Supporting Information

Nanobody-Based Apolipoprotein E Immunosensor for Point-of-Care Testing

Xiang Ren[†#], Junrong Yan[§#], Dan Wu[†], Qin Wei[†*], Yakun Wan[‡*]

[†] Key Laboratory of Interfacial Reaction & Sensing Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, Shandong, China.

[‡] CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Pudong, Shanghai, 201203, P.R. China.

[§] Institute of Life Sciences, Southeast University, Nanjing 210000, P.R. China.

*Corresponding author:

Tel.: 86-531-82767872; Fax: 86-531-82765969.

Email: sdjndxwq@163.com (Q. Wei);

ykwan@simm.ac.cn (Y. Wan).

# These authors have equally contributed to this work.

EXPERIMENTAL SECTION

Chemicals and materials.

ApoE was purchased from Huibiao Biological Technology Co., Ltd. (China). Freund's adjuvant, HRP, GOD, bovine serum albumin (BSA), goat alkaline phosphatase-conjugated anti-mouse IgG, Bis (p-nitrophenyl) phosphate (BNPP) and Tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (USA). Sucrase was purchased from Shanghai yuanye Bio-Technology Co., Ltd (China). Mouse anti-HA tag antibody was obtained from Covance (USA). Ficoll-paqueTM plus was provided by GE healthcare (USA). Pst I and Not I were obtained from NEB (USA). The Superscript II First-Strand Synthesis System RT-PCR was provided by Invitrogen (USA). P123, sucrose, glucose, TiCl4, Ti(OBu)4, HAuCl4, H2O2, ethanol, sodium citrate, glutaraldehyde (GA), 3-aminopropyltrimethoxysilane (APTMS), 3-aminopropyltriethoxysilane (APTES) were all obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Chitosan was from Lvshen Bioengineering Co., Ltd. (Nantong, China). 1-butyl-3-methyl-imidazolium tetrafluoroborate (BMIM·BF4, >99%) was purchased from Lanzhou institute of chemical physics (China). All other chemicals were of analytical reagents grade and used without further purification. Ultrapure water was used throughout the experiments.

Apparatus.

Ultra-filtration columns were purchased from Millipore (USA). 96-well Maxisorp plate was purchased from Thermo Scientific NUNC (Denmark). Bactrian camel was provided by "Joint Center for Nanobody Research & Development between SEU and Egens Bio". Absorbance determination was carried out with Bio-Rad iMarkTM (Bio-Rad, USA). Surface plasmon resonance imaging (SPRi) binding assay was performed on PlexArray® HT system (Plexera LLC, Beijing). The camera was purchased from Olympus (Japan). X-ray powder diffraction (XRD) patterns were acquired with a Bruker D8 Focus diffractometer (Germany) using Cu Karadiation (40 kV, 40 mA) of wavelength 0.154 nm. Scanning electron microscope (SEM) and Energy Dispersive X-ray Spectroscopy (EDS) images were obtained with a JEOL JSM-6700F microscope (Japan). HRTEM images were obtained from a JEOL JEM-2100F (Japan).

Bactrian camel immunization.

A 1-year-old male bactrian camel was subcutaneously immunized once a week with 1 mg of pure ApoE per injection. The ApoE was conjugated in Freund's incomplete adjuvant the first time and Freund's incomplete adjuvant for the following six times of injections. Three days after the seventh immunization, on day 45, 100 mL of peripheral blood was collected in BD Vacutainer Single-use Containers (BD, UK).

Lymphocyte isolation, RNA extraction and library construction.

