Electronic Supplementary Information

Discrimination of hemoglobin with subtle difference by aptamer based sensing array

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Experimental details

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S-1. Materials and devices

Human glycated hemoglobin (hHbA1c), human hemoglobin (hHb) and anti-human hemoglobin (monoclonal antibody) were purchased from Fitzgerald (USA). Human serum albumin (HSA) and human immunoglobulin G (IgG) were purchased from Beijing Dingguo Changsheng biotech Co., Ltd (Beijing, China). Bovine hemoglobin and porcine hemoglobin were purchased from Ruibio (USA). Ovine hemoglobin and mouse hemoglobin were purchased from Uscn life science Inc. Myoglobin was purchased from Abcam, Inc. (Cambridge, MA, USA). Graphene oxide (GO) was obtained from Institute of Coal Chemistry, Chinese academy of sciences. Protein A (SPA) magnetic beads were purchased from Pierce. Streptavidin-coated sepharose beads were obtained from GE Healthcare (Piscataway, NJ). The DNA pool was synthesized and purified by Takara Biotechnology (Dalian, China), and all of the other DNAs used in the experiment were synthesized from Sangon Biotech (Shanghai, China) Co., Ltd. (Keep in 4 ^{o}C before use). All of the chemical reagents were of analytical grade or higher. Ultrapure water (18.2 MΩ.cm) was used throughout.

S-2. Design of DNA pool

40-base randomized domain was placed in the middle of a FAM-labeled DNA pool, then, a 19-nt and 20-nt constant regions were placed at both ends of pool. The FAM-labeled forward primer (FP) and biotin-labeled reverse primer (RP) were used for PCR amplification. Forward primer and reverse primer were used for cloning and sequencing. DNA sequences used in this selection were shown in Table S1.

Table S1. Sequences of DNA used in this selection

Name	Sequence
ssDNA pool	5′-FAM-GACAGGCAGGACACCGTAA-N_{40}-CTGCTACCTCCCTCCTCTTC-3′
FP	5′-GACAGGCAGGACACCGTAA-3′
RP	5′-GAAGAGGAGGGAGGTAGCAG-3′
RP-biotin	5′-biotin-GAAGAGGAGGGAGGTAGCAG-3′
FP-FAM	5′-FAM-GACAGGCAGGACACCGTAA-3′

*FP: forward primer; RP: reverse primer

S-3. hHbA1c immobilization on protein A magnetic beads

Firstly, 50 μL protein A magnetic beads were washed with 150 μL PBST composed of 0.05 % Tween 20 in 20 mM PBS buffer (pH=7.4, 0.15mM NaCl). The washed protein A magnetic beads were incubated with 750 nM anti-human hemoglobin monoclonal antibody for 2 h and then washed with PBST to remove unbound antibody. Next, the antibody captured magnetic beads were incubated with 750 nM hHbA1c for 2 h and then washed with PBST to remove unbound hHbA1c.

S-4. Selection of aptamers against hHbA1c

(1) Selection of aptamers

The aptamer selection process began with positive selection (Fig. S1). Firstly, 1.3 nmol ssDNA pool dissolved in 100 μL 1× binding buffer (BB, 20 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) was heated at 95 °C for 5 min and rapidly cooled down to 0 °C in an ice bath for 5 min, and then incubated for another 5 min at room temperature. Subsequently, the treated pool incubated with hHbA1c immobilized magnetic beads for 2 h, the unbound ssDNAs were removed in a magnetic particle concentrator separator. Finally, the magnetic beads were heated to 95 °C to collect the bound ssDNAs.

Fig. S1 Schematic of aptamer selection by magnetic beads technology.

(2) PCR amplification

A PCR mixture containing 25 μL 2× Power Taq Master Mix, 1 μL of 10 μM FAM-

labeled forward primer, and 1 μL of 10 μM biotinylated reverse primer was then combined with 10 μL of collected products and ultrapure water to bring the total volume to 50 μL. The thermal cycling conditions were as follows: 94 °C for 5 min, followed by 10 cycles of a rapid three-step PCR (30 s denaturation at 94 °C, 30 s annealing at 58.5 °C, 30 s extension at 72 °C), and finally extension at 72 °C for 7 min. During the extension step of each round, 10 μL of PCR mixture was collected and resolved on a 2.5 % agarose gel electrophoresis (AGE) to find the optimal PCR amplification cycle number. Finally, the collected DNA products were all PCR amplified at the optimized cycle number.

