Label-Free photoelectrochemical Immunosensor for Neutrophil Gelatinase-Associated Lipocalin based on the Use of Nanobodies

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Bactrian Camel Immunization and Library Construction

A healthy 1-year-old bactrian camel (*Camelus bactrianus*) received six subcutaneous injections at weekly intervals of 1 mg NGAL. For the first injection, NGAL was mixed with an equal volume of Freund's complete adjuvant, and all subsequent boosts were with Freund's incomplete adjuvant. Thirty-eight days after the first injection, peripheral blood lymphocytes (PBLs) were extracted from 100 ml of blood sample using lymphocyte isolation sterile solution for the preparation of library construction. The Nb library was constructed as described in our previous studies. Briefly, total mRNA was extracted from about 10^7 lymphocytes and from which 39 μg mRNA was used to synthesize the cDNA with $oligo_{(dT)}$ primer. Then a two-step nested PCR was used to amplify Nb gene fragments. The first PCR was performed using the synthetic cDNA as the template with the primers CALL001 and CALL002. PCR products were analyzed by agarose gel electrophoresis and the ~ 600 bp fragments was re-extracted to use as the template for the second PCR. The primers VHH-Back and PMCF are used to amplify the VHH repertoire and the final products were extracted by agarose gel purification. The purified PCR fragments were ligated into the phage-display phagemid pMECS after the digestion by restriction enzymes *Pst* I and *Not* I, and subsequently electrotransformed into competent *Escherichia coli* (*E. coli*) TG1 cells. The transformed cells were cultured at 37 ºC overnight on solid 2×TY medium (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl) with 2% glucose and 100 μg mL^{-1} ampicillin. The library capacity was measured by counting the colonies number after gradient dilution and for the meantime, 24 colonies were randomly chosen to determine the correct insertion rate by PCR amplification. All the other colonies were scraped into 2×TY medium supplemented with 1% glucose and 50% glycerol, and finally were stored at -80 ºC as the library stock.

Nbs Selection, Expression and Purification

The anti-NGAL Nbs were selected by bio-panning. The Nbs were expressed on phage after infected with VCSM13 helper phages. Phages expressing NGAL-specific Nbs on their coat proteins were enriched by three consecutive rounds of bio-panning. 95 Individual colonies were randomly selected from the second and third round of panning, and the colonies were grown in terrific broth (TB) medium. The expression of Nbs was induced by adding 1 mM IPTG. Positive clones expressing anti-NGAL Nbs were identified by performing periplasmic extract ELISA

(PE-ELISA). The positive clones identified were sequenced. Amino acids sequence of Nbs genes were compared and classified into different families based on diverse amino acids sequence in CDR3 region.

The plasmids of clones were extracted from TG1 cells and electro-transformed into *E. coli* WK6 cells. The cells were grown in TB medium and when the OD_{600} reached 0.6-0.9, Nbs were expressed by inducing with 1 mM IPTG in shaker flasks overnight at 28 ºC. The periplasmic extracted proteins were released and a Ni-nitrilotriacetic acid (NI-NTA) Superflow Sepharose column with imidazole solution was used to purify the Nbs. The purified Nbs were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), concentrated by Ultra-filtration column and dialyzed in PBS buffer.

Affinity Determination

The affinities of the selected Nbs to NGAL were measured with the kinetic analysis by SPR experiment using PlexArray® HT system. Briefly, 10 μL of Nbs (10 μg mL^{-1}) were immobilized on PlexArray® Nanocapture® Sensor Chips. Four different concentrations of NGAL (9.7 nM, 29.2 nM, 87.6 nM and 263 nM) were injected with a flow rate of 3 μL per minute. These binding sensorgrams signals were recorded and calculated the k_a and k_d values to determine the equilibrium dissociation constant (K_D).

Thermostability Analysis

Thermostability of the Nb, as well as the Ab, was assessed by incubating at 37 ºC for various time (0, 6, 12, 24, 48, 72, 96 h), and temperatures of 25 ºC, 30 ºC, 37 ºC, 45 ºC and 75 ºC, and 95 ºC for 10 min and 60 min, respectively. Wells of high-binding microtiter plates were coated with 10 μg mL^{-1} of NGAL. Blocked with 1% skim milk for 2 h, then 100 μL of Nb or Ab solution (10 μg mL^{-1}) was added into wells to incubate for 1 h. After incubation, the secondary antibody (mouse anti-HA tag antibody/HRP or goat anti-mouse IgG/HRP) was added for 1 h. After 5 times of washing with PBST, chromogenic tetramethylbenzidine substrate solution was added and the absorbance at 450 nm was measured with an ELISA reader.