Peripheral blood lymphocytes (PBLs) were isolated by density gradient using Ficoll-paqueTM plus according to the manufacturer's instructions. Total RNA was extracted using TRIZOL reagent (Takara, Japan) from about 1×107 PBLs. The Superscript II First-Strand Synthesis System RT-PCR was used to synthesize the cDNA with the extracted RNA as the templates. Afterwards, two-step nested PCR was used to amplify the DNA fragments encoding the VHH. In the first PCR, the VH and VHH regions were amplified by using the synthesized cDNA as the template with primers CALL001 and CALL002.26 The PCR products were analyzed by agarose gel electrophoresis and the bands near 700 bp were re-extracted for the secondary PCR amplification with VHH-Back and PMCF27 as the primers, which contains Pst I and Not I restriction enzymes sites, respectively. Then the PCR products were run on agarose gel and the DNA band around 400 bp was extracted by agarose gel purification. The purified fragments were inserted into the phagemid vector pMECS after the digestion by restriction enzymes Pst I and Not I. Subsequently, the recombinant plasmids were electro-transformed into competent E. coli TG1 cells. The transformants were cultured on lysogeny broth (LB) medium containing 2% glucose and 100 μg mL-1 ampicillin, and the cells were cultured at 37 ºC overnight. The library size was determined by calculating the colonies number after gradient dilutions. Furthermore, 24 colonies were randomly chosen to measure the correct insertion rate by PCR extension with primers VHH-Back and GIII.27 Finally, the colonies were preserved in LB medium supplemented with 1% glucose and 15% glycerol.

Selection of high affinity anti-ApoE colonies.

Bio-panning was performed as our previous study described.[1] About 2×10^{11} phage particles were used for each bio-panning against ApoE coated on the 96-well Maxisorp plate. After three rounds of bio-panning, 95 individual colonies were randomly picked and grown in terrific broth (TB) medium. The 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of Nbs overnight at 28 ºC. The positive colonies expressing anti-ApoE Nbs were identified by periplasmic extraction ELISA (PE-ELISA) according to our previous study.[1] Briefly, 96-well plates were coated with 100 μL of ApoE (2 μg mL^{-1}) and 100 mM $NaHCO_3$ (pH 8.2) as the controls. Cell extracts were

transferred into wells (100 μL well^{-1}) and incubated for 1 h. Then, the mouse anti-HA tag antibody was added to incubate for 1 h followed by incubating the goat anti-mouse IgG-alkaline phosphatase for another 1 h. After being washed with PBS/Tween (PBS with 0.05% Tween-20, pH 7.4) for 5 times, the BNPP was added and finally the plate was read at 405 nm by Bio-Rad imark^{TM}. The identified ApoE-positive colonies were sequenced and the Nbs were classified into different families based on the great diversity of amino acids sequences in complementarity determining region (CDR) 3 regions.

Expression and purification of Nbs.

The plasmids of the identified Nbs families were extracted from TG1 cells and electro-transformed into WK6 cells. Nbs were expressed by the induction of 1 mM IPTG overnight at 28 ºC. The periplasmic extracted proteins were released by osmotic shock using TES buffer (0.5 M sucrose, 0.2 M Tris-HCl pH 8.0 and 0.5 mM EDTA) and 4/TES buffer to incubate overnight at 4 ºC, respectively. ApoE-specific Nbs were produced as C-terminal hemagglutinin (HA)-tag and hexahistidine (His6)-tag proteins. The fusion protein was purified by a His-Select column (Sigma-Aldrich, USA) with a 500 mM imidazole solution as the elution buffer. The purified Nbs were moved to Ultra-filtration columns to remove the imidazole molecules with PBS solution by centrifugation. Finally, these Nbs were diluted to 1 mg mL^{-1} and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The epitope mapping and SPR bingding assay (Supporting Information) were also conducted to evaluate the Nbs.

Epitope mapping.

To etermine the Nbs that recognize the different epitopes of ApoE, we firstly coupled the Nbs with HRP as our previous studies described.[2,3] Anti-ApoE Nbs (100 μL, 10 μg mL^{-1}) were coated on a 96-well plate at 4 ºC overnight. After being washed with PBS/Tween-20 and blocking with 3% bovine serum albumin (BSA), 100 μL of ApoE (2 μg mL^{-1}) was added to the wells and PBS buffer was added into the control wells without ApoE. After 1 h, five different Nbs coupled with HRP (HRP-Nbs) (1 μg mL^{-1}) were added to the wells. After several extensive washes, 100 μL of TMB solution was added and incubated for 5-10 min at RT. The color reaction was stopped by 2 M H_2SO_4 solution, and finally the absorbance was read at 450 nm.

