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Nanozyme tags enabled chemiluminescence imaging immunoassay for multiplexed cytokine monitoring

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Experimental

Materials and Reagents. Cupric nitrate trihydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3 \text{H}_2\text{O}$) and sodium sulfide nonahydrate ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) were from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). The monoclonal primary and secondary antibodies Ab₁ and Ab₂ for IFN- γ (3.34 and 3.27 mg/mL, respectively) and IL-4 (2.01 and 3.07 mg/mL, respectively), purified recombinant IFN- γ and IL-4 antigens (0.33 and 0.784 mg/mL, respectively) from Escherichia Coli, and chicken serum samples were purchased from Wuhan Gene Create Biological Engineering Co., Ltd. (china). Thermosetting insulating paint AC-3G was bought from JUJO Chemical Co. (Japan). Luminol and p-iodophenol (PIP) were obtained from Acros (Belgium) and Alfa Aesar (China), respectively. N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (N-hydroxy-2,5-pyrrolidinedione, NHS), bovine serum albumin (BSA), chitosan and 3-glycidoxypropyltrimethoxysilane (GPTMS 98%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). A stock luminol solution (0.01 M) was

prepared in 100 mL of 0.1 M NaOH. PIP stock solution (0.01 M) were prepared by dissolving 110 mg PIP in DMSO and then diluted with water to 50 mL. Prior to use, luminol and PIP stock solutions were mixed and diluted using 0.1 M Tris-HCl buffer, pH 8.5. The CL substrate solution contained luminol (5 mM), PIP (0.6 mM) and H₂O₂ (4 mM). Phosphate buffered saline (PBS, 0.01M, pH 7.4) was used to dilute antibodies and antigens of chicken cytokines. Blocking buffer (0.01 M PBS, pH 7.4, containing 1% BSA), was used to block any residual reactive sites on immunosensor array, while washing buffer (PBST, 0.01 M PBS, pH 7.4, containing 0.05% Tween-20) was used for avoiding non-specific adsorption of immunological reagents. Deionized water was employed in all experiments, and all commercial chemicals were of analytical grade for analysis and used as received.

Apparatus. CL imaging signals were recorded by FluorChemE imaging system (Protein Simple Co., America). The kinetic behavior of CL reaction catalyzed by nanozyme tags on the sandwich immunocomplex was studied by the IFFM-D Luminescent Analyzer (Remax Analytical Instrument Co., Ltd., China). Scanning electron micrographs (SEM) were obtained with a Hitachi S-4800 scanning electron microscope (Japan) at an acceleration voltage of 15 KV. Transmission electron micrographs (TEM) were performed by a Philips Tecnai12 electron microscope (The Netherlands) located operating voltage at 120 kV. UV-visible absorption spectrum measurements were carried out on UV-3600 Spectrophotometer (Shimadzu Co., Japan). X-ray diffractometer (XRD) pattern was recorded in D8 Advance X-ray diffractometer (Bruker Co., Germany). The dynamic light scattering (DLS) measurements were obtained by laser light scattering instrument (ALV Co., Germany).

Preparation of CuSNPs-Ab₂ catalytic probes. Carboxyl group activated CuSNPs were synthesized according to the previous literature.^{S1} In brief, thioglycolic acid (15 mL) was added to Cu(NO₃)₂ (2mM, 50mL) solution, and then adjusted to pH 9.0 with NaOH aqueous solution. After bubbling in a nitrogen atmosphere for 30 min, Na₂S (5 mM, 50 mL) was dropwise added into mixture solution, and kept under nitrogen atmosphere within 24 hours to obtain a dark green colloid solution. Ethanol and deionized water were finally used to wash the resultant product three times so as to remove residual ions and surfactant.