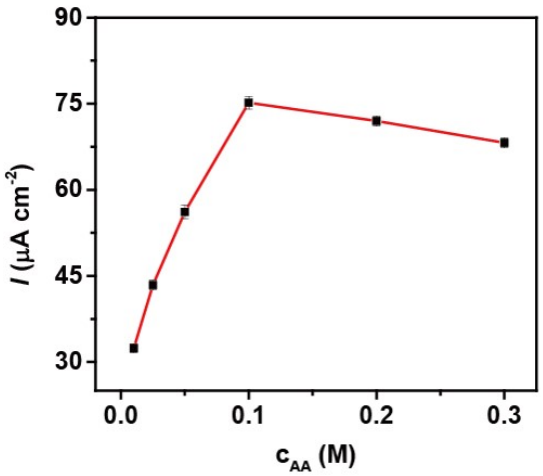

Figure S1. A concentration-dependent photocurrent response of ITO/TiO_2/CoPc electrode in 0.1 M PBS (pH 7.4) at -0.2 V with visible light excitation.

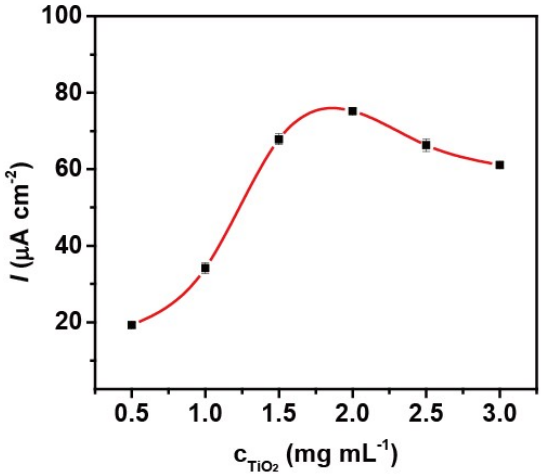

Figure S2. Effects of varied concentrations of TiO_2 suspension on photocurrent responses of ITO/TiO_2/CoPc electrode in 0.1 M PBS (pH 7.4) containing 0.1 M AA at -0.2 V with visible light excitation.

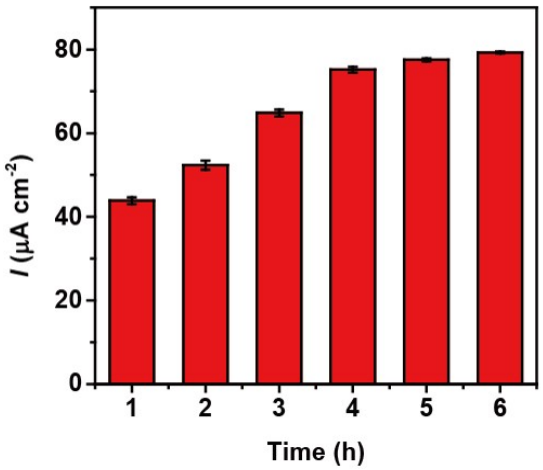

Figure S3. Time effect of the ITO/TiO_2 electrode immersed in a CoPc solution on the photocurrent responses of a TiO_2/CoPc film.

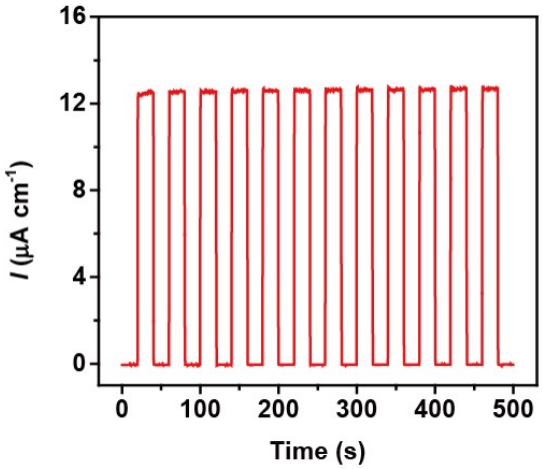

Figure S4. Time-based photoelectrochemical responses of the ITO/TiO_2/CoPc/CS/SA/BSA/BiNb/NGAL (10 ng mL^{-1}) electrode measured in 0.1 M PBS (pH 7.4) containing 0.1 M AA at -0.2 V with visible light excitation.

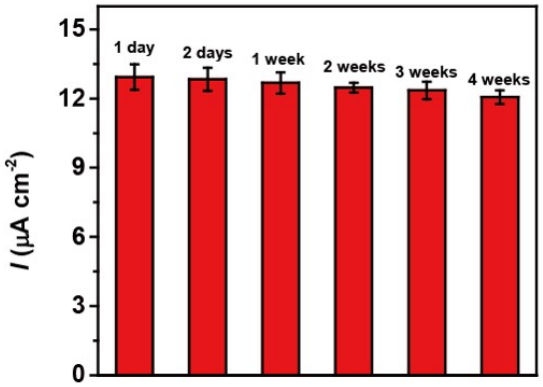

Figure S5. Stability test of the PEC immunosensor for detection of 10 ng mL^{-1} NGAL (n = 3).

Table S1 Photoelectrochemical Detection of NGAL in Serum Samples by the Proposed Immunosensor

	NGAL concentration (ng mL^{-1})			
sample	added	found	recovery	RSD
1	0.010	0.021	110%	5.12%
2	1.0	2.06	106%	3.79%
3	10.0	19.96	99.6%	4.63%
4	50.0	96.6	93.2%	6.12%
5	100.0	111.2	112%	2.89%