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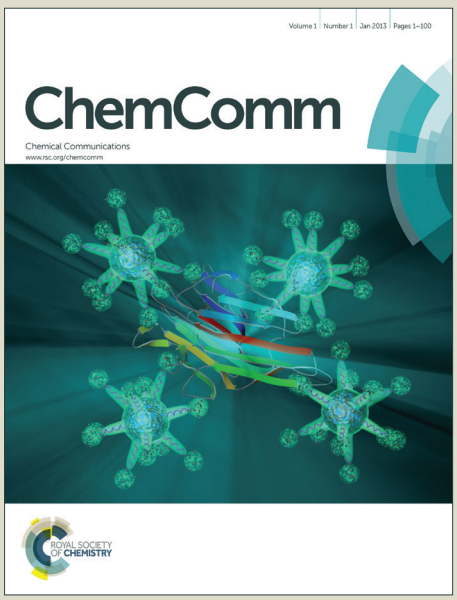





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# Discrimination of hemoglobin with subtle differences by aptamer based sensing array

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**Discrimination of hemoglobin with subtle differences was achieved by aptamer based sensing array. Linear discriminant analysis (LDA) showed that the sensing array can discriminate human hemoglobin from hemoglobins of different species.**

Blood is the most commonly encountered body fluid at crime scenes.[1] Sometimes it is very important to determine if the sample is human blood, and at other times some specific species need to be determined.[2] Usually, hemoglobin has been chosen for blood identification since this protein is specific for blood and present in large amounts.[3] However, hemoglobins of most vertebrates have similar structures with a tetrameric globular protein consisting of two α and β polypeptide chains. The number of amino acids varies slightly among species, and the molecular weight varies around 65,000.[4] Therefore, the analysis of different hemoglobins remains challenging due to the subtle differences in hemoglobin structures. A wide array of hemoglobin-based assays have been used for species identification, including mass spectrometry (MS),[3] immunochemical assays,[5] high-performance liquid chromatography (HPLC),[6] and Raman spectroscopy.[7] However, immunochemical assays may exhibit cross-reaction with animal blood. In addition, immunoassays suffer from reagent instability. MS, HPLC, and Raman spectroscopy are usually costly, time consuming, and require highly trained personnel. Therefore, a more stable and cost-effective assay is urgently needed for hemoglobins discrimination of different species.

Linear discriminant analysis (LDA) is a well-known statistical method which can be used to separate classes of objects or assign new objects to appropriate classes. The discriminants are linear combinations of the measured variables, e.g., sensor responses. This method maximizes the ratio of between-class and within-class variances in any particular data set, allowing response patterns to be quantitatively differentiated.[8] Over the past several years, pattern array-based sensing approaches combined with LDA had been successfully used for the discrimination and recognition of protein analytes.[9-15] In these sensing arrays, proteins were chosen according to different sizes and charges. Therefore, it is of great importance to develop a system for discrimination of proteins with subtle differences. Recently, Tomita et al. reported an enzyme-based sensing array for the discrimination of homologous proteins, which relies on the changes of enzyme activity after binding with proteins.[16]

In recent years, aptamers, as a kind of alternative probes, have been used for the discrimination of different analytes by sensing array.[17, 18] Aptamers are short single-stranded oligonucleotides (DNA or RNA) obtained by in vitro process, i.e. SELEX (systematic evolution of ligands by exponential enrichment).[19, 20] Numerous high-affinity and specific aptamers have been generated against a variety of targets.[21-24] Aptamers offer several favorable advantages. Firstly, they are stable and resistant to harsh environment. Secondly, the production of aptamers is cost-effective and reproducible. Thirdly, aptamers can easily bear labels at each end of the strand.[25]

Herein, an aptamer sensing array for hemoglobins discrimination of different species was proposed. Our sensing system employed a competitive assay, which relied on the differences of affinity between aptamers and hemoglobins (Fig. 1). Fluorescence of FAM labeled aptamers is firstly quenched by graphene oxide (GO). After the addition of hemoglobin to aptamer/GO complex, fluorescence is then recovered.

DNA aptamers used in this study were obtained by SELEX (see details in Section S-4 in ESI). The selection efficiency of DNA pool was significantly increased with the increasing of SELEX rounds (Fig.S2 in ESI). When the selection efficiency reached a plateau, the DNA pools were cloned and sequenced (see details in Section S-5 in ESI). According to the results of sequencing, eight aptamers named $9^{th}$-1, $9^{th}$-3, $9^{th}$-7, $10^{th}$-1, $10^{th}$-2, $10^{th}$-6, $10^{th}$-7 and $10^{th}$-9 were chosen for further binding affinity studies based on the repeated sequences and similarity of secondary structure. After the affinity and specificity determination (see details in Section S-6 in ESI), four aptamers named $9^{th}$-1, $10^{th}$-2, $10^{th}$-6 and $10^{th}$-9 exhibited apparent affinity for human hemoglobin (Table 1), and the $K_d$ values were almost consistent when measured on different days (see details in Section S-7 in ESI). Different from the random designed nucleic acid, the aptamers achieved by SELEX showed binding ability to human hemoglobin, which was important for the discrimination of human hemoglobin from hemoglobins of other species.

