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Multiplex immunoassay of chicken cytokines via highly-sensitive chemiluminescent imaging array

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Abstract

Quantitative detection of multiple chicken cytokines is a good evaluation of cell-mediated immunity in chickens after disease infection or vaccination. However, current assay methods for chicken cytokines cannot meet the needs of clinical diagnosis due to unsatisfactory sensitivity and low assay throughput. Herein, a sensitive chemiluminescence (CL) imaging immunosensor array has been developed for high-throughput detection of multiple chicken cytokines. The chicken cytokines immunosensor array was prepared by assembling different cytokine capture antibodies onto a disposable silanized glass chip, where horseradish peroxidase and antibody-conjugated gold nanoparticles were used as multienzymatic amplification probe for CL imaging signal amplification. By using a sandwich assay mode, the amplified CL signals from each sensing array cell were collected for quantitation. Using chicken interleukin-4 and chicken interferon-γ as model cytokines, this novel multiplexed and amplified method demonstrated simultaneous measurement of the two chicken cytokines in the linear ranges of 0.008–0.12 ng/mL and 0.005–0.20 ng/mL, respectively, which yields limits of detection down to 2 pg/mL and 3 pg/mL. The CL imaging array method reported here also demonstrated high specificity, good repeatability, and high stability and accuracy, providing a novel multiplex immunoassay strategy for highly sensitive and high-throughput detection of chicken cytokines and further disease diagnosis in poultry.

Graphical Abstract

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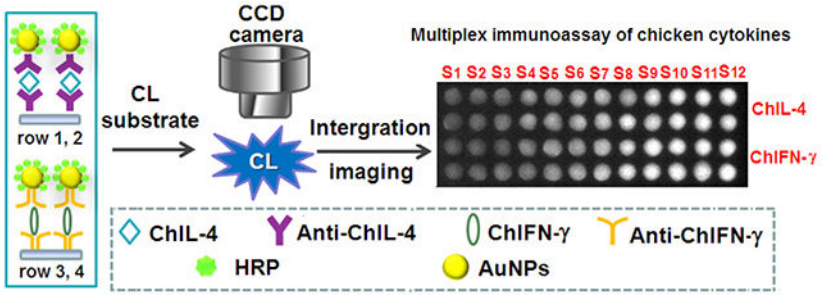
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2018.10.050.



Keywords

Cytokines; Sensor array; Multiplex immunoassay; Chemiluminescence imaging; Nanoparticle-signal amplification

1. Introduction

Cytokines are a broad range of biologically active small molecule proteins secreted by a variety of different cells, which can be classified as interferon, interleukin, tumor necrosis factor, growth factor and so on [1,2]. Cytokines play pivotal roles in diverse physiological functions such as inflammation, cell differentiation, promoting chemotaxis, and generating an acute phase response [3]. In chickens, the level of cytokines, specifically interleukin-4 (ChIL-4) and chicken interferon-γ (ChIFN-γ), have been proven to be an excellent indicator for evaluating cell-mediated immunity (CMI) in vaccinated chickens or chickens infected by common virus, bacterial and parasitic diseases [4,5]. Redundancy, interdependence, and antagonism within the in vivo cytokine network of an organism often confound the interpretation of data from measuring and monitoring a single cytokine [3]. Therefore, a reliable approach for evaluating in the current levels of immune response in vaccinated or infected chickens, and so monitor for disease outbreaks, would be to simultaneously determine multiple cytokines in body fluid of chickens.

Various methods have been developed for quantitative detection of chicken cytokine including biological assays [6], enzyme-linked immunosorbent assays (ELISA) [7], electrochemical impedance immunoassays [8] and chemiluminescent (CL) immunoassays [9,10]. These standard assay methods have been confined to single component methodologies and time-consuming operation, however, so only one species of chicken cytokine can be determined per assay run. Due to the limited specificity and usefulness of monitoring a single chicken cytokine in evaluating CMI in chicken, development of multiplexed methodologies to determine a panel of cytokines in complex samples is urgently needed for improving the accuracy and effectiveness of clinical diagnoses. Additionally, cytokines are small molecular proteins, and exist in body fluid in extremely low concentrations. As a result, the current assay methods can lack the sensitivity necessary to detect the low-abundance chicken cytokines in clinical samples in a reliable manner [11].