(3) ssDNA generation

The biotinylated, double-stranded PCR products were incubated with 200 μL of GE Streptavidin agarose microbeads for 0.5 h at room temperature. ssDNAs were generated by adding 200 mM NaOH and incubating for 10 min at room temperature, after which the supernatant was collected, desalted and characterized by UV-visible measurement at 260 nm.

(4) Counter selection

After the 3rd round, a counter selection was carried out. The purified ssDNA pool (120 pmol) of 3rd round was incubated with the hHb immobilized magnetic beads for 2 h, the unbound ssDNAs were collected. Then the collected DNA products were incubated with the hHbA1c immobilized magnetic beads for another 2 h, the unbound

ssDNAs were removed in a magnetic particle concentrator separator, the magnetic beads were heated to 95 °C to collect the bound ssDNAs.

(5) Selection efficiency determination

After 11 rounds of selection, the selection efficiency of each round was measured by fluorescence method.[1] The fluorescence value of 100 nM ssDNA pool of each round (F0) was firstly measured after preparation. Then, the ssDNA pool of each round was incubated with hHbA1c immobilized magnetic beads individually, and the fluorescence value of supernatant (F1) was measured. Then the selection efficiency was calculated using the following equation, the selection efficiency of DNA pool to hHbA1c was significantly increased with increasing of the SELEX round, and the signal reached a plateau in the 9th round. The results of 7th to 11th round were shown in Fig. S2.

$$\text{Selection efficiency} = \frac{F0\text{-}F1}{F1} \times 100\%$$

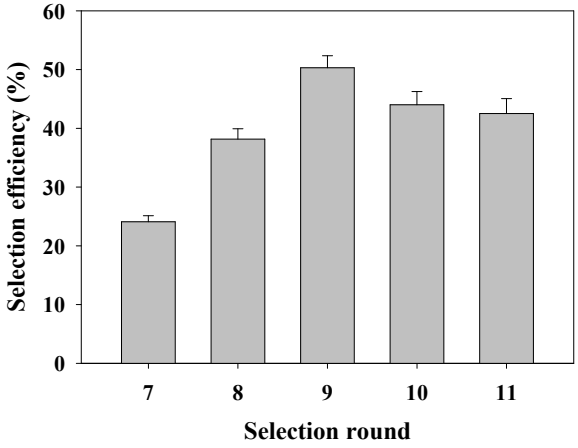


Fig. S2 Selection efficiency of DNA pool from the 7th to 11th round.

S-5. Cloning and sequencing of selected aptamers

The DNA pools of the 9th and 10th rounds were PCR amplified with unlabeled forward and reverse primers at the optimized PCR cycle number determined by the pilot PCR. The PCR products were cloned into *Escherichia coli* using the TOPO TA cloning kit, and 100 colonies of each round were randomly picked and sequenced at the Sangon Biotech (Shanghai, China). The secondary structures of selected aptamers were analyzed by the Internet tool Mfold. Sequence alignment was performed by DNAMAN software. Repeated sequences were chosen and then classified into eight groups based their similarity of secondary structure as shown in Fig. S3.

Group1

9th-5:

5′-
GACAGGCAGGACACCGTAAATCCCACCGGACAAACCAAGTTCAGGTACACTTGACAG
GTCTGCTACCTCCCTCCTCTTC-3′

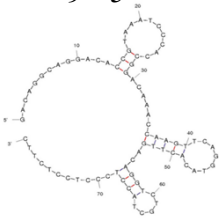
10th-7:

5′-
GACAGGCAGGACACCGTAACCCGGTTATAGGCGCGTTCGTTAAGCGTGTTCCATTGG
GACTGCTACCTCCCTCCTCTTC-3′

10th-8:

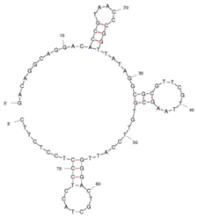
5′-
GACAGGCAGGACACCGTAAGTAACAGGTTGAGAACTCTTGGTGGTCGAGTTTCTGGG
GGCTGCTACCTCCCTCCTCTTC-3′