In order to identify the Nbs that recognized the distinct epitopes of ApoE, we performed the paired test by double Nbs sandwich method. Firstly, the five ApoE-specific Nbs were coupled with HRP. In the sandwich assay, Nbs without coupling HRP were used to capture the ApoE, and HRP-Nbs were used as the chromogenic antibodies. If two Nbs recognize the different epitopes, the chromogenic Nb could

successfully bind on another epitope of ApoE, and then the HRP molecules would catalyze the color reaction. However, when they recognized the same epitope, the HRP-Nbs could not be caught. Thus, no signal or a very weak signal would be detected. By performing the paired test, among of the five anti-ApoE Nbs, Nb05 and Nb40 recognized the distinct epitopes of ApoE.

SPR binding assay.

Briefly, the anti-ApoE Nbs were spotted on the NanoCapture 3D-Carboxyl sensor chip surface for 1 h to immobilize. Then the chip was incubated in 2 mL of Ethanolamine-HCl (1 M, pH 8.5) for 20 min to block the residual activated N-Hydroxy succinimide (NHS) groups. Afterwards, the chip was loaded into PlexArray® HT, and PBS/Tween was flooded as a running buffer at a constant flow rate. Different concentrations of ApoE (1, 3, 9, 27 and 81 nM) in PBS/Tween-20 were injected with a flow rate of 2 μL s^{-1} to record the signals. The whole assays were conducted at 25 ºC, and signals were recorded as sensorgrams.

PlexArray® HT and NanoCapture 3D-Carboxyl sensor chip were from Plexera® LLC, Beijing, China. All binding data were finally analyzed using Plexera Data Analysis Module (DAM). Binding curves were fitted with 1:1 langmiur binding model for a binding kinetics of Nbs-ApoE interaction.

Chromogenic reaction

For the chromogenic reaction, the color-developing agent is essential. The APTMS-GA complex can exhibit red color and can be oxidized by H_2O_2. The quantity of the two contents was selected to adjust the best proportion (Figure S1). The APTMS-GA (4:6, v/v) was selected as the color-developing agent. First of all, ITO was aminated by a reported method with some modification. The carved ITO (4 mm × 4 mm) was immersed in anhydrous toluene (50 mL) in a flask and heated to 70 °C. Then APTES (1 mL) was rapidly added dropwise into the solution and the reaction was refluxed for 3 h at a constant temperature of 70 °C. The aminated ITO was dried by flowing N_2. Then, 6 μL of APTMS-GA complex was added on the ITO surface to form the chromogenic layer. To insure the APTMS-GA becomes stable and uniform, the ionic liquid BMIM·BF_4 (IL) was incubated on the surface which is prepared by the addition of IL (10 mg/mL) and chitosan (CS, 0.5%). Then 6 μL Au NPs were added on the former layer to connect the former IL-CS and the latter protein.

Synthesis of materials.

Au/TiO_2 nanocomposites were prepared according to a reported method.[4] Transparent sols were prepared by mixing 1.0 g P123, 3.0 g $Ti(OBu)_4$, 1.7 g $TiCl_4$, and 3.7 mL of $HAuCl_4$ (1%, w/w) with 20.0 mL of ethanol at room temperature and casting the sols in a petri dish to form a thin gel layer. After aging at 40 °C for 24 h and 100 °C for 12 h, the gels were calcined at 350 °C for 4 h in air using a heating rate of 0.5 °C/min. Then the Au/TiO_2 nanocomposites were obtained. Gold nanoparticles (Au NPs) were synthesized by the reduction of sodium citrate. Typically, 1 mL of $HAuCl_4$ (1%, w/w) was added in 100 mL of ultrapure water under magnetic stirring. Then 2.5 mL of sodium citrate was put in the solution when boiled. When the color turned wine red, the solution was heated for 5 min and the Au NPs were synthesized well.

Characterization of Au-TiO_2.

In this immunosensor, Au-TiO_2 was used as a support to amplify the loading quantity of GOD which can catalyze the glucose to generate an obvious signal for the ApoE detection. Figure S4A is the XRD patterns of Au-TiO_2 which illustrates the synthesis was successful. By the Debye–Scherrer analysis, the sharp, strong peaks confirmed the samples well crystallized. The labeled "*" in the XRD patterns indicated the as-synthesized TiO_2 was the anatase structure. The labeled "□" in the patterns were the characteristic diffraction peaks of Au, which illustrates the Au-TiO_2 was successful. The EDS also verify the fact (Figure S5). In Figure S4B, the SEM image was shown with a rough surface and the bright points were the Au NPs attached on TiO_2. The rough surface can provide more active sites to support Au NPs and to connect more biomolecules. To confirm the concrete information of Au-TiO_2, TEM and HRTEM were characterized (Figure S4C, S4D). From the TEM image, the depth alternate contrast indicates the structure was mesoporous which was the better candidate in the immunosensor fabrication. Both nanoparticle and framework are highly crystallized as evidenced from the well resolved Au (111) (0.25 nm) and TiO_2 (101) (0.36 nm) crystalline lattices as shown in Figure S4C and S4D.