In recent years, multiplex immunoassays have attracted considerable interest in various fields due to the growing demand and straightforward advantages of high sample

throughput, short assay time, low sample consumption and cost [12-17]. Particularly, great efforts have been made to develop spatially-resolved sensing arrays to simultaneously detect different targets on one substrate [18,19]. Among them, CL-based arrays coupled with a charge-coupled device (CCD) detector have garnered increasing attention in detection of multiple biomarkers and pathogenic bacteria [20-22]. Additionally, nanoparticle (NP)-based signal amplification strategies have been exploited for the development of sensitive immunoassay methods [23-29]. Among various NPs for signal amplification, gold NPs (AuNPs) have become attractive carriers for fabrication of amplification probes via the bioconjugation of large numbers of enzyme molecules and secondary antibodies to the NP surface [30-33].

In this work, a high-performance multiplex immunoassay method was firstly proposed for simultaneous detection of chicken cytokines by combination of CL array sensor with a cooled low-light CCD coupled to a AuNP-based signal amplification strategy (illustrated in Scheme 1). Two probes were prepared by immobilization of horseradish peroxidase (HRP) and secondary antibody onto AuNPs for highly sensitive detection of ChIL-4 and ChIFN-γ. The chicken cytokines immunosensing array was fabricated by immobilizing different capture antibodies on a disposable silanized glass chip with 48 sensing cells (4 row × 12 column), which can be used to simultaneously detect two cytokines in 24 samples. The multiplexed array should solve the problem of poor data quality arising from monitoring only a single cytokine, and the increased signal from the immobilized nanoparticles should improve the sensitivity issues seen in standard methodologies. This work provides a promising strategy for high-throughput and sensitive detection of multiple chicken cytokines and further disease diagnosis in poultry.

2. Materials and methods

2.1. Chemicals and materials

The monoclonal capture antibodies Ab_1 and Ab_2 of ChIL-4 (2.01 and 3.07 mg/mL concentrations) and ChIFN-γ (3.34 and 3.27 mg/mL concentrations), and purified recombinant ChIL-4 and ChIFN-γ antigens from Escherichia Coli (0.784 and 0.33 mg/mL concentrations) were purchased from Wuhan GeneCreate Biological Engineering Co., Ltd. (china). Thermosetting insulating paint AC-3G was from JUJO Chemical Co. (Japan). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$) and trisodium citrate were bought from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Luminol and p-iodophenol (PIP) were obtain from Acros (Belgium) and Alfa Aesar (China), respectively. Bovine serum albumin, chitosan and 3-glycidoxypropyltrime thoxysilane (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 dimethylsulphoxide and then diluted with water to 50 mL. Prior to use, luminol and PIP stack solutions were mixed and diluted using 0.1 M pH 8.5 Tris-HCL buffer. The CL substrate solution contained luminol (5 mM), PIP (0.6 mM) and H_2O_2 (4 mM). Phosphate buffered saline (PBS, 0.01 M, pH 7.4) is used to dilute antibodies and antigens of chicken cytokines. Blocking buffer is 0.01 M PBS (pH 7.4) containing 1% BSA, which is used to block any residual reactive sites on sensors array. Washing buffer, which

is used to avoid nonspecific adsorption of immunological reagents, was 0.01 M PBS (pH 7.4) spiked with 0.05% Tween-20. Deionized water was employed in all experiments, and all commercial chemicals were analytical grade and used as received.

2.2. Instruments

Chemiluminescent signals in all measurements were measured with a cooled low-light CCD (Protein Simple Co., America). The IFFM-D Luminescent Analyzer (Remax Analytical Instrument Co., Ltd., China) was used to study the kinetic behavior of CL reaction catalyzed by HPR label on the sandwich immunocomplex. UV–visible spectra were recorded on a UV-3600 spectrophotometer (Shimadzu. Japan). Scanning electron micrographs (SEM) were obtained with a Hitachi S-4800 scanning electron microscope (Japan) at an acceleration voltage of 15 KV.