9th-5



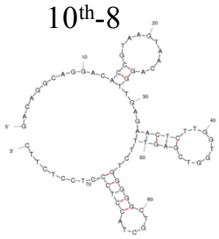
ΔG = -5.05 kcal/mol

10th-7



ΔG = −5. 70 kcal/mol

10th-8



ΔG = -2.42 kcal/mol

Group2

9th-2:

5′-

GACAGGCAGGACACCGTAATCCAATTCATGGACTACGTGGACAGGAGATTTAAATCG

ATCTGCTACCTCCCTCCTCTTC-3′

9th-4:

5′-

GACAGGCAGGACACCGTAAAGAATGGTTGAGTCGCAGCCGTATTCCGCCGTTCATGA

GTCTGCTACCTCCCTCCTCTTC-3′

9th-7:

5′-

GACAGGCAGGACACCGTAACTCCCTACGACATGCCTTAGCGTTGGGTTCGCACGCTCT

ACTGCTACCTCCCTCCTCTTC-3′

10th-3:

5′-

GACAGGCAGGACACCGTAATCCCGCCGGCTAAGGTGCAGGACAGTTTCTTAGACAAA

AGCTGCTACCTCCCTCCTCTTC-3′

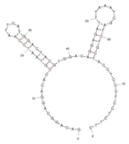
10th-10:

5′-

GACAGGCAGGACACCGTAAGGAGCGGCTATTCATGTGTGTGGGTCACTTAACTGACA

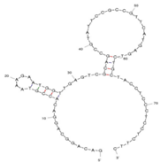
CGCTGCTACCTCCCTCCTCTTC-3′

9th-2



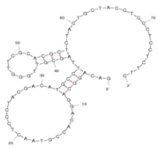
ΔG = -5.22 kcal/mol

9th-4



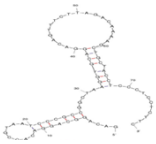
ΔG = -1.32 kcal/mol

9th-7



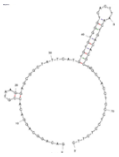
ΔG = -4.73 kcal/mol

10th-3



ΔG = -3.35 kcal/mol

10th-10



ΔG = -4.21 kcal/mol

Group3

9th-6:

5′-

GACAGGCAGGACACCGTAACCTTCTCGCAGTTCGTGATCGTCTCTACCAACGTGAACC

ACTGCTACCTCCCTCCTCTTC-3′

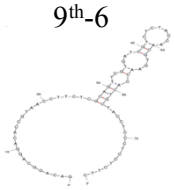
10th-9:

5′-

GACAGGCAGGACACCGTAAGCGCGAGTAAGGGTACGTCTAGTAACTCCTCGGTACGG

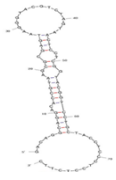
TCCTGCTACCTCCCTCCTCTTC-3′

9th-6



ΔG = -0.94 kcal/mol

10th-9



ΔG = -7.41 kcal/mol

Group4

10th-4:

5′-

GACAGGCAGGACACCGTAACGTAGCCAATTCGCCGCGTCGTCAAATAACTTCCATTG

CTCTGCTACCTCCCTCCTCTTC-3′

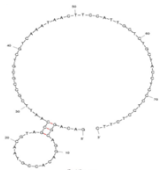
10th-11:

5′-

GACAGGCAGGACACCGTAAGTGCGCGCTTTGTATCCCTCCCCGTTCAAGATTAGGTG

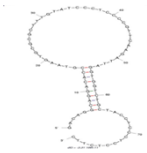
GTCTGCTACCTCCCTCCTCTTC-3′

10th-4



ΔG = -1.16 kcal/mol

10th-11



ΔG = -3.31 kcal/mol

Group5

9th-1:

5′-

GACAGGCAGGACACCGTAATTCCGGAATGTGCTGCTTTCCCCTGACTGTTTGGGAACC

CCTGCTACCTCCCTCCTCTTC-3′

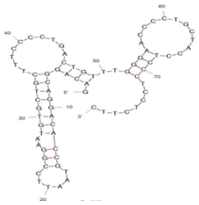
9th-3:

5′-

GACAGGCAGGACACCGTAATCCAACAGGGTGGGGGGGGGTGGGTCATTTGGATTATCT

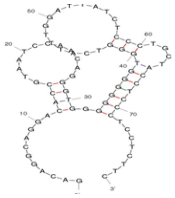
CCCTGCTACCTCCCTCCTCTTC-3′

9th-1



ΔG = -22.85 kcal/mol

9th-3



ΔG = -4.53 kcal/mol

Group6

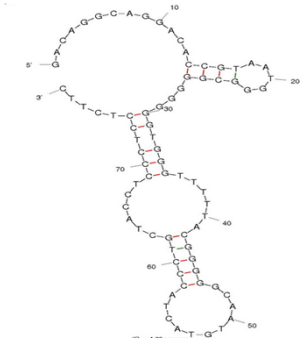
10th-2:

5′-

GACAGGCAGGACACCGTAATGGGCGGGGGGGTGGGTTTTTACGGGGGCAATGTACTA

CCCTGCTACCTCCCTCCTCTTC-3′

10th-2



ΔG = -4.02 kcal/mol

Group7

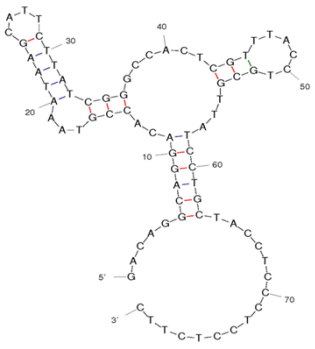
10th-1:

5′-

GACAGGCAGGACACCGTAAATAAGCATTCTTATCGGGCCACTCGTTTACCTGCGTTAT

CCTGCTACCTCCCTCCTCTTC-3′

10th-1



ΔG = -2.16 kcal/mol

Group8

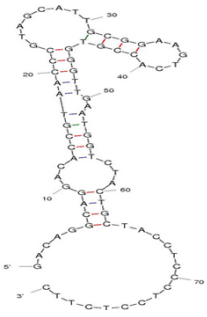
10th-6:

5′-

GACAGGCAGGACACCGTAACCCGTAGCATTGCGGAAGTCACCGTGGGTTGAATGGTC

TACTGCTACCTCCCTCCTCTTC-3′

10th-6



ΔG = -2.97 kcal/mol

Fig. S3 The secondary structures of eight groups of the repeated sequences.

S-6. Affinity and specificity determination

(1) Quenching efficiency of graphene oxide

Firstly, 4 pmol aptamer dissolved in 1× binding buffer (20 mM Hepes, 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), was heated at 95 °C for 5 min and rapidly cooled down to 0 °C in an ice bath for 5 min, and then mixed with a serious of different concentrations of GO solution (from 20 μg/mL to 200 μg/mL) at 37 °C for 2 h. The final volume was 200 μL. Subsequently, the fluorescence intensity of aptamer (F_0) before mixed with GO was measured at 521 nm, and the fluorescence intensity of GO/aptamer mixture (F) was also measured. Then, the quenching efficiency $(F_0-F)/F_0$ of GO was calculated. As shown in Fig. S4, the quenching efficiency was nearly saturated when the concentration of GO was 120 μg/mL. Hence, 120 μg/mL GO was chosen for affinity and specificity determination.

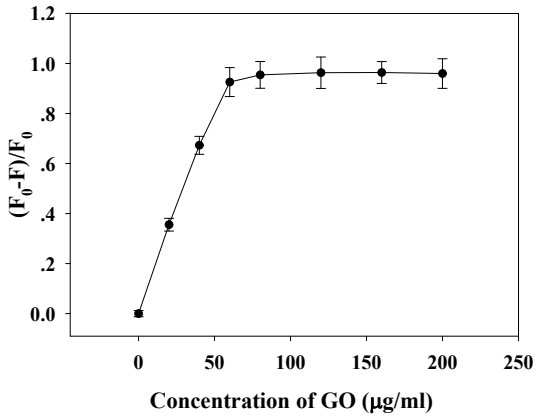


Fig. S4 The determination of quenching efficiency (F0-F)/F0 of GO by fluorescence method.

(2) Optimization of incubation time

In order to well control the experimental condition, incubation time of aptamers and targets were optimized. Firstly, 20 nM FAM-labeled aptamer dissolved in 1× binding buffer, was heated at 95 °C for 5 min and rapidly cooled down to 0 °C in an

ice bath for 5 min, and then incubated with 120 μg/mL GO. The fluorescence intensity of GO/aptamer mixture was then measured with excitation and emission wavelengths of 488 nm and 521 nm. Subsequently, 20 μg/mL hHbA1c was mixed with the GO/aptamer mixture, and the fluorescence intensity of the mixture was measured every 10 min. As shown in Fig. S5, the recovery of fluorescence intensity was almost completely finished in 70 min. Hence, 70 min was chosen as the incubation time of aptamers and proteins.

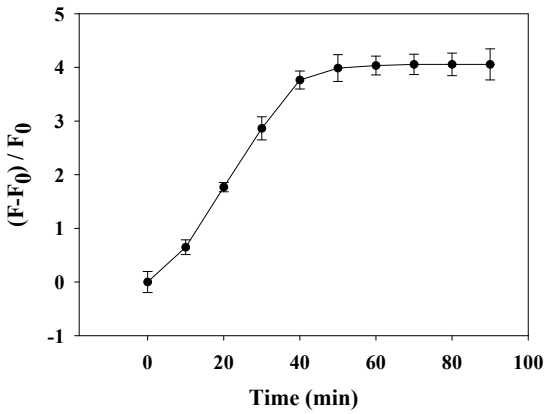


Fig. S5 Optimization of incubation time of aptamers and proteins.

(3) Affinity determination

The binding affinity of individual ssDNA aptamers to hHbA1c was tested using GO-based fluorescence method as shown in Fig. S6. Firstly, 20 nM FAM-labeled aptamer dissolved in 1× binding buffer, was heated at 95 °C for 5 min and rapidly cooled down to 0 °C in an ice bath for 5 min, and then incubated with 120 μg/mL GO. The final volume was 200 μL. the fluorescence intensity of GO/aptamer mixture was then measured by fluorometry using excitation and emission wavelengths of 488 nm and 521 nm. Subsequently, eight concentrations of hHbA1c, ranging from 0 to 60

μg/mL, were mixed with the GO/aptamer mixture individually at 37 °C for 70 min. The fluorescence intensity of the mixture was determined. Finally, the K_d values of aptamers were calculated from the calibrated curve fitting using the equation $Y = B_{max}X / (K_d + X)$. As shown in Fig. S7, four aptamers exhibited the apparent affinity for hHbA1c.

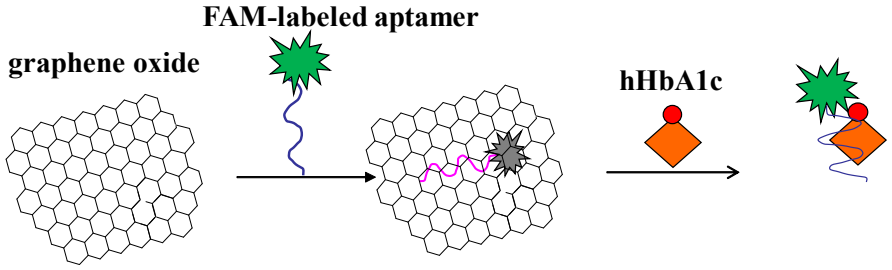


Fig. S6 Schematic of affinity determination of selected aptamers by GO-based fluorescence method.

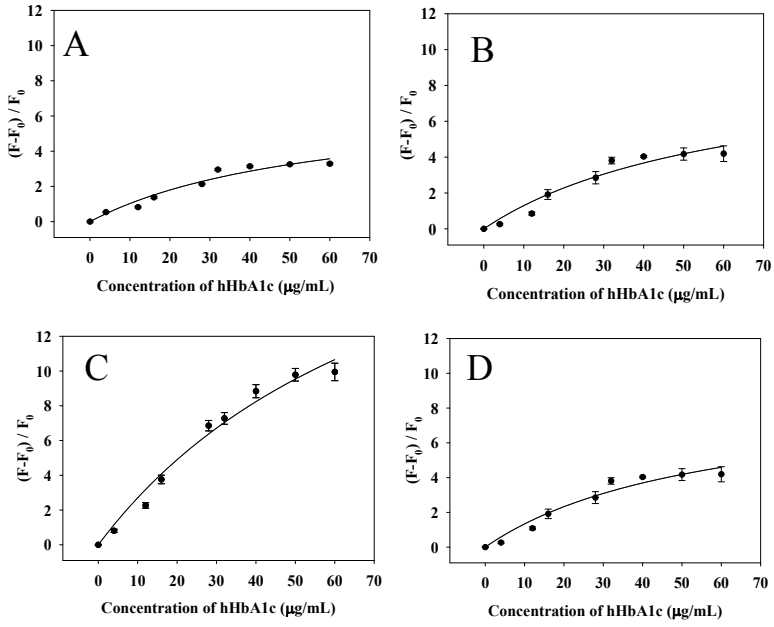


Fig. S7 Identification of binding affinity of selected aptamers. (A) 9th-1, (B) 10th-2, (C) 10th-6, (D) 10th-9.