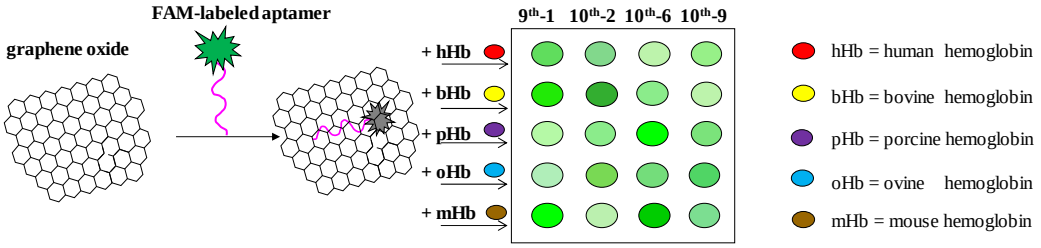


**Fig. 1** Schematic illustration of fluorescence sensing array for discrimination of hemoglobins. Fluorescence pattern generation through differential release of FAM-labeled aptamers from GO.

**Table 1** Sequences of aptamers used in sensing array

| Aptamer | Sequence of random region (5′→3′) | $K_d$ of hHbA1c / nM | $K_d$ of hHb / nM |
|---|---|---|---|
| 9th-1 | TTCCGGAATGTGCTGCTTTCCCCTGACTGTTTGGGAACCC | 57.98 ± 1.30 (n=5) | 73.94 ± 1.65 (n=5) |
| 9th-3 | TCCAACAGGGTGGGGGGGGTGGGTCATTTGGATTATCTCC | — | — |
| 9th-7 | CTCCCTACGACATGCCTTAGCGTTGGGTTCGCACGCTCTA | — | — |
| 10th-1 | ATAAGCATTCTTATCGGGCCACTCGTTTACCTGCGTTATC | — | — |
| 10th-2 | TGGGCGGGGGGGTGGGTTTTTACGGGGGCAATGTACTACC | 64.27 ± 1.48 (n=5) | 85.34 ± 2.18 (n=5) |
| 10th-6 | CCCGTAGCATTGCGGAAGTCACCGTGGGTTGAATGGTCTA | 85.73 ± 1.84 (n=5) | 116.99 ± 2.01 (n=5) |
| 10th-7 | CCCGGTTATAGGCGCGTTCGTTAAGCGTGTTCCATTGGGA | — | — |
| 10th-9 | GCGCGAGTAAGGGTACGTCTAGTAACTCCTCGGTACGGTC | 55.09 ± 1.83 (n=5) | 47.64 ± 2.13 (n=5) |

For the sensing array strategy, five hemoglobins including human hemoglobin (hHb), bovine hemoglobin (bHb), porcine hemoglobin (pHb), ovine hemoglobin (oHb) and mouse hemoglobin (mHb) were chosen as sensing targets. In order to determine the detailed fluorescence response of aptamers to different concentrations of hemoglobins, fluorescence titration experiments were performed (Fig. 2). Fluorescence of FAM-labeled aptamers was firstly quenched by GO. When hemoglobins were gradually added, their fluorescence was then recovered. Fluorescence recovery depends on the affinity of aptamers for the hemoglobins. We found that these four aptamers exhibited different responses to different hemoglobins. The results demonstrated the possibility that aptamers can be employed as signal molecules for pattern sensing of hemoglobins.

The pattern sensing tests were conducted at 20 μg/mL proteins using conventional 96-well microplates (see details in Section S-8 in ESI). Since human serum albumin (HSA) is the main protein in plasma and myoglobin (Myo) has similar structure with hemoglobin, HSA and Myo might be the main influences in hemoglobin detection. Therefore, HSA and Myo were included in the fluorescence sensing array. Proteins interacted differently with these four aptamers resulting in a variable amount of displaced fluorescence. We investigated the fluorescence behavior of aptamer/GO complex after interacting with different proteins by monitoring the changes of fluorescence intensity at 521 nm. The raw data were provided in Table S2 of ESI. The responses were compiled into a training matrix, and distinct fluorescence responses were observed for each protein. In brief, twelve replicates were obtained for each protein in each sensing array, producing 336 data points (4 aptamers × 7 proteins × 12 replicates) for the array. At a given concentration, each of the analytes generated a different response pattern (Fig. 3A). And then the data were analyzed using LDA in SYSTAT (version 11.5). LDA has been used to differentiate the response patterns of different analytes.[10, 17, 26-30]