2.3. Preparation of chicken cytokines immunosensor array

The immunosensor array used a 4 row × 12 column format containing 48 total sensing cells. Four chicken cytokines in a single sample can be simultaneously detected by each column, and 12 samples can be simultaneously detected by the 12 columns. Here, two chicken cytokines, ChIL-4 and ChIFN-γ, were used as model cytokines to illustrate the proposed sensor array-based CL imaging multiplex immunoassay.

Firstly, a microscope glass slide was dipped in piranha solution (H_2SO_4/30% H_2O_2 7:3 in volume) 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. Then the activated glass slide was washed with toluene and alcohol 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 × 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 keep the antibody and other solution for immunoassay. Two capture antibodies (5 μL, 100 μg/mL) for ChIL-4 and ChIFN-γ were individually dropped in different silanized sites of the glass slide, and incubated overnight at 4 °C. After the slides rinsed with washing buffer, 5 μL blocking solution was added to each sensing site for 8 h to block the unreacted epoxy group. Finally, the immunosensor array was washing three times with washing buffer and stored in PBS at 4 °C before use. All washing steps were manually done using a washing bottle.

2.4. Fabrication of Ab_2-AuNP-HRP probe

AuNPs were synthesized according to the classical citrate reduction method [34]. In brief, 100 mL of aqueous $HAuCL_4$ solution (0.01%, w/w) was boiled under vigorous stirring, and then 2.5 mL of trisodium citrate solution (1%) was quickly added dropwise to the boiling solution under continuous stirring until the color from the yellow to dark red. The resultant solution was cooled down to room temperature under constant stirring. Ab_2-AuNP-HRP probe was prepared according to previous literature with some minor revision [35]. Firstly, 10 μL of 100 μg/mL Ab_2 and 30 μL of 4.0 mg/mL HRP were added into 1.0 mL of AuNPs solution (pH 9.0). After being shaken gently for 2 h, the solution was centrifuged

at 10000 rpm for 30 min at 4 °C so as to remove the excess Ab_2 and HPR. The obtained Ab_2-AuNP-HRP probe was further washed with PBS three times and re-suspended in 1.0 mL of 0.01 M PBS containing 1% BSA and stored at 4 °C for subsequent use.

2.5. Multiplex CL immunoassay

The CL multiplex imaging immunoassay for simultaneous detection of multiple chicken cytokines is depicted in Scheme 1. To obtain the calibration curves of ChIL-4 and ChIFN-γ, different concentration of chicken cytokines (5 μL) were added to the sensing sites on corresponding detection rows. After incubation for 30 min, the sensing slide was rinsed with PBST and dried in nitrogen. 5 μL of Ab_2-AuNP-HRP probes were added into corresponding sensing sites, and incubated for 25 min. Next, 5 μL CL substrate was introduced into the sensing cells to trigger the CL reaction. The 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). The CL intensity of each spot was calculated as the mean pixel intensity with a circle of a given diameter around center.

3. Results and discussion

3.1. Characterization of cytokines immunosensor array and Ab_2-AuNP-HRP probe

Scanning electron microscope was used to characterize the primary processing steps of immunosensor array fabrication for the multiplex CL immunoassay. As seen from SEM of the glass slide treated with piranha solution (Fig. S1a, Supporting information), an initial smooth and homogeneous surface was observed. After treatment of the glass slide with GPTMS, the surface shows a homogeneous epoxy silane layer with which to do further functionalization (Fig. S1b, Supporting information). After Ab_1, the primary antibody of the sandwich assay, was incubated for immobilization the on epoxy group-activitated glass slide, the SEM image (Fig. S1c, Supporting information) displayed obvious aggregation of the loaded protein biomolecules with a regular distribution, indicating Ab_1 was successfully modified on the sensing sites.