Color intensity measurement.

When the sensor is well fabricated, the color intensity can be recorded by a camera. And the photos were quantified by the software Image J. In order to measure the mean gray values of the covered ITO surface, a uniform region of the sensor was employed. The signal for an individual sensor was calculated to evaluate the target influence.

Cost comparison

In the traditional comparable electrochemical immunosensor using electrochemical working station (~$7000), and three-electrode system including working electrode (GCE, ~$50), reference electrode (Ag/AgCl electrode ~$20) or saturated calomel electrode (~$20)), and auxiliary electrode (platinum wire, ~$80) were used.

In this work, the expensive electrochemical working station can be ignored. A cellphone is Ok (~$400), and three-electrode system including working electrode (ITO, ~$1/dm^2), reference electrode (Ag/AgCl electrode ~$20) or saturated calomel electrode (~$20)), and auxiliary electrode (platinum wire, ~$80) were used. The inorganic materials and bio-materials are the same in two method. Thus, the POCT immunosensor is much cheaper (save ~$6500) than the traditional electrochemical immunosensor.

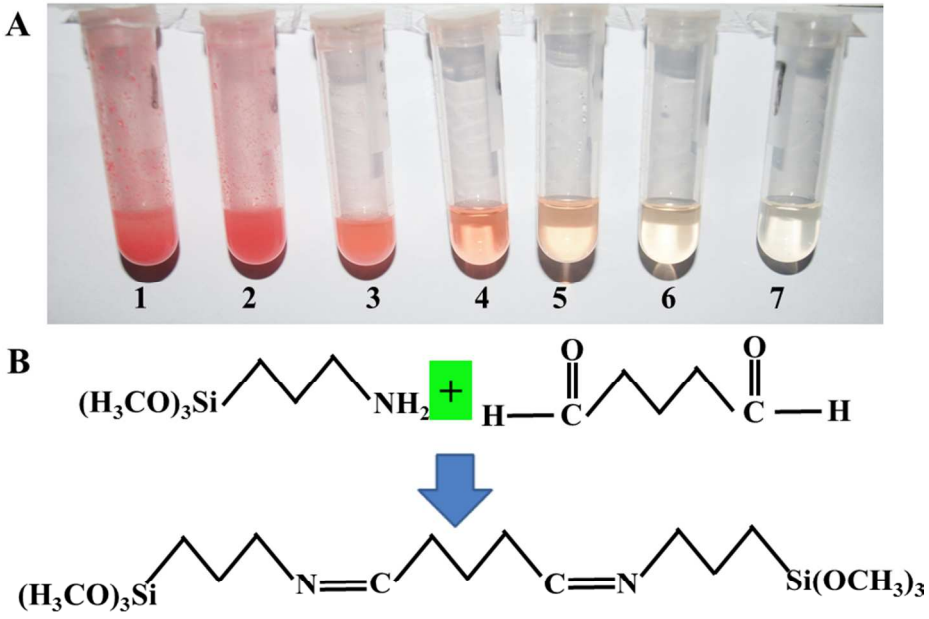

Figure S1. (A) APTMS-GA complex solution with different ratios, 1-7 represent GA solution (0.25%) keep 80%, 70%, 60%, 50%, 40%, 30%, 20% in volume and APTMS ethanol solution (5%) keep the opposite volume respectively. (B) the colorimetric reaction between GA and APTMS.