The above-prepared copper sulfide suspension (0.5 mg/mL, 1 mL) was centrifuged, and dispersed in a

mixture solution of EDC (20mg/mL, 100 μ L) and NHS (10 mg/mL, 100 μ L) to activate carboxyl groups on the surface of the CuSNPs. After centrifugation to remove the supernatant, Ab₂ (0.1 mg/mL, 2 mL) was added to the previously activated CuSNPs and mixed under gently stirring overnight at 4 °C. Excessive Ab₂ was removed via centrifugation at 4 °C for three times (8000 rpm, 30 min). The resulting CuSNPs-Ab₂ nanozyme probe was finally dispersed in 2.0 mL of 0.01M PBS containing 1.0% BSA, and stored in a refrigerator at 4 °C.

Preparation of the immunosensor array. The cytokines immunosensor array used a 4 rows \times 12 columns format containing 48 total sensing cells (Scheme 1). Four target analytes in a single sample can be simultaneously detected by each column, and 12 samples can be simultaneously detected by the 12 columns. Here, ChIFN- γ and ChIL-4 were used as model cytokines to illustrate the proposed nanozyme-labeled CL imaging multiplex immunoassay.

Firstly, a microscope glass slide was dipped in piranha solution (H₂SO₄/30% H₂O₂, 7:3 v/v concentration) for 12 h to produce abundant hydroxyl groups on its surface, and then rinsed with water and dried in a nitrogen atmosphere. The pretreated glass slide was silanized with 1% GPTMS/toluene overnight at room temperature to form epoxy groups on the surface and subsequently washed with toluene and ethanol so as to remove the physically absorbed GPTMS, followed by drying with nitrogen. Using screen-printing technology, a layer of hydrophobic photoinactive film with 48 cells in a 4 \times 12 format was finally printed on the microscope glass slide (2 mm diameter, 4 mm edge-to edge separation) by a film template with 48 plots. These cells can be utilized to contain the antibody and other required solutions for immunoassay performance. Two capture antibodies (5 μ L, 200 μ g/mL) for IFN- γ and IL-4, respectively, were individually dropped in different silanized cells of the glass slide, and incubated for 30 min at room temperature and then overnight at 4 °C. After the modification with specific capture antibodies, slides were rinsed with washing buffer and 5 μ L blocking solution was added to each sensing site and incubated for 8 h to block the unreacted epoxy group. Finally, the immunosensor array was washed three times with washing buffer and stored in PBS at 4 °C before use.

Multiplex chemiluminescent imaging nanozyme immunoassay. The process of multiplex CINIA

for detection of multiple chicken cytokines is shown in [Scheme 1](#). Different concentration of chicken cytokines (5 μL) were added to the sensing sites on corresponding detection rows. After incubation for 25 min at room temperature, the sensing slide was rinsed with PBST and dried under nitrogen, followed by incubation with 5 μL of the specific CuSNPs-Ab₂ probes for 30 min at room temperature. Finally, 5 μL of CL substrate was introduced into the sensing cells to trigger the CL reaction and resulted CL signals from each sensing well were simultaneously collected by CCD with dynamic integration of 15 min. Spots were automatically identified by self-contained data acquisition software (Alpha View SA) and CL signal of each spot was calculated as the mean pixel intensity with a circle of a given diameter around center.

Characterization of cytokines immunosensor array

Scanning electron microscope was used to characterize the primary processing steps of immunosensor array fabrication for the multiplex CL immunoassay. As it can be seen from SEM of the glass slide treated with piranha solution ([Fig. S1a](#)), an initial smooth and homogeneous surface was observed. After the treatment with GPTMS, the surface of the glass slide shows a homogeneous epoxy silane layer which can be further functionalized ([Fig. S1b](#)). After the immobilization of Ab₁ on epoxy group-activated glass slide, the SEM image ([Fig. S1c](#)) display obvious aggregation of the loaded protein biomolecules, indicating that primary capture antibody involved in the sandwich assay was successfully modified on the sensing sites.