UV–vis absorption spectroscopy was used to characterize the nanoparticle modifications of the Ab_2-AuNP-HRP probe; namely, the addition of secondary antibody Ab_2 and enzyme HRP. As shown in Fig. 1a, the absorption spectrum of single AuNPs shows the characteristic absorption peak at 527 nm. Compared with the unmodified AuNPs (curve i in Fig. 1a), the Ab_2-AuNP-HRP probe shows a strong absorption peak at 280 nm, which can be ascribed to typical protein absorption (curve ii in Fig. 1a), indicating the existence and successful incorporation of protein molecules on the surface of the AuNPs. Additionally, a wider absorption peak with a slight red shift was observed from the UV–vis absorption spectrum of Ab_2-AuNP-HRP probe. This is likely due to some marginal aggregation of AuNPs occurred after the modification of Ab_2 and HRP onto these nanoparticles.

3.2. Effect of chemiluminescent signal amplification by Ab_2-AuNP-HRP probe

In order to test the magnitude of the signal amplification enhancement due the Au nanoparticles in the Ab_2-AuNP-HRP probe, the proposed CL cytokine immunosensor array

was used to examine the performance of Ab_2-AuNP-HRP and Ab_2-HRP probes with a sandwich immunoassay under the same testing conditions. As seen from Fig. 1b, the Ab_2-AuNP-HRP probe shows an obvious CL signal amplification, as the CL signal intensity is 6.5-fold larger than that of Ab_2-HRP probe. This signal amplification phenomenon is due to the increased surface area of the Au nanoparticles for the attachment point for the HRP, rather than the secondary antibody itself. This allows many more HRP to participate in the sandwich and significantly increases the ratio of HRP to Ab_2, This, in turn, results in a vastly increased chemiluminescent signal response per immobilized cytokine, which greatly improves the sensitivity of the multiplex cytokine immunoassay.

3.3. Choice of exposure time of CCD for signal collection and incubation times

A static experiment of the CL reaction catalyzed by Ab_2-AuNP-HRP labeled to sandwich immunocomplexes was used to generate a kinetic curve of CL intensity over time (Fig. S2, Supporting information). This information was used to examine and determine the optimal exposure time for CL signal collection. Upon the addition of CL substrate to sensing cells, the CL reaction quickly reached its maximum intensity within 2 min, and then slowly decreased to 80% of the maximum value after 15 min. To maximize the sensitivity of multiplex detection of cytokines, the CCD imaging needs a long exposure time to collect the relatively weak CL signals from each sensing cell. Therefore, an exposure time of 15 min was used for dynamic integration to collect the CL imaging signals.

Appropriate incubation time for each step is an important factor in affecting the performance of CL imaging immunoassay. To improve the operation of multiplex assay, various incubation times were investigated at room temperature by use of 0.05 ng/mL ChIL-4 and ChIFN-γ. For the first incubation, the surface capture of the cytokines by their respective primary antibodies anti-ChIL-4 and anti-ChIFN-γ, the subsequent CL intensity value increased with increasing incubation time up to a maximum at 30 min (Fig. 2a). For the second step, formation of the sandwich via the attachment of the Ab_2-AuNP-HRP probe to the exposed Ab_1-cytokine-immuno-complex, the maximum CL value was similarly reached with only a 25 min incubation (Fig. 2b). Thus, 30 and 25 min were chosen as the two incubation times in order to form the fully saturated sandwich complex in the multiplex immunoassay of chicken cytokines.