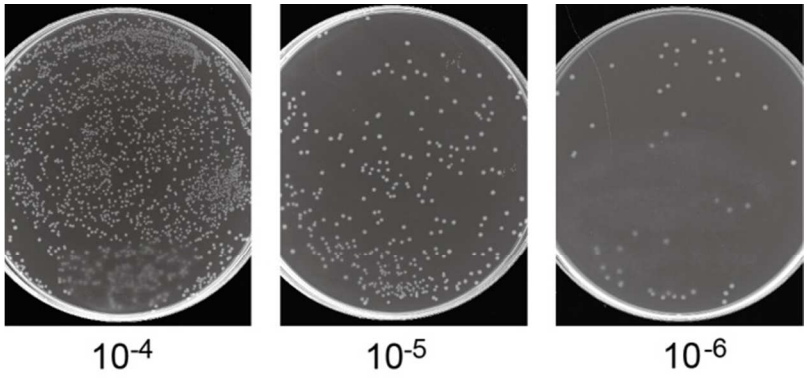

Figure S2. Calculation of the library size. The library size was calculated by counting the colonies number after gradient dilutions.

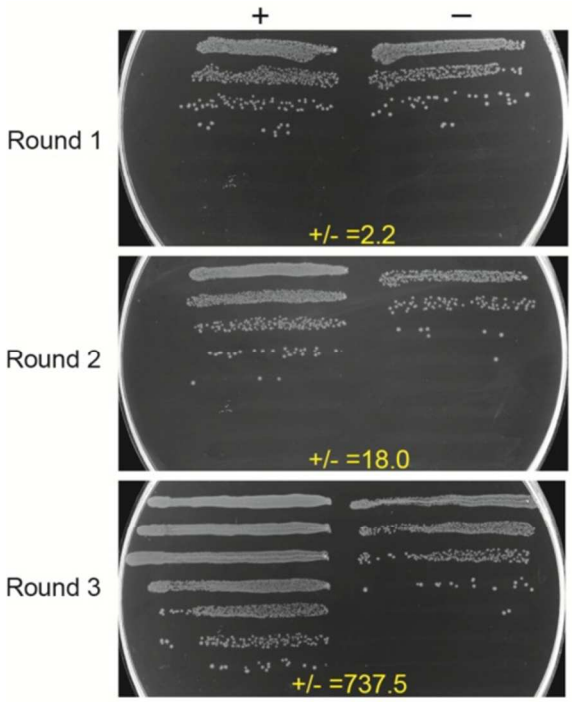

Figure S3. Determination of the relative enrichment in bio-panning. In each round of bio-panning, the relative enrichment of phage particles eluted from the wells coated with ApoE (+) versus those without ApoE (-) was measured. The enriching times increased to 737.5-fold after the third round.

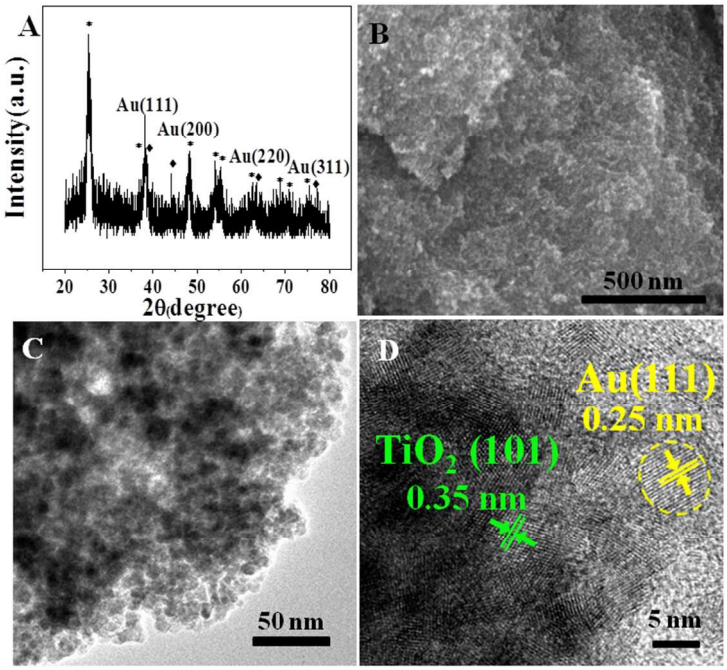

Figure S4. Characterization of the prepared materials. (A) XRD patterns of the Au/TiO_2. (B) SEM of the Au/TiO_2. (C) TEM of the Au/TiO_2. (D) HRTEM of Au/TiO_2.

