zhang, Y.X. Cheng, L.T. Jin, Electrochem. Commun. 8 (2006) 305–310.
[39] J. Zhang, K. Seo, C. Jeon, Anal. Bioanal. Chem. 375 (2003) 539–543.
[40] E. Alvarez de Eulate, L. Serls, D.W. Arrigan, Anal. Bioanal. Chem. 405 (2013) 3801–3806.