3.4. Performance of the multiplex cytokine immunoassay

Under the now optimized conditions, the calibration curves of the proposed multiplex cytokines CL imaging immunoassay were obtained and shown in Fig. 3. After the two-step incubation and addition of chemiluminescent substrate, the array sensing sites showed an increased light intensity (Fig. 3a), indicating the extent of the CL reaction. The brightness of each spot is positively correlated to the concentration of chicken cytokines. The obtained CL intensities linearly increased with increasing concentrations of chicken cytokines in the range of 0.008–0.12 ng/mL for ChIL-4 and 0.005–0.20 ng/mL for ChIFN-γ (Fig. 3b). The detection limits for the two cytokines were calculated to be 0.002 for ChIL-4 and 0.003 ng/mL for ChIFN-γ at a signal-to-noise ratio of 3. This indicates that using the increased surface area of the Au nanoparticles for extra HRP binding was an effective means of amplifying the CL signal. The increased sensitivity also means that immune response

in the chickens can be detected earlier and more reliably, improving the effectiveness of measures to prevent spreading of disease. The quantitative comparison between the proposed CL imaging multiplex immunoassay and the single-component assay methods reported previously for chicken cytokines was shown in Table S1 (Supporting information). This multiplex CL cytokine immunoassay possesses significant advantages in sensitivity, sample throughput and assay speed, which is of great importance in development of immunoassay method and clinical testing.

The whole assay process on one sensing array, including two-step incubation (55 min), exposure for CCD-based signal collection (15 min), and washing, can be completed within 70 min. In other words, this setup allows for 48 samples to be simultaneously measured in 70 min, so the long-term throughput capacity of the proposed immunoassay method is 41 samples per hour for single cytokine detection. Additionally, this array could easily be expanded to however large a sample size is required by the user.

3.5. Detection of multiple cytokines in serum samples

In order to investigate the direct practical applicability of this multiplex immunoassay method to clinical settings, recovery experiments were carried out by spiking standard cytokine antigens to clinical chicken serum samples. As shown in Table 1, the recovery values for ChIL-4 and ChIFN-γ by multiplex immunoassay method were 96.3%–108.0% and 94.00%–106.3%, respectively. These recoveries represent an acceptable accuracy in the quantitative analysis of multiple cytokines in real samples.

3.6. Specificity, reproducibility and stability of the multiplex CL immunoassay

Any multiplexed analysis requires that there be no cross-reactivity between the analytes and their probes, as this could quickly complicate the data. In order to determine any cross-reactivity between the cytokines and non-specific antibodies, various known concentrations of ChIFN-γ antigen were added to a constant concentration of ChIL-4 antigen, and ChIL-4 antigen was added to a definite concentration of ChIFN-γ incubation solution. The overall changes of CL intensity with the increasing competing cytokine concentration (0.02–0.1 ng/mL) in different solution were shown in Fig. 4a. The maximum variation in chemiluminescent signal for ChIL-4 and ChIFN-γ on the sensing array were less than 3.7% and 1.7%, respectively, suggesting that the cross-reaction of cytokines with non-specific antibodies is negligible. The proposed immunosensor array has good selectivity for recognizing the intended target cytokine from the cytokine mixture, a crucial feature in effective multiplexed assays.

In addition, the reproducibility of the CL immunosensor array was investigated by the intra-and inter-assay coefficients of variation (CVs) with five measurements at a standard concentration of 0.05 ng/mL for ChIL-4 and ChIFN-γ. The obtained values of intra- and inter-assay CVs on the immunosensor array were 2.8% and 6.3% for ChIL-4 and 3.7% and 5.9% for ChIFN-γ, respectively. These results indicate an acceptable detection and fabrication reproducibility. When the immunosensor array chip was not in use, it was stored in PBS at 4 °C. No obvious decreases in CL signals were observed after the storage

for at least four weeks (Fig. 4b), indicating the acceptable storage life of the developed immunosensor array chip.