(4) Specificity determination

In order to identify the specificity of selected aptamers to hHbA1c, six targets (hHb, Myoglobin, HSA, Hemoglobin antibody, Glucose and IgG) were respectively reacted with GO/aptamer complex at the concentration of 20 μg/mL, and the fluorescence intensity change of each target was calculated. The results of GO-based fluorescence method indicated that aptamers had good affinity to hHb, and the results were shown in Fig. S8.

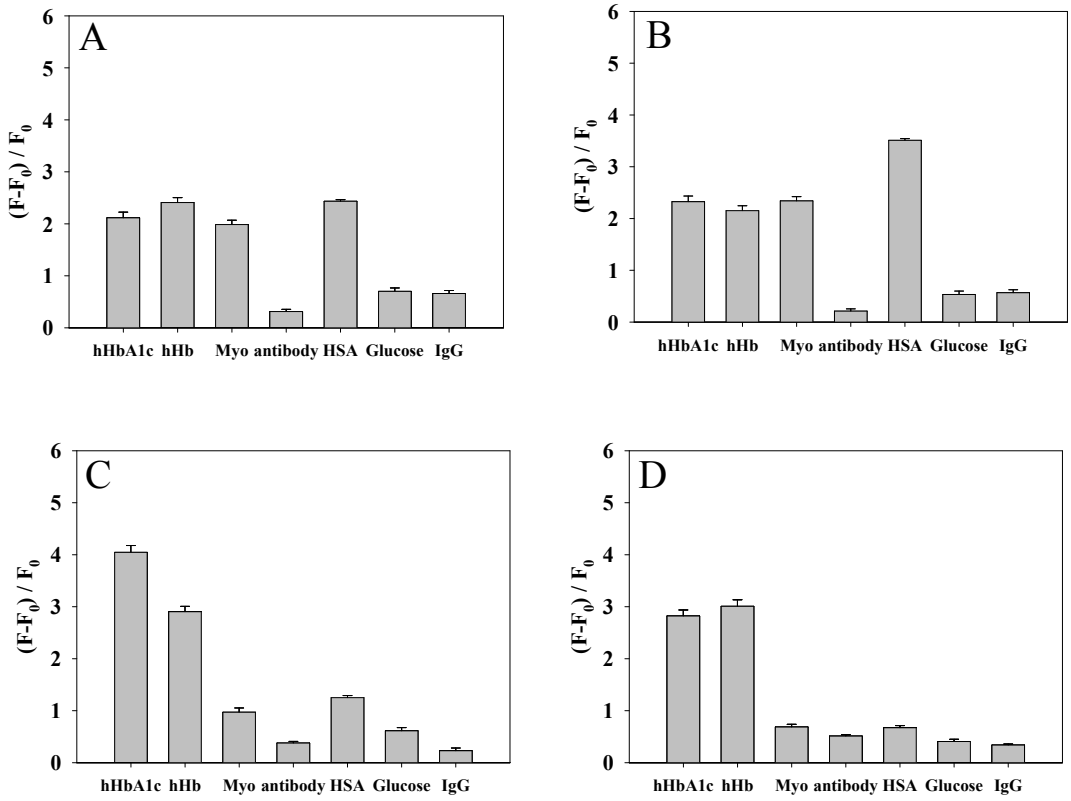


Fig. S8 Identification of specificity of selected aptamers. (A) 9th-1, (B) 10th-2, (C) 10th-6, (D) 10th-9. Protein concentration: 20 μg/mL.

S-7. Stability determination

To estimate the stability of aptamers, we measured their K_d values on different days. The K_d values of aptamers to hHbA1c and hHb were both determined by GO-based fluorescence method as shown in Fig. S5. Firstly, 20 nM FAM-labeled aptamer dissolved in 1× binding buffer, was heated at 95 °C for 5 min and rapidly cooled down to 0 °C in an ice bath for 5 min, and then incubated with 120 μg/mL GO. The final volume was 200 μL. The fluorescence intensity of GO/aptamer mixture was then measured by fluorometry using excitation and emission wavelengths of 488 nm and 521 nm. Subsequently, different concentrations of hHbA1c and hHb, ranging from 0 to 60 μg/mL, were mixed with the GO/aptamer mixture individually at 37 °C for 70 min. The fluorescence intensity of the mixture was determined. The K_d values of aptamers were calculated from the calibrated curve fitting using the equation $Y = B_{max}X / (K_d + X)$. In all, we measured the K_d values over 5 days. The results were shown in Fig. S9.

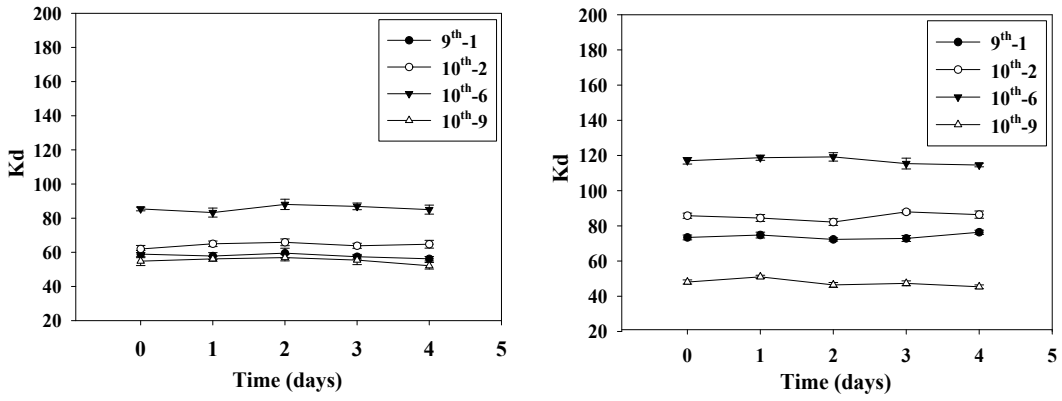


Fig. S9 changes of K_d values of different aptamers on different days. (A) Changes of K_d values of different aptamers to hHbA1c, (B) changes of K_d values of different aptamers to hHb.

S-8. Fluorescence pattern sensing array

In a 96-well plate, 4 μL of aptamer (diluted in 1× binding buffer, 1 μM) was pipetted into each well, and 12 μL of GO (2 mg/mL) was added, then, 2 μL of protein was added to each well, with final concentration of 20 μg/mL. After incubation for 70 min, the fluorescence intensity of FAM-labeled aptamers at 521 nm was recorded. This process was repeated for the seven protein targets. Thus, the seven proteins were tested against the four kinds of aptamers arrays twelve times to give a 4 aptamers × 7 proteins × 12 replicates training data matrix. The raw data matrix was processed using classical linear discriminate analysis (LDA) in SYSTAT (version 11.5). LDA is a well-known statistical method for recognizing the linear combination of features that characterizes or separates two or more classes of objects or events. This method maximizes the ratio of between-class and within-class variances in any particular data set, allowing response patterns to be differentiated. Since HSA and myoglobin might influence the detection of hemoglobin, we carried them in the sensing array, and the raw data were shown in Table S2.

Table S2 Training matrix of $(F-F_0)/F_0$ of aptamers sensing array (9^{th}-1, 10^{th}-2, 10^{th}-6 and 10^{th}-9) against different proteins at 20 μg/mL.

Protein	9^{th}-1	10^{th}-2	10^{th}-6	10^{th}-9
hHb	2.4375	1.9695	2.8594	3.0981
hHb	2.3739	2.3869	2.7353	2.8638
hHb	2.0468	2.1371	2.6873	3.1870
hHb	2.5993	2.0964	2.7224	2.9035
hHb	2.3732	1.9795	2.7634	2.7940
hHb	2.0743	2.3792	3.0548	3.2809
hHb	2.3862	2.1768	3.1737	2.8512
hHb	2.2707	2.0857	2.8432	2.7505
hHb	2.6936	1.9182	2.9461	3.1589

hHb	2.7836	2.4038	3.1744	2.7053
hHb	2.2865	2.2523	2.6573	3.3047
hHb	2.3787	2.0753	3.2632	3.0881
bHb	0.6462	3.4701	0.5989	2.3784
bHb	0.6830	3.0643	0.6351	2.1167
bHb	0.6472	3.0285	0.6073	1.9051
bHb	0.7026	2.9784	0.6684	2.4918
bHb	0.7051	3.1636	0.6732	2.1212
bHb	0.6704	2.9679	0.6835	2.1783
bHb	0.6749	3.5748	0.5936	2.4740
bHb	0.6382	3.6514	0.6913	2.0825
bHb	0.6738	3.2873	0.6337	2.3784
bHb	0.6842	3.1812	0.6522	2.0769
bHb	0.6942	3.0187	0.6273	2.1463
bHb	0.7208	2.9786	0.6418	2.2069
pHb	0.5173	0.6904	0.6153	0.3578
pHb	0.4628	0.6698	0.5164	0.3783
pHb	0.4845	0.7005	0.5049	0.3875
pHb	0.4648	0.6692	0.5802	0.3905
pHb	0.5243	0.7656	0.5575	0.3296
pHb	0.5397	0.6431	0.5206	0.3275
pHb	0.4983	0.6784	0.5623	0.3782
pHb	0.5017	0.7063	0.5118	0.3896
pHb	0.5319	0.6895	0.5749	0.3214
pHb	0.4836	0.6543	0.6031	0.3319
pHb	0.5194	0.7483	0.5168	0.3483
pHb	0.4986	0.6571	0.5248	0.3791
oHb	0.4163	1.7516	0.5806	0.9745
oHb	0.4375	1.6359	0.5421	1.1976
oHb	0.4185	1.7894	0.4693	0.8953
oHb	0.4614	1.8245	0.5529	1.0568
oHb	0.3968	1.8183	0.4685	0.9378
oHb	0.4147	1.7245	0.5732	0.9983
oHb	0.4471	1.8537	0.4986	1.1274
oHb	0.4053	1.7143	0.4984	0.8532
oHb	0.4595	1.8352	0.5247	0.7931
oHb	0.4265	1.7198	0.4882	1.2086
oHb	0.4583	1.8129	0.4771	0.8529
oHb	0.4009	1.6027	0.4853	0.7641
mHb	0.4680	0.7742	0.4332	1.5938

mHb	0.4977	0.7382	0.4392	1.7127
mHb	0.4482	0.7209	0.5027	1.6463
mHb	0.4396	0.8083	0.4086	1.6482
mHb	0.4264	0.7394	0.4109	1.5873
mHb	0.4532	0.6638	0.4084	1.6168
mHb	0.5005	0.7816	0.4693	1.7264
mHb	0.4742	0.8193	0.5296	1.6021
mHb	0.4196	0.6993	0.4274	1.5983
mHb	0.4286	0.7437	0.4097	1.7073
mHb	0.4982	0.8195	0.4753	1.6043
mHb	0.5018	0.7942	0.4641	1.5732
Myo	1.8974	2.6956	1.0964	0.6699
Myo	2.1583	2.7109	0.8452	0.6826
Myo	1.8532	2.3846	0.8531	0.9985
Myo	2.0081	2.5167	1.0016	0.5108
Myo	2.2456	2.2795	1.0238	0.6434
Myo	2.0642	2.1853	0.8304	0.7551
Myo	1.7953	2.6749	0.9502	0.6214
Myo	2.3824	2.5792	0.8274	0.7257
Myo	2.1471	2.2795	1.0892	0.6789
Myo	1.7853	2.7943	0.8665	0.7524
Myo	1.8642	2.1357	1.1074	0.6381
Myo	1.6847	2.4783	1.1515	0.5493
HSA	2.5246	3.7938	1.3262	0.7551
HSA	2.3598	3.7842	1.3096	0.7648
HSA	2.0842	3.6831	1.1704	0.7894
HSA	2.5683	3.5831	1.3877	0.7162
HSA	2.1753	3.8604	1.2975	0.6753
HSA	2.6421	3.5579	1.2479	0.7465
HSA	2.3842	3.4780	1.2764	0.6943
HSA	2.4589	3.3678	1.2215	0.6576
HSA	2.6583	3.9075	1.2796	0.6838
HSA	2.1357	3.7642	1.1368	0.6562
HSA	2.3454	3.4494	1.2136	0.6155
HSA	2.2648	3.4674	1.3591	0.7377

References

S1. *A. Wochner and J. Glokler, BioTechniques, 2007, **42**, 578-582.*