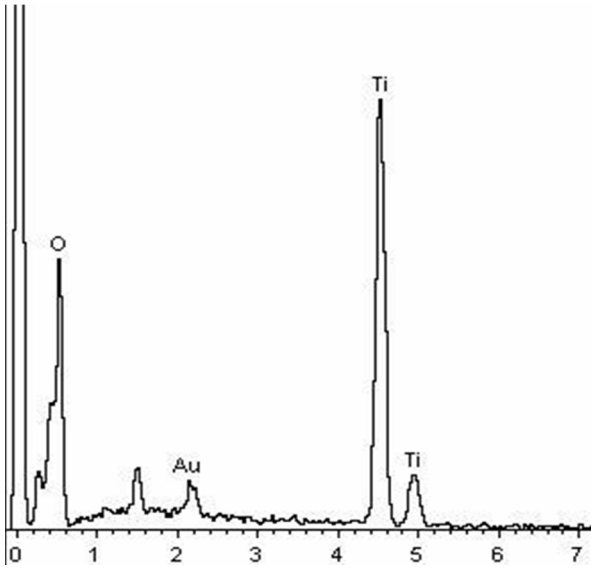

Figure S5. EDS diagram of the as-synthesized Au-TiO_2.

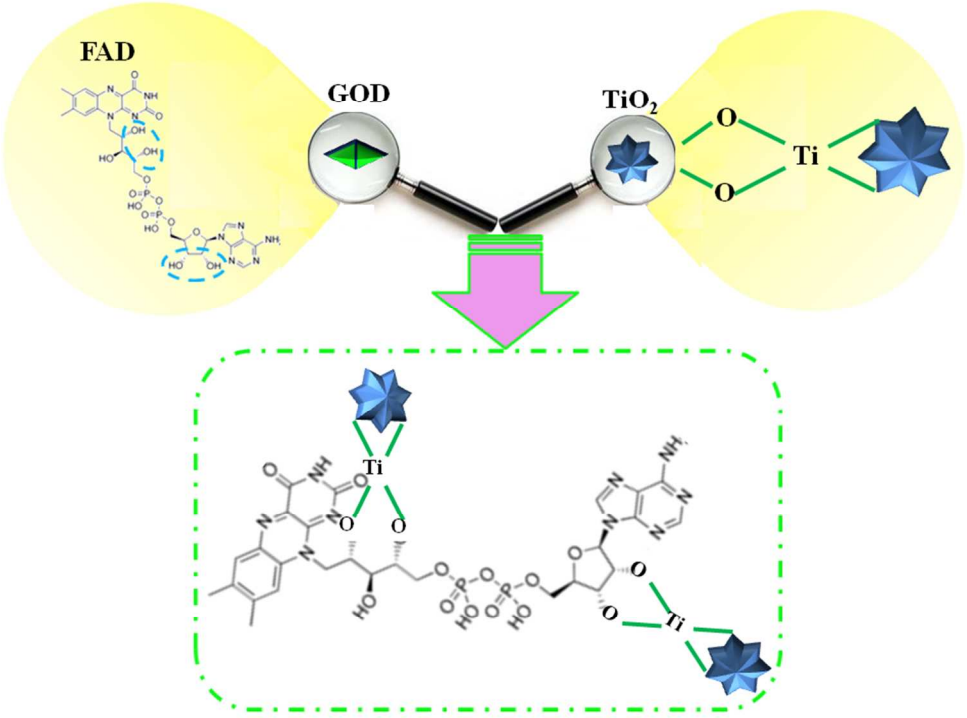

Figure S6. The combination of TiO_2 and FAD from the GOD.