4. Conclusions

In this work, we reported the first multiplex immunoassay method for simultaneous detection of multiple chicken cytokines. This novel multiplex assay strategy was performed based on a chicken cytokine immunosensor array and CL imagining system. The chicken cytokine immunosensor array can be easily fabricated and was characterized with various means. The use of AuNPs-based multi-enzyme amplification probe in the multiplex assay resulted in much lower detection limit than other standard methodologies. Compared with traditional single-component chicken cytokine assay methods with a single enzyme tag, the proposed multiplex assay methods show significant advantages such as higher sensitivity, higher throughput, lower cost, less consumption, and more rapid assay speed, and easier operation. Furthermore, the proposed multiplex imaging immunoassay method has acceptable reproducibility and stability, and can be used to measure cytokines in practical chicken samples with satisfactory recoveries. This methodology should meet the standards needed to both improve clinical treatment timing and effectiveness and control disease outbreaks in chicken farms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Multiplex immunosensor array for chicken cytokines was proposed for the first time.
- Multiple chicken cytokines were simultaneously detected by sensitive CL imaging system.
- The multiplex cytokines immunoassay has much better performance compared with current methods.
- This work provides a novel strategy for sensitive and high-throughput multiplex immunoassay.

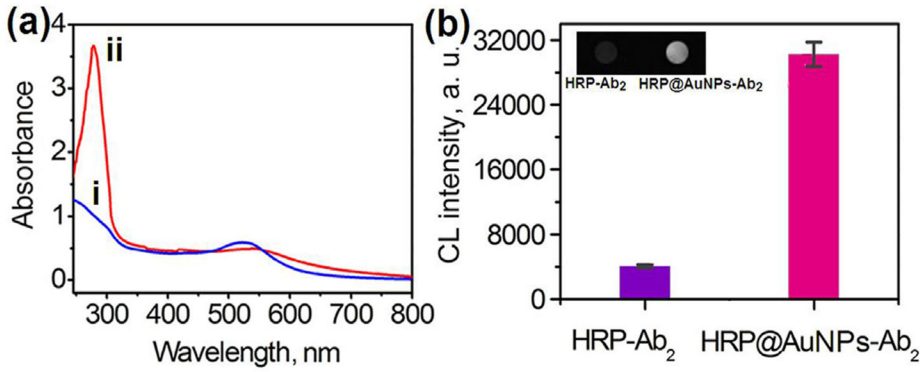


Fig. 1.

(a) UV–vis spectra of AuNPs (i) and Ab_2-AuNP-HRP probe (ii); (b) efficiency of signal amplification of Ab_2-AuNP-HRP probe compared with Ab_2-HRP probe on the immunosensor array under the same conditions.

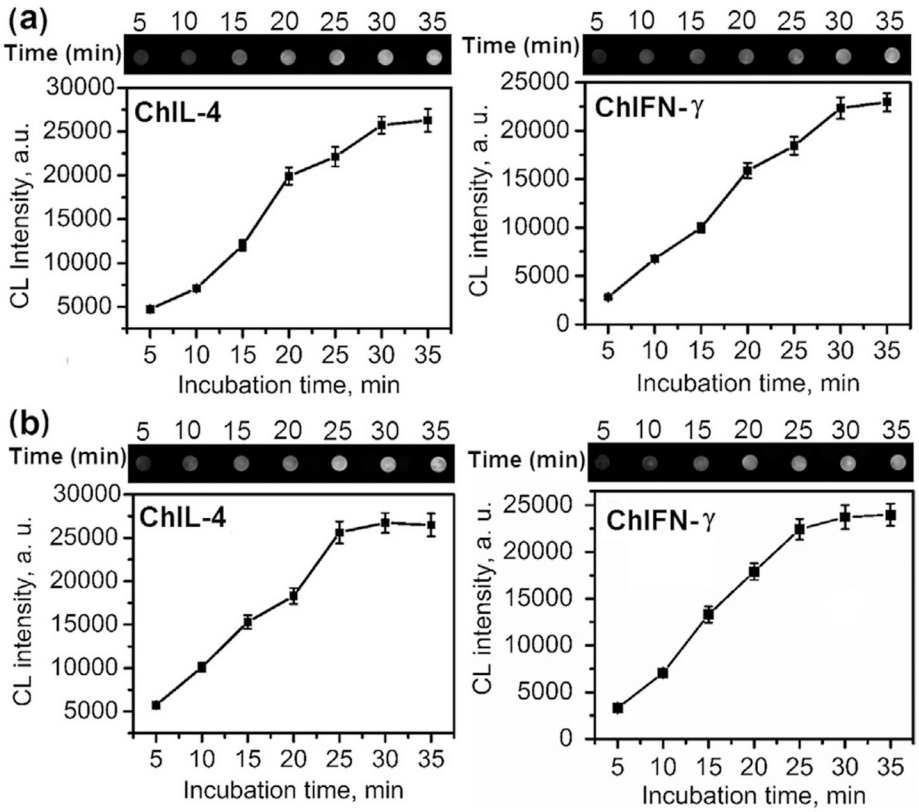


Fig. 2.