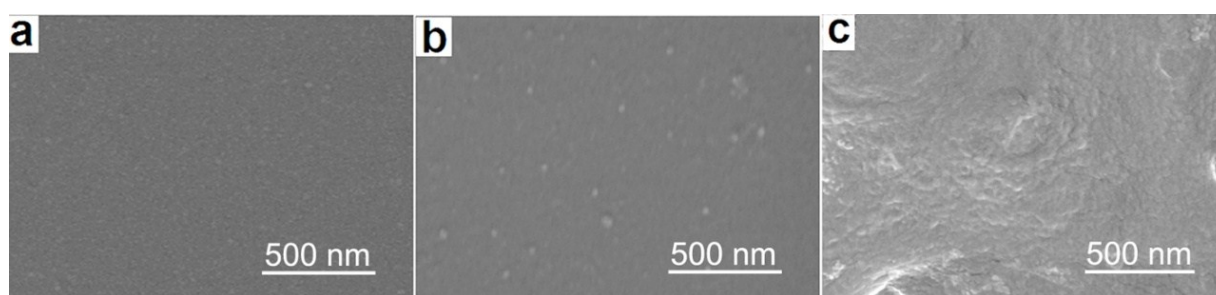


Fig. S1 SEM images of piranha solution-treated (a), GPTMS silylanized (b) and antibodies-immobilized sensing array (c).

Dynamic light scattering (DLS) analysis of CuSNPs

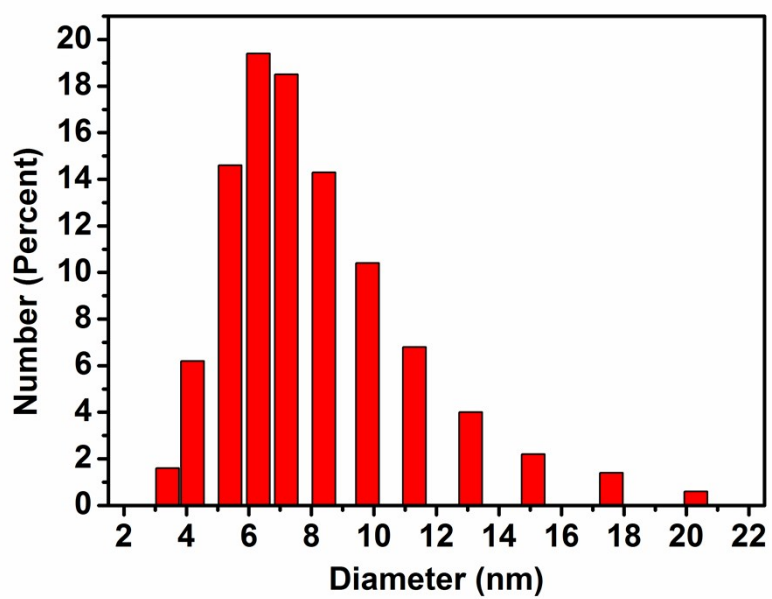


Fig. S2 The dynamic light scattering (DLS) analysis of CuSNPs

Exposure time for CL imaging signal collection

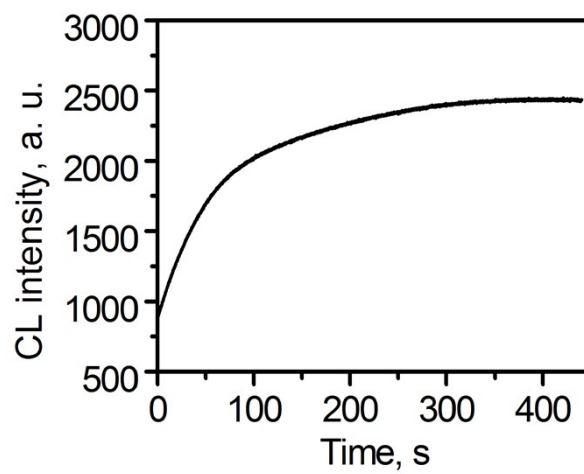


Fig. S3 The kinetic curve of CL reaction catalyzed by CuSNPs-labeled to sandwich immunocomplexes at 30 ng/mL concentration of ChIL-4.