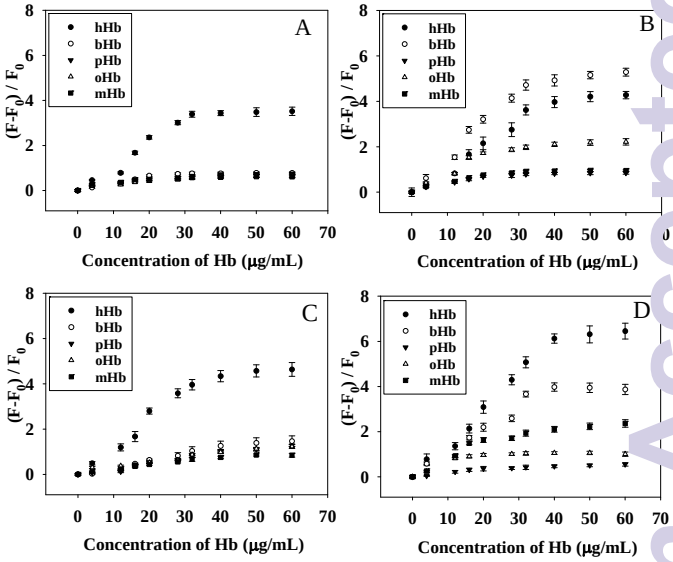


**Fig. 2** Fluorescence intensity changes (λex/em = 488/521 nm) of aptamer/GO mixture with the gradual addition of various concentrations of hemoglobins. (A) 9th-1, (B) 10th-2, (C) 10th-6, (D) 10th-9. The error bars indicate the standard deviations of three parallel experiments.

As shown in Fig 3B, despite slight differences between these hemoglobins, a clear separation of the clusters of replicates was observed from the three-dimensional LDA score plot. The three canonical factors were 64.8%, 24.7% and 9.5%. All proteins were clustered to seven distinct groups and five hemoglobins were grouped close in the LDA plot, indicating that the subtle difference in the proteins generated marked changes in response. In addition, HSA and Myo could be well distinguished with different hemoglobins.

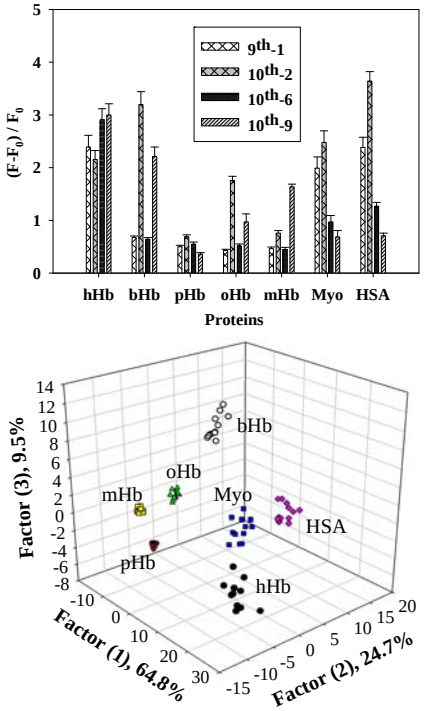


**Fig. 3** Pattern-based sensing of proteins using aptamers. (A) Fluorescence response patterns of aptamers in the 96-well microplate on addition of proteins (20 μg/mL). The fluorescence intensities were recorded at λex/em = 488/521 nm. (B) Canonical score plot for the fluorescence patterns as obtained from LDA against different proteins (hHb, bHb, pHb, oHb, mHb, Myo and HSA) at a concentration of 20 μg/mL.

We believe that the discriminatory ability of this approach can be explained as follows. On the one hand, the subtle interspecies differences in the hemoglobin structure result in the differences in binding affinity between aptamer and protein. On the other hand, DNA aptamers of different sequences and structures interact differentially with GO by various supramolecular interactions including hydrogen bonding and electrostatic interaction.

In summary, the discrimination of hemoglobins with similar characteristics was achieved by aptamer based sensing array. In addition to the high differentiability, our strategy has potential applicability for other analytes, such as cells and particles, by obtaining corresponding aptamers. We believe that our present study will broaden the application field of aptamer for proteins detection.