(a) Effects of incubation time for the reaction between Ab_1 and chicken cytokines on CL intensity for 0.05 ng/ml ChIL-4 and ChIFN-γ (n = 5 for each point); (b) effects of incubation time for the reaction between Ab_1-cytokine-immunocomplex and HRP@AuNP-Ab_2 probe on CL intensity for 0.05 ng/ml ChIL-4 and 0.05 ng/mL ChIFN-γ (n = 5 for each point).

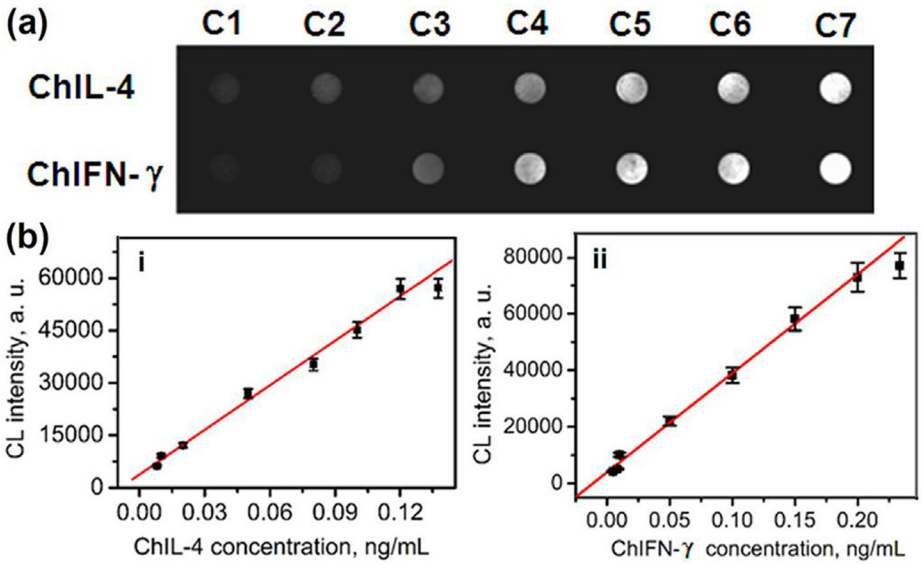


Fig. 3.

(a) Image of CL array and (b) calibration curves for multiplex immunoassay of chicken cytokines. C1–C7 represent concentrations of 0.005, 0.008, 0.01, 0.05, 0.1, 0.15 and 0.2 ng/ml for ChIL-4 (i) and ChIFN-γ (ii) (n = 5 for each point).