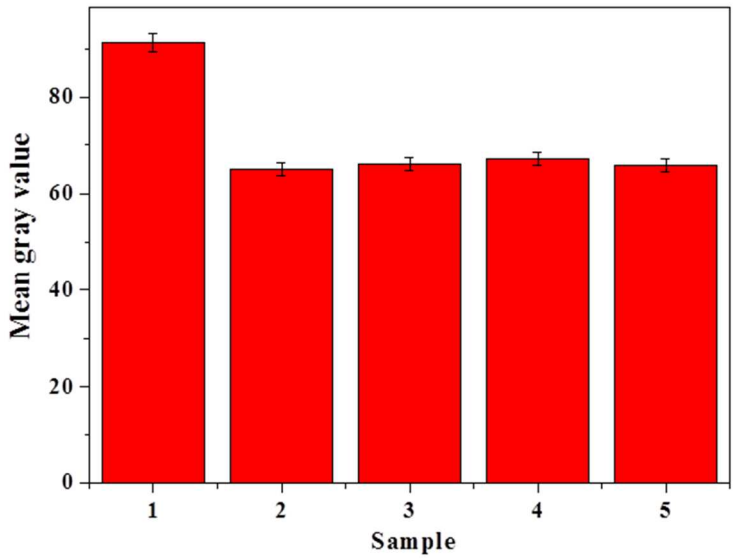

Figure S7. Specificity of the immunosensor (1) 1 ng/mL ApoE, (2) 10 ng/mL BSA, (3) 10 ng/mL AFP, (4) 10 ng/mL CEA, (5) 10 ng/mL PSA.

Table S1. Properties of anti-ApoE single-domain antibodies.

VHH	M_W [a] (kDa)	pI [b]	ε [c] (g/L)	Yield (mg/L)
Nb05	15.94	6.14	1.969	10.0
Nb19	14.45	8.98	1.205	10.5
Nb21	16.56	7.18	1.986	11.0
Nb24	14.45	8.58	1.689	9.0
Nb40	15.74	7.17	1.835	10.5

[a]Molecular weight (Mw) and extinction coefficient (ε) include HA and His_6 tag. [a,b,c]Mw, ε and theoretical isoelectric point (pI) were calculated by the ExPAsy ProtParam Tool.

Table S2. ApoE detection in serum samples by the immunosensor.

Sample No.	Found in diluted serum (ng/mL)	Added ApoE (ng/mL)	RSD (%)	Recovery (%)
1	3.32, 3.21, 3.25, 3.08, 3.10	1.00	1.1	103
2	4.58, 4.45, 4.57, 4.49, 4.39	1.00	1.2	94.0

Table S3. Comparisons of the developed colorimetric immunosensor with ELISA kit for the determination of ApoE in diluted human serum sample.

Sample number[a]	ELISA (ng/mL)	Mean (ng/mL)	s	RSD (%)	This method (ng/mL)	Mean (ng/mL)	s	RSD (%)	Relative errors (%)	F[b] value	t[b] test
1	3.32, 3.21 3.25, 3.08 3.10	3.19	0.10	3.1	3.08, 3.25, 3.07, 3.32, 3.14	3.17	0.11	3.5	-0.63	1.21	0.41
2	4.58, 4.45, 4.57, 4.49, 4.39	4.50	0.081	1.8	4.52, 4.43, 4.61, 4.39, 4.51	4.49	0.086	1.9	-0.22	1.13	0.26

[a] The sample was diluted (1:10000) with PBS before testing;

[b] The *t*- and *F*- values refer to the proposed ELISA kit. The theoretical values at 95% confidence limits: $F = 6.39$, $t = 2.57$.

References:

(1) Zhu, M.; Hu, Y.; Li, G.; Ou, W.; Mao, P.; Xin, S.; Wan, Y. Combining Magnetic Nanoparticle with Biotinylated Nanobodies for Rapid and Sensitive Detection of Influenza H_3N_2. *Nanoscale Res. Lett.* **2014**, *9*, 528–528.

(2) Wang, P.; Li, G.; Yan, J.; Hu, Y.; Zhang, C.; Liu, X.; Wan, Y. Bactrian Camel Nanobody-based Immunoassay for Specific and Sensitive Detection of Cry1Fa Toxin. *Toxicon* **2014**, *92C*, 186–192.

(3) Yan, J.; Li, G.; Hu, Y.; Ou, W.; Wan, Y. Construction of A Synthetic Phage-Displayed Nanobody Library with CDR3 Regions Randomized by Trinucleotide Cassettes for Diagnostic Applications. *J Transl. Med.* **2014**, *12*, 343–343.

(4) Li, H.; Bian, Z.; Zhu, J.; Huo, Y.; Li, H.; Lu, Y. Mesoporous Au/TiO_2 Nanocomposites with Enhanced Photocatalytic Activity. *J. Am. Chem. Soc.* **2007**, *129*, 4538–4539.