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**Notes and references**

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*Electronic Supplementary Information (ESI) available: chemicals and materials, experimental details, conditions optimization, data as noted in the text. See DOI: 10.1039/c000000x/*

1. B. A. Schweers, J. Old, P. W. Boonlayangoor and K. A. Reich, *Forensic Sci. Int.: Gen.*, 2008, **2**, 243-247.
2. K. Virkler and I. K. Lednev, *Anal. Chem*., 2009, **81**, 7773–7777.
3. K. V. Steendam, M. D. Ceuleneer, M. Dhaenens, D. V. Hoofstat and L. Deforce, *Int. J. Legal Med.*, 2013, **127**, 287-298.
4. S. F. Perry and B. Tufts, *Fish respiration*, Academic Press, California, 1998.
5. M. N. Hochmeister, B. Budowle, R. Sparkes, O. Rudin, C. Gehrig, M. Thali, L. Schmidt, A. Cordier and R. Dirnhofer, *J. Forensic Sci*., 1999, **44**, 597-602.
6. J. Andrasko and B. Rosen, *J. Forensic Sci*., 1994, **39**, 1018-1025.
7. G. McLaughlin, K. C. Doty and I. K. Lednev, *Anal. Chem*., 2014, **8**, 11628-11633.
8. P. C. Jurs, G. A. Bakken and H. E. McClelland, *Chem. Rev*., 2000**, 10**, 2649-2678.
9. O. R. Miranda, C. C. You, R. Phillips, I. B. Kim, P. S. Ghosh, U. H. F. Bunz and V. M. Rotello, *J. Am. Chem. Soc*., 2007, **129**, 9856-9857.
10. Y. Lu, H. Kong, F. Wen, S. Zhang and X. Zhang, *Chem. Commun*., 2013, **49**, 81-83.
11. S. Tomita and K. Yoshimoto, *Chem. Commun.*, 2013, **49**, 10430-10[illegible].
12. C. C. You, O. R. Miranda, B. Gider, P. S. Ghosh, I. B. Kim, B. Erdogan, S. A. Krovi, U. H. F. Bunz and V. M. Rotello, *Nat. Nanotechnol*., 2007, **2**, 318-323.
13. M. De, S. Rana, H. Akpinar, O. R. Miranda, R. R. Arvizo, U. H. F. Bunz and V. M. Rotello, *Nat. Chem*., 2009, **1**, 461-465.
14. O. R. Miranda, H. T. Chen, C. C. You, D. E. Mortenson, X. C. Yang, U. H. F. Bunz and V. M. Rotello, *J. Am. Chem. Soc.*, 2010, **132**, 52[illegible] 5289.
15. H. Pei, J. Li, M. Lv, J. Wang, J. Gao, J. Lu, Y. Li, Q. Huang, J. Hu and C. Fan, *J. Am. Chem. Soc*., 2012, **134**, 13843-13849.
16. S. Tomita, T. Soejima, K. Shiraki and K. Yoshimoto, *Analyst*, 201[illegible] **139**, 6100-6103.
17. S. Wang, H. Kong, X. Gong, S. Zhang and X. Zhang, *Anal. Chem*., 2014, **86**, 8261-8266.
18. Y. Lu, Y. Liu, S. Zhang, S. Wang, S. Zhang and X. Zhang, *Anal. Chem*., 2013, **85**, 6571-6574.
19. A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818-822.
20. C. Tuerk and L. Gold, *Science*, 1990, **249**, 505-510.
21. G. Mayer, *Angew. Chem. Int. Ed*., 2009, **48**, 2672-2689.
22. M. Mascini, I. Palchetti and S. Tombelli, *Angew. Chem. Int. Ed*., 201[illegible] **51**, 1316-1332.
23. S. D. Jayasena, *Clin. Chem*., 1999, **45**, 1628-1650.
24. W. Tan, M. J. Donovan and J. Jiang, *Chem. Rev*., 2013, **113**, 2842-2862.
25. Q. Wang, W. Liu, Y. Xing, X. Yang, K. Wang, R. Jiang, P. Wang and Q. Zhao, Anal. Chem., 2014, **86**, 6572-6579.
26. J. Liu, G. Li, X. Yang, K. Wang, L. Li, W. Liu, X. Shi and Y. Guo, *Anal. Chem*., 2015, **87**, 876-883.
27. T. Minami, N. A. Esipenko, B. Zhang, L. Isaacs and P. Anzenbacher, Jr., *Chem. Commun*., 2014, **50**, 61-63.
28. W. Chen, Q. Li, W. Zheng, F. Hu, G. Zhang, Z. Wang, D. Zhang and X. Jiang, *Angew. Chem. Int. Ed*., 2014, **126**, 13954-13959.
29. T. Ema, K. Okuda, S. Watanabe, T. Yamasaki, T. Minami, N. A. Esipenko and P. Anzenbacher, Jr., *Org. Lett*., 2014, **16**, 1302-1305.
30. C. Pezzato, B. Lee, K. Severin and L. J. Prins, *Chem. Commun*., 2015, **49**, 469-471.