Incubation time of CL imaging nanozyme immunoassay

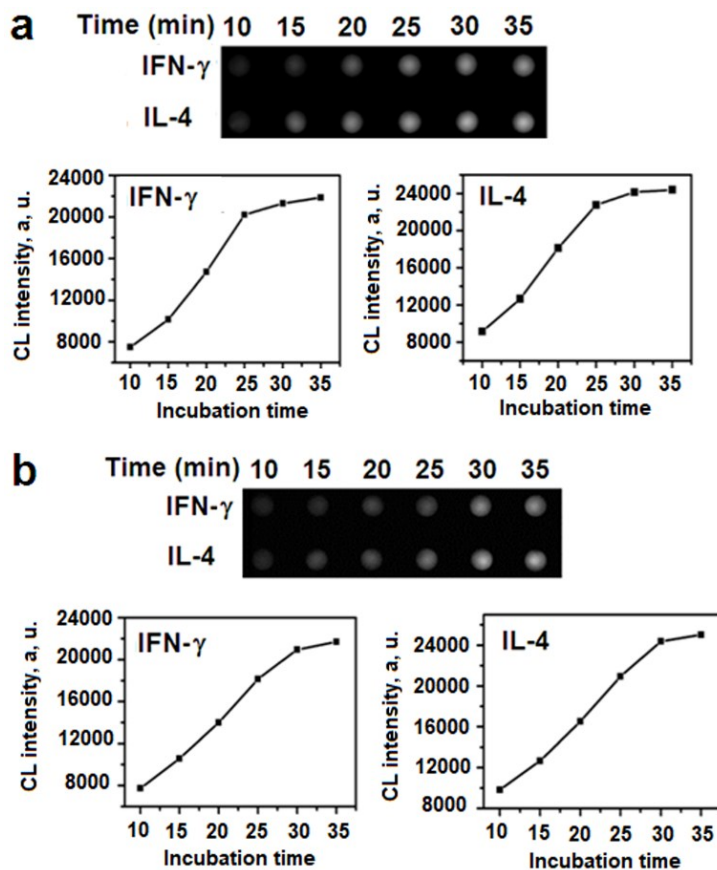


Fig. S4 (a) Effects of incubation time for the reaction between Ab₁ and chicken cytokines on CL intensity for 30 ng/mL IFN- γ and IL-4, respectively, (b) effects of incubation time for the reaction between Ab₁-cytokine-immunocomplex and CuSNPs-Ab₂ probe on CL intensity for 30 ng/mL IFN- γ and IL-4, respectively.

Table S1 Comparison between the CINIA method and current single-component assay methods for chicken cytokines

Methods	Cytokines	Detection mode	Incubation time	Linear range (ng/mL)	Detection limit (pg/mL)	Reference
CINIA	IFN- γ IL-4	Multi-component	55 min	0.01-60 0.01-60	2.9 3.2	This work
Bioassay	–	Single-component	48-72 h	–	–	[21a]
ELISA	IFN- γ	Single-component	2 h	10–100	500	[21b]
ECII	IL-4	Single-component	45 min	0.1–1000	100	[23]
CLIA	IFN- γ	Single-component	40 min	0.001–0.1	0.87	[24]
CLIA	IL-4	Single-component	30 min	0.05–70	20	[22]

CINIA: chemiluminescent imaging nanozyme immunoassay

Bioassay: in vitro virus neutralization assay

ELISA: enzyme-linked immunosorbent assay

CLIA: chemiluminescent immunoassay

ECII: Electrochemical impedance immunoassay

References

S1 Y. D. Zhu, J. Peng, L. P. Jaing and J. J. Zhu, *Analyst* 2014, **139**, 649.