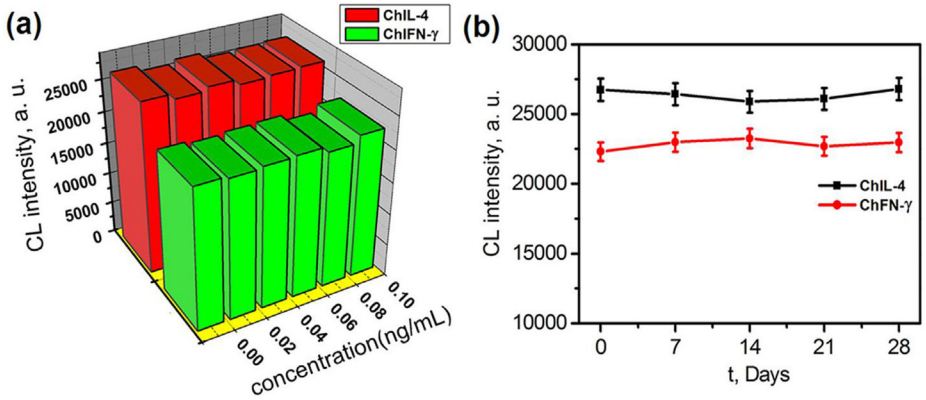
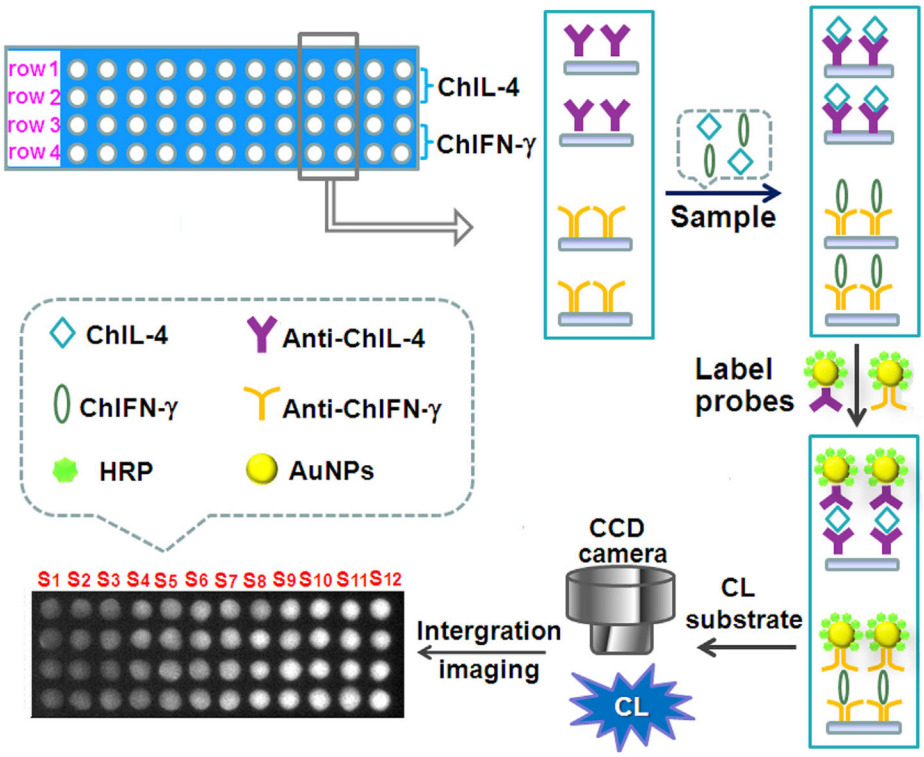


Fig. 4.

(a) Effect of addition of varying concentration of competing cytokine on CL intensity for immunoassay of 0.05 ng/mL ChIL-4 and ChIFN-γ and (b) CL signals of the immunosensor array measured after the storage of different days at 0.05 ng/mL concentration of cytokine antigen.



Scheme 1.

Schematic illustration of chicken cytokine immunosensor array with 4 × 12 sensing cells and CL imaging immunoassay of multiple chicken cytokines. Each column can be used for simultaneous detection of up to four targets in a single sample, and S1–S12 represent 12 samples.

Table 1

Results of recovery for ChIL-4 and ChIFN-γ using proposed method (n = 5).

Sample	Added (ng/mL)		Detected (ng/mL)		Recovery (%)	
	ChIL-4	ChIFN-γ	ChIL-4	ChIFN-γ	ChIL-4	ChIFN-γ
1	0.0100	0.0100	0.0108	0.0094	108.0	94.00
2	0.0200	0.0400	0.0211	0.0382	105.5	95.50
3	0.0400	0.0800	0.0387	0.0815	96.70	101.9
4	0.0800	0.1200	0.0823	0.1275	102.8	106.3
5	0.1000	0.1600	0.0963	0.1549	96.